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Activation of cathepsin D by glycosaminoglycans

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We have previously shown that heparin can increase the activity of the proenzyme form of Alzheimer's β-site amyloid precursor protein cleaving enzyme 1 (BACE1). Cathepsin D (CD) is a member of the aspartic protease family and has sequence similarity to BACE1. Therefore, we examined whether heparin and other glycosaminoglycans (GAGs) can influence the activity of CD. Heparin and other GAGs were found to stimulate the activity of recombinant proCD. Desulfation of heparin almost abolished the stimulation, indicating that sulfate groups were important for the stimulatory effect. In addition, the stimulation was dependent on the length of the GAG chain, as larger GAGs were more potent in their ability to stimulate proCD than shorter fragments. In the presence of heparin, limited autocatalytic proteolysis of the proenzyme was increased, suggesting that heparin increases the activity of proCD by accelerating the conversion of proCD, which has little activity, to pseudoCD, an active form lacking residues 1–26 of the prodomain. Furthermore, the activity of spleen-derived mature CD, which lacks the entire 44 amino acid residue prodomain, was also increased by heparin, indicating that the catalytic domain of CD contains at least one region to which GAGs bind and stimulate enzyme activity. Because heparin also stimulated the activity of pseudoCD, proenzyme activation was probably accelerated by the interaction of heparin with the catalytic domain of pseudoCD. However, it is possible that heparin may also activate the proenzyme directly. On the basis of this study, we propose that GAGs may regulate CD activity *in vivo*.

Introduction

Cathepsin D (CD, EC 3.4.23.5) is a member of the family of aspartic proteases. It is a major lysosomal protease involved in protein degradation [1,2]. In addition, CD has a function in the activation or inactivation of a range of proteins, such as enzymes, hormones and biologically active peptides. Interest in CD as a drug target stems from its possible involvement in a diverse range of pathological conditions, including breast cancer and neurodegenerative diseases.

CD matures via a series of post-translational modifications [3]. It is synthesized as a preproenzyme of 412 amino acid residues. The signal peptide is removed within the endoplasmatic reticulum and after transport of the proenzyme to an acidic compartment, it is activated by proteolytic removal of a 44 amino acid residue prodomain. The mature form of CD may exist in a single- or two-chain form, both of which are equally active [4]. The two-chain form of human CD is

Abbreviations
BACE1, β-site amyloid precursor protein cleaving enzyme 1; CD, cathepsin D; CSE, chondroitin sulfate E; *F*, relative fluorescence; GAG, glycosaminoglycan; HS, heparan sulfate; LH, lung heparin; MH, mucosal heparin; SEM, standard error of the mean.

formed by excision of seven amino acid residues from the N-terminal domain, resulting in the noncovalent association of a C-terminal 34 kDa heavy chain and an N-terminal 14 kDa light chain. In addition, CD contains phosphorylated N-linked oligosaccharides involved in targeting of the enzyme to lysosomes via mannose-6-phosphate receptors [3].

ProCD is overexpressed and secreted at high levels in many cancer-derived cell lines and has been shown to have mitogenic effects on cancer cells, fibroblasts and keratinocytes that are independent of the catalytic activity of the enzyme [1,2,5,6]. Thus, proCD may have a function apart from its role as the precursor of lysosomal CD. Recently, proCD was also shown to be secreted by activated microglia and the proenzyme has been proposed to have a role in inflammation-mediated neurodegeneration [7]. CD has also been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, in which defects in the endosomal–lysosomal system are observed in the brain, including an upregulation of CD mRNA and protein [8,9]. Very recently, CD has attracted attention as a potential target for decreasing the concentration of α-synuclein in Parkinson's disease and other synucleinopathies [10–12].

We have found that the glycosaminoglycan (GAG) heparin can increase the activity of the proenzyme form of the aspartic protease BACE1, i.e. β-site amyloid precursor protein cleaving enzyme 1 [13]. BACE1 is a membrane-anchored enzyme that catalyses the first step in the production of β-amyloid, the protein that accumulates in the brain of Alzheimer's disease patients [14]. For this reason, BACE1 is a major target for Alzheimer's disease drug development. We found that proBACE1 can be activated by low concentrations of heparin, a highly sulfated analogue of heparan sulfate (HS), supporting the view that GAGs may play an important role in the regulation of BACE1 activity *in vivo* [13]. Our results suggested that the activation of proBACE1 required the presence of the prodomain. A preparation of BACE1 lacking the prodomain was not stimulated by heparin. We also found that a peptide homologous to the BACE1 prodomain bound heparin, supporting the hypothesis that proBACE1 was activated via binding of heparin to the prodomain.

Commonly, heparin-binding sites correspond to surface-exposed shallow pockets of positive charge containing certain patterns of primarily basic and hydrophobic residues [15,16]. Basic residues that are more distant in sequence can also be important as they may be brought spatially close through the folding of the protein. Alignment of the amino acid sequences of the prodomains of BACE1 and CD shows that an N-terminal pattern of basic and hydrophobic residues (I/V)R(L/I)PL(R/H) is conserved. Following this motif, the N-terminal region (residues 1–14) contains three additional basic residues. As there is also experimental evidence suggesting that heparin can bind to the CD prodomain [17], we hypothesized that proCD might also be activated by GAGs.

GAGs are generally covalently linked to a protein core to form proteoglycans [16]. Proteoglycans are abundantly expressed at cell surfaces and in the extracellular matrix to regulate a wide range of cellular processes, including cell proliferation, differentiation and migration. HS is a common GAG that is composed of alternating units of uronic acids and glucosamine residues, which can be substituted with sulfate groups in various positions. Heparin is a highly sulfated form of HS. The sulfated saccharide domains on GAGs are important in providing docking sites for protein ligands, such as growth factors, extracellular matrix proteins and enzymes [15,16]. Importantly, because proCD may encounter GAGs extracellularly, on the cell surface or intracellularly during trafficking through the Golgi apparatus and the endosomal–lysosomal system [1,3,18,19], it is feasible that GAGs may regulate proCD activity during physiological or pathophysiological conditions.

The aim of this study was to examine the effect of heparin on the activity of proCD and mature CD. We report here for the first time that both proCD and the mature enzyme are activated by low concentrations of GAGs *in vitro*, indicating a high-affinity interaction between CD and GAGs that may be of importance *in vivo*.

Results

Effect of GAGs on proCD activity

We have previously shown that proBACE1 can be activated by a low concentration of heparin (1 $\mu g \cdot mL^{-1}$) [13]. Therefore, to investigate the effect of heparin on CD, the ability of recombinant human proCD to cleave a peptide substrate was first examined in the absence or presence of 1 $\mu g \cdot mL^{-1}$ porcine mucosal heparin (MH) or the more highly sulfated bovine lung heparin (LH) (Fig. 1A). In the absence of heparin, little activity was observed over the first 40 min of incubation. This initial lag phase has previously been shown to be the period when the essentially inactive zymogen is converted to a fully active form called pseudoCD [20,21]. Thereafter, substrate peptide cleavage increased to 0.6 ± 0.01

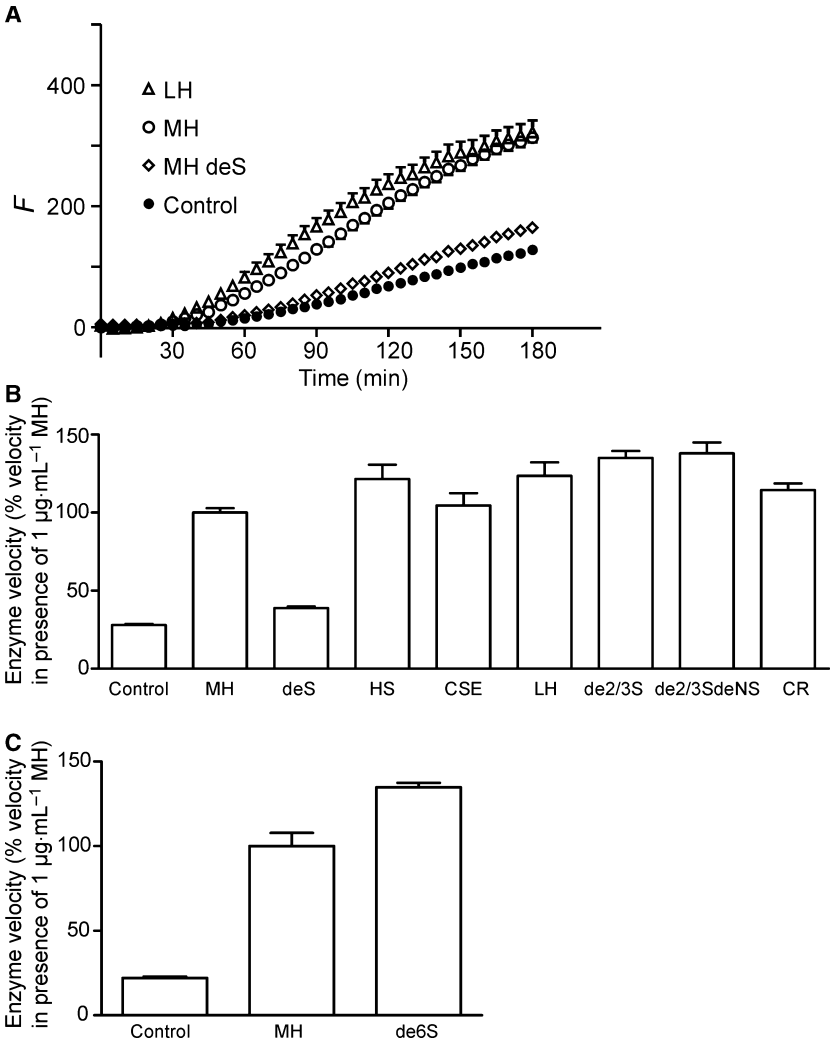

Fig. 1. Effect of GAGs on enzyme activity of recombinant proCD. Enzyme activity was monitored by the increase in *F* during hydrolysis of 10 μM substrate peptide in the presence of: (A) 0 $\mu g \cdot mL^{-1}$, 1 $\mu g \cdot mL^{-1}$ MH, 1 $\mu g \cdot mL^{-1}$ LH and 1 $\mu g \cdot mL^{-1}$ desulfated MH. Data from all incubations are expressed as means ± SEM, $n = 3$. Enzyme activity in the presence of MH or LH was significantly different from controls lacking heparin ($P < 0.0001$, Student's *t*-test). (B, C) Enzyme velocity in the presence of 1 $\mu g \cdot mL^{-1}$ GAG expressed as a percentage of the velocity in the presence of 1 $\mu g \cdot mL^{-1}$ MH. Data from all incubations are expressed as means ± SEM, $n = 3$. Control, no GAG; deS, desulfated MH; de2/3S, de-2/3O-sulfated MH; de2/3S/deNS, de-2/3O-/de-N-sulfated re-N-acetylated MH; de6S, de-6O-sulfated MH; CR, carboxyl reduced MH.

$\Delta F \cdot min^{-1}$ [mean ± standard error of the mean (SEM), where F = relative fluorescence]. The addition of heparin significantly decreased the duration of the lag phase by 15 min (MH) or 20 min (LH). In addition, the activity following the lag phase was substantially increased in the presence of both types of heparin (2.6- and 3.4-fold for MH and LH, respectively). Similar results were obtained with an alternative substrate peptide for CD (Fig. S1).

As sulfate groups on heparin are known to be important for GAG binding properties, we examined the effect of desulfated MH on enzyme activity. Desulfated MH had no significant effect on the activity of recombinant human proCD (Fig. 1A), showing that the ability of heparin to stimulate enzyme activity was dependent on sulfation of the heparin.

To investigate the role of negative charge density and backbone structure further, we compared the effect of bovine kidney HS, which is composed of the same disaccharide units as heparin, but exhibits a lower overall degree of sulfation than heparin (on average approximately < 1 versus 2.7 sulfate groups per disaccharide unit) [15], and chondroitin sulfate E (CSE), which is composed of disaccharide units containing glucuronic acid and *N*-acetylgalactosamine with approximately 1.5 sulfate groups per disaccharide [22]. Compared with heparin, we found that HS and CSE had an equal (CSE) or slightly higher (HS) tendency to stimulate the activity of the recombinant proCD (Fig. 1B). This indicated that the difference in carbohydrate backbone structure between heparin (or HS) and CSE was not critical for the stimulatory effect of these GAGs. Furthermore, a high degree of sulfation in heparin was not essential, as HS, with its lower degree of sulfation, was at least as potent as heparin in increasing enzyme activity.

Heparin can carry sulfate groups on the 2O-position of the uronic acid residues or on the 6O-, N- and/or 3O-positions of glucosamine residues [15]. CSE from

squid cartilage is predominately sulfated at the 4O- and 6O-positions of *N*-acetylgalactosamine [22]. To gain further insight into the role of sulfation in specific positions and the role of negative charge in general for the effect of heparin on recombinant human proCD, the effect of four chemically modified derivatives of heparin: de-2/3O-sulfated, de-2/3O-/de-N-sulfated re-N-acetylated, de-6O-sulfated or carboxyl reduced MH was examined. The differentially desulfated forms of heparin and the carboxyl reduced heparin all retained their ability to stimulate the CD zymogen (Fig. 1B, C), showing that the presence of the carboxyl group was not critical for the stimulatory effect. Because total desulfation of heparin almost abolished the ability of MH to stimulate enzyme activity (Fig. 1A), the results indicated that the effect was dependent on sulfation, but that the specific position of the sulfate group in the heparin molecule appeared to be less important.

Effect of heparin on autocatalytic cleavage of proCD

The activation of proCD at acidic pH *in vitro* involves the autocatalytic removal of residues 1–26 of the prodomain [20,21,23]. The resulting enzyme, referred to as pseudoCD, is a fully catalytically active form retaining 18 residues (27–44) of the prodomain. As heparin decreased the duration of the lag phase of activity (Fig. 1), this suggested that the GAG may stimulate the rate of autocatalytic cleavage of the zymogen to pseudoCD. To investigate this possibility, we incubated recombinant human proCD in the absence or presence of heparin at pH 4. Aliquots taken at different time points were then analysed on silver-stained SDS/PAGE. Unincubated recombinant proCD migrated as a doublet on the gel with an apparent molecular mass of 46–50 kDa (Fig. 2A). This doublet probably comprised two forms of proCD with different degrees of glycosylation, as sequence analysis as reported by the manufacturer indicated that the preparation contained only the single-chain proenzyme form. After a 1 h incubation of the control, a protein with an apparent molecular mass of 43 kDa, consistent with the molecular mass of pseudoCD, was detected and after 3 h of incubation the enzyme had mainly been converted to the 43 kDa form. In the presence of heparin, we found that most of the enzyme had already been processed to a 43 kDa form within 1 h of incubation. Because the detection of enzyme activity requires a period of preincubation at acidic pH, and because the activity increases in parallel with the formation of pseudoCD, it has been concluded that pseudoCD is the active enzyme species at low pH

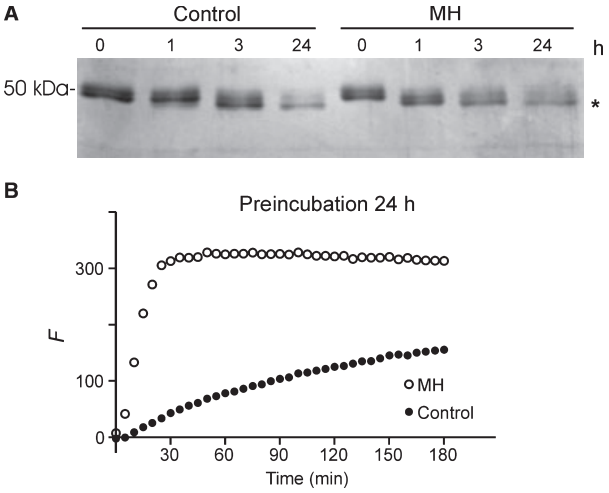

Fig. 2. Effect of MH on autocatalytic cleavage of recombinant proCD and on the activity of the enzyme. (A) Silver-stained SDS/PAGE showing the autocatalytic cleavage of proCD. Recombinant proCD (2.5 μg in 100 μL, pH 4) was incubated in the absence (control) or presence of 1 $\mu g \cdot mL^{-1}$ MH and aliquots of 20 μL were analysed after 0–24 h incubations as indicated. *43 kDa form. (B) The activity of recombinant human proCD that had been preincubated for 24 h [8 μL of the reaction in (A)] was monitored in the absence (control) or presence of 1 $\mu g \cdot mL^{-1}$ MH.

in vitro and that the nonprocessed zymogen has little or no catalytic activity [20,21]. Our result suggested that heparin accelerated the conversion of proCD to pseudoCD.

To determine whether residues 1–26 of the prodomain were required for the heparin-induced activation of recombinant human proCD, we examined the effect of MH on enzyme activity after the enzyme had been converted to pseudoCD by preincubation for 24 h (Fig. 2A). The addition of heparin to the preincubated enzyme preparation activated the enzyme $\sim$ 4.5-fold (Fig. 2B). Following preincubation, no lag phase was observed, consistent with the view that activation of the enzyme by cleavage of residues 1–26 had occurred prior to the addition of MH. Because pseudoCD lacks residues 1–26 of the prodomain, the result suggested that other regions of the enzyme were required for complete activation of proCD by heparin.

Effect of heparin on the activity of mature CD

The results suggested that residues 1–26 of the prodomain were not necessary for heparin-induced stimulation of the enzyme. Therefore, we also examined whether mature CD (lacking the entire prodomain) could be activated by heparin. For this study, we used a preparation of CD from human spleen. N-terminal

sequence analysis of the enzyme was consistent with an N-terminal domain beginning with the residues GPIPE, as expected for the mature enzyme (data not shown). Further analysis of the enzyme preparation on silver-stained SDS/PAGE showed that spleen CD was primarily in the two-chain form (Fig. 3A). Incubation of the enzyme at acidic pH for up to 1 h in the absence or presence of MH (1 $\mu g \cdot mL^{-1}$) had no detectable effect on the apparent molecular mass of the heavy and light chains, indicating that mature CD was stable and not subject to autocatalysis under these conditions. Next, the effect of MH on enzyme activity was measured. Similar to the results with recombinant proCD (Fig. 1B), 1 $\mu g \cdot mL^{-1}$ MH stimulated mature spleen CD ($\sim$ 7-fold) (Fig. 3B), whereas desulfated MH had no effect on enzyme activity (data not shown). Similarly to pseudoCD, no lag phase was observed in the reaction with mature CD.

To determine whether the increase in activity was due to an effect on the V_{max} or K_M of the enzyme reaction, the ability of 1 $\mu g \cdot mL^{-1}$ MH to stimulate the activity of mature spleen CD was measured over a range of substrate concentrations (1–30 μM) (Fig. 3C). The results showed that the V_{max} of CD was increased 15-fold in the presence of MH (mean ± SEM: 1.5 ± 0.2 and 24.2 ± 2.7 $\Delta F \cdot min^{-1}$ in the absence or presence of MH, respectively), whereas MH had no effect on K_M (mean ± SEM: 5.7 ± 2.4 and 7.3 ± 1.9 μM in the absence or presence of MH, respectively). Taken together, the results showed that similar to pseudoCD, mature CD can be stimulated by low concentrations of MH. The observation that the amount of activation of pseudoCD and two-chain CD by MH was similar indicated that the effect of heparin on pseudoCD was not dependent on the presence of the remaining portion of the prodomain (residues 27–44). The finding that MH activated not only the inactive zymogen, but also the mature enzyme indicated that the enzyme has at least one binding site for heparin outside the prodomain.

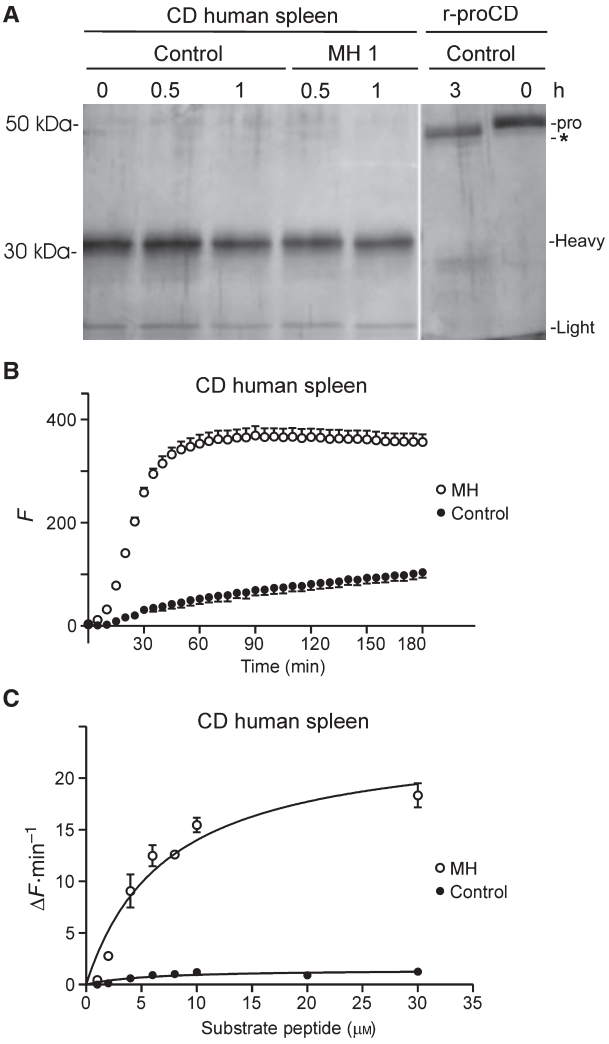

Fig. 3. Effect of MH on the activity of mature CD from human spleen. (A) Silver-stained gel after SDS/PAGE. CD (2.5 μg in 100 μL, pH 4) was incubated in the absence (control) or presence of 1 $\mu g \cdot mL^{-1}$ MH and aliquots of 20 μL were analysed after 0–1 h incubations as indicated. For comparison, recombinant proCD (r-proCD) incubated for 0 and 3 h is also shown from another part of the same gel. The positions of proCD, the 43 kDa form (*), heavy and light chains of CD are indicated. (B) *F* during CD hydrolysis of 10 μM substrate peptide in the absence (control) or presence of 1 $\mu g \cdot mL^{-1}$ MH. Data are expressed as means ± SEM, $n = 3$. (C) Velocity ($\Delta F \cdot min^{-1}$) of product formation by CD as a function of substrate concentration in the absence (control) or presence of 1 $\mu g \cdot mL^{-1}$ MH. Data for 1–30 μM substrate peptide are expressed as means ± SEM, $n = 2$. The curves shown are calculated from a best fit of the data to the Michaelis–Menten equation.

The role of polysaccharide chain length

We next investigated the role of polysaccharide chain length on the amount of activation of recombinant proCD. Because our previous results showed that proCD and mature CD were markedly activated by $\sim$ 50 nM MH (1 $\mu g \cdot mL^{-1}$ of 17–19 kDa MH), we examined the effect of 50 nM di-, tetra-, hexa- and octasaccharides and longer (3–4 and 4–6 kDa) MH or (15 and 22 kDa) HS moieties on enzyme activity. Of the oligosaccharides tested, only the hexa- and octasaccharides stimulated the activity of recombinant proCD, with the octasaccharide being the most potent (Fig. 4A, C). With increased chain length, the activity increased from 1.6 ± 0.05 $\Delta F \cdot min^{-1}$ (mean ± SEM) for 3–4 kDa to 3.8 ± 0.1 $\Delta F \cdot min^{-1}$ (mean ± SEM)

for the 22 kDa derivative (Fig. 4B, C). In addition, the lag phase decreased with increasing GAG size (Fig. 4C, inset). Therefore, these experiments showed that at a concentration of 50 nM, a hexasaccharide was the smallest heparin-derived sugar that was able to activate the CD zymogen. Furthermore, the potency of the stimulatory effect of GAGs increased with polysaccharide chain length. Of the GAGs tested, 22 kDa HS resulted in the highest activation of proCD, indicating that it was the most effective in accelerating the conversion from proCD to pseudoCD.

Next we examined the effect of polysaccharide chain length on the stimulation of the two-chain mature spleen CD. Similar to recombinant proCD, we observed that the activity of the two-chain enzyme was stimulated by the hexa- and octasaccharides. There was little effect of the di- and tetrasaccharides when used at a concentration of 50 nM (Fig. 5A, B). Optimal stimulation of the two-chain enzyme was achieved by 3–4 kDa MH and a further increase in chain length did not increase the stimulatory effect. The result suggested that 3–4 kDa MH, which corresponds to

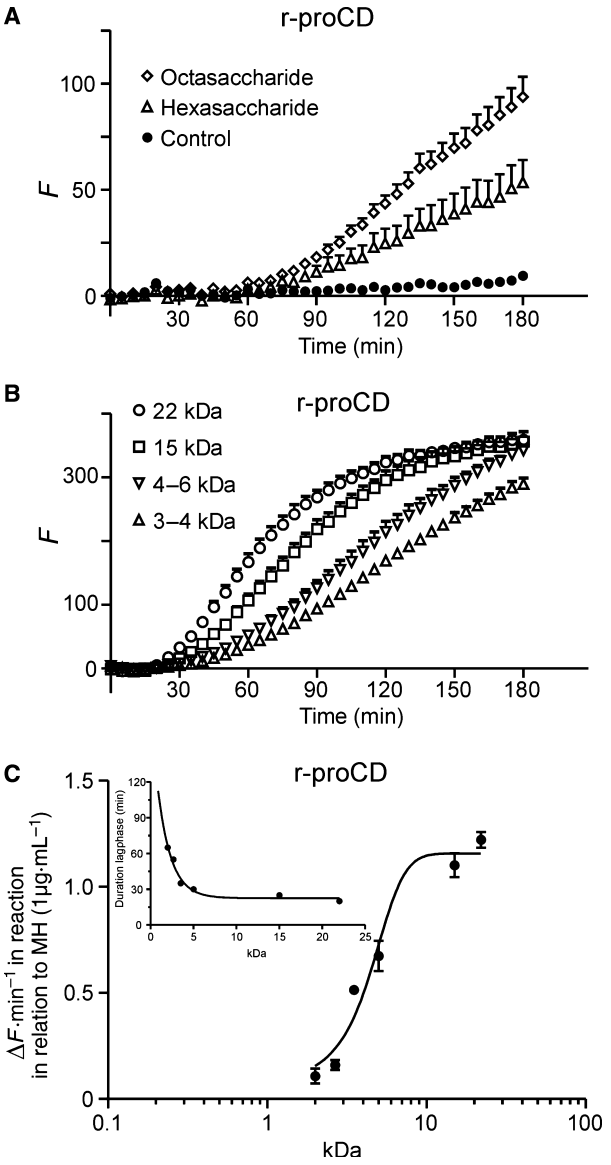

Fig. 4. Effect of polysaccharide chain length on recombinant proCD (r-proCD) activity. The figure shows the increase in *F* during r-proCD hydrolysis of 10 μM substrate peptide in the absence (control) or presence of 50 nM hexa- or octasaccharides (A) or in the presence of a 50 nM concentration of higher molecular mass GAGs: 3–4 kDa heparin, 4–6 kDa heparin, 15 kDa HS or 22 kDa HS (B). Data from all incubations are expressed as means ± SEM, $n = 3$. (C) Enzyme velocity in the presence of 1 $\mu g \cdot mL^{-1}$ GAGs of different molecular mass expressed as a percentage of the velocity in the presence of 1 $\mu g \cdot mL^{-1}$ MH. Data from all incubations are expressed as means ± SEM, $n = 3$. The inset in (C) shows the duration of the lag phase as a function of polysaccharide chain length.

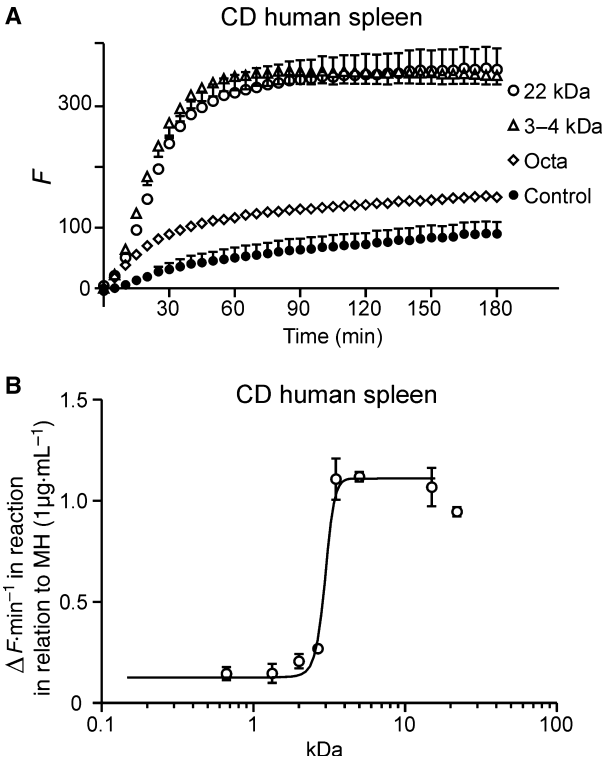

Fig. 5. Effect of polysaccharide chain length on the activity of mature CD from human spleen. The figure shows the increase in *F* during CD hydrolysis of 10 μM substrate peptide in the absence or presence of 50 nM octasaccharide, 3–4 kDa heparin, 22 kDa HS (A). Data from all incubations are expressed as means ± SEM, $n = 3$. The enzyme velocity in the presence of 1 $\mu g \cdot mL^{-1}$ GAGs of different molecular mass expressed as a percentage of the velocity in the presence of 1 $\mu g \cdot mL^{-1}$ MH is also shown (B). Data from all incubations are expressed as means ± SEM, $n = 3$.

approximately six to eight disaccharide units per molecule, was sufficient for optimal stimulation of mature CD by GAGs under our experimental conditions.

Discussion

This study has demonstrated that GAGs can increase the activity of CD *in vitro*. In this regard, our study has shown that CD belongs to a group of enzymes including BACE1, ADAM12, the cathepsins B, L and S that are regulated by GAGs *in vitro* [13,24–27]. We found that not only was the activity of the CD proenzyme stimulated by GAGs, but the mature (prodomain-cleaved) enzyme was also stimulated. In addition, we found that heparin could increase the limited proteolysis of proCD at acidic pH, concomitant with an increased rate of substrate peptide cleavage. This increased rate of autocatalytic activation may have been due to direct stimulation of a small amount of active enzyme in the preparation. We found that pseudoCD and the mature enzyme were stimulated by heparin to a similar degree, indicating that GAGs could interact with the protease domain. The stimulation of mature enzyme was due to an increase in enzyme V_{max}.

It has been previously reported that the presence of the C-terminal part of the propeptide in pseudoCD did not significantly alter the basal enzymatic and structural properties of the enzyme as compared with the mature two-chain form [23]. Therefore, our findings suggest that activation of the zymogen is, at least in part, caused by the binding of GAGs to newly formed pseudoCD molecules. Their activation may in turn accelerate the process of autocatalytic conversion of proCD to pseudoCD, thereby accounting for the GAG-induced decrease in the duration of the lag phase. However, our data are also consistent with the possibility that heparin can activate the zymogen directly, as we have proposed for BACE1 [13]. Studies of the binding of heparin oligosaccharides to well-known heparin-binding proteins, including the protease thrombin and fibroblast growth factor, have reported binding affinities (K_D) in the range of nM to low μM [16]. The observation that low concentrations of heparin (50 nM) activated CD suggests that the interaction between the enzyme and heparin is of high affinity. However, the identity of the heparin-binding residues in CD remains to be determined.

In comparison with the mature enzyme, which was optimally stimulated by 3–4 kDa MH, activation of the zymogen was more sensitive to the increasing polysaccharide chain lengths of HS up to 22 kDa. Activity detected after incubation of proCD in the presence of the 3–4 kDa MH derivative was < 50% of the activity observed in the presence of 22 kDa HS. The higher potency of the longer chains to activate the zymogen might be explained by the fact that there are additional residues within the prodomain that may contribute to binding and that, as a consequence, the binding region for the zymogen is larger than that of the mature enzyme. Because the longer chains were not more potent than the 3–4 kDa fragment in stimulating the mature enzyme, our results are consistent with the existence of an additional binding region in the prodomain. Support for the conclusion that the prodomain contains a GAG-binding site comes from a recent study on the capacity of prodomain peptides to inhibit mature CD [17]. In that study, heparin decreased the inhibitory effect of a peptide homologous to the N-terminal part of the prodomain (residues 1–14) and the effect was shown to be dependent on basic residues in this peptide, thus suggesting that residues 1–14 of the prodomain contain a binding site for GAGs.

In previous kinetic studies of the conversion of proCD to pseudoCD, a two-phase mechanism has been suggested [21]. In the initial phase, the first molecules of pseudoCD are formed at a low rate and in the following phase the process is autocatalytically accelerated by newly formed pseudoCD molecules. It is possible that there is a mechanism by which the binding of GAGs to the N-terminal part of the prodomain may accelerate the reaction in the first rate-limiting step in the activation of the zymogen, in which the conversion of proCD to active pseudoCD is initiated. Because the N-terminal part of the prodomain is inhibitory and may retain an inhibitory capacity after its cleavage by interacting with the active site [17], GAGs may also act by sequestering the autocatalytically cleaved fragment containing residues 1–26 away from the catalytic cleft and thereby increase the rate of activation. Whether GAGs directly increase the activity of proCD by weakening the interaction between the prodomain and the catalytic cleft, and/or render the prodomain more accessible for cleavage, and/or play a role in the sequestration of the cleaved 1–26 fragment remain to be determined. In CD-deficient fibroblasts, the generation of pseudoCD by autoactivation is not required for the intracellular maturation of CD expressed by transfection or for the maturation of endocytosed proCD [28]. However, *in vivo*, pseudoCD species are formed by mechanisms involving cysteine proteases and other unidentified proteases that cut at different sites within the CD prodomain. Little is known about the activation of secreted proCD, which may be secreted both under pathological [1,7] and physiological [29] conditions. ProCD and mature CD were shown to be secreted by explants of various mouse

tissues, such as kidney and heart under physiological conditions [29]. It is noteworthy that the secreted CD was found to be active, reportedly due to membrane proton pumps generating a local acidification of the environment [29]. Whether extracellular GAGs may play a role in the activation of secreted CD remains to be determined.

The interaction between enzyme and GAGs was found to be dependent on sulfation of the GAG molecule. HS, which has a relatively low overall degree of sulfation, was as effective as the more extensively sulfated heparin and CSE in activating CD, indicating that neither the difference in carbohydrate backbone structure between MH/HS and CSE nor a higher degree of overall sulfation was critical for the stimulatory effect. We also did not observe a requirement for sulfation at specific positions in the carbohydrate backbone. This finding suggests that the overall net negative charge of the GAG may be more important than the precise location of the sulfate groups.

The finding that CD can be stimulated by low concentrations of GAGs raises the possibility that GAGs play a role in activating the enzyme *in vivo*. It is worth noting that both CD and HS have for more than 20 years been implicated in cancer progression [1,2,30]. In view of the results of the present study, it might be speculated that HS could be involved in the dysregulation of CD that has been observed in various cancers. In any case, an increased understanding of how GAGs regulate the activity of CD may aid in the development of drugs beneficial for pathological conditions in which CD is involved.

Materials and methods

Materials

Recombinant human proCD, beginning at leucine 21 and containing a C-terminal His-tag, was obtained from R&D Systems (1014-AS, BioScientific, Gymea, Australia). CD prepared from human spleen (catalogue number 219394) and bovine LH (catalogue number 375093) were from Calbiochem (Merck, Kilsyth, Australia). Heparin from porcine intestinal mucosa (MH, catalogue number H3393), 4–6 kDa porcine MH (H8537) and bovine kidney HS (catalogue number H7640) were obtained from Sigma-Aldrich (Sydney, Australia). Porcine mucosal 15 kDa HS (HS1098) was from Celsus Laboratories (Cincinnati, OH, USA), porcine mucosal 22 kDa HS (ORG553) was a generous gift from Organon (Oss, The Netherlands) and squid cartilage CSE (400678-1A) was from Seikagaku (Tokyo, Japan). Heparin-derived di-, tetra-, hexa- and octasaccharides were obtained from Dextra Laboratories (Reading, UK). The substrate peptide I MCA-SEVNLDAEFRK(DNP)RR-NH_2 was purchased from MP Biomedicals (Seven Hills, NSW, Australia), substrate peptide II MCA-PLGL-Dpa-AR-NH_2 from R&D Systems and microplates (OptiPlate-96 F) from PerkinElmer Life Sciences (Boston, MA, USA). The Bio-Rad Silver Stain kit was from Bio-Rad Laboratories (Regents Park, Australia).

Preparation and chemical modification of heparin derivatives

Totally desulfated heparin was prepared using the method of Nagasawa *et al*. [31] by treatment of the pyridinium salt form with dimethyl sulfoxide/water (9 : 1 by volume) for 2 h at 100 °C. 2/3O-desulfated heparin was prepared using the method of Jaseja *et al*. [32] by dissolving heparin in 0.2 M NaOH and lyophilization. The N-desulfated derivative was prepared using the method of Inoue and Nagasawa [33]. The pyridinium salt form was treated with dimethyl sulfoxide/water (95 : 5 by volume) for 1.5 h at 50 °C. The N-desulfated compound was N-acetylated with acetic anhydride using the method of Yates *et al*. [34]. Porcine MH was decarboxylated ($\sim$ 80%) by treatment with 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide and subsequent sodium borohydride reduction as described previously [35]. Heparin was chemically de-6O-sulfated using the method of Toida *et al*. [36] using *N*, *O*-bis (trimethylsilyl) acetamide. A 3–4 kDa heparin was prepared by free radical degradation using the method of Volpi *et al*. [37].

CD activity assay

CD activity was measured using a quenched-fluorescence assay with the substrate peptide I homologous to the Swedish amyloid precursor protein mutant β-cleavage site [38,39]. Previously, CD has been shown to cleave peptides that encompass the EVNLDAEF sequence with a similar catalytic efficacy as BACE1 [39–42]. Assays were performed in a buffer of 0.1 M sodium acetate pH 4, 5% dimethylsulfoxide using 100 ng enzyme (unless otherwise specified) and 10 μM substrate peptide in a total volume of 100 μL in black 96-well plates. Heparin was dissolved in water and sterile filtered with a 0.45 μm syringe filter. The increase in *F* produced by substrate hydrolysis was monitored at 37 °C in a fluorescence microplate reader (FLUOstar, BMG Lab Technologies, Offenburg, Germany) or at room temperature using a FLUOstar 403 (BMG Lab Technologies) with excitation and emission wavelengths of 320 and 405 nm, respectively. Background fluorescence (substrate incubated in the absence of enzyme) was subtracted at each time point. Enzyme activity was calculated from the change in *F* per min ($\Delta F \cdot min^{-1}$) during the linear phase of the reaction representing the highest rate of cleavage. Graphs and statistical calculations were made using GRAPHPAD PRISM software (version 4.00, GraphPad Software Inc., La Jolla, CA, USA).

Gel electrophoresis and western blotting

Samples (~ 500 ng protein) were mixed with an equal volume of 2× SDS sample buffer and heated to 95 °C for 5 min prior to gel loading. The proteins were separated by 10% SDS/PAGE [43] and stained using Bio-Rad Silver Stain reagent. An image of the signals obtained by silver staining was acquired and relative molecular mass was determined using an AlphaImager with ALPHAEASE software (Quantum Scientific, Glebe, Australia).

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Supporting information

The following supplementary material is available:
Fig. S1. Effect of MH on enzyme activity of recombinant proCD.

This supplementary material can be found in the online version of this article.

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