Characterisation and Functional Analysis of Histidine-Rich Glycoprotein

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A thesis submitted for the degree of Doctor of Philosophy at The Australian National University

May 2004
Dedicated to my parents,
Kerryne and Bill Jones
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Declaration

The experimental work presented in this thesis constitutes original work by myself unless otherwise stated in the text. Peng Jian assisted with mouse injections. Eloisa Pagler assisted with insect cell culture. Dr. Craig Freeman assisted in the purification of HRG from human plasma.

This thesis conforms to The Australian National University guidelines and regulations. The work contained within has not been submitted for the purpose of obtaining any other degree at this or other universities.

Allison Louise Jones

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Division of Immunology and Genetics
The John Curtin School of Medical Research
Australian National University

May 2004
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<thead>
<tr>
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<th>Description</th>
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azido-bis (3-ethylbenzthiazoline-6sulphonic acid) diammonium</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>Ala</td>
<td>Alanine</td>
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<td>Amp</td>
<td>Ampicillin</td>
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<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>Arg</td>
<td>Arginine</td>
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<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C-</td>
<td>Carboxyl-terminal</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster designation</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CFSE</td>
<td>Carboxy-fluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CPI</td>
<td>Cysteine proteinase inhibitor</td>
</tr>
<tr>
<td>CSA</td>
<td>Human cystatin SA</td>
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<tr>
<td>CSC</td>
<td>Human cystatin C</td>
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<td>Human cystatin SN.</td>
</tr>
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<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>DDW</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DEPC-MQ</td>
<td>Diethyl pyrocarbonate Milli-Q filtered double distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EthBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>Ex</td>
<td>Exon</td>
</tr>
<tr>
<td>FcγRI</td>
<td>high affinity receptor for the Fc portion of IgG (CD64)</td>
</tr>
<tr>
<td>FcγRII</td>
<td>low affinity receptor for the Fc portion of IgG (CD32)</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptors</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FPLC</td>
<td>Fast performance liquid chromatography</td>
</tr>
<tr>
<td>g</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan(s)</td>
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<td>GHHPH</td>
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<tr>
<td>Gln</td>
<td>Glutamine</td>
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<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
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<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<td>GvHD</td>
<td>Graft versus host disease</td>
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<tr>
<td>H</td>
<td>Heavy chain</td>
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<td>HBSS</td>
<td>Hank's buffered saline solution</td>
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<td>HCC</td>
<td>Human cystatin C</td>
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<td>HHMWK</td>
<td>Human high molecular weight kininogen</td>
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<td>His</td>
<td>Histidine</td>
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<td>HMDM</td>
<td>Human monocyte derived macrophages</td>
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<td>HMWK</td>
<td>High molecular weight kininogen</td>
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<td>HPRG</td>
<td>Histidine-proline-rich glycoprotein</td>
</tr>
<tr>
<td>hr</td>
<td>hour(s)</td>
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<td>HRG</td>
<td>Histidine-rich glycoprotein</td>
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<td>HRP</td>
<td>Horse radish peroxidase</td>
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<td>HRR</td>
<td>Histidine-rich region</td>
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<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<td>IC</td>
<td>Immune complex</td>
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<td>IFNγ</td>
<td>Interferon gamma</td>
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<td>IIC</td>
<td>Insoluble immune complex</td>
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<td>Interleukin-2 receptor</td>
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<td>Ill</td>
<td>Isoleucine</td>
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<td>IMAC</td>
<td>Immobilized metal ion-affinity chromatography</td>
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<td>ip</td>
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<td>kb</td>
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<td>Dissociation constant</td>
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<td>Kilo Daltons</td>
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<td>Litre</td>
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<td>L</td>
<td>Light chain</td>
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<td>Luria broth</td>
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<td>Monoclonal antibody</td>
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<td>MAC</td>
<td>Membrane attack complex</td>
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<td>Met</td>
<td>Methionine</td>
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<tr>
<td>mg</td>
<td>Milligram(s)</td>
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</table>
µl  Microlitre(s)
min  Minute(s)
ml  Millilitre(s)
MOI  Multiplicity of infection
mRNA  Messenger ribonucleic acid
n  Nano
N-  Amino-terminal
N1  The first cystatin-like domain of HRG
N1N2  Amino-terminal cystatin-like domains of HRG
NHS  N-hydroxysuccidimide
Ni-NTA  Nickle nitrilotriacetic acid
OPA  One-Phor-All buffer
PA  Plasminogen activator
PAI  Plasminogen activator inhibitor
PAGE  Polyacrylamide gel electrophoresis
PBL  Peripheral blood leukocytes
PCR  Polymerase chain reaction
PE  Phycoerythrin
PgR  Plasminogen receptor
Phe  Phenylalanine
PMSF  Phenylmethylsulphonyl fluoride
Pro  Proline
PRR  Proline-rich region of HRG
RA  Rheumatoid arthritis
RNA  Ribonucleic acid
RT-PCR  Reverse transcriptase polymerase chain reaction
SAM-HRP  Sheep anti-mouse Ig horse-radish peroxidase
SAR-HRP  Sheep anti-rabbit Ig horse-radish peroxidase
SDS  Sodium-dodecyl sulphate
Ser  Serine
Sf-9  Spodoptera frugiperda derived insect cell line
SLE  Systemic lupus erythematosus
SMC  Smooth muscle cell
SMP  Skim milk powder
SRBC  Sheep red blood cells
Thr  Threonine
t-PA  Tissue-type plasminogen activator
Trp  Tryptophane
TSP  Thrombospondin
Tyr  Tyrosine
u-PA  Urokinase-type plasminogen activator
UV  Ultraviolet
Val  Valine
Zn²⁺  Zinc ion
Abstract

Histidine-rich glycoprotein (HRG) is an abundant plasma glycoprotein whose precise physiological function remains unclear. It has been proposed that HRG may regulate various physiological processes such as tumour angiogenesis, cell adhesion and migration, coagulation, complement activation, immune complex clearance and phagocytosis of apoptotic cells. However, many of the basic characteristics describing the interaction of HRG with its ligands remain undefined, and as such, do not facilitate further understanding of the mechanisms underlying HRG function. In order to overcome some of these deficiencies, the aim of this thesis was to characterise the cell surface ligands for HRG and the domain of HRG that binds to cells, to identify and characterise novel HRG ligands, and to further examine the functional significance of the interaction of HRG with both viable and dying cells.

Experiments described in Chapter 3 used flow cytometry techniques to demonstrate that HRG binds to most cell lines in a Zn$^{2+}$ dependent manner but fails to bind to CHO cells that lacked cell surface GAGs. Subsequent treatment of GAG+ve CHO cells with heparanase, but not chondroitinase ABC, abolished HRG binding, while blocking studies with various GAG species indicated that only heparin was a potent inhibitor of HRG binding. These data suggest that heparan sulphate is the predominate cell surface ligand for HRG, and that mammalian heparanase is a potential regulator of HRG binding. Recombinant full-length HRG and the amino-terminal N1N2 domain of HRG were produced in baculovirus infected insect cells, and were used to show that the N1N2 domain binds specifically to cell surface heparan sulphate. In contrast, synthetic peptides corresponding to the HRR domain of HRG did not interact with cells or block HRG binding. Furthermore, the binding of full-length HRG, but not the N1N2 domain, was potentiated by physiological concentrations of Zn$^{2+}$. Based on these findings a model is proposed whereby the N1N2 domain of HRG binds to cell surface heparan sulphate, with the interaction of Zn$^{2+}$ with the HRR indirectly enhancing cell surface binding.

Studies outlined in Chapter 4 revealed that HRG acts as a serum adaptor protein and tethers plasminogen to the surface of cells. Experiments utilising the BIAcore biosensor showed that immobilised HRG has the ability to interact with plasminogen with high affinity, and that the interaction is dissociable by lysine. Furthermore, plasminogen binding studies using both full-length recombinant HRG and recombinant HRG lacking the C-terminal lysine residue, showed that the HRG-plasminogen complex does not involve the C-terminal lysine of HRG. Flow cytometry studies also indicated that HRG could dramatically enhance the binding of plasminogen to the surface of tumour cells, but only under conditions of low pH and/or elevated Zn$^{2+}$. In addition, tumour cells coated with HRG under these same conditions were found to be
more metastatic in vivo. Based on these studies, a model is proposed in which HRG acts as a serum adaptor molecule that binds to cells at sites of tissue injury, tumour growth and angiogenesis, providing a high affinity receptor for tethering degradative enzymes, such as plasminogen or heparanase, to the cell surface, and thereby enhancing the migratory potential of the cells.

Chapter 5 describes experiments which examined the interaction of HRG with FcγR. Recombinant human FcγRI and human HRG were produced by baculovirus infected insect cells, and were used in studies to show that HRG binds with high affinity to FcγRI. In addition, Chapter 5 outlines studies using both biosensor and cell surface binding analyses, which show that HRG binds to FcγRIIa, the low affinity receptor for IgG. Furthermore, it was apparent that HRG only binds to the His131, and not to the Arg131 polymorphic form of FcγRIIa. Stably transfected cell lines expressing both polymorphic forms of FcγRIIa at their surface were produced in GAG-ve CHO cells, and were used to confirm that HRG binds to FcγRIIa-his131. Additional biosensor studies also suggested that HRG binds to the IgA receptor, FcαR. Thus, utilising recombinant proteins and stable transfected cell lines, data presented in Chapter 5 provides, for the first time, direct evidence that HRG binds to FcγRI, FcγRIIa-his131 and FcαR. These data suggest that HRG regulate immune complex clearance mediated through leukocyte FcRs, and therefore may play a role in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.

Finally, Chapter 6 describes experiments that examined the interaction of HRG with viable, early-stage apoptotic and necrotic cells. Using confocal microscopy and flow cytometry, studies described in Chapter 6 confirmed that HRG binds to heparan sulphate on the surface of viable cells via its N1N2 domain, but also demonstrated that HRG binds poorly to early-stage apoptotic cells. In contrast, HRG binds very strongly to an undefined intracellular ligand within necrotic cells, via its N1N2 domain. Heparin blocking studies and cell binding studies using cell lines lacking GAGs, indicated that the intracellular HRG ligand is unrelated to heparan sulphate. Furthermore, a phagocytosis assay utilising necrotic Jurkat T cells and phagocytic THP-1 cells labelled with different fluorescent dyes, revealed that the presence of physiological concentrations of HRG significantly increased the rate of phagocytosis of the necrotic cells by the monocytic cell line. These data suggest that HRG may play an important physiological role in vivo by facilitating the uptake and clearance of necrotic cells, but not apoptotic cells, by monocytes/macrophages.
Publications resulting from thesis


Meetings abstract


Chapter one

Literature Review:
Histidine-Rich Glycoprotein

This chapter provides a comprehensive overview of the molecular, structural, biological and clinical properties of the plasma protein, histidine-rich glycoprotein, and reviews the role of HRG in various physiological processes.
1.1 Introduction

Histidine rich glycoprotein (HRG), a ~75 kDa single polypeptide chain and α₂-plasma glycoprotein, was first isolated in 1972 (Haupt and Heimburger, 1972; Heimburger et al., 1972). HRG is found in the plasma of many vertebrates including human, mouse, rabbit, rat, chicken and cow. HRG is synthesised in liver parenchymal cells and secreted into human plasma at a relatively high concentration of 100-200 µg/ml (~2 µM), suggesting HRG plays an important physiological role. Perhaps the most distinctive feature of HRG arises from its high content of histidine and proline residues, each of which account for ~13% of total amino acids (Koide et al., 1986b). HRG is known to bind a variety of ligands, can interact with different cell surface receptors and appears to regulate numerous biological functions, however to date no precise functional role has been attributed to HRG. Many biological properties of HRG have been thoroughly investigated and this review aims to summarise the molecular, structural, biological and clinical properties of HRG as well as reviewing the role of HRG in various physiological processes.

1.2 HRG protein structure

1.2.1 Discovery and cloning

HRG was first isolated and characterised from human serum by Heimburger et al., (1972) as an α₂-glycoprotein with a molecular weight of ~60 kDa. Full-length native HRG has since been shown to migrate at ~75 kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels, the 15 kDa discrepancy in size arising from protease cleavage of HRG in original preparations (Rylatt et al., 1981). Subsequently HRG was also isolated and characterised from human plasma (Koide et al., 1982; Lijnen et al., 1980) and platelets (Leung et al., 1983). Both plasma and platelet HRG appear immunohistochemically identical (Leung et al., 1983). Studies of the domain structure of HRG were limited until the primary structure of HRG was determined from the nucleotide sequence of its complementary DNA (cDNA) (Koide et al., 1986b). The cloning of HRG revealed unique and interesting repeat patterns within the protein, most specifically 12 tandem repeats of a dominant 5 amino acid peptide within the histidine-rich region. HRG is a multi-domain molecule consisting of two cystatin-like domains at the amino-terminus (termed N1 and N2), a histidine-rich region (HRR) flanked by two proline-rich sequences (PRR), and a carboxyl-terminal (C)
domain (Figure 1.1). Although the role of HRG \textit{in vivo} remains somewhat unclear, nucleotide and amino acid sequence comparisons between HRG and analogous proteins have prompted possible functional roles for HRG, while analysis of the gene structure has provided insights into the evolution of the gene and protein.

1.2.2 Primary structure

The primary structure of human HRG, as determined from its 2067 nucleotide sequence, consists of 507 amino acids. A complete nucleotide and amino acid sequence of HRG is presented in Appendix I (Koide \textit{et al.}, 1986b). The first cystatin domain (N1) in HRG consists of residues 1 to 112, while the second cystatin domain (N2) consists of amino acid residues 113 to 225. The histidine-rich region lies between residues 330 to 389, with the first flanking proline-rich region (PRR1) located between residues 225 to 314 and the second flanking proline-rich region between residues 398 and 439 (PRR2). The C-terminal domain is located between residues 440 to 507. The C-terminal amino acid consists of a lysine residue at position 507. The modular domain structure of HRG, the location of internal disulphide bonds and various carbohydrate attachment sites are represented as a schematic diagram in Figure 1.1. Perhaps the most striking feature of HRG arises from its unusually high content of histidine and proline residues, each of which exceeds 12% of total amino acid content and is most concentrated within the HRR and PRRs (Haupt and Heimburger, 1972; Koide \textit{et al.}, 1985; Lijnen \textit{et al.}, 1980). In fact, this high content of both histidine and proline residues has resulted in HRG also being termed ‘histidine-proline rich glycoprotein’ (HPRG) (Borza \textit{et al.}, 1996). The high level of histidine amino acids is likely to account for the hydrophilic nature of HRG, while high proline levels may form bending and twisting conformations providing a flexible framework for the histidine-rich region structure (Kyte and Doolittle, 1982).

The leader sequence of 18 amino acids in HRG contains a typical region rich in hydrophobic amino acids (Blobel \textit{et al.}, 1979). This leader sequence is removed through cleavage of an alanine-valine peptide bond by a signal peptidase. A number of stop codons precede the initiation methionine amino acid at position –18, indicating there is not a second methionine start site upstream from position –18 (Koide \textit{et al.}, 1986b). Human HRG is composed of approximately 14% carbohydrate (Haupt and Heimburger,
1972; Koide et al., 1986b). In most humans six glycosylation sites are conserved (resulting in form-1 HRG), with three glycosylation sites present in the N1 domain, one in the N2 domain and two in the HRR (Figure 1.1).

Figure 1.1. Modular structure of histidine-rich glycoprotein (HRG). Human HRG is comprised of three principal domain structures, namely the N1 and N2 amino-terminal domains represented in blue, the central histidine-rich region domain (HRR) represented in green, and the C-terminal domain at the carboxyl-terminus represented in yellow. The histidine-rich region is flanked by two proline-rich sequences (PRR) coloured pink. Two internal disulphide bonds link the domains (represented by red solid lines); residue 6 in the N1 domain links to residue 486 in the C-terminal domain and residue 185 in the N2 domain links to residue 399 in PRR2. There are four intra-domain disulphide bonds; residues 60 and 71 in the N1 domain, residues 87 and 108 in the N1 domain, residues 200 and 223 in the N2 domain and between two undefined cysteine residues in PRR1. These disulphide bonds render various domains of the molecule resistant to proteolytic cleavage. In most humans, six glycosylation sites are conserved (HRG form-1), with three being present in the N1 domain, one in the N2 domain and two in the HRR. A polymorphism exists whereby Ser186 is substituted with Pro186, resulting in the loss of a glycosylation site in the N2 domain (HRG form-2). The N1 domain is composed of amino acid residues 1 - 112, N2: 113 - 225, PRR1: 225 - 314, HRR: 330 - 389, PRR2: 398 - 439 and the C-terminal domain: 440 - 507.
A polymorphism exists whereby Ser186 is substituted for Pro186, resulting in loss of the glycosylation site in the N2 domain (form-2 HRG) (Hennis et al., 1995d). Four of the putative carbohydrate attachment sites are described with the known carbohydrate consensus sequence Asn-X-Thr/Ser including; Asn45, Asn107, Asn326 and Asn327 (Koide et al., 1986b). A fifth carbohydrate attachment site at Asn69 has also been described with the consensus sequence Asn-Asp-Cys, which is known to correlate with carbohydrate attachment sites in Protein C (Koide et al., 1986b). These described carbohydrate attachment sites presumably account for the 14% carbohydrate mass within HRG.

Recombinant human HRG has been expressed in *Spodoptera frugiperda* (Sf-9 insect cells) by infection with recombinant baculovirus (Schinke et al., 1997). The apparent molecular mass of recombinant HRG (66 kDa) is somewhat smaller than plasma-derived HRG (75 kDa), apparently due to incomplete N-glycosylation within the Sf-9 expression system. Although recombinant HRG is partially glycosylated, it appears to lack terminal sialic acid resulting in the reduced molecular mass.

### 1.2.3 Domain structure

One of the striking features of the amino acid sequence of HRG is that it contains internal repeat sequences that can be classified into 5 different types (types I-V) (Figure 1.2). Over half the amino acids in HRG are present in one of the different types of internal repeats, most of which are successive and contiguous (Koide et al., 1986b). Type I, IV and V are triplication type repeats while types II and III are duplication repeats. Type V repeats include the interesting 12 tandem repeats of 5 amino acids (Koide et al., 1986b). Perhaps the most striking feature within HRG arises when the 12 tandem repeats of the penta-peptide sequence (between amino acids 330 - 389) are arranged in blocks (Figure 1.3a). Similarly, when the nucleotide sequence of this region is arranged in blocks of 15 (5 codons) (Figure 1.3b), the striking repetition of the consensus nucleotide sequence is highlighted. Of the 12 tandem amino acid repeats, the last 5 repeated sequences are identical representing exact repetitions of a consensus ‘glycine-histidine-histidine-proline-histidine’ motif. It is possible this region evolved via successive gene duplication from a short stretch of 15 nucleotides (1328-1342) coding for 5 original amino acids. An ancestral gene coding ‘gly-his-his-pro-his’ may have been initially duplicated generating a gene coding for 10 amino acids. Duplication of the latter sequence would generate a gene coding for a repeat of 20 amino acids.
composed of 4 tandem repeats of the 5 original amino acids, resulting in the first of the type V repeats. Further duplication of the 20 amino acid repeats would yield the second and third type V repeats (Koide et al., 1986b). The modular structure of HRG, comprised of linked domains, yields distinct structural properties (Figure 1.1). The N-terminal domain is composed of 2 cystatin-like regions termed N1 and N2, while the middle section of the molecule is comprised of a histidine-rich region sandwiched between two proline-rich sequences that account for the unusually high histidine and proline content, each constituting ~13% of total amino acids. The final domain is the C-terminal region of somewhat undefined structure and function.

<table>
<thead>
<tr>
<th>Repeat</th>
<th>Amino acids</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>(16-38) ALD LIN KRR RRD GYLF QLRLRIFA DA</td>
<td>(166-188) ARVRGEGGTGYFVDFS YRNCPRH</td>
</tr>
<tr>
<td></td>
<td>(143-165) ALE KYKEENDDFASFRV DRIERV</td>
<td></td>
</tr>
<tr>
<td>Type II</td>
<td>(100-121) DL RV - IDF NCT TSSV S S ALANT K</td>
<td>(122-142) DSV PLL IDF E D E R Y R K Q - AN - K</td>
</tr>
<tr>
<td></td>
<td>(143-165) ALE KYKEENDDFASFRV DRIERV</td>
<td></td>
</tr>
<tr>
<td>Type III</td>
<td>(226-244) FD P Q E HEN IN - GVPPHLGHHP</td>
<td>(245-265) FHWG GHERSSTTKP PP KPHG S</td>
</tr>
<tr>
<td></td>
<td>(246-265) FHWG GHERSSTTKP PP KPHG S</td>
<td></td>
</tr>
<tr>
<td>Type IV</td>
<td>(266-285) RDHSHH - PHKPHENHCPPP - PPDE</td>
<td>(286-306) RDHSHGPPPLP - QGPPPLLLPMSC</td>
</tr>
<tr>
<td></td>
<td>(246-265) FHWG GHERSSTTKP PP KPHG S</td>
<td></td>
</tr>
<tr>
<td>Type V</td>
<td>(330-349) DLHPHKHSHHHEQ HP HGHHPH</td>
<td>(350-369) AHPHEHDTHRQ HPHGHPHHP</td>
</tr>
<tr>
<td></td>
<td>(370-389) GHP HGHHPHGHHPHHP</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.2. Classification of the 5 types of internal repeat sequences present within human HRG. Identical residues in each of the type I – V repeat sequences are enclosed in coloured boxes. Identical residues in type I repeats are coloured purple, type II coloured green, type III coloured yellow, type IV coloured blue and type V coloured pink. Gaps were inserted for maximal homology in the amino acid sequences. Type I, IV and V repeats are triplications while type II and III are duplication repeats. The single letter code for amino acids is as follows: Ala - A, Asn - N, Asp - D, Cys - C, Gln - Q, Glu - E, Gly - G, His - H, Ile - I, Leu - L, Lys - K, Met - M, Phe - F, Pro - P, Ser - S, Thr - T, Trp - W, Tyr - Y, Val - V. Adapted from Figure 3, Koide et al. (1986b).
These structural features suggest HRG is a typical modular protein with independent functions being assigned to each domain. It should be noted that with many plasma proteins, it is often observed that ‘binding sites’ are located near the $N$-terminal end of the molecule while biologically ‘active’ sites are associated with the $C$-terminus of the molecule. For example, the lysine-binding site of plasmin is located at the $N$-terminal region of the molecule, whereas the active catalytic site of plasmin is located at the $C$-terminus (Sottrup-Jensen, 1978). From these and other observations, it is possible that the HRG ligand binding sites are located within the $N$-terminal domains, but that the biologically active sites of HRG could be located within the HRR and/or $C$-terminal region. The structure of the individual domains is discussed in more detail below.

Figure 1.3. Tandem repeats in the histidine-rich region of human HRG cDNA and the derived amino acid sequences. (a) Amino acid residues between 330 and 389 are arranged into a block sequence of 5 columns and 12 rows highlighting conservation within the tandem repeat sequences. Amino acids identical with the consensus amino acid sequence "glycine, histidine, histidine, proline, histidine" are enclosed in yellow boxes. (b) Nucleotides between 1163 and 1342 are arranged into a block sequence of 15 columns (5 codons) and 12 rows. Nucleotides identical with the consensus sequence "GGA CAC CAT CCC CAT" are enclosed in blue boxes. Adapted from Figure 4, Koide et al. (1986b).
1.2.3.1 Histidine and proline-rich regions

Histidine residues are highly concentrated in the small region between amino acids 330-389 forming the ‘histidine-rich’ region of the protein (Koide et al., 1986b). Proline residues are more widely distributed, although many are concentrated in the ‘proline-rich’ region immediately prior to and following the histidine-rich region. These proline and histidine-rich regions (residues 225 - 439) contain very few hydrophobic and basic amino acids and may form bending and twisting conformations providing a structural framework for the histidine-rich region (Koide et al., 1986b).

Amino acid sequence comparison between histidine-rich regions of HRG and histidine-rich regions of both human and bovine high molecular weight kininogen (HMWK) reveal ~ 50% homology (Figure 1.4a), with many non-identical residues differing by only one base in the respective codon. The histidine-rich proteins HRG and HMWK also both share low hydrophobic amino acid content, leading to suggestions that the histidine-rich regions of the two molecules may be evolutionary and functionally related.

Figure 1.4. Comparison of amino acid sequences of human HRG domains with proteins that share homology. (a) Comparison of the histidine-rich region amino acid sequences of human HRG and human and bovine high molecular weight kininogen (HMWK). Amino acids 334 – 391 within the HRR of human HRG are compared with amino acids 445 – 506 in human HMWK (HHMWK) and amino acids 445 – 505 in bovine HMWK (BHMWK). Amino acid residues identical with those of HRG are enclosed in green boxes. (b) Comparison of the amino acid sequences of two proline-rich regions in human HRG (amino acids 271 – 303 and amino acids 398 – 425) with those of proline-rich peptides and proteins from parotid saliva. Amino acid residues that are identical with those of the PRR in HRG are enclosed in orange boxes. Gaps were inserted for maximal homology. The sequences of proline-rich peptides (abbreviated as P-D and P-F) were taken from Saitoh et al. (1983a; 1983b) respectively and those of proline-rich proteins (abbreviated as PRP and PRP-1) were taken from Azen et al. (1984) and Ziemen et al. (1984) respectively. (c) Comparison of the amino acid sequences of the N-terminal domain of human HRG and human and bovine HMWK. Amino acids 30 – 75 within the N1 domain of HRG are compared with amino acids 54 – 98 in HHMWK and amino acids 53 – 97 in BHMWK. Amino acid residues identical with those of HRG are enclosed in blue boxes. Gaps were inserted for maximal homology. The numbering of the amino acid residues for human and bovine HMWK was taken from Takagaki et al. (1985) and Kitamura et al. (1983) respectively. (d) Comparison of the N-terminal sequence of human HRG (amino acids 1 – 146) with the N-terminal sequence of human antithrombin III (amino acids 24 – 139). Amino acid residues that are similar to human HRG are enclosed in yellow boxes. Gaps were inserted for maximal homology. The antithrombin sequence was derived from Petersen (1979).
(a) Histidine-rich region

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td></td>
<td>HHMWK (445-474)</td>
<td>H K H - E R D Q - G H G H Q R G H G L G H G H E Q Q H G L G H G</td>
</tr>
<tr>
<td></td>
<td>BHMWK (445-474)</td>
<td>H K H - K H D Q - G H G H G S H G L G H G Q K Q H G L G H G</td>
</tr>
<tr>
<td></td>
<td>HRG (364-391)</td>
<td>H G H H P H G H H P - - G H P - H G H H H P H H P H H P - -</td>
</tr>
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</table>

(b) Proline-rich region

<table>
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<tr>
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<th>HRG (271-303)</th>
<th>P H K P H E H G P P P P P D E R D H S H G P P L - - - Q P G P P P -</th>
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<tr>
<td></td>
<td>P - D (23-54)</td>
<td>P G K P - - Q G P P P P G G N P Q Q P Q A P P A - G K P Q P G P P P -</td>
</tr>
<tr>
<td></td>
<td>PRP (110-140)</td>
<td>P G N P - - Q G P P P P Q G P P Q P G P P P Q</td>
</tr>
<tr>
<td></td>
<td>P - P (27-50)</td>
<td>- P P P Q G P P P - - Q G G N K - P Q G P P P P G K P Q - - G F</td>
</tr>
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</table>

(c) Cystatin-like domain

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<tr>
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<th>HRG (30-55)</th>
<th>F Q L L R I A D A H L D R V E N T T Y Y L V L D V</th>
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<tr>
<td></td>
<td>HHMWK (54-78)</td>
<td>F V L Y R I T E A T K T - V G S D T F Y S F K Y E I</td>
</tr>
<tr>
<td></td>
<td>BHMWK (53-77)</td>
<td>F V L Y R I T E V A R M - D N P D T F Y S L K Y Q I</td>
</tr>
<tr>
<td></td>
<td>HRG (56-75)</td>
<td>Q E S D C S V L S R K Y W N D C E P P D</td>
</tr>
<tr>
<td></td>
<td>HHMWK (79-98)</td>
<td>K E G D C P V Q S G K T W Q D C E Y K D</td>
</tr>
<tr>
<td></td>
<td>BHMWK (78-97)</td>
<td>K E G D C P F Q S N K T W Q D C D Y K D</td>
</tr>
</tbody>
</table>

(d) Antithrombin III

<table>
<thead>
<tr>
<th></th>
<th>HRG (1-36)</th>
<th>V S F T D C S A V E P E A - E - K A L D L I N K R R R D G Y L F Q L L R I A</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HRG (65-91)</td>
<td>R K Y W N D C E P P D S - - - - - - R R P S E I V I G Q C K V I A</td>
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<tr>
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<td>AT3 (24-59)</td>
<td>R S P - E K K A T E D E G S E Q K I P E A T N R R V W E L S K A N S - R F A</td>
</tr>
<tr>
<td></td>
<td>HRG (37-64)</td>
<td>D A H L D R V E N T T V Y Y L V L D - V Q E S D C S - V L S</td>
</tr>
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<td></td>
<td>HRG (92-112)</td>
<td>- - - - - - - - - - T R - - - - H S H S - Q D L R V T D W N C T T S</td>
</tr>
<tr>
<td></td>
<td>AT3 (60-82)</td>
<td>- - - - - - T T F Y Q H L A D S K N D N D N I - F L S - P L S</td>
</tr>
<tr>
<td></td>
<td>HRG (113-133)</td>
<td>V S S A L A N T K - - - - - - D S P V - L I D F F E - D T -</td>
</tr>
<tr>
<td></td>
<td>HRG (134-146)</td>
<td>E R - - - - - - - - - - Y R K Q A N K A L E K</td>
</tr>
<tr>
<td></td>
<td>AT3 (113-139)</td>
<td>E K T S D Q I H F F F A K L N C R L Y R K - A N K S S - K</td>
</tr>
</tbody>
</table>
HMWK binds kaolin in the kaolin-mediated initiation of blood coagulation (Sugo et al., 1980), and due to high sequence homology between HRG and HMWK it has been suggested HRG may share a similar function to kininogen acting as a modulator of contact activation of blood coagulation (Koide et al., 1986b).

Alternatively, amino acid sequence comparison between HRG and other histidine-rich proteins, such as that of the malaria parasite (73% histidine), show very little sequence homology (Ravetch et al., 1984), suggesting a role for HRG more likely related to histidine-rich proteins like HMWK of which it shares high sequence homology, rather than simple high histidine content proteins such as that present in the malaria parasite. Amino acid sequence comparison between proline-rich regions of HRG and proline-rich proteins and peptides (Azen et al., 1984; Saitoh et al., 1983a; Saitoh et al., 1983b) from parotid saliva also show up to ~ 50% sequence homology (Figure 1.4b). The characteristic sequence of ‘gln-gly-pro-pro-pro’ in proline-rich regions of saliva proteins also exists in HRG as gln/his-gly-pro-pro-pro. Due to the high sequence homology it appears likely the two proline-rich regions within HRG may also form elongated bending and twisting conformations similar to the much studied proline-rich proteins from saliva (Koide et al., 1986b). Data from electrophoretic and sedimentation behaviour studies on HRG indicate histidine residues become protonated below pH 6, causing a conformational change that can be transmitted to the N- and C-terminal domains (Borza et al., 1996). The isoelectric point of HRG is ~ 6.45 suggesting that at physiological pH HRG remains negatively charged.

An important functional role of the numerous histidine residues within the HRR involves mediation of haem and metal binding. Two imidazole rings (each from a single histidine amino acid residue) in the ‘gly-his-his-pro-his’ sequence form a configuration ideal for metal and haem binding (Figure 1.5) and are likely to account for the functional metal binding properties of HRG (Morgan, 1985). Modelling studies predict the tertiary structure of the tandem histidine-repeat motif to be an elongated twisted binding structure (Figure 1.5c), whereby imidazole binding units are positioned on the outer region of the structure allowing access for metal ions to bind (Borza et al., 1996). The interaction between HRG and metals or haem is dependent both on pH and intact histidine residues, lending support to the idea that the histidine residues are an integral structural component of HRG mediating metal binding (Morgan, 1985). Despite HRG binding metal ions and haem, HRG does not appear to play a critical role in serum transport of these ligands under normal physiological conditions. It appears more likely that HRG metal binding modifies the conformation of the molecule and thereby regulates subsequent biological activity.
1.2.3.2 The cystatin domains

The cystatin supergene family are more generally known as cysteine proteinase inhibitors (CPI) and are widely distributed in mammalian tissues and body fluids. The superfamily is classified into three families (Koide and Odani, 1987);

a) cystatins of various origins (one domain)
b) kininogens (3 domains)
c) stefin family

CPI and HRG share ~ 17% amino acid sequence homology and ~ 32% nucleotide sequence homology (Koide and Odani, 1987), primarily in the N-terminal region. The N1 and N2 domains of HRG are extensively homologous to domains 1 and 3 of HMWK (Koide and Odani, 1987). N1 exhibits most homology to domain 1 of kininogen, which is unique in the cystatin superfamily in that it does not possess inhibitory activity. Figure 1.4c shows amino acid sequence comparison between the cystatin domains of HRG and human and bovine HMWK, highlighting amino acid conservation between the N-terminal domains of the proteins.

Other similarities shared between HRG and the cystatin superfamily include the location of disulphide bonds, with the two disulphide bonds present in cystatins and kininogen being conserved within the N-terminal domains of HRG (Koide and Odani, 1987) (Figure 1.9). The crystal structure of related cystatin and cystatin-like proteins (Figure 1.6) probably gives the best indication and projection of the likely structure of the N-terminal domain of HRG. Apart from kininogen, other non-inhibitor members of the cystatin superfamily include ovalbumin, angiotensinogen, barley endosperm protein and thyroxine-binding (Koide and Odani, 1987). Since no cystatin-like inhibitory activity has ever been reported for HRG, it too may also be classified as a non-inhibitor member of CPI. Although HRG is often classified as a kininogen-like member of the CPI it has been suggested that HRG should be classified as a new family within the cystatin superfamily, as it consists of only 2, not 3 cystatin-like domains. Therefore it may be an intermediate between the cystatin (one domain) and kininogen (three domains) family in the genealogy of mammalian CPI (Koide and Odani, 1987). Studies on the primary structure of HRG show the N-terminal sequence also shares homology with antithrombin III (Koide et al., 1986a; Koide et al., 1982) (Figure 1.4d). Both these
plasma proteins bind heparin with high affinity, although HRG does not show any thrombin inhibitory activity nor heparin co-factor activity (Koide et al., 1982). Although the two plasma proteins may not be functionally related, it has been suggested that the heparin-binding site within HRG may be located within the \( N \)-terminal domain, similar to the heparin-binding site in antithrombin III (Koide et al., 1982). However, other studies suggest that the HRG heparin-binding site is located within the HRR. The putative heparin-binding site of HRG is discussed further in section 1.5.9.3.

Figure 1.5. Structure of a histidine amino acid and projected structure of the histidine-rich region of human HRG. (a) Chemical structure of a histidine amino acid, noting the imidazole ring structure formed from two nitrogens within the ring structure. Two of these ring structures form the basic metal binding unit. (b) Space filled (left) and stick (right) representation of a histidine amino acid highlighting spatial structure of the amino acid. (c) Projection of the helical structure of the histidine-rich region of HRG noting that the imidazole side chains are projected on to the outer edge of the structure forming the numerous metal-binding ring units.
Figure 1.6. Crystal structure and projected structure of various cystatin molecules that share high sequence homology with the N-terminal domains of HRG. The amino-terminal domains of HRG share high sequence homology with members of the cystatin superfamily. Thus, HRG probably shares a similar 3-dimensional structure with the cystatin superfamily members, particularly in regard to the amino-terminal domain. The crystal structure of members of the cystatin family is well studied. (a) 3-dimensional molscript cartoon of a cystatin-like module prepared by Annalisa Pastore (EMBL) is shown in pink (http://www.bork.embl-heidelberg.de/Modules/3d/). (b) Human cystatin C (HCC), a potent inhibitor of class C1 cysteine proteases, is shown in green and blue as an inactive dimer (http://www.man.poznan.pl/CBB/research.html). (c) Cystatin-like: the X-ray crystal structure of chicken egg white cystatin is shown in purple (Bode et al., 1988). Each of the cystatins described show a helical domain with numerous β-sheets.
1.2.4 Secondary structure

The secondary structure of HRG has not been entirely solved, however estimations from its primary sequence predict 8% α-helices, 14% β-sheets, 46% β-turns and 32% random coils (Koide et al., 1986b). The N-terminal structure appears primarily composed of β-sheet and some α-helix content, which is similar to high sequence homology related proteins like HCC (Figure 1.6b) (Borza et al., 1996). The C-terminal portion of the molecule (amino acids 440-507) is predicted to contain mostly random coil structure with no helical structure. Of the three domains, the histidine-rich region quantifies as a ‘low complexity’ region where hydrophilic sequences are relatively mobile and are likely to generate relatively extended coils or helical structures. The lack of hydrophobic amino acids in the histidine-rich domain precludes the formation of a compact, globular structure. Indeed far-UV CD spectrum studies suggest the presence of a polyproline (II) helix as expected from the high content of proline, β-turns and random coil structures (Borza et al., 1996). Overall the predicted secondary structure suggests multifunctional properties due to the organization of the polypeptide chain into separate modular domains (Koide et al., 1986b).

Human HRG contains 16 half-cysteine residues of which 12 are involved in putative disulphide bridges (Figure 1.1). Disulphide bridges occur between residue 6 in N1 and residue 486 in the C-terminal domain, residues 60 and 71 in the N1 domain, residues 87 and 108 in the N1 domain, residues 200 and 223 in the N2 domain, between two undefined residues within PRR1 and residue 185 in the N2 domain and 399 in PRR2. These disulphide bridges are likely to provide structural stability for HRG as well as linking the modular domains of HRG together even after plasmin digestion (Figure 1.7).

1.2.5 Proteolytic cleavage

Studies indicate that HRG is readily cleaved by serine proteases such as plasmin from its native form (75 kDa) into fragments ranging in size from 10 – 65 kDa (Smith et al., 1985). Indeed, lower recoveries of native HRG are obtained from serum than from plasma due to the proteolytic action of serum proteases (Rylatt et al., 1981). Recent studies suggest the dominant plasmin cleavage sites flank the HRR, cleaving HRG at Arg295 within PRR1 and at Lys413 within PRR2 (based on the rabbit amino acid numbering system) as shown in Figure 1.7 (Borza et al., 1996). This cleavage results in
the release of the HRR leaving N- and C-terminal fragments connected by a disulphide bridge between residues 6 and 486 (Borza et al., 1996) which can be separated by further denaturation and reduction. The C-terminal region is easily digested into smaller fragments, whereas the N-terminal domain remains relatively resistant to further proteolysis due to the stabilising effect of two intra-domain disulphide bridges.

**Intact HRG**

**Plasmin cleaved HRG**

**N- and C-terminal fragments**

**HRR fragment**

Figure 1.7. Major plasmin cleavage sites within human HRG. The modular domain structure of human HRG is linked together by inter-and intra-domain disulphide bonds. Plasmin (a serine protease) has the degradative potential to cleave HRG into two or more fragments. Based on the rabbit amino acid numbering system, the major plasmin cleavage sites flank the HRR at Arg295 and Lys413. This results in the formation of two primary fragments, a HRR containing a residual portion of the PRRs, and a larger part of the molecule containing the N-terminal domains and the C-terminal domains as well as fragments of the proline-rich regions. The larger N- and C-terminal fragment is relatively resistant to initial proteolysis due to the presence of inter-chain disulphide bonds, however upon further proteolysis and reduction, the C-terminal domain is easily digested into smaller fragments whereas the N-terminal fragment is relatively resistant to further proteolysis due to the stabilising effect of two-intra domain disulphide bonds.
1.3 HRG gene structure and evolution

The human HRG gene has been mapped to chromosome 3, position 3q28-q29 (Koide, 1988; van den Berg et al., 1990) and spans approximately 11 kb. This region of chromosome 3 is home for two other members of the human cystatin gene superfamily namely α2-HS glycoprotein and kininogen. Fluorescence in situ hybridisation studies have shown that the HRG gene and the two human cystatin gene superfamily members map at 3q27 within one megabase of each other (Rizzu and Baldini, 1995), supporting suggestions that HRG may be evolutionarily related to the cystatin gene superfamily. Evidence suggests that in humans HRG exists as a single copy gene. Murine data using Southern blot analysis of digested mouse genomic DNA probed with full-length mouse HRG cDNA reveals a simple hybridisation band pattern consistent with the murine HRG gene being a single copy gene (Hulett and Parish, 2000). The human HRG gene consists of eight exons and seven introns (Figure 1.8) (Hennis et al., 1994; Koide, 1988; Koide et al., 1986b; van den Berg et al., 1990). Originally the gene was thought to contain nine exons and eight introns, however PCR of genomic DNA using oligonucleotides designed from exon VIII and exon IX showed the putative intron between amino acids 439 and 440 was absent (Hennis et al., 1994). The first intron (intron₁) occurs in the 5' untranslated region of the gene. The leader sequence (residues -18 to -1) and part of the first cystatin domain are encoded by exon II. Two introns (intron₂ and intron₃) are located within the N1 domain, making the N1 domain consist of 3 exons (exon II, III and IV). Intron₄ occurs at the boundary between N1 and N2. The N2 domain is also encoded by three exons (exon V, VI and VII) separated by intron₅ and intron₆. Intron₇ is present at the end of the N2 domain (Koide, 1988). There appears to be no introns within the HRR and C-terminal domain, with the single exon VIII encoding the HRR, the PRRs flanking either side of the HRR as well as the C-terminal domain. Analysis of human HRG cDNA as derived from mRNA shows it to consist of 2067 nucleotides, including 121 nucleotides of 5' non-coding sequence, 54 nucleotides coding for the leader sequence of 18 amino acids, 1521 nucleotides coding for the mature protein of 507 amino acids, a stop codon of TTA, and 352 nucleotides of 3' non-coding sequence followed by a polyA-tail of 16 nucleotides (Koide et al., 1986b).
Figure 1.8. Structure of the human HRG gene. The human HRG gene is composed of 8 exons and 7 introns. The first intron (Intron 1) occurs in the 5’ untranslated region of the gene. The leader sequence (encoded by residues -18 to -1) and a small portion of the N1 domain is encoded by exon II. The N1 domain (amino acid residues 1 - 112) is encoded by three exons in total (exons II, III, IV) while the N2 domain (amino acid residues 113 – 229) is also encoded by three exons (exons V, VI, VII). The exons encoding the N-terminal domains are shown in blue. The PRRs shown in pink (amino acids 255 – 314 and 398 – 439), the HRR is shown in green (amino acid residues 330 – 389) and the C-terminal domain shown in yellow (amino acid residues 440 – 507) are all encoded by the single exon VIII.

1.3.1 Evolution of the HRG gene

Several evolutionary implications arise from an analysis of the HRG gene and protein. The many tandem repeats strongly suggest the HRG gene arose by endo-duplication of primordial mini-domains or modules which correspond with previously described (section 1.2.3) type I-V repeats (Koide, 1988). Comparisons between the gene structure of HRG and members of the cystatin supergene family show evolutionary similarity, as discussed below.

1.3.1.1 Gene structure of the cystatin domains

Each cystatin domain (N1 and N2) of HRG is coded for at the gene level by three exons and two introns. The location of introns within cystatin domains of HRG and members of the cystatin supergene family (cystatin and kininogens) occur in essentially the same positions (Figure 1.9a). These similarities are consistent with HRG belonging to the cystatin supergene family while also suggesting that the HRG gene is evolutionarily related to the cystatin supergene family (Hennis et al., 1994; Koide, 1988).
**Figure 1.9. Comparison between the gene structure of human HRG and cystatin superfamily members.** Comparison of the intron location within the HRG and cystatin superfamily genes. The intron localisation of the cystatin domains (N1 and N2) of human HRG is very similar to the intron localisation of the first two cystatin domains of kininogen and various cystatins. Intron positions for each individual gene are indicated by solid red triangles. Gaps are included for the best alignment of amino acid sequences (Koide and Odani, 1987). N1: human HRG N1 domain (first cystatin domain), N2: human HRG N2 domain (second cystatin domain), K1: human kininogen domain 1, K2: human kininogen domain 2, K3: human kininogen domain 3 (Kitamura et al., 1985). CSN: human cystatin SN, CSA: human cystatin SA, CSC: human cystatin C (Saitoh et al., 1988). Disulphide bridges in cystatin and kininogen (Sueyoshi et al., 1987) are shown by blue solid lines, while potential disulphide bridges are shown by broken blue lines.
Another similarity exists between HRG and kininogen whereby the HRR (which in both proteins is situated C-terminal to the cystatin domains) of these proteins is encoded by only one exon. In addition to homologous gene structure, the colocalisation of the HRG gene and the kininogen gene to a similar region of the human genome substantiates the evolutionary relatedness of HRG to the cystatin superfamily (Hennis et al., 1994).

1.3.2 HRG polymorphisms and skeletal muscle HRG

1.3.2.1 HRG polymorphisms

Two particular isoforms of HRG have been found to commonly exist in the population differing by a molecular weight of ~ 2 kDa (Hennis et al., 1995d). Sequencing of genomic DNA from homozygous individuals showed the resulting polymorphism occurs in exon 5. Form-1 HRG (77 kDa) contains a serine residue at amino acid position 186, whereas form-2 HRG (75 kDa) contains a proline residue at amino acid position 186. The presence of serine at position 186 introduces an N-glycosylation site making form-1 more highly glycosylated than form-2 and accounting for the 2 kDa difference (Hennis et al., 1995d). The frequency of the polymorphisms within the population (N = 36) was found to be 0.35 and 0.65 for form-1 HRG and form-2 HRG respectively.

1.3.2.2 Skeletal muscle HRG

A ~ 75 kDa novel polypeptide was isolated from purified rabbit and then human (~ 85 kDa) skeletal muscle adenosine monophosphate (AMP) deaminase, an important enzyme that interacts with myosin and titin in relation to muscle function (Ranieri-Raggi et al., 1997; Sabbatini et al., 1999). This protein shows striking similarity to rabbit and human HRG (Borza et al., 1996). AMP deaminase is composed of 4 near identical subunits (Boosman and Chilson, 1976; Coffee and Kofke, 1975), and contains two different protein species; one being the deaminase catalytic subunit, while the other subunit shows similarity to plasma HRG (Ranieri-Raggi et al., 1997). Interestingly, a small degree of divergence occurs between AMP-HRG and rabbit HRG in the region containing residues 472-477 and thus the peptide is identified as a muscle specific form of HRG, herein referred to as muscle-HRG (Ranieri-Raggi et al., 1997). Studies indicate that a possible mechanism for the production of muscle-HRG involves
alternative splicing of the HRG primary transcript, producing mRNA encoding a variant containing a unique N-terminus spliced onto the mature HRG polypeptide (Ranieri-Raggi et al., 1997). The existence of muscle-HRG was confirmed in human skeletal muscle myofibrils using specific plasma HRG antibodies (Ranieri-Raggi et al., 1997; Sabbatini et al., 1999). Muscle fibres are classified as type I (slow twitch), and type II (fast twitch). Type II fibres are further divided into two subtypes; IIA (fast-twitch oxidative-glycolytic), and IIB (fast-twitch glycolytic) (Sabbatini et al., 1999). Muscle specific HRG is clearly localised in type IIB (fast-twitch glycolytic) muscle fibres which are well known to contain the highest level of AMP deaminase among muscle fibres (Raggi et al., 1969). It is unknown whether muscle-HRG contributes to the structure of skeletal muscle AMP deaminase or whether it simply binds the enzyme (Sabbatini et al., 1999). Due to the multi-domain structure of HRG, muscle HRG may function in skeletal muscle by participating in protein-protein interactions linking the catalytic site of AMP deaminase with both myosin and titin (Ashby and Frieden, 1977; Soteriou et al., 1993). Muscle-HRG may possibly play a role in the rapid clearance of AMP deaminase from the circulation by binding to hepatocyte cell surface heparan-sulphates and thereby aiding internalisation of the enzyme (Ranieri-Raggi et al., 1997).

### 1.3.3 Comparison of HRG molecules between species

Comparisons between human, mouse, rat, and rabbit HRG amino acid and nucleotide sequences provides insights into conserved regions within HRG that are likely to play an important role in the structure and function of the molecule. The predicted mouse and rat HRG amino acid sequences contain 5 or 6 N-linked glycosylation sites that are conserved in both species. Rat HRG has a 6th glycosylation site not found in mouse HRG. A putative signal peptide sequence is conserved in both mouse and rat at their respective N-termini between amino acids 1-18. Alignment of amino acid sequences from mouse, rat, rabbit and human HRG show a high degree of sequence conservation between species. Table 1.1 below indicates the percentage of HRG amino acid sequence homology between the different domains of human, rabbit, rat and mouse HRG.

The overall domain structure of mouse, rat and rabbit HRG is similar to that proposed for human HRG with each comprising of three overall domains; an N-terminal domain containing 2 cystatin-like regions (N1 and N2), a histidine-rich domain (HRR) flanked
by two proline rich regions (PRR1 and PRR2) and a C-terminal domain, retaining the basic 3-domain modular structure between all species.

**Table 1.1** Percent homology of HRG amino acid sequence between domains of various mammalian species

<table>
<thead>
<tr>
<th>Species</th>
<th>Leader</th>
<th>N1</th>
<th>N2</th>
<th>HRR</th>
<th>PRR1</th>
<th>PRR2</th>
<th>C</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>M v Rt</td>
<td>100</td>
<td>87.4</td>
<td>82.9</td>
<td>68.6</td>
<td>80.4</td>
<td>80.0</td>
<td>80.1</td>
<td>83.9</td>
</tr>
<tr>
<td>M v H</td>
<td>72.2</td>
<td>69.4</td>
<td>68.4</td>
<td>67</td>
<td>40</td>
<td>57.7</td>
<td>69.1</td>
<td>63.2</td>
</tr>
<tr>
<td>M v Rb</td>
<td>-</td>
<td>64.9</td>
<td>63.2</td>
<td>58.1</td>
<td>46.6</td>
<td>53.3</td>
<td>64.7</td>
<td>60.5</td>
</tr>
<tr>
<td>Ra v H</td>
<td>72.2</td>
<td>68.6</td>
<td>70.0</td>
<td>65.0</td>
<td>41.7</td>
<td>61.9</td>
<td>64.7</td>
<td>63.3</td>
</tr>
<tr>
<td>Ra v Rb</td>
<td>-</td>
<td>64.0</td>
<td>66.7</td>
<td>48.6</td>
<td>43.3</td>
<td>53.3</td>
<td>58.8</td>
<td>61.7</td>
</tr>
<tr>
<td>H v Rb</td>
<td>-</td>
<td>72.9</td>
<td>69.2</td>
<td>47.3</td>
<td>51.6</td>
<td>55.6</td>
<td>66.2</td>
<td>65.3</td>
</tr>
</tbody>
</table>

Adapted from Table 1, Hulett and Parish (2000), M = mouse, Rt = rat, Rb = rabbit, H = human

The HRRs also show a high degree of conservation, although they display greater divergence than the N- and C-terminal domains due to differing numbers of pentapeptide repeat sequences between species (Table 1.2). Of all the species, rabbit HRG appears the most divergent due to a slightly different repeat motif. The sequence 'gly-his-his-pro-his' predominates in mouse, rat and human HRG, where it is repeated 8, 6 and 6 times respectively (exact consensus), whereas 'gly-his-pro-pro-his' and 'gly-pro-pro-pro-his' are repeated 5 and 6 times in rabbit HRG (Hulett and Parish, 2000).

**Table 1.2** Variation between the number of motif a repeats in the histidine-rich region between human, rabbit, rat and mouse HRG.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of repeats of motif sequence a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>13</td>
</tr>
<tr>
<td>Rat</td>
<td>11</td>
</tr>
<tr>
<td>Human</td>
<td>11</td>
</tr>
<tr>
<td>Rabbit</td>
<td>14</td>
</tr>
</tbody>
</table>

Adapted from Table 2, Hulett and Parish (2000)

*aDefined as motif containing at least 3 conserved residues of the gly-his his pro-his motif

The PRRs (in particular PRR1) are generally the most divergent of the defined regions within HRG. Interestingly there is very little sequence identity between species in regions that intervene between domains such as between N2 and PRR1 and also between PRR1 and HRR, implying these regions are unlikely to play an important
functional role and may act as a spacer region between the domains (Hulett and Parish, 2000).

Studies using bovine HRG have defined 6 inter-chain disulphide bonds likely to play an important structural role. In human HRG, 3 disulphide bridges are situated in the N-terminal domain between residues 60-71, 87-108 and 200-223, one between the N1 domain and the C-terminal domain (6-486), one between the N2 domain and PRR2 (185-399) and one between undefined residues within PRR1 (Hulett and Parish, 2000). The 12 cysteine residues that form disulfide bonds in mouse are also conserved in rabbit and rat HRG whereas 10/12 are conserved in human HRG (Cys254 is substituted for Pro254) suggesting these cysteine residues also form disulphide bonds likely to play important structural roles (Hulett and Parish, 2000).

Recently a histidine-rich glycoprotein with high affinity for cadmium was isolated from the blood of the blue mussel *Mytilus edulis*. Molecular properties such as molecular weight and isoelectric point, carbohydrate content, high histidine composition and cadmium binding capacity indicate the aquatic invertebrate form of HRG shares many similarities with mammalian HRG. The high level of histidine strongly suggests imidazole rings present within the histidine structure may act as a primary chelation site resulting in cadmium binding (Nair and Robinson, 1999). It is yet to be determined whether this histidine-rich protein shares any sequence homology with mammalian HRG.

### 1.4 Sites of synthesis and plasma levels of HRG

#### 1.4.1 **Synthesis of HRG**

Liver parenchymal cells are thought to be the site of HRG protein synthesis, a claim supported by a 900kb HRG specific PCR product being amplified only from human liver (Corrigan *et al.*, 1990; Drasin and Sahud, 1996; Koide *et al.*, 1986b). Other studies suggest HRG may also be expressed by some immune cells such as monocytes and macrophages in the mouse (Sia *et al.*, 1982) and in human megakaryocytes (Leung *et al.*, 1983). A small proportion of plasma HRG (0.14%) has been shown to exist on the surface of platelets (Leung *et al.*, 1983). Whether or not platelets synthesise HRG themselves or simply acquire plasma HRG remains controversial (Hulett and Parish,
A high circulating concentration of HRG in plasma (100-200 µg/ml) would certainly ensure that circulating immune cells are exposed to significant levels of HRG. Additional studies have also demonstrated that HRG is able to bind to many classes of cell surface receptors, suggesting it is probably more likely that immune cells simply acquire HRG from plasma. Studies in mice showed that HRG mRNA is specifically localised and in the liver as both Northern blot and RT-PCR studies failed to detect HRG mRNA in the spleen, thymus, lymph nodes, bone marrow, PBL, heart, lung, kidney, brain or testis (Hulett and Parish, 2000). Blood clearance studies indicate HRG in human plasma has a half-life of approximately three days thereby suggesting a rapid turnover of HRG in vivo (Lijnen et al., 1981).

1.4.2 HRG plasma levels in normal populations

1.4.2.1 Normal HRG levels

HRG plasma levels are found to be highly variable in the population fluctuating between 47-147% of a mean plasma concentration of ~100 µg/ml (~2 µM) for normal healthy adults (Corrigan et al., 1990; Morgan, 1978; Saito et al., 1982). HRG levels in children are lower than adults and are age dependent, with plasma HRG levels at birth being ~20% of adults, and plasma HRG levels gradually increasing with age (Table 1.3).

<table>
<thead>
<tr>
<th>Age of infant</th>
<th>Plasma HRG µg/ml (mean +/- SD)</th>
<th>% of normal (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonate (&lt;30 days old)</td>
<td>16 ± 5</td>
<td>20</td>
</tr>
<tr>
<td>1-3 months</td>
<td>29 ± 12</td>
<td>34</td>
</tr>
<tr>
<td>3-12 months</td>
<td>40 ± 11</td>
<td>46</td>
</tr>
<tr>
<td>1-2 years</td>
<td>49 ± 9</td>
<td>57</td>
</tr>
<tr>
<td>2-3 years</td>
<td>63 ± 9</td>
<td>73</td>
</tr>
<tr>
<td>Adults over 21 years</td>
<td>86 ± 19</td>
<td>100</td>
</tr>
</tbody>
</table>

Adapted from Corrigan et al. (1990)

Studies of HRG plasma levels in twins (Boomsma et al., 1993) indicate a simple additive genetic model of inheritance. Genetic factors explain 70% of variance in plasma HRG levels with a 3% age effect and a 27% effect from individual environmental factors (Hennis et al., 1995b).
Of the genetic influences, approximately 80% of variability is accounted for by the HRG phenotype. HRG form-1 homozygotes have HRG levels 50% higher, while HRG form-2 homozygotes have plasma HRG levels 50% lower than the mean. Heterozygotes have an intermediate level of the two isoforms and are defined as the 100% level of plasma HRG. The remaining 20% of genetic variability in plasma HRG levels has been attributed to residual genetic factors such as blood groups (Hennis et al., 1995b). The mean HRG level of blood-type AB (~125%) appears significantly greater than blood-type A (~103%) and O (~105%) (Drasin and Sahud, 1996).

HRG plasma levels usually increase linearly with increasing age. Levels are very low at birth (Table 1.3) and gradually increase until reaching mature levels at about 3-4 years of age (Corrigan, Jeter et al. 1990). As adults, mean HRG plasma levels continue to slowly rise with HRG plasma levels at age 60 being significantly greater than at age 40 (Drasin and Sahud, 1996).

1.4.2.2 Congenital HRG deficiency

Various cases of congenital HRG deficiency have been reported. In one family five members across three generations had low (20-35%) plasma HRG levels (Shigekiyo et al., 1993). Molecular analysis showed the cause to be a single nucleotide substitution; namely guanine (G) to adenine (A) at position 429 causing an amino acid change from Gly85 to Glu85 in the N1 domain. This uncommon substitution provides a conformational change in the mutant HRG protein resulting in impaired (~20%) secretion of HRG into the plasma (Shigekiyo et al., 1998). This family and others with congenital HRG deficiency (20-35% of normal) fail to show any abnormalities in routine laboratory assays of haemostatic function, immunologic function and plasma trace element levels, suggesting that a plasma HRG level 20% of normal is probably sufficient for physiological function (Shigekiyo et al., 1995). Other studies of families with congenital HRG deficiency describe some family members suffering from thrombosis, which has been attributed to the HRG deficiency, a conclusion that contradicts other studies which show that increased HRG levels are associated with thrombosis (Souto et al., 1996). Whether HRG plays a role in thrombosis by possibly regulating fibrinolysis is unclear (discussed in more detail in section 1.4.3.2).
1.4.2.3 HRG levels during pregnancy

During pregnancy most coagulation factors and activators show elevated plasma levels. HRG is one of the few plasma proteins reported to show a marked reduction (~50%) during the last trimester (Halbmayer et al., 1992). Levels are also low in the immediate period postpartum, although HRG plasma levels usually return to normal within five days of delivery (Haukkamaa et al., 1983; Omri et al., 1988). Plasma HRG levels are also found to be decreased to ~75% of normal levels in females using oestrogen based oral contraceptives (Jespersen and Kluf, 1982). Oestrogens reduce plasma HRG levels in a dose dependent manner with high doses reducing HRG by up to 30% of normal levels (Hennis et al., 1995a).

1.4.3 HRG plasma levels in clinical populations

Levels of plasma HRG are found to decrease during acute states of disease with HRG levels usually returning to normal upon recovery. It has been concluded that HRG acts as a negative acute phase reactant whereby plasma levels of HRG are markedly reduced during the acute non-specific response to tissue injury by either decreased synthesis or increased catabolism (Jespersen et al., 1984; Saigo et al., 1990). The reason why HRG levels decrease during periods of acute inflammation is yet to be determined.

1.4.3.1 Liver cirrhosis

Plasma HRG levels are usually reduced in patients with liver disease, although the severity of liver cirrhosis appears to directly modulate HRG levels. Interestingly patients with mild cirrhosis show small but significantly increased HRG levels. In contrast, patients with moderate and advanced cirrhosis show ~50% lower HRG levels compared to normal populations (Leebeek et al., 1989; Saito et al., 1982). Liver cirrhosis patients have a reduced capacity to synthesise proteins as is commonly illustrated by decreased levels of albumin and it is possible that HRG levels are low in liver cirrhosis patients simply due to impaired liver protein synthesis.

1.4.3.2 Thrombosis

Thrombosis occurs when fibrin clots form in areas of low blood flow in haemostatic vessels caused by either a decrease in the inhibition of coagulation or a delay in the
resolution of fibrin. Borza and Morgan (1998) suggest that stagnant blood flow may favour the development of local acidosis resulting in enhanced HRG binding to heparin-like substrates, subsequently neutralising the natural anticoagulant effect of heparin. In addition, the quantity of circulating plasminogen available for binding and dissolving fibrin may also be regulated by HRG. It is estimated that up to 50% of circulating plasminogen is bound to HRG, allegedly reducing the amount of free plasminogen circulating in blood available to dissolve fibrin clots (Lijnen et al., 1980). Thus an excess plasma level of HRG may induce a pro-thrombotic effect via neutralising the biological activity of heparin and the generation of plasmin from plasminogen. Indeed Jespersen et al. (1984) found that patients with deep vein thrombosis (DVT) could be identified by having a level of plasma HRG consistently higher than a group of patients without DVT. A patient with abnormally high HRG levels (~220% of normal) was found to suffer from retinal vein occlusion, which is most likely related to thrombosis occurring in retinal veins (Kuhli et al., 2003). Engesser et al. (1987) also observed a family with abnormally high HRG levels (~180%) which correlated with a positive family history of thrombosis. Studies also show that patients with thrombophilia have a 6-9% incidence of elevated HRG levels. From these trends, many suggest HRG functions as an anti-fibrinolytic agent, mediated through its binding to both natural heparin-like molecules and plasminogen, subsequently blocking their fibrinolytic effect and thus playing an important modulating role in the disease of thrombosis (Borza and Morgan, 1998).

As mentioned above, high levels of HRG have been claimed to contribute to the hypo-fibrinolytic state observed in patients suffering from thrombosis. Conversely, in vitro studies indicate the amount of plasmin generated by plasma from patients having abnormally high levels of HRG (up to 280%) is similar to control HRG (100%) levels, implying HRG does not affect binding and activation of plasminogen at the fibrin surface and providing evidence that excess plasma HRG is unrelated to the incidence of thrombosis (Angles-Cano et al., 1993). Thus, further investigations are required to elucidate whether increased HRG levels are a contributing factor to thrombosis. At present it is unclear whether the association between increased HRG levels and thrombosis is coincidental or causal (Hennis et al., 1995c; Hoffmann et al., 1993).
1.4.3.3 AIDS, immunosuppression and transplantation

Patients with AIDS have significantly lower plasma HRG levels (~50%) compared to healthy adults. Similarly, HRG levels are low in patients with:

a) end stage renal disease, after renal transplantation and immunosuppressive steroid therapy
b) asthma
c) chronic obstructive pulmonary disease (COPD) following treatment with steroids.

Other serum glycoproteins synthesised by the liver, such as hemopexin, are not low in these patients suggesting HRG levels are selectively decreased in all of the above mentioned patients treated with immunosuppressive steroids (Morgan, 1986).

Following bone marrow transplantation (BMT), graft versus host disease (GvHD) remains an unfortunate secondary clinical problem. HRG has been shown to inhibit both T-cell activation and production of inflammatory cytokines such as IFNγ, suggesting that HRG can mediate an anti-inflammatory effect. In patients with acute GvHD, HRG levels were significantly decreased (~ 50% pre treatment) three weeks after BMT (Mauz-Korholz et al., 1995). This fall in HRG plasma levels between week 2 and 3 post BMT has been suggested to be of useful diagnostic value as it can predict the occurrence of acute GvHD and thus allow early detection and treatment of the disease (Mauz-Korholz et al., 1995).

1.5 HRG ligands

HRG is known to bind a variety of ligands including heme and divalent cations such as Zn$^{2+}$, plasminogen, plasmin, fibrinogen, thrombospondin, IgG, FcγR, C1q, heparin and heparan sulphate. Table 1.4 lists the various HRG ligands, indicating the affinity and stoichiometry with which they bind HRG as well as the domain of HRG with which they interact with. The sections below will discuss in more detail each of the important classes of HRG ligands.
### Table 1.4 Ligands that bind HRG

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\sim K_d$</th>
<th>Stoichiometry</th>
<th>HRG domain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haem</td>
<td>1.5 µM</td>
<td>1:1 – 1:10</td>
<td>HRR</td>
<td>(Katagiri et al., 1987; Morgan, 1978)</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>1-4 µM</td>
<td>1:1 – 1:10</td>
<td>HRR</td>
<td>(Morgan, 1981)</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>0.5 – 1.1 µM</td>
<td>1:1</td>
<td>N-, C-</td>
<td>(Kluft and Los, 1988)</td>
</tr>
<tr>
<td></td>
<td>60 nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmin</td>
<td>0.9 µM</td>
<td>1:1</td>
<td>N-, C-</td>
<td>(Leung, 1986)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>6.7 nM</td>
<td>?</td>
<td>?</td>
<td>(Leung, 1986)</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>?</td>
<td>1:3</td>
<td>?</td>
<td>(Walz et al., 1987)</td>
</tr>
<tr>
<td>IgG</td>
<td>0.6 – 250 nM</td>
<td>?</td>
<td>N-</td>
<td>(Gorgani et al., 1999c)</td>
</tr>
<tr>
<td></td>
<td>(see Table 1.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FcγR</td>
<td>?</td>
<td>?</td>
<td>N-</td>
<td>(Chang et al., 1994)</td>
</tr>
<tr>
<td>C1q</td>
<td>7.8 nM</td>
<td>?</td>
<td>N-</td>
<td>(Gorgani et al., 1997)</td>
</tr>
<tr>
<td>Heparin and heparan</td>
<td>7 nM</td>
<td>1:1</td>
<td>N- HRR</td>
<td>(Lijnen et al., 1983d)</td>
</tr>
<tr>
<td>sulphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* soluble HRG  
*b* immobilised HRG

#### 1.5.1 Haem

Early studies revealed that HRG interacts with haem with a stoichiometry of $\sim 1:1$ and $K_d \sim 1.5$ µM (Morgan, 1978), although conformation changes within HRG allow a further 10 additional haem units to bind. Thus, the 'gly-his-his-pro-his' motif which in human HRG is repeated 12 times, is approximately equal to the number of haem binding sites, suggesting that each individual motif repeat represents a single histidine dependent heme binding unit (Katagiri et al., 1987). The haem–HRG interaction is highly dependent on pH, with maximum binding occurring between pH 6.5 and 7.0, precisely when histidine side chains become protonated, lending support to the view that the haem binding site is localised within the histidine-rich region (Borza et al.,...
1996; Katagiri et al., 1987). Despite HRG binding to haem, the physiological relevance of this interaction remains unclear.

1.5.2 \( \text{Zn}^{2+} \) and other divalent metal cations

\( \text{Zn}^{2+} \) is an important trace element within the immune system, essential for the function of more than 300 metalloenzymes (Wellinghausen et al., 1997). Imidazole side chains of histidine are known to be important for coordination with metal ions (Sundberg, 1974). HRG interacts with \( \text{Zn}^{2+} \) with a \( K_d \) between 1-4 \( \mu \text{M} \) and a stoichiometry \( \sim 1:1 \) - \( 1:10 \) (Morgan, 1978). Studies indicate the HRR of HRG is most likely the domain responsible for \( \text{Zn}^{2+} \) binding due to its high level of histidine residues, with two histidine residues being required to form a metal binding unit (Figure 1.5). The interaction between HRG and \( \text{Zn}^{2+} \) is abolished when histidine residues are chemically removed from HRG, supporting the model that histidine residues are essential for \( \text{Zn}^{2+} \) binding (Morgan, 1981).

Furthermore, the HRR containing fragment released following plasmin digestion of rabbit HRG retains its ability to bind \( \text{Zn}^{2+} \) ions in a pH dependant manner, providing additional evidence that \( \text{Zn}^{2+} \) binding sites of HRG are located within the HRR. Furthermore, it appears that haem and metal ions compete for the same histidine binding sites within the HRR (Morgan, 1978). A conformational change occurs in the HRR of HRG following metal binding or when the pH falls below 6, this conformational change being transmitted to other parts of the HRG molecule via disulphide bonds. Thus, HRG can undergo a conformational change under specific inducible conditions, such as \( \text{Zn}^{2+} \) binding or low pH, providing a regulatory mechanism for the function of HRG (Borza and Morgan, 1998).

In fact, HRG interacts \textit{in vitro} with a variety of divalent metal cations including; copper, mercury, cadmium, nickel and cobalt, with an affinity of \( \sim 1 \mu \text{M} \). HRG, however, was found to not interact with magnesium, manganese or lead (Morgan, 1981). HRG binds metal ions in the presence of other metal binding serum proteins such as albumin and transferrin, leading Morgan (1981) to postulate that HRG may play a role in the transport or homeostasis of metals \textit{in vivo} (Guthans and Morgan, 1982). Studies by Failla et al. (1982), however, provide data which conflict with the hypothesis that HRG acts as a physiologically important metal ion transporter. Both in normal human plasma and plasma from a family with elevated \( \text{Zn}^{2+} \) levels, the majority of \( \text{Zn}^{2+} \)
ions were found to remain bound to albumin and not to HRG. These studies suggest that although HRG binds to metals in vivo, HRG is unlikely to play a major role in the transport of divalent metal ions. Other possible functions relating to the HRG-Zn\(^{2+}\) interaction are discussed below.

Possibly one of the more physiologically important functions involving the HRG-Zn\(^{2+}\) interaction is the modulation of HRG binding to glycosaminoglycans (GAGs). Briefly, HRG binding to cell surface GAGs is potentiated by the presence of Zn\(^{2+}\) (discussed in more detail in section 1.5.9). Horne et al. (2001) showed that HRG interacts with Zn\(^{2+}\) with only a moderate affinity (K\(_d\) ~ 4 µM). Given this moderate affinity and the relatively stable but low plasma concentrations of Zn\(^{2+}\), they suggested that only ~ 1% of HRG binding sites for Zn\(^{2+}\) are occupied in normal plasma. However, Zn\(^{2+}\) concentrations are known to substantially increase in the vicinity of thrombin-activated platelets, where approximately 40% of platelet Zn\(^{2+}\) is secreted during thrombin activation, resulting in a 30-60 fold increase in the local Zn\(^{2+}\) concentration (Aktulga, 1974; Gorodetsky et al., 1993). Thus, in the milieu of activated platelets, HRG becomes exposed to high local concentrations of Zn\(^{2+}\) resulting in high affinity binding to cell surface GAGs (Horne et al., 2001). It seems unlikely that Zn\(^{2+}\) alone regulates the HRG–GAG interaction, but more likely acts synergistically with local acidosis to promote cell surface binding. Zn\(^{2+}\) shift the pH dependence of HRG–GAG binding towards physiological pH values so that smaller decreases in local pH are required to achieve equivalent binding in the presence of Zn\(^{2+}\) (Borza and Morgan, 1998).

There are numerous conflicting studies regarding the importance or not of Zn\(^{2+}\) in enhancing the binding of HRG to both heparin and heparan sulphate (Burch et al., 1987; Kazama and Koide, 1992; Lane et al., 1986; Lijnen and Collen, 1983). Similarly, there are variable reports on the effect or not of Zn\(^{2+}\) on the binding of HRG to the surface of cells, peripheral T cells in particular (Saigo et al., 1989), as well as components of the complement cascade (Chang et al., 1992a). A possible explanation for so many conflicting reports on the regulatory role of Zn\(^{2+}\) may arise from differing experimental conditions, specifically, the availability of free Zn\(^{2+}\) in buffer systems. Bovine serum albumin (BSA) is commonly used in buffer systems and is known to sequester free Zn\(^{2+}\). Subtle differences between serum concentrations in buffers, as well as other factors such as quality of deionised water, could all contribute to the resulting
different concentrations of free $\text{Zn}^{2+}$, and possibly accounting for some of the conflicting experimental results.

It is well established that histidine forms stable complexes with transition metal ions. Taking advantage of this property the IMAC (immobilized metal ion-affinity chromatography) purification system was developed. In particular, nickel complexed to nitrilotriacetic acid (NTA) is an improved chelation agent that interacts with proteins containing exposed histidine residues, such as hexa-histidine tagged recombinant proteins, with high affinity (Mori et al., 2003b). Native rabbit HRG contains 53 histidine residues, with 34 located within the HRR. Thus, due to this high histidine content, Mori et al. (2003b) postulated that rabbit HRG should directly bind Ni-NTA without requiring the additional hexa-histidine affinity tag and subsequently developed a novel quantification method for rabbit HRG utilising this high affinity interaction between HRG and Ni-NTA. Indeed Ni-NTA appears a suitable probe for both quantification and purification of rabbit HRG, with Borza and Morgan (1997; 1998) already having used Ni-NTA to purify rabbit HRG for some years.

1.5.3 Plasminogen

Plasminogen plays a key role in the fibrinolytic system and is also one of the numerous HRG ligands. A brief overview of the fibrinolytic system is given below (Figure 1.10) while a more detailed study of plasminogen and its interaction with HRG is described in Chapter 4 of this thesis.

Vascular integrity is sustained by a complex system of circulating and cell-associated haemostatic factors that control local platelet deposition, the conversion of soluble fibrinogen to an insoluble fibrin polymer and ultimately the dissolution of fibrin matrices – a process known as fibrinolysis (Degen, 2001). Plasminogen activation is the key event in the fibrinolytic system resulting in the dissolution of blood clots (Parry et al., 2000). The fibrinolytic system is comprised of an inactive proenzyme, plasminogen, that can be converted to the active enzyme plasmin, which in turn degrades fibrin into soluble fibrin degradation products, schematically represented in Figure 1.10 (Lijnen, 2001). Two distinct physiologic plasminogen activators (PA) have been identified; tissue-type PA (t-PA) and urokinase-type PA (u-PA). Inhibition of the fibrinolytic
system occurs at both the activation level by plasminogen activator inhibitors (PAI-1 and PAI-2) and also at the plasmin level primarily by $\alpha_2$-antiplasmin inhibitor (Dobrovolsky and Titaeva, 2002). The physiological importance of the fibrinolytic system is demonstrated by an association between abnormal fibrinolysis resulting in a tendency towards bleeding or thrombosis. Thus, to maintain homeostasis a coordinated balance between coagulation, whereby the fibrin clot is formed, and fibrinolysis, whereby the fibrin clot is dissolved, must be achieved.

Plasminogen is able to provide degradative potential and thus, also plays a key role in a variety of other activation cascades such as the activation of metalloproteinases. Indeed, plasmin has been implicated in wound healing, tissue remodelling, angiogenesis, embryogenesis, pathogen and tumour cell invasion, and tumour metastasis (Parry et al., 2000). Interestingly both eukaryotic cancer cells and prokaryotic pathogenic microorganisms recruit plasmin' proteolytic activity to their cell surface to facilitate cell invasion and migration through tissue layers (Parry et al., 2000). HRG was first found to interact with plasminogen during studies which attempted to purify one of the plasminogen inhibitors, $\alpha_2$-antiplasmin, from plasma (Lijnen et al., 1980). Subsequently, Saez et al. (1995) provided the first direct evidence for an association between HRG and plasminogen by in vitro interaction studies. It should be noted that in general, proteins and peptides (even those with C-terminal lysine residues) do not exhibit binding to plasminogen (Winn et al., 1980).

Plasminogen is composed of a heavy and light chain, the heavy chain consisting of the pre-activation peptide and five triple loop structures termed 'kringles' which contain lysine binding sites (LBS) that specifically bind certain amino acids with antifibrinolytic properties such as lysine and 6-aminohexanoic acid (Miyashita et al., 1988; Rickli and Otavsky, 1975). LBS are involved in the interaction of plasminogen with fibrin (Landmann, 1973; Thorsen, 1975; Wiman and Wallen, 1977) and play a crucial role in the regulation of fibrinolysis by binding lysine residues on fibrin, $\alpha_2$-antiplasmin and cell surfaces during the physiological lysis of fibrin (Wiman and Collen, 1978). Plasminogen contains one high affinity LBS ($K_d \approx 9 \mu M$) and four to five sites with lower affinity ($K_d \approx 5 \text{mM}$) (Markus et al., 1978). Antifibrinolytic amino acids (such as lysine) also have the property of inducing a marked conformational change in the plasminogen molecule resulting in an enhanced activation rate by u-PA.
Figure 1.10. Overview of the fibrinolysis system. Plasmin is a serine protease that plays a central role in the dissolution of blood clots (fibrinolysis), in tissue remodelling and pericellular proteolysis either directly or indirectly via activation of growth factors and other proteinase cascades. Plasmin itself stimulates its own production in an important feedback mechanism. Plasminogen (shown in blue) binds to a cell surface via a plasminogen receptor (PgR) (shown in orange) and is activated by one of the specific plasminogen activators (t-PA or u-PA) to form the active serine proteinase plasmin. The degradative potential of plasmin is capable of binding and degrading fibrin clots, degrading the ECM and performing pericellular proteolysis. Plasmin plays an indirect role in the fibrinolytic system by activating growth factors such as TGFβ and mobilising metalloproteinases. Plasmin is rapidly inactivated by α2-antiplasmin and α2-macroglobulin when it is no longer surface associated. Plasminogen activator inhibitors (PAI-1 and PAI-2) can inactivate plasmin production at the plasminogen activator level. Abbreviations used: α2-AP: α2-antiplasmin, α2-M: α2-macroglobulin, ECM: extracellular matrix, PAI-1: plasminogen activator inhibitor type 1, PgR: plasminogen receptor, Pro-MMP: pro-matrix metalloproteinases, Pro-uPA: pro-chain urokinase plasminogen activator, t-PA: tissue type plasminogen activator, TGFβ: transforming growth factor β, TIMPs: tissue inhibitors of metalloproteinases, u-PAR: urokinase receptor.
Physiological concentrations of HRG (~1.8 μM) result in significantly reduced plasminogen binding to fibrin and α₂-antiplasmin (Lijnen et al., 1980), suggesting HRG probably interacts with LBSs on plasminogen via HRG’s numerous lysine residues. In addition, free lysine has been shown to inhibit the interaction between HRG and plasminogen, while modification of lysine residues on HRG also causes inhibition of the HRG-plasminogen interaction (Saez et al., 1995). Extensive modification of arginine or histidine residues on HRG does not inhibit plasminogen binding, leading Saez et al. (1995) to conclude that one or more lysine residues of HRG are involved in the association between HRG and plasminogen. In this regard, HRG has several lysine residues (Lys15, Lys22, Lys66, Lys121, Lys138, Lys142, Lys148, Lys257, Lys427, Lys507 in the human protein) that are conserved in human, rabbit and bovine HRG proteins. The plasminogen binding site within HRG is likely to involve the N- and C-terminal domains, since immobilised N- and C-terminal domains (but not the HRR domain) can bind plasminogen and stimulate activation (Borza and Morgan, 1997). A disulphide bridge connects the N- and C-terminal domains of the protein implying that both domains in vivo may participate in binding plasminogen (Saez et al., 1995). In particular, lysine residues in the C-terminal domain of HRG are found to be essential for plasminogen binding (Borza and Morgan, 1997; Saez et al., 1995).

HRG interacts with plasmin by an apparently single association-dissociation constant of ~0.9 μM and with plasminogen with a Kₐ of ~0.5 - 1.1 μM (Kluft and Los, 1988; Lijnen et al., 1980) making HRG one of the most important plasminogen ligands in plasma, where it has been estimated that ~50% of plasminogen circulates bound to HRG. HRG has been shown to bind reversibly to plasminogen in a uniform manner (in respect to complex formation) by Kluft and Los (1988) which contrasts with Jacobsson (1983), who showed plasma HRG did not uniformly bind to the kringle I domain isolated from plasminogen. Plasminogen appears to bind HRG independently of heparin indicating that the plasminogen binding sites on HRG are probably distinct from the
heparin binding sites (Saez et al., 1995). Numerous studies have confirmed an ~ 1:1 stoichiometry of binding between HRG and plasminogen (Saez et al., 1995). By binding to plasminogen, HRG is thought to reduce the concentration of free circulating plasminogen, thus inhibiting plasminogen binding to fibrin and resulting in retarded fibrinolysis. Studies by Horne et al. (2000), however, could not detect an effect of HRG on the rate of fibrin-dependent plasmin production and concluded that HRG is not mechanistically analogous to the antifibrinolytic amino acids in its impact on fibrinolysis. Indeed, several studies (Borza and Morgan, 1997; Silverstein et al., 1985b) found that immobilised HRG (as opposed to soluble HRG) has a much higher affinity for plasminogen ($K_d \sim 60$ nM) resulting in an up to ~ 100-fold enhancement in plasminogen activation by t-PA, suggesting that HRG has a pro-fibrinolytic effect. Not only was plasminogen activation enhanced compared to the fluid phase, but over 70% of the plasmin formed remained surface associated where it was protected from inhibition by $\alpha_2$-antiplasmin (Silverstein et al., 1985b). These findings are consistent with a physiological model where HRG has a higher affinity for plasminogen when HRG is immobilised on cells via interaction with cell surface heparan sulphate. Evidence suggests that HRG plays a physiological role in regulating fibrinolysis and proteolytic events via plasminogen binding and activation in non-fibrin containing microenvironments, although further work is required to validate this phenomenon and determine its functional relevance.

Cell surface binding sites for components of the fibrinolytic system such as plasminogen, provide a mechanism for local regulation of fibrinolysis by promoting enhanced plasminogen activation (Miles and Plow, 1985), by protecting cell-bound plasmin from $\alpha_2$-antiplasmin inhibition (Plow et al., 1986), and by localising plasmin activity (Miles and Plow, 1985; Plow et al., 1986). No high affinity cell surface receptors for plasminogen are known to exist, although low affinity receptors ($K_d \sim 1 \mu$M) are widely distributed on many circulating peripheral blood cells such as endothelial cells, monocytes, macrophages and some tumour cells (Brownstein et al., 2001; Miles and Plow, 1987) as well as some cultured cells (Plow et al., 1986). Candidate low affinity plasminogen binding sites on cells include gangliosides (cell membrane glycolipids) (Miles et al., 1989), $\alpha$-enolase (Miles et al., 1991), annexin II (a peripheral membrane protein) and the cell surface platelet integrin GPIIb-IIIa. Vassalli et al., (1991) provided evidence that cell surface proteins with carboxyl-terminal lysine
residues can act as plasminogen binding sites. Plasminogen cell surface receptors are thus loosely defined and represent general cell surface proteins that bind plasminogen with low affinity.

1.5.4 Fibrinogen

HRG specifically interacts with fibrinogen with high affinity, with an apparent $K_d$ of ~ 6.7 nM (Leung, 1986). Competitive inhibition studies using soluble fibrinogen suggest native fibrinogen has a high affinity for HRG in the fluid phase (Leung, 1986). While the stoichiometry of the HRG fibrinogen interaction remains undefined, data suggests a significant proportion of plasma HRG may bind fibrinogen and circulate as HRG-fibrinogen complexes. It should be noted the molar ratio of fibrinogen to HRG is ~ 10:1 in plasma and therefore it remains likely that most plasma fibrinogen molecules are not complexed to HRG. The effect of other plasma proteins such as plasminogen (which interacts with both HRG and fibrinogen) have on the HRG–fibrinogen complex remains to be determined (Lijnen et al., 1980; Lucas et al., 1983).

HRG specifically binds to and is incorporated into fibrin clots with an apparent $K_d$ of ~ 0.25 μM. This incorporation into plasma clots results in a significant decrease in serum HRG levels as compared with plasma levels (Leung, 1986). While various assay systems were employed to show HRG binding to fibrinogen (ELISA and rocket electrophoresis) and fibrin (direct incorporation of radiolabelled HRG into fibrin clots) these data suggest the affinity of HRG for fibrin may be lower than that for fibrinogen (Leung, 1986). Using radiolabelled proteins, it was demonstrated that the incorporation of HRG into clots had no effect on the extent of conversion of fibrinogen to fibrin in clots. Interestingly, the incorporation of HRG into fibrin clots had a significant effect on the final structure of the fibrin gel. Based on the final opacity density of the fibrin gel, which indicates the average mass:length ratio of fibrin fibres within the gel (Carr and Hermans, 1978), it was found that fibrin was distributed over more, but thinner fibrils in the presence of HRG. The effect of HRG on gel structure was concentration-dependent and was observed when fibrinogen and HRG were present at molar concentrations of 11:1 (which is similar to their molar ratio in plasma) supporting physiological relevance. The biological significance of HRG interacting with fibrinogen and fibrin may not be limited to the fibrin polymerisation process. Fibrin has been shown to form...
on the surface of thrombin-activated platelets (Leung et al., 1983), and has also been detected on the surface of macrophages (Morgan, 1985), thus it is possible that the interaction of HRG with fibrinogen may extend to a role in cell-cell interactions involving platelets and macrophages (Leung, 1986).

1.5.5 Thrombospondin

Thrombospondin is a 450 kDa filamentous multifunctional α-granule ‘adhesive’ glycoprotein of human platelets that binds to fibrinogen, fibronectin, heparin and HRG (Baenziger et al., 1971; Lawler et al., 1978). Adhesive proteins are known to be important mediators of cell-cell and cell-matrix interactions (Walz et al., 1987) and thrombospondin is thought to play a role in regulating thrombotic events at vessel surfaces.

Thrombospondin forms a high affinity complex with HRG (Walz et al., 1987) and plasminogen (Kₐ ~ 35 nM) both via LBS (Silverstein et al., 1984; Walz et al., 1987), and interestingly the HRG-thrombospondin complex is still able to bind plasminogen and heparin. Walz et al. (1987) first suggested HRG, plasminogen and thrombospondin form a tri-molecular complex on the cell surface (Figure 1.11). This was confirmed by Silverstein and Nachman (1987) who showed that the tri-molecular complex formed by HRG, thrombospondin and plasminogen is able to bind cell surface heparan sulphate and serve as a substrate for plasminogen activation to plasmin (Silverstein et al., 1985a). In binding assays, neither of the ligands inhibited complex formation of the other with thrombospondin, strongly suggesting that thrombospondin contains independent binding sites for plasminogen and HRG (Silverstein et al., 1985a). Although studies did not permit accurate measurement of stoichiometric relations, sucrose density ultracentrifugation studies showed ~ 3 HRG and ~ 3 plasminogen molecules per molecule of thrombospondin (Leung et al., 1984). Although plasma thrombospondin circulates at very low levels, significant concentrations are present in the microenvironment of plasminogen activation (Silverstein et al., 1984).

The physiological significance of a surface associated plasminogen–HRG–thrombospondin complex is complicated, although appears to allow efficient activation of plasminogen in microenvironments that do not contain fibrin. In view of
the broad distribution of thrombospondin in different cell systems including endothelial cells (Mosher et al., 1982), smooth muscle cells (McPherson et al., 1981; Raugi et al., 1982), fibroblasts and monocytes (Jaffe et al., 1985), as well as the ECM (Jaffe et al., 1983), it seems reasonable to propose that the trimolecular complex could assemble in focal areas independent of the presence of fibrin. At sites of inflammation, tumour cell migration and implantation synthesis and secretion of plasminogen activators is known to be increased (Wilson and Dowdle, 1978). Thus, plasminogen immobilised on thrombospondin in association with HRG may serve to generate plasmin in a fibrin free environment in a kinetically enhanced manner, thus possibly playing a role in physiological and pathological events such as ovulation, implantation, inflammation, and tumour cell invasion (Silverstein et al., 1984).

Figure 1.11. Trimolecular complex formed between HRG, plasminogen and thrombospondin on the cell surface. Model depicting the multimolecular surface assembly of a thrombospondin, plasminogen and HRG complex on the cell surface. Thrombospondin is represented in green, HRG represented in blue and plasminogen represented in red. The model shows independent binding sites on thrombospondin for HRG and plasminogen along with a secondary plasminogen – HRG interaction. Such complex formation may modulate plasminogen activation as well as heparin and HRG function. Adapted from Figure 8, Silverstein and Nachman (1987).
1.5.6 IgG

Immunoglobulin (Ig) molecules consist of two identical heavy (H)- and light (L)-chains with the variable regions of the H-chains and L-chains associating to form the antigen-binding site of the antibody. Nine types of H-chains exist in humans (\(\mu, \delta, \gamma_1, \gamma_2, \gamma_3, \gamma_4, \alpha_1, \alpha_2\) and \(\varepsilon\)) which define a range of Ig classes and subclasses, with the functional role of almost all H-chains being well characterised. In many species two types of L-chains termed \(\kappa\) and \(\lambda\) exist, differing remarkably in amino acid homology and, unlike H-chains, their function remains unknown. Gorgani et al. (1999c) showed that HRG binds IgG with a different affinity for each of the various IgG subclasses and interestingly, has differential binding affinity for the two L-chain subclasses.

HRG binds human IgG with high affinity (\(K_d \sim 85\) nM), as determined using surface plasmon resonance (Gorgani et al., 1997). The HRG-IgG interaction is potentiated up to 5-fold in the presence of Zn\(^{2+}\) ions, whereas the binding affinity is reduced in the presence of EDTA, probably due to EDTA chelating trace metal ions present in the buffer (Gorgani et al., 1997). HRG shows the highest affinity for human IgG1\(\kappa\) and IgG2\(\kappa\), followed by IgG3\(\kappa\) and then the L-chain containing \(\lambda\) subclasses (Table 1.5), whereas HRG has a much lower affinity for IgM.

Table 1.5 Binding affinity of HRG for various IgG and IgM subtypes.

<table>
<thead>
<tr>
<th>HRG binding to immobilised Ig</th>
<th>~ (K_d) nM</th>
<th>~ (K_d) nM (+20 (\mu M) Zn(^{2+}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1(\kappa)</td>
<td>3</td>
<td>0.6</td>
</tr>
<tr>
<td>IgG2(\kappa)</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>IgG3(\kappa)</td>
<td>148</td>
<td>92</td>
</tr>
<tr>
<td>IgG4(\kappa)</td>
<td>268</td>
<td>167</td>
</tr>
<tr>
<td>IgM(\kappa)</td>
<td>1990</td>
<td>7754</td>
</tr>
<tr>
<td>IgG1(\lambda)</td>
<td>189</td>
<td>266</td>
</tr>
<tr>
<td>IgG2(\lambda)</td>
<td>112</td>
<td>150</td>
</tr>
<tr>
<td>IgG3(\lambda)</td>
<td>109</td>
<td>113</td>
</tr>
<tr>
<td>IgG4(\lambda)</td>
<td>96</td>
<td>103</td>
</tr>
</tbody>
</table>

Adapted from Table 1, Gorgani et al. (1999c)

* \(\kappa\) indicates that IgG subclass contains \(\kappa\) L-chains

* \(\lambda\) indicates that IgG subclass contains \(\lambda\) L-chains
In fact, κ L-chain containing forms of IgG1 and IgG2 have about a 10-fold greater affinity for HRG than λ L-chain containing forms, the differential affinity of HRG for κ and λ L-chains providing the first evidence for a functional difference between these two L-chains. The HRG-IgG interaction is thought to occur predominantly via the F_{(ab)} region of IgG, while it is also suggested that the IgG binding site of HRG is located within the N-terminal domain. Furthermore, the presence of 20 µM Zn^{2+} greatly potentiates some HRG-IgG interactions (Table 1.5) (Gargani et al., 1997).

1.5.7  \textit{Fc}γ receptors

Fcγ receptors (FcγR) play a key role in host defence mechanisms by providing a link between the humoral immune response and cell mediated effector systems. FcγR bind the Fc domain of IgG molecules providing an interaction between cells that express FcγR on their surface and opsonized antigen in IgG containing immune complexes (IC). Granulocytes, monocytes, macrophages and NK cells express FcγR on their plasma membranes. In humans, three classes of FcγR exist that bind cytophilic IgG (IgG1 and IgG3), namely FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (Figure 1.12).

FcγRI is a high affinity receptor capable of binding monomeric IgG1, IgG3 and IgG4, but not IgG2. FcγRII and FcγRIII are low affinity receptors interacting with only IgG in complexed or aggregated form. Human FcγRII is expressed by a variety of cells such as granulocytes, monocytes/macrophages, platelets, B cells, endothelial cells of the placenta and some T cell subsets. The FcγRIIa subset is expressed on neutrophils and monocytes/macrophages and initiates phagocytosis, ADCC (antibody dependent cellular cytotoxicity) and cellular activation. Studies provide evidence that a structural and functional polymorphism at position 131 of this receptor leads to a point mutation resulting in a switch from an arginine to a histidine residue in the proximal Ig-binding domain that greatly affects receptor affinity and specificity (Warmerdam et al., 1991). FcγRIIa-his131 exhibits an affinity for IgG2 not seen with the FcγRIIa-arg131 resulting in possible consequential functional differences. These polymorphisms could become important during FcγR-IgG IC clearance. Relatively inefficient IC clearance may induce subsequent inflammation as observed in a number of autoimmune diseases and may hamper the containment of bacterial infections (Van Sorge et al., 2003). This concept may be especially important in cases where IgG2 represents the dominant
Table 1.6. Leukocyte FcγR subclass distribution, signalling properties, receptor heterogeneity and ligand specificity.

<table>
<thead>
<tr>
<th>Receptor Class</th>
<th>FcγRI</th>
<th>FcγRIla</th>
<th>FcγRIIa</th>
<th>FcγRIIIa</th>
<th>FcγRIIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution</td>
<td>Myeloid cells</td>
<td>Myeloid cells, neutrophils</td>
<td>B-cells, monocytes, macrophages</td>
<td>Macrophages, monocytes, NK cells</td>
<td>Neutrophils, eosinophils</td>
</tr>
<tr>
<td>Signalling motif</td>
<td>ITAM</td>
<td>ITIM</td>
<td>ITAM</td>
<td>ND</td>
<td>CR3, FcγRIIα</td>
</tr>
<tr>
<td>Accessory signalling subunit</td>
<td>γ</td>
<td>γ</td>
<td>ND</td>
<td>β, γ, ζ</td>
<td></td>
</tr>
<tr>
<td>Allotype</td>
<td>R131/H131</td>
<td>V158/F158</td>
<td>NA1/NA2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human IgG subclass specificity</td>
<td>R131:3=3&gt;&gt;&gt;2,4</td>
<td>R131:3=1&gt;&gt;&gt;2,4</td>
<td>H131:3,1=3&gt;&gt;&gt;4</td>
<td>1=3&gt;&gt;&gt;2,4a</td>
<td>1=3&gt;&gt;&gt;2,4b</td>
</tr>
</tbody>
</table>

Adapted from Table 2 (Van Sorge et al., 2003)

ND = not determined

* FcγRIIA-V158 has a higher affinity for hlgG1 and 3 than FcγRIII-F158, and binds hlgG4

* FcγRIIB-NA1 has a higher affinity for hlgG3 than FcγRIIB-NA2, and hlgG1- or hlgG3-opsonised particles are more efficiently ingested upon interaction with former allotype

Figure 1.12. Structural diversity and heterogeneity of human Fcγ receptors (FcγR). There are three classes of FcγR in humans that bind IgG with all receptors belonging to the immunoglobulin (Ig) superfamily, i.e., FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). The extracellular region is composed of disulphide linked (S-S) domains. FcγRI, FcγRIIA and FcγRIIB are all activating receptors characterised by the presence of an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain of the receptor (FcγRIla) or associated with the receptor as an accessory signalling subunit (γ and/or ζ chains associated with FcγRI and FcγRIIIa). FcγRIIB is an inhibitory receptor containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. FcγRIIB is linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Functional polymorphisms in FcγRII and FcγRIIIa are indicated by black circles. Adapted from Figure 1, Dijstelbloem et al. (2001).
subclass. IgG2 is the most important subclass elicited by encapsulated bacteria in humans whilst autoantibodies directed against Clq and phospholipids are also usually comprised of the IgG2 subclass. Alternatively, an overly efficient FcγR-IgG interaction may trigger detrimental inflammatory responses.

Based on inhibition studies, HRG has indirectly been shown to bind to FcγR (Chang et al., 1994; Gorgani et al., 1999b). Chang et al. (1994) demonstrated that HRG can bind murine inflammatory peritoneal macrophages (which express FcγR), binding which is not inhibited by heparin, implying HRG binds a receptor on macrophages independent of heparan sulphates, with the N-terminal region of HRG being found to modulate the interaction. Gorgani et al. (1999b) also suggested that HRG may interact with human FcγRI, with pre-treatment of the monocytic cell line, THP-1 (which express human FcγRI) with human IgG1 (the native ligand for FcγRI) inhibiting HRG binding by up to 35%. Similarly, pre-treatment of THP-1 cells with HRG strongly inhibited subsequent binding of IgG, presumably by HRG blocking FcγRI sites normally available to bind IgG (Gorgani et al., 1999b). Zn^{2+} appeared not to affect the inhibitory effect of HRG on IgG binding. Additionally, IgG did not interfere with the binding of HRG to the T-lymphocyte cell lines Jurkat and MT4, which lack FcγRI. These data suggest that HRG binds FcγRI on the surface of cells.

1.5.8 Complement

HRG was first discovered by Heimburger et al. (1972) when it co-purified with the first component of complement (Clq), although they did not provide further evidence that the two proteins could interact. Gorgani et al. (1997) showed that HRG interacts with Clq, via two distinct high affinity binding sites (K_d ~ 7.8 nM, and 37 nM), and located the Clq binding sites within the N-terminal fragment of HRG. Interestingly the addition of Zn^{2+} markedly inhibited the reaction, suggesting Zn^{2+} potentiates HRG binding to some ligands such as IgG while inhibiting HRG binding to other ligands such as Clq. The inhibitory effect of Zn^{2+} may be due to an induced conformational change in the HRG molecule resulting in a loss of Clq binding sites. The fluid phase complement membrane attack complex (MAC) is composed of complement components C5b, C6, C7, C8, C9 and serum S-protein. Chang et al., (1992a) showed that immobilised HRG binds many of the MAC components including C8, C9, factor D, and S-protein.
suggesting that HRG may play a role in regulating the formation of the MAC in the fluid phase and may be capable of modulating complement functional efficiency. However, the molecular basis of these interactions is unclear and the functional significance is unknown.

1.5.9  **Heparin and heparan sulphate**

1.5.9.1 Heparan sulphate

A glycosaminoglycan is a linear heteropolysaccharide possessing a characteristic disaccharide repeat sequence. One monosaccharide of the disaccharide repeat is the amino sugar D-glucosamine or D-galactosamine, whereas the other unit is typically, although not always, a uronic acid residue of either D-glucuronic acid or D-iduronic acid. Both units are variably N- and O-sulphated, both of which add heterogeneity to these complex macromolecules. The most common GAG structures are heparan sulphate, chondroitin sulphate A and C, dermatan sulphate (chondroitin sulphate B), keratan sulphate, hyaluronic acid and heparin. The heparan sulphate structure is shown in Figure 1.13. Heparan sulphate is structurally related to heparin, although it is much less sulphated than heparin as well as having a more varied structure (Capila and Linhardt, 2002). Most GAG chains are covalently attached at their reducing end through an O-glycosidic linkage to a serine residue or N-linked to asparagine in a core protein, resulting in a macromolecule termed a proteoglycan (Jackson *et al.*, 1991).

Heparan sulphate proteoglycans are ubiquitously expressed on animal cell surfaces and are generally more abundant on the cell surface than most receptors (Bernfield *et al.*, 1999). A major function of cell surface proteoglycans involves regulation of cell adhesion and migration, dynamic processes that are mediated through interactions between the GAG chain and ECM components such as laminin, collagen and fibronectin. Proteoglycans also occur as integral components of basement membranes in most mammalian tissues (Jackson *et al.*, 1991). Cell surface proteoglycans on endothelial cells play a crucial role in the maintenance of non-thrombotic blood vessels.

Proteoglycans and GAGs play a critical role in the pathophysiology of basement membrane related diseases including diabetes, atherosclerosis, and tumour metastasis. In addition, cell specific growth factors and enzymes that are immobilised in the ECM
and at the cell surface are bound to GAGs. As such, GAGs localise proteins and enzymes at their site of action and can regulate protein and gene expression (Jackson et al., 1991).

1.5.9.2 Heparin

The pharmaceutical product heparin is derived from the heparan sulphate proteoglycan within mast cell granules (Bernfield et al., 1999). Heparin is a highly sulphated polysaccharide that has numerous important biological activities. Heparin is widely used as an anticoagulant drug based on its ability to inhibit serine proteases in the blood coagulation cascade (Capila and Linhardt, 2002). Specifically, heparin acts as an anticoagulant by binding the proteinase inhibitor antithrombin III thereby inducing a conformational change in antithrombin III that facilitates inactivation of coagulation enzymes (Bjork and Lindahl, 1982).

Figure 1.13. Structure of heparin and heparan sulphate glycosaminoglycans. Heparin is a linear, unbranched, highly sulphated polymer consisting of repeating units of 1→4-linked pyranosyluronic acid and 2-amino-2-deoxyglucopyranose (glucosamine) residues. The uronic acid residues typically consist of 90% L-idopyranosyluronic acid (L-iduronic acid) and 10% D-glucopyranosyluronic acid (D-glucoronic acid). Heparin has the highest negative charge density of any known biological macromolecule due to the high content of negatively charged sulpho and carboxyl groups. Heparin exists primarily as a helical structure and unlike proteins, is not known to display or fold into any particular tertiary structure. During heparin biosynthesis, heparin chains are attached to a unique core protein, serglycin, found only in mast cells and some haematopoietic cells. Tissue proteases act on this core protein to release the peptidoglycan heparin, which is short lived and immediately processed by β-endoglucuronidase to smaller ~15 kDa polysaccharide chains called heparin. Heparan sulphate is structurally related to heparin but is much less substituted with sulpho groups than heparin and has a more varied structure. Like heparin, heparan sulphate is a repeating linear copolymer of uronic acid 1→4-linked to glucosamine. While D-glucuronic acid predominates in heparan sulphate, it can contain substantial amounts of L-iduronic acid. Heparan sulphates generally contain approximately one sulpho group per disaccharide compared to 2.7 sulpho groups per disaccharide in heparin. Heparan sulphate chains are polydisperse but are generally longer than heparin chains, having an average molecular weight of about 30 kDa, which ranges from 5-50 kDa. Heparan sulphate is biosynthesised as a proteoglycan through the same pathway as heparin, however the heparan sulphate GAG chain remains connected to its core protein. Heparan sulphate is ubiquitously distributed on cell surfaces and is also a common component of the ECM. Two types of core proteins, the syndecans (an integral membrane protein) and the glypicans (a GPI-anchored protein) commonly carry heparan sulphate GAG chains and correspond to the two major families of heparan sulphate proteoglycans.
Heparin

![Heparin molecule diagram](image)

**major sequence**

![Heparin molecule diagram](image)

**variable sequence**

$X = H$ or $SO_3$

$Y = Ac, SO_3$ or $H$

**Helical conformation of a heparin dodecasaccharide sequence having the major disaccharide repeating structure shown above with sulphur atoms shown in yellow, oxygen atoms shown in red, nitrogen atoms shown in blue and hydrogen atoms in cyan.**

Heparan sulphate

![Heparan sulphate molecule diagram](image)

**major sequence**

![Heparan sulphate molecule diagram](image)

**variable sequence**

SYNDECANS - transmembrane

GLYPICANS - GPI linked
Heparin cofactor II is another plasma protein that inhibits thrombin rapidly in the presence of either heparin or dermatan sulphate and is similar to antithrombin III in structure and mechanism of action (Tollefsen and Pestka, 1985). Antithrombin III inhibits all of the serine proteases of the intrinsic coagulation system whereas heparin cofactor II specificity is restricted to thrombin (Griffith et al., 1983; Wunderwald et al., 1982). The anticoagulant properties of heparan sulphate are similar to that of heparin and largely depend on the activation of antithrombin III.

1.5.9.3 Interaction with HRG

Heimburger et al. (1972) first described the interaction between HRG and heparin. Linjen et al. (1983b) confirmed the interaction between HRG and heparin, further describing the HRG-heparin interaction to be of high affinity with HRG also neutralising the anticoagulant activity of heparin. HRG binds heparin, thereby preventing activation of both antithrombin III and heparin cofactor II and thus neutralising the anticoagulant effect of heparin (Lijnen et al., 1983a; Tollefsen and Pestka, 1985). These studies suggest HRG may play an important role in the neutralization of heparin in plasma. In purified systems HRG and heparin interact with a 1:1 stoichiometry to form a high affinity complex with a $K_d$ of ~ 7 nM (Lijnen et al., 1983a).

A covalent antithrombin-heparin complex also reacts with HRG with high enough affinity ($K_d$ ~ 30 nM) to counteract the anticoagulant activity of heparin (Lijnen et al., 1983a). Similarly, heparin binding studies using rabbit HRG in a purified system revealed a $K_d$ ~ 55 nM (Peterson et al., 1987). Conflicting studies by Shimada et al. (1989) suggest that HRG does not interfere with the interaction of endothelial cell GAGs with antithrombin III, implying that HRG does not play an important role in the modulation of the anticoagulant activity of endothelial cell GAG in vivo. The majority of studies, however, show that HRG does interact with heparin and heparan sulphates with high affinity, although whether this interaction in vivo plays a significant role in blood homeostasis is unclear.

Many groups have investigated the location of the heparin-binding site within HRG and conflicting results arising from these studies are discussed below. Studies using rabbit HRG and heparin show the interaction is dependent on pH (Burch et al., 1987; Peterson...
et al., 1987) and that chemical modification of histidine residues abolishes the heparin-HRG interaction indicating histidine residues are instrumental in forming and maintaining the HRG-heparin complex. The interaction between heparin and HRG also appears dependent on Zn$^{2+}$ (Lane et al., 1986), suggesting that the interaction between HRG and heparin involves divalent cations. In further support of the heparin binding site being linked to the histidine-rich region, a histidine-rich protein from the malarial parasite *Plasmodium falciparum* and a histidine-rich fragment of hemopexin have been shown to also interact tightly with heparin (Burch et al., 1987). Hydrogen bonding between protonated histidines and the negatively charged sulphate groups of heparin could represent the principal mode of interaction and could explain the sensitivity of the heparin-HRG interaction to ionic strength and pH (Burch et al., 1987). Conversely, other groups argue that the heparin-binding site within HRG is analogous to the antithrombin III heparin-binding site, since both HRG and antithrombin III are known to bind heparin with high affinity. High sequence homology between the N-terminal region of HRG and antithrombin III (~40%) led Koide et al. (1986a; 1986b) to suggest that the heparin binding site of HRG is similar to antithrombin III, and located in the N-terminal domain. The mechanism of binding between heparin and HRG appears unresolved, with good evidence supporting the heparin-binding site being located both at the N-terminal domain mediated though lysine residues and at the HRR mediated via histidine residues. Nonetheless, HRG remains an efficient competitor of the antithrombin III-heparin interaction resulting in heparin’s neutralisation of anticoagulant activity in plasma (Lijnen et al., 1983a). HRG is reported to be the only plasma protein that significantly competes with antithrombin III for heparin binding (Zammit et al., 1993). Fu and Horn (2002) suggested that if HRG could be administered safely in adequate doses it might be used as an antidote for heparin overdose. The primary heparin antidote is currently protamine, a cationic protein derived from salmon sperm whose side-effects include hypotension, anaphylaxis and pulmonary vasoconstriction (Carr and Silverman, 1999). Fu and Horn (2002) further investigated the possibility of HRG acting as the antidote for heparin and found that high (600 µg/ml) levels of HRG combined with Zn$^{2+}$ resulted in total neutralization of 0.2 to 4.5 units/ml of heparin. Further research is necessary to pursue the possibility of HRG as an antidote for heparin to determine whether the requisite doses could be achieved without toxicity.
1.6 HRG cell surface receptors

HRG binds many cell types including human peripheral blood macrophages, T-lymphocytes, murine erythrocytes, macrophages, platelets as well as numerous fibroblastic, endothelial and tumour cell lines including U937 (a monocytoid cell line), Jurkat T lymphoma and Epstein-Barr virus-transformed human B cells (Saigo et al., 1989). HRG does not interact with cell types such as human red blood cells, Burkitt lymphoma (human B cell line) and COS-7 cells (Saigo et al., 1989). Despite the ability of HRG to bind to a diverse range of cell types, HRG cell surface receptors are not clearly defined. Three broad classes of cell surface receptors have been identified and are discussed in more detail below.

1.6.1 Heparan sulphate glycosaminoglycans

Heparan sulphate glycosaminoglycans are ubiquitously expressed on most cells surfaces. Given that HRG binds heparan sulphate with high affinity (discussed in section 1.5.9) it would be anticipated that a significant proportion of HRG cell surface binding is mediated via cell surface heparan sulphate GAGs. Indeed, studies by Parish et al. (1984) demonstrated that human HRG interacts with anionic carbohydrates, probably heparan sulphates, on murine erythrocytes. Studies involving a fragment of HRG lacking its HRR resulted in loss of binding to murine erythrocytes lending support to the suggestion that the HRR plays a key role in modulating binding (Lijnen et al., 1983c). Although it is assumed that the majority of HRG cell surface binding is mediated via cell surface heparan sulphate there is a lack of studies confirming this interaction. Chapter three of this thesis further examines the interaction of HRG with cell surface GAGs.

1.6.2 T cells

Studies with human peripheral blood T lymphocytes suggests that heparan sulphate independent binding of HRG to cell surfaces can occur (Saigo et al., 1989). Kinetic analysis studies indicated both a high affinity (K_d ~19 nM) and a low affinity (K_d ~500 nM) class of binding sites of human T cells for HRG. Unlike HRG binding to most other cell types, the presence of Zn^{2+} did not significantly enhance HRG binding to T lymphocytes (Saigo et al., 1989). Whether HRG binding to T-lymphocytes is independent of Zn^{2+} or whether the concentration of Zn^{2+} tested was too low (2 µM was
used compared to the more common 20 µM Zn\(^{2+}\) ion concentration found to enhance HRG binding to cell surfaces) is debatable. A 50-fold molar excess of heparin or pretreatment of T lymphocytes with bacterial heparanase also did not significantly interfere with HRG binding suggesting the binding site was independent of cell surface heparan sulphate (Saigo et al., 1989). Thus, this report showed that the binding sites on human T lymphocytes for HRG are relatively specific, independent of divalent cations and mediated by distinct cell surface molecules unrelated to heparan sulphate.

A 56 kDa protein isolated from T lymphocyte lysates was identified as the specific HRG T cell surface receptor. It was shown that the receptor was not CD2, but a 50-55 kDa surface protein on T lymphocytes that mediates T cell rosette formation with sheep erythrocytes. Although HRG also interferes with human T cell rosette formation, it appears that HRG does not bind CD2 but somehow blocks rosetting between T cells and sheep erythrocytes by binding this unidentified 56 kDa receptor (Saigo et al., 1989). It was found that an anti-CD2 antibody did not interfere with HRG binding to T cells and the 56 kDa protein was not immunoprecipitating by an anti-CD2 antibody (Saigo et al., 1989). Thus HRG appears to bind to human T lymphocytes at a site distinct from both CD2 and heparan sulphate, yet is still able to interfere with CD2 mediated binding of erythrocytes by T cells (Shatsky et al., 1989).

More recent studies by Olsen et al. (1996) further investigated HRG binding to T cells using the human T cell lines, Jurkat and MT4 as well as a mouse CD4\(^{+}\) T cell line D10. In these studies, physiological concentrations of Zn\(^{2+}\) (~ 20 µM) were found to profoundly potentiate (3-6 fold) HRG binding to T lymphocytes (Kazama and Koide, 1992; Olsen et al., 1996). Interestingly, Zn\(^{2+}\) inhibited HRG binding to cells at concentrations above 100 µM, indicating that Zn\(^{2+}\) levels need to be within the physiological range for optimal HRG binding. It was suggested that increased HRG binding occurs as a result of a conformational change mediated throughout the HRG molecule via disulphide bonds following Zn\(^{2+}\) binding to the HRR (Koide et al., 1986b; Morgan, 1985). Potentiation of HRG binding to cell surfaces in vitro occurs within the physiological concentration range of Zn\(^{2+}\) suggesting Zn\(^{2+}\) is likely to play an important regulatory role mediating HRG activity under physiological conditions. In addition, Olsen et al. (1996) found that heparin largely inhibited the binding of HRG to human peripheral blood T lymphocytes contrasting with the results of Saigo et al. (1989).
Heparin inhibited HRG binding both in the presence and absence of Zn$^{2+}$, suggesting that HRG interacts with T cells via surface GAGs with Zn$^{2+}$ playing a modulating role. Presumably the conflicting observations obtained regarding the effects of Zn$^{2+}$ and heparin on HRG binding reflect differences in the T cell types used, HRG preparations and experimental techniques. More specifically, heparan sulphate may be a more important ligand on activated and/or transformed T lymphocytes (Olsen et al., 1996) than on resting peripheral blood T lymphocytes (Saigo et al., 1989).

Olsen et al. (1996) also found that HRG can be actively internalised by T-cells. Incubation of the human T-cell line, MT4, at 37°C for 24 hrs in the presence of HRG and Zn$^{2+}$ resulted in the majority of cell-associated HRG being internalised. MT4 proliferation and protein tyrosine phosphorylation induced by CD3 cross linking by a CD3-specific mAb was not affected. Whether internalisation of the HRG-receptor complex can explain the reported inhibitory effect of HRG on the proliferation of peripheral blood T cells (Shatsky et al., 1989) is not clear.

1.6.3 Fcγ receptors

Granulocytes, monocytes, macrophages and NK cells express FcγR on their plasma membranes. As discussed in section 1.5.7, HRG has indirectly been shown to bind FcγR monocytic cells. Chang et al. (1992b) demonstrated that HRG binds inflammatory peritoneal macrophages via a cell surface receptor distinct from heparan sulphate that was postulated to be human FcγRII, the low affinity Fcγ receptor. Studies by Gorgani et al. (1999b) suggested that HRG can interact with the high affinity Fcγ receptor (human FcγRI) on THP-1 cells, a monocytic cell line. The inhibition studies described in this paper provide strong indirect evidence that HRG can indeed bind to cell surfaces independent of heparan sulphate, with the most likely candidate receptor being FcγR, possibly both FcγRI and FcγRII. Many parameters regarding the HRG–FcγR interaction remain yet to be studied.
1.7 Functional effects of HRG

1.7.1 pH sensor and Zn$^{2+}$ detector

Studies indicate that the interaction between HRG and Zn$^{2+}$ is highly dependent on pH (Morgan, 1981). Morgan (1981) hypothesised that the unique, conserved HRR domain of HRG acts as a pH sensor, providing a mechanism for regulating HRG activity. In their model, histidine side chains of HRG become protonated when local pH falls during conditions such as ischemia or hypoxia. The HRR becomes positively charged allowing HRG to subsequently strongly bind negatively charged GAGs on the cell surface. This pH sensor phenomenon may become physiologically relevant as it allows HRG to function as an adapter molecule, co-immobilising soluble proteins, such as plasminogen, onto cell surface GAGs only under specific conditions. In support of this concept, in vitro studies showed that immobilised, but not soluble, HRG stimulates plasminogen activation by t-PA (Borza and Morgan, 1997). Thus, in a physiological setting, cell-surface GAGs can bind HRG in vivo when the local pH is lowered, a process that may result in plasmin generation within a confined and regulated local environment that does not extend systemically.

The pH dependence of the HRG-GAG interaction is likely to be physiologically relevant as the local pH can drop by as much as one pH unit under conditions of hypoxia or ischemia (Hess and Manson, 1984; LaManna, 1996), or 0.5 of a pH unit during an inflammatory response due to lactic acidosis (Punnia-Moorthy, 1987). Thus, HRG may function as a pH sensor that binds cell surface GAGs only when local pH decreases or when local Zn$^{2+}$ increases, such as during tissue injury or tumour metastasis (Borza and Morgan, 1998). Thus HRG is in a unique position among plasma proteins to perform this regulated binding to cell surface GAGs due to its high content of histidine residues in the HRR domain.

Other proteins have also been documented to function as a pH sensor. In fact another histidine-rich protein, hisactophilin from Dictyostelium (which binds actin) acts as a pH sensor whereby the binding of this protein to the negatively charged inner plasma membrane is regulated by intracellular pH (Hanakam et al., 1996). HRG appears to be the first plasma protein to which a pH sensor role has be described.
Due to the HRR of HRG acquiring a positive charge upon protonation, HRG can be described as a facultative polycation (Borza and Morgan, 1997). Cationic polyelectrolytes have been described as mediating numerous biological effects including antimicrobial activity, enhancement of vascular permeability, and modulation of leukocyte adherence, chemotaxis and phagocytosis (Ginsburg, 1987). Polyhistidine acts as an opsonic agent (Ginsburg et al., 1982), and a potent inducer of superoxide generation and respiratory burst in polymorphonuclear leukocytes (Ginsburg, 1989; Ginsburg et al., 1987). It seems likely that many of the biological actions of HRG are related to its polycationic nature, implying that the functional effects of HRG are regulated by pH and expressed only under physiological conditions that produce local acidosis.

Various physiological environments exist that support local conditions of high Zn$^{2+}$ levels and low pH. The Zn$^{2+}$ concentration in platelets is estimated to be $\sim 500 \mu$M, with $\sim 40\%$ of platelet Zn$^{2+}$ being secreted into the local environment upon thrombin stimulation (Aktulga, 1974; Gorodetsky et al., 1993), suggesting sites of platelet degranulation/activation result in extracellular Zn$^{2+}$ concentrations much greater than in cell free plasma. Thus areas within the vicinity of platelet degranulation may support HRG cell surface binding through increases in the local Zn$^{2+}$ concentration. Early studies suggest HRG to be an intrinsic platelet protein, whereby following thrombin stimulation HRG becomes surface expressed on the platelet and is subsequently released into the surrounding medium. The physiological significance of platelet HRG release remains unclear since HRG plasma levels of $\sim 100 \mu$g/ml appear to be far greater than platelet HRG levels which constitute only $\sim 0.14\%$ of the blood content on a volume basis (Leung et al., 1989). However, large numbers of platelets become concentrated in and are an integral part of fibrin clots. Thus, HRG released by platelets following thrombin stimulation may achieve a high local concentration and thus play a significant role in modulating fibrinolysis in the microenvironment of the platelet plug (Leung et al., 1989). In support of this view, recent studies indicate that HRG only binds platelets under conditions of low pH and following the addition of transition metals, and binds poorly under conditions of physiological pH and in the absence of transition metals (Horne et al., 2001). Excess Zn$^{2+}$ eventually suppresses HRG binding to platelets similar to the effect of excess Zn$^{2+}$ inhibiting HRG binding to lymphocytes. Excess Zn$^{2+}$ may reflect competition between Zn$^{2+}$-protein complexes and free Zn$^{2+}$, or
simply that high Zn\textsuperscript{2+} concentrations alter cellular membranes resulting in loss of binding (Horne et al., 2001). From the numerous studies regarding the regulation of HRG binding to cell surfaces, it becomes apparent that local pH and the presence of Zn\textsuperscript{2+} probably plays a key role in regulating the activity of HRG.

1.7.2 Modulation of cell proliferation

1.7.2.1 Inhibition of T-cell proliferation

It has been reported that physiological concentrations of human HRG inhibit the proliferation of anti-CD3 activated human peripheral blood T lymphocytes by up to 80-90\% provided HRG is added during the initial 2 hrs of activation, apparently by interfering with early events during T lymphocyte activation (Shatsky et al., 1989). Inhibition of proliferation is proposed to occur via inhibition of interleukin-2 receptor (IL-2R) expression and IFN\textgamma release. HRG does not exhibit anti-proliferative activity when added to the T cells at later stages of activation or when the T cells have been previously activated (Shatsky et al., 1989). It is possible that HRG mediates inhibition of T cell proliferation by binding to T cells via the previously described 56 kDa cell surface protein (Saigo et al., 1989) and not via cell surface heparan sulphate.

1.7.2.2 Modulation of cell proliferation via heparan sulphate

In contrast to the inhibition of T-cell proliferation, studies with other cell types suggest that HRG modulates cell proliferation by interacting with cell surface GAGs. HRG has been reported to induce proliferation of cultured arterial smooth muscle cells by inhibiting the anti-proliferative effects of heparin (Hajjar et al., 1987). Other studies indicate that human HRG inhibits proliferation of BALB/c 3T3 fibroblasts by interacting with cell surface heparan sulphate and subsequently inhibiting the binding of acidic and basic fibroblast growth factors (FGFs) to these cells. These findings support a role for HRG in modulating the release of FGFs from the ECM and basement membranes and also in inhibiting the interaction of FGFs with cell surface heparan sulphates. There is ample evidence that heparan sulphate plays a crucial role in stabilising the interaction of certain FGFs with FGF receptors, thus facilitating transmembrane signalling by these growth factors. Thus, by inhibiting the interaction between certain FGFs and cell surface heparan sulphate, HRG interferes with one of the
key steps involved in triggering cell division by FGFs (Brown and Parish, 1994). Thus HRG has been reported to both inhibit and enhance cell proliferation by interacting with heparan sulphate on cell surfaces.

### 1.7.3 Modulation of cell adhesion

Long term treatment (72 hrs) of peritoneal macrophages with physiological concentrations of HRG results in the loss of adherence of these cells to plastic wells, this effect most probably being mediated through reduced synthesis of cell-surface adhesive proteins such as the sialic acid specific receptor, sialoadhesin (Chang et al., 1992b). Physiologically, HRG mediated down-regulation of sialoadhesin may contribute to decreased binding of opsonised erythrocytes to macrophages, with a resultant decrease in phagocytosis (Chang et al., 1992b). Furthermore, Olsen et al. (1996) found that HRG inhibits the adhesion properties of murine T cell lines to plastic culture dishes coated with components of the ECM (i.e., laminin, collagen and fibronectin), whilst promoting homotypic adhesion between the cells. The presence of physiological concentrations of Zn$^{2+}$ potentiated this effect, resulting in a reduction in murine T cells adhesion to the ECM components.

Lamb-Wharton and Morgan (1993) also reported that HRG can modulate T cell adhesion. Incubation of stimulated (various mitogenic lectins) MOLT-3 cells (human T lymphoblastic cell line) with HRG promoted their adhesion to a plastic culture dish, whilst also inducing a change in morphology. HRG induced MOLT-3 cells either to produce extended cellular processes or to become elongated at the poles. Various monosaccharides such as mannose, glucose, fucose and N-acetylglucosamine, inhibited HRG induced MOLT-3 cell attachment to varying degrees. The changes in adherence status and morphology observed in MOLT-3 cells are similar to those of leukocyte cells preparing to migrate through the microvascular system to sites of inflammation. The generation of an effective immune or inflammatory response in vivo is well known to require recruitment of circulating lymphocytes to the appropriate site followed by subsequent cell activation and proliferation. Initial cell-cell and cell-ECM adhesive interactions are essential to this process, with HRG appearing to be involved in initial T lymphocyte adhesion to the activated surfaces of endothelial cells and platelets (Lamb-Wharton and Morgan, 1993).
HRG isolated from the sera of many vertebrates was found to be the plasma protein that could inhibit the formation of autorosettes (Haupt and Heimburger, 1972; Hsu et al., 1980; Kolb, 1977; Rylatt et al., 1981; Sia et al., 1982). Autorosetting is a phenomenon that exists in the mouse whereby a lymphocyte population binds autologous erythrocytes (Baxley et al., 1973; Sandilands et al., 1974). Although the mechanism by which HRG inhibits autorosetting is unclear Parish et al. (1984) found that in the murine system, HRG has a high affinity for sulphated polysaccharides and probably blocks autorosetting by masking anionic carbohydrate acceptor sites on erythrocytes. In the human system, spontaneous rosette formation occurs between human T cells and sheep red blood cells (SRBS) (NB: not autorosetting but xenographic sheep red blood cells binding to human lymphocytes), and HRG inhibits rosetting between human T cells and SRBC by masking the erythrocyte binding receptors on the T lymphocytes, suggesting the molecular mechanism of rosette formation in the human and mouse systems may be different.

Thus, HRG (especially in the presence of Zn$^{2+}$) appears to play an important role regulating T cell interactions and other aspects of T cell function. It is conceivable that in vivo such actions of HRG may include regulatory effects on T cell activation and/or in modulating the passage of T cells through endothelium during an inflammatory response (Olsen et al., 1996).

1.7.4 Regulation of cell invasion

1.7.4.1 Regulation of angiogenesis

The vascular endothelium consists of a highly ordered monolayer of quiescent, non-migrating cells that can be induced to migrate and replicate. During angiogenesis, new capillary blood vessels are formed from pre-existing vessels in response to angiogenic stimuli. Microvascular endothelial cells locally degrade their basement membrane and subsequently invade the surrounding ECM forming a capillary sprout that develops into a functional vessel following the formation of a lumen (D'Amore and Thompson, 1987; Zetter, 1988). To breach the mechanical barriers imposed by the basement membrane and surrounding ECM, endothelial cells use proteolytic enzymes concentrated at the moving front of the cell surface (Moscatelli and Rifkin, 1988; Pepper and Montesano, 1990). Plasminogen activators are key mediators converting inactive plasminogen to the
active plasmin protease that is capable of directly degrading matrix components as well as activating other matrix degradation enzymes such as metalloproteinases (Pepper et al., 1993). Plasminogen activation rates remain very low in solution, but are greatly enhanced when plasminogen becomes cell bound. Plasminogen does not have any known high affinity cell surface receptors on endothelial cells, introducing a possible role for HRG as a receptor by its ability to link plasminogen to endothelial cell surfaces, thereby providing both a means of enhancing plasminogen activation and also localising proteolysis to the cell surface. The role of HRG in promoting angiogenesis is not experimentally confirmed, and further work is required to elucidate this pro-angiogenic role of HRG.

Thrombospondin (TSP) is known to be a potent inhibitor of angiogenesis. TSP is secreted by activated platelets and a variety of normal vascular cells (including endothelial and smooth muscle cells), and has been shown to inhibit endothelial cell proliferation, migration and tube formation in response to multiple angiogenic stimuli. Although TSP interacts with a number of distinct cellular receptors, CD36 has been recognised as the critical angiostatic receptor for TSP (Dawson et al., 1997; Jimenez et al., 2000). The binding of TSP to CD36 is mediated by a type I peptide sequence, 'cysteine-serine-valine-threonine-cysteine-glycine' (CSVTCG), the same type-1 repeat which has been shown to possess anti-angiogenic activity (Asch et al., 1993). The TSP-I binding site in CD36 is known as a CLESH-1 motif. There are two regions in HRG with significant homology to the CLESH-1 motif of the TSP binding site in CD36. In fact, studies have shown that binding between HRG and TSP is mediated through type I motifs. Simantov et al. (2001) proposed a model whereby HRG inhibits the anti-angiogenic effects of TSP (Figure 1.14). Interaction of type-I repeats in TSP-I with a CLESH-1 domain present in CD36 leads to a cascade of anti-angiogenic signalling events mediated through CD36. HRG can interfere with the interaction between TSP-I and CD36 preventing the anti-angiogenic signal, thus possibly acting as a natural modulator of angiogenesis by antagonising the effect of endogenous TSP (Simantov et al., 2001). Interestingly, HRG may also provide a mechanism by which tumours escape or become resistant to the anti-angiogenic effects of TSP.

Angiogenesis is particularly relevant in the pathogenesis of breast cancer, where degree of angiogenesis is directly correlated with rate of metastasis, with the level of
angiogenesis in breast cancers being an independent prognostic factor (Weidner et al., 1992; Weidner et al., 1991). Immunohistochemical studies of breast cancer specimens show HRG co-localizes with TSP in the tumour matrix and that the interaction masks the anti-angiogenic epitope of TSP. Therefore, in areas where TSP is an important inhibitor of angiogenesis, HRG may serve as a modulator of TSP activity by promoting angiogenesis (Simantov et al., 2001). Conversely, other studies have shown that rabbit HRG is able to inhibit human umbilical vein endothelial cell (HUVECs) tube formation stimulated by fibroblast growth factor-2 (FGF-2), suggesting an anti-angiogenic effect mediated through HRG. The anti-angiogenic activity of rabbit HRG appears to be localised to the HRR (Juarez et al., 2002). HUVECs incubated with HRG show an increase in activation of caspase-3, suggesting apoptosis of activated endothelial cells may be one of the mechanisms underlying HRG’s antiangiogenic activity (Juarez et al., 2002). Furthermore, very recent studies by Olsson et al. (2004) show that HRG has potent antiangiogenic properties, reducing the growth of the aggressive fibrosarcoma by >60% in mice. HRG treatment appeared to mediate its effect by reducing tumour angiogenesis, leading to increased apoptosis and reduced proliferation in the tumours. Olsson et al. (2004) suggested that HRG acts by decreasing the attachment of endothelial cells to vitronectin and reducing endothelial cell migration.

Figure 1.14. Model of inhibition of the anti-angiogenic effect of thrombospondin-1 by HRG. Angiogenesis induced by bFGF is inhibited by thrombospondin-1 through the interaction of the thrombospondin type-1 repeat with the CLESH-1 domain of the signalling receptor CD36 (centre). HRG also contains a CLESH-1 motif and upon binding thrombospondin-1, inhibits the interaction of thrombospondin-1 with CD36 thereby inhibiting the antiangiogenic effect of thrombospondin (right). Adapted from (Simantov et al., 2001).
1.7.4.2 Regulation of cell migration by HRG

Generation of cell surface proteases is fundamental to a wide variety of in vivo biological processes, both pathological and physiological, such as tumour cell invasion, angiogenesis, embryogenesis and monocyte/macrophage migration to sites of inflammation. Cells of one tissue type are required to invade and penetrate neighbouring tissues or matrices (Brownstein et al., 2001).

Through the generation of plasmin and in conjunction with other enzymes such as heparanase, collagenases and elastases, these proteolytic enzymes catalyse the degradation of most proteins of the extracellular space including laminin, thrombospondin, fibronectin and fibrinogen (Estreicher et al., 1990). Directed cell migration requires localised proteolysis whereby high affinity cell surface receptors polarise enzymes (such as plasminogen and heparanase) to the leading front of migrating cells, focusing ECM degradation to the point of cell invasion (Estreicher et al., 1990; Ploplis and Castellino, 2000). However, to date, high affinity cell surface receptors for many enzymes, such as plasminogen, remain undefined.

1.7.5 Regulation of coagulation and fibrinolysis

1.7.5.1 Coagulation

HRG has been found to modulate various components of the coagulation cascade. HRG can inhibit binding of heparin (a potent anticoagulant) to monocytes, resulting in a failure of heparin to inhibit the procoagulant activity of monocytes at sites of inflammation and thrombosis (Leung et al., 1989). In vitro studies employing plasma have also found that HRG binds to the anticoagulant GAGs, heparin and dermatan sulphate, inhibiting their ability to prolong the thrombin time (Lijnen et al., 1984). Furthermore, a number of studies have reported a procoagulating role for HRG based on the ability of HRG to bind to heparin in the fluid phase and block the anticoagulant activity of heparin.

Antithrombin III is a plasma proteinase inhibitor that functions as a principal regulatory protein in blood coagulation. The inhibitory effect of antithrombin III is vastly accelerated in the presence of heparin. HRG and antithrombin III both bind heparin with high affinity. In fact, HRG can inhibit heparin binding to antithrombin III and thus...
block its protease inhibitory effect (Lijnen et al., 1983d). Clinical studies support HRG interacting with heparin in vivo and regulating the anticoagulant effects of heparin administration in humans by inhibiting the interaction of heparin with antithrombin III (Leung et al., 1984; Lijnen and Collen, 1983; Lijnen et al., 1984).

Various amino acids have been identified as important heparin binding sites in antithrombin III, namely Lys114, Lys125, Arg129 and Lys136 which appear to be the four most important residues (Arocas et al., 2000; Schedin-Weiss et al., 2002a; Schedin-Weiss et al., 2002b) whilst Phe122 and Phe121 also contribute to the interaction through contacts between their phenyl rings (Jairajpuri et al., 2003). The N-terminal region of HRG shares a homologous sequence to the N-terminal region of antithrombin III (~ 40%), suggesting the putative heparin binding site of HRG is also located within the N1 or N2 domain (Koide et al., 1982; Koide et al., 1985). An alignment between the N-terminal regions of various species of HRG and antithrombin III is shown in Figure 1.4d. Koide et al. (1986a) observed that although there was high sequence homology between the N-terminal regions of HRG and antithrombin III there is no apparent homology between the remaining 70% of the molecule suggesting the two are evolutionarily related only in their heparin binding region or domain.

Contact activation of the blood coagulation system and activation of the plasma kallikrein-kinin system both result from the same complex series of proteolytic reactions involving factor VII, plasma prekallikrein, high molecular weight kininogen and an activating surface (Kaplan and Silverberg, 1987). The exact mechanism of initiation is unknown, but the interaction of factor XII, plasma prekallikrein and kininogen with an activating surface leads to the formation of factor XIIa and plasma kallikrein through limited mutual proteolysis (Vestergaard et al., 1990). Various surfaces have been used to mediate contact activation in vitro, although surfaces participating in contact activation in vivo are poorly characterised. Factor XII binds directly to negatively charged surfaces via kininogen with which it circulates in plasma as a ~ 1:1 stoichiometric complex (Revak and Cochrane, 1976; Wiggins et al., 1977). One of the mechanisms by which contact activation is inhibited involves competitive neutralisation by β2-glycoprotein I of the negative charges on the activating surface. High molecular weight kininogen inhibits contact activation of blood coagulation in the same way as β2-glycoprotein (Halkier and Magnusson, 1988).
As previously discussed, the HRRs of HRG and kininogen share a high degree of sequence homology. This region in kininogen is known to be essential for the coagulation cofactor activity due to its kaolin binding properties (Ikari et al., 1981). Indeed, bovine HRG has the ability to inhibit contact activation of factor XII and plasma pre-kallikrein in human plasma. It is possible the inhibition results from binding of HRG to the negative charges on the activating surface. Assessment of the biological significance of the observed inhibition of contact activation by HRG is not clear, as surfaces involved in contact activation in vivo are not well characterised, as well as the precise physiological role of the contact activation system being uncertain.

1.7.5.2 Fibrinolysis

Many studies have investigated whether HRG can act as an antifibrinolytic agent due to its high affinity ($K_d$ = 0.5 - 1.1 µM) interaction with plasminogen, the key enzyme in the fibrinolytic system. HRG reportedly binds to LBS on plasminogen via lysine residues in the N- and C-terminal domain thus inhibiting plasminogen from interacting with fibrinogen and $\alpha_2$-antiplasmin. Although there is no direct evidence, it has been proposed by numerous groups that HRG bound to plasminogen inhibits the interaction of plasminogen with fibrin clots thus indirectly inhibiting plasmin mediated fibrinolysis (Lijnen et al., 1980). This proposed antifibrinolytic effect of HRG would be expected to potentiate clotting in the microenvironment of fibrinolysis. Clinical studies supporting an antifibrinolytic effect of HRG include patients with lymphocytic leukaemia undergoing L-asparaginase therapy where thrombotic diathesis is a side effect, have decreased plasma levels of HRG and plasminogen (Vellenga et al., 1984). However, many other clinical studies fail to support an antifibrinolytic role for HRG. For example, plasma HRG levels are normal in patients with pulmonary thromboembolism undergoing streptokinase therapy.

In contrast to the anti-fibrinolytic effect of HRG, other studies (Borza and Morgan, 1997; Silverstein et al., 1985b) have demonstrated that surface immobilised HRG can act as a pro-fibrinolytic agent, enhancing fibrinolysis. Soluble HRG binds plasminogen with moderate affinity ($K_d$ ~1 µM) whereas immobilised HRG binds plasminogen with 50-100 fold higher affinity. This high affinity interaction of immobilised HRG with plasminogen has been shown to accelerate plasminogen activation by plasminogen
activators ~ 30-fold, thereby enhancing the generation of plasmin and resulting in higher amounts of plasmin formation in the microenvironment of fibrin clots (Borza and Morgan, 1997). These findings suggest HRG is likely to potentiate plasminogen activation when immobilised onto cells via interaction of its heparin-binding domain with cell surface heparan sulphates. Additional studies suggest that HRG is incorporated into fibrin clots through its high affinity interaction with fibrinogen ($K_d \sim 6.7$ nM) (Leung, 1986). Although HRG does not appear to affect the conversion of fibrinogen to fibrin, HRG becomes incorporated into fibrin clots and promotes the formation of thinner and more distributed fibrils. It appears HRG can thus influence the stability and general structure of fibrin clots in the vicinity of active haemostasis and thrombosis.

1.7.6 Enhancing clearance of insoluble immune complexes

Certain autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are associated with the production of autoantibodies (Mountz et al., 1994). Autoantibodies can cross-link with their cognate antibodies to form insoluble immune complexes (IIC) that may precipitate in target tissues such as the joint synovial membrane, resulting in a lack of nutrient transport and consequent tissue injury (Sittampalam and Wilson, 1984). The effective clearance of circulating immune complexes is necessary to limit tissue injury, and protective measures may include mechanisms that inhibit the formation of IIC as well as those which promote solubilization of formed IIC (Charlesworth et al., 1982). The mechanisms by which such IIC are cleared from the circulation and tissues are not well understood, although binding of IIC to Fc receptors and complement receptors on mononuclear phagocytic cells is known to be important. The binding of IgG to FcR on mononuclear cells triggers responses including phagocytosis and uptake and clearance of IIC. Large IIC activate and incorporate complement which results in the IIC binding C3b receptors on erythrocytes, are subsequently transported to the spleen and/or liver where they are released and then phagocytosed by resident macrophages and monocytes via receptors for Fc, C3b and C3d (Ehlenberger and Nussenzweig, 1977; Emlen et al., 1992; Taylor et al., 1997).
Gorgani et al. (1999a; 1997) found that physiological concentrations of HRG (in particular the N-terminal domain of HRG) can inhibit the formation of IIC, specifically IIC resulting from the interaction of autoantibodies (RF) with human IgG, suggesting that HRG may play a role in regulating IIC formation in vivo. The formation of IIC occurs in two phases (Gorgani et al., 1996); stage-1 consists of a slow increase in the radii of ICs until the radii reaches a critical size (~100 nm) followed by stage-2 where the radii of the ICs rapidly increase in size leading to the formation of very large IIC. HRG is thought to inhibit only the first phase when immune complexes are smaller than the critical 100 nm size. HRG also probably inhibits IIC formation by masking epitopes on IgG recognised by RF (Gorgani et al., 1999a). HRG was also found to promote solubilization of already formed IIC. However, HRG is more (~40%) effective at inhibiting the formation of IIC compared to promoting solubilization. Gorgani et al. (1999a) postulated that HRG may play an important role in the formation, solubilization and subsequent clearance of IIC in vivo.

1.7.7 Modulation of Fcγ receptor mediated phagocytosis

HRG has been shown to biphasically modulate the expression of FcγR and its associated phagocytic function on murine peritoneal macrophages. After 1-2 hrs of HRG treatment, FcγR expression is rapidly up-regulated on inflammatory peritoneal macrophages resulting in enhanced phagocytosis of opsonised sheep erythrocytes (Chang et al., 1994; Chang et al., 1992b). After 16-48 hrs of treatment, or continuation in culture after the short 1-2 hr treatment without further HRG treatment, FcγR expression is down-regulated on macrophages resulting in significantly reduced phagocytosis of opsonised sheep erythrocytes (Chang et al., 1994; Chang et al., 1992b). Chang et al. (1994) demonstrated the N-terminal domain of HRG is important for regulating macrophage phagocytic function, although they also suggest that the C-terminal domain plays a role in the regulation of cellular function. Molecular mechanisms by which HRG modulates macrophage FcγR expression and phagocytosis are not yet elucidated, although it is reasonable to assume that HRG regulates macrophages via a receptor-dependent signal transduction mechanism (Chang et al., 1994). These studies suggest a role for HRG in regulating macrophage FcγR expression and FcγR mediated phagocytosis, although the mechanisms by which HRG mediates its
time-dependant effect on macrophage expression of FcγR and phagocytic activity is unclear.

Further investigation showed that HRG causes FcγRII to be specifically down-regulated resulting in less efficient phagocytosis, whereas expression of FcγRI appears less affected by HRG (Chang et al., 1992b). These studies indicate HRG may play a role in regulating protein synthesis in macrophage and monocytic cell lines. A reduction of FcγR expression suggests a reduced rate of FcγR translocation from the endoplasmic reticulum to the cell membrane or an enhanced turnover rate for the FcγR protein (Chang et al., 1994). Heparin has been shown to down-regulate FcγRI mediated phagocytosis of IgG2a but up-regulate FcγRII mediated phagocytosis of IgG2b (Yamada et al., 1989). Inhibition of FcγRI phagocytic function by heparin is associated with intracellular casein kinase II activity and interaction of FcγRI with cytoskeletal proteins. However, other studies suggest that HRG regulates macrophage function via a different signalling pathway to heparin. Similarly, IFNγ failed to block HRG function supporting the view that HRG and heparin exert their effects via different signalling pathways in macrophages (Chang et al., 1992b).

Gorgani (1999b) observed that HRG appeared to interact with FcγRI on monocytes (THP-1 cells) and block monomeric IgG binding, although interestingly, did not affect the binding of IC. However, if HRG was incorporated into IgG containing IC, HRG could enhance the binding and uptake of IC by monocytes, probably via its heparan sulphate binding domain. The presence of 20 μM Zn^{2+} was essential for the effect of HRG to enhance the uptake of IC. Collectively, these observations indicate that HRG could act as an important regulator of FcγRI mediated phagocytosis of IC.

More recent studies by Gorgani et al. (2002) showed HRG can increase the rate of phagocytosis of apoptotic cells by human monocyte derived macrophages (HMDM). Based on these observations, they postulated that HRG forms a bridge between monocytic and apoptotic cells via FcγRI on HMDM and naked DNA on apoptotic cells. They also suggested that HRG-dependant removal of apoptotic cells occurs under normal physiological conditions whereas in pathological conditions, such as acute or chronic inflammatory disorders, thrombospondin and C-reactive protein / complement
dependant mechanisms also contribute to the clearance of apoptotic cells (Gorgani et al., 2002).

SLE represents the prototype systemic autoimmune disease and is characterised by circulating IC and inflammatory complications in multiple organs. Impaired clearance of apoptotic cells and IIC in SLE patients is thought to be due to dysfunctional FcγR on mononuclear phagocytes, this defect playing an important role in the pathology of the disease (Salmon et al., 1984). A lack of IIC clearance may lead to IIC deposition in peripheral tissues causing localised inflammation and subsequent damage of target organs. Gorgani et al. (2002) suggest that the exposure of DNA on apoptotic cells may provide a signal for the clearance of the cells by macrophages through the bridging action of HRG. Physiological levels of HRG, by virtue of this capacity to promote the clearance of apoptotic cells, may also protect individuals from the development of systemic autoimmunity. This theory is supported by HRG levels being low in the sera of human SLE patients (Castel, 1983). However, future work with HRG knockout mice and mice predisposed to SLE should provide more evidence to support this hypothesis.

### 1.7.8 Inhibition of apatite formation

Blood contains calcium and phosphate ions at relatively high concentrations, raising the possibility that spontaneous formation of apatite could occur under (patho)physiological conditions. Plasma proteins such as albumin and fetuin have been shown to inhibit apatite formation (Schinke et al., 1996). HRG can also inhibit the formation of apatite calcifications in vitro (Schinke et al., 1997). Under physiological conditions it is possible that HRG may accumulate at sites of tissue damage and prevent ectopic calcification by inhibiting apatite precipitation.

### 1.7.9 Vascular smooth muscle hyperplasia

Vascular smooth muscle cell (SMC) hyperplasia is known to be an important component in endothelial cell injury and the pathogenesis of arteriosclerosis and restenosis. Heparin is known to suppress SMC growth in vitro, however attempts to use heparin as a therapeutic anti-restenotic drug do not correlate with favourable clinical outcomes in angioplasty trials (Mori et al., 2003a). HRG (in the presence of physiological concentrations of Zn²⁺) has the ability to reverse heparin-induced
inhibition of SMC proliferation by binding heparin \textit{in vivo}. Thus, it is possible that HRG may be one of the unidentified factors that reverses the inhibitory effect of heparin on SMC proliferation in arterial microenvironments \textit{in vivo} (Hajjar \textit{et al}., 1987; Mori \textit{et al}., 2003a).

\textbf{1.7.10 Heparanase activity inhibition}

Heparanase is an endoglycosidase that cleaves the hepan sulphate side chains of hepan sulphate proteoglycans, assisting in the disassembly of the ECM and facilitating cell migration (Freeman and Parish, 1997). Heparanase activity has been shown to correlate with the metastatic potential of murine and human melanoma cell lines (Nakajima \textit{et al}., 1988). HRG is known to interact with the heparanase substrate by masking the heparanase cleavage site on hepan sulphate chains. Freeman and Parish (1997) used this property of HRG to develop a rapid and simple quantitative assay for the detection of mammalian heparanase activity. After heparanase digestion, radiolabelled hepan sulphate fragments do not bind to chicken HRG coupled to sepharose, whereas the remaining intact and partially degraded substrate can bind to the HRG-sepharose column allowing a rapid separation of the cleaved product from the substrate and subsequent quantitation of heparanase activity (Freeman and Parish, 1997).

Thus, in a physiological environment, soluble HRG may bind to and mask the vascular wall and ECM hepan sulphate, thereby preventing hepan sulphate degradation from heparanase. Heparanase not only cleaves hepan sulphate chains, but also plays the important role regulating the release of growth factors from the ECM. Thus, HRG may play a role in protecting growth factor release from hepan sulphate matrices, preventing the release of growth factors that could subsequently trigger various cellular adhesion and migratory phenotypes, possibly playing a role in the pathogenesis of tumour cell metastasis.
Chapter two

Materials and Methods

This chapter details the materials and methods used for experiments outlined in this thesis. Furthermore, this chapter describes the design and production of recombinant HRG and HRG fragment expression constructs as well as production of recombinant HRG proteins. In addition, this chapter also outlines the mapping of HRG-specific monoclonal antibody epitopes.
2.1 General reagents and equipment

Oligonucleotides were obtained from the Biomolecular Resource Facility, The John Curtin School of Medical Research, Canberra, Australia, or Invitrogen, Carlsbad, CA. Native human plasminogen fractionated into the two isoforms plasminogen-I and plasminogen-II, and angiostatin, was prepared and kindly supplied by Dr. Phil Hogg, Centre for Thrombosis and Vascular Research, The University of New South Wales, Sydney, Australia. The pgsA-745 cell line was a kind gift from Dr Eva Lee, The John Curtin School of Medical Research, Australian National University, Canberra, Australia. Soluble recombinant FcγRIIa-his131, FcγRIIa-arg131 and FcαRI were obtained from Professor Mark Hogarth, The Austin Research Institute, Melbourne, Australia. Mouse anti-human HRG monoclonal antibody (mAb) ascites preparations (AGEN, Brisbane, Australia) were assigned the following mAb codes; HRG-1, HRG-2, HRG-3, HRG-4 and HRG-5, detailed in Table 2.1. All media was prepared by Media Facility within The John Curtin School of Medical Research, Australian National University, Canberra, Australia, and dissolved in double distilled Milli-Q water (DDW) and filtered through 0.22 μM Millipore filters. PSN antibiotics (1000x): penicillin G 30 mg/ml, streptomycin sulphate 50 mg/ml, neomycin sulphate 50 mg/ml. DMEM: H16 powder 9.99 g/l, NaHCO₃ 3.7 g/l. HBSS (10x): NaCl 100 g/l, KCl 5 g/l, MgSO₄.7H₂O 1.25 g/l, MgCl₂.6H₂O 1.25 g/l, CaCl₂ 1.4 g/l, glucose, 12.5 g/l, phenol red 1 g/l, Na₂HPO₄.2H₂O 3.9 g/l, KH₂PO₄ 3 g/l. Luria broth (LB): tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l. Phosphate buffered saline (PBS): NaCl 800 mg/ml, Na₂HPO₄.2H₂O 125 mg/ml, NaH₂PO₄.H₂O 35.3 mg/ml. RPMI-1640: RPMI-1640 powder 10.44 g/l, NaHCO₃ 2 g/l.

Table 2.1 Details of HRG specific mAbs (AGEN, Brisbane, Australia).

<table>
<thead>
<tr>
<th>HRG mAb</th>
<th>Antibody code</th>
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<tbody>
<tr>
<td>HRG-1</td>
<td>G-15-31DH/108</td>
</tr>
<tr>
<td>HRG-2</td>
<td>G-13-3-2Ab/83</td>
</tr>
<tr>
<td>HRG-3</td>
<td>G-12-3-4Ab/94</td>
</tr>
<tr>
<td>HRG-4</td>
<td>G-15-3-4B3/148</td>
</tr>
<tr>
<td>HRG-5</td>
<td>G-14-3-4D5/65</td>
</tr>
</tbody>
</table>
2.2 Purification of proteins

2.2.1 Purification of plasma-derived HRG

Native human HRG was purified from fresh human plasma as previously described (Rylatt et al., 1981). Briefly, a phosphocellulose column was prepared by adding a mixture of 500 ml 0.2 M HCl and 500 ml ethanol to 35 g Whatman P-11 phosphocellulose (Whatman Ltd., Kent, United Kingdom) and stirred gently using a glass rod. After washing sequentially with 1 l of DDW, 1 l of 0.1 M NaOH and 1 l of 1 mM EDTA, the phosphocellulose was suspended in 500 ml of 0.5 M NaCl loading buffer (0.5 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 6.8) and poured into a 20 cm BioRad (BioRad, Richmond, CA) column fitted to a P1 Peristaltic Pump (Pharmacia, Uppsala, Sweden) and equilibrated with 0.5 M NaCl loading buffer for 24 hr at 4°C. The column was washed with 100 ml of 0.5 M NaCl loading buffer containing 50 mg/ml of bovine serum albumin (BSA), followed by washing with 100 ml of 0.5 M NaCl loading buffer and then 2 L of 2 M NaCl elution buffer (2 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 6.8), before final equilibration with 0.5 M NaCl loading buffer. Fresh human plasma was provided by Red Cross House, The Canberra Hospital, Canberra, Australia, and centrifuged for 10 min at 10000 g in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Dupont, Wilmington, DE) and filtered through Whatman No 1 (Balston Ltd., London, United Kingdom) filter paper. Plasma was mixed with NaCl and EDTA to final concentrations similar to the loading buffer, and with protease inhibitors aprotinin (Boehringer, Mannheim, Germany) (2 µg/ml), phenylmethysulfonyl fluoride (PMSF) (Sigma, St. Louis, MI) (100 µg/ml) and 4-(2-aminoethyl)-benzensulfonyl fluoride hydrochloride (AEBSF) (ICN Pharmaceutical Inc., Costa Mesa, CA) (100 µg/ml). The plasma was passed through the equilibrated column and unbound protein was removed by extensive washing of the column with 0.5 M NaCl loading buffer. Bound HRG was then eluted from the column using 2 M NaCl elution buffer. Eluent was collected into ~ 1 ml fractions and analysed for protein content using the Bradford assay. Fractions containing protein were pooled and concentrated using a Diaflo Concentrator 202 through YM30 Diaflo Ultrafiltration Membrane (Amicon Inc., Beverly, MA). Purified HRG was aliquoted and stored at -80°C until use with the purity of the preparations being monitored by SDS-PAGE and Western blotting (Figure 2.1).
2.2.2 Purification of human plasminogen

Native human plasminogen was purified from fresh human plasma as previously described (Moroi and Aoki, 1976; Rickli and Otavsky, 1975; Wiman, 1980). Fresh human plasma was provided by Red Cross House, The Canberra Hospital, Canberra, Australia, was centrifuged at 14000 g for 15 min in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Dupont, Wilmington, DE) and then filtered through Whatman No 1 (Balston Ltd., London, United Kingdom) filter paper. Lysine-Sepharose 4B (Amerham-Pharmacia Biotechnologies, Uppsala, Sweden) was mixed with PBS and poured into a 20 cm BioRad Column (BioRad, Richmond, CA) and equilibrated with 100 ml of 0.1 M sodium phosphate (pH 7.4). 200 ml of plasma was passed through the column and then the column was extensively washed with 0.3 M sodium phosphate (pH 7.4). Plasminogen was eluted from the column with 0.1 M ε-amino-caproic acid pH 7.4, and concentrated to ~10 ml using a Diaflo Concentrator 202 through a YM30 Diaflo Ultrafiltration Membrane (Amicon Inc., Beverley, MA) before being stored at -80°C until use, with purity being monitored as for HRG (Figure 2.1). Angiostatin, a truncated form of plasminogen, was provided as a kind gift by Dr. Phil Hogg, Centre for Thrombosis and Vascular Research, The University of New South Wales, Sydney, Australia.

**Figure 2.1.** Coomassie blue and Western blot analysis of native human HRG and plasminogen. Native HRG (~75 kDa) was analysed by SDS-PAGE (4-20% gel) under reducing (R) and non-reducing (NR) conditions while plasminogen (~92 kDa) was analysed under reducing conditions. Both gels were then stained with 0.1% Coomassie Brilliant Blue. Western blot analysis of native HRG and plasminogen used a HRG specific mAb, HRG-4 for HRG samples, and a rabbit polyclonal plasminogen antibody for plasminogen samples.
2.2.3 Synthesis of L1-L5 peptides

The HRR within human HRG is comprised of 12 tandem repeats with the dominant consensus repeat sequence being the 5 amino acid motif GHHPH. Synthetic peptides were produced comprised of 1, 2, 3, 4 or 5 repeats of this 5 amino acid motif, termed L1, L2, L3, L4 and L5 respectively. L1, L2 and L3 peptides were also prepared that were biotinylated at the N-terminus. The L1-L5 peptides were provided as a kind gift by Dr. Joe Altin, School of Biochemistry and Molecular Biology, Australian National University, Canberra, Australia.

2.2.4 BioRad Bradford protein assay

An adaptation based on the Bradford assay was used to estimate protein concentration. Typically, protein samples were diluted in PBS to a total volume of 50 µl in the wells of a 96 well plate, and 50 µl of BioRad protein assay reagent concentrate dye (BioRad, Hercules, CA) diluted 1:4 in DDW was added. The optical density was measured at 405 nm (reference wavelength 495 nm) on a Thermomax microplate reader with the resultant data being analysed by SoftMaxPro software (Molecular Devices Corporation, Sunnyvale, CA). A standard curve was determined using known concentrations of BSA to allow the protein concentration of unknown protein samples to be estimated.

2.2.5 Sodium dodecysulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were boiled for 10 min in 20 µl of sodium dodecysulfate (SDS) reducing or non-reducing sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 10% dithiothreitol, 4% SDS), with the non-reducing buffer not containing dithiothreitol, and then subjected to electrophoresis on a 4-20% (w/v) precast polyacrylamide gradient MiniGel (Gradipore, Sydney, Australia) or, on a 10% (w/v) precast polyacrylamide MiniGel (Gradipore, Sydney, Australia). Prestained molecular weight standards (5 µl) (BioRad, Hercules, CA) were run simultaneously to confirm the molecular weight of the different protein samples. After electrophoresis, protein bands were identified by staining for 60 min with 0.1% (w/v) Coomassie Brilliant Blue (BioRad, Hercules, CA) diluted in 10% (v/v) methanol and 10% (v/v) glacial acetic acid in DDW, followed by extensive destaining in 10% (v/v) methanol, 10% (v/v) glacial acetic acid in DDW.
2.2.6 Western blotting

Following SDS-PAGE electrophoresis, proteins were transferred electrophoretically (60 volts, 2 hr) using a Mini-Protean II apparatus (BioRad, Hercules, CA) from the polyacrylamide gel onto a nitrocellulose membrane (BioRad, Hercules, CA) using a transfer buffer containing 48 mM Tris, 39 mM glycine in 20% (v/v) methanol. The membrane was blocked overnight at 4°C with 5% (w/v) skim milk powder in PBS or 3% (w/v) BSA in PBS. Full-length HRG or C-mutant HRG were detected using the HRG-specific mAb, HRG-4 (AGEN, Brisbane, Australia) and the N1 and N1N2 domains were detected using the hexa-histidine specific mAb, His6 (Roche Diagnostics, Mannheim, Germany). Plasminogen was detected using a plasminogen polyclonal antibody (DAKO, Glostrup, Denmark). FcγRI was detected using either a CD64-specific mAb, 10.1 (BD Biosciences Pharmingen, San Jose, CA), or a CD64 polyclonal antibody, N19 (Santa Cruz Biotechnology Inc, CA).

Table 2.2 Specific primary and secondary antibody protocols for Western blotting.

<table>
<thead>
<tr>
<th>Protein detection</th>
<th>Blocking buffer a</th>
<th>Primary antibody / dilution (supplier)</th>
<th>Secondary Ab b / dilution (supplier)</th>
<th>Washing buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRG</td>
<td>PBS/5% SMP</td>
<td>HRG-4 or HRG-5 / 1:3000 (AGEN, Brisbane, Australia)</td>
<td>SAM-HRP / 1:1000 (Chemicon Australia, Melbourne, Australia)</td>
<td>PBS/0.1% Tween-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His6</td>
<td>PBS/3% BSA</td>
<td>His6 / 1:1000 (Roche Diagnostics, GmbH, Mannheim, Germany)</td>
<td>SAM-HRP / 1:1000 (Chemicon Australia, Melbourne, Australia)</td>
<td>PBS/0.1% Tween-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasminogen</td>
<td>PBS/5% SMP</td>
<td>Plasminogen / 1:500 (DAKO A/S, Glostrup, Denmark)</td>
<td>SAR-HRP / 1:1000 (Chemicon Australia, Melbourne, Australia)</td>
<td>PBS/0.1% Tween-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD64</td>
<td>PBS/3% BSA</td>
<td>CD64-biotin/ 1:200 (BD Biosciences Pharmingen, San Jose, CA)</td>
<td>strep-HRP / 1:1000 (Chemicon Australia, Melbourne, Australia)</td>
<td>PBS/0.1% Tween-20</td>
</tr>
</tbody>
</table>

a SMP: skim milk powder, BSA: bovine serum albumin
b SAM-HRP: sheep anti mouse-horse radish peroxidase, SAR-HRP: sheep anti rabbit-horse radish peroxidase, strep-HRP: streptavidin-horse radish peroxidase
The membrane was incubated with the primary antibody diluted in PBS/1% BSA for 90 min at room temperature and then washed extensively for 1 hr with PBS/0.1% Tween-20. The membrane was then incubated with the secondary antibody diluted in PBS/1% BSA for 60 min at room temperature and then washed extensively for 1 hr with PBS/0.1% Tween-20. Table 2.2 outlines the specific primary and secondary antibody protocols used for Western blotting. Chemiluminescence was detected using ECL Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, United Kingdom) according to the manufacture’s instructions. Briefly, 1 ml of detection reagent I and 1 ml of detection reagent II was mixed and poured onto the membrane for 60 seconds. Excess detection reagent was drained from the membrane and the membrane then wrapped between two pieces of plastic film. In the dark, Super-RX-Fuji Medical X-ray film (Fuji Photofilm Co. Ltd., Tokyo, Japan) was exposed to the membrane for 10-120 seconds in a closed cassette before processing in a Kodak X-OMAT M20 film processor (Kodak Pty., Ltd., Sydney, Australia).

2.3 Cell culture

2.3.1 Sf-9 insect cells

The Spodoptera frugiperda derived insect cell line Sf-9 was cultured as a monolayer in Sf-900 II serum free medium (Gibco-BRL, Gaithersburg, MA) at 27°C. Cells were passaged by gently aspirating cells from the flask with medium using a 10 ml pipette. Cells were seeded at 2 x 10^5 cells/ml and were subcultured after 2-3 days when cell density reached ~ 1 x 10^6 cells/ml. Cell viability was checked regularly using trypan blue staining (section 2.3.3) and was consistently > 98%.

2.3.2 Mammalian cell lines

Various mammalian cell lines were routinely cultured in medium supplemented with 10% FCS (Table 2.3) and incubated at 37°C in a Hepa-Filtered IR Incubator (Forma Scientific Inc., Marietta, OH) humidified atmosphere containing 5% CO_2. Cells were passaged approximately every three days according to the rate of cell growth, and were subcultured at a cell concentration of 2 x 10^5 cells/ml and allowed to grow to a maximum cell concentration of ~ 1 x 10^6 cells/ml.
Table 2.3 Details of cell lines, medium, and supplements used throughout this thesis.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source organism</th>
<th>Growth properties</th>
<th>Medium</th>
<th>% FCS</th>
<th>Antibiotics</th>
<th>Supplement</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16-F10</td>
<td><em>Mus musculus</em>, skin melanoma</td>
<td>metastatic, adherent</td>
<td>RPMI-1640</td>
<td>10%</td>
<td>PSN</td>
<td>-</td>
<td>Dr Robin Anderson, Peter Mac Callum Cancer Institute, Melbourne, Australia</td>
</tr>
<tr>
<td>B16-F1</td>
<td><em>Mus musculus</em>, skin melanoma</td>
<td>metastatic, adherent</td>
<td>RPMI-1640</td>
<td>10%</td>
<td>PSN</td>
<td>-</td>
<td>As above</td>
</tr>
<tr>
<td>CHO-xl</td>
<td><em>Cricetulus griseus</em>, Chinese hamster ovary</td>
<td>fibroblast, adherent</td>
<td>DMEM</td>
<td>10%</td>
<td>PSN</td>
<td>21 µg/ml proline</td>
<td>Hulett/Hogarth The Austin Research Institute, Melbourne, Australia</td>
</tr>
<tr>
<td>PgsA-745 (GAG+ve)</td>
<td><em>Cricetulus griseus</em>, Chinese hamster ovary</td>
<td>CHO-xl mutant lacking cell-surface GAG</td>
<td>DMEM</td>
<td>10%</td>
<td>PSN</td>
<td>21 µg/ml proline</td>
<td>Dr Eva Lee, John Curtin School of Medical Research, Canberra, Australia</td>
</tr>
<tr>
<td>HT-1080</td>
<td><em>Homo sapiens</em>, fibrosarcoma of connective tissue</td>
<td>epithelial, adherent</td>
<td>DMEM</td>
<td>10%</td>
<td>PSN</td>
<td>-</td>
<td>Dr Phil Hogg, The University of New South Wales, Sydney, Australia</td>
</tr>
<tr>
<td>COS-7</td>
<td><em>Cercopithecus aethiops</em>, African green monkey, kidney</td>
<td>fibroblast, adherent</td>
<td>RPMI-1640</td>
<td>10%</td>
<td>PSN</td>
<td>-</td>
<td>ATCC</td>
</tr>
<tr>
<td>U937</td>
<td><em>Homo sapiens</em>, histiocytic lymphoma</td>
<td>lymphoblast</td>
<td>RPMI-1640</td>
<td>10%</td>
<td>PSN</td>
<td>-</td>
<td>ATCC</td>
</tr>
<tr>
<td>THP-1</td>
<td><em>Homo sapiens</em>, monocytic cell</td>
<td>floating aggregates</td>
<td>RPMI-1640</td>
<td>10%</td>
<td>PSN</td>
<td>-</td>
<td>Dr Hilary Warren, The Canberra Hospital, ACT, Australia</td>
</tr>
<tr>
<td>MT4</td>
<td><em>Homo sapiens</em>, T-lymphoma</td>
<td>non-adherent</td>
<td>RPMI-1640</td>
<td>10%</td>
<td>PSN</td>
<td>-</td>
<td>ATCC</td>
</tr>
<tr>
<td>HR9</td>
<td><em>Mus musculus</em>, endodermal</td>
<td>adherent</td>
<td>DMEM</td>
<td>10%</td>
<td>PSN</td>
<td>daily ascorbic acid 50 µg/ml, 1% gelatin plates</td>
<td>Dr Angus Tester, The University of New South Wales, Sydney, Australia</td>
</tr>
<tr>
<td>Jurkat T cells</td>
<td><em>Homo sapiens</em>, T-cell leukemia</td>
<td>non-adherent</td>
<td>RPMI-1640</td>
<td>10%</td>
<td>PSN</td>
<td>-</td>
<td>ATCC</td>
</tr>
<tr>
<td>HUVEC</td>
<td><em>Homo sapiens</em>, umbilical vein, endothelial</td>
<td>adherent</td>
<td>M199 basic</td>
<td>10%</td>
<td>PSN</td>
<td>L-Glutamine, Endothelial cell growth supplement, heparin</td>
<td>Primary isolates, The Canberra Hospital, Canberra, Australia</td>
</tr>
</tbody>
</table>
2.3.3 **Cell counting and viability**

A 20 µl cell solution from a known volume was diluted 1:1 with 0.1% Trypan Blue (BDH Poole Chemicals, London, England) loaded onto a haemocytometer and viewed under a standard Olympus B x40 light microscope (Olympus Optical Co. Ltd, Tokyo, Japan) to perform a cell count and viability count.

2.3.4 **Preparation of cell lysates**

Cell lysates were typically prepared from $2 \times 10^6$ cells. Cells were pelleted in an Eppendorf tube by centrifugation at 320 g for 5 min at 4°C and then resuspended in 100 µl of lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.2, 2 mM EDTA, 1% (v/v) NP-40 detergent), containing a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and placed on ice for 15 min. Samples were then vigorously vortexed for 1 min before centrifugation at 15800 g for 15 min at 4°C. The supernatants were collected and stored at −20°C until use.

2.4 **Immunofluorescence flow cytometry**

Cell lines were analysed for human HRG or plasminogen binding or heparan sulphate cell surface expression by immunofluorescence flow cytometry. Table 2.4 outlines antibody and conjugate staining protocols used for each of the cell binding assays. Typically, cells were resuspended to $5 \times 10^6$ cells/ml in PBS/0.1%BSA ± 20 µM Zn$^{2+}$, and 100 µl of cells were added to a 96 V-well plate. Cells were incubated with plasma-derived human HRG (100-200 µg/ml) and/or plasma-derived human plasminogen (150 µg/ml) diluted in PBS/0.1%BSA/20 µM Zn$^{2+}$ pH 7.2, for 60 min at 4°C. Cells were then washed three times in PBS/0.1%BSA pH 7.2 and HRG or plasminogen binding was detected using the HRG specific mAbs, HRG-4 or HRG-5 (AGEN, Brisbane, Australia) and a polyclonal plasminogen antibody (DAKO A/S, Glostrup, Denmark) respectively, diluted in PBS/0.1%BSA and incubated with cells for 30 min at 4°C. Cells were then washed three times in PBS/0.1%BSA before incubating the cells for 30 min at 4°C with appropriate secondary antibodies diluted in PBS/0.1%BSA. Finally, cells were again washed three times in PBS/0.1%BSA and analysed by flow cytometry using an LSR Flow Cytometer (BD Biosciences, San Jose, CA), using forward scatter, side scatter, FL-1, FL-2 and FL-3 detectors. Flow cytometry data was analysed using Cell Quest Pro.
software (BD Biosciences, San Jose, CA). Single colour controls were used to set up compensation parameters. Each sample was typically repeated in triplicate and each experiment was repeated 2-3 times unless otherwise stated. Samples that were incubated with the primary antibody and secondary conjugate in the absence of HRG and/or plasminogen were used as negative controls. In some experiments, cells were treated for 2 hr at 37°C with mammalian heparanase (2 units/ml), bacterial heparinase III (2 units/ml) (Sigma, St.Louis, MO) or chondroitinase ABC (2 units/ml) (Sigma, St.Louis, MO) diluted in PBS/0.1% BSA. Human heparanase was provided as a kind gift from Dr. Craig Freeman, The John Curtin School of Medical Research, Australian National University, Canberra, Australia. In other experiments, human HRG (100 µg/ml) was co-incubated with 0.5-100 µg/ml of different GAGs including bovine lung heparin (3.1 kDa, 4.5 kDa, 10.6 kDa, 12.5 kDa, 16.7 kDa) or chondroitin sulphate A, B, C or E (Sigma, St. Louis, MO). Also, in some cases, cells were incubated with the biotinylated peptides L1-L3 (100 µM), with peptide binding to cells being detected by streptavidin R-phycoerythrin conjugate (Caltag Laboratories, Burlingame, CA).

Table 2.4 Antibody staining protocols for immunofluorescence flow cytometry analysis.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
<th>Detection channel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(raised in) / dilution (supplier)</td>
<td>(raised in) / dilution (supplier)</td>
<td></td>
</tr>
<tr>
<td>human HRG</td>
<td>HRG-4, HRG-5 (mouse) / 1:200 (AGEN, Brisbane, Australia)</td>
<td>Sheep anti-mouse Ig F(ab')2-FITC (sheep) / 1:40 (Amrad Biotech, Melbourne, Australia)</td>
<td>FL-1</td>
</tr>
<tr>
<td>plasminogen</td>
<td>Plasminogen (rabbit) / 1:200 (Dako A/S, Glostrup, Denmark)</td>
<td>Sheep anti-rabbit Ig F(ab')2-FITC (sheep) / 1:40 (Amrad Biotech, Melbourne, Australia)</td>
<td>FL-1</td>
</tr>
<tr>
<td>heparan sulphate</td>
<td>F58-10E4 (mouse) / 1:50 (Seikagaku Corp., Tokyo, Japan)</td>
<td>Sheep anti-mouse Ig F(ab')2-FITC (sheep) / 1:40 (Amrad Biotech, Melbourne, Australia)</td>
<td>FL-1</td>
</tr>
<tr>
<td>hFcγRII</td>
<td>CD32 - 8.26 (mouse) / 1:500 (lerino et al., 1993)</td>
<td>Sheep anti mouse Ig F(ab')2-FITC (sheep) / 1:40 (Amrad Biotech, Melbourne, Australia)</td>
<td>FL-1</td>
</tr>
<tr>
<td>hFcγRI</td>
<td>CD64 (a) 10.1-FITC (mouse) / 1:50 (BD Biosciences Pharmingen, San Jose, CA)</td>
<td>-</td>
<td>FL-1</td>
</tr>
<tr>
<td></td>
<td>(b) 10.1-biotin (mouse) / 1:500 (BD Biosciences Pharmingen, San Jose, CA)</td>
<td>streptavidin-PE / 1:1000 (Caltag Laboratories, Burlingame, CA)</td>
<td>FL-2</td>
</tr>
<tr>
<td></td>
<td>(c) N19 (goat) / 1:300 (Santa Cruz Biotechnology Inc, CA)</td>
<td>Donkey anti-goat Ig F(ab')2-FITC (donkey) / 1:40 (Chemicon Australia, Melbourne, Australia)</td>
<td>FL-1</td>
</tr>
</tbody>
</table>
2.5 Induction and detection of necrotic and apoptotic cells

2.5.1 Induction and detection of necrotic cells

Jurkat T-cells were induced into necrosis by exposure to hyperthermic conditions. Cells were resuspended to a cell concentration of $1 \times 10^7$ cells/ml in RPMI-1640/10% FCS and placed into a 56°C water-bath for 45 min. Necrotic cells were detected by incubating cells with the cell viability dye, 7-AAD (0.3 µg/ml) (Molecular Probes, Inc., Leiden, The Netherlands) in PBS/0.1% BSA (pH 7.2) for 15 min at 4°C in the dark. Cells were then analysed by immunofluorescence flow cytometry using an LSR Flow Cytometer (Becton Dickinson Biosciences, San Jose, CA), with forward scatter, side scatter, FL-1 and FL-3 data being collected. Cell-Quest Pro (Becton Dickinson Biosciences, San Jose, CA) software was used to analyse flow cytometry data. Necrotic cells were detected by gating on 7-AAD (FL-3) positive cells (Figure 2.2). HRG binding to necrotic cells was performed as described in section 2.4.

![Figure 2.2. Detection of viable, apoptotic and necrotic cells by flow cytometry.](image)

Viable (control) cells remain 7-AAD (FL-3) and Annexin-V (FL-2) negative. Early-stage apoptotic cells are Annexin-V (FL-2) positive, but remain 7-AAD (FL-3) negative. Necrotic cells are 7-AAD (FL-3) positive, but remain Annexin-V (FL-2) negative.
2.5.2 Induction and detection of apoptotic cells

Jurkat T-cells were induced into apoptosis using camptothecin (Sigma, St. Louis, MO). Jurkat T-cells were resuspended to 3 x 10⁵ cell/ml and 4 ml added to a 16 x 15 mm petri dish. Apoptosis was induced at 37°C in a humidified atmosphere of 5% CO₂ at 0, 2, 4 and 6 hr time-points by the addition of 1 µM camptothecin. Cells were then harvested and centrifuged at 400 g for 5 min. Detection of apoptotic cells was performed using an apoptosis detection kit (Oncogene Research Products, Cambridge, MA). Cells were resuspended in 100 µl of cold binding buffer (from apoptosis detection kit) and added to a 96 U-well plate. Annexin-V-PE (BD Pharmingen, San Diego, CA) (1 µl) was added to each well and incubated at room temperature for 15 min. Cells were centrifuged at 400 g for 5 min and then resuspended in 100 µl of cold binding buffer containing 7-AAD (0.3 µg/ml) (Molecular Probes Inc., Leiden, The Netherlands) and incubated for 15 min at 4°C in the dark. Cells were then analysed for apoptosis induction by flow cytometry using an LSR Flow Cytometer (BD Biosciences, San Jose, CA) collecting forward scatter, side scatter, FL-1, FL-2 and FL-3 data. Once conditions for inducing apoptosis were established, (i.e, cells shifting from Annexin-V (FL-2) negative to Annexin-V (FL-2) positive, but remaining 7-AAD (FL-3) negative) (Figure 2.2), the capacity of HRG to bind to camptothecin-induced apoptotic cells was determined at each of the 0, 2, 4 and 6 hr time-points. HRG binding was detected as described in section 2.4.

2.6 Phagocytosis assay

THP-1 cells were used as the phagocytic cell and were labelled with SNARF (Molecular Probes, Leiden, The Netherlands). Jurkat T-cells were used as the necrotic cell and were labelled with carboxy-fluorescein diacetate succinimidyl ester (CFSE). Cells were resuspended to 1 x 10⁸ cells/ml in RPMI-1640 only, SNARF was diluted to 50 µM and CFSE was diluted to 10 µM in RPMI-1640 only. Equal volumes of THP-1 cells and diluted SNARF were mixed, giving a final concentration of 5 x 10⁷ cells/ml and 25 µM SNARF, while equal volumes of Jurkat T-cells and diluted CFSE were mixed giving a final concentration of 5 x 10⁷ cells/ml and 5 µM CFSE, and allowed to react in the dark for 15 min in a 37°C waterbath. Cells were then washed twice in RPMI-1640/5%FCS to remove unbound SNARF and CFSE. THP-1 cells were then resuspended to 1 x 10⁶ cell/ml, while Jurkat T-cells were induced into necrosis as previously described in section 2.5.1, after which they were resuspended to 1 x 10⁷
cells/ml. The phagocytosis assay was then performed at 37°C in a humidified atmosphere of 5% CO₂ using pre-warmed 96 U-well plates, by incubating 50 µl/well of SNARF-labelled THP-1 cells with 50 µl/well of CFSE-labelled necrotic Jurkat T-cells (at a cell ratio of 10:1) for 0 - 80 min, with and without plasma-derived HRG (100 µg/ml) and/or 12 kDa heparin (100 µg/ml). Cells were immediately placed on ice and analysed by flow cytometry as described in section 2.4. Rate of phagocytosis was calculated as the number of CFSE + and SNARF + THP-1 cells (quadrant 2, Figure 2.3) as a percentage of the total number of SNARF + THP-1 cells (quadrant 1 + quadrant 2, Figure 2.3).

2.7 Confocal microscopy

Glass microscope slides were prepared by hole punching a 2 cm x 2 cm square of double-sided masking tape and mounting it on the centre of slides. Stained cells were made up to 1 x 10⁸ cells/ml and mounted on slides by placing a drop of cell suspension into the middle of the tape hole. Cover slides were used to seal the hole and cells were visualised using a Nikon Eclipse TE 300 confocal microscope with Nikon Super High Pressure Mercury Lamp power supply (Nikon Corporation, Tokyo, Japan) and a Radiance 2000 Laser Scanning System (Bio-Rad, Hercules, CA). Images were scanned and analysed using Photoshop 7.0 (Adobe Systems Inc., San Jose, CA).

Figure 2.3. Assessment of the phagocytosis of necrotic Jurkat T-cells by the human monocytic cell line, THP-1, by flow cytometry. At time 0, most THP-1 cells are SNARF + (FL-2 +) and most necrotic Jurkat T-cells are CFSE + (FL-1 +), with only a small percentage of cells SNARF + / CFSE + (FL-1 + and FL-2 +) (quadrant 2). During the process of phagocytosis (time 80 min), THP-1 cells bind and ingest necrotic Jurkat T-cells resulting in THP-1 cells becoming CFSE +, that is they become SNARF + (FL-2 +) and CFSE + (FL-1 +) double positive cells. Rate of phagocytosis was measured as the percentage of SNARF + THP-1 cells that were CFSE +.
2.8 Enzyme-linked immunoadsorbent assay (ELISA)

ELISAs were performed by coating 96 well PVC microtitre plastic plates (Dynex Technologies Inc., Chantilly, VA) overnight at 4°C with the protein to be immobilised (50 µl/well, ~ 5 µg/ml) in a 0.05 M Na₂CO₃/NaHCO₃ buffer (pH 9.6) (Sigma, St Louis, MO). Plates were then washed three times (200 µl/well) with PBS/0.1% Tween-20 (Sigma, St. Louis, MO) and then blocked for 120 min at room temperature with 3% (w/v) BSA in PBS or 5% (w/v) skim milk powder (SMP) in PBS (200 µl/well). The blocking buffer was removed by gently flicking the plate and the binding protein was added at 50 µl/well in triplicate, with each triplicate serially diluted from ~ 1:20 up to 1:10000 in PBS/1% BSA and then incubated for 60 min at room temperature. Plates were then washed three times with 200 µl/well of PBS/0.1% Tween-20. Protein specific mAbs were diluted in PBS/1% BSA and incubated (50 µl/well) in the wells for 60 min at room temperature, then washed three times with 200 µl/well of PBS/0.1% Tween-20. The secondary antibody conjugates, sheep anti-mouse Ig horse-radish peroxidase (SAM-HRP) or sheep anti-rabbit Ig horse radish peroxidase (SAR-HRP) (Amrad Biotech, Melbourne, Australia) diluted 1:1000 in PBS/1% BSA were then added (50 µl/well) and incubated in the plates for 60 min at room temperature. Plates were washed three times with 200 µl/well of PBS/0.1% Tween-20. Plate bound peroxidase was detected using the peroxidase substrate, 2,2’-azido-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) and measuring the absorbance of the enzymatic product at 405 nm (reference wavelength 490 nm) on a Thermomax microplate reader, with the resultant data being analysed by SoftMaxPro software (Molecular Devices Corporation, Sunnyvale, CA).

2.9 Tumour cell metastasis

B16F1 mouse melanoma cells were preincubated for 60 min at 4°C with plasma-derived human HRG (100 µg/ml) diluted in PBS/0.1%BSA/20 µM Zn²⁺ (pH 6.5). Cells were washed in PBS/0.1%BSA/20 µM Zn²⁺ (pH 6.5) and resuspended in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD). Recipient C57BL/6 mice were injected iv with 0.5-1.0 x 10⁵ melanoma cells preincubated with or without human HRG. Mice were sacrificed on day 21 and analysed for number of lung metastases using a disecting microscope.
2.10 Surface plasmon resonance

A BIACore 2000 instrument (Pharmacia Biosensor, Uppsala, Sweden) was used to measure binding by surface plasmon resonance. CM5 sensor chips and coupling reagents were purchased from Pharmacia Biosensor. The running buffer (PBS) was routinely degassed and filtered through a 0.45 µm Millipore filter.

2.10.1 Immobilisation of HRG

The carboxymethylated dextran surface of flow-cells on the CM5 chip were activated by injecting a mixture of 35 µl of 0.05 M 1-ethyl-3(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC) and 35 µl of 0.05 M N-hydroxysuccinimide (NHS) diluted in DDW at 5 µl/min. Either plasma-derived or recombinant HRG each were immobilised onto the NHS-ester activated surface by injecting ~ 10 µl of HRG (100 µg/ml) in 10 mM sodium acetate (pH 4.5) at 5 µl/min. Remaining NHS-esters were deactivated by injecting 35 µl of 1 M ethanolamine (pH 8.5) at 5 µl/min. A typical sensogram outlining immobilisation of HRG is depicted in Figure 2.4a.

2.10.2 Protein binding assays

Typically a flow rate of 10 µl/min was used throughout the binding assays. Different concentrations of plasminogen (0-2000 nM, 100 µl), human heparanase (0.2-10 µg/ml, 40 µl), angiostatin (0.5-2 µM, 30 µl) and recombinant FcγRI, FcγRII, FcαRI (0-2000 nM, 30 µl) and control proteins (Ni-NTA purified Sf-9 cell culture supernatant) diluted in PBS were injected into the flow-cells of the biosensor, with binding and dissociation each monitored for 3 - 10 min. An example of a binding curve showing association, dissociation and regeneration is depicted in Figure 2.4b. Experiments used an automated program to control triplicate injections of each binding protein. Flow-cell 1 was used as a blank control reference cell with the background binding to the dextran matrix detected in flow-cell 1 being subtracted from responses in flow-cells 2, 3 and 4. Binding curves were analysed using the BIA Evaluation program (Pharmacia Biosensor, Uppsala, Sweden).
2.10.3 Regeneration

HRG-plasminogen/angiostatin complexes were effectively disrupted by injecting 100 mM $L$-lysine for 1 min at a flow-rate of 100 µl/min. HRG-FcR and HRG-heparanase complexes were effectively disrupted by injecting 50 mM HCl for 1 min at a flow-rate of 100 µl/min.

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**Figure 2.4. Typical biacore sensorgrams.** (a) Sensorgram outlining immobilisation procedure, highlighting EDC/NHS injection that activates the surface of the dextran chip, immobilisation of ~ 2200 response units (RU) of HRG followed by deactivation of the flow cell surface by an ethanolamine injection. (b) Typical binding curve of an analyte (plasminogen) binding to an immobilised ligand (HRG), highlighting the association and dissociation curves and the regeneration process that uses an injection of 100 mM $L$-lysine to disrupt the HRG-plasminogen complex and allows the baseline to return to normal.
2.11 Molecular biology

2.11.1 Total RNA preparation
Pelleted cells were resuspended in 1 ml of Trizol reagent (Life Technologies, Grand Island, NY) and then homogenised by passing through a 19 gauge and 21 gauge needle. Samples were mixed with 100 µl of chloroform, vortexed for 1 min, and incubated at 4°C for 5 min before being centrifuged at 15800 g for 15 min at 4°C. The top aqueous layer was transferred to a new tube without disturbing the proteinaceous interface. Isopropanol (500 µl) was added and the samples incubated at 4°C for 5 min before being centrifuged at 15800 g for 15 min at 4°C. After discarding the supernatant, samples were then washed with 70% ethanol in DEPC-DDW by centrifugation at 15800 g for 5 min at 4°C. The resulting pellet was dried at room temperature for 5-10 min and then resuspended in 30-50 µl of DEPC-DDW. Samples were stored at −70°C.

2.11.2 1st strand cDNA synthesis
RNA samples were first diluted to 1-5 µg in a total volume of 8 µl in DEPC-DDW. cDNA was then generated using a cDNA first strand synthesis kit (Amersham Pharmacia Biosciences, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Briefly, RNA samples were incubated at 65°C for 10 min and then immediately placed on ice. To each sample was added 5 µl of bulk first strand mix, 1 µl of dithiothreitol and 0.2 µg of oligo Norl(T)₁₈ oligonucleotide primer (1 µl diluted 1:25 in DEPC-DDW). Samples were then incubated at 37°C for 60 min and stored at −20°C.

2.11.3 Spectrophotometry
The concentration of RNA, oligonucleotide DNA and plasmid DNA was determined by spectrophotometric analysis. For each sample, 5 µl of nucleic acid was diluted to 500 µl in DDW. After setting up a reference of plain DDW, 500 µl of each sample was then loaded into a thoroughly washed quartz cuvette of 1 cm path length (Amersham Biosciences, NJ). Absorbance at 260 nm, 280 nm and 320 nm, concentration and the $A_{260}/A_{280}$ ratios were then recorded using a Smart Spec 3000 spectrophotometer (Bio-Rad, Hercules, CA).
2.11.4  **Polymerase chain reaction (PCR)**

Preparation of all PCRs was done in a dedicated, UV-irradiated PCR hood. Table 2.5 details all oligonucleotides used in PCR reactions. Typical 20 µl reactions contained: 2.0 µl of 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl) (Invitrogen, Carlsbad, CA), 0.6 µl of 50 mM MgCl₂ (final concentration 1.5 mM) (Invitrogen, Carlsbad, CA), 3.2 µl of 1.25 mM dNTPs (final concentration 0.2 mM) (Pharmacia Biotech, Buckinghamshire, United Kingdom), 1.0 µl of each of two 5 pM/µl (~ 50 ng) oligonucleotides, 1.0 µl of template (~ 150-300 ng), 0.1 µl (0.5 unit) of Taq polymerase or platinum Taq polymerase (Invitrogen, Carlsbad, CA) and 11.1 µl of DDW. PCR conditions varied between experiments, but usually involved: 1 cycle of 94°C for 2 min, then 30 cycles of 94°C for 30 seconds, 58°C – 65°C for 30 seconds and 72°C for 60 seconds, then 1 cycle of 72°C for 10 min. PCRs were carried out using a PC960C Cooled Thermal Cycler (Corbett Research, Mortlake, Australia).

2.11.5  **DNA agarose gel electrophoresis**

To each sample was added ~ 10% (v/v) gel loading buffer containing 30% glycerol and 0.25% bromomethyl blue (MBI Fermentas, Hanover, IN) running dye in DDW. Samples were then loaded into wells of 1% (w/v) agarose (BioRad, Hercules, CA) 40 mM Tris-acetate, 1 mM EDTA (TAE) gels containing 100 ng/ml of ethidium bromide (Sigma, St. Louis, MO) and immersed in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Electrophoresis was performed using either a Pharmacia Biotech DNA 100 gel tank (Pharmacia Biotech, Buckinghamshire, United Kingdom) or a BioRad DNA Sub-Cell gel tank (BioRad, Hercules, CA) attached to a BioRad Power Pac 300 (BioRad, Hercules, CA), typically running 70-100 volts across the gel. Either 1 kb⁺, 1 kb or 100 bp ladder DNA markers (Fermentas, Hanover, IN) were run in parallel to confirm band size. Gels were then exposed to UV illumination using a UV Transilluminator (UVP Inc, Upland, CA) and images were captured using a Mitsubishi Video Copy Processor P67UA (Mitsubishi Electric, Singapore) (Figure 2.5).

2.11.6  **Cloning into pCR3.1**

PCR amplified DNA fragments excised as bands from agarose gels were extracted using a Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) as per the manufacturer's
instructions. Gel purified DNA was then immediately ligated into the cloning vector pCR3.1 using a Eukaryotic TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Tag polymerase has a non-template dependent activity that adds a single deoxyadenosine (A) to the 3' end of duplex molecules. The linearised vector pCR3.1 contains single 3' deoxythymidine (T) residues, allowing PCR inserts to ligate efficiently with the vector. Briefly, ~60 ng (~2 µl) of linearized vector pCR3.1, ~100 ng (~6 µl) of purified cDNA, 1 µl of 10x T4 DNA ligase buffer (USB Corporation, Cleveland, OH) and 3 units of T4 DNA ligase (USB Corporation, Cleveland, OH) were mixed to a total of 10 µl and incubated at 14°C overnight. The pCR3.1 ligation mixture was then transformed onto One Shot TOP10F' competent cells (Invitrogen, Carlsbad, CA) by heat shock as per the manufacturer’s instructions. Briefly, 2 µl of each ligation reaction was gently added to 50 µl of competent bacterial cells, recently thawed on ice. To each transformed sample was added 250 µl of SOC medium (2% (w/v) bacto tryptone, 0.55% (w/v) bacto yeast extract, 10 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose, pH 7.0).

**Figure 2.5. PCR screen of bacmid DNA by agarose gel electrophoresis.** Using oligonucleotide primers M13P-F and M13P-R, PCR amplification of empty bacmids (from blue colonies) gives a specific 300 bp product, while PCR amplification of human FcγRI-his6 cDNA gives a specific 3200 bp product.
Samples were then incubated at 37°C for 1 hr in a G10 Gyrotory Shaker shaking incubator (New Brunswick Scientific Co Inc., NJ), after which they were spread in volumes varying between 20 µl and 200 µl on to luria broth (LB) agar plates containing 50 µg/ml ampicillin (amp) and plates were incubated overnight at 37°C. Bacterial colonies were then picked with a sterile pipette tip and inoculated into Eppendorf tubes containing 1 ml of LB-amp and incubated overnight at 37°C on a shaking incubator. Plasmid DNA was then purified by alkaline lysis extraction. Tubes were centrifuged at 400 g for 5 min and the supernatant was discarded. To each tube was added 100 µl of solution I (25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose), 200 µl of solution II (10 M NaOH, 10%SDS) and 150 µl of solution III (3 M KOAc, 11.5% (v/v) glacial acetic acid). Samples were then centrifuged at 15800 g for 5 min and the supernatant was transferred to a new Eppendorf tube. An equal volume of isopropanol was added and tubes were mixed gently before centrifuging at 15800 g for 10 min. Samples were then washed with 1 ml of 70% ethanol by centrifugation at 15800 g for 5 min and air-dried before re-suspension in 20 µl of DDW. Purified plasmids were then examined to determine whether they contained an insert of the correct size by restriction enzyme digest analysis using 2 units of EcoRI (MBI Fermentas, Hanover, IN) and 10 µl of purified plasmid with the appropriate buffers (MBI Fermentas, Hanover, IN) incubated at 37°C for at least 60 min. Samples were then subjected to gel agarose electrophoresis as described in 2.11.5. On the basis of gel results, colonies yielding plasmids containing inserts of the correct size were then re-picked with sterile pipette tips, inoculated into tubes containing 10 ml of LB-amp and incubated overnight at 37°C on a shaking incubator. Plasmids from competent cells were then purified using a Wizard Plus SV Minipreps DNA purification system kit (Promega, Madison, WI). Purified plasmids were then examined to confirm that they contained an insert of the correct size by restriction enzyme digest analysis and gel electrophoresis as described above. Those samples with inserts of correct size were stored at -20°C.

2.11.7 Cloning into pFastBac-1

Ligations of cDNA into the pFastBac-1 (Life Technologies, Grand Island, NY) vector required both the vector and cDNA insert to be cut with appropriate restriction enzyme pairs, such as BamHI and XhoI (FcγRI cDNA) or EcoRI and KpnI (HRG cDNA). Preparations of restricted inserts and vector typically used ~ 1-2 µg of cDNA, 3 µl of
One-Phor-All (OPA) buffer (Amersham-Pharmacia Biotechnologies, Uppsala, Sweden), 2 units each of the appropriate restriction enzyme made up to 30 µl with DDW, and incubated in a 37°C water bath for 120 min. 1 unit of calf intestinal phosphatase (CIP) (Amersham-Pharmacia Biotechnologies, Uppsala, Sweden) was added to the pFastBac reaction vessel and incubated at 37°C for 30 min. The restriction enzymes were then heat killed by incubating at 85°C for 10 min. Digested cDNA was gel purified using a Qiaquick gel extraction kit (Qiagen, Valencia, CA) as per the manufacturer’s instructions and then immediately ligated into the cloning vector, pFastBac, in a 20 µl reaction using ~ 1:10 ratio of vector:insert, such as ~ 20 ng of purified digested pFastBac vector in 2 µl, and 200 ng of purified digested cDNA in 8 µl, 2 µl of 10x T4 DNA ligase buffer, 3 units of T4 DNA ligase and 7 µl of DDW and incubated at 14°C overnight. The pFastBac ligation mixture was then transformed onto One Shot TOP10F' competent cells (Invitrogen, Carlsbad, CA) and positive colonies were picked, plasmid purified and screened for the correct insert as described above. The pFastBac expression cassette was then transformed into a baculovirus shuttle vector (bacmid) propagated in DH10Bac competent cells (Life Technologies, Grand Island, NY). LB plates were prepared containing 50 µg/ml of kanamycin (kana), 7 µg/ml of gentamycin (genta) and 10 µg/ml of tetracycline (teta). 35 µl of 100 µg/ml X-gal and 20 µl of 40 µg/ml IPTG was spread onto the surface of each triple antibiotic plate. 10 ng of purified cloned pFastBac was gently added to 100 µl of competent DH10Bac cells recently thawed on ice, and allowed to incubate on ice for 30 min before being heat shocked at 42°C for 45 seconds. To each transformed sample was added 900 µl of SOC medium. Samples were then incubated at 37°C for 4 hr in a shaking incubator after which they were diluted 1:10 in LB and spread in volumes varying between 20 µl and 200 µl on to the kana/genta/teta/X-gal/IPTG LB agar plates. Plates were then incubated overnight at 37°C. pFastBac plasmids that integrated into the mini-Tn-7 attachment site on the bacmid disrupted the expression of the lacZα peptide so that colonies containing the recombinant bacmid appeared white in a background of blue colonies that harboured the unaltered bacmid. Thus, white bacterial colonies were easily distinguished, labelled and picked with a sterile pipette tip and inoculated into aerated 15 ml tubes containing 2 ml of LB genta/kana/teta and incubated overnight at 37°C on a shaking incubator. Bacmid DNA was then purified by bacmid alkaline lysis extraction. Tubes were centrifuged at 15800 g for 5 min and the supernatant was discarded. To each tube was added 300 µl of bacmid solution I (15 mM Tris pH 8.0, 10
mM EDTA, 100 µg/ml RNAse), 300 µl of bacmid solution II (0.2 N NaOH, 1% SDS) and gently mixed and incubated at room temperature for 5 min. 300 µl of 3 M sodium acetate was slowly added to samples and placed on ice for 10 min before centrifugation at 15800 g for 10 min. The supernatant was mixed with 800 µl of isopropanol and placed on ice for 10 min before centrifugation at 15800 g for 15 min. Samples were then washed with 1 ml of 70% ethanol and were air-dried before re-suspension in 40 µl of DDW. Purified bacmid DNA preparations were then examined to determine whether they contained an insert of the correct size by PCR using M13P-F and M13P-R oligonucleotides (Table 2.5), and subjecting the PCR amplified product to gel agarose electrophoresis followed by Southern blotting. The PCR program was similar to that described in 2.11.4 except that the extension time was longer (300 seconds at 72°C) to accommodate larger chain extension. PCR amplifications of 300 bp indicated an empty bacmid, 2300 bp indicated a bacmid containing empty pFastBac, while 2300 bp plus the length of the insert indicated a recombinant bacmid (Figure 2.5) suitable for use in the baculovirus expression system.

2.11.8 Sequencing

Sequencing of material cloned into either the pCR 3.1 or pFastBac cloning vectors was performed using the Big Dye v3.1 dye primer sequencing strategy (Perkin Elmer Applied Biosciences, Foster City, CA) according to the manufacturer’s protocol. Briefly, ~200-400 ng of purified plasmid DNA, 4 µl of BigDye master mix and 1 µl of 3.2 pM/µl of either T7 (forward), BGH (reverse), M13P-F (forward) or M13P-R (reverse) oligonucleotide primers (MBI Fermentas, Hanover, IN) were combined in a 10 µl PCR reaction in capillary tubes using a FTA 4000 Thermal Sequencer (Corbett Research, Mortlake, Australia) with the following program: 1 cycle of 94°C for 5 min, then 30 cycles of 96°C for 10 seconds, 50°C for 30 seconds and 60°C for 4 min. To each completed PCR reaction, 1 µl of 3 M sodium acetate pH 5.2 and 30 µl of cold 95% ethanol was added. Samples were then centrifuged at 15800 g for 15 min at room temperature and the supernatant was discarded. The pellet was then washed twice with 70% cold ethanol, air-dried, stored at ~20°C and sequenced at the BRF, The John Curtin School of Medical Research, Australian National University, Canberra, Australia using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were analysed using EditView1.0.1 (Perkin Elmer Applied Biosystems, Foster City, CA).
Table 2.5  Oligonucleotides used in PCR amplification of HRG, FcγRI, FcγRII cDNAs.

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<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>RE b</th>
<th>Brief description:</th>
<th>Tag</th>
<th>oligonucleotide hybridisation position c</th>
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<td>22</td>
<td>66</td>
<td>-</td>
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<td>FcγRlla 694-715</td>
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<td>22</td>
<td>66</td>
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<td>FcγRlla 694-715</td>
<td></td>
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<td>58</td>
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<td>'5' end FcγRI</td>
<td>FcγRI 21-39</td>
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<td>3' end of EC region hFcyRI</td>
<td>His6</td>
<td>FcγRI 893-912</td>
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a For oligonucleotides containing sequence complementary to both FcγRI and FcγRlla cDNAs, red sequence is complementary to the FcγRI sequence, blue sequence is complementary to the FcγRlla cDNA. Green sequence identifies non-homologous regions containing restriction enzyme recognition sites.

b Oligonucleotide hybridisation positions refer to nucleotide sequences of the HRG, FcγRI or FcγRlla cDNAs (see Appendix 1).

c Complementary to pFastBac vector (Life Technologies, Grand Island, NY) directed at sequences on either side of the mini-attR site within the lacZa -complementation region of the bacmid.

1 Complementary to pCR3.1 vector (Invitrogen, Carlsbad, CA) directed at sequences either side of the multiple cloning region of the vector.

RE: restriction enzyme
2.11.9  Southern blotting

After PCR amplified bacmid DNA samples had been subjected to 0.8% (w/v) agarose TAE gel electrophoresis, the gel was depurinated by incubating in concentrated HCl diluted 1:40 in DDW for 20 min, followed by a 30 min incubation in cracking solution (1.5 M NaCl, 0.5 M NaOH), and a 30 min incubation in neutralising solution (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl), before overnight transfer to Hybond N+ membrane in 10xSSC (1.5 M NaCl, 171 mM sodium citrate, pH 7.0). The membrane was cross-linked for 7 min under UV light and then soaked in 5 ml of pre-hybridisation solution (50% (v/v) formamide, 5x denhardts, 5x SSC, 0.1% (v/v) SDS, 100 µg/ml of salmon sperm DNA) for at least 60 min at 42°C. The radioactive probe was prepared using a Megaprime DNA labelling systems kit (Amersham Biosciences, Buckinghamshire, United Kingdom). 5 µl of purified EC FcyRI cDNA was added to 5 µl of primers (from the kit) and boiled for 5 min. 10 µl of labelling buffer, 2 µl of enzyme, 23 µl of DDW and 5 µl of 32P-dCTP were added and centrifuged at 15800 g for 5 min. Samples were then incubated at 37°C for 10 min before stopping the reaction by adding 5 µl of 0.2 M EDTA. Samples were purified through a G-50 micro column (ProbeQuant, Rohm and Haas, DE), eluted in 400 µl of TE, boiled for 5 min and placed immediately on ice. The denatured FcyRI-32P-dCTP probe was added to the pre-hybridised filters in hybridisation bottles and incubated at 42°C overnight in a hybaid oven. The washing procedure involved rinsing the membrane in 2xSSC/0.05%SDS, washing the membrane in 2xSSC/0.05%SDS two times for 30 min each at room temperature, followed by two final washes in 0.2xSSC/0.1%SDS for 30 min at 55°C. The membrane was then exposed to X-ray film in a cassette and placed at -70°C for 1.5 hr before processing as described in 2.2.5.

2.12  Expression of recombinant proteins using 'Bac-to-Bac' Baculovirus expression system

2.12.1  Transfection of Sf-9 cells with recombinant bacmid DNA

The donor plasmid pFastBac has been developed to be compatible with the Bac-to-Bac Baculovirus Expression System (Life Technologies, Grand Island, NY). Sf-9 cells were seeded at 9 x 10^5 cells per 35 mm well in 2 ml of Sf-900 II (Gibco-BRL, MD) serum free medium and allowed to attach for 30 min at 27°C. In tube A, ~ 1-2 µg of recombinant bacmid DNA was added into a total of 100 µl of Sf-900 II. In tube B, 5 µl
of Cellfectin Reagent (Invitrogen Life Technologies, CA) was added into a total of 100 µl of Sf-900 II. The contents of tube A and B were mixed gently and incubated at room temperature for 45 min. Meanwhile, cells were washed with 2 ml of Sf-900 II. For each transfection, 800 µl of Sf-900 II was added to each tube containing the lipid-DNA complex and mixed gently. Medium was removed from the cells and the DNA-lipid complex was added drop-wise to the cells and incubated at 27°C. The transfection mixture was removed after 5 hr and replaced with 2 ml of Sf-900 II. Virus was harvested after 72 hr (Figure 2.6).

Figure 2.6. Strategy for generation of recombinant proteins using the Baculovirus expression system (Life Technologies, Grand Island, NY).
2.12.2 **Harvesting recombinant baculovirus**

Supernatant containing recombinant baculovirus was harvested 72 hr post transfection, and was centrifuged at 400 g for 5 min to remove cell debris. The baculovirus containing supernatant was filtered through a 22 µm syringe filter and stored at 4°C protected from light. Stocks of baculovirus supplemented with 5% FCS were also stored at -70°C.

2.12.3 **Amplifying recombinant baculovirus**

*Sf*-9 cells were seeded at 3.0 x 10^5 cells/ml in 175 cm tissue culture flasks in 40 ml of Sf-900 II. The monolayer was infected at a multiplicity of infection (MOI) of approximately 0.01-0.1; routinely a 40 ml culture was infected with 0.4 ml of viral stock that was 2 x 10^7 pfu/ml for a MOI of 0.1. Typically, recombinant baculovirus was amplified three times before used to produce recombinant protein.

2.12.4 **Production and purification of recombinant proteins**

*Sf*-9 cells were seeded at 3.0 x 10^5 cells/ml in 175 cm tissue culture flasks in 40 ml of Sf-900 II and infected with 4 ml of three-times amplified recombinant baculovirus for 3-5 days. The recombinant protein expressed by *Sf*-9 cells was released into the supernatant, allowing recombinant protein to be purified from the supernatant under non-denaturing conditions. After 3-5 days of infection, the supernatant was harvested into sterile 50 ml tubes and centrifuged at 900 g for 5 min at 4°C to remove the debris. The harvested supernatant was then dialysed against PBS in pre-boiled cellulose membrane dialysis tubing (Sigma, St. Louis, MO) overnight at 4°C to optimise the pH of the supernatant for recombinant protein purification. Dialysed supernatant was centrifuged at 900 g for 10 min and poured into fresh tubes. Ni-NTA agarose was pre-washed in PBS before 250 µl of 50% Ni-NTA agarose slurry (Qiagen, Hilden, Germany) was added to 50 ml of dialysed baculovirus containing supernatant and rotated on a RSM6 daisy wheel (Ratek Instruments Pty. Ltd., Boronia, Australia) at 4°C for 2 hr. The Ni-NTA agarose was then extensively washed with cold PBS and then transferred to a poly-prep chromatography column (BioRad, Hercules, CA) and equilibrated with PBS. Recombinant protein was eluted using 200 mM cold imidazole diluted in PBS. The hexa-histidine tag on the recombinant N1, N1N2 and FcγRI allowed purification by Ni-NTA chelation chromatography, whereas the recombinant
HRR of full-length HRG contains sufficient histidine residues to allow Ni-NTA agarose affinity purification. Eluted fractions were immediately collected on ice and fractions containing protein were pooled and re-dialysed against PBS overnight at 4°C before being aliquoted and stored at −80°C. Recombinant proteins were checked for purity by Western blotting, by Coomassie blue staining of SDS-PAGE gels and by ELISA.

2.13 Production of recombinant full-length HRG, C-mutant HRG, N1 fragment and the N1N2 domain

Two sources of human HRG were used for the experiments described throughout this thesis. Firstly, HRG purified from plasma (purification procedure described in section 2.2.1), and secondly, recombinant HRG produced by baculovirus infected *Sj*-9 insect cells. Plasma-derived human HRG provided a ready source of full-length native HRG at high concentration, a preparation useful for most experiments. Recombinant HRG provided another valuable (but more scarce) source of full-length HRG, free from contaminating human plasma proteins, and protected from proteolysis by plasma proteins such as plasmin. Additionally, production of recombinant HRG facilitated the production of mutated and truncated forms of the protein that could be used to probe the function of various HRG domains. Two forms of full-length HRG were produced, both full-length HRG and HRG lacking the C-terminal lysine residue, which was used to analyse the importance of the C-terminal lysine residue for plasminogen binding (further described in Chapter 4). Earlier sequence homology studies have suggested that the N1N2 domain of HRG contains a heparin-binding motif and may represent the region of HRG that interacts with cell surface heparan sulphate. In order to test this possibility, the amino-terminal domain N1N2, and the first cystatin fragment N1 were also produced. Until now, most reports describing the role of HRG domains was based on indirect studies that used impure fragments of full-length native HRG cleaved by various plasma proteases. In this thesis, the *Sj*-9 (insect cell line) was used to produce recombinant HRG and fragments for use in *in vitro* cell surface binding assays to provide definitive evidence regarding the function of the amino-terminal domain.
Figure 2.7. Strategy for the generation of HRG and HRG fragment expression constructs. (a) Schematic representation of full-length HRG cDNA shown with oligonucleotide primers (ALJ-1 to ALJ-5) used in PCR reactions positioned at their priming sites with 5’ to 3’ direction indicated by arrows. The oligonucleotide ALJ-1 contains an EcoRI site at the 5’ end, oligonucleotides ALJ-2, ALJ-3, ALJ-4 and ALJ-5 contain KpnI sites at their 5’ end. Oligonucleotides ALJ-2 and ALJ-3 contain a hexa-histidine tag engineered immediately prior to the KpnI site at the 5’ end. ALJ-4 oligonucleotide does not contain the final three nucleotides coding for the C-terminal lysine residue of HRG. PCR with oligonucleotide pairs (ALJ-1 + ALJ-2), (ALJ-1 + ALJ3), (ALJ-1 + ALJ-4) or (ALJ-1 + ALJ-5) was used to produce the four fragments or full-length forms of HRG cDNA as indicated. L: Leader, N1: first cystatin domain, N2: second cystatin domain, HRR: histidine-rich region, C: C-terminal domain. (b) Generation of HRG expression constructs. HRG cDNAs were cloned into pCR3.1 before being digested with EcoRI and KpnI and subcloned into the pFastBac vector that had been linearised with EcoRI and KpnI.
2.13.1 Generation of recombinant human full-length HRG and HRG fragment expression constructs

Using the pUC9-HRG plasmid construct as a template (Koide et al., 1986b), polymerase chain reaction with oligonucleotides ALJ-1 and ALJ-5 was used to amplify a cDNA encoding the full-length human HRG comprising the leader (L), first cystatin (N1) domain, second cystatin (N2) domain, histidine-rich region (HRR), and C-terminal (C) domain coding regions (Figure 2.7a). A mutant form of HRG (C-mut HRG) cDNA comprising the full-length HRG coding region lacking the C-terminal lysine residue 507 was generated by amplification with oligonucleotides ALJ-1 and ALJ-4. The N1 coding region was generated by amplification with oligonucleotides ALJ-1 and ALJ-2, while a cDNA encoding the first two cystatin domains (N1N2) was generated by amplification with oligonucleotides ALJ-1 and ALJ-3. Both N1 and N1N2 were engineered to contain a hexa-histidine tag at the 3′ end of the molecules through the inclusion of 6 tandem histidine codons in oligonucleotides ALJ-2 and ALJ-3 (Figure 2.7a). All HRG and HRG fragment cDNAs were cloned into the eukaryotic expression vector pCR3.1 (Figure 2.7b), and then spliced out by digesting with EcoRI and Kpnl restriction enzymes, and subcloned into the mammalian expression vector pFastBac (Figure 2.7b).

2.14 Epitope specificity of HRG-specific mAbs

Five mouse monoclonal antibodies (mAbs) were available that were specific for human HRG and were supplied by AGEN, Brisbane, Australia (Table 2.1). Studies were undertaken to map the mAb epitope recognition sites within human HRG. Using the four recombinant human HRG proteins (full-length HRG, C-mutant HRG, N1N2, and N1), and plasma-derived human HRG, direct and competitive inhibition ELISA studies were performed to map the mAb binding domains within HRG (Figure 2.8a). ELISA studies confirmed that all five of the HRG-specific mAbs detected plasma-derived HRG. The HRG-2, HRG-4 and HRG-5 mAbs detected recombinant full-length HRG, C-mutant HRG and the N1N2 domain. The first cystatin fragment of HRG, N1, was not recognised by any of the HRG mAbs, while HRG-1 and HRG-3 only bound to plasma derived HRG. These results suggest that the epitopes recognised by the HRG-2, HRG-4 and HRG-5 mAbs is probably located within the N2 cystatin-like domain of HRG, since the N1N2 domain, but not the N1 fragment, reacted with these mAbs, although it is possible that both N1 and N2 domains are required for some of these epitopes. Table
2.6 summarises the ability of the different HRG preparations to inhibit the binding of the five HRG-specific mAbs to plastic immobilised plasma-derived HRG, with IC-50 values being presented. These combined ELISA studies indicate that the HRG-specific mAbs bind to HRG with high affinity, with HRG-2, HRG-4 and HRG-5 being the most useful mAbs for future studies as they bind to all recombinant HRG proteins except the small N1 fragment. To further investigate the mAb epitope recognition site of HRG-1, recombinant full-length HRG was digested with plasmin. Interestingly, plasmin cleaved recombinant full-length HRG, but not intact recombinant full-length HRG, was recognised by the HRG-1 mAb (Figure 2.8b), indicating that HRG-1 probably recognises an epitope dependent upon HRG being cleaved by plasmin. The nature of the epitope recognised by the HRG-3 mAb in plasma-derived HRG remains to be determined.

Table 2.6  Ability of the different HRG preparations to inhibit HRG specific mAbs binding to plastic immobilised plasma-derived HRG.

<table>
<thead>
<tr>
<th>HRG-specific mAb</th>
<th>Plasma derived HRG</th>
<th>Full-length recombinant HRG</th>
<th>C-mutant recombinant HRG</th>
<th>N1 domain</th>
<th>N1N2 domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRG-1</td>
<td>250 ng/ml</td>
<td>&gt;100 µg/ml</td>
<td>&gt;100 µg/ml</td>
<td>&gt;100 µg/ml</td>
<td>&gt;100 µg/ml</td>
</tr>
<tr>
<td>HRG-2</td>
<td>7 ng/ml</td>
<td>20 ng/ml</td>
<td>40 ng/ml</td>
<td>&gt;100 µg/ml</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>HRG-3</td>
<td>200 ng/ml</td>
<td>&gt;100 µg/ml</td>
<td>&gt;100 µg/ml</td>
<td>&gt;100 µg/ml</td>
<td>&gt;100 µg/ml</td>
</tr>
<tr>
<td>HRG-4</td>
<td>15 ng/ml</td>
<td>80 ng/ml</td>
<td>250 ng/ml</td>
<td>&gt;100 µg/ml</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>HRG-5</td>
<td>60 ng/ml</td>
<td>80 ng/ml</td>
<td>1 µg/ml</td>
<td>&gt;100 µg/ml</td>
<td>1 µg/ml</td>
</tr>
</tbody>
</table>

*Concentration of recombinant protein that inhibits HRG-specific mAbs (HRG-1 to HRG-5) binding to plastic immobilised plasma-derived HRG by 50% as measured by ELISA.
Figure 2.8. Mapping epitopes recognised by human HRG-specific mAbs. (a) ELISA measuring ability of five different HRG-specific mAbs, HRG-1, HRG-2, HRG-3, HRG-4 and HRG-5 to bind to plastic immobilised plasma-derived full-length HRG, recombinant full-length HRG, C-mutant HRG, N1 fragment and the N1N2 domain. Sf-9 cell culture supernatants were purified using Ni-NTA agarose and were included as a negative control. (b) ELISA measuring the ability of the HRG-1 mAb to bind to plastic immobilised plasmin cleaved recombinant full-length HRG and intact recombinant full-length HRG.
2.15 Stable transfection of mammalian cell lines

Fifty percent confluent pgsA-745 cells (GAG deficient CHO cells) in a 35 mm 6 well plate were stably transfected with cDNA expression constructs (i.e., pCR3.1-FLFcγRI, pCR3.1-FcγRII, pCR3.1:FcγRII-his131, pCR3.1:FcγRII-arg131) using FuGENE 6 (Roche Diagnostic Corporation, IN) transfection reagent (Table 2.7). Cells were incubated for 48 hr with a transfection mixture (100 µl added to each 35 mm well) consisting of 3 µl of FuGENE and 2 µg of cDNA diluted in OptiMem1 (Gibco BRL, NY). Cells were then passaged from each 35 mm well into 3 x 100 mm petri dishes, and 0.9 mg/ml of geneticin (Gibco BRL, NY) (selection pressure) applied. Single colonies were harvested 10-12 days after the addition of the selection reagent, and were maintained in 0.9 mg/ml geneticin selective medium until adequate numbers of cells were available to allow screening for positive cell surface expression of FcγRI and FcγRIIa by immunofluorescence flow cytometry (section 2.4). Cells were routinely cultured in DMEM supplemented with 10% foetal calf serum, PSN, and 21 µg/ml proline.

Table 2.7 Properties of expression constructs used.

<table>
<thead>
<tr>
<th>Expression construct</th>
<th>Description of cDNA insert within plasmid vector</th>
<th>Tag</th>
<th>Cloning restriction sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR3.1-HRG</td>
<td>hHRG: Full length human HRG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR3.1-C-mutHRG</td>
<td>C-mut: Full length human HRG lacking lysine 507</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR3.1-N1</td>
<td>N1: First cystatin domain of human HRG</td>
<td>His6</td>
<td></td>
</tr>
<tr>
<td>pCR3.1-N1N2</td>
<td>N1N2: First two cystatin domains of human HRG</td>
<td>His6</td>
<td></td>
</tr>
<tr>
<td>PFastBac-HRG</td>
<td>hHRG: Full length human HRG</td>
<td></td>
<td>EcoRI and Kpnl</td>
</tr>
<tr>
<td>PFastBac-C-mutHRG</td>
<td>C-mut: Full length human HRG lacking lysine 507</td>
<td></td>
<td>EcoRI and Kpnl</td>
</tr>
<tr>
<td>PFastBac-N1</td>
<td>N1: First cystatin domain of human HRG</td>
<td>His6</td>
<td>EcoRI and Kpnl</td>
</tr>
<tr>
<td>PFastBac-N1N2</td>
<td>N1N2: First two cystatin domains of human HRG</td>
<td>His6</td>
<td>EcoRI and Kpnl</td>
</tr>
<tr>
<td>pCR3.1-FcγRI</td>
<td>FcγRI: Full length human FcγRI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFastBac-ECFcγRI</td>
<td>ECFcγRI: Extracellular domains of human FcγRI</td>
<td>His6</td>
<td>BamHI and XhoI</td>
</tr>
<tr>
<td>pCR3.1-FcγRI-II</td>
<td>FcγRII-11: TM +CT FcγRII spliced to EC FcγRI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR3.1-FcγRII-his131</td>
<td>FcγRII-his131: FL FcγRII with His 131 polymorphism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR3.1-FcγRII-arg131</td>
<td>FcγRII-arg-131: FL FcγRII with Arg131 polymorphism</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter three

Histidine-Rich Glycoprotein Binds to Cell Surface Heparan Sulphate via its Amino-Terminal Domain Following Zn\(^{2+}\) Chelation

3.1 Abstract

Histidine-rich glycoprotein is an \(\alpha_2\)-glycoprotein found in mammalian plasma at high concentrations (~ 150 \(\mu\)g/ml), and is distinguished by its high content of histidine and proline residues. Structurally, HRG is a modular protein consisting of an amino-terminal cystatin-like domain (N1N2), a central histidine-rich region (HRR) flanked by proline-rich sequences, and a C-terminal domain. HRG binds to cell surfaces and numerous ligands, such as plasminogen, fibrinogen, thrombospondin, C1q, heparin and IgG, suggesting that it may act as an adaptor protein by either targeting ligands to cell surfaces, or by cross linking soluble ligands. Despite the suggested functional importance of HRG, the cell binding characteristics of the molecule are poorly defined. In this chapter, HRG was shown to bind to most cell lines in a Zn\(^{2+}\) dependent manner but failed to interact with the CHO cell line, pgsA-745, which lacks cell surface glycosaminoglycans (GAGs). Subsequent treatment of GAG+ve CHO cells with mammalian heparanase or bacterial heparinase III, but not chondroitinase ABC, abolished HRG binding. Furthermore, blocking studies with various GAG species indicated that only heparin was a potent inhibitor of HRG binding. These data suggest that heparan sulphate is the predominate cell surface ligand for HRG, and that mammalian heparanase is a potential regulator of HRG binding. Using recombinant forms of full-length HRG and the amino-terminal N1N2 domain, it was shown that the N1N2 domain binds specifically to immobilised heparin and to cell surface heparan sulphate. In contrast, synthetic peptides corresponding to the Zn\(^{2+}\) binding HRR domain of HRG did not interact with cells. Furthermore, the binding of full-length HRG, but not the N1N2 domain, was potentiated by physiological concentrations of Zn\(^{2+}\). Based on these data, a model is proposed whereby the N1N2 domain of HRG binds to cell surface heparan sulphate, and that the interaction of Zn\(^{2+}\) with the HRR indirectly enhances cell surface binding, possibly by inducing a conformational change in the molecule.
3.2 Introduction

HRG is an approximately 75 kDa α2-plasma glycoprotein synthesised in the liver and found in the plasma of most vertebrates at the relatively high concentration of 100-200 µg/ml (~2 µM). The most distinctive feature of HRG arises from its high content of histidine and proline residues, which each account for ~13% of total amino acids (Koide et al., 1986b). HRG is predicted to be a multi-domain protein consisting of two cystatin-like domains at the amino-terminus (termed N1 and N2), a central histidine-rich region (HRR) flanked by two proline-rich sequences, and a C-terminal (C) domain (Figure 1.1). HRG is known to bind a variety of ligands, including haem and Zn\(^{2+}\) (Guthans and Morgan, 1982; Morgan, 1981; Morgan, 1985), plasminogen (Ichinose et al., 1984; Lijnen et al., 1980), fibrinogen (Leung, 1986), thrombospondin, IgG (Gorgani et al., 1999c; Gargani et al., 1997), C1q (Gorgani et al., 1997) and heparin (Burch et al., 1987; Lijnen et al., 1983a; Peterson et al., 1987), and can interact with various cell surface receptors including Fcγ receptors (FcγR) (Gorgani et al., 1999b; Gorgani et al., 2002), and an undefined T-cell receptor (Saigo et al., 1989). Thus, potentially HRG may regulate numerous biological processes such as haemostasis, fibrinolysis, thrombosis, angiogenesis, leukocyte migration and cancer metastasis.

Despite considerable interest in HRG, many of the fundamental characteristics regarding HRG cell surface binding remain undefined. For example, despite the identification of numerous soluble ligands for HRG, the cell surface ligands for HRG are not well characterised. Indirect evidence suggests that negatively charged glycosaminoglycans (GAGs) may mediate cell surface HRG binding, as heparin is a potent inhibitor of HRG binding to cells (Borza and Morgan, 1998; Brown and Parish, 1994). Zn\(^{2+}\) is also known to interact with HRG, most probably via the proposed metal chelation sites located within the HRR (Guthans and Morgan, 1982; Morgan, 1981; Morgan, 1985), with this interaction enhancing the binding of HRG to cells (Olsen et al., 1996). Similarly, the location of the cell surface binding domain within HRG is unclear. The ability of Zn\(^{2+}\) to enhance cell surface binding, and the assumption that heparin binds to the HRR and inhibits binding, has led to the hypothesis that the HRR interacts with cell surfaces (Borza and Morgan, 1998; Borza et al., 1996; Burch et al., 1987; Peterson et al., 1987). Evidence supporting the view that the HRR of HRG interacts with heparin/heparan sulphate includes the observation that chemical modification of the histidine residues of HRG abolishes heparan sulphate binding.
In contrast, it has also been suggested that a heparin-binding sequence may be located in the N1N2 domain which raises the possibility that this domain of HRG may interact with cells (Brown and Parish, 1994; Koide et al., 1982; Koide et al., 1985).

In this chapter, the cell surface binding properties of HRG have been characterised using recombinant full-length HRG and recombinant N1N2 domain together with various approaches to remove GAGs from cell surfaces. Clear evidence is provided supporting the view that heparan sulphate is the dominant cell surface ligand for HRG, with the interaction being mediated through the N1N2 domain and not the HRR of HRG. Indeed, ELISA studies confirmed that the N1N2 domain specifically binds to immobilised heparin with comparable affinity to full-length HRG. Furthermore, cell surface binding of full-length HRG, but not the N1N2 domain, is potentiated by the presence of physiological concentrations of Zn$^{2+}$. Based on these data, a model is proposed whereby HRG binds to cell surface heparan sulphate via its N1N2 domain with low affinity that is enhanced following Zn$^{2+}$ binding to the HRR. Thus, HRG may play an important physiological and/or pathological role by binding to cell surfaces in local environments that contain high levels of Zn$^{2+}$, such as sites of inflammation or during tumour metastasis and angiogenesis.
3.3 Results

3.3.1 HRG binding to cell surfaces is Zn$^{2+}$ dependent

Previous studies have suggested that the binding of HRG to cell surfaces is highly Zn$^{2+}$ dependent, with physiological concentrations of Zn$^{2+}$ (~20 µM) being particularly efficacious (Olsen et al., 1996). Initial experiments used immunofluorescence flow cytometry to analyse the effect of 20 µM Zn$^{2+}$ on the binding of plasma-derived HRG to six different mammalian cell lines having widely differing tissue and species origins, namely, mouse melanoma cells (B16F1), human T-cells (MT4 and Jurkat), monkey kidney fibroblasts (COS-7), human fibrosarcoma cells (HT1080) and human umbilical vein endothelial cells (HUVEC) (Figure 3.1). HRG bound to five of the six cell lines tested in a highly Zn$^{2+}$ dependent manner, with the exception of HUVEC, which exhibited negligible HRG binding both in the presence or absence of Zn$^{2+}$.

![Figure 3.1](image-url)  
*Figure 3.1. Role of Zn$^{2+}$ in the binding of human HRG to cell surfaces.* Various cell types were incubated with plasma-derived HRG (100 µg/ml) in the presence or absence of 20 µM Zn$^{2+}$, and analysed for HRG binding by immunofluorescence flow cytometry. Filled histograms represent background binding of the HRG-specific mAb, HRG-4, to cells in the absence of HRG, open histograms represent HRG binding to cells as detected by HRG-4.*
3.3.2 Human HRG interacts with cell surface heparan sulphate

It has been postulated previously that cell surface GAGs, such as heparan sulphate, are able to interact with HRG (Borza and Morgan, 1998; Brown and Parish, 1994). In order to test this hypothesis directly, advantage was taken of CHO cell lines that either express cell surface GAGs (CHO-KI) or lack cell surface GAGs (pgsA-745) due to a deficiency in xylosyl-transferase (Esko et al., 1986; Esko et al., 1985). It was found that GAG expressing (GAG+ve) CHO cells bound HRG in a Zn$^{2+}$ dependent manner, but that GAG deficient (GAG-ve) CHO cells did not bind HRG either in the presence or absence of 20 µM Zn$^{2+}$ (Figure 3.2a and 3.2b). These results indicate that HRG binds to cell surface GAGs and this interaction is enhanced by physiological concentrations of Zn$^{2+}$ (20 µM). Previous studies have reported that HRG interacts with the GAG, heparin (Burch et al., 1987; Lijnen et al., 1983a; Peterson et al., 1987). Thus ELISA studies were carried out to analyse the effect of Zn$^{2+}$ on the binding of full-length HRG to plastic immobilised heparin. Consistent with previous results (Borza and Morgan, 1998), HRG interacted with immobilised heparin and this interaction was enhanced ~4-fold in the presence of 20 µM Zn$^{2+}$ (Figure 3.2c). Cell surface GAGs are comprised of a mixture of heparan sulphate, chondroitin sulphate A and C, dermatan sulphate (chondroitin sulphate B) and hyaluronic acid (Capila and Linhardt, 2002). In order to define the specific cell surface GAGs that interact with HRG, cells were treated enzymatically to remove heparan sulphate and chondroitin sulphates A, B and C from GAG+ve CHO cells. Initially, using a heparan sulphate-specific mAb and immunofluorescence flow cytometry, GAG+ve CHO cells were verified for expression of high levels of cell surface heparan sulphate, while GAG-ve CHO cells were shown to not express heparan sulphate (Figure 3.3a). The enzymatic activity of mammalian heparanase, bacterial heparinase III and chondroitinase ABC was verified by demonstrating by FPLC that the enzymes could cleave heparan sulphate or chondroitin 6-sulphate chains, respectively, into smaller fragments (data not shown). GAG+ve CHO cells that were treated with either mammalian heparanase or bacterial heparinase III were completely depleted of surface heparan sulphate (Figure 3.3b and 3.3c). Subsequently, HRG binding to these mammalian heparanase and heparinase III treated cells was found to be markedly reduced (~ 85 - 90%) (Figure 3.3d and 3.3e). In contrast, chondroitinase ABC treatment had no effect on HRG binding (Figure 3.3d and 3.3e). These results indicate that HRG binds specifically to cell surface heparan
sulphate and not chondroitin sulphates, and that heparan sulphate is the principal cell surface receptor for HRG on cells.

Additional binding-inhibition experiments with a range of soluble GAGs were consistent with heparan sulphate being the GAG receptor on cells for HRG. Thus, various sized fragments of bovine lung heparin (4.5 kDa – 16.7 kDa preparations) were potent inhibitors of the interaction of HRG with GAG+ve CHO cells, although very low molecular weight heparin (3.1 kDa) was a relatively ineffectual inhibitor (Figure 3.4 and Table 3.1). In contrast, chondroitin sulphates A, B, C and E were totally inactive as inhibitors (Figure 3.4 and Table 3.1).

Table 3.1  Ability of different GAGs to inhibit cell surface binding of HRG to CHO-K1 cells.

<table>
<thead>
<tr>
<th>GAG</th>
<th>IC-50 (µg/ml) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin 3.1 kDa</td>
<td>50</td>
</tr>
<tr>
<td>Heparin 4.5 kDa</td>
<td>6</td>
</tr>
<tr>
<td>Heparin 10.6 kDa</td>
<td>3</td>
</tr>
<tr>
<td>Heparin 12.5 kDa</td>
<td>3</td>
</tr>
<tr>
<td>Heparin 16.7 kDa</td>
<td>3</td>
</tr>
<tr>
<td>Chondroitin sulphate A</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Chondroitin sulphate B</td>
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<tr>
<td>Chondroitin sulphate C</td>
<td>&gt;100</td>
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<tr>
<td>Chondroitin sulphate E</td>
<td>&gt;100</td>
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* Concentration of GAG that inhibits HRG binding to GAG+ve CHO cells by 50% as measured by immunofluorescence flow cytometry.

Figure 3.2. Role of cell surface GAGs and Zn²⁺ in the binding of human HRG to cell surfaces. (a) CHO cell lines that express cell surface GAGs (GAG+ve CHO) or lack cell surface GAGs (GAG-ve CHO) were incubated with 100 µg/ml of plasma-derived HRG, in the presence or absence of 20 µM Zn²⁺, and then analysed for HRG binding by immunofluorescence flow cytometry. Filled histograms represent background binding of the HRG-specific mAb, HRG-4, to cells in the absence of HRG; open histograms represent HRG binding to cells detected by the HRG-specific mAb, HRG-4. (b) Numerical values showing HRG binding as fold increase in median fluorescence above background for GAG-ve and GAG+ve CHO cells, with or without 20 µM Zn²⁺. Vertical bars represent SEM (n=3). (c) ELISA showing the binding of full-length plasma-derived HRG (0.1 - 53 nM), diluted in PBS/1%BSA containing 20 µM Zn²⁺ or 1 mM EDTA, to plastic immobilised heparin. HRG binding was detected using the HRG-specific mAb, HRG-4. Vertical bars represent SEM (n=3).
a

GAG-ve CHO cells
GAG+ve CHO cells

No Zn\(^{2+}\)
20 \(\mu\)M Zn\(^{2+}\)

events
HRG

b

HRG binding

<table>
<thead>
<tr>
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<th>GAG-ve</th>
<th>GAG+ve</th>
<th>GAG-ve</th>
<th>GAG+ve</th>
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<tr>
<td>20 (\mu)M Zn(^{2+})</td>
<td><img src="image" alt="Graph" /></td>
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<tr>
<td>No Zn(^{2+})</td>
<td><img src="image" alt="Graph" /></td>
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c

Absorbance (405nm)

![Graph](image)

[HRG] nM

- - 20 \(\mu\)M Zn\(^{2+}\)
- 1 mM EDTA
3.3.3  The HRR of HRG does not bind to cells

It has been suggested previously that the HRR of HRG interacts with heparin/heparan sulphate (Borza and Morgan, 1998; Borza et al., 1996; Burch et al., 1987). To test this hypothesis, peptides corresponding to the histidine repeat sequence within the HRR of HRG were synthesised and used in binding studies. The peptides comprised one (L1), two (L2), three (L3), four (L4) or five (L5) repeats of the dominant penta-peptide motif GHHHPH that is tandemly repeated in the HRR of human HRG. Immunofluorescence flow cytometry analysis indicated that biotinylated preparations of the L1, L2 and L3 peptides did not bind to GAG+ve CHO cells, either in the presence or absence of 20 μM Zn²⁺ (Figure 3.5a), with streptavidin-PE being used to monitor peptide binding. Furthermore, HRG blocking experiments revealed that high concentrations (100 μM) of peptides L1, L2, L3, L4 or L5 failed to inhibit full-length HRG binding to GAG expressing CHO cells and B16F1 melanoma cells (Figure 3.5b). These data contrast with previous indirect evidence that the HRR binds heparan sulphate (Borza and Morgan, 1998; Borza et al., 1996; Burch et al., 1987), and imply that the cell surface binding domain of HRG may be located in another region of the molecule.

Figure 3.3. Human HRG interacts with cell surface heparan sulphate and not chondroitin sulphate. (a) Cell surface expression of heparan sulphate by the GAG expressing (GAG+ve) and GAG deficient (GAG-ve) CHO cell lines was assessed by immunofluorescence flow cytometry using a heparan sulphate-specific mAb. (b) The effect of mammalian heparanase or bacterial heparinase III treatment on heparan sulphate expression by the GAG expressing CHO cell line. (c) Fold change in median fluorescence representing heparan sulphate expression on GAG expressing CHO cells following mammalian heparanase or bacterial heparinase III treatment. Data mean ± SEM of 3 determinations. (d) Representative flow cytometry histograms showing the effect of mammalian heparanase or chondroitinase ABC treatment of HRG binding to GAG+ve CHO cells. (e) HRG binding (fold increase in median fluorescence above background) following the mammalian heparanase or chondroitinase ABC treatments. Data mean ± SEM of 3 determinations. Cells were incubated with 100 μg/ml of plasma-derived HRG in the presence of 20 μM Zn²⁺. With flow cytometry histograms, the filled histograms represent background binding of either the heparan sulphate or HRG specific mAb, F58-10E4 and HRG-4 respectively, to cells; open histograms represent heparan sulphate expression or HRG binding as detected by the appropriate mAb.
GAG+ve CHO cells

GAG-ve CHO cells

Mammalian heparanase

Bacterial heparinase III

GAG+ve CHO cells

Mammalian heparanase

Chondroitinase ABC

HRG binding

Control

Mammalian Heparanase

Chondroitinase ABC
3.3.4 Production of recombinant full-length human HRG and the amino-terminal domain, N1N2.

Earlier sequence homology studies have suggested that the N1N2 domain of HRG contains a heparin-binding motif and may represent the region of HRG that interacts with cell surface heparan sulphate. In order to test this possibility, recombinant full-length human HRG and the amino-terminal fragment of HRG, N1N2 (Figure 3.6a), was produced in insect cells using a baculovirus expression system. A hexa-histidine tag was engineered onto the C-terminus of N1N2 to allow purification of the recombinant protein by Ni-NTA chelation chromatography. The recombinant full-length HRG was made untagged as it contains sufficient histidine residues in the HRR to allow Ni-NTA agarose affinity purification. Western blot analysis of purified recombinant full-length HRG showed a single band at the anticipated molecular weight of 75 kDa, and the N1N2 preparation showed a prominent band at the appropriate molecular weight of 35 kDa using the HRG-specific mAb, HRG-4 (Figure 3.6b). A Sf9 cell culture supernatant was purified using Ni-NTA agarose and was included as a negative control. ELISA studies (Figure 3.6c) indicated that full-length HRG and the N1N2 domain are both recognised by the HRG-specific mAb, HRG-4, demonstrating that the epitope recognised by HRG-4 is located within the N1N2 domain of HRG.

Figure 3.4. GAGs block HRG binding to GAG expressing CHO cells. GAG+ve CHO cells were incubated with plasma derived HRG (100 µg/ml) and either with or without 100 µg/ml of various GAGs including bovine lung heparin (3.1 kDa – 16.7 kDa), and chondroitin sulphate A, B C and E, in the presence of 20 µM Zn²⁺, and then analysed for HRG binding by immunofluorescence flow cytometry. Results are calculated from median fluorescence, and show percent of HRG binding compared to control HRG binding in the absence of any GAGs.
Figure 3.5. Peptides corresponding to the histidine-rich repeat sequence of human HRG do not bind to GAG expressing CHO cells and do not block HRG binding to GAG expressing CHO cells. (a) GAG+ve CHO cells were incubated in the presence or absence of 20 µM Zn\(^{2+}\) with biotinylated peptides which correspond to one (L1), two (L2) or three (L3) repeats of the consensus histidine penta-peptide of the HRR of HRG (GHHPH) (100 µM), with peptide binding being detected by using R-phycoerythrin conjugated streptavidin and immunofluorescence flow cytometry. Results are shown as fold increase of peptide binding above background, and a value of 1 represents background median fluorescence of streptavidin-PE (shown as dotted line). Binding of HRG (100 µg/ml) as detected by the HRG-specific mAb, HRG-4, is presented as a positive control. (b) GAG+ve CHO cells and B16F1 melanoma cells were incubated with HRG (100 µg/ml) and 20 µM Zn\(^{2+}\), in the presence or absence of peptides corresponding to 1-5 repeats of the consensus histidine penta-peptide (L1-L5) (100 µM), and then analysed for HRG binding using the HRG-specific mAb, HRG-4 by immunofluorescence flow cytometry. Results are shown as percent HRG binding compared to control (dotted line) HRG binding in the absence of any GAGs, with vertical bars representing SEM (n=3).
3.3.5 The N1N2 domain of HRG binds to cells

ELISA studies showed that the recombinant N1N2 domain of HRG binds to plastic immobilised heparin with similar affinity to the recombinant full-length HRG in the presence of 20 µM Zn\(^{2+}\) (Figure 3.7a). Immunofluorescence flow cytometry studies were performed to determine whether the N1N2 domain binds to cells. Firstly, it was established that recombinant full-length HRG produced in insect cells bound to GAG+ve CHO cells. Indeed, the recombinant full-length HRG, as with plasma-derived HRG, bound to GAG expressing CHO cells in a Zn\(^{2+}\) inducible manner (Figure 3.7b and 3.7d), and the binding was totally inhibited by 100 µg/ml of 12.5 kDa heparin (Figure 3.7b). As expected, recombinant full-length HRG did not bind to GAG deficient CHO cells (Figure 3.7b and 3.7d). Similarly, N1N2 bound to GAG+ve CHO cells in the presence of 20 µM Zn\(^{2+}\), and interestingly, also bound equally well to these cells in the absence of 20 µM Zn\(^{2+}\) (Figure 3.7c and 3.7d). Similar to full-length HRG, the binding of N1N2 to GAG expressing CHO cells was also inhibited by 100 µg/ml of 12.5 kDa heparin, suggesting that the N1N2 domain interacts with the same putative cell surface receptor as full-length HRG, namely heparan sulphate (Figure 3.7c). Consistent with this observation, N1N2 did not bind to GAG deficient CHO cells (Figure 3.7c and 3.7d).

Figure 3.6. Production of recombinant full-length human HRG and the amino-terminal region of HRG (N1N2). (a) Schematic representation of full-length recombinant HRG and the amino-terminal fragment of HRG, N1N2. N1N2 contains a hexa-histidine tag engineered onto the C-terminus of the molecule. Inter- and intra- domain disulfide bonds are represented by thick red lines. Recombinant proteins were produced by baculovirus expression in the Sf-9 insect cell line. (b) Western blot analysis of Ni-NTA agarose purified recombinant HRG and N1N2 region on reducing SDS-PAGE gels. Recombinant HRG (75 kDa) and the N1N2 domain (35 kDa) were detected by a HRG-specific mAb, HRG-4. A Sf-9 cell culture supernatant was purified using Ni-NTA agarose and was run as a negative control. (c) ELISA assay showing that plastic immobilised recombinant HRG and N1N2 domain bind the HRG-specific mAb, HRG-4, with Sf-9 proteins, as in panel (b), included as a control. Vertical error bars represent SEM (n=3). (d) Coomassie blue stained SDS-PAGE gel analysis of full-length HRG (75 kDa) and the amino-terminal domain, N1N2 (35 kDa). (e) Sample ELISA assay showing that recombinant HRG binds to the HRG-specific mAb, HRG-4 in a concentration dependent manner under non-denaturing conditions.
a

Full-length HRG

N1 N2 P1 HRR P2 C
1 112 113 229 255 314 330 389 398 439 440 507

N1N2 his x 6

N1 N2 his x 6
1 112 113 229

b
c

75 kDa

35 kDa

Absorbance (405 nm)

HRG N1N2 Sf-9

control

d
e

full-length HRG

75 kDa

N1N2

35 kDa

Absorbance (405 nm)

[Recombinant HRG] µg/ml

0.005 0.05 0.5 5
Figure 3.7. Ability of recombinant human HRG and the N1N2 domain of HRG to bind to cell surfaces. (a) ELISA depicting the ability of full-length recombinant HRG and the N1N2 domain of HRG (0.1-106 nM), diluted in PBS/1%BSA/20 µM Zn²⁺, to plastic immobilised heparin. HRG and N1N2 binding was detected using the HRG-specific mAb, HRG-4. Vertical bars represent SEM (n=3). (b) Binding of recombinant full-length HRG (50 µg/ml) to GAG+ve CHO cells in the presence or absence of 20 µM Zn²⁺ (left panel), in the presence or absence of 100 µg/ml of 12.5 kDa bovine lung heparin and 20 µM Zn²⁺ (center panel), or to GAG-ve CHO cells in the presence of 20 µM Zn²⁺ ± heparin (right panel) as assessed by immunofluorescence flow cytometry. Filled histograms represent background binding of the HAG-specific mAb, HRG-4, to cells in the absence of HRG; open histograms represent HRG binding. (c) Representative flow cytometry histograms of binding of the N1N2 domain (50 µg/ml) to GAG+ve and GAG-ve CHO cells as in panel (b). (d) Quantitative comparison of the Zn²⁺ dependence of binding of full-length recombinant HRG or the N1N2 domain of HRG to GAG+ve or GAG-ve CHO cells. HRG/N1N2 binding expressed as fold increase in median fluorescence relative to background with error bars representing SEM (n=3).
Figure a shows a graph with absorbance at 405nm plotted against [HRG/N1N2] nM. The graph includes data points for HRG and N1N2.

Figure b includes three panels showing histograms of GAG+ve CHO cells, GAG+ve CHO cells with heparin, and GAG-ve CHO cells. Each panel has data for HRG and N1N2.

Figure c includes three panels showing histograms of GAG+ve CHO cells, GAG+ve CHO cells with heparin, and GAG-ve CHO cells. Each panel has data for N1N2.

Figure d shows a bar graph comparing HRG/N1N2 binding. The bars represent Full-length HRG and N1N2 for GAG+ve CHO cells and GAG-ve CHO cells.
3.4 Discussion

Despite numerous studies suggesting that HRG can interact with cell surfaces, the basic cell binding characteristics of the molecule remain poorly defined. This chapter demonstrated that human HRG, at physiological concentrations (~100 µg/ml), binds to cell surface heparan sulphate via its amino-terminal domain (N1N2), and that binding is greatly potentiated by the presence of physiological concentrations (~20 µM) of Zn\(^{2+}\). Similarly, it was shown that full-length HRG binds to immobilised heparin, an interaction that is enhanced ~ 4-fold in the presence of 20 µM Zn\(^{2+}\), and that the N1N2 domain of HRG binds to immobilised heparin with a comparable affinity to full-length HRG.

It has been postulated previously that HRG binds to negatively charged GAGs on cell surfaces (Brown and Parish, 1994; Parish et al., 1984), however, no direct evidence was provided to support this claim. Our results reveal that HRG cannot bind to the CHO cell line, pgsA-745, which lacks cell surface GAGs, although HRG readily binds to the parent cell line, CHO-K1 that is not GAG deficient. Furthermore, treatment of the CHO-K1 cell line with mammalian heparanase or bacterial heparinase III, processes that remove cell surface heparan sulphate, essentially abolished HRG binding, whereas chondroitinase ABC treatment had no effect. Combined with the observation that heparin was the only GAG tested that inhibited HRG binding to cells, these data support the conclusion that heparan sulphate is an important cell surface ligand for HRG. HRG has been reported to interact with other specific cell surface receptors, such as FcγR (Chang et al., 1992b; Gorgani et al., 1999b; Gorgani et al., 2002), and an undefined T-cell receptor (Saigo et al., 1989), however, ubiquitously expressed heparan sulphate is likely to be the predominant HRG receptor on cells. Of particular interest was the finding that treatment of cells with mammalian heparanase resulted in the complete removal of cell surface heparan sulphate, with a resultant reduction in HRG binding. This result implies that the local production of heparanase may provide an endogenous mechanism for regulating the interaction of HRG with cell surfaces.

Studies were also conducted to define the domain of HRG that binds to cells. The results collected in this chapter of study contrast with previous circumstantial evidence which suggests that HRG binds to cells via its HRR and that heparin binds to the HRR (Borza and Morgan, 1998; Borza et al., 1996; Burch et al., 1987; Peterson et al., 1987).
These previous studies relied on the observation that Zn\(^{2+}\), which binds to chelation sites in the HRR, regulates cell surface binding, but no direct evidence has been published demonstrating that the HRR binds to cells. In order to resolve the issue, synthetic peptides representing multiple repeats (1-5) of the dominant penta-peptide sequence in the HRR of human HRG were examined for whether they interacted with cells. Contrary to earlier predictions, it was found that all 5 synthetic peptides were unable to inhibit HRG binding to cells, even when used at a 50-fold molar excess, and biotinylated versions of three of the peptides (1-3 penta-peptide repeats) also did not bind to CHO-K1 cells. Recent studies in this laboratory also indicate that these same synthetic peptides containing multiple penta-peptide repeats exhibit anti-angiogenic activity \textit{in vitro} (A. Bezos and C. Parish, unpublished), which is in agreement with studies by Simantov \textit{et al.} (2001), and Olsson \textit{et al.} (2004), suggesting that the HRR plays an important role in targeting angiogenesis, and not mediating the binding of HRG to cells. Since it has also been hypothesised that the N1N2 region of HRG contains a heparin-binding site (Koide \textit{et al.}, 1986a), recombinant forms of both full-length HRG and the amino-terminal N1N2 domain of HRG were produced to directly investigate the cell surface binding properties of the N1N2 domain. The results clearly indicate that the N1N2 domain binds to immobilised heparin and to cells in a heparin-inhibitable manner and that, unlike full-length HRG, this binding is not dependent on Zn\(^{2+}\).

The sequence of the \textit{N}-terminal domain of HRG was analysed for classic heparin-binding motifs, although none could be identified (Cardin and Weintraub, 1989). However, not all heparin-binding proteins exhibit classic heparin-binding motifs, as in some cases the protein conformation brings together basic residues that are actually distant in the linear sequence. Thus the spatial orientation of basic residues rather than sequence proximity remains an important factor in determining heparin-binding capacity. Antithrombin III represents one such heparin-binding protein that does not contain a classic heparin-binding motif, where it appears that both linearly contiguous basic heparin-binding residues as well as remote basic residues are appropriately positioned in the structure of the protein to bind heparin (Huntington \textit{et al.}, 1996). The heparin-binding domain of antithrombin III is located within its \textit{N}-terminal domain and, interestingly, HRG shares ~40% sequence identity with this region of antithrombin III (Koide \textit{et al.}, 1986a; Koide and Odani, 1987; Koide \textit{et al.}, 1985). The heparin-binding
domain of antithrombin III has been extensively studied, and Arg46, Arg47, Lys114 and Lys125 have been identified as key basic residues that bind to negatively charged heparin (Koide et al., 1986a; Schedin-Weiss et al., 2002a; Schedin-Weiss et al., 2002b). Interestingly, a number of these residues are also conserved in HRG. Arg46 and Arg47 are conserved in human, mouse, rat and rabbit HRG, Lys125 is conserved in human, mouse and rat HRG, whereas Lys114 is substituted with a conserved basic residue, Arg in human HRG. Collectively, the sequence alignment between HRG and antithrombin III suggests that Lys22, Arg23, Arg77, Arg78, Arg135 and Lys146 in human HRG could possibly constitute heparin-binding residues. However, it can only be speculated that these basic amino acids constitute the heparin-binding region within HRG, and clearly, further work is needed to define the specific heparin/heparan sulphate binding residues within the N1N2 domain of HRG.

One of the most intriguing aspects of the current study was the observation that the binding of full-length HRG to most cells is highly $\text{Zn}^{2+}$ dependent, whereas binding of the N1N2 domain was unaffected by physiological concentrations of $\text{Zn}^{2+}$. Divalent cations, in particular $\text{Zn}^{2+}$, are known to interact with the HRR of HRG (Morgan, 1978; Morgan, 1981; Morgan, 1985). Kazama and Koide (1992) first noted that physiological concentrations of $\text{Zn}^{2+}$ ($\sim 20 \mu \text{M}$) could enhance the ability of HRG to bind and neutralise heparin in an *in vitro* system. Olsen et al. (1996) then investigated the effect of $\text{Zn}^{2+}$ on cell surface HRG binding, and showed that $\text{Zn}^{2+}$ strongly potentiated the binding of HRG to various T-cell lines. Similarly, the current study found that physiological concentrations of $\text{Zn}^{2+}$ ($\sim 20 \mu \text{M}$) potentiated the binding of HRG to 5 out of 6 cell lines tested, in all cases resulting in at least a $\sim 10$-fold increase in HRG binding. The one exception was HUVEC, which failed to interact with HRG, either in the presence or absence of $\text{Zn}^{2+}$, raising the interesting possibility that vascular endothelial cells may lack the heparan sulphate motif recognised by HRG. Whether this is due to HRG recognising a specific heparan sulphate sequence that is absent on endothelial cells, or due to the requirement for a second receptor remains to be determined. Based these findings, a model is proposed (Figure 3.8), whereby full-length HRG binds with low affinity to cell surface heparan sulphate in the absence of physiological concentrations of $\text{Zn}^{2+}$ via its N1N2 domain. Following chelation of $\text{Zn}^{2+}$ by the HRR, however, a change is induced in the molecule that results in the protein binding with higher affinity to cell surface heparan sulphate. This enhanced binding
could be due to the interaction of Zn\(^{2+}\) with the HRR inducing a conformation change within the protein that results in enhanced N1N2 binding to heparan sulphate. Alternatively, Zn\(^{2+}\) could mediate cross-linking of HRRs in adjoining HRG molecules, the resultant dimeric/multimeric HRG complexes having an increased avidity for cell surface heparan sulphate.

Collectively, this study provides new insights into the cell surface binding characteristics of HRG. Physiologically, HRG binding to cell surface heparan sulphate may be regulated by high local Zn\(^{2+}\) concentrations that can occur at sites of tissue injury where degranulating platelets locally release high levels of Zn\(^{2+}\) from intracellular stores (Aktulga, 1974; Gorodetsky et al., 1993). The modular structure of HRG could provide a mechanism for the molecule to bind to cells via its N1N2 domain, and then cross-link other ligands, such as plasminogen, to cell surfaces. Thus HRG could act as an adaptor molecule, tethering ligands to cells at sites of high local concentrations of Zn\(^{2+}\). Armed with this important basic understanding of the interaction of HRG with cell surfaces, further studies described in subsequent chapters of this thesis are aimed at identifying the functional role of HRG as an extracellular adaptor molecule.

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**Figure 3.8. Proposed model depicting the binding of HRG to cell surfaces.** HRG binds to cell surface heparan sulphate chains via its N1N2 amino-terminal domain with low affinity in the absence of physiological concentrations of Zn\(^{2+}\) (top panel). In the presence of physiological Zn\(^{2+}\) concentrations, the HRR binds Zn\(^{2+}\) which induces a conformational change in HRG that is transmitted throughout the molecule. The change in conformation allows a more kinetically favourable interaction of the N1N2 domain with cell surface heparan sulphate molecules (bottom panel). Disulphide bonds are indicated by red lines.
Plasminogen has been implicated in extracellular matrix degradation by invading cells, but a high affinity cell surface receptor for the molecule has not been identified. Previous studies have reported that the plasma protein HRG interacts with plasminogen and cell surfaces, raising the possibility that HRG may immobilise plasminogen/plasmin to cell surfaces. This chapter describes experiments that show, based on optical biosensor analyses, that immobilised HRG interacts with soluble plasminogen with high affinity and with an extremely slow dissociation rate. Furthermore, the HRG-plasminogen interaction is lysine dissociable and predominately involves the amino-terminal domain of HRG, but not the C-terminal lysine of the molecule. HRG is also shown to tether plasminogen to cell surfaces, with this interaction being potentiated by elevated Zn\(^{2+}\) levels and low pH, conditions which prevail at sites of tissue injury, tumour growth and angiogenesis. In fact, tumour cells coated with HRG under conditions of low pH and elevated Zn\(^{2+}\) were found to be more metastatic in vivo. Based on these data, a model is proposed whereby HRG acts as a soluble adaptor molecule that binds to cells at sites of tissue injury, tumour growth and angiogenesis, providing a high-affinity receptor for tethering plasminogen to the cell surface, and thereby enhancing the migratory potential of cells.
4.2 Introduction

Plasminogen and plasmin are two plasma proteins generally known to play a pivotal role in fibrinolysis (Collen, 1999; Lijnen, 2001). Plasminogen is a 92 kDa modular glycoprotein consisting of a pre-activation peptide, five kringle domains and a catalytic C-terminal serine proteinase domain (Parry et al., 2000), with healthy adults having a plasma concentration of 150-200 µg/ml (~1.5-2 µM) (Miyashita et al., 1988). Two isoforms of plasminogen occur which differ in their extent of glycosylation (Hayes and Castellino, 1979a; Hayes and Castellino, 1979b; Hayes and Castellino, 1979c). Plasminogen-I is di-glycosylated, carrying two carbohydrate moieties, and is thus 2 kDa larger than plasminogen-II, which carries only one carbohydrate moiety (Miyashita et al., 1988). Specifically, plasminogen-I has 9 or 10 monosaccharide units linked N-glycosidically at Asn288, and 3 or 4 residues linked O-glycosidically at Thr345, whereas plasminogen-II carries only the Thr345 attached carbohydrate (Hayes and Castellino, 1979a; Hayes and Castellino, 1979b).

Plasmin, the active form of plasminogen, is the proteolytic enzyme responsible for degrading fibrin clots and plays an important role in maintaining vascular homeostasis (Collen, 2001). Recent data also indicate that the plasminogen system aids directional cell migration associated with embryogenesis, development, tissue remodelling (Dano et al., 1985; Plow et al., 1995; Vassalli et al., 1991), inflammation, angiogenesis and tumour metastasis (Chapman et al., 1982). This role in cell migration depends on the capacity of plasmin either to directly degrade a number of extracellular matrix proteins or to activate other proteases with matrix degrading capabilities (Brunner et al., 1998; Chapman, 1997; Estreicher et al., 1990; Lahteenmaki et al., 2000; Ploplis and Castellino, 2000). Efficient cell migration and/or invasion usually requires that degradative enzymes be expressed on the surface of cells, rather than being secreted into the extracellular environment. However, the fact that high affinity plasminogen receptors have not yet been identified on cells raises the question of how plasminogen is tethered onto the cell surface. A number of low affinity (K_d ~ 1 µM) receptors for plasminogen have been defined, such as α-enolase (Miles et al., 1991), gangliosides (Miles et al., 1989), and annexin-II (Brownstein et al., 2001). It has been argued that since plasminogen is present in plasma at ~ 2 µM, it could theoretically associate with cells via these low affinity receptors. However, it is known that plasmin is rapidly inactivated unless it remains bound to cells (Lijnen, 2001), and thus a low affinity
interaction with high rates of dissociation would result in plasminogen being rapidly inactivated. This would suggest the existence of high affinity plasminogen receptors.

Previous studies have shown that an important ligand for HRG is plasminogen/plasmin. Since, as discussed in Chapter 3, HRG can also bind to cell surfaces, an interesting possibility is that HRG can act as an adaptor protein and tether plasminogen/plasmin to cell surfaces. Thus, Chapter 4 describes studies which examine the interaction between HRG and plasminogen using both optical biosensor and cell surface binding assays. These investigations provide the first evidence that HRG can tether plasminogen to the surface of cells, specifically at low pH and in the presence of 20 µM Zn\(^{2+}\), conditions that usually occur at sites of tissue injury, inflammation, tumour growth and angiogenesis. Based on these findings, a model is proposed whereby under conditions of low pH and elevated free Zn\(^{2+}\), HRG binds to cell surfaces and acts as a high affinity receptor for plasminogen. This would, in theory, enhance the efficiency of conversion of plasminogen to plasmin, protect plasmin from inactivation and provide a mechanism for polarising the proteolytic activity of plasmin on the cell surface, resulting in enhanced migratory potential.
4.3 Results

4.3.1 Effect of Zn\(^{2+}\) and pH on the binding of human HRG to cell surfaces

Previous studies have shown that the binding of HRG to surface immobilised heparin is dependent on both Zn\(^{2+}\) and pH, with Borza and Morgan (1998) suggesting that Zn\(^{2+}\) and pH act synergistically to affect the conformation and thus function of HRG. In light of this observation, studies were undertaken to test the effect of Zn\(^{2+}\) and pH on HRG binding to cell surfaces. The metastatic mouse melanoma cell line, B16F1, was used in the cell surface binding studies. Dose response experiments indicated that the potentiating effect of Zn\(^{2+}\) on HRG binding to B16F1 cells plateaued between 5-50 µM but was near maximal at ~ 20 µM Zn\(^{2+}\) (Figure 4.1a), with HRG binding being enhanced ~ 6-fold. In contrast, at high Zn\(^{2+}\) concentrations (100 µM), HRG binding was decreased to less than that observed in the absence of any added Zn\(^{2+}\) (Figure 4.1b). A similar effect of Zn\(^{2+}\) concentration on HRG binding was observed with CHO-KI cells (data not shown). The effect of pH on HRG binding to cell surfaces in the presence of an optimal Zn\(^{2+}\) concentration (20 µM) was also investigated and it was found that binding was minimal at pH 8.0 but steadily increased as the pH was reduced to 6.0 (Figure 4.2a and 4.2b). In fact there was a ~250-fold increase in the level of cell surface bound HRG across this pH range. Thus, it appears that both Zn\(^{2+}\) and pH directly affect the interaction of HRG with cell surfaces, with optimal HRG binding occurring in the presence of ~ 20 µM Zn\(^{2+}\) and at pH 6.0.

4.3.2 Soluble plasminogen binds to immobilised HRG

Previous studies identified plasminogen as an important HRG ligand (Ichinose et al., 1984; Lijnen et al., 1980). Using the optical biosensor, this interaction was further characterised by monitoring the binding and dissociation of soluble plasminogen-I and plasminogen-II to immobilised HRG (Figure 4.3a). These binding studies showed that the interaction of soluble plasminogen-I and II, which differ by one glycosylation site (Hayes and Castellino, 1979a; Hayes and Castellino, 1979b; Hayes and Castellino, 1979c), with immobilised HRG, is essentially identical, and is dependent on the plasminogen concentration over the range tested of 32-2000 nM (3-185 µg/ml). The almost flat dissociation curve (Figure 4.3a) indicates an extremely slow off-rate, implying a high affinity interaction (see analysis below). In fact, even when dissociation
was allowed to occur for many hours, plasminogen remained tightly bound to HRG (data not shown). Nevertheless, bound plasminogen could be very effectively displaced by exposure to 100 mM L-lysine, indicating that lysine can disrupt HRG-plasminogen complexes and hence the interaction is likely to be mediated by lysine residues (Figure 4.3b). Alternatively, lysine may disrupt the plasminogen-HRG complex by binding to plasminogen and inducing a conformation change which subsequently results in the complex dissociating.

**Figure 4.1.** The effect of Zn²⁺ on HRG binding to B16F1 melanoma cells. (a) B16F1 cells were incubated with plasma derived HRG (100 µg/ml) in PBS/0.1%BSA (pH 7.2) in the presence of 0, 20 or 100 µM Zn²⁺, and then analysed for HRG binding by using the HRG-specific mAb, HRG-4 and immunofluorescence flow cytometry. Representative flow cytometry histograms show HRG binding at 0, 20 and 100 µM Zn²⁺. Shaded histograms represent background binding of the HRG-specific mAb, HRG-4, to cells in the absence of HRG; open histograms represent HRG binding. (b) Numerical values showing HRG binding to B16F1 cells as fold increase in median fluorescence above background at different Zn²⁺ concentrations (0-100 µM). Data mean ±SEM (n=3).
Figure 4.2. The effect of pH on HRG binding to B16F1 melanoma cells. (a) B16F1 melanoma cells were incubated with plasma-derived HRG (100 µg/ml) in PBS/0.1%BSA/20 µM Zn^{2+} at different pHs (6.0 - 8.0), and then analysed for HRG binding by immunofluorescence flow cytometry. Representative flow cytometry histograms show HRG binding at pH 6.0, 6.5, 7.0, 7.5 and 8.0. Shaded histograms represent background binding of the HRG-specific mAb, HRG-4; open histograms represent HRG binding. (b) Numerical values showing HRG binding to B16F1 cells between pH 6.0 - 8.0 as fold increase in median fluorescence above background. Data mean ±SEM (n=3).
An analysis of the binding curves for the interaction between soluble plasminogen and HRG using the BiaEvaluation program showed that the data could be described by both a bivalent analyte model and a two state conformation model. The bivalent analyte model assumes that each molecule of the analyte (plasminogen-I or plasminogen-II) can interact bivalently with one or two immobilised ligand (HRG) molecule(s). Binding to the first HRG molecule is defined by a single set of rate constants \( (K_{a1} \text{ and } K_{d1}) \), and binding to the second HRG molecule is defined by a second set of rate constants \( (K_{a2} \text{ and } K_{d2}) \), thus allowing the model to take cooperative effects into account (Table 4.1).

Such co-operative effects could well explain the extremely slow off-rates that are observed (Figure 4.3b). The two-state conformation model on the other hand, assumes that one molecule of plasminogen binds to one or more molecules of immobilised HRG, and that this is then followed by a conformation change (represented as * in the equation below) in the complex, which stabilises the interaction. Again, two sets of kinetic constants are produced (Table 4.1), involving two-step association and dissociation, shown below.

\[
\begin{align*}
\text{Association} & : \\
A + B & \Rightarrow AB \quad \text{(step 1)} \\
AB & \Rightarrow AB^* \quad \text{(step 2)} \\
\text{Dissociation} & : \\
AB^* & \Rightarrow AB \quad \text{(step 1)} \\
AB & \Rightarrow A + B \quad \text{(step 2)}
\end{align*}
\]

Both the bivalent analyte and two-state conformation models estimate an apparent \( K_d \) value for the interaction of \( \sim 200-300 \) nM, with the most notable feature of the interaction between immobilised HRG and soluble plasminogen being the extremely slow dissociation/off rate, indicating a high affinity, highly stable interaction.

**Table 4.1** Association rate \( (k_a) \) and dissociation rate \( (k_d) \) constants for the Bivalent Analyte model and Two-State Conformation model for describing the interaction of soluble plasminogen-I and plasminogen-II with immobilised HRG.

<table>
<thead>
<tr>
<th>Model</th>
<th>( k_{a1} )</th>
<th>( k_{d1} )</th>
<th>( k_{a2} )</th>
<th>( k_{d2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bivalent Model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasminogen-I</td>
<td>( 4.60 \times 10^4 ) (Ms)(^{-1} )</td>
<td>( 1.88 \times 10^{-1} ) s(^{-1} )</td>
<td>( 1.78 \times 10^5 ) RU(^{-1} )</td>
<td>( 1.21 \times 10^3 ) s(^{-1} )</td>
</tr>
<tr>
<td>Plasminogen-II</td>
<td>( 5.22 \times 10^4 ) (Ms)(^{-1} )</td>
<td>( 2.20 \times 10^{-1} ) s(^{-1} )</td>
<td>( 1.30 \times 10^5 ) RU(^{-1} )</td>
<td>( 1.31 \times 10^3 ) s(^{-1} )</td>
</tr>
<tr>
<td><strong>Two-state Model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasminogen-I</td>
<td>( 3.11 \times 10^5 ) (Ms)(^{-1} )</td>
<td>( 1.94 \times 10^{-1} ) s(^{-1} )</td>
<td>( 5.20 \times 10^5 ) s(^{-1} )</td>
<td>( 2.48 \times 10^3 ) s(^{-1} )</td>
</tr>
<tr>
<td>Plasminogen-II</td>
<td>( 2.72 \times 10^5 ) (Ms)(^{-1} )</td>
<td>( 1.89 \times 10^{-1} ) s(^{-1} )</td>
<td>( 4.54 \times 10^5 ) s(^{-1} )</td>
<td>( 2.51 \times 10^3 ) s(^{-1} )</td>
</tr>
</tbody>
</table>
Figure 4.3. Binding of soluble plasminogen to immobilised HRG using an optical biosensor. (a) The binding of human plasminogen-I and plasminogen-II to human plasma-derived HRG was examined using a BIAcore 2000 biosensor. Plasma-derived human HRG was covalently attached to the carboxymethylated dextran surface of a CM5 sensor chip and human plasminogen-I or plasminogen-II at the indicated concentrations (diluted in PBS, pH 7.2) was injected into the flow-cells, with binding and dissociation being monitored in each case for 10 min. A flow-cell within the CM5 sensor chip with no immobilised HRG served as a control. The figure depicts representative sensorgrams for each plasminogen concentration used. (b) Analysis of the binding of plasminogen-I and plasminogen-II to immobilised plasma-derived HRG using the BiaEvaluation program. Actual data is shown in coloured lines, and fitted data is shown as black lines. Fitted data are comparable with two model types, either a Bivalent Analyte, or a Two-State Conformation model.
b

Plasminogen-I
Bivalent Analyte Model

Plasminogen-I
Two-State Conformation Model

Plasminogen-II
Bivalent Analyte Model

Plasminogen-II
Two-State Conformation Model

response units

seconds
4.3.3  

Plasminogen binding to the surface of B16F1 cells is dramatically enhanced in the presence of HRG

Although earlier studies have shown that plasminogen can interact with HRG, there have been no previous indication that HRG can potentiate the interaction of plasminogen with the cell surface. Using flow cytometry, it was found that plasminogen (150 µg/ml) binding to the surface of B16F1 cells is greatly enhanced (up to 14-fold) when the cells have been either pre-coated with plasma-derived HRG (100 µg/ml) (Figure 4.4a, HRG/plasminogen), or when plasminogen is co-incubated with HRG (Figure 4.4a, HRG+plasminogen). There was no difference between HRG mediated plasminogen-I or plasminogen-II binding (Figure 4.4a and 4.4b). The effect of HRG on plasminogen binding to B16F1 cells was maximal when the cells were co-incubated with a mixture of plasminogen and HRG, rather than pre-coating the cells with HRG prior to plasminogen exposure, $P < 0.01$ (Figure 4.4b). HRG also enhanced the binding of plasminogen to cells over a wide range of plasminogen concentrations (0.1-200 µg/ml) (data not shown), although these studies showed that in the presence of physiological concentrations of HRG (100 µg/ml, 1.3 µM), maximal plasminogen binding occurred with 150 µg/ml (~ 1.6 µM) of plasminogen, which is the physiological plasma concentration of this molecule. Further studies aimed at characterising the effect of soluble HRG on plasminogen I or plasminogen-II cell surface binding showed that excess soluble HRG (>5-times molar excess of HRG over plasminogen) (1.6 µM plasminogen, 8 µM HRG) did not inhibit cell surface plasminogen binding (Figure 4.5a). Even relatively low concentrations of HRG (0.3 µM) were sufficient to support near maximum plasminogen binding, suggesting that HRG is an efficient adaptor molecule for binding plasminogen to the cell surface. Initial binding studies indicated that pH affects HRG cell surface binding (Figure 2). Using flow cytometry, it was found that pH regulated the ability of HRG to potentiate the binding of plasminogen to cells (Figure 4.5c). Thus, plasminogen binding steadily increased with decreasing pH, the lowest plasminogen binding occurring at pH 8.0, and the highest at pH 6.0. At all pHs tested, plasminogen binding was virtually totally dependent on the presence of HRG (Figure 4.5d). These data suggesting that pH modulates plasminogen binding by regulating the interaction of HRG with the cell surface.
Figure 4.4. Plasminogen binding to the surface of cells is dramatically enhanced in the presence of HRG. (a) B16F1 cells were pre-incubated with or without plasma derived HRG (100 µg/ml) in PBS/0.1%BSA (pH 7.2, 20 µM Zn²⁺), washed with PBS/0.1%BSA, incubated with plasminogen-I or plasminogen-II (150 µg/ml) in PBS/0.1%BSA (pH 7.2), and then analysed for plasminogen binding by immunofluorescence flow cytometry (upper panels – HRG/plasminogen). Alternatively, B16F1 cells were incubated simultaneously with plasma-derived HRG (100 µg/ml) and plasminogen-I or plasminogen-II (150 µg/ml) in PBS/0.1%BSA (pH 7.2, 20 µM Zn²⁺), and then analysed for plasminogen binding by immunofluorescence flow cytometry (lower panels – HRG+plasminogen). Representative flow cytometry histograms show plasminogen binding ± HRG, with shaded histograms representing background binding of the polyclonal plasminogen Ab to cells without plasminogen, open blue histograms representing plasminogen binding with HRG, and open orange histograms representing plasminogen binding without HRG. (b) Quantitative comparison, based on data from (a), of the effect of pre-incubating cells with HRG (HRG/plasminogen) versus the simultaneous incubation of cells with HRG and plasminogen (HRG+plasminogen) on plasminogen binding to cells. Plasminogen binding is expressed as fold increase in median fluorescence relative to background, with error bars being SEM (n=3). For each treatment condition, white histograms represent plasminogen binding in the absence of HRG, and black histograms represent plasminogen binding in the presence of HRG.
a

Plasminogen-I

Plasminogen-II

0.3 μM

1.3 μM

8.0 μM

Events

Plasminogen

db

Plasminogen binding

0 0.3 1.3 8.0

[HRG] μM
Figure 4.5. Plasminogen-I and plasminogen-II bind to the surface of cells in the presence of excess soluble HRG. (a) B16F1 cells were incubated with plasminogen-I or plasminogen-II (150 µg/ml, 1.6 µM) and 0, 0.3, 1.3 or 8 µM plasma-derived HRG in PBS/0.1%BSA (pH 7.2, 20 µM Zn²⁺), and then analysed for plasminogen binding by immunofluorescence flow cytometry. Representative flow cytometry histograms show plasminogen binding ± HRG, with shaded histograms representing binding of the polyclonal plasminogen Ab to cells in the absence of plasminogen, and open blue histograms representing plasminogen binding with HRG, and open orange histograms representing plasminogen binding without HRG. (b) Numerical values showing plasminogen binding as fold increase in median fluorescence above background, with error bars being SEM (n=3). Black histograms represent plasminogen-I binding, and white histograms represent plasminogen-II binding. (c) B16F1 cells were incubated with plasminogen (150 µg/ml) and either with (black histogram) or without (white histogram) plasma-derived HRG (100 µg/ml) in PBS/0.1%BSA/20 µM Zn²⁺ at pH 6.0, 6.5, 7.0, 7.5 and 8.0, and then analysed for plasminogen binding by immunofluorescence flow cytometry. Plasminogen binding is shown as fold increase in median fluorescence above background, with error bars being SEM (n=3).
4.3.4 **Analysis of the regions of HRG and plasminogen involved in the HRG-plasminogen interaction**

Lysine residues have been predicted to play an important role in the interaction between HRG and plasminogen, the biosensor studies outlined in Figure 4.3 supporting this view. It has been suggested that the C-terminal lysine residue on HRG is a likely candidate as one of the key plasminogen binding residues (Saez *et al*., 1995). On the other hand, it has been proposed that the amino-terminal region of HRG may interact with plasminogen (Saez *et al*., 1995). In order to test these possibilities, recombinant full-length HRG, C-mutant HRG lacking the C-terminal lysine residue, the amino-terminal fragment of HRG, termed N1N2, and the first cystatin domain of HRG, termed N1 were produced in insect cells using a baculovirus expression system (Figure 4.6a). A hexa-histidine tag was engineered onto the C-terminus of N1 and N1N2 to allow purification of the recombinant proteins by Ni-NTA chelation chromatography (Figure 4.6a). The recombinant full-length HRG and C-mutant HRG was made untagged as they contain sufficient histidine residues in their HRR to allow Ni-NTA agarose affinity purification. Western blot analysis of purified recombinant full-length HRG (75 kDa), C-mutant HRG (75 kDa), and the N1N2 domain (35 kDa) showed a single band at the appropriate molecular weight using the HRG-specific mAb, HRG-4, whereas N1 (18 kDa) and N1N2 (35 kDa) also showed dominant bands at the anticipated molecular weight using the hexa-histidine tag specific mAb, His6 (Figure 4.6b). N1 was not detected by HRG-4 (data not shown). A *Sf*-9 cell culture supernatant was purified using Ni-NTA agarose and was included as a negative control. ELISA studies (Figure 4.6c) indicated that full-length HRG, C-mutant HRG, and the N1N2 domain were recognised by the HRG-specific mAb, HRG-4, while the first cystatin fragment of HRG, N1, was only detected by a hexa-histidine specific mAb, His6, suggesting that the epitope recognised by the HRG-4 mAb is located within the N2, and not the N1 portion of the N1N2 domain. Furthermore, these studies indicate that the HRG-4 mAb interacts with both denatured (Western blotting studies) and native (ELISA data) forms of HRG, C-mutant HRG and N1N2.

The recombinant full-length HRG and C-mutant HRG were then tested for their ability to bind plasminogen. The two forms of HRG were immobilised onto the surface of a CM5 sensor chip, and the binding of soluble plasminogen was determined by the
Figure 4.6. (a) A schematic representation of the different recombinant forms of HRG, namely full-length HRG, C-mutant HRG which lacks a terminal lysine residue, the N1N2 amino-terminal domain of HRG and a N1 fragment, with both N1 and N1N2 containing a C-terminal hexa-histidine tag to aid purification. Inter- and intra- domain disulfide bonds are represented as red lines. Recombinant proteins were produced by baculovirus expression in the Sf-9 insect cell line. (b) Western blot analysis of Ni-NTA agarose purified recombinant full-length HRG, C-mutant HRG, and the N1 and N1N2 fragments of HRG on reducing SDS-PAGE gels. Recombinant full-length HRG (75 kDa) and C-mutant HRG (75 kDa) were detected by a HRG specific mAb (HRG-4), whereas the N1 fragment (18 kDa) was detected by a mAb specific for its hexa-histidine tag (His6). In contrast, the N1N2 domain (35 kDa) was detected by both the HRG-4 and His6 mAbs. Ni-NTA purified Sf-9 cell culture supernatant was included as a negative control. (c) ELISA assay showing that recombinant full-length HRG, C-mutant HRG and the N1N2 domain (~5 µg/ml), when immobilised to plastic in their native state, bind HRG-4, whereas the N1 fragment binds His6. Ni-NTA purified Sf-9 cell culture supernatant was included as a negative control.
**Diagram a:**

- **Full-length HRG**
  - Graph shows response units over time in seconds for different concentrations of HRG.
  - Concentrations range from 0nM to 2000nM.

- **C-mutant HRG**
  - Graph shows response units over time in seconds for different concentrations of HRG.
  - Concentrations range from 0nM to 31.3nM.

**Diagram b:**

- **Full-length HRG**
  - Graph shows absorbance at 405nm for different concentrations of HRG.
  - Concentrations range from 0.002 µg/ml to 2.0 µg/ml.

- **C-mutant HRG**
  - Graph shows absorbance at 405nm for different concentrations of HRG.
  - Concentrations range from 0.002 µg/ml to 2.0 µg/ml.
Figure 4.7. Analysis of plasminogen binding to recombinant full-length HRG, C-mutant HRG and the HRG fragments, N1 and N1N2. (a) Recombinant full-length HRG and C-mutant HRG were immobilised on the surface of different flow-cells of a CM5 sensor chip, and human plasminogen diluted in PBS (pH 7.2) at the indicated concentrations, was then injected into the biosensor flow-cells, with binding and dissociation each being monitored for 10 min. A flow-cell within the chip with no immobilised HRG served as a control. The figure depicts representative sensorgrams for each plasminogen concentration. (b) Plasmaderived plasminogen was immobilised on ELISA plates overnight and, following blocking of non-specific binding, various concentrations of recombinant full-length and C-mutant HRG (0.1-2 µg/ml) were allowed to bind to the plates. HRG binding was detected with the HRG-specific mAb, HRG-4, using ABTS detection reagents, and with the optical density being measured at 405 nm. (c) Plasma-derived plasminogen was immobilised on ELISA plates overnight, and following blocking of non-specific binding, 2 µg/ml of recombinant full-length HRG, N1 fragment or N1N2 domain was allowed to bind to the plates, with binding of full-length HRG or the N1N2 domain being detected using the HRG specific mAb, HRG-4, or binding of the N1 fragment being detected using the hexa-histidine specific mAb, His6. Antibody binding was detected as in (b). Data mean ± SEM of 3 determinations.
biosensor. Resultant biosensor sensorgrams (Figure 4.7a) showed that the binding of plasminogen to full-length HRG is essentially identical to the binding of plasminogen to C-mutant HRG. Due to high background binding of soluble HRG we were, unfortunately, unable to use the biosensor to examine the binding of soluble HRG to immobilised plasminogen. As an alternative approach, we immobilised plasminogen in the wells of plastic microtitre plates and used an ELISA to measure the binding of both full-length HRG and C-mutant HRG to the immobilised plasminogen. Again, these experiments indicated that plasminogen binds to C-mutant and full-length HRG with similar affinity, and thus the C-terminal lysine residue on HRG does not appear to be essential for plasminogen binding (Figure 4.7). To further investigate the plasminogen-binding domain within HRG, the recombinant N1 fragment or the N1N2 domain was tested for binding to immobilised plasminogen. ELISA studies indicated that N1N2 exhibited significant binding to plasminogen \( (P < 0.01) \), although binding appears to be somewhat lower than full-length recombinant HRG, whereas the smaller fragment, N1, failed to bind to plasminogen \( (P = NS) \) (Figure 4.7c). Biosensor studies with immobilised N1N2 or N1 and soluble plasminogen were unsuccessful due to the poor immobilisation of these HRG fragments to the surface of the biosensor chips.

Angiostatin is a fragment of plasminogen that exhibits anti-angiogenic activity, which consists of the first four kringle domains and part of the fifth kringle domain. Since the present study has shown that plasminogen binds with high affinity to HRG, it was of interest to determine whether a truncated form of plasminogen also exhibited HRG binding. Based on optical biosensor studies, it was found that angiostatin did not bind with high affinity to immobilised HRG, when compared to plasminogen, which bound HRG with high affinity (Figure 4.8a). Similarly, immunofluorescence flow cytometry studies indicated that the presence of physiological concentrations of HRG (100 µg/ml) could dramatically enhance the binding of plasminogen-I and plasminogen-II to the surface of B16F1 melanoma cells, whereas HRG did not promote the binding of angiostatin to the surface of B16F1 cells (Figure 4.8b). It should be noted that ELISA studies, using immobilised angiostatin, demonstrated that the polyclonal anti-plasminogen antibody used in the cell binding studies reacted strongly with angiostatin (data not shown).
Figure 4.8. Angiostatin, a fragment of plasminogen, does not bind to HRG. (a) Plasma-derived HRG was immobilised onto the surface of a CM5 sensor chip, and human angiostatin or plasminogen (0.5-2 µM) diluted in PBS (pH 7.2) was then injected into the biosensor flow-cell, with binding and dissociation each being monitored for 3 min. A flow-cell within the chip with no immobilised HRG served as a control. The figure depicts representative sensorgrams of 2 µM angiostatin and 2 µM plasminogen binding. (b) B16F1 cells were incubated simultaneously with angiostatin, plasminogen-I or plasminogen-II (150 µg/ml), and either with or without plasma-derived HRG (100 µg/ml) in PBS/0.1% BSA (pH 7.2, 20 µM Zn²⁺), and then analysed for angiostatin/plasminogen binding by immunofluorescence flow cytometry. Angiostatin/plasminogen binding is expressed as fold increase in median fluorescence relative to background Ab binding, with error bars being SEM (n=3). For each treatment condition, white histograms represent plasminogen binding in the absence of HRG, and black histograms represent plasminogen binding in the presence of HRG.
4.3.6  

**Heparanase, another degradative enzyme, also binds to HRG**

Plasmin is a serine protease that aids ECM degradation during cell migration, with studies described in this chapter indicating that HRG can tether plasminogen/plasmin to cell surfaces and, consequently, aid cell invasion. Additional studies were, therefore, undertaken to assess the potential of HRG to interact with other ECM degrading enzymes, specifically the heparan sulphate cleaving endoglycosidase, heparanase. Preliminary optical biosensor data actually demonstrated that soluble heparanase can bind to immobilised HRG (Figure 4.9). Kinetic analysis using BiaEvaluation software suggests an apparent $K_d \sim 5 \times 10^{-8} \text{ M} (~50 \text{ nM})$. Further studies aimed at characterising the interaction between HRG and heparanase on the cell surface will be carried out when a reliable heparanase antibody is available for flow cytometry studies.

![Figure 4.9. Soluble heparanase binds to immobilised HRG.](image)

*Figure 4.9. Soluble heparanase binds to immobilised HRG. Plasma derived HRG was immobilised onto the surface of a CM5 sensor chip, and human heparanase diluted in PBS (pH 7.2) at the indicated concentrations, was then injected into the biosensor flow-cells, with binding and dissociation each being monitored for 4 min. A flow-cell within the CM5 sensor chip with no immobilised HRG served as a control. The figure depicts representative sensorgrams for each heparanase concentration.*
4.3.7 Effect of HRG on melanoma metastasis

Since HRG has the ability to potentiate the interaction of plasminogen with cell surfaces, and plasmin has been implicated in tumour metastasis (Brunner et al., 1998; Estreicher et al., 1990; Lahteenmaki et al., 2000), the effect of pre-coating the metastatic melanoma mouse cell line, B16F1, with HRG under condition of low pH (pH 6.5) and elevated Zn$^{2+}$ (20 µM) on subsequent tumour metastasis was examined. Mice were injected iv with 0.5 x 10$^5$ or 1.0 x 10$^5$ tumour cells/mouse and then lungs were examined for tumour metastasis after 21 days. The results show that recipient mice exhibited significantly higher numbers of lung metastasis when the tumour cells were pre-coated with physiological concentrations (100 µg/ml) of human HRG (Figure 4.10). In fact, pre-incubation of the cells with HRG augmented the average number of lung metastasis by 3-6 fold.

![Figure 4.10. Coating the surface of tumour cells with HRG increases their metastatic potential.](image)

**Figure 4.10. Coating the surface of tumour cells with HRG increases their metastatic potential.** Plasma-derived human HRG (100 µg/ml) was incubated with B16F1 melanoma cells in PBS/0.1%BSA (pH 6.5, 20 µM Zn$^{2+}$), pH and Zn$^{2+}$ conditions that promote significantly higher HRG cell surface binding than occurs in normal plasma. Cells were washed and resuspended in serum free medium (RPMI-1640 medium) before injecting 0.5 x 10$^5$ or 1.0 x 10$^5$ cells into the tail vein of female C57BL/6 mice. Mice were sacrificed 21 days following tumour cell injection and analysed for the number of tumour metastasis identifiable as foci located on the surface of the lungs. Data presented as percent control metastases with vertical bars representing SEM (n=14). Data pooled from 3 separate experiments.
4.4 Discussion

For ECM degrading enzymes, such as plasmin, to optimally aid cell invasion they need to be tethered to the surface of invading cells. Many cell types express specific receptors for urokinase plasminogen activator (uPAR), however to date, no high affinity cell surface receptor for plasminogen has been described, although low affinity (K_d ∼1 µM) generic receptors including annexin II (Brownstein et al., 2001), α enolase (Miles et al., 1991), and gangliosides (Miles et al., 1989) have been identified. Experiments described in this chapter show for the first time that the soluble plasma protein, HRG, can act as an adaptor protein that tethers plasminogen to cell surfaces in a highly stable manner. Interestingly, the binding of HRG (and thus plasminogen) to the cell surface appears to be dependent on acidic pH and enhanced levels of free Zn^{2+} (Figure 4.1 and 4.2). In this regard, Zn^{2+} stored in platelet granules is rapidly released into the plasma following thrombin stimulated platelet degranulation (Aktulga, 1974; Gorodetsky et al., 1993), while pH can drop as much as one pH unit during hypoxia or ischemia, or 0.5 of a pH unit during an inflammatory response due to lactic acidosis. Thus, at sites of inflammation, angiogenesis and wound healing, acidic pH and elevated free Zn^{2+} provide a mechanism for plasmin activity to be selectively expressed on cell surfaces.

Metal divalent cations, in particular, Zn^{2+}, are known to interact with the HRR of HRG (Morgan, 1978; Morgan, 1981; Morgan, 1985). The high concentration of histidine residues located within the HRR also results in HRG having an ionic charge that is sensitive to pH in the range between pH 6-7 as the histidine residues become protonated (Borza and Morgan, 1998). Using optical biosensor studies, Borza and Morgan (1998) found that HRG binding to immobilised heparin was strikingly pH sensitive, with maximum binding occurring at pH < 6.0. Poor HRG binding was observed at physiological pH in the absence of Zn^{2+}, although the interaction was promoted by addition of free Zn^{2+}, and the pH dependence was shifted toward alkaline pH by Zn^{2+} (Borza and Morgan, 1998). The HRR was suggested to act like a pH sensor, whereby Zn^{2+} and pH act synergistically in regulating HRG function. Consistent with these findings, the present study shows that pH can profoundly alter HRG cell surface binding, whereby HRG binding to B16F1 cells is greatly potentiated at pH 6.0 but reduced at pH 8.0 (Figure 4.2). Of particular relevance here is the finding described in Chapter 3, that heparan sulphate is the predominant cell surface ligand for HRG, with Zn^{2+} regulating this interaction and the amino-terminal N1N2 domain of HRG
interacting with this ligand (Figure 3.8). Hence, a model is proposed whereby the HRR of HRG binds Zn$^{2+}$ and enhances HRG cell surface binding to heparan sulphate via its N1N2 domain. Under such conditions, the modular domain structure of HRG would allow cell surface bound HRG to then co-immobilise other molecules, such as plasminogen, to the cell surface.

Previous studies based on chemical modification of lysines, suggest that HRG binds to plasminogen via HRG lysine residues (Saez et al., 1995). The biosensor studies described herein support the notion that the plasminogen – HRG interaction is dependent on the well described lysine binding sites (LBS) (Plow et al., 1995) of plasminogen as free L-lysine reversed the interaction. Since C-terminal lysine residues of many proteins are often involved in plasminogen binding, recombinant HRG lacking the C-terminal lysine residue was produced. Surprisingly, there appeared to be no difference in the binding of plasminogen to C-mutant HRG and full-length HRG (Figure 4.7a and 4.7b), implying that this residue does not play a role in the interaction. On the other hand, the amino-terminal N1N2 domain of HRG, but not a N1 fragment, interacted with plasminogen (Figure 4.7c), although lysine residues within other regions of HRG may also participate in plasminogen binding.

The heavy chain of plasminogen contains five triple loop structures termed ‘kringles’ which are held in a loop structure by three disulphide bridges (Miyashita et al., 1988), and contain one high affinity LBS ($K_d \sim 9 \mu M$) and four or five low affinity LBS ($K_d \sim 5 mM$) that play a crucial role in the regulation of fibrinolysis by interacting specifically with lysine residues on fibrin, $\alpha_2$-antiplasmin and cell surfaces during the physiological lysis of fibrin. Previous studies suggest that HRG binds to the high affinity LBS (Lijnen et al., 1980). In this study, analysis of optical biosensor data suggests that immobilised HRG interacts bivalently with plasminogen, the interaction being found to have extremely slow off-rates (Figure 4.3), suggesting that co-operative binding and/or a change in conformation contributes to the high affinity interaction. It seems highly likely that the two putative HRG binding sites located within plasminogen consist of a high affinity and a low affinity LBS. The present finding that excess soluble HRG does not interfere with the interaction of plasminogen with cell bound HRG (Figure 4.5a and 4.5b), strongly suggests that plasminogen contains at least two HRG binding sites whereas HRG only contains one site. Thus, a multivalent array of HRG displayed on a
cell surface would be able to interact cooperatively with soluble plasminogen to form a highly stable complex. In fact, the demonstration that angiostatin binds with only low affinity to HRG (Figure 4.8a) suggests one of the HRG binding sites is LBS-5, as angiostatin lacks the fifth kringle domain of plasminogen (Figure 4.11).

In blood, plasminogen circulates in a globular (closed) conformation, probably as a safeguard mechanism to prevent uncontrolled plasmin generation. When bound to a cell or fibrin surface through its LBS, it adopts an extended ‘open’ conformation that is more rapidly activated to form plasmin (Borza and Morgan, 1997; Miyashita et al., 1988; Parry et al., 2000; Walther et al., 1975). Borza and Morgan (1997) previously showed, using in vitro techniques, that plasminogen is more rapidly activated to plasmin when associated with immobilised HRG, but not soluble HRG. Cell bound plasmin also remains protected from inactivation by α₂-antiplasmin and α₂-macroglobulin, whereas soluble plasmin is rapidly inhibited by these proteins (Lijnen, 2001), ensuring that the generation and activity of plasmin remains localised to the microenvironment of either the clot or the polarised cell surface. Generation of cell surface proteases is fundamental to a wide variety of in vivo biological processes, with the plasminogen activator/plasmin system known to be important for proteolysis during cellular migration (Brunner et al., 1998; Chapman, 1997; Estreicher et al., 1990; Lahteenmaki et al., 2000; Ploplis and Castellino, 2000; Reich, 1978). Processes including tumour cell invasion, angiogenesis, embryogenesis and leukocyte migration to a site of inflammation, require cells to invade and penetrate neighbouring tissues (Brownstein et al., 2001). Directed cell migration requires localised proteolysis, with u-PAR being rapidly polarised to the leading edge of migrating cells to focus the plasmin mediated ECM degradation (Estreicher et al., 1990; Ploplis and Castellino, 2000), and indeed, increased u-PAR expression correlates with the prognosis of many invasive human cancers (Blasi, 1999; Sappino et al., 1991; Scherrer et al., 1999). Through the generation of plasmin, plasminogen activators catalyse the degradation of most proteins of the extracellular space, including laminin, thrombospondin, fibronectin and fibrinogen (Estreicher et al., 1990). Plasmin can also activate other proteases such as matrix metalloproteinases (Brownstein et al., 2001). Expression of the powerful degradative potential of plasmin heavily depends on plasminogen being associated with the cell surface via a high affinity receptor. In this regard it is interesting to note that tumour cells pre-coated with HRG are more metastatic than their untreated counterparts.
(Figure 4.10), with the cell bound HRG potentially allowing the recruitment of endogenous plasminogen (or other degradative enzymes like heparanase) to the tumour cell surface. However, further studies using plasminogen deficient mice as the tumour cell recipients are required to validate this hypothesis. Thus HRG may potentially provide the means for tumour cells to 'hijack' the degradative potential of plasmin and use its proteolytic ability to aid tumour cell metastasis and tumour angiogenesis. Indeed, cancer related morbidity and mortality are closely linked to the capacity of tumour cells to invade and metastasise (Sanderson, 2001). Further investigation into the interaction of HRG with its ligands is paramount to understanding the function of HRG in haemostasis and tumour cell angiogenesis and migration.

Figure 4.11. Schematic diagram of the tertiary structure of human plasminogen.

The heavy chain of plasmin is indicated by (-----), while the light chain is indicated by (--). The sites of cleavage by plasminogen activators as well as by plasmin are shown. Plasmin is formed by cleavage of the Arg560-Val561 bond by plasminogen activators. Disulphide bonds (\[\text{---}\]), position of the Cys168-Cys296 inter-kringle disulphide bond (\[\text{^\text{C}}\]), carbohydrate moieties (-CHO) and the amino acids forming the active site in plasmin (His, Asp, Ser) are shown. Adapted from Miyashita et al. (1988).
Chapter five

Characterisation of Histidine-Rich Glycoprotein as a Novel Ligand for Leukocyte Fe Receptors

5.1 Abstract

Leukocyte Fc receptors (FcR) link the cellular and humoral arms of the immune system by connecting the antigen-specific interactions of antibodies to the non-specific effector mechanisms of Fc receptor bearing cells. There are three classes of IgG binding FcγR, namely FcγRI, FcγRII and FcγRIII. Previous studies have reported that HRG may interact with FcγRI, the high affinity receptor for IgG. This study utilises recombinant FcγRI, and both recombinant and plasma-derived HRG to provide the first direct evidence that HRG binds to FcγRI, using both ELISA and optical biosensor approaches. In addition, the findings in this chapter also show that HRG binds to FcγRIIa, the low affinity receptor for IgG, using both biosensor and cell surface binding analysis. Interestingly, the interaction between HRG and FcγRIIa appears to be dependent on a FcγRIIa polymorphism that comprises either an arginine or histidine residue at amino acid position 131. The His131 form of FcγRIIa is able to bind IgG2 isotypes, and is the only FcγR capable of binding and efficiently ingesting IgG2 immune complexes; whereas the Arg131 form of FcγRIIa is unable to bind IgG2. Biosensor studies indicated that HRG binds to only the His131 form of FcγRIIa, and not the Arg131 form. Similarly, stable transfected GAG-ve CHO cells expressing either of the polymorphic forms of FcγRIIa also indicated that HRG can bind to cell surface expressed FcγRIIa-his131. Furthermore, biosensor studies suggest that HRG also binds to the IgA receptor, FcαR. Thus, utilising recombinant proteins and stable transfected cell lines, this chapter presents for the first time, evidence that HRG binds to FcγRI, FcγRIIa-his131 and FcαR. These data suggest that HRG may play an important role in regulating immune complex clearance mediated through leukocyte FcR, and therefore autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE).
5.2 Introduction

Leukocyte FcR are cell membrane receptors specific for the Fc portion of immunoglobulins (Ig). FcR form an important connection between the cellular and humoral arms of the immune system by linking the antigen-specific interactions of antibodies to the non-specific effector mechanisms of FcR-bearing cells. Three classes of receptors for the Fc portion of IgG have been defined, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (Ravetch and Kinet, 1991). Functionally, these receptors are either activating (FcγRI, FcγRIIa and FcγRIII), or inhibitory (FcγRIIb). All leukocyte FcγR contain a unique ligand binding α-chain that belongs to the Ig superfamily. However, in order to induce signalling, most FcγR are dependent on the presence of associated promiscuous signalling subunit(s). FcγRI-, FcγRIII- and FcαR-induced signalling depends on the presence of the common FcR γ- or CD3 ζ-signal transducing chain homodimer that contain ITAM sequences in their cytoplasmic tails (Figure 1.12). FcγRIIa and FcγRIIib are the only FcγR class that contains signalling motifs in their own cytoplasmic tail, and thus do not require the presence of a separate signalling subunit for initiation of leukocyte responses (Van Sorge et al., 2003). FcγRIIa has an ITAM signalling motif, while FcγRIIib has an ITIM signalling motif within their cytoplasmic tail. On the other hand, FcγRI (van Vugt et al., 1996), and FcγRIIIa (Hibbs et al., 1989), both require association with the FcR γ-chain for stable cell surface expression, the signalling subunit also being reported to contribute to the high affinity binding properties of FcγRI.

FcγRI has three Ig-like domains in its extracellular ligand-binding domain, whereas FcγRII and FcγRIII have only two Ig-like domains. The additional Ig-like domain in FcγRI is responsible for its unique ability to bind monomeric IgG with high affinity. FcγRI is a heavily glycosylated 72 kDa protein and is anchored into the plasma membrane by a single transmembrane-domain. FcγRI is the only FcγR capable of binding monomeric IgG with high affinity, $K_d \sim 1 \times 10^{-8}$ M. FcγRII is a 40 kDa integral membrane glycoprotein expressed on granulocytes, macrophages, monocytes, B cells (FcγRIIib only) and platelets. FcγRII has low affinity, $K_d < 1 \times 10^{-7}$ M, for monomeric IgG and mediates a variety of biological functions upon clustering by immune complexes (Tate et al., 1992; van de Winkel and Anderson, 1991). FcγRIII is a heavily glycosylated receptor that runs as a broad band of ~ 50 - 70 kDa on SDS-PAGE gels,
and is expressed on neutrophils, eosinophils, macrophages, mast cells and NK cells (Van Sorge et al., 2003; Dijstelbloem et al., 2001). The inhibitory FcγRIII is expressed on all haematopoietic cells except T and NK cells. FcγRIII is not expressed on blood monocytes, but is expressed on monocyte-derived macrophages and peritoneal macrophages (Van Sorge et al., 2003; Dijstelbloem et al., 2001).

The receptor for IgA, FcαR (CD89) is expressed on macrophages/monocytes, eosinophils and granulocytes, as well as tonsilar, splenic and alveolar macrophages (Geissmann et al., 2001; Ravetch and Kinet, 1991; van Egmond et al., 2000). A low affinity interaction (Kd ~ 1 x 10⁻⁶ M) occurs between FcαR and IgA (Monteiro and Van De Winkel, 2003; Wines et al., 1999). IgA is the most abundant serum antibody and the predominate antibody at mucosal sites. IgA binding to FcαR triggers the cellular aspects of IgA-mediated immunity against pathogens, both in the circulation and at the mucosal interface (Monteiro and Van De Winkel, 2003).

Many infections can generate specific IgG subclass responses and therefore the ability of different polymorphic forms of FcγRs to recognise and bind these specific IgG subclasses affects the ability of phagocytes to effectively eliminate opsonized pathogens. In particular, CD32 shows a biallelic polymorphism at position 131, containing either a histidine or arginine residue that affects binding and uptake of antigens opsonised with different IgG2. The polymorphism is located in the second extracellular Ig-like domain, and the histidine to arginine substitution alters the affinity of the receptor for IgG2. Neutrophils from individuals that are homozygous for the FcγRIIa-his131 allele are able to phagocytose and kill IgG2-opsonised bacteria more effectively than neutrophils from individuals that are homozygous for the Arg131 allele (Bredius et al., 1993; Rodriguez et al., 1999; Salmon et al., 1992; Sanders et al., 1994; Wilson et al., 1995). As such, this polymorphism is extremely relevant to FcγRIIa function in health and disease, especially in situations where IgG2 is the predominant antibody subclass generated. Indeed, IgG2 is the most important subclass elicited by encapsulated bacteria in humans including important human pathogens such as Neisseria meningitidis, Haemophilus influenzae and Streptococcus pneumoniae (Van Sorge et al., 2003).
HRG has been suggested to interact with FcγRI, however all studies have used indirect binding approaches. Pre-incubation of HRG with the FcγRI expressing monocytic cell line, THP-1, effectively blocked subsequent IgG binding (Gorgani et al., 1999b). HRG has also been shown to opsonise apoptotic cells and increase the efficiency of their phagocytosis by monocyte-derived macrophages through FcγRI (Gorgani et al., 2002). However, as yet, no direct binding studies or detailed characterisation of the interaction has been performed. In this chapter, recombinant soluble FcγRI was produced to further characterise the proposed interaction between HRG and FcγRI. The recombinant protein was used in both ELISA and optical biosensor studies to demonstrate that HRG binds to FcγRI with high affinity, $K_d \approx 1 \times 10^{-7}$ M (~ 100 nM), providing the first direct evidence supporting this interaction. In addition, soluble polymorphic recombinant forms of FcγRIIa were used in biosensor studies to investigate the interaction between FcγRIIa and HRG. Results indicate that HRG binds to the His131 form of FcγRIIa, but not the Arg131 form. GAG-ve CHO cells stably transfected to express either FcγRIIa-his131 or FcγRIIa-arg131 were also produced, providing independent evidence that HRG binds to cell surface expressed FcγRIIa-his131. Finally, recombinant soluble FcαR was also used in biosensor studies, with results indicating that HRG binds the IgA receptor with moderate affinity, $K_d \approx 8.5 \times 10^{-7}$ M (~ 850 nM).
5.3 Results

5.3.1 Generation of FL human FcγRI and chimeric FcγRI-II expression constructs and production of FcγRI/FcγRI-II stable transfected GAG-ve CHO cells

In order to further characterise the previously reported association between FcγRI and HRG (Gorgani et al., 1999b; Gorgani et al., 2002), it was attempted to produce stable transfected cell lines expressing cell surface FcγRI. Polymerase chain reaction using oligonucleotides FCG-1 and FCG-3 was used to amplify full-length (FL) human FcγRI cDNA encoding the leader (L), extracellular domain 1 (EC1), extracellular domain (EC2) and extracellular domain 3 (EC3), transmembrane (TM) and cytoplasmic tail (CT) coding regions (Figure 5.2a). The FL FcγRI cDNA was cloned into the eukaryotic expression vector pCR3.1 to produce pCR3.1-FLFcγRI (Figure 5.2a). The expression construct pCR3.1-FLFcγRI was transfected into the GAG-ve CHO cell line, pgsA-745. GAG-ve CHO cells lack cell surface GAGs due to a xylosyltransferase deficiency, and subsequently do not exhibit heparan sulphate mediated HRG binding, providing an ideal platform to analyse FcγR mediated HRG binding. Stable transfected clones were selected in the presence of 0.9 mg/ml Genetecin (G418). G418 resistant clones were analysed for cell surface CD64 expression using the CD64 specific mAb, 10.1, and polyclonal CD64 antibody, N19, using immunofluorescence flow cytometry. Unfortunately, none of the 10 analysed clones exhibited CD64 cell surface expression (Figure 5.1b). Subsequently, PCR using FcγRI specific FCG-1 and FCG-3 oligonucleotide primers on cDNA generated from total RNA isolated from the clonal cell lines produced a 1500 bp amplified product of the expected size for FL FcγRI cDNA (data not shown), suggesting that FcγRI mRNA was being produced, but FcγRI protein was not being expressed at the cell surface. Endogenous cell surface expressed FcγRI is normally associated with the γ- or ζ- signalling subunits, and the absence of these signalling subunits in this model may have contributed to the lack of cell surface expressed FcγRI. As FcγRIIa transfected GAG-ve CHO cells were able to be generated that exhibited high levels of FcγRIIa cell surface expression (Figure 5.4b), this prompted the design of a chimeric FcγR comprised of an EC FcγRI domain (3 Ig-like domains), and a FcγRIIa TM and CT, to produce a chimeric receptor containing the FcγRI ligand binding domain that should be efficiently expressed at the cell surface. Chimeric receptor cDNA was generated between EC FcγRI cDNA and the TM and CT
FcyRIIa cDNA (see Appendix 2 for nucleotide and predicted amino acid sequence of FcyRI). The chimeric FcyRI-II cDNA was generated by splice overlap extension (SOE) (Horton et al., 1989) PCR, using three PCR reactions as detailed (Figure 5.1a). To generate the FcyRII cDNA, PCR was used to produce two cDNA fragments; one comprising the L, EC1, EC2 and EC3 coding regions of FcyRI (nucleotides 21 to 885, encoding amino acids 8 to 295), generated by amplification with oligonucleotides FCGR-1 and yRI-IIR on hFcyRI cDNA; and a second comprising the TM and CT coding regions of FcyRII (nucleotides 531 to 957, encoding amino acids 170 to 281), generated by amplification with oligonucleotides yRI-IIF and EG5 on FcyRII cDNA; which were spliced together and amplified in a third SOE PCR using oligonucleotides FCGR-1 and EG5 (Figure 5.1a). The chimeric FcyRI-II cDNA was cloned into the eukaryotic expression vector pCR3.1 producing pCR3.1-FcyRI-II. GAG-ve CHO cells were transfected with pCR3.1-FcyRI-II chimeric expression construct (Figure 5.1a), and stable transfected clones were selected and amplified in the presence of 0.9 mg/ml Genetecin. G418 resistant clones were analysed for cell surface CD64 expression using the CD64 specific mAb, 10.1, and a goat polyclonal CD64 antibody, N19, and immunofluorescence flow cytometry. Again, none of the 10 analysed clones exhibited CD64 cell surface expression (Figure 5.1b), indicating a problem with the receptor being expressed at the cell surface. It had been hoped to test the binding of HRG to either the stable transfected FcyRI or FcyRII expressing GAG-ve CHO cells, however unfortunately, due to the inability to produce FcyRI/FcyRII expressing GAG-ve CHO cells, these studies were unable to be performed.

Figure 5.1. Strategy for the generation of FcyRI-II chimeric expression construct, and production of FcyRI and FcyRII stable transfected cell lines in GAG-ve CHO cells. (a) Fragments encoding (i) L, EC1, EC2 and EC3 of FcyRI generated by PCR amplification with oligonucleotides FCGR-1 and yRI-IIR on FcyRI cDNA and (ii) TM and CT of FcyRII generated by PCR amplification using oligonucleotides yRI-IIF and EG5 on FcyRII cDNA; were spliced together in a third splice overlap extension (SOE) PCR reaction using oligonucleotides FCGR-1 and EG5. The FcyRII chimeric cDNA was then cloned into the pCR3.1 expression vector to produce the pCR3.1-FcyRII expression construct. Arrows indicate direction of transcription. (b) pCR3.1-FLFcyRI and pCR3.1-FcyRII were transfected into GAG-ve CHO cells (pgsA-745) to produce stable GAG-ve CHO cell lines expressing FL FcyRI and FcyRII. The G418 resistant cell lines were screened for cell surface expression of FcyRI (CD64) using the CD64 specific mAb, 10.1 and immunofluorescence flow cytometry. Representative histograms are shown, with filled histograms representing background binding of the secondary conjugate, and open histograms representing CD64 cell surface expression.
a  FcγRI cDNA  

FCGR-1  
5' L EC1 EC2 EC3 TM CT 3'  

→  γRI-IIR  

PCR 1  

5' L EC1 EC2 EC3 3'  

+  5' 19 CT 3'  

→  FcγRII cDNA  

FCGR-2  
5' L EC1 EC2 EC3 CT 3'  

→  pCR3.1-FcγRI-II  

b  FcγRI  

FcγRII  

CD64  

CD64
Both of these cell populations produced the relevant messenger RNA (mRNA) (data not shown), however, the fact that the receptor failed to be expressed at the cell surface suggests that there is a block between the mRNA level and protein expression at the cell surface. Further work is continuing in attempt to resolve the problem of expressing FcyRI on the cell surface of GAG-ve CHO cells.

5.3.2 Generation of his-tagged EC human FcyRI expression construct and production of recombinant EC FcyRI

As described in section 5.3.1, attempts at producing stable cell lines expressing cell surface FcyRI were unsuccessful. In order to resolve the issue of whether HRG could directly interact with FcyRI, recombinant extracellular (EC) FcyRI was therefore produced using a baculovirus expression system in insect cells. A cDNA encoding an extracellular fragment of human FcyRI comprising the L, EC1, EC2 and EC3 coding regions, lacking the TM and CT coding regions, was generated by amplification with oligonucleotides FCGR-1 and FCGR-2 and engineered to contain a hexa-histidine tag at the 3′ end through the inclusion of 6 tandem histidine codons in oligonucleotide FCGR-2 (Figure 5.2a).

Figure 5.2. Strategy for the generation of FL and EC FcyRI expression constructs, and production of recombinant EC FcyRI. (a) Schematic representation of FL FcyRI cDNA shown with oligonucleotide primers FCGR-1, FCGR-2 and FCGR-3 used in PCR reactions positioned at their priming sites with 5′ to 3′ direction indicated by arrows. Oligonucleotide FCGR-2 contains a hexa-histidine tag (his6) engineered at the 5′ end. PCR with oligonucleotide pairs FCGR-1 + FCGR-2 and FCGR-1 + FCGR-3 was used to produce EC and FL FcyRI cDNA respectively. EC FcyRI encodes the leader domain, EC domain 1 (EC1), EC domain 2 (EC2) and EC domain 3 (EC3) coding regions. FL FcyRI comprises the leader, EC domain 1 (EC1), EC domain 2 (EC2), EC domain 3 (EC3), transmembrane (TM) and cytoplasmic tail (CT) coding regions. EC and FL FcyRI cDNAs were cloned into the eukaryotic expression vector pCR3.1 to produce pCR3.1-ECFcyRI and pCR3.1-FLFcyRI respectively. pCR3.1-ECFcyRI was then digested with BamHI and Xhol and subcloned into the mammalian expression vector pFastBac that had been linearised with BamHI and Xhol to produce pFastBac-ECFcyRI. (b) Western blot analysis of Ni-NTA agarose purified recombinant EC FcyRI on reducing SDS-PAGE. Recombinant EC FcyRI (~ 45 kDa) was detected by a mAb specific for the hexa-histidine tag (his6), and a FcyRI (CD64) specific mAb (10.1). THP-1 cell lysates were run as a positive control (FL FcyRI, ~ 70 kDa). A Sf-9 cell culture supernatant was purified using Ni-NTA agarose and was run as a negative control. (c) Sample ELISA assay showing plastic immobilised recombinant EC FcyRI binds to the CD64-specific mAb, 10.1 in a concentration dependent manner under non-denaturing conditions, with Sf-9 proteins, as in (b) included as a negative control.
a) FcγRI cDNA

EC FcγRI cDNA  FL FcγRI cDNA

5' 3'

pCR3.1-ECFcγRI  pCR3.1-FLFcγRI

Xhol

BamHI pFastBac-ECFcγRI

b) THP-1  SF-9  FcγRI  SF-9  FcγRI

70 kDa  45 kDa

10.1  His6

c) Absorbance (405nm)

[FcγRI] µg/ml

0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 0.5

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
The EC FcγRI cDNA was then cloned into the eukaryotic expression vector pCR3.1 to produce pCR3.1-ECFcγRI. Subsequently, EC FcγRI cDNA was excised from pCR3.1-ECFcγRI using \textit{BamHI} and \textit{XhoI} restriction digestion, and subcloned into the mammalian expression vector pFastBac that had been linearised with \textit{BamHI} and \textit{XhoI} to produce pFastBac-ECFcγRI (Figure 5.2a).

Recombinant human EC FcγRI was produced using the ‘Bac-to-Bac’ baculovirus expression system in \textit{Sf}9 insect cells. The recombinant bacmid construct was generated using the pFastBac-ECFcγRI expression construct. EC FcγRI bacmid DNA was verified that it contained the EC FcγRI insert by both PCR and Southern blot analysis (data not shown). A hexa-histidine tag was engineered onto the C-terminus of EC3 to allow purification of the recombinant protein by Ni-NTA chelation chromatography. Western blot analysis of Ni-NTA purified recombinant EC FcγRI showed a dominant band at the expected molecular weight of ~ 45 kDa using the CD64-specific mAb, 10.1, and the hexa-histidine tag-specific mAb, His6 (Figure 5.2b). Cell lysates from the CD64 expressing cell line, THP-1, were run contemporaneously as a positive control, and showed a dominant band corresponding to ~ 70 kDa, the expected molecular weight of FL FcγRI. Ni-NTA purified \textit{Sf}9 cell culture supernatant was included as a negative control. ELISA experiments indicated that plastic immobilised recombinant EC FcγRI was specifically detected by the CD64 mAb, 10.1, under non-denaturing conditions (Figure 5.2c).

\textbf{5.3.3 Analysis of HRG binding to soluble EC FcγRI}

To further characterise the interaction between FcγRI and HRG, both ELISA-based and optical biosensor binding studies were performed. Initially, soluble recombinant EC FcγRI was immobilised onto plastic ELISA plates and recombinant HRG binding was monitored using the HRG specific mAb, HRG-4 (Figure 5.3a). Binding was significant and specific, supporting previous evidence suggesting that HRG binds to FcγRI, and showing for the first time a direct interaction between recombinant HRG and recombinant FcγRI. Further studies were carried out using an optical biosensor by monitoring the binding of soluble recombinant EC FcγRI to immobilised plasma-derived HRG (Figure 5.3b). Analysis of these binding studies using BIAevaluation
software showed that the interaction of soluble FcγRI and HRG was a high affinity interaction with an apparent $K_d \sim 1 \times 10^{-7}$ M ($\sim 100$ nM).

**Figure 5.3. Analysis of HRG binding to soluble EC FcγRI.** (a) Insect cell derived recombinant human EC FcγRI was immobilised on ELISA plates overnight and, following blocking of non-specific binding, various concentrations of recombinant HRG (0-10 µg/ml) was allowed to bind to the plates. HRG binding was detected with the HRG-specific mAb, HRG-4. Data mean ± SEM (n=3). (b) Plasma-derived HRG was immobilised on the surface of a CM5 sensor chip, and recombinant human EC FcγRI diluted in PBS (pH 7.2) at the indicated concentrations was then injected into the flow-cells, with binding and dissociation each being monitored for 7 min. A flow-cell within the CM5 sensor chip with no immobilised HRG served as a negative control. The figure depicts representative sensorgrams for each FcγRI concentration.
5.3.4 Generation of his/arg131 polymorphism FcγRIIa expression constructs, and production of FcγRII-his/arg131 stable transfected GAG-ve CHO cells

Based on the ability of FcγRI to bind HRG, it was of interest to determine if other related leukocyte Fc receptors could also interact with HRG. The low affinity receptor for IgG, FcγRIIa is one such candidate receptor. Two polymorphic forms of hFcγRIIa have been described, where there is a histidine or arginine residue at position 131. In order to examine HRG binding to both cell surface expressed polymorphic forms of FcγRIIa, FcγRIIa-his131 and FcγRIIa-arg131 expression constructs were produced from an existing hFcγRIIa-his-131 cDNA construct by simple PCR amplification, or by SOE PCR mutagenesis, respectively. The sequences of the oligonucleotides used to produce the two polymorphic forms of hFcγRIIa and their positions of hybridisation with FcγRIIa cDNAs are outlined in Table 2.5. For Arg131, SOE PCR was performed as follows (Figure 5.4a): Two PCR reactions were used to amplify the FcγRIIa fragments to be spliced together using oligonucleotides NR1 with ARG-1, and ARG-2 with EG5.

Figure 5.4. Strategy for the generation of FcγRIIa-his131 and FcγRIIa-arg131 expression constructs, and production of FcγRIIa-his131 and FcγRIIa-arg131 stable transfected cell lines in GAG-ve CHO cells. (a) A schematic diagram of FcγRIIa-his131 cDNA is shown, with oligonucleotide primers Arg-1, Arg-2, EG5 and NR1 used in PCR reactions positioned at their priming sites with 5′ to 3′ direction indicated by arrows. The closed pink circle within oligonucleotides Arg-1 and Arg-2 represents a nucleotide change from adenosine to guanidine at position 505 resulting in an amino acid change from histidine to arginine at position 131. PCR with oligonucleotide pairs, NR1 and Arg-1, and Arg2 and EG5, was used to produce the two fragments of FcγRIIa cDNA as indicated, which were then spliced together in a third SOE PCR reaction using oligonucleotides NR1 and EG5. Both the His131 and Arg131 forms of FcγRIIa cDNA were cloned into the pCR3.1 expression vector to produce pCR3.1-FcγRIIa-his131 and pCR3.1-FcγRIIa-arg131. The nucleotide sequences of each of these expression constructs were verified to confirm the polymorphism; the relevant region of each of the sequencing traces are shown. (b) pCR3.1-FcγRII-his131 and pCR3.1-FcγRII-arg131 were transfected into GAG-ve CHO cells (pgsA-745) to produce stable transfected GAG-ve cell lines expressing FcγRIIa-his131 and FcγRIIa-arg131. They were screened for cell surface expression of FcγRII (CD32) using the CD32 specific mAb, 8.26 and immunofluorescence flow cytometry. Representative histograms are shown, with filled histograms representing background binding of the secondary conjugate and open histograms representing CD32 cell surface expression.
a

**FcγRIIa-his131 cDNA**

![Diagram showing the sequence and PCR amplification of FcγRIIa-his131 cDNA](image)

**FcγRIIa-arg131 cDNA**

![Diagram showing the sequence and PCR amplification of FcγRIIa-arg131 cDNA](image)

b

**Histograms showing expression of FcγRIIa-his131 and FcγRIIa-arg131**

![Histogram of CD32 expression for FcγRIIa-his131](image)

![Histogram of CD32 expression for FcγRIIa-arg131](image)
A third SOE PCR reaction was performed to splice the two fragments together and amplify the spliced product using oligonucleotides NR1 and EG5. FcγRIIa-his131 or FcγRIIa-arg131 cDNAs were sub-cloned into the eukaryotic expression vector pCR3.1 to produce pCR3.1-FcγRII-his131 and pCR3.1-FcγRII-arg131 respectively (Figure 5.4a). GAG-ve CHO cells were transfected with pCR3.1-FcγRII-his131 and pCR3.1-FcγRII-arg131 expression constructs (Figure 5.4a) and stable transfected clones were selected in the presence of 0.9 mg/ml G418. Selected clones were analysed for cell surface CD32 expression using the CD32 specific mAb, 8.26 (Ierino et al., 1993) and immunofluorescence flow cytometry (Figure 5.4b). Stable cell lines that exhibited high levels of cell surface expression of each FcγRIIa-his131 or FcγRIIa-arg131 were selected and used for future experiments.

5.3.5 Analysis of HRG binding to cell surface FcγRIIa

Studies analysing the interaction between HRG and FcγRIIa were carried out utilising the GAG-ve CHO stable transfected FcγRIIa-his131 or FcγRIIa-arg131 cell surface expressing cell lines. HRG binding studies were carried out by incubating the transfected cells with plasma-derived HRG (100 µg/ml) in PBS/0.1%BSA/20 µM Zn²⁺ (pH 7.2). HRG binding was detected using the HRG specific mAb, HRG-4 and immunofluorescence flow cytometry. GAG+ve and GAG-ve CHO cells were used as positive and negative controls respectively. Representative histograms are shown, with filled histograms representing background binding of the HRG-specific mAb, HRG-4, and open histograms representing HRG binding. As expected, HRG did not bind to GAG-ve CHO cells, but exhibited high levels of binding to GAG+ve CHO cells (Figure 5.5a and Figure 5.5b). Interestingly, HRG bound specifically to FcγRIIa-his131 expressing GAG-ve CHO cells (Figure 5.5c). It is difficult to determine whether HRG specifically bound to FcγRIIa-arg131 expressing cells, as the mouse IgG1 isotype, HRG-4 mAb bound to FcγRIIa-arg-131 expressing cells, note the double peak in the filled histogram (Figure 5.5d). This result is not unexpected, as human FcγRIIa-arg131 (known as the high responder) is documented to bind mouse IgG1 isotypes, whereas human FcγRIIa-his131 (known as the low responder) does not bind to mouse IgG1 (Warmerdam et al., 1991). Although there is a small shift in HRG binding to FcγRIIa-arg131 expressing cells between the open and filled histograms, it is difficult to determine whether this is specific HRG binding. All of the HRG-specific mAbs
available to this laboratory are a mouse IgG1 isotype. An alternative method of HRG detection was also tested, whereby biotinylated-HRG was to be used instead of a mouse IgG mAb (HRG-4). However, biotinylated-HRG also introduced problems into the HRG detection system, as biotinylated-HRG preparations bound to GAG-ve CHO cells, introducing high levels of background binding. Nonetheless, this analysis shows that HRG binds specifically to surface expressed FcyRIIa-his131.

**Figure 5.5. Analysis of HRG binding to cell surface expressed FcyRIIa-his131 and FcyRIIa-arg131.** GAG-ve (a), GAG+ve (b), GAG-ve CHO cells stably transfected with FcyRIIa-his131 (c), or FcyRIIa-arg131 (d), were analysed for plasma-derived HRG (100 µg/ml) binding using the HRG specific mAb, HRG-4, and immunofluorescence flow cytometry. Representative histograms are shown, with filled histograms representing background binding of the HRG-specific mAb, HRG-4 in the absence of HRG, and open histograms representing HRG binding.
5.3.6 Analysis of HRG binding to soluble FcγRIIa

Both polymorphic forms of FcγRIIa were provided as a kind gift from Dr. Mark Hogarth, The Austin Research Institute, Melbourne, Australia. Both polymorphic forms of soluble hFcγRIIa were verified for Fc binding function on the biosensor by binding to immobilised Sandoglobulin on a CM5 chip (data not shown). Optical biosensor studies were carried out to analyse the binding between immobilised HRG and soluble FcγRIIa. As previously described, plasma-derived HRG was immobilised onto the surface of a CM5 sensor chip, and each of the soluble recombinant FcγRIIa-his131 and FcγRIIa-arg131 was injected into the flow-cells at the indicated concentration (Figure 5.6), with binding and dissociation monitored for 5 min each. The interaction was dissociated by a one min injection of 50 mM HCl, pH 3.0. Interestingly, FcγRIIa-his131 bound to immobilised HRG with high affinity, whereas FcγRIIa-arg131 did not bind to HRG at all, indicating a strong difference in HRG binding between the polymorphisms. Analysis of these binding studies using BIAevaluation software indicated that the interaction between soluble FcγRIIa-his131 and HRG could be described by a simple 1:1 interaction, with an apparent $K_d \sim 5 \times 10^{-7}$ M ($\sim 500$ nM).

5.3.7 Analysis of HRG binding to soluble FcαR

Based on the ability of HRG to bind to FcγRI and FcγRIIa, further studies were carried out to determine whether HRG could bind additional leukocyte FcRs, namely the IgA receptor, FcαR. FcαR was provided as a kind gift from Dr. Mark Hogarth, The Austin Research Institute, Melbourne, Australia. It was shown using optical biosensor studies that FcαR binds to immobilised HRG with high affinity (Figure 5.6). Analysis of these binding studies using BIAevaluation software indicated that the interaction between soluble FcαR and HRG could be described by a simple 1:1 interaction, with an apparent $K_d \sim 8.5 \times 10^{-7}$ M ($\sim 850$ nM).
Figure 5.6. Analysis of HRG binding to polymorphic forms of soluble EC FcγRlla, and FcoR. Plasma derived HRG was immobilised on the surface of a CM5 sensor chip, and recombinant human EC FcγRlla-his131, EC FcγRlla-arg131 or EC FcoR diluted in PBS (pH 7.2) at the indicated concentrations was then injected into the flow-cells, with binding and dissociation being monitored for 5 min each. A flow-cell within the CM5 sensor chip with no immobilised HRG served as a control. The figure depicts representative sensorgrams for each FcγRlla or FcoR concentration.
5.4 Discussion

This chapter examines the interaction of HRG with hFcγRI (the high affinity receptor for IgG), both polymorphic forms of hFcγRIIa (the low affinity IgG receptor), and FcαR (the IgA receptor). HRG has been implicated to interact with FcγRI through indirect binding studies which suggested that human HRG could bind to human FcγRI on the monocytic cell line, THP-1 (Gargani et al., 1999b). The pre-incubation of THP-1 cells with HRG blocked binding of monomeric IgG to these cells, HRG presumably binding to FcγRI and blocking the IgG binding site (Gargani et al., 1999b).

Subsequently, Gargani et al. (2002) showed that physiological concentrations of HRG opsonised apoptotic cells, enhancing their uptake via FcγRI mediated phagocytosis by human monocyte derived macrophages. It was shown that HRG mediated this effect by forming a bridge between the apoptotic cells and macrophage FcγRI, suggesting that HRG may play a physiological role regulating the uptake and clearance of immune complexes and necrotic/apoptotic cells in vivo. The results from these studies prompted a more detailed examination aiming to characterise the interaction of HRG with hFcγR. In order to perform direct binding studies, soluble extracellular recombinant hFcγRI and recombinant HRG were produced in the insect cell line, Sf-9, using the baculovirus expression system. Production of recombinant EC FcγRI provided a pure source of FcγRI free of other FcγR, while recombinant HRG also provided a source of HRG free from plasma proteins, such as IgG.

Initially, using an ELISA based approach, it was shown that recombinant HRG could directly bind to plastic immobilised recombinant FcγRI (Figure 5.3a). Further studies utilising surface plasmon resonance on also confirmed the interaction and indicated that plasma-derived native HRG binds to FcγRI with high affinity (K_d ~ 1 x 10^-7 M). These data provided for the first time direct evidence that HRG binds to FcγRI. In order to investigate the physiological relevance of HRG binding to hFcγRI, hFcγRI cDNA was stably transfected into GAG-ve CHO cells (Esko et al., 1986; Esko et al., 1985). GAG-ve CHO cells were chosen as the model cell line as they lack cell surface GAGs, and thus do not normally exhibit heparan sulphate mediated HRG cell surface binding, providing an ideal cell line in which to study HRG binding to FcγR without background binding to cell surface heparan sulphate. Unfortunately, despite numerous efforts to express hFcγRI at the cell surface of GAG-ve CHO cells, a stable cell line expressing detectable levels of FcγRI was not achieved. Although positive G418 resistant clones
expressing FcγRI mRNA were selected from GAG-ve CHO cells transfected with hFcγRI cDNA, no cell surface expression of FcγRI was observed using either a FcγRI-specific mAb, 10.1, or polyclonal Ab, N19, by flow cytometry. One explanation for the lack of cell surface expression of FcγRI could be that efficient expression of the receptor requires the presence of the accessory signalling subunit, FcR-γ, which is absent in this system. In order to overcome the lack of cell surface expression of FcγRI, chimeric FcγRI-II expression constructs containing the EC portion of FcγRI and the TM and CT of FcγRIIa were transfected into GAG-ve CHO cells. It was anticipated that chimeric hFcγRI-II receptors would be transported efficiently to the cell surface, but still retain the specific EC FcγRI binding domains. In fact, transfection studies by Hulett et al. (1991) using chimeric mouse FcγRI-II (EC portion of FcγRI and the TM and CT of FcγRII) had previously confirmed that chimeric FcγRI-II is efficiently transported to the cell surface, and exhibited identical IgG binding affinity when compared to wild type FcγRI. However, unfortunately this strategy too, was unsuccessful at expressing FcγRI-II at the cell surface. Whether the lack of FcγRI cell surface expression was due to a peculiarity of the GAG-ve CHO cell line, or whether FcγRI requires association with the FcR γ-chain to be efficiently expressed at the cell surface in this particular cell line, remains yet to be determined. PCR analysis of total RNA obtained from cells transfected with FcγRI and FcγRI-II, indicated that mRNA of both receptors was being transcribed, but that the corresponding FcγRI/FcγRI-II protein was not present on the cell surface. Ongoing work is being carried out in order to resolve the issue of expressing hFcγRI at the surface of GAG-ve CHO cells. Indeed, perhaps co-transfecting GAG-ve CHO cells with both FcγRI and the signalling FcRγ-chain would provide the necessary cellular machinery for transporting and maintaining efficient FcγRI cell surface expression. Nonetheless, the biosensor and ELISA studies have confirmed previous indirect suggestions that HRG interacts with FcγRI, and provides the first direct evidence using two independent methods that HRG binds to FcγRI. These findings lay the foundation for further studies to define the functional significance of the FcγRI-HRG interaction.

Previous work by Gorgani et al. (1999a) showed that HRG binds to immune complexes and regulates the formation of insoluble immune complexes. In fact, it was shown that HRG could inhibit the formation of insoluble immune complexes, raising the interesting possibility that HRG may play a regulatory role in immune complex formation and
clearance *in vivo*. If HRG can affect the formation and clearance of insoluble immune complexes, it is possible that HRG may provide endogenous protection from the development of diseases such as rheumatoid arthritis, that arise from the formation of insoluble immune complexes, by monitoring the clearance of pathological insoluble immune complexes. The proposed role of HRG in regulating immune complex clearance through FcγRI suggested that studies investigating the role of other FcRs involved in binding and clearing immune complexes should be undertaken. Thus, based on the ability of FcγRI to bind HRG, another leukocyte receptor, FcγRIIa, the low affinity receptor for IgG, was also investigated for its potential HRG binding capabilities. As described, a polymorphism exists in FcγRIIa whereby an arginine or histidine residue is present at amino acid position 131, which confers the ability to bind hIgG2. FcγRIIa-his131 is known as the 'low responder', and exhibits binding to human IgG2, but does not exhibit binding to mouse IgG1 isotypes. FcγRIIa-arg131 known as the 'high responder' and, on the other hand, does not exhibit binding to human IgG2, but does bind mouse IgG1 isotypes (Salmon *et al.*, 1992; Warmerdam *et al.*, 1991). Interestingly, FcγRIIa-his131 is the only FcγR capable of significant interaction with IgG2 and thus is the only FcγR able to mediate efficient neutrophil phagocytosis of IgG2 opsonised particles. Importantly, neutrophils from individuals who are homozygous for FcγRIIa-arg131 cannot efficiently take up and phagocytose IgG2 opsonised particles, which include important human pathogens such as *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* (Salmon *et al.*, 1992; Sanders *et al.*, 1994).

In order to characterise the interaction of HRG with the polymorphic forms of FcγRIIa, stable transfected GAG-ve CHO cells were made expressing either the His131 or the Arg131 form of the FcγRIIa at the cell surface (Figure 5.4b). Unlike the FcγRI transfected cell lines, there was no problem with FcγRIIa being expressed at the cell surface, and high levels of cell surface CD32 were detected using the CD32 specific mAb, 8.26 (Figure 5.4b). Stable cell surface expression and the ability of FcγRIIa to signal upon receptor ligandation does not require the association of the FcR-γ subunit due to the presence of either ITAM (FcγRIIa) or ITIM (FcγRIIb) signalling motifs present within the cytoplasmic tail of FcγRIIa or FcγRIIb respectively (Dijstelbloem *et al.*, 2001). This could explain, in part, why FcγRIIa, but not FcγRI, was able to be efficiently expressed on the surface of transfected GAG-ve CHO cells. HRG binding
studies were carried out on the FcγRIIa His/Arg131 polymorphic expressing cells. Plasma-derived HRG was found to bind to FcγRIIa-his131 expressing cells, whereas the assessment of HRG binding to FcγRIIa-arg131 cells was somewhat more complicated. The HRG binding detection system incorporating mouse IgG1 mAbs (HRG-1 to HRG-5) was not ideal for measuring binding to FcγRs. Significantly, mouse IgG1 isotypes are known to bind to hFcγRIIa-arg131, but not to hFcγRIIa-his131 (Warmerdam et al., 1991). Thus, the HRG-specific mAbs (all of IgG1 isotype) bind to FcγRIIa-arg131 expressing cells, but not FcγRIIa-his131 expressing cells, causing background problems for the FcγRIIa-arg131 binding studies (see double peak in filled histogram in Figure 5.5b). Despite the high level of background binding, specific HRG binding appears negligible on FcγRIIa-arg131 expressing cells. However, it is impossible to definitively conclude that HRG does not bind to FcγRIIa-arg131 expressing cells. However, given the negative HRG binding to soluble FcγRIIa-arg131 on the biosensor (Figure 5.6a), the compiled data does suggest a binding model whereby HRG binds differentially to polymorphic forms of FcγRIIa. In order to resolve the above discussed technical difficulties regarding HRG detection using mouse IgG mAbs on FcγRIIa expressing cells, biotinylated preparations of HRG were made to eliminate the requirement of mouse mAbs. However, biotinylated preparations of HRG also introduced background-binding problems, with biotinylated-HRG binding to mock transfected control GAG-ve cells (data not shown).

To further investigate the potential interaction of between HRG and FcγRIIa, both polymorphic forms of soluble hFcγRIIa were verified for Fc binding function on the optical biosensor by binding soluble FcγRIIa to sandoglobulin immobilised on a CM5 chip. Subsequent HRG binding studies using the biosensor provided the first direct evidence that soluble recombinant FcγRIIa could bind to immobilised HRG (Figure 5.6). Interestingly, HRG binding was dependant on the hFcγRIIa his/arg131 polymorphism, HRG binding with high affinity to FcγRIIa-his131, but not binding at all to FcγRIIa-arg131. This raises significant interest regarding the physiological function of HRG in relation to an IgG2 immune response. That HRG binds to FcγRIIa-his131, the only FcγR capable of binding and ingesting IgG2 opsonised particles, raises the possibility that HRG plays an important regulatory role in the phagocytosis of IgG2 immune complexes. Since HRG has been reported to bind insoluble immune complexes (Gorgani et al., 1999b), it could be speculated that HRG may provide a regulatory role
for individuals that express the His131 isoforms, for example, homozygous or heterozygous for His131 (His/His or His/Arg respectively), by binding to IgG2 immune complexes, and regulating their uptake and clearance via FcγRIIa-his131 on phagocytic cells. However, since HRG does not interact with the Arg131 polymorphic form, and individuals that homozygous for the Arg131 form (Arg/Arg) lack function of IgG2 immune complex clearance, HRG would not be expected to mediate regulation of IgG2 immune complex clearance in these individuals. On the other hand, both polymorphic forms of FcγRIIa also mediate the binding and uptake of other IgG isotypes such as IgG1 and IgG3, and thus HRG may also possibly regulate the binding and clearance of these particular isotypic immune complexes. The frequency of this allotypic polymorphism varies among ethnic groups; eastern asians are more frequently of His131/His131 (~ 85%) compared to caucasians (~ 30%) (Lehrnbecher et al., 1999; Osborne et al., 1994). The ethnic variation in the FcγRIIa allotype frequency may in part explain radical differences in the susceptibility to certain diseases, in particular those associated with IgG2 humoral responses.

In addition, the IgA receptor, hFccαR, was also assessed for HRG binding using the optical biosensor. Real time analysis revealed that soluble hFccαR bound with moderate affinity, $K_d \sim 8.5 \times 10^{-7} \text{M} (~ 850 \text{nM})$ to immobilised HRG. Thus, this finding that describes a moderate affinity interaction between HRG and FccαR raises the interesting possibility that HRG may play a potential regulatory role in regulating IgA immune complex binding to FccαR expressing cells, such as neutrophils, monocytes and eosinophils. Further investigation of the functional importance of HRG binding to the IgA receptor in vivo could provide novel insights into understanding the regulation of immune complex clearance at mucosal surfaces.

It is interesting to note that another serum protein, C-Reactive protein (CRP), implicated to play a role in immune complex formation and clearance, also binds FcγR like HRG. CRP is a member of the pentraxin family of proteins and is an acute phase serum protein in humans (Ballou and Kushner, 1992), and is rapidly synthesised by the liver in response to inflammatory cytokines, with serum levels increase up to 1000-fold within 24 - 48 hrs (Ballou and Kushner, 1992). CRP is part of the ancient innate immune system, and opsonises its ligands for ingestion by neutrophils and macrophages in a similar manner to antibodies within the adaptive immune response (Kilpatrick and
Volanakis, 1985; Mortensen et al., 1976). It has been demonstrated that CRP binds to monocytes through a distinct receptor (CRP-R), and also FcγRI and FcγRIIa (Bharadwaj et al., 1999; Marnell et al., 1995). Thus, CRP exhibits similarities to HRG in that both these plasma proteins have been shown to interact with FcγRI and FcγRIIa. Interestingly, both proteins show differential binding to the polymorphic forms of FcγRIIa. CRP binds to the Arg131 form, while HRG appears to bind to the His131 form of FcγRIIa. Stein et al. (2000) found that CRP bound to monocytes from donors homozygous for the for the FcγRIIa-arg131 allele, but that minimal binding occurred to cells from donors homozygous for the FcγRIIa-his131 allele. Functionally, CRP has been shown to opsonize particles (necrotic and apoptotic cells) resulting in an increased rate of phagocytosis of these cells by macrophages (Bodman-Smith et al., 2002; Mold et al., 2002; Wakeland et al., 2001). However, CRP did not increase phagocytosis by macrophages derived from FcγRI-chain deficient mice (which lack functional FcγRI and FcγRIII), indicating that CRP promotes the uptake and ingestion of necrotic and apoptotic cells through FcγRI and/or FcγRIII, but not FcγRII. Interestingly, the His/Arg131 polymorphism in human FcγRIIa exhibits differential binding to mouse IgG isotypes, with hFcγRIIa-arg131 exhibiting binding to mouse IgG1, whereas hFcγRIIa-his131 does not bind mouse IgG1. Studies by Saeland (2001) did not agree with previous work showing that CRP binds to FcγRIIa on phagocytic cells, and suggested that the observed binding may be attributed to the polymorphic variation for mouse IgG1 isotypes, since mouse IgG1 mAbs were used to detect CRP binding to FcγRIIa. Correspondingly, the IgG1 mouse isotype anti-CRP antibodies bound only to the FcγRIIa-arg131, and not FcγRIIa-his131. Indeed, further studies by Hundt et al. (2001) also suggest that CRP does not bind to hFcγR, and demonstrate that highly purified CRP preparations do not bind to FcγR on white blood cells, but that partially purified CRP preparations (that contained IgG contamination as demonstrated by immunoblotting) do bind hFcγR, hence attributing CRP binding to FcγR to IgG contamination. Thus, whether CRP binds to hFcγRI, and to Arg131 polymorphic forms of hFcγRIIa, remains somewhat controversial.

Based on the concerns raised from the CRP binding studies, an important technical consideration is to ensure that adequate controls and purified proteins are used for HRG binding studies. In particular, we have used recombinant HRG for binding studies, particularly for the FcγRI (the only FcγR able to bind monomeric IgG) binding studies,
as it is possible that despite every precaution that plasma-derived HRG preparations may contain contaminating IgG. Similarly, biosensor analysis between soluble FcγRI and FcγRIIa to immobilised HRG eliminated the requirement for mouse IgG isotypes, while also providing a real time analysis between the two proteins without the need for any detection or labelling antibodies/conjugates. It is noted that the FcγRIIa cell surface binding studies which incorporated mouse IgG1 HRG-specific mAbs, were not ideal for assessing the differential binding of HRG to the Arg131 polymorphic form of FcγRIIa, however, they did provide clear evidence that HRG specifically interacts with cell surface expressed FcγRIIa-his131.

SLE is an immune complex mediated disease with a broad spectrum of autoimmune phenomena and clinical symptoms, however what causes the disease is unknown. In autoimmune diseases such as SLE, it has been shown in vivo and in vitro, that abnormalities in FcγR mediated immune complex clearance contribute to the pathology of the disease (Davies et al., 1992; Frank et al., 1979). A major site of immune complex clearance occurs in the mononuclear phagocytic system of the liver and spleen, and impaired FcγR function inhibits the removal of immune complexes through FcγR dependent mechanisms by splenic phagocytes. In addition, it has been suggested that FcγR gene polymorphisms also play an important role in determining pathogenesis of the disease (Manger et al., 2002). A recent analysis has confirmed the previously reported association between FcγRIIa-arg131 allele and susceptibility to SLE (Karassa et al., 2002). Similarly, FcγRIIa-arg131 has also been associated with lupus nephritis (Norsworthy et al., 1999). The homozygous FcγRIIa-arg131 phenotype (when associated with another polymorphism in FcγRIIIb) has also been found to be associated with meningococcal meningitis (Fijen et al., 1993). Thus, HRG may play some role in either the susceptibility or protection of autoimmune disease development, as it has been shown to play a functional role in phagocytosis of apoptotic and necrotic cells, and interestingly, also shows differential binding to polymorphic forms of FcγRIIa. However, it should be noted that as HRG does not bind to the Arg131 polymorphic form of FcγRIIa, it cannot regulate immune complex binding and clearance through this particular isoform.

It should also be considered that over-efficient FcγR-IgG interactions may trigger detrimental inflammatory responses. Such interactions may be of relevance for
autoantibody interaction with FcγRIIa on platelets, and in localised cytotoxic responses, which may play a role in tissue-specific autoimmune disease. Similarly, over-efficient activation of neutrophils via ligation of their FcγR has the potential to result in host tissue damage in autoimmune diseases such as rheumatoid arthritis. Neutrophils have enormous capacity to inflict host damage via their ability to secrete reactive oxygen metabolites and granule enzymes, and are often found in large numbers in inflamed tissues such as diseased rheumatoid joints. Relatively inefficient immune complex clearance may lower the threshold for complex deposition in peripheral tissues and the induction of subsequent inflammation, as observed in a number of autoimmune diseases, and may hamper the containment of bacterial infections. Since HRG binding to FcγRIIa is dependent on FcγRIIa polymorphisms, the presence of HRG may influence the vigour of the inflammatory response and may contribute to differences in susceptibility to infectious and autoimmune diseases.

In conclusion, in this chapter is presented data that identifies HRG as a novel ligand for a number of the leukocyte FcR; FcγRI FcγRIIa and FcαR. In particular, previous indirect studies suggesting that HRG binds to FcγRI have been confirmed using direct biosensor and ELISA approaches, utilising recombinant forms of both FcγRI and HRG. Secondly, HRG has also been shown to bind to the His131 polymorphic form of FcγRIIa, but not to the Arg131 form, using both an optical biosensor and cell surface binding studies. Thirdly, HRG was also shown to interact with the IgA receptor, FcαR. Further work is obviously necessary to define the functional role of these interactions. FcγRI and FcγRIIa are known to play pivotal roles in mediating phagocytosis of apoptotic, necrotic and opsonised immune complexes, suggesting that HRG may play a physiological role in regulating the uptake and clearance of these particles through these receptors. The differential binding of HRG to polymorphic forms of FcγRIIa could have important ramifications for homozygous individuals for either allelic form. Furthermore, the interaction of HRG with FcγRI, and the His131 polymorphic form of FcγRIIa raises the possibility that HRG plays a regulatory role in autoimmune diseases, such as SLE. In addition, as HRG has been shown to bind the IgA receptor, it could be speculated that HRG may also play a role in regulating immune responses at mucosal surfaces. Overall, these binding studies provide a solid framework for future investigation into defining the functional roles of the HRG interaction with leukocyte FcR.
Chapter six

Histidine-Rich Glycoprotein Acts as an Opsonin for Necrotic Cells

6.1 Abstract

Cells that become necrotic through tissue damage, tumour growth or microbial attack, or cells that enter the apoptotic pathway during normal cellular turnover, are usually rapidly cleared from the circulation and tissues. Phagocytic cells circulate with body fluids or associate with particular organs, and bind and engulf apoptotic and necrotic cells. In addition, phagocytosis of necrotic cells may activate innate and adaptive immune responses. Previous studies have reported that HRG opsonises late-stage apoptotic cells, by increasing the rate of binding and ingestion of these cells by macrophages. This chapter describes experiments which examined the interaction of HRG with viable, early-stage apoptotic and necrotic cells. Using confocal microscopy and immunofluorescence flow cytometry, this chapter confirms that HRG binds to heparan sulphate on the surface of viable cells via its N1N2 domain, but also demonstrates that HRG binds poorly to early-stage apoptotic cells. In contrast, HRG binds very strongly to an undefined intracellular ligand within necrotic cells, via its N1N2 domain. Heparin blocking studies, and cell binding studies using cell lines lacking GAGs, indicate that the intracellular HRG ligand is unrelated to heparan sulphate. Furthermore, a phagocytosis assay utilising necrotic Jurkat T cells and phagocytic THP-1 cells labelled with different fluorescent dyes, revealed that the presence of physiological concentrations of HRG significantly increased the rate of phagocytosis of the necrotic cells by the monocytic cell line. These data suggest that HRG may play an important physiological role in vivo by facilitating the uptake and clearance of necrotic cells, but not apoptotic cells, by monocytes/macrophages.
6.2 Introduction

Cell death is vital for the morphological shaping of tissues during development and for the sculpting of functionally appropriate cellular repertoires (Cecconi et al., 1998; Surh and Sprent, 1994), while also protecting the body from viral infections and pathogenic microorganisms. Selective cell death continues to play a role in the homeostasis of mature tissues, such as the deletion of immune cells in the attenuation of an immune response (Webb et al., 1990), and the elimination of cells that have become functionally inappropriate, including virally infected and transformed cells (Kagi et al., 1995). As well as providing a first line of defence against microbial pathogens, the innate immune system is also responsible for the recognition of necrotic and apoptotic cells and self-antigens that are released from such cells (Franc et al., 1999). Apoptosis and necrosis are two forms of cell death characterised by distinct morphologies. Rapid and efficient phagocytic removal of dying cells is a key feature of apoptosis, whereas the role and extent of phagocytosis in the clearance of necrotic cells is not well documented.

Apoptosis is characterised by an orderly sequence of internal events, including chromatin condensation that precedes the loss of cellular integrity (Harvey et al., 2000). Apoptotic cells exhibit surface exposure of phosphatidylserine and alteration of membrane carbohydrates. Multiple ligands and receptors have been implicated in the recognition and uptake of apoptotic cells by phagocytes prior to membrane lysis, thus preventing release of potentially toxic and immunogenic intracellular substances into tissues. In addition, the binding and/or uptake of apoptotic cells inhibits proinflammatory cytokine production (Huynh et al., 2002). When there are disturbances in either apoptosis or the phagocytosis of apoptotic cells, antibodies against subsequently exposed nucleosomes may be formed, leading the development of autoimmune diseases such as systemic lupus erythematosus (SLE). In contrast to apoptosis, necrotic cell death has previously been defined as a disordered mode of cell death, occurring either in cases of severe and acute injuries such as sudden shortage of nutrients and abrupt anoxia or in extreme injuries such as exposure to heat, detergents, strong bases, and irradiation. Necrotic cell death is marked by rapid disorganised swelling and rupture of the cell, and is associated with pathological tissue injury and inflammatory responses. Necrotic cells too, are usually rapidly cleared from the circulation by phagocytic cells. More recently, the existence of a necrotic-like cell death
pathway regulated by an intrinsic death program distinct from that of apoptosis has become apparent (Kitanaka and Kuchino, 1999; Proskuryakov et al., 2003).

Phagocytic cells circulate within the body fluids or associate with particular organs, and have the specialised capacity to ingest large particles (1 µm or more in diameter). They are often the first cells of the immune system to encounter an invader and participate in both engulfment of the pathogen and activation of the innate and adaptive immune responses. Phagocytes also serve a scavenging function, by engulfing ‘worn out’ cells or cellular debris.

Studies by Gargani et al. (1999b) have demonstrated that HRG can bind to IgG containing immune complexes, and subsequently potentiates the binding of immune complexes to phagocytic THP-1 cells, indicating that HRG may be an important regulator of immune complex uptake and clearance by monocytes. Interestingly, the same study concluded that HRG binds to FcyRI expressed on the surface THP-1 cells, subsequently blocking monomeric IgG binding. In addition, Gargani et al. (2002) showed that HRG potentiated the ingestion of apoptotic cells by mature human monocyte-derived macrophages, and went on to suggest that HRG acts a bridge between DNA exposed within apoptotic cells and FcyRI on macrophages, implying that HRG may be a key mediator of apoptotic cell clearance by macrophages.

The studies described in this chapter were aimed at further investigating the role of HRG in the phagocytosis of dying cells, specifically the influence of HRG on the uptake and clearance of necrotic cells. The data presented herein indicate that HRG binds avidly to necrotic cells, but not to apoptotic cells, and that this binding is not mediated via the HRG heparin binding site or cell surface heparan sulphate, as high concentrations of heparin did not interfere with HRG binding to necrotic cells and HRG bound strongly to necrotic GAG-ve CHO cells that lack cell surface heparan sulphate. Additional experiments revealed that HRG binds to an undefined cytoplasmic ligand within necrotic cells via its N1N2 domain, with this interaction substantially increasing the rate of phagocytosis of necrotic cells by monocytes. Thus, this chapter demonstrates for the first time that HRG may represent a key plasma protein that facilitates the ingestion of necrotic cells by phagocytes.
6.3 Results

6.3.1 Binding of HRG to necrotic cells is heparan sulphate independent

The studies described in Chapter 3 and Chapter 4 demonstrated that HRG binds to cell surface heparan sulphate on viable cells via its N1N2 domain, and that this binding is almost entirely abolished by high concentrations of heparin. This chapter aimed to characterise the interaction of HRG with non-viable cells, namely necrotic and early-stage apoptotic cells. GAG+ve and GAG-ve CHO cells were induced into a necrotic state using two different methods; firstly, exposure of the cells to the detergent saponin (0.02%), and secondly, by exposing the cells (resuspended in RPMI-1640 medium supplemented with 10% FCS) to hyperthermic conditions (56°C) for 45 min. Cells were then analysed for viability using the cell viability and DNA intercalating dye, 7-AAD, with viability being assessed by fluorescence flow cytometry (Figure 6.1a). Both methods of inducing cell necrosis were 100% successful. From hereon, the hyperthermic method was used to induce cells into a necrotic state, and was used in all future experiments. In addition, necrotic cells stained with the cell viability dye, 7-AAD, were analysed by confocal fluorescence microscopy, and it was found that 7-AAD was taken up by the nuclei of essentially all cells subjected to hyperthermic treatment, confirming they were indeed necrotic (Figure 6.1b). Both viable and necrotic GAG+ve and GAG-ve CHO cells were analysed for their capacity to bind HRG. In agreement with previous findings, viable GAG+ve CHO cells bound plasma-derived human HRG (100 µg/ml), binding which was almost entirely abolished by the presence of 12.5 kDa heparin (100 µg/ml). As reported in Chapter 3, HRG exhibited little binding to viable GAG-ve CHO cells either in the presence or absence of heparin (Figure 6.2a and 6.2b). Interestingly, HRG bound to necrotic GAG+ve and GAG-ve CHO cells at levels 2-3 fold higher than for viable GAG+ve CHO cells (Figure 6.2a and 6.2b). This binding was only partially inhibited by heparin (~20 - 30%), suggesting that the majority of HRG binding to necrotic cells is mediated though a heparan sulphate independent ligand.
Figure 6.1. Induction of necrosis in CHO cells. (a) GAG+ve and GAG-ve CHO cells were induced into a necrotic state using two methods, namely treatment of the cells with a detergent (0.02% saponin) (top panel), or exposure of the cells to hyperthermic conditions (56°C) for 45 min (bottom panel). Cells were analysed by immunofluorescence flow cytometry for necrosis using the viability cell dye, 7-AAD. Filled histograms represent 7-AAD uptake by control viable cells and open histograms depict 7-AAD uptake by the necrotic cells. (b) Necrotic GAG+ve CHO cells were produced by exposure of the cells to hyperthermic conditions (56°C) for 45 min, and then analysed for 7-AAD uptake by confocal microscopy. The left panel represents a bright field image of necrotic GAG+ve cells, the right panel depicts 7-AAD uptake (red) by the nuclei of the necrotic cells.
Figure 6.2. Ability of full-length HRG to bind to viable and necrotic CHO cells. (a) Binding of full-length plasma-derived HRG (100 µg/ml) in PBS/0.1%BSA/20 µM Zn²⁺ (pH 7.2) to viable (top panel) or hyperthermia induced necrotic (bottom panel) GAG+ve or GAG-ve CHO cells in the presence or absence of 100 µg/ml of 12.5 kDa heparin. HRG binding was detected using the HRG specific mAb, HRG-4. Representative flow cytometry histograms are shown, with filled histograms representing background binding of the HRG-specific mAb, HRG-4 to cells in the absence of HRG, pink histograms representing HRG binding, and blue histograms representing HRG binding in the presence of heparin. (b) Quantitative comparison of the binding of HRG to necrotic and viable cells in the presence or absence of heparin, with data being expressed as fold increase in HRG binding (median fluorescence) relative to background ± SEM (n=3).
Figure 6.3. Ability of the N1N2 domain of HRG to bind to viable and necrotic CHO cells. (a) Binding of the N1N2 domain of HRG (50 μg/ml) in PBS/0.1%BSA/20 μM Zn²⁺ (pH 7.2) to viable (top panel) or hyperthermia induced necrotic (bottom panel) GAG+ve or GAG-ve CHO cells in the presence or absence of 100 μg/ml of 12.5 kDa heparin. N1N2 binding was detected using the HRG specific mAb, HRG-4. Representative flow cytometry histograms are shown, with filled histograms representing background binding of the HRG-specific mAb, HRG-4 to cells in the absence of N1N2, and open histograms representing N1N2 binding in the presence or absence of heparin. (b) Quantitative comparison of the binding of the N1N2 domain to necrotic and viable cells in the presence or absence of heparin, with data being expressed as fold increase in median fluorescence (N1N2 binding) relative to background ± SEM (n=3).
6.3.2 The N1N2 domain of HRG binds to necrotic cells in a heparan sulphate independent manner

Immunofluorescence flow cytometry studies were performed to determine whether the N1N2 domain of HRG also binds to necrotic cells. In agreement with previous results (Chapter 3), the N1N2 domain bound to GAG+ve CHO cells, binding that was essentially inhibited by 12.5 kDa heparin (100 µg/ml). Consistent with this observation, the N1N2 domain of HRG did not bind to GAG deficient CHO cells (Figure 6.3a and 6.3b). However, the N1N2 domain of HRG exhibited comparable binding to both necrotic GAG+ve and GAG-ve CHO cells, binding that was not blocked by heparin (Figure 6.3a and 6.3b). Collectively, these data suggest that the N1N2 domain of HRG interacts with the same ligand expressed by necrotic cells as full-length HRG, and that this ligand is unrelated to heparan sulphate.

6.3.3 HRG and the N1N2 domain of HRG bind to intracellular ligand(s) in necrotic cells

Confocal microscopy was used to visualise the binding of HRG and the N1N2 domain of HRG to both viable and necrotic cells. GAG+ve and GAG-ve CHO cells were induced into a necrotic state by hyperthermia and analysed by confocal microscopy for HRG binding using the HRG-specific mAb, HRG-4 (Figure 6.4). Viable GAG+ve CHO cells exhibited HRG binding (green) that appears to be predominantly localised to the outer surface of all viable cells. As expected, uptake of the cell viability dye, 7-AAD (red), is absent from the viable cells and present in the necrotic cells. As expected, viable GAG-ve cells did not exhibit binding of either HRG or uptake of 7-AAD (data not shown). Conversely, HRG binding to necrotic GAG+ve and GAG-ve CHO cells appeared to be localised within the cytoplasm, but not the nucleus (Figure 6.4), with the nuclei of necrotic cells exhibiting significant uptake of the fluorescent DNA binding dye, 7-AAD (Figure 6.4). These confocal microscopy experiments were repeated using the N1N2 domain of HRG, with similar trends being obtained (Figure 6.5). Again, viable GAG+ve CHO cells exhibited N1N2 domain binding (green) that was mostly localised to the outer cell surface. In contrast, the N1N2 domain did not bind to viable GAG-ve cells (data not shown). As with full-length HRG, the N1N2 domain exhibited binding to both necrotic GAG+ve and GAG-ve CHO cells to a ligand within the cytoplasm. It is difficult to determine whether the binding of the N1N2 domain is
localised only within the cytoplasm, or whether binding extends into the nucleus. Indeed, the confocal images shown here suggest that the N1N2 domain of HRG may also interact with ligands localised within the nucleus. However, staining within the nucleus appears somewhat lighter than staining within the cytoplasm, and may be due to non-specific background staining (Figure 6.5). Thus, these data suggest that the N1N2 domain of HRG probably interacts with the same putative necrotic cell ligand(s) as full-length HRG, and that these ligand(s) are located within the cytoplasm, or perhaps in both the cytoplasm and the nucleus.

![Figure 6.4. Confocal images of HRG binding to viable and necrotic CHO cells.](image)

Both viable and hyperthermia induced necrotic GAG+ve CHO cells and necrotic GAG-ve CHO cells were analysed for HRG binding (100 µg/ml) in PBS/0.1%BSA/20 µM Zn²⁺ (pH 7.2) using the HRG-specific mAb, HRG-4, and for cell viability using the cell viability dye, 7-AAD, and then examined by bright field and fluorescence confocal microscopy. HRG binding to cells is represented as green, and necrotic cells are shown as red, due to 7-AAD uptake in their nuclei. Representative confocal images are shown, with GAG-ve viable cell data being omitted as these cells did not bind HRG or take up 7-AAD.
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**Figure 6.5.** Confocal images of the binding of the N1N2 domain of HRG to viable and necrotic CHO cells. Both viable and hyperthermia induced necrotic GAG+ve CHO cells and GAG-ve CHO cells were analysed for N1N2 binding (50 µg/ml) in PBS/0.1%BSA/20 µM Zn²⁺ (pH 7.2) using the HRG-specific mAb, HRG-4, and for cell viability using the cell viability dye, 7-AAD, and then examined by bright field and fluorescence confocal microscopy. N1N2 binding to cells is depicted as green, and necrotic cells are shown as red, due to 7-AAD uptake in the nuclei. Representative confocal images are shown, with GAG-ve viable cell data being omitted as these cells did not bind N1N2 or take up 7-AAD.
6.3.4 HRG binds poorly to early-stage apoptotic cells

So far in this thesis it has been established that HRG binds to the surface of viable cells via heparan sulphate, and to an intracellular ligand within necrotic cells. Thus, the capacity of HRG to bind to early-stage apoptotic (pre-necrotic) cells was investigated. Jurkat T cells were induced into an apoptotic state by 1 µM camptothecin treatment for up to 6 hr at 37°C. Cells were then analysed by immunofluorescence flow cytometry for Annexin-V binding and uptake of the viability dye, 7-AAD. Untreated cells (viable cells) did not exhibit uptake of the cell viability dye, 7-AAD, or binding of Annexin-V (Figure 6.6a). With increasing incubation time with camptothecin (2, 4, and 6 hr), a population of cells appeared that gradually exhibited Annexin-V binding but remained 7-AAD negative, this cell population having entered the apoptotic pathway. Indeed, 35% of camptothecin treated cells became apoptotic after 6 hrs of treatment (Figure 6.6a and 6.6b).

The ability of HRG to bind to viable, early-stage apoptotic or necrotic cells, and the effect of heparin on this binding was then investigated. Jurkat cells were either induced into a necrotic state by hyperthermic treatment, induced into an apoptotic state by 6 hr camptothecin treatment, or left untreated (viable cells). Each of these groups was then incubated with plasma-derived HRG (100 µg/ml) in PBS/0.1% BSA/20 µM Zn²⁺ (pH 7.2) with or without 12.5 kDa heparin (100 µg/ml) and then analysed for HRG binding using the HRG-specific mAb, HRG-4, and immunofluorescence flow cytometry. Cells were also analysed for uptake of the cell viability dye, 7-AAD, and Annexin-V binding in order to determine the gating regions used for each of the viable, early-stage apoptotic and necrotic cell populations (Figure 6.7a). 'Viable' cells were classified as cells that remained 7-AAD and Annexin-V negative, early-apoptotic cells were classified as cells that were 7-AAD negative and Annexin-V positive, while necrotic cells were classed as cells that were 7-AAD positive and Annexin-V negative. Viable cells exhibited substantial levels of HRG binding that could be almost entirely inhibited by heparin. In contrast, early-stage apoptotic cells exhibited low levels of HRG binding that was marginally affected by heparin, while necrotic cells bound high levels of HRG that was also only slightly affected by heparin (Figure 6.7b and 6.7c). Thus it appears that Jurkat cells behave in a similar manner to GAG+ve CHO cells in that when they are necrotic, they bind HRG in a heparan sulphate independent manner. These binding data also suggest that the ligand recognised by HRG in necrotic cells is not exposed
until the cell membrane has become permeable, allowing HRG access to cytoplasmic ligands in necrotic cells. Early-stage apoptotic cells have intact cell membranes, and thus do not appear to allow HRG access to the cytoplasmic ligands.

**Figure 6.6. Induction of apoptotic Jurkat T cells.** (a) Apoptosis was induced in Jurkat T cells with 1 µM camptothecin over a 6 hr time-course. Apoptosis was detected using the apoptotic marker Annexin-V-PE, while cell necrosis was monitored using the cell viability dye, 7-AAD. Cells were analysed by immunofluorescence flow cytometry, with early-stage apoptotic cells exhibiting Annexin-V binding, but remaining 7-AAD negative (bottom right quadrant). Percentage of total cells are shown in each quadrant. (b) Percentage of apoptotic cells ± SEM (n=3) induced by 1 µM camptothecin over a 6 hr time-course, as detected in (a).
6.3.5 **HRG enhances the phagocytic uptake of necrotic cells by THP-1 cells**

Recent studies by Gorgani *et al.* (2002) showed that HRG enhances the ingestion of late-stage apoptotic cells by human monocyte-derived macrophages. Gorgani *et al.* (2002) suggested that HRG acts as a bridge between FcγRI expressed on macrophages, and DNA on apoptotic cells. In this study, the effect of HRG on the uptake and phagocytosis of necrotic cells by phagocytic THP-1 cells was investigated. A phagocytic assay was designed whereby THP-1 phagocytic cells were labelled with the intracellular dye, SNARF (red), and Jurkat T cells were labelled with the intracellular dye CFSE (green), before induction into necrosis. Labelled cells were then mixed at a ratio of 10:1 necrotic cells to phagocytic cells, and incubated with or without plasma derived HRG (100 µg/ml) at 37°C for up to 80 min. Cells were then immediately placed at 4°C to inhibit further phagocytosis before immediate analysis of the cells by flow cytometry. Cells were analysed for CFSE (necrotic Jurkat cell) and SNARF (phagocytic THP-1 cell) staining (Figure 6.8a). At time 0, the majority of cells were either CFSE positive or SNARF positive, indicating that no interaction had taken place between the cells. As time progressed, cells gradually became positive for both CFSE and SNARF, indicating that SNARF labelled THP-1 cells were binding and ingesting the labelled necrotic Jurkat T cells and becoming double positive cells. The rate of phagocytosis was calculated as the number of double positive THP-1 cells as a percentage of the total number of THP-1 cells. It was found that the presence of physiological concentrations of HRG (150 µg/ml) resulted in a significantly higher percentage of THP-1 cells binding and ingesting necrotic cells at all time points tested (Figure 6.8a and 6.8b). HRG not only increased the overall number of THP-1-containing necrotic cells from ~40% to ~75%, but also accelerated the rate of ingestion by ~2-fold (Figure 6.8b) in a HRG concentration-dependent manner (Figure 6.8c). Furthermore, heparin (100 µg/ml) did not interfere with the ability of HRG to enhance the phagocytosis of necrotic cells (Figure 6.8d). Control experiments revealed that in either the presence or absence of HRG (100 µg/ml) there was negligible binding of viable Jurkat T cells to THP-1 cells after 80 min incubation at 37°C (3-4%), indicating that HRG selectively opsonised necrotic cells for ingestion by THP-1 cells (data not shown).
Figure 6.7. HRG binding to viable, early-stage apoptotic and necrotic Jurkat T cells. Apoptotic (1 µM camptothecin, 6 hr) or necrotic (56°C, 45 min) Jurkat T cells were incubated with plasma derived HRG (100 µg/ml) in PBS/0.1%BSA/20 µM Zn²⁺ (pH 7.2) in the presence or absence of 100 µg/ml of 12.5 kDa heparin, with HRG binding being detected using the HRG-specific mAb, HRG-4. Cells were analysed by immunofluorescence flow cytometry with apoptosis being detected using the apoptosis marker Annexin-V-PE, and cell necrosis being monitored by the cell viability dye 7-AAD. (a) Appropriate gates were determined for viable cells (7-AAD and Annexin-V negative), early-stage apoptotic cells (7-AAD negative, Annexin-V positive) and necrotic cells (7-AAD positive, Annexin-V negative). Representative flow cytometry dot plots are shown for each of the appropriate gates, with viable, early-stage apoptotic and necrotic cell populations being circled in red. (b) Representative flow cytometry histograms showing HRG binding to viable, early-stage apoptotic and necrotic cells, with filled histograms representing background binding of the HRG-specific mAb, HRG-4 to cells in the absence of HRG, and open histograms representing HRG binding with or without heparin (c) Numerical values showing HRG binding to viable, early-stage apoptotic and necrotic Jurkat T cells, with data expressed as fold increase in median fluorescence above the background of each cell population, with filled histograms representing HRG binding in the absence of heparin, and grey histograms representing HRG binding in the presence of heparin. Error bars represent SEM (n=3).
a

Viable

Apoptotic

Necrotic

7-AAD

Annexin-V

b

Viable

Apoptotic

Necrotic

HRG

c

HRG binding

heparin viable

heparin apoptotic

heparin necrotic
a

control (no HRG) | HRG (100µg/ml)

0 mins
3.1% | 2.9%
20 mins
18% | 27%
40 mins
24.9% | 62%
60 mins
31% | 71%
80 mins
37.2% | 72%

SNARF (THP-1) → CFSE (Jurkat)
Figure 6.8. Effect of HRG on phagocytosis of necrotic Jurkat T cells by the monocytic THP-1 cell line. (a) A phagocytosis assay was developed using the intracellular fluorescent dyes SNARF and CFSE. THP-1 cells were labelled with SNARF (red), while necrotic Jurkat T cells were labelled with CFSE (green). Phagocytic cells (THP-1) were mixed with necrotic cells (Jurkat) at a ratio of 1:10, and incubated at 37°C for up to 80 min in the presence or absence of plasma derived HRG (100 µg/ml), before analysis by immunofluorescence flow cytometry. Rate of phagocytosis was determined as the percentage of SNARF+ phagocytic cells that had ingested CFSE+ necrotic cells, i.e., top right hand quadrant in each flow cytometry dot plot as a percentage of the total of both top left and right quadrants. (b) Numerical representation of phagocytosis rate in the presence or absence of plasma derived HRG (100 µg/ml), expressed as percentage CFSE+/SNARF+ THP-1 cells ± SEM (n=3). (c) The effect of HRG concentration (3-200 µg/ml) on the phagocytosis of necrotic Jurkat T cells following incubation for 60 min at 37°C, data being expressed as percentage CFSE+/SNARF+ THP-1 cells. (d) Effect of 12.5 kDa heparin (100 µg/ml) on HRG enhanced uptake of necrotic Jurkat T cells by phagocytic THP-1 cells following an 80 min incubation at 37°C. Results are shown as percentage control phagocytosis in the absence of HRG.
6.4 Discussion

This chapter examines the interaction of HRG with viable, early-stage apoptotic and necrotic cells. It was found that physiological concentrations of human HRG bound strongly to necrotic cells in a heparan sulphate independent manner and increased the phagocytosis of necrotic Jurkat cells by the monocytic cell line, THP-1, thus implicating a role for HRG in regulating the binding, uptake and clearance of necrotic cells in vivo.

Necrotic cell death occurs in cases of severe and acute injury due to a shortage of nutrients, or exposure to heat, detergents, strong bases, irradiation or abrupt anoxia. Usually necrotic cells are extremely efficiently cleared from the circulation, however, if they are not rapidly cleared, as occurs in pathological tissue, they can induce an inflammatory response (Dijstelbloem et al., 2001; Gaipl et al., 2004). Initial experiments examined the interaction of HRG with necrotic cells. HRG was shown to exhibit heparin inhibitable binding to viable GAG+ve CHO cells, and negligible binding to GAG-ve CHO cells (Figure 6.2). This result confirmed previous results described in Chapter 3 which demonstrated that HRG binds to cell surface heparan sulphate on viable cells. However, an interesting result arising from the current analysis was that HRG binds to a heparan sulphate independent ligand on both necrotic GAG+ve and GAG-ve CHO cells. Thus, despite GAG-ve CHO cells lacking cell surface GAGs due to a deficiency in xylosyltransferase, HRG exhibited high levels of binding to necrotic GAG-ve cells, binding that was not abolished by the presence of 100 μg/ml of 12.5 kDa heparin (Figure 6.2). Although heparin did somewhat reduce the binding of HRG to necrotic cells, the remaining HRG binding in the presence of heparin was still significantly greater than HRG binding to viable cells. Confocal microscopy studies revealed that, unlike viable cells where HRG primarily bound to the cell surface, HRG binding to necrotic cells appeared to be localised intracellularly, being predominantly in the cytoplasm rather than the nucleus (Figure 6.4).

The N1N2 domain of HRG also bound necrotic GAG+ve and GAG-ve CHO cells in a similar manner to full-length HRG (Figure 6.3), suggesting that the N1N2 domain of HRG is the domain within HRG that interacts with both cell surface heparan sulphate (Chapter 3), and the intracellular ligand in necrotic cells. Confocal microscopy images suggested that the N1N2 domain of HRG binds to ligands in both the cytoplasm and
nucleus of necrotic cells unlike full-length HRG which was shown to bind to only the cytoplasm of necrotic cells (Figure 6.4). This discrepancy in HRG and N1N2 domain binding could be explained by N1N2 having a significantly different conformation and size to full-length HRG, allowing the molecule to more readily diffuse into the nucleus of necrotic cells and bind nuclear proteins. An alternative explanation is that the N1N2 domain of HRG does not bind to the nuclear region within the cells, but that the poor resolution of the confocal image does not differentiate clearly between the intracellular regions. For example, the N1N2 domain staining within the cell nuclei in necrotic GAG+ve and GAG-ve cells is somewhat less bright than binding to the cytoplasmic domain (Figure 6.5), perhaps suggesting that the N1N2 domain only binds to the cytoplasmic ligand, and that there is background staining within the nucleus. Nonetheless, this study has shown that the N1N2 domain of HRG is the domain of HRG that binds to some currently undefined intracellular ligand(s) within necrotic cells.

Further studies are obviously required to define the intracellular HRG ligand(s), although Gorgani et al. (2002) reported that the ability of HRG to enhance the binding of late-stage apoptotic cells to macrophages was partially inhibited by DNA suggesting that DNA may be a ligand. On the other hand, preliminary unpublished studies by J. Altin, and C. R. Parish (Pers. Comm.) suggest that HRG binds to histones, a result consistent with some nuclear uptake of the N1N2 domain by necrotic cells. Recent studies by Gorgani et al. (2002) indicated that HRG binds to late-stage apoptotic cells. It is interesting to note, however, that late-stage apoptotic cells lose the integrity of their cell membrane, and thus begin to resemble cells that are often defined as necrotic. Hence, in this chapter experiments are described which characterised the binding of HRG to early-stage apoptotic cells, such apoptotic cells having an intact plasma membrane. An apoptosis assay was established that utilised Jurkat T cells as the model cell line. Camptothecin was used to induce the cells into an apoptotic state (Figure 6.6a), and after 6 hrs, 35% of the Jurkat T cells had become apoptotic, as indicated by Annexin-V binding. Importantly, these early, 6-hr induced apoptotic cells retained their cell membrane integrity, and did not take up the cell viability dye, 7-AAD. HRG binding studies were carried out on viable, early-apoptotic and necrotic Jurkat T cells (cell populations outlined in Figure 6.7a). Interestingly, HRG binding to early-stage apoptotic cells was much lower than HRG binding to viable cells (Figure 6.7b and 6.7c). In addition, the residual HRG binding to early-stage apoptotic cells appeared to
be unaffected by the presence of heparin, whereas HRG binding to viable cells was almost entirely abolished by heparin. It is well established that there are substantial chances in the extracellular composition of the plasma membrane of early-stage apoptotic cells (Harvey et al., 2000). The HRG binding data is consistent with these earlier findings and implies that heparan sulphate is lost from the surface of apoptotic cells. In contrast to early-stage apoptotic cells, HRG binding to necrotic cells was significantly higher than HRG binding to viable cells, and also was only partially inhibited by heparin. Thus, HRG interacts differently with viable, early-stage apoptotic and necrotic cells. Binding to viable cells is heparin inhibitable, and is mediated by interaction of the N1N2 domain of HRG with cell surface heparan sulphate. In contrast, early-stage apoptotic cells have undergone cell membrane changes, including exposure of phosphatidylserine and alteration of membrane carbohydrates, including heparan sulphate, and thus exhibit low levels of HRG cell surface binding. However, once cells become necrotic, either by becoming late-stage apoptotic cells or following exposure to toxic conditions such as hyperthermia, the plasma membrane becomes permeable and allows high level binding of HRG, via its N1N2 domain to unidentified intracellular ligands that are not heparan/heparan sulphate related.

Subsequent experiments described in this chapter investigated the functional relevance of HRG binding to necrotic cells by analysing effect of HRG on the uptake and ingestion of necrotic cells by phagocytic cells. The monocytic cell line, THP-1 was used as the phagocytic cell, while Jurkat T cells were induced into a necrotic state and used as the cell to be recognised and ingested by the THP-1 cells. The cells were mixed together at a ratio of 10:1 necrotic cells to phagocytic cells, either in the presence or absence of 100 µg/ml HRG, at 37°C for up to 80 min. It was found that HRG had a striking effect on the phagocytosis of the necrotic cells, increasing both the level and rate of uptake of the cells by about 2-fold. Such results are in agreement with the general findings of Gorgani et al. (2002), who showed that HRG binds and opsonises late-stage apoptotic cells. However, one of key features arising from this study is that HRG binds and opsonises necrotic cells, and not early-stage apoptotic cells, highlighting that the cell membrane needs to become permeable in order to allow HRG to bind to intracellular ligands. Thus, theoretically HRG may play a role in the uptake and clearance of both necrotic and late-stage apoptotic cells, although in reality late-stage apoptotic cells rarely exist in vivo as there are highly efficient clearance
mechanisms for eliminating early-stage apoptotic cells. Thus, a major in vivo function of HRG is probably to facilitate the clearance of necrotic cells by macrophages. Heparin did not significantly interfere with the ability of HRG to enhance the phagocytosis of necrotic cells, suggesting that the mechanism by which HRG enhances phagocytosis does not involve recognition of heparan sulphate on either necrotic or phagocytic cells. Clearly, further work is required to elucidate the mechanism by which HRG increases the efficiency of uptake of necrotic cells by monocytic cells. Indeed, a HRG 'knockout' mouse has been designed in this laboratory, and is in the final production stage. Using these HRG deficient mice, it will be of considerable interest to determine the role that HRG plays in vivo in the clearance of necrotic cells and the consequences of HRG deficiency on the development of autoimmune diseases such as SLE and RA.
Chapter seven

Final Discussion and Future Directions

This chapter summarises the findings of experiments described in Chapter 3, 4, 5 and 6 of this thesis, and discusses the results obtained in the context of past and current literature. Furthermore, future research directions arising from these studies are addressed.
7.1 Introduction

HRG is a multi-domain protein, with each domain capable of binding to one or more different ligands. In fact, HRG has the ability to simultaneously bind multiple ligands, thus acting as a linking or ‘adapter’ molecule. Results reported in Chapter 3 and Chapter 4 of this thesis suggest a model whereby HRG binds to cell surface heparan sulphate via its N1N2 domain under conditions of low pH and elevated Zn$^{2+}$, and that HRG can simultaneously bind, and thus tether, plasminogen to the cell surface. In addition, results described in Chapter 5 and Chapter 6 of this thesis support a model in which HRG aids the uptake and phagocytosis of necrotic cells by monocytes by binding to an unidentified intracellular ligand on necrotic cells, and providing a link between necrotic cells and phagocytic cells, possibly via FcγR on the surface of monocytes. In both suggested models, HRG acts as an adapter molecule by bringing together and linking ligands to cells. This synopsis, therefore, discusses these models in the light of past and current literature, and explores some of the potential research avenues made possible through this work.

7.2 HRG tethers degradative enzymes to the surface of tumour cells and increases their metastatic potential

Numerous studies have reported that HRG binds to the surface of cells (Chang et al., 1992b; Gorgani et al., 1999b; Kazama and Koide, 1992; Olsen et al., 1996; Saigo et al., 1989). Indeed, Chapter 3 also provides evidence that HRG binds to the surface of 5 different cell lines with differing species and tissue of origin. However, prior to the studies reported in this thesis, it was unclear with which domain HRG bound to cells. Many previous studies supported a model which involved the HRR binding to cell surfaces (Borza and Morgan, 1998; Borza et al., 1996; Burch et al., 1987; Morgan, 1981; Peterson et al., 1987). Meanwhile, other groups have argued that HRG is likely to bind to the cell surface via its N-terminal domain (N1N2), due to high levels of sequence homology of this region of the molecule with other heparin binding proteins, such as antithrombin III (Brown and Parish, 1994; Koide et al., 1982; Koide et al., 1985). Nonetheless, experiments performed in Chapter 3 definitively show, for the first time, that HRG binds to cell surface heparan sulphate via its N1N2 domain and not via its HRR. This result was, on the whole, unexpected, since there is a considerable amount of indirect data from previous reports supporting the view that the HRR probably binds heparan sulphate. In addition, Chapter 3 provides an extensive analysis...
of the cell surface ligand for HRG, and concludes that HRG binds to cell surface heparan sulphate proteoglycans, and not to other GAGs such as the chondroitin sulphates. Numerous groups have reported an interaction between HRG and heparin, and have thus suggested that HRG binds to negatively charged heparan sulphate on cell surfaces. Nevertheless, until this study, no group had reported a direct interaction of HRG with cell surface heparan sulphate. Thus, the results outlined in Chapter 3 represent an important contribution to our basic understanding of the interaction of HRG with cell surfaces and provides information which underpins future studies of HRG function, particularly at the cellular level.

Another key feature of the findings presented in Chapters 3 and 4 is the confirmation of the previously reported effects of Zn\(^{2+}\) and pH on HRG binding to heparin/heparan sulphate (Borza and Morgan, 1998; Burch et al., 1987; Horne et al., 2001; Kazama and Koide, 1992; Lane et al., 1986). Early studies demonstrated that HRG interacts with heparin and heparan sulphate (Heimburger et al., 1972; Lijnen et al., 1983a; Lijnen et al., 1983b), while Borza and Morgan (1998) subsequently reported that the formation of HRG - heparin complexes is, in fact, enhanced by low pH and physiological concentrations of Zn\(^{2+}\). Olsen et al. (1996) also reported that HRG binding to the surface of T cells was markedly enhanced by the presence of 20 µM Zn\(^{2+}\). Based on their studies, Borza and Morgan (1998) proposed that HRG has the unique ability to act as a pH sensor molecule, whereby HRG becomes protonated at acidic pH, thus gaining an increased capacity to bind to cell surface heparan sulphate in areas of local acidosis. Borza and Morgan (1998) also concluded that Zn\(^{2+}\) and acidic pH work synergistically in enhancing the interaction of HRG with heparin/heparan sulphate. However, none of their studies examined the interaction of HRG with cell surfaces. Thus, the cell surface binding studies that were performed in Chapter 3 and Chapter 4 were based on key ideas introduced by Borza and Morgan (1998). One notable difference between Borza and Morgan’s model of HRG binding and the model proposed in Chapter 3, however, is that they postulate that HRG binds to heparan sulphate via its HRR, whereas the model described in Chapter 3 (Figure 3.8) proposes that the N1N2 domain of HRG binds to cell surface heparan sulphate. Importantly, this new model acknowledges that although the HRR does not directly bind to the cell surface, it plays an indirect role by acting as a key regulator of heparan sulphate binding by the N1N2 domain. Indeed, Zn\(^{2+}\) and acidic pH act on the HRG molecule via the HRR, this interaction probably inducing a
conformation change that is mediated throughout the molecule to the N1N2 domain, possibly via inter-domain disulphide bonds. This effect of Zn$^{2+}$ and/or pH on the HRR ultimately determines how effectively the N1N2 domain binds to cell surface heparan sulphate, and as such, both the HRR and the N1N2 domain participate in the recognition of cell surface heparan sulphate by HRG.

It has been previously reported that HRG binds to many soluble ligands, including plasminogen (Gorgani et al., 1999b; Gorgani et al., 1997; Guthans and Morgan, 1982; Leung, 1986; Lijnen et al., 1980; Morgan, 1981; Morgan, 1985; Peterson et al., 1987). Other groups have also highlighted the notion that immobilised HRG appears to have a higher affinity for plasminogen than soluble HRG (Silverstein et al., 1985b). This raises the important issue of whether cell surface immobilised HRG can act as a cell surface receptor for plasminogen. Indeed, plasminogen lacks any known high affinity cell surface receptors. The studies carried out in Chapter 4 unequivocally show, for the first time, that HRG can simultaneously bind to cell surface heparan sulphate and plasminogen, ultimately linking plasminogen to the cell. Surface immobilised plasminogen is more able to be rapidly converted to its active form, plasmin, than soluble plasminogen, while surface associated plasminogen also remains protected from inactivation by the plasminogen inactivators, PAI-1 and PAI-2 (Lijnen, 2001). Perhaps most importantly, surface associated plasminogen is able to focus its degradative potential to the leading edge of a migrating cell. The experiments described in Chapter 4 used the metastatic mouse cell line, B16F1, to show that HRG has the ability to tether plasminogen to cells under specific conditions of elevated Zn$^{2+}$ and low pH. In vivo mouse studies showed that tumour cells coated with HRG produced a greater number of tumour foci in the lung compared to mice injected with tumour cells without HRG treatment. From these studies, it can be postulated that HRG coated tumour cells become more metastatic by recruiting endogenous plasminogen and/or heparanase to their cell surface, resulting in a more metastatic phenotype, although additional experiments such as using plasminogen and/or heparanase deficient mice as tumour cell recipients are needed to validate this view. Interestingly, the conditions that promoted high levels of HRG cell surface binding (high local Zn$^{2+}$ and low pH) also regulated plasminogen binding (Chapter 4). Elevated Zn$^{2+}$ concentrations and low pH usually occur in local areas of tissue injury, wound healing, angiogenesis, and tumour growth. Thus, in a physiological setting, it can be postulated that HRG tethers plasminogen to
the cell surface in microenvironments exhibiting elevated local Zn$^{2+}$ concentrations and low pH, allowing cell surface associated plasminogen to participate in degradative events associated with tissue injury, wound healing and leukocyte migration. In a more pathological setting, HRG may bind to tumour cells, and recruit endogenous degradative enzymes like plasminogen or heparanase to the cell surface, resulting in more metastatic tumour cells (Figure 7.1). This would suggest that HRG has positive effects on tumour metastasis and possibly tumour growth. Recent papers by Juarez et al. (2002) and Olsson et al. (2004) contradict this notion, as both report that HRG, in particular the HRR of HRG, is a potent inhibitor of tumour angiogenesis.

Figure 7.1. Schematic representation of HRG tethering degradative enzymes to the surface of a tumour cell and facilitating ECM degrading activity. HRG is shown in blue, and binds to cell surface heparan sulphate (green circles) on the surface of tumour cells via its N1N2 domain, with HRG binding to cells being enhanced by elevated Zn$^{2+}$. Simultaneously, HRG binds degradative enzymes such as plasminogen/plasmin or heparanase, tethering them to the cell surface and resulting in enhanced conversion of plasminogen to plasmin by uPA, protecting cell bound plasmin from inactivation, and polarising the ECM degrading activity to the leading edge of the cell. This focused matrix degrading activity facilitates tumour cell invasion, tumour-associated angiogenesis and tumour cell intravasation.
Thus, evidence exists which suggests that HRG exhibits both pro- and anti-tumour effects. Perhaps these conflicting roles of HRG can be explained by proposing that proteolytic fragments of HRG containing the HRR exhibit anti-angiogenic effects, whereas the full-length molecule participates in events associated with promoting angiogenesis and metastasis.

7.3 HRG binds to necrotic cells and enhances their rate of phagocytosis

Another major finding resulting from studies reported in this thesis is that HRG can opsonise necrotic cells, resulting in an increased rate of binding and uptake of these cells by monocytes. It should be noted that little is known about the clearance of necrotic cells from tissues, whereas the clearance of apoptotic cells is well documented. Nonetheless, the efficient uptake of dying cells by phagocytes is essential to avoid induction of chronic inflammation, with some human autoimmune responses being thought to be driven by autoantigens arising from inappropriate clearance of apoptotic or necrotic cells (Mountz et al., 1994). Apoptotic cells externalise phosphatidylserine, which marks them as ‘apoptotic’ and triggers their phagocytosis by macrophages (Fadok et al., 2001; Henson et al., 2001). Necrotic cells do not express substantial levels of phosphatidylserine until after membrane destruction, and are thus not readily recognised by phosphatidylserine receptors on phagocytes until their intracellular contents are released into the extracellular space (Shacter et al., 2000). Nevertheless, Brouckaert et al. (2004) showed that both apoptotic and necrotic cells can be recognized and phagocytosed by macrophages, although the uptake of apoptotic cells appears more efficient than the phagocytosis of necrotic cells. Interestingly, in both cases phagocytosis occurred through a phosphatidylserine-dependent mechanism, suggesting that externalization of phosphatidylserine is a general trigger for clearance of dying cells by macrophages. Electron microscopy studies, however, showed morphological differences in the engulfment and uptake of necrotic and apoptotic cells by macrophages. Apoptotic cells appear to be taken up as condensed membrane-bound cells of various sizes, whereas necrotic cells are internalized as small cellular particles that have lost membrane integrity (Brouckaert et al., 2004; Krysko et al., 2003). In addition, Clq and DNase have been shown to play a role in the disposal of necrotic cell-derived chromatin (Gaipl et al., 2004). Furthermore, the serum opsonin mannose-binding lectin has been shown to interact with necrotic cells and facilitate necrotic cell
uptake by macrophages (Nauta et al., 2003). This finding introduces the concept that plasma opsonins exist that interact with necrotic cells and facilitate their uptake by macrophages.

Based on the results reported in Chapter 6, HRG may represent another plasma opsonin for necrotic cells. Similar studies have been previously reported by Gargani et al. (2002), who showed that HRG can opsonise late-stage apoptotic cells, increasing their rate of uptake and clearance by human monocyte derived macrophages. Late-stage apoptotic cells, however, are unlikely to remain in the circulation or in tissues during normal, disease-free physiological conditions, as early-stage apoptotic cells are very efficiently cleared by macrophages. Thus, HRG would normally act as an opsonin for necrotic, not late-stage apoptotic cells. In fact, binding studies performed in Chapter 6 revealed that HRG exhibits little or no reactivity with early-stage apoptotic cells. On the other hand, HRG binds strongly to an undefined intracellular ligand within necrotic cells that is possibly located within the cytoplasm, whereas it interacts with cell surface heparan sulphate on viable cells. Further studies are obviously required to fully characterise the intracellular HRG ligand within necrotic cells. Preliminary studies in this laboratory suggest, however, that HRG may interact with histones (J. Altin and C. R. Parish – Pers. Comm.), and as such, histones may represent a prime candidate for the undefined intracellular HRG ligand in necrotic cells.

Phagocytosis and clearance of immune complexes is normally mediated by Fc and C1q receptors expressed on phagocytic cells. As previously discussed, HRG appears to function primarily as an adaptor protein that is able to tether a ligand to a cell surface receptor. Thus, it is possible that HRG interacts with an intracellular necrotic ligand, links the necrotic cell to FcR on the surface of phagocytes, and thus facilitates phagocytic clearance of necrotic cells in vivo (Figure 7.2). In fact, it has previously been reported that HRG interacts with FcR (Gorgani et al., 1999b; Gorgani et al., 2002). It should be noted, however, that all previous studies reporting an interaction between HRG and FcγRI utilised indirect cell binding methods. Chapter 5 describes direct analyses of HRG interacting with FcγRI, FcγRIIa and FccαR. Both recombinant HRG and recombinant FcγRI were produced in baculovirus infected insect cells, and biosensor, ELISA and flow cytometry studies were employed to analyse the HRG - FcγRI interaction. Clear evidence was obtained that HRG binds to FcγRI. HRG also
exhibited binding to the low affinity receptor for IgG, FcγRIIa, although it appears that HRG binds preferentially to the His131 and not the Arg131 polymorphic form of FcγRIIa. In addition, HRG exhibited binding to the IgA receptor, FcαR. It is interesting to note that HRG has also been shown to play a crucial role in the recognition and clearance of immune complexes (Gargani et al., 1999a). Since HRG opsonises necrotic cells, it is tempting to postulate that this effect is mediated via necrotic cell bound HRG interacting with FcγRI on the surface of phagocytic cells (Figure 7.2). Obviously, further work is required to elucidate the validity of this model. Furthermore, it would be of interest to determine whether the differential binding of HRG to each of the polymorphic forms of FcγRIIa is of functional relevance. Of significance is the observation that neutrophils derived from individuals who are homozygous for the Arg131 FcγRIIa allele are unable to effectively process IgG2 opsonised bacteria (Bredius et al., 1993; Rodriguez et al., 1999; Salmon et al., 1992; Sanders et al., 1994; Wilson et al., 1995). Whether this functional polymorphism is at least partially due to the differential ability of HRG to bind to the two forms of FcγRIIa remains to be determined.

**Figure 7.2. Schematic representation of HRG opsonising necrotic cells.** HRG, shown in blue, binds to an unidentified intracellular receptor within necrotic cells, and simultaneously binds to phagocytic cells, possibly via FcγRI, or other FcγR, resulting in an enhanced rate of uptake and clearance of the necrotic cell.
Thus, under normal physiological conditions, HRG may function *in vivo* as a circulating surveillance molecule, assisting in the normal phagocytosis and clearance of necrotic cells and immune complexes. This would provide endogenous protection from spontaneous inflammation and resultant tissue damage, by ensuring that dying cells are efficiently cleared. Autoimmune diseases, such as SLE, are associated with impaired clearance of immune complexes and the production of autoantibodies against intracellular antigens expressed by dying cells (Salmon *et al.*, 1984). Thus, if HRG plays a key role in the clearance of necrotic cells and immune complexes, then impaired HRG function could be associated with the development or increased severity of such autoimmune diseases.

### 7.4 Future directions

HRG is a multi-domain molecule and interacts with many ligands and receptors. Thus, it is not surprising that although a number of important questions regarding HRG function have been addressed in this thesis, many more questions have arisen.

Firstly, some fundamental aspects of HRG binding to cell surface heparan sulphate still require further investigation. In particular, the amino acid residues within the N1N2 domain that bind to heparan sulphate need to be elucidated. Site directed mutagenesis studies would assist in the identification of such residues, whilst crystallisation of the individual domains, and the whole molecule, would greatly assist our understanding of the structure of the molecule, and how it interacts with its various ligands. It is important too, to identify the regions and amino acid residues in plasminogen and heparanase that interact with HRG. For example, mutagenesis studies would allow the identification of the precise LBS residues within the plasminogen molecule that interact with HRG.

The significance of HRG tethering degradative enzymes, such as plasminogen or heparanase, to the surface of tumour cells also requires further investigation. Matrigel migration assays in transwells could be used to determine whether degradative enzymes such as plasmin and heparanase, that are associated with tumour cells via HRG, aid tumour cell invasion. Similarly, it would be interesting to assess whether HRG deficient mice (HRG −/−), which are currently in the final stages of production in this laboratory, exhibit an increased resistance to tumour growth and impaired tumour metastasis.
Immunohistological analysis of tumour and normal tissue sections may also provide insights into the localisation of HRG within healthy and diseased tissue. For example, studies undertaken in Chapter 3 showed that HRG did not exhibit binding to human endothelial (HUVEC) cells. Thus, it would be of interest to determine whether HRG also fails to associate with vascular endothelial cells \textit{in vivo}.

Further research should also attempt to understand the anti-angiogenic effects of HRG, which are reported to be mediated via the HRR of the molecule (Juarez \textit{et al.}, 2002; Olsson \textit{et al.}, 2004). Recombinant forms of the HRR could be produced using baculovirus infected insect cells, and used in further studies to understand the effects of the HRR on angiogenesis and tumour cell growth. Of particular interest are the findings reported in this thesis which suggest that the HRR does not interact with cells. In fact, preliminary studies described in Chapter 3 suggest that full-length HRG may not even interact with endothelial cells. How then does the HRR mediate its anti-angiogenic effect? Is it via Zn$^{2+}$ or other metal ion depletion?

Further work also needs to investigate the opsonisation of necrotic cells by HRG. Of particular importance is the identification of the intracellular ligand which HRG binds to in necrotic cells. Previous work in this laboratory suggests that the 'mystery ligand' may be histones, thus initial studies should examine the interaction between HRG and histones and whether histones block the binding of HRG to necrotic cells. These are simple experiments that could rapidly resolve the role, if any, of histones in this process. Unfortunately, due to time constraints, these studies were unable to be completed before the due date of this thesis. Similarly, it is important to identify the HRG receptor on phagocytic cells that facilitates necrotic cell binding and uptake. Studies reported in Chapter 5 and 6 suggest that HRG does not interact with heparan sulphate on phagocytic cells, and that it possibly interacts with the phagocytic cells via FcγRI, FcγRIIa or FcαR. However, more comprehensive studies need to be carried out to ascertain whether these receptors actually mediate the uptake and resulting clearance of necrotic cells. Phagocytic assays that incorporate FcR blocking antibodies should rapidly establish whether these receptors play a role. More physiological studies could monitor the \textit{in vivo} clearance of fluorescently labelled necrotic cells and immune complexes in HRG$^{-/-}$ mice. If HRG plays a crucial role in both these processes then impaired clearance would be observed in the HRG deficient animals. In fact, HRG$^{-/-}$
mice should provide many clues as to the normal physiological functions of HRG. For example, these mice should be monitored for their susceptibility to autoimmune diseases, such as SLE, as again one would predict that HRG \textsuperscript{-/-} mice will be more susceptible to such autoimmune diseases compared to wild type mice. Clearly, much of the work in this thesis has laid the foundation for future studies which will unequivically establish the physiological relevance of this intriguing protein in both health and disease.
References


weight heparin and for the involvement of histidine residues in heparin binding. 

*Biochemistry* 26, 7477-82.


abnormalities in routine laboratory assays of hemostatic function, immunologic function, and trace elements [see comments]. *J Lab Clin Med* **125**, 719-23.


Appendix 1  Nucleotide and amino acid sequence of human Histidine-Rich Glycoprotein

tta aag gga tgg ttt taa aac gaa
leu lys gly trp phe asn lys

N1
N2
HRR
C-terminal

cDNA and deduced amino acid sequence of human HRG. The protein start codon is represented by blue type, while the stop codon is shown in red and marked with STOP. The N1 and N2 cystatin-like domains are boxed with blue shading, and separated by a vertical black line, the HRR is boxed with green shading, while the C-terminal domain is boxed in yellow shading.
Appendix 2  Nucleotide and amino acid sequence of human FcγRI.

**EC1**  **EC2**  **EC3**

TM + CT

cDNA and deduced amino acid sequence of human FcγRI. The protein start codon is represented by blue type, while the stop codon is shown in red and marked with STOP. The three extracellular domains are boxed with blue shading, while the transmembrane and cytoplasmic tail regions are boxed with green shading.