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Regulation of histidine-rich glycoprotein (HRG) function via plasmin-mediated proteolytic cleavage

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The plasminogen/plasmin system is involved in a variety of normal physiological and pathological processes, including tissue remodelling, angiogenesis and tumour metastasis. Plasminogen activators and receptors for plasminogen/plasminogen activators are essential for the processing of plasminogen to form the active serine protease plasmin. Plasmin can in turn positively or negatively regulate further plasminogen activation via plasmin-mediated cleavage of receptors and activators. HRG (histidine-rich glycoprotein), a relatively abundant (approx. 100–150 μg/ml) plasma glycoprotein, has a multi-domain structure that can interact with many ligands, including Zn^{2+}, heparin, HS (heparan sulfate) and plasminogen. HRG has been shown to function as an adaptor molecule to tether plasminogen to GAG (glycosaminoglycan)-bearing surfaces and to regulate plasminogen activation via various mechanisms. As HRG itself is sensitive to plasmin cleavage, the present study examines in detail the cleavage of human HRG by plasmin and the effect of this cleavage on various functions of HRG. HRG fragments, generated by plasmin cleavage, are held together by disulfide linkages and are not released from the molecule under non-reducing conditions. Plasmin-mediated cleavage partially inhibited HRG binding to cell surface HS, but enhanced HRG binding to necrotic cells and to plasminogen. However, both intact and plasmin-cleaved HRG enhanced the binding of plasminogen to heparin-coated surfaces to a similar extent. Furthermore, the presence of heparin, Zn^{2+} or acidic pH was found to protect HRG from plasmin cleavage. Thus proteolytic cleavage of HRG by plasmin may provide a feedback mechanism to regulate the effects of HRG on the plasminogen/plasmin system and other functions of HRG.

Key words: heparan sulfate (HS), histidine-rich glycoprotein (HRG), plasmin, plasminogen, proteolytic cleavage.

INTRODUCTION

Serum protein-based biological systems, such as the complement, coagulation and fibrinolytic pathways, often rely heavily on the proteolytic cleavage of serum proteins to regulate successive enzymatic cascades or to generate specific protein fragments that can perform various functions. In particular, the plasminogen/plasmin system, which plays an important role in the dissolution of fibrin clots and remodelling of the ECM (extracellular matrix), utilizes a variety of proteolytic cleavage-based mechanisms to either positively or negatively regulate the formation of the serine protease plasmin from its zymogen plasminogen [1]. Briefly, plasmin is formed via the proteolytic processing of the plasma zymogen plasminogen by activators such as urokinase-type or tissue-type plasminogen activators (u-PA or t-PA) [1]. Plasmin can in turn regulate plasminogen activation via a number of different mechanisms, namely: (i) plasmin-mediated cleavage of single chain u-PA and t-PA to generate a more active two-chain enzyme [2,3]; (ii) proteolytic cleavage of fibrin by plasmin to expose C-terminal lysine residues that enhance plasminogen binding to fibrin and activation by t-PA [4]; and (iii) plasmin-mediated cleavage of the u-PAR (u-PA receptor) to reduce the cell surface localization of u-PA for plasminogen activation [5].

HRG (histidine-rich glycoprotein) is a secreted single-polypeptide-chain protein, with a molecular mass of approx. 75 kDa, that is found in human plasma at the relatively high concentration of approx. 100–150 μg/ml [6]. HRG has a multi-domain structure consisting of two N-terminal regions with homology with cystatin-like domains, together termed the N1N2 domain, a central HRR (histidine-rich region), flanked by two PRRs (proline-rich regions; PRR1 and PRR2), and a C-terminal domain [6]. HRG also contains four intra-domain and two inter-domain disulfide bridges that link the C-terminal domain and the HRR to the N1N2 domain [7]. There are many ligands of HRG, such as Zn^{2+}, haemin, heparin, HS (heparan sulfate), IgG and fibrinogen [6,8], with HRG also interacting strongly with the lysine-binding sites on plasminogen [9], possibly via its C-terminal lysine residues [10,11]. Initially, HRG was proposed to be an anti-fibrinolytic agent, acting by blocking plasminogen from interacting with binding partners that are important for plasmin activation [9], such as fibrinogen, fibrin, integrin $\alpha_M\beta_2$ and annexin 2 [1]. However, there are conflicting reports in this area with different studies demonstrating that HRG either inhibits, or has no effect on, fibrinogen-dependent plasminogen activation [12,13]. In stark contrast, HRG has been suggested to function as a soluble plasminogen receptor that aids plasminogen activation by tethering plasminogen to GAG (glycosaminoglycan)-coated surfaces [12] and to cell surfaces [14].

Human HRG has been shown to be susceptible to proteolytic cleavage *in vitro* by serine proteases, such as plasmin and kallikrein, but not thrombin [15]. Extensive proteolysis of human HRG has also been observed in patients that have received streptokinase therapy, which increases plasmin activity *in vivo*

Abbreviations used: CHO, Chinese-hamster ovary; ECM, extracellular matrix; FGF, fibroblast growth factor; GAG, glycosaminoglycan; HRG, histidine-rich glycoprotein; HRR, histidine-rich region of HRG; HRP, horseradish peroxidase; HS, heparan sulfate; N1N2, the N-terminal domains of HRG; Ni-NTA, Ni^{2+}–nitrilotriacetic acid; PE, phycoerythrin; PRR, proline-rich region of HRG; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

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[15]. Furthermore, the ability of plasmin to cleave HRG at specific sites between different domains of the molecule has been utilized to isolate various intact domains of HRG and characterize their structure and function, with rabbit HRG having been studied in some detail [10,16–18]. Although the release of the HRR of HRG following plasmin cleavage was suggested to play an important role in mediating the anti-angiogenic [19], and possibly the anti-microbial, effects of the molecule [20], the physiological significance of plasmin-mediated cleavage of human HRG has not been thoroughly examined. To this end, in the present study human HRG was proteolytically cleaved by plasmin, a treatment that generated HRG fragments that are held together by disulfide bonds. Although initial plasmin treatment did not release any HRG fragments under non-reducing conditions, plasmin-mediated cleavage partially reduced HRG binding to cell surface HS and potentiated HRG binding to necrotic cells and to plasminogen. However, plasmin cleavage had no major effect on the ability of HRG to tether plasminogen to heparin-coated surfaces. In addition, the presence of heparin, Zn^{2+} or acidic pH was found to protect HRG from plasmin cleavage. Therefore, proteolytic cleavage of HRG by plasmin may play an important role in regulating HRG function.

EXPERIMENTAL

Antibodies and reagents

An N1N2-domain specific anti-(human HRG) monoclonal antibody, HRG-4, was provided by AGEN Biomedical. Domain-specific rabbit anti-(human HRG) antibodies (0115, 0116 and 0119) were produced in-house as previously described [19]. The use of animals to generate domain-specific rabbit anti-(human HRG) antibodies was approved by the Uppsala University. Rabbit anti-(human plasminogen) antibody was supplied by Dako. Sheep anti-mouse Ig–HRP (horseradish peroxidase) antibody, sheep anti-rabbit Ig–HRP antibody, sheep $F(ab')_2$ anti-mouse Ig–FITC antibody and sheep $F(ab')_2$ anti-mouse Ig–PE (phycoerythrin) antibody were purchased from Chemicon and Ni-NTA (Ni^{2+}-nitrilotriacetate)–HRP was purchased from Kirkegaard and Perry Laboratories. Human plasminogen and biotinylated heparin were gifts from Dr Allison Jones and Dr Craig Freeman (The John Curtin School of Medical Research, Australian National University, Canberra, Australia) respectively. BSA, ExtrAvidin®, bovine lung heparin and human plasmin were purchased from Sigma–Aldrich.

Cell lines

The GAG-bearing CHO (Chinese-hamster ovary)-K1 and the GAG-deficient CHO pgsA-745 cell lines were cultured in 50 % Dulbecco's modified Eagle's medium and 50 % Ham's nutrient mixture F-12 (Invitrogen). Jurkat T-cells were cultured in RPMI-1640 medium (Invitrogen). All mammalian cell culture media were supplemented with 10 % (v/v) fetal calf serum, 5 mM L-glutamine, 30 μg/ml penicillin G, 50 μg/ml streptomycin sulfate and 50 μg/ml neomycin sulfate. Mammalian cell lines were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. To generate primary necrotic cells, Jurkat T-cells were resuspended at a concentration of 2×10^6 cells/ml and exposed to hyperthermic conditions (56 °C) for 30 min.

Purification of human plasma-derived HRG

HRG was purified from human plasma according to a previously described method [21,22]. The use of human plasma was approved by the Australian Red Cross Blood Service, the Australian National University and La Trobe University. Briefly, human plasma was passed through a phosphocellulose column (Whatman) equilibrated with a solution containing 0.5 M NaCl, 10 mM sodium phosphate buffer and 1 mM EDTA at pH 6.8. Bound HRG was eluted with a solution containing 2.0 M NaCl, 10 mM sodium phosphate buffer and 1 mM EDTA at pH 6.8. Traces of human IgG were removed by passing plasma-derived HRG through a HiTrap™ Protein G column (Amersham Biosciences).

Generation of plasmin-cleaved HRG

Plasmin-cleaved HRG used in the present study was generated via the same procedure with minor modifications, depending on the application. For the initial analysis of the proteolytic cleavage of HRG by plasmin and the preparation of samples for Edman N-terminal sequencing, plasmin-mediated digestion of HRG was performed at 37 °C using 1 mg/ml HRG and 50 μg/ml plasmin in PBS (pH 7.2). To examine plasmin-mediated cleavage of HRG *ex vivo*, 1 μl of human plasma was diluted to 10 μl in PBS (pH 7.2) containing 1, 3 and 9 μg of plasmin and then incubated at 37 °C for 120 min. For ELISA- and flow cytometry-based binding assays and Western blot analysis using the HRG-4 N1N2-domain specific anti-(human HRG) antibody, proteolytic cleavage of HRG was performed at 37 °C using 300 μg/ml HRG and 30 μg/ml plasmin in PBS (pH 7.2). Following plasmin treatment, HRG preparations were diluted in appropriate buffer containing 50 μg/ml aprotinin (Boehringer) to inhibit further digestion of HRG by plasmin during the subsequent binding assay. To investigate the effect of heparin (12.5 kDa), pH and Zn^{2+} on the proteolytic cleavage of HRG by plasmin, 30 μg/ml HRG and 6 μg/ml plasmin were diluted in PBS and incubated at 37 °C for 60 min under the different conditions.

SDS/PAGE and Coomassie Brilliant Blue protein staining

Protein samples were boiled in reducing or non-reducing SDS sample buffer [125 mM Tris/HCl, pH 6.8, containing 20 % (v/v) glycerol, 4 % (w/v) SDS], with the reducing buffer containing 50 mM dithiothreitol. Samples were subjected to PAGE on a 4–20 % (w/v) gradient gel (Gradipore). Unless otherwise stated, all samples were subjected to SDS/PAGE under reducing conditions. Protein samples were subsequently stained with Coomassie Brilliant Blue [0.2 % Coomassie Brilliant Blue, 50 % (v/v) methanol, 7.5 % (v/v) acetic acid] and then destained with destaining buffer [40 % (v/v) methanol, 7 % (v/v) acetic acid].

N-terminal sequencing

Following SDS/PAGE and Coomassie Brilliant Blue staining, selected protein bands were excised from the polyacrylamide gel and subjected to Edman N-terminal sequencing (performed by the Australian Proteome Analysis Facility).

Western blotting

Following SDS/PAGE, protein samples were transferred electrophoretically from the polyacrylamide gel on to a nitrocellulose membrane (Amersham Biosciences) using a Mini-Protean II apparatus (Bio-Rad) in transfer buffer containing 25 mM Tris base, 192 mM glycine and 20 % (v/v) methanol. The membrane was blocked for 16 h at 4 °C with 5 % (w/v) skimmed milk powder diluted in PBS. Intact and plasmin-cleaved HRG were detected using domain-specific mouse and rabbit anti-(human HRG) antibodies and Ni-NTA–HRP and by chemiluminescence

using ECL® (enhanced chemiluminescence) Western blotting detection reagents (GE Healthcare).

Enzyme-linked immunoabsorbent assay

ELISAs were performed by coating U-bottomed 96-well microtitre plates (Dynex) with the protein to be immobilized in PBS for 16 h at 4 °C. Plates were washed with PBS containing 0.02 % Tween-20 and then incubated with PBS containing 3 % (w/v) BSA for 3 h at 4 °C to block non-specific binding. In some experiments 10 μg/ml biotinylated heparin, diluted in PBS conataining 1 % (w/v) BSA, was added to 10 μg/ml ExtrAvidin®-coated plates for 90 min at 4 °C prior to addition of PBS containing 3 % (w/v) BSA. Binding proteins diluted in PBS containing 1 % (w/v) BSA were then added and the plates incubated for 90 min at 4 °C. Plate-bound HRG and plasminogen were detected using the HRG-4 N1N2-domain specific antibody and a rabbit anti-(human plasminogen) antibody respectively, followed by secondary antibody detection with HRP-conjugated antibodies. Plate-bound HRP was detected using a peroxidase substrate (Kirkegaard and Perry Laboratories). The absorbance of the enzymatic product at 405 nm was measured using a Thermomax microplate reader (Molecular Devices). The results were analysed using SoftMaxPro 4.0 software (Molecular Devices).

Immunofluorescence flow cytometry

Viable and necrotic cells were analysed for HRG binding by immunofluorescence flow cytometry. Typically, 2×10^5 cells were incubated with 100 μg/ml intact or plasmin-cleaved HRG diluted in PBS containing 0.1 % BSA (pH 6.6 or pH 7.2), with or without Zn^{2+}, for 30 min at 4 °C. Cells were washed three times with PBS containing 0.1 % BSA (pH 7.2) and then cell-bound HRG was detected using the HRG-4 N1N2-domain specific antibody, followed by secondary antibody detection with FITC- or PE-conjugated antibodies. Cells were resuspended in PBS, pH 7.2, containing 0.1 % BSA and 1 μg/ml Hoechst 33258 (Calbiochem) and immediately analysed by immunofluorescence flow cytometry using a LSR1 Flow Cytometer and Cell Quest Pro software (BD Biosciences). The resultant flow cytometry results were analysed using FlowJo software (Tree Star). Cells treated in the same manner in the absence of binding proteins were used as negative controls to set up appropriate laser voltage. Cells were gated appropriately based on forward and side scattering. Living and dead cells were distinguished on the basis of Hoechst 33258 negative and positive staining respectively.

Plasmin activity assay

The chromogenic plasmin substrate *N*-(*p*-tosyl)-Gly-Pro-Lys (Sigma–Aldrich) was used to assess plasmin activity. Briefly, samples were prepared in 50 μl of PBS (pH 7.2, 6.6 or 6.0) containing 1 mg/ml *N*-(*p*-tosyl)-Gly-Pro-Lys and incubated for 60 min at 37 °C. Samples were then transferred into a U-bottomed 96-well microtitre plate and the absorbance of the enzymatic product was measured at 405 nm on a Thermomax microplate reader. Results were analysed using SoftMaxPro 4.0 software.

RESULTS

Proteolytic cleavage of human HRG by plasmin

As the plasmin cleavage sites in human HRG have not been defined, the ability of plasmin to cleave human HRG was initially examined by SDS/PAGE under both non-reducing and reducing conditions. Under non-reducing conditions, plasmin-cleaved (30–120 min at 37 °C) HRG showed no major decrease in molecular mass compared with untreated (0 min) HRG, whereas multiple protein bands were observed under reducing conditions with plasmin-cleaved but not with untreated HRG (Figure 1A). These results demonstrate that plasmin can effectively cleave human HRG at multiple sites with a cleavage pattern similar to that previously described for rabbit HRG [16] and human HRG [15] when analysed by SDS/PAGE under reducing conditions. In addition, plasmin-cleaved fragments of HRG remain predominately bound together by disulfide bonds and are not released from the rest of the molecule under non-reducing conditions. However, it is worth noting that under non-reducing conditions a small proportion of the HRG subjected to the extensive plasmin-cleavage (120 min at 37 °C) showed a clear reduction in molecular mass compared with the untreated HRG (Figure 1A). These results suggest that certain plasmin-generated fragments of HRG are not linked to the rest of the molecule via disulfide bonds and can be released from HRG under non-reducing conditions, although such fragments are only generated following prolonged plasmin treatment. Furthermore, proteolytic cleavage of HRG can also occur in human plasma *ex vivo* by the addition of exogenous plasmin (Figure 1B), indicating that an increase in plasmin activity in plasma can generate plasmin-cleaved fragments of HRG.

To determine the domains of HRG that are present in each fragment of plasmin-cleaved HRG, domain-specific rabbit anti-(human HRG) antibodies (0115, 0116 and 0119) were used to detect intact and plasmin-cleaved HRG under reducing conditions by Western blot analysis. As shown in Figure 1(C), the N-terminal-specific anti-(human HRG) antibody (0116) bound strongly to intact HRG and plasmin-cleaved HRG fragments that are ~37 kDa or above, indicating that all these fragments contain the N-terminal domain of HRG. It is worth noting that these N-terminal fragments of HRG appear to migrate as doublets on SDS/PAGE under reducing conditions (Figure 1C), possibly representing different glycoforms of HRG. Moreover, the HRR-specific anti-(human HRG) antibody (0119) only detected intact HRG and the largest plasmin-cleaved HRG fragment of ~60 kDa, indicating that the epitope recognized by the HRR-specific anti-(human HRG) antibody (0119) is absent in all the other plasmin-cleaved HRG fragments (Figure 1C). Similarly, the C-terminal-specific anti-human HRG antibody (0115) detected intact HRG and the ~12 kDa fragment (Figure 1C), indicating that the 12 kDa plasmin-cleaved HRG fragment contains the C-terminal domain.

The proteolytic cleavage of human HRG by plasmin was further investigated by Edman N-terminal sequence analysis of intact and selected plasmin-cleaved HRG fragments acquired under reducing conditions, in order to define the plasmin cleavage sites and predict the domains of HRG present in the fragment of interest. Consistent with the antibody-binding results (Figure 1C), and with previous studies on rabbit HRG [16], the N-terminal sequence of intact and the ~37 kDa fragment of HRG was identical (Figure 2A), suggesting that all plasmin-cleaved HRG fragments above 37 kDa are likely to contain the same N-terminus and each successive fragment from 75 to 37 kDa represents progressive plasmin-mediated cleavage of HRG from the C-terminus. The ~12 kDa fragment that was released after plasmin treatment of HRG at 37 °C for 30 min contains an N-terminal sequence of RRGPGKG (Figure 2A), indicating a plasmin cleavage site on the carboxy side of Arg^{421} and therefore this fragment contains the C-terminal domain and parts of the PRR2 of HRG (see Supplementary Figure S1, available at http://www.BiochemJ.

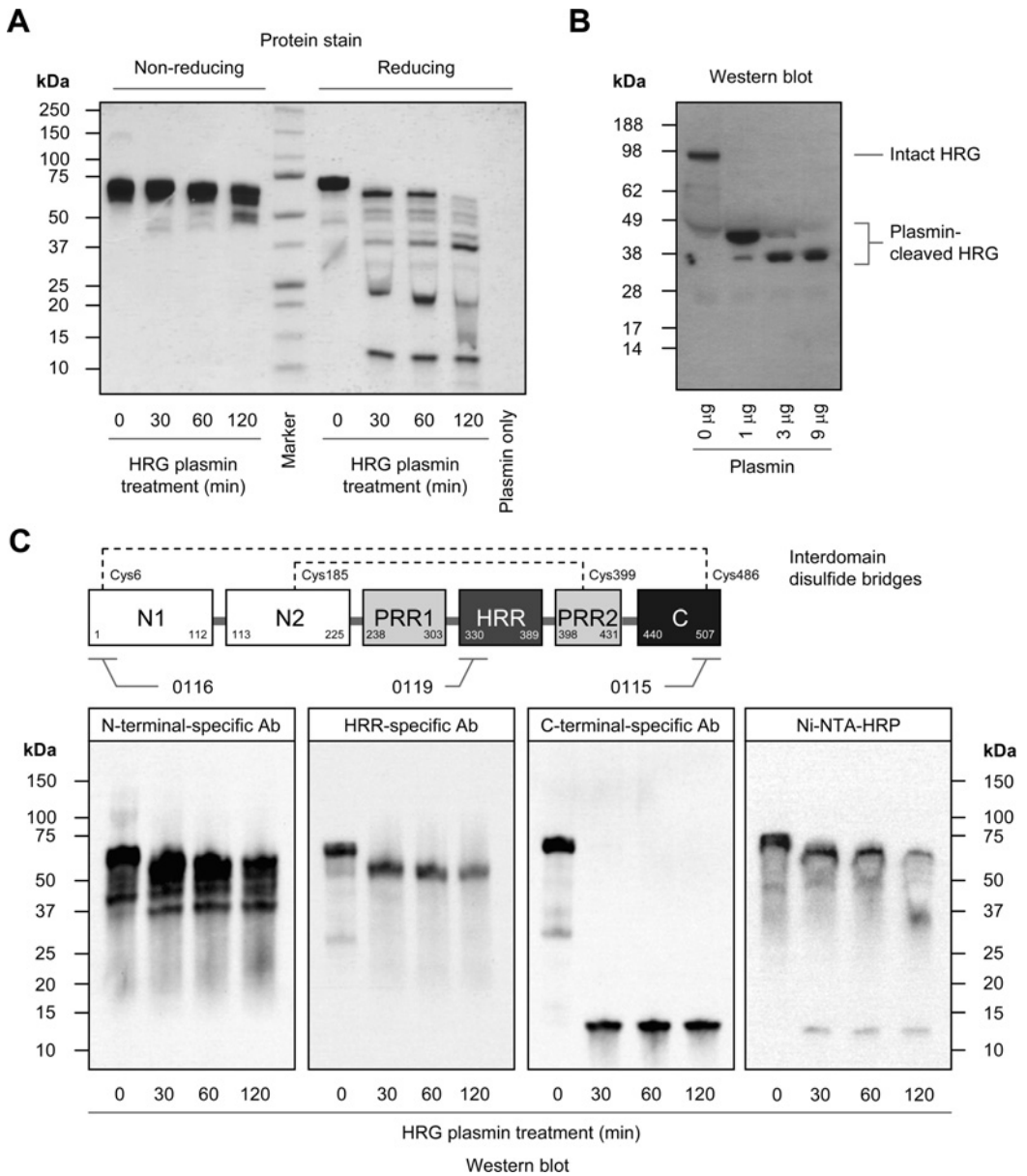

Figure 1 Determination of the plasmin-cleaved fragments of human HRG

(**A**) SDS/PAGE analysis of the proteolysis of HRG (10 μg, 1 mg/ml) by plasmin (0.5 μg, 50 μg/ml) at 37 °C for 0, 30, 60 and 120 min in the presence (Reducing) or absence (Non-reducing) of dithiothreitol, with plasmin alone being included as a negative control. Protein bands stained with 0.2 % Coomassie Brilliant Blue. (**B**) Western blot analysis of the proteolysis of HRG in 1 μl human plasma by 1, 3 and 9 μg exogenous plasmin at 37 °C for 120 min using monoclonal HRG-4 anti-(human HRG) antibody, specific to the N1N2 domain. (**C**) Western blot analysis of the proteolysis of HRG (750 ng, 1 mg/ml) by plasmin (37.5 ng, 50 μg/ml) at 37 °C for 0, 30, 60 and 120 min using the domain-specific rabbit anti-(human HRG) antibodies 0115, 0116 and 0119. The specificity of each antibody is shown schematically in the upper panel. N1, N-terminal domain 1; N2, N-terminal domain 2; PRR1, proline-rich region 1; HRR, histidine-rich region; PRR2, proline-rich region 2; C, C-terminal domain. Histidine-rich sequences present in 3 μg of intact and plasmin-cleaved HRG were also detected using HRP-conjugated Ni-NTA (Ni-NTA-HRP).

org/bj/424/bj4240027add.htm, for the amino acid sequence of human HRG). Although no conclusive N-terminal sequence was obtained from the ~20 kDa plasmin-cleaved HRG fragment, histidine was found to be the most abundant amino acid in the first Edman sequencing cycle, followed by glycine, leucine and proline (Figure 2A). These results suggest that the ~20 kDa fragment probably contains the HRR domain and has a frayed N-terminus. Furthermore, the presence of a frayed N-terminus in this ~20 kDa fragment could potentially disrupt the epitope recognized by the HRR-specific anti-(human HRG) antibody (0119) (Figure 1C). These results are consistent with the presence of multiple internal plasmin cleavage sites within the HRR, and hence the difficulties encountered in isolating different domains of human HRG following plasmin digestion [17]. To validate whether the ~20 kDa fragment contains the HRR of HRG, Ni-NTA–HRP was used to detect histidine-rich sequences present in various cleaved fragments of HRG. Surprisingly, similar to the HRR-specific anti-human HRG antibody (0119), Ni-NTA–HRP was found to bind predominately to intact HRG and the largest plasmin-cleaved HRG fragment of ~60 kDa, but not the ~20 kDa fragment (Figure 1C). Thus the present study cannot determine whether the ~20 kDa fragment contains the HRR

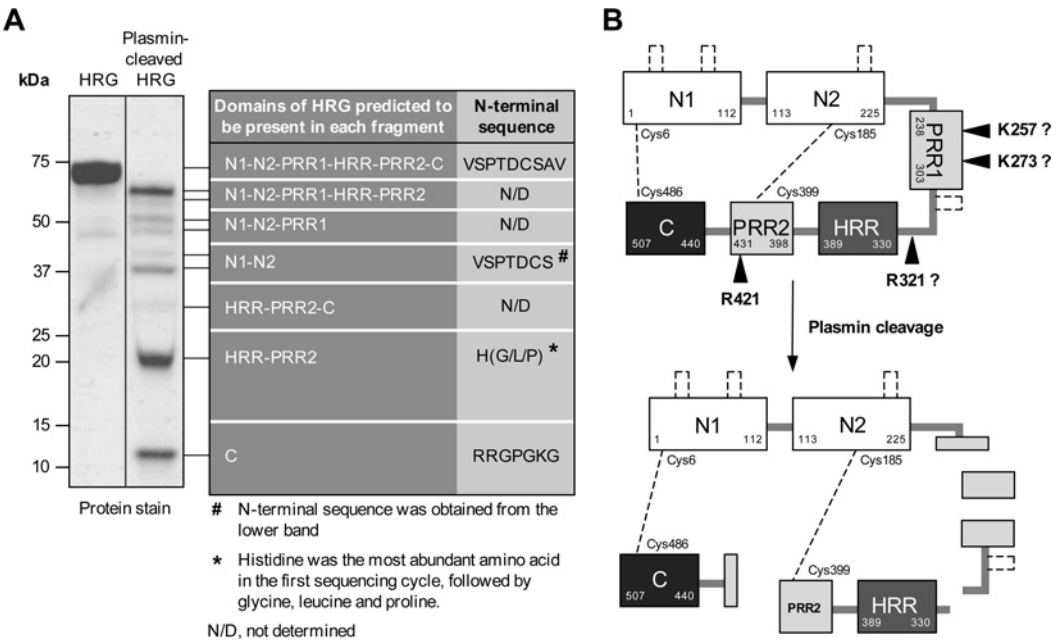

Figure 2 Determination of plasmin cleavage sites in human HRG by N-terminal sequencing

HRG was subjected to plasmin cleavage as in Figure 1(A). (**A**) N-terminal amino acid sequence analysis of intact and plasmin-cleaved HRG fragments obtained under reducing conditions. Edman N-terminal sequencing results for each HRG fragment are indicated in the Figure corresponding to the results acquired in Figure 1(A). On the basis of the N-terminal sequencing results and domain-specific antibody and Ni-NTA binding results in Figure 1(B), domains of HRG predicted to be present in each HRG fragment are also indicated. (**B**) Schematic representation of the proteolysis of human HRG by plasmin, with black triangle indicating potential plasmin cleavage sites. N1, N-terminal domain 1; N2, N-terminal domain 2; PRR1, proline-rich region 1; HRR, histidine-rich region; PRR2, proline-rich region 2; C, C-terminal domain.

of HRG. Furthermore, it is worth noting that a faint band was also detected at ~30 kDa under reducing conditions (Figure 1A). However, owing to the low yield of this fragment, its N-terminal sequence was not determined. Nevertheless, as the HRR- and C-terminal-specific anti-(human HRG) antibodies (0119 and 0115) were both able to recognize the ~30 kDa fragment (Figure 1C), this fragment may contain the HRR and C-terminal domain of HRG (Figure 2A). Therefore based on the results in the present paper, and previous studies on rabbit HRG [16,17] and human HRG [19], the predicted outcome of plasmin-mediated cleavage of HRG is shown in Figure 2(B).

In order to monitor the effect of plasmin cleavage on various functions of HRG, an antibody-based detection method was developed. As the N-terminal domains of HRG (i.e. the two cystatin-like domains, N1 and N2) are relatively resistant to proteases [16] and most plasmin-cleaved fragments of HRG are held together by the N1N2 domain under non-reducing conditions (see Figures 1 and 2), the HRG-4 N1N2-domain-specific antibody was used to detect intact and plasmin-cleaved HRG in various assays. Western blot analysis under reducing conditions identified several bands, indicating a stepwise reduction in the molecular mass of the N-terminus of HRG following plasmin cleavage (Figure 3A; Bands 1–4). These results confirm that the generation of the lower-molecular-mass bands, and the loss of the higher molecular mass bands, represents the progressive plasmin-mediated cleavage of HRG from the C-terminus. The predicted identity of the N-terminal fragments of HRG under reducing conditions are also shown in Figure 3(A). Furthermore, the HRG-4 antibody effectively detected native intact (0 min) and plasmin-cleaved (30 and 120 min at 37 °C) HRG immobilized on ELISA wells to a similar extent (Figure 3B). This implies that, under native conditions, the epitope on HRG recognized by the HRG-4 antibody is not sensitive to plasmin cleavage. Therefore, this antibody-based detection method provides a very sensitive means to detect intact and extensively plasmin-cleaved HRG.

Plasmin cleavage reduces HRG binding to cell surface HS

The cleavage of HRG by plasmin has been shown to generate intact domains of HRG that have preserved functional properties [10,16]. Although plasmin-cleaved HRG was predominantly used to isolate the different domains of HRG and to characterize the functions of each domain in isolation from the rest of the molecule [10,16–18], plasmin cleavage can potentially play a physiological role in regulating the normal function of HRG by modulating HRG binding to various ligands. HRG binding to cell surface HS has been shown to play an important role in regulating growth factor binding to HS [23], as well as in tethering plasminogen to the cell surface [12,14]. Thus the effect of plasmin cleavage on HRG binding to cell surface HS was initially examined using viable CHO-K1 cells as the model cell line [21]. As shown in Figure 4(A), 100 μg/ml HRG bound strongly to viable CHO-K1 cells and showed no detectable binding to the pgsA-745 GAG-deficient CHO cell line, indicating that HRG binding to CHO-K1 cells is mediated via cell surface GAGs, such as HS [21]. HRG treated with plasmin at 37 °C for 0, 30 and 120 min was used to represent intact HRG, HRG that has been cleaved predominately at position Arg^{421} or extensively cleaved HRG respectively. Strikingly, HRG binding to cell surface HS was reduced by >50 % when HRG was treated with plasmin for either 30 or 120 min at 37 °C (Figure 4B). These results suggest that plasmin cleavage can potentially destroy the HS-binding site and/or modify the conformation of HRG to reduce HRG binding to cell surface HS. It is worth noting that initial (30 min at 37 °C) or extensive (120 min at 37 °C) plasmin cleavage of HRG had a similar level of inhibitory effect (approx. 55 %) on HRG binding

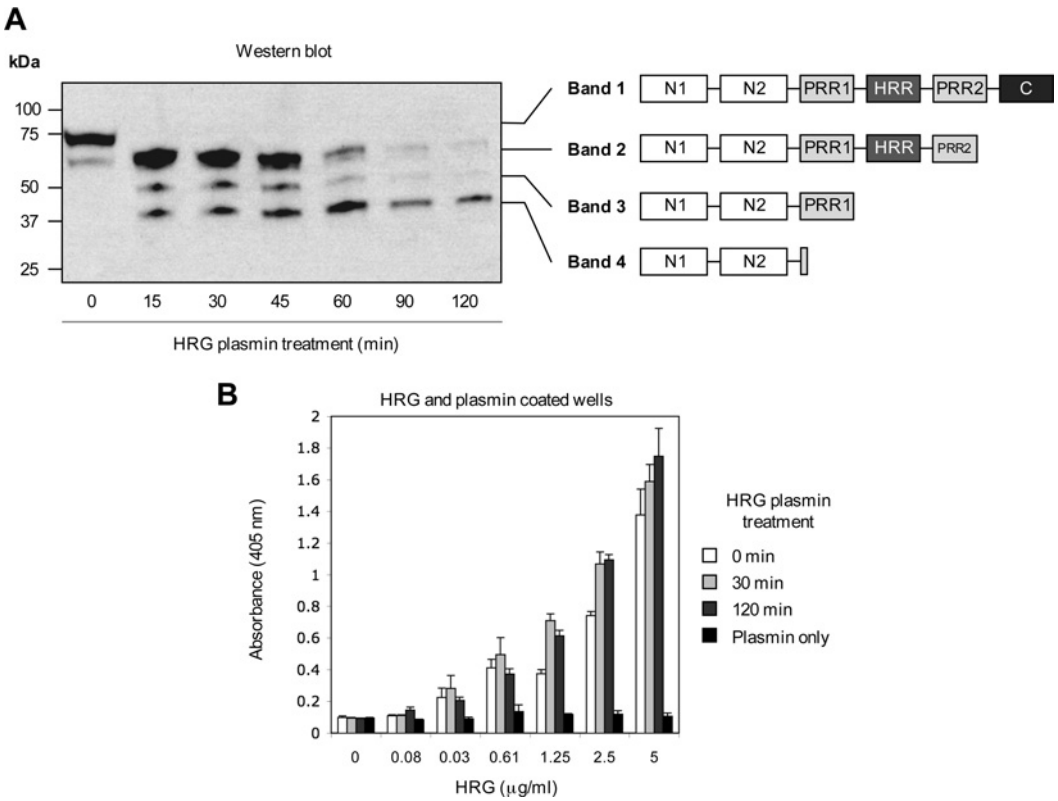

Figure 3 Analysis of plasmin-cleaved HRG using a monoclonal antibody specific for the N1N2 domain

(**A**) Western blot analysis of the proteolysis of 300 ng (300 μg/ml) HRG by 30 ng (30 μg/ml) plasmin at 37 °C for 0 to 120 min using the HRG-4 N1N2-domain-specific antibody. The predicted identity of each N-terminal fragment of HRG under reducing conditions is shown on the right (Bands 1–4). (**B**) Analysis of the ability of 0.6 ng/ml HRG-4 N1N2-domain specific antibody, to bind to ELISA wells pre-coated with various concentrations (0.08 to 5 μg/ml) of intact HRG (white bars, 0 min) and plasmin-cleaved HRG for 30 and 120 min (light grey and dark grey bars respectively) at 37 °C. Plasmin alone is included as a negative control (black bars). Error bars represent the S.E.M. over three replicates.

to CHO-K1 cells (Figure 4B), indicating that a single plasmin cleavage at position Arg^{421} is adequate to significantly reduce HRG binding to cell surface HS and further plasmin cleavage had no additional effect. Furthermore, the presence of heparin (12.5 kDa, 100 μg/ml) completely abolished the ability of intact, as well as plasmin-cleaved HRG, to bind to CHO-K1 cells (Figure 4B), indicating that both intact and plasmin-cleaved HRG can still bind to heparin, which competes for cell surface HS binding.

Previous studies have demonstrated that the binding of HRG to cell surfaces can be potentiated by the presence of Zn^{2+} [21,24] or by a low pH [14]. Therefore, the ability of plasmin-cleaved HRG to bind to CHO-K1 cells in the presence of Zn^{2+} or at a acidic pH was also investigated. Consistent with previous studies, the presence of physiological concentrations of Zn^{2+} (20 μM) or pH 6.6 enhanced the binding of HRG to cell surface HS (Figure 4C). Interestingly, plasmin treatment (30 and 120 min at 37 °C) not only reduced HRG binding to CHO-K1 cells, but also abolished the ability of Zn^{2+} or acidic pH to enhance HRG binding (Figure 4C), suggesting that an intact molecule is required for HRG to respond to regulatory factors such as Zn^{2+} and pH. Thus plasmin cleavage can potentially inhibit the ability of HRG to function as a pH and Zn^{2+} sensor in response to tissue injury.

In additional experiments, the ability of intact and plasmin-cleaved HRG to bind to necrotic Jurkat T-cells was also examined. In contrast with cell surface HS binding, plasmin-mediated cleavage significantly enhanced the binding of HRG to necrotic cells (Figure 4D). These results suggest that plasmin cleavage at sites of tissue injury may potentiate HRG binding to necrotic cells. Moreover, consistent with the results of Jones et al. [25], the interaction between HRG and necrotic cells is likely to be independent of cell surface HS as, despite plasmin cleavage affecting the binding of HRG to cell surface HS on viable cells, plasmin cleavage had no inhibitory effect on HRG binding to necrotic cells.

Plasmin cleavage may modulate the ability of HRG to regulate the plasminogen/plasmin system

HRG has been shown to bind to plasminogen in a number of studies [9–12,14] and to modulate the activation of plasminogen into plasmin [10,12]. The ability of HRG to tether plasminogen to GAG-coated surfaces has also been proposed to play an important role in facilitating plasminogen activation [12,14]. As HRG itself is sensitive to plasmin cleavage, activation of plasminogen into plasmin may in turn promote the generation of plasmin-cleaved HRG and alter the ability of HRG to regulate the plasminogen/plasmin system. Therefore the effect of plasmin cleavage on the binding of HRG to plasminogen was initially investigated. Unlike cell surface HS binding, initial plasmin cleavage (30 min at 37 °C) markedly enhanced HRG binding to plasminogen immobilized on ELISA wells, whereas extensive plasmin cleavage (120 min at 37 °C) of HRG resulted in plasminogen binding returning to control levels (Figure 5A). These results suggest that a single plasmin cleavage at Arg^{421} may alter the conformation of the molecule and expose C-terminal lysine residues to aid plasminogen binding. In contrast, extensive plasmin cleavage may further modify the conformation of HRG

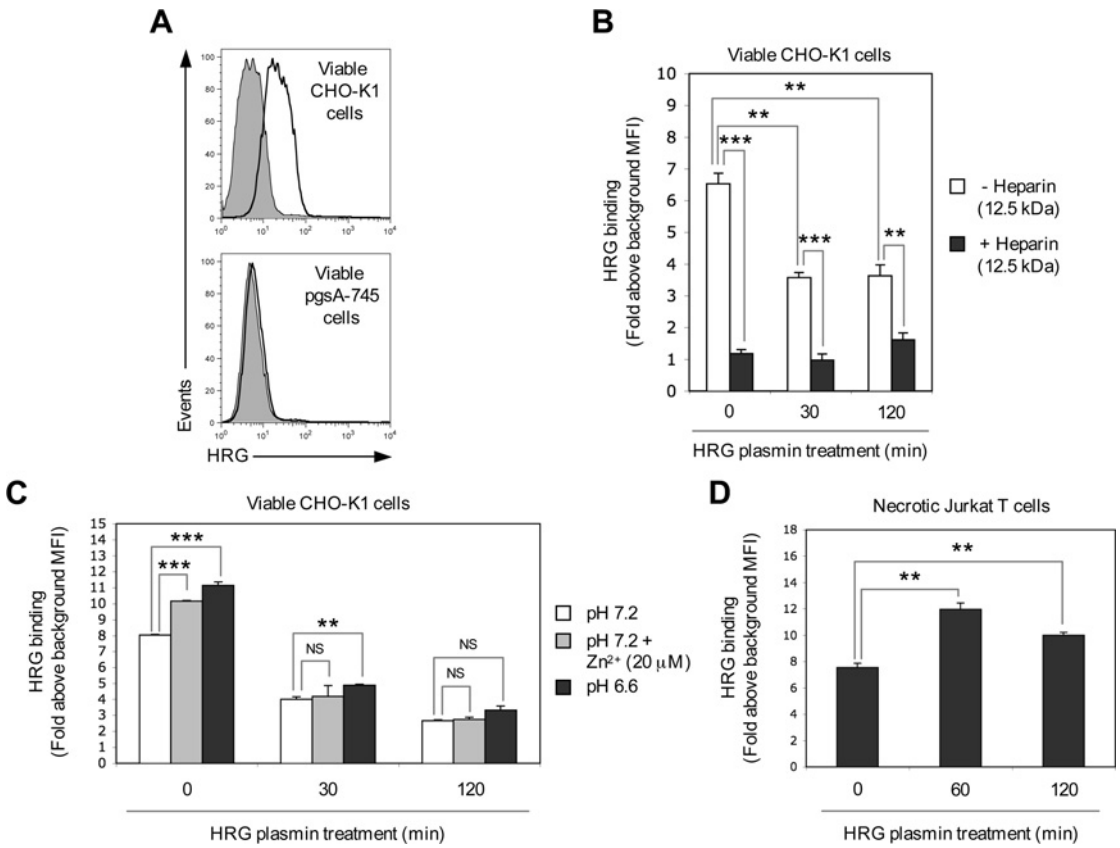

Figure 4 Plasmin cleavage reduces the ability of HRG to bind to cell surface HS but enhances HRG binding to necrotic cells

HRG (100 μg/ml) was subjected to plasmin cleavage and its binding to cell surface HS or necrotic cells then assessed. (**A**) Ability of 100 μg/ml intact HRG to bind to viable CHO-K1 cells and a GAG-deficient CHO cell line (pgsA-745) as detected by flow cytometry using an anti-human HRG antibody (HRG-4) and a FITC-conjugated secondary antibody. Representative flow cytometry histograms are shown. The filled histograms represent primary antibody only and secondary antibody only controls. Open histograms represent HRG binding. (**B** and **C**) Binding of intact (0 min) or plasmin-cleaved HRG (30 and 120 min) to viable CHO-K1 cells in (**B**) the presence (black bars) or absence (white bars) of heparin and (**C**) Zn^{2+} (grey bars) or acid pH (pH 6.6; black bars). (**D**) Ability of intact (0 min) or plasmin-cleaved HRG (30 and 120 min) to bind to heat-induced necrotic Jurkat T cells (56 °C, 30 min). HRG binding in (**B**–**D**) was analysed by flow cytometry using the HRG-4 anti-(human HRG) antibody and a PE-conjugated secondary antibody. Results are expressed as fold binding above MFI (median fluorescence intensity). Error bars represent the S.E.M. from three replicates. $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant.

and/or remove C-terminal lysine residues to abolish the enhanced plasminogen binding induced by initial plasmin cleavage.

To examine the effect of plasmin cleavage on the ability of HRG to tether plasminogen to GAG-coated surfaces, intact (0 min) or plasmin-cleaved (30 and 120 min at 37 °C) HRG was either pre-coated on heparin-coated plates, prior to assessing binding of plasminogen (Figure 5B), or was incubated simultaneously with plasminogen on heparin-coated wells (Figure 5C). As expected, plasminogen binding to heparin-coated wells was markedly enhanced by intact HRG using either binding procedure (Figures 5B and 5C). Surprisingly, plasmin cleavage had minimal effect on the ability of HRG to tether plasminogen to the heparin-coated wells (Figure 5B and 5C), indicating that both intact and plasmin-cleaved HRG can enhance the binding of plasminogen to GAG-coated surfaces.

Regulation of plasmin-mediated cleavage of HRG by heparin, Zn^{2+} and pH

As plasmin cleavage can regulate various functions of HRG, factors that may modulate the proteolysis of HRG by plasmin were further examined. The binding of growth factors and chemokines such as FGF (fibroblast growth factor) [26] and eotaxin [27] to heparin/HS has been shown to protect these molecules from plasmin-mediated degradation. Thus the effect of heparin on the proteolytic cleavage of HRG by plasmin was investigated to evaluate the sensitivity of HRG to plasmin cleavage when bound to cell surface HS or free heparin. As shown in Figure 6(A), heparin (12.5 kDa, 2 and 10 μg/ml) alone did not interfere with plasmin activity as measured by the cleavage of the chromogenic substrate *N*-(*p*-tosyl)-Gly-Pro-Lys. However, the presence of heparin attenuated plasmin-mediated cleavage of HRG, as indicated by the lack of lower-molecular-mass N-terminal fragments of HRG (Figure 6B). These results suggest that the binding of HRG to heparin can protect HRG from plasmin cleavage, possibly by masking exposed plasmin cleavage sites.

Furthermore, an increase in the local concentration of Zn^{2+}, and a decrease in pH, at sites of tissue injury have been proposed to regulate various functions of HRG, such as HRG tethering plasminogen to cell surfaces [14] and HRG exerting antimicrobial effects [20,28]. Thus the effect of Zn^{2+} and acidic pH on the proteolysis of HRG by plasmin was investigated. Acidic pH, in particular pH 6.0, and to a much lesser extent the presence of 10 and 20 μM Zn^{2+} ions, reduced the enzymatic activity of plasmin against the chromogenic substrate (Figure 6C), but also resulted in a substantial reduction in plasmin-mediated cleavage of HRG (Figure 6D). These results suggest that an increase in the local Zn^{2+} concentration and/or a decrease in pH may delay plasmin-mediated cleavage of HRG by directly reducing plasmin activity but, in the case of Zn^{2+}, may also render HRG less susceptible

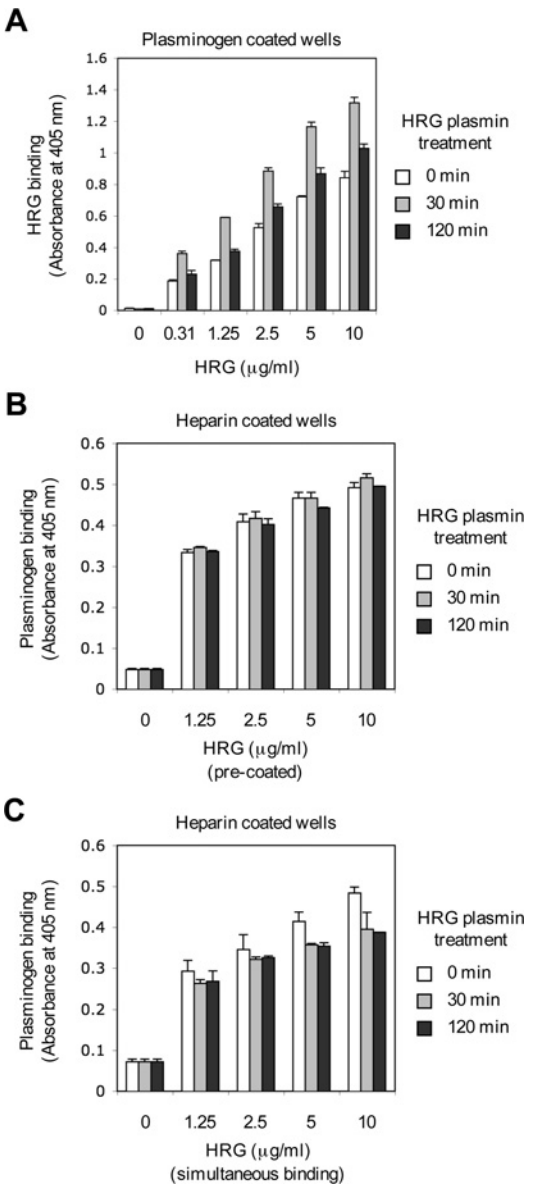

Figure 5 Plasmin cleavage enhances HRG binding to plasminogen but had no effect on HRG-mediated tethering of plasminogen to immobilized heparin

(**A**) Effect of plasmin cleavage (0, 30 and 120 min at 37 °C) on HRG binding to immobilized human plasminogen measured by ELISA using wells pre-coated with 1 μg/ml of human plasminogen. Error bars represent the S.E.M. for three replicates. (**B** and **C**) Analysis of the ability of intact (0 min) and plasmin-cleaved (30 and 120 min at 37 °C) HRG to tether human plasminogen to heparin-coated ELISA wells by (**B**) pre-coating heparin-coated wells with HRG prior to assessing binding of human plasminogen (5 μg/ml) or (**C**) by simultaneous incubation of HRG and human plasminogen (5 μg/ml) with heparin-coated wells. ELISA wells were pre-coated with 10 μg/ml ExtrAvidin® and 10 μg/ml biotinylated heparin and then analysed for plasminogen binding by ELISA under the different conditions. Error bars represent the range of duplicate samples.

to plasmin cleavage, possibly via a conformational change in the HRG molecule.

DISCUSSION

On the basis of the modular structure of HRG and the ability of HRG to bind to a variety of different ligands [6], it has been proposed that HRG may function as an adaptor molecule that interacts with multiple ligands simultaneously via several independent binding sites [16]. Thus HRG can potentially regulate numerous biological processes such as cell adhesion, angiogenesis, coagulation, fibrinolysis and clearance of immune complexes, necrotic cells and pathogens. Importantly, the functions of HRG are often regulated by pH and Zn^{2+} [6], these factors modulating HRG activity at sites of tissue injury when local pH decreases [29,30] or when local Zn^{2+} concentrations increase due to the release of Zn^{2+} from degranulating platelets [31]. Apart from local Zn^{2+} concentrations and pH, it has been suggested that proteolytic degradation of HRG may be required for HRG to exert its anti-angiogenic activity [19]. Although the ability of proteases such as plasmin and kallikrein to cleave HRG was initially reported by Smith et al. [15], the results presented in this study provide the first detailed analysis of the proteolytic cleavage of human HRG by plasmin and demonstrate the potential effects of plasmin-mediated cleavage on the functional activity of HRG (summarized in Figure 7).

In contrast with kallikrein-mediated cleavage of HRG, which degrades HRG extensively and rapidly [15], HRG is cleaved at specific sites by plasmin, generating distinct fragments that are relatively resistant to further plasmin cleavage, with the exception that an internal ~20 kDa fragment is degraded following prolonged plasmin treatment (Figure 1). Most importantly, plasmin cleavage occurs between the various domains of HRG, which may preserve the function of these domains (e.g. the N1N2, HRR and C-terminal domain) (Figures 1 and 2). However, unlike the closely related cystatin superfamily member high-molecular-mass kininogen [32,33], the majority of the plasmin-generated fragments of HRG remain bound to the N1N2 domain of HRG via disulfide bridges (Figures 1 and 2), suggesting that plasmin cleavage alone is unlikely to release various potentially active fragments of HRG. Nevertheless, the results from the present study suggest that plasmin cleavage alone can modulate the ability of HRG to bind to various ligands, possibly by altering the conformation of HRG and/or disrupting ligand binding sites. We show a single plasmin cleavage at Arg^{421} on HRG was sufficient to reduce HRG binding to cell surface HS by over 50 % (Figure 4B). As the Arg^{421} plasmin cleavage site is located distantly from the proposed HS/heparin-binding sites on HRG (i.e. the N1N2 domain [21] and HRR [34] domain of HRG), the decrease in HRG binding to cell surface HS following plasmin cleavage at a single site is likely to be the result of a conformational change in HRG, rather than being due to a direct disruption of the HS/heparin-binding sites. It is worth noting that plasmin cleavage only partially reduced the binding of HRG to HS (Figure 4B), suggesting that one or both HS/heparin-binding sites on HRG can remain partly functional following plasmin cleavage. However, some indirect evidence in the present study suggests that plasmin cleavage is likely to modify the function of the HS/heparin-binding site located in the HRR rather than that in the N1N2 domain of HRG. For example, excessive plasmin cleavage, which preserved the integrity of the N1N2 domain but degraded the predicted HRR fragment of HRG (Figure 1 and 2), resulted in no further reduction in HRG binding to cell surface HS compared with HRG that was cleaved predominately at Arg^{421} (Figure 4B). Furthermore, plasmin cleavage also rendered HRG unresponsive to acidic pH or Zn^{2+} (Figure 4C), which are factors known to potentiate the HS/heparin-binding properties of the HRR [34], but not the N1N2 domain of HRG [21]. Thus the residual binding of HRG to cell surface HS and heparin following plasmin cleavage is probably mediated via the N1N2 domain of HRG.

As HS binding is required for HRG to regulate various biological processes [6,8], the ability of plasmin cleavage to modulate HRG binding to HS is likely to have a major impact

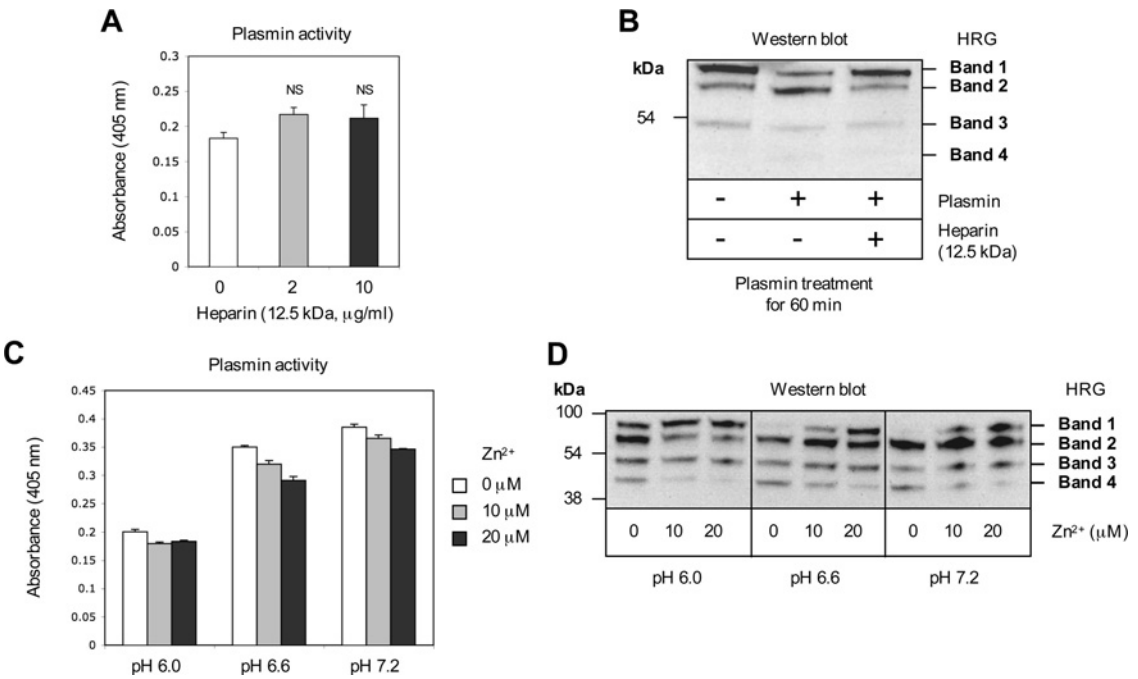

Figure 6 The presence of heparin, Zn^{2+} and acidic pH delays proteolytic cleavage of HRG by plasmin

(**A**) Effect of heparin (12.5 kDa, 2 or 10 μg/ml) on plasmin enzymatic activity (2 μg/ml) as measured by the cleavage of the chromogenic substrate *N*-(*p*-tosyl)-Gly-Pro-Lys (1 mg/ml) at 37 °C for 60 min. Error bars represent the S.E.M. for three replciates. NS, not significant. (**B**) Western blot analysis of the proteolysis of 300 ng HRG (30 μg/ml) by 60 ng plasmin (6 μg/ml) at 37 °C for 60 min in the presence (+) or absence (−) of heparin (12.5 kDa, 50 ng, 5 μg/ml). (**C**) Effect of pH and variuous concentrations of Zn^{2+} on plasmin enzymatic activity (2 μg/ml) as measured by the cleavage of the chromogenic substrate *N*-(*p*-tosyl)-Gly-Pro-Lys (1 mg/ml) at 37 °C for 60 min. Error bars represent the S.E.M. for three replicates. (**D**) Western blot analysis of the proteolysis of 300 ng HRG (30 μg/ml) by 60 ng plasmin (6 μg/ml) at 37 °C for 60 min in the presence of 0, 10 or 20 μM Zn^{2+} and at different pH (pH 6.0, 6.6 or 7.2). N-terminal fragments of HRG in (**B**) and (**D**) were analysed by SDS/PAGE under reducing conditions and were detected using the HRG-4 anti-(human HRG) antibody. Predicted N-terminal fragments of HRG (Bands 1–4) are as in Figure 3(A).

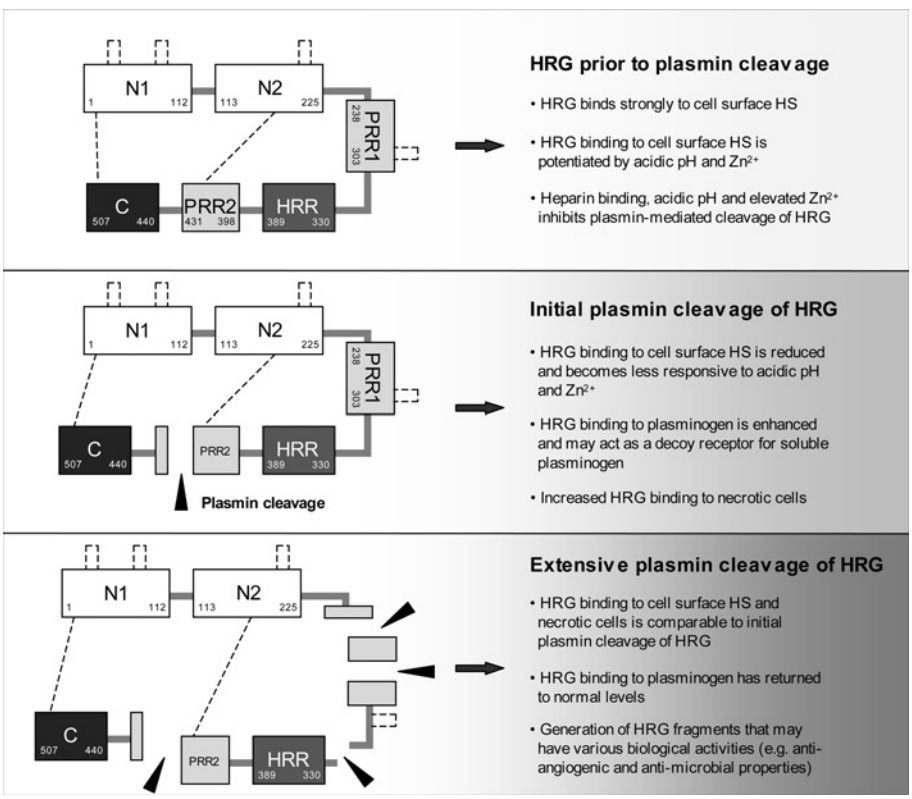

Figure 7 Regulation of HRG function by plasmin-mediated proteolytic cleavage

on the function of HRG. For example, plasmin cleavage may affect the ability of HRG to compete with other HS/heparin-binding proteins, such as FGF-2 or heparanase, from interacting with cell surface HS and the ECM. Previous studies have also demonstrated that HRG can aid the activation of plasminogen to plasmin by tethering plasminogen to GAG-presenting surfaces [12,14]. Thus proteolytic cleavage of HRG by plasmin may act as a negative feedback mechanism to limit plasminogen activation by reducing the amount of intact HRG that can efficiently tether plasminogen to GAG-presenting surfaces. Interestingly, in a similar manner to plasmin-cleaved fibrin [4], initial plasmin cleavage enhanced the binding of HRG to plasminogen (Figure 5A), possibly via the exposure of a C-terminal lysine residue. This is consistent with previous studies where 'plasmin-clipped' rabbit HRG also shows an enhancement in plasminogen binding compared with intact HRG [10]. As plasmin-cleaved HRG retained the ability to bind plasminogen (Figure 5A), but interacted with cell surface HS less efficiently (Figure 4B), plasmin-cleaved HRG may also act as a decoy receptor for plasminogen, which could compete with intact HRG, or other plasminogen receptors, for plasminogen binding to cell surfaces. Surprisingly, plasmin cleavage did not have any apparent effect on the ability of HRG to tether plasminogen to heparin-coated surfaces (Figure 5B and 5C), although this may be due to the high density of immobilized heparin masking the partial loss in cell surface HS binding exhibited by plasmin-cleaved HRG.

As mentioned above, several studies have suggested that the HRR of HRG (in particular peptide fragments derived from the HRR of HRG) exhibit anti-angiogenic [17–19,35,36], anti-microbial [20,28,37] and endotoxin-neutralizing properties [38]. Interestingly, unlike in rabbit HRG, a potential plasmin cleavage site at Arg^{360} within the HRR of human HRG may allow the release of a HRG fragment in the absence of disulfide cleavage (corresponding to amino acid residues 322–360 following plasmin cleavage at Arg^{321} and Arg^{360}) that is similar to the well-characterized HRG-derived heparin-binding/anti-angiogenic peptide, HRGP330 (HRG residues 330–364) [34–36]. Although this predicted HRR fragment of HRG was not apparent in this study when plasmin-cleaved HRG was analysed under both non-reducing and reducing conditions (Figure 1), further investigation is needed to determine whether the release of the HRR of HRG requires both proteolytic and disulfide cleavage. Interestingly, the unpublished observations discussed in Rydengard et al. [20] suggest that elastase is capable of digesting HRG and may generate peptides derived from the HRR of HRG.

The experimental results in the present study also demonstrate that factors such as heparin, Zn^{2+} and pH, may co-operatively regulate the ability of plasmin to cleave HRG (Figure 6). Consistent with a previous study by Smith et al. [15], the binding of heparin by HRG reduced the sensitivity of HRG to plasmin cleavage (Figure 6B). Similarly, acidic pH and the presence of Zn^{2+} directly inhibited plasmin activity (Figure 6C), as well as substantially reducing the cleavage of HRG by plasmin (Figure 6D). Indeed, the ability of Zn^{2+} to inhibit plasmin activity has been shown previously to abolish the proteolytic cleavage of fibrinogen by plasmin [39]. In addition, under conditions of tissue injury when the local concentration of Zn^{2+} is elevated [31] and the pH is acidic [29,30], HRG can bind more effectively to cell surface HS [14,21], thereby masking plasmin cleavage sites and possibly further delaying the proteolytic cleavage of HRG by plasmin. On the basis of these results, the ability of acidic pH and elevated Zn^{2+} levels to reduce plasmin-mediated cleavage of HRG represents a novel indirect mechanism of regulating the function of HRG.

It is worth noting that as intact HRG was used in most of the published studies that define HRG functions, proteolytic cleavage is unlikely to be essential for HRG to perform its role in many biological processes. However, disassembling the modular domain structure of HRG through plasmin-mediated cleavage, and possibly inter-domain disulfide bond cleavage may modulate the functional activity of HRG in multiple biological systems. For example, under normal physiological conditions, a large proportion of HRG may form high-affinity complexes with ligands, such as with IgG and plasminogen in plasma and with HS present on cell surfaces and in the ECM. These complexes can potentially sequester HRG away from interacting with other ligands or inducing an anti-angiogenic, anti-microbial or endotoxin-neutralizing effect via the HRR of HRG. Therefore, the combination of proteolytic and disulfide cleavage may release fragments of HRG containing different domains that can perform independent functions. Alternatively, the release of differently cleaved fragments may also abolish the ability of HRG to function as an adaptor molecule, such as by tethering plasminogen to GAG-presenting surfaces [12,14].

In summary, the experimental results in the present study demonstrate that various functions of HRG can be regulated by plasmin cleavage, which may in turn affect the ability of HRG to modulate the plasminogen/plasmin system. As proteolytically cleaved HRG can persist in the circulation [15], cleavage of HRG by proteases such as plasmin may provide an elegant means of regulating the multi-functional properties of HRG, rather than simply controlling the turnover of the protein. Further investigations are needed to examine the role of plasmin cleavage, as well as proteolytic cleavage by other proteases, such as kallikrein and elastase, in regulating the function of HRG in immunity, tumour progression and vascular biology.

AUTHOR CONTRIBUTION

Ivan Poon designed research, performed research, analysed data and wrote the paper. Anna-Karin Olsson contributed new reagents. Mark Hulett and Christopher Parish designed research, analysed results and wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Regulation of histidine-rich glycoprotein (HRG) function via plasmin-mediated proteolytic cleavage

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Figure S1 The amino acid sequence of human HRG

N-terminal amino acid sequencing results acquired from intact and plasmin-cleaved HRG fragments are indicated in red and the domains of HRG are highlighted in different colours. Cysteine residues that mediate interdomain disulfide bridges are highlighted in bold and with asterisks. N1N2, N-terminal domains 1 and 2; PRR 1/2, proline-rich region 1 or 2; HRR, histidine-rich region; C, C-terminal domain.

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