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Proteinase activity regulation by glycosaminoglycans

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Abstract

There are few reports concerning the biological role and the mechanisms of interaction between proteinases and carbohydrates other than those involved in clotting. It has been shown that the interplay of enzymes and glycosaminoglycans is able to modulate the activity of different proteases and also to affect their structures. From the large number of proteases belonging to the well-known protease families and also the variety of carbohydrates described as widely distributed, only few events have been analyzed more deeply. The term "family" is used to describe a group of proteases in which every member shows an evolutionary relationship to at least one other protease. This relationship may be evident throughout the entire sequence, or at least in that part of the sequence responsible for catalytic activity. The majority of proteases belong to the serine, cysteine, aspartic or metalloprotease families. By considering the existing limited proteolysis process, in addition to the initial idea that the proteinases participate only in digestive processes, it is possible to conclude that the function of the enzymes is strictly limited to the cleavage of intended substrates since the destruction of functional proteins would result in normal tissue damage. In addition, the location as well as the eventual regulation of protease activity promoted by glycosaminoglycans can play an essential role in the development of several physiopathological conditions.

Key words
- Proteinases
- Heparin binding
- Glycosaminoglycan
- Macromolecules

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Introduction

The interaction between macromolecules as a result of their colocalization is a traditional aspect when considering *in vivo* conditions; however, regarding *in vitro* analysis, this aspect has been neglected as a technical impossibility or even as a cause of artifact generation. Proteinases and glycosaminoglycans belong to two of the four existing macromolecule groups, which also include lipids and nucleic acids. Their widespread distribution, colocalization and physicochemical properties provide the essential features that allow their interaction and possible functional linkage. Due to the structural and functional complexity present in proteinases as well as in the glycosaminoglycan families, it is interesting to summarize some of their characteristics. Also, some of the interactions between proteases and glycosaminoglycans known, their respective mechanisms and possible biological role are presented in Table 1.

Proteinases

Proteinases form a large and diverse family of enzymes. The term family is used to describe a group of enzymes in which each member shows an evolutionary relationship to at least another one, either throughout the whole sequence or at least in the part of the sequence responsible for catalytic activity. Enzymes were initially considered only as proteins acting in digestive processes; however, they have been demonstrated to be involved in almost every aspect of the biological machinery. By introducing one water molecule into an amide bond of a peptide, cleavage occurs through a reaction involv-

Table 1. Some macromolecular interactions involving proteases, their inhibitors and glycosaminoglycans and related effects.

	Ligand	Structural/functional effect	Biological role
Proteases			
Papain	Heparin, heparan sulfate	Structural change, protection against alkaline inactivation, enhancement of catalytic activity (1)	Physicochemical model
Cathepsin B	Heparin, heparan sulfate	Structural change, protection against alkaline inactivation, enhancement of catalytic activity (2)	Tumor growth, metastasis
Cathepsin K	Chondroitin sulfate	Increases the stability, enhances catalytic activity (3)	Bone reabsorption
Cathepsin G	Heparin	Reduction of protease inhibition by alpha1-antichymotrypsin, α_1-PI and eglin C (4)	Inflammation and pulmonary diseases
Neutrophil elastase	Heparin	Reduction of protease inhibition by α_1-PI (5)	Inflammation and pulmonary diseases
Thrombin	Heparin	Structural change (6)	Hemostatic defects
Chimase	Heparin	Reduction of protease inhibition by natural inhibitors (7)	Inflammation and pulmonary diseases
Inhibitors			
Cystatin	Heparin	Imbalance of cysteine protease activity regulation	
Antithrombin	Heparin	Structural change, enhancement of inhibitor efficiency (8,9)	Hemostasis
Kallistatin	Heparin	Steric blocking (10), reduction of inhibitor efficiency	Inflammation and pulmonary diseases
Antitrypsin-1	Heparin	Enhancement of inhibitor binding	Inflammation and pulmonary diseases
TIMP-3	Heparin, heparan sulfate	Interaction with extracellular matrix components (11)	Tumor growth, angiogenesis,
Secretory leukocyte protease inhibitor	Heparin	Enhancement of inhibitor efficiency (12)	Inflammation
Mucus proteinase inhibitor	Heparin	Enhancement of inhibitor efficiency (4)	Inflammation and pulmonary diseases

TIMP-3: tissue inhibitor of metalloproteinase 3; α_1-PI: α_1-proteinase inhibitor.

ing nucleophilic attack on the carbonyl carbon of the scissile bond, but the actual mechanism varies as different subtypes are described (13). The structural features and catalytic mechanisms of the proteinases form the basis for their classification, where the catalytic amino acid residues of each type determine the family to which the proteinase belongs. Most of the known proteases belong to the serine, cysteine, aspartic or metalloprotease families (see Ref. 14 for a detailed discussion of proteinase classification and nomenclature).

Proteinases are also classified by their substrate specificity, which implies the recognition of peptide bonds or residues at the amino or carboxyl terminus of the molecule as well as side chains of the surrounding amino acids at the bond to be cleaved. Since these enzymes can select biologically important substrates, they are related to different actions on different aspects, yielding the necessary balance between specificity and diversity.

Limited proteolysis, in addition to the initial concept that proteinases participate only in digestion processes, is important to direct the cleavage of intended substrates and can also be up- or down-regulated. In spite of possible physiological roles of this regulation, the cleavage of functional proteins would result in normal tissue damage. In this respect, it is noteworthy to evaluate the presence of factors that are able to control the activity of proteinases. These molecules comprise other proteins, peptides, ions, nucleic acids, lipids, coenzymes (vitamins, oxidants, reducing agents), and sugars (Table 1).

Glycosaminoglycans

Large numbers of proteins in animal tissues occur immobilized in the extracellular space, on the cell surface or in the extracellular matrix. Some are anchored through interactions with other proteins. However, current research increasingly implicates proteoglycans as scaffold structures, designed to accommodate proteins by noncovalent binding to their glycosaminoglycan side chains. In particular, heparan sulfate proteoglycans are recognized as ubiquitous protein ligands. Binding of proteins to heparan sulfate chains may serve a variety of functional purposes, from simple immobilization or protection against proteolytic degradation to distinct modulation of biological activity of unexpected regularity (15).

Heparan sulfate is a ubiquitous glycosaminoglycan of animal cells (16). These classes of compounds are heteropolysaccharides made up of repeating units of disaccharides, a uronic acid residue, either D-glucuronic acid or L-iduronic acid, and D-glucosamine with *N*- and 6-*O*-sulfates and *N*-acetyl substitutions (17). Heparan sulfate occurs on the cell surface and in the extracellular matrix as proteoglycans. Most of the cellular heparan sulfate derives from the syndecan and glypican proteoglycans. The syndecan family is associated with the cell membranes via transmembrane core proteins (18), and the glypican family is anchored by glycosylyl phosphatidylinositol-anchor core proteins (19). Also, heparan sulfate proteoglycans are present in basement membranes, representing the perlecan family (20).

Heparan sulfate and heparin are particularly important among glycosaminoglycans in their ability to bind a large number of different proteins. Heparin-like glycosaminoglycans play a complex role in the extracellular matrix, regulating a wide variety of biological processes, including hemostasis, inflammation, angiogenesis, growth factors, cell adhesion, and others (21). Some of these functions are summarized in Table 1.

Regulation of enzymatic activity

Pericellular proteolytic activity is known to be the result of interplay between different molecules such as receptors, tissue inhibitors, and activation factors. In addition, it is

now clear that in many tissue-remodeling processes, including embryo development, morphogenesis, reproduction and cancer invasion, different cell populations synthesize the individual components that are involved in matrix degradation. To complicate matters even further, there are variations in which cell type synthesizes which of the components. This variation occurs according to different biological processes, so that a given cell type has a specific role in a given biological process (22).

Heparin and heparin-like glycosaminoglycans have long been known as regulators of the proteolytic activity of some very important serine proteases (12,23,24). In the coagulation cascade, for instance, several types of zymogen activation, e.g., limited proteolysis of inactive precursors, occur only in the presence of, or are significantly improved by, interactions taking place at the cellular or basement membrane level where heparin-like glycosaminoglycans and proteoglycans are present among other molecules (25). Moreover, it has been shown that the activation of factor XI by thrombin or factor XIa is increased in the presence of glycosaminoglycans (26). On the other hand, it was also demonstrated that the inhibitory activity of antithrombin-III (ATIII) towards thrombin is increased in the presence of glycosaminoglycans. Two mechanisms have been reported to contribute to the potentiation of ATIII activity: 1) colocalization of the proteins in the same heparin chain (6), and 2) a conformational change in ATIII induced upon its binding to heparin (27).

Structural modifications induced by heparin binding

Glycosaminoglycans have long been known to bind to either peptides or protein domains that exhibit certain preferred conformations (28), or to induce conformational changes upon their binding to peptides or proteins (27). This behavior may be related to the ability of glycosaminoglycans to act as modulators for some proteins by changing their catalytic activity (29), by protecting the enzyme against alkaline pH-induced inactivation (2) or by permitting some binding to occur, by exposing binding regions on the target protein (9).

Heparin, a highly sulfated glycosaminoglycan present in mast cells and implicated in physiopathological processes such as cell proliferation and differentiation and in coagulation inhibition, has been extensively studied as a model of the effect of glycosaminoglycans on protein and peptide structure (30) as well as a modulator of enzymatic activity (6). Heparin has the highest negative charge density of any known mammalian macromolecule.

Cardin and Weintraub (31) identified two clusters of positive charges in known heparin-binding proteins in which amino acids tend to be arranged in the *XBBXBX* or *XBBBXXBX* patterns, where B stands for an amino acid with basic charge, usually arginine or lysine, and X represents an uncharged or hydrophobic amino acid. Molecular modeling of these consensus sites predicts the arrangements of these amino acids into either α-helices or ß-strands. This permits the clustering of non-contiguous basic amino acids on one side of the helix domain, thus forming a charged domain to which glycosaminoglycans could bind, as observed for some of the proteases and inhibitors listed in Table 1.

Glycosaminoglycan participation in extracellular matrix degradation: proteinases and protein inhibitor interactions

Serine protease interactions

Human polymorphonuclear leukocytes contain a number of proteolytic enzymes including neutrophil elastase (NE), a 30-kDa glycoprotein that belongs to the class of

serine proteinases. NE cleaves extracellular matrix proteins including elastin, interstitial collagen, fibronectin, proteoglycan and laminin, as well as plasma proteins such as antithrombin, fibrinogen, and components of the immune system. The concentration of NE in the azurophilic granules is thought to be in the millimolar range. Uncontrolled release of this potent enzyme may lead to degenerative connective tissue diseases such as lung emphysema and rheumatoid arthritis (32). The proteinase inhibitors responsible for the control of NE activity are α_1-proteinase inhibitor (α_1-PI), α_2-macroglobulin, and mucus proteinase inhibitor. *In vivo*, the inhibition of NE by mucus proteinase inhibitor and α_1-PI may be affected by compounds present at the site where the inhibition reaction takes place, with oxidants released by polymorphonuclear neutrophils present at sites of inflammation reducing the capacity of NE inhibition by α_1-antitrypsin (33) and α_1-PI (34). On the other hand, when heparin is administered to some patients, it will bind NE and decrease its rate of inhibition by α_1-PI (5). Negatively charged molecules such as DNA released from short-lived cells such as neutrophils are also able to affect the enzymatic activity of NE (35).

Other serine proteases, playing major roles in other physiological processes, have also their proteolytic activity or inhibition changed by heparin. Human tissue kallikrein (hK1), for example, the enzyme responsible for the release of kallidin (Lys-bradykinin) from kininogen, does not have its proteolytic activity altered by heparin. On the other hand, kallistatin, the specific kallikrein inhibitor of the serpin family, has its inhibitory activity towards hK1 deeply reduced by heparin, whose binding to this serpin is different from its binding to ATIII (another serpin) (10,36).

Matrix metalloprotease interactions

The tissue inhibitors of metalloproteinases (TIMP) are specific protein inhibitors of the matrix metalloproteinases (MMP), a group of zinc-dependent enzymes that include collagenases, gelatinases, and stromelysins. Four human forms have been cloned: TIMP-1 (37), TIMP-2 (38), TIMP-3 (39), and, more recently, TIMP-4 (40). TIMP-1 and TIMP-2 are secreted by many cell types in culture and are found in body fluids and tissue extracts. TIMP-3 is unique in that it appears to be a component of the extracellular matrix (11) and occurs in relatively small amounts, possibly being expressed during specific cellular events (41). Of the four known TIMP, TIMP-3 is distinguished by its tighter binding to the extracellular matrix. Glycosaminoglycans such as heparin, heparan sulfate, chondroitin-4/6-sulfate, dermatan sulfate and sulfated compounds such as suramin and pentosan efficiently extract TIMP-3 from the postpartum rat uterus. Enzymatic treatment by heparinase III or chondroitinase ABC also releases TIMP-3, but neither one alone produces complete release. Confocal microscopy shows colocalization of heparan sulfate and TIMP-3 in the endometrium subjacent to the lumen of the uterus. TIMP-3 binding may be important for the cellular regulation of MMP activity (42).

The interaction of heparin with gelatinase A is mediated by ionic interactions, being disrupted by 0.5 M NaCl. In the interaction with matrilysin, heparin increased twofold the rate of interaction of this protease with TIMP-3. TIMP-3 does not contain any of the reported linear heparin-binding motifs, but a motif defined by the three-dimensional structure could exist (43). It is likely that TIMP-3 interacts with cell surface and extracellular matrix glycosaminoglycans via the large number of positively charged residues in TIMP-3, and that this is the basis for its location in the extracellular matrix both *in vivo* and in cell culture. Hence, colocalization of TIMP-3 with proenzymes in the pericellular environment may be a mechanism for increasing the rate of MMP inhibition

and regulating extracellular matrix breakdown during morphogenetic processes.

Colocalization of TIMP-3 in the pericellular environment via binding to the extracellular matrix, including heparan sulfate proteoglycans, would place this inhibitor in a key position to inhibit MMP produced by endothelial cells, thus regulating degradation of the extracellular matrix and release of the angiogenic factors required for migration and angiogenesis.

Cysteine protease interactions

The effect of glycosaminoglycans on the activity of different proteinases from the cysteine proteinases family, namely papain (1), cathepsin K (3), and cathepsin B (2), has been described. In mammals, lysosomal cysteine proteinases have been implicated in diseases involving tissue remodeling states, such as inflammation (23) and tumor metastasis (44). Nevertheless, the activity of cathepsin D, another lysosomal cathepsin, but pertaining to the aspartyl proteinase family, that is overexpressed in breast tumors, towards small synthetic peptides is not regulated by heparin (45). The participation of cysteine proteases in parasite infection has also been observed (46).

Papain was the first cysteine protease reported to interact with glycosaminoglycans (1). This interaction was proposed as a model for mammalian cysteine proteinase-glycosaminoglycan interaction and regulation of the function of this class of proteinases *in vivo*. It was observed in this study that papain exhibited high-affinity binding to heparin, being eluted at 1.0 M NaCl from a heparin-Sepharose column. This interaction was specific, since this binding was disrupted by the previous addition of 100 µM free heparin to the papain solution. These data show that papain binding to heparin is mediated mainly by electrostatic interactions. The presence of heparin significantly changes the papain structure by increasing the α-helix content of the enzyme, and the binding event can be monitored by circular dichroism analysis. This binding results in a powerful increase in the affinity of the enzyme for the substrate. It was also shown that the interaction between heparin or heparan sulfate with papain is specific, since other sulfated glycosaminoglycans, namely dermatan sulfate and chondroitin sulfate, were not able to increase the affinity of papain for the substrate, nor did they change the α-helix content in papain.

The observation of the interaction between a cysteine proteinase and glycosaminoglycans was later extended to another lysosomal cysteine proteinase, cathepsin K. It is currently known that cathepsin K is the predominant cysteine proteinase in osteoclast-mediated bone remodeling, and the proteinase is thought to be involved in the pathogenesis of diseases with excessive bone and cartilage resorption (3). Chondroitin-4-sulfate was shown to increase cathepsin K stability in a specific and selective manner. In kinetic terms, as a combined effect on cathepsin K activity and stability parameters, a 200-fold increase was observed associated with the presence of chondroitin-4-sulfate. The observed increase of activity involved the cleavage of soluble as well as insoluble type I and II collagens.

Recently, cathepsin B, that also belongs to the papain superfamily, was also demonstrated to be affected by the presence of glycosaminoglycans (2). The main feature that distinguishes cathepsin B from other cysteine proteinases is the presence of a large insertion loop structure termed occluding loop, which covers the active site. It has been shown that lysosomal cysteine proteinases, especially cathepsin B, can participate in tumor invasion by degrading extracellular matrix components (44). This can take place either intracellularly, by heterophagosomal activity of the tumor cell, or extracellularly (47) by cell surface-associated cathepsin B (48). It has been demonstrated that the pres-

ence of cathepsin B on the plasma membrane results in focal dissolution of extracellular matrix proteins and enables the tumor cell to invade (49). Trafficking and targeting of lysosomal enzymes are mostly mediated by mannose-6-phosphate receptor pathways (50). However, several reports show that this class of receptors is not sufficient for targeting of lysosomal enzymes along intracellular routes, either by an alteration in these receptors (51) or by changes in glycosylation pattern of lysosomal enzymes, as observed for cathepsin B in carcinoma cells (52). Indeed, mannose-6-phosphate-independent targeting has been proposed for cathepsin B in normal cells (53) and in a human colon carcinoma cell line (54). High levels of cathepsin B and qualitative changes in cathepsin B protein expression, including an abnormal pattern of glycosylation, may be important in maintaining the malignant phenotype in carcinoma cells. Alterations in cathepsin B expression, processing and cellular localization have been observed in several human tumor tissues, and clinical investigations have shown that cathepsin B is a highly predictive indicator for prognosis and diagnosis in cancer (55).

As mentioned above, the presence of cathepsin B on the plasma membrane results in focal dissolution of extracellular matrix proteins and enables the tumor cell to invade the tissue. Recent results suggest that the cell surface heparan sulfate can anchor the membrane forms of cathepsin B, and such complexation affects the cathepsin B activities. The coupling of cathepsin B to heparan sulfate on the cell surface can potentiate the endopeptidase activity of the enzyme by increasing 5-fold its half-life at physiological pH (2). The endopeptidase activity of cathepsin B is related to the degradation of extracellular matrix proteins. These results agree with the observation that the membrane-bound forms of cathepsin B are highly resistant to inactivation at neutral pH. As previously mentioned, the mechanism of secretion and insertion of cathepsin B on the plasma membrane is not fully understood. Mannose-6-phosphate-independent targeting has been proposed for cathepsin B. So, according to this scenario, the cell surface heparan sulfate proteoglycans may anchor a pool of the membrane forms of cathepsin B. Moreover, the cell surface heparan sulfate proteoglycans are in a constant turnover as a result of their continuous secretion and endocytosis. It has been shown that some proteins bound to heparan sulfate glycosaminoglycan chains are endocytosed together with proteoglycans, e.g., fibroblast growth factor, thrombospodin and lipoprotein lipases (21). It is interesting to observe that in the lysosomal vesicles there is a high concentration of cathepsin B and that heparan sulfate is also present in this compartment during its intracellular traffic. These observations suggest that the mechanism of insertion of cathepsin B on the plasma membrane and its cellular traffic may depend on heparan sulfate proteoglycans present on the cell surface.

The modulatory effect exerted by proteoglycans on cysteine proteinase activities is expected to lead to new insights in the understanding of some molecular systems present in pathological states, providing new targets for drug therapy.

Discussion

Protease activity is under a fine control since the systems susceptible to its enzymatic activities, such as the cascade of enzymes involved in the cell death response (56), coagulation (57), and hypertension (58), can be considered to be responsible for homeostasis.

Except for digestive processes occurring in the lumen of the intestine, proteases are always accompanied by a specific inhibitor with the sole function to regulate the enzyme's activity. Such digestive enzymes are also subjected to an activity control, which

consists of the storage of the enzyme as an inactive precursor (zymogen) and/or the change in pH in different regions of the intestine, leading to different enzymatic activities being preferred. On the other hand, tissue or plasma enzymes are normally less concentrated than digestive enzymes and must play their role more rapidly. This leads to an increase in the enzyme's specificity, followed by the development of a more specific inhibitor responsible for assuring that the enzyme would only play the desired role with no further damage to the other proteins. This fine tuning can be assured by inhibitors alone or by other factors associated with the enzyme control, such as glycosaminoglycans, which would give the system much more flexibility in terms of action and counteraction. It is noteworthy to observe, from a structural viewpoint, the nonlinear features of some heparin-binding sites present in kallistatin that cannot be found among the sites suggested for other protein inhibitors such as TIMP-3. By comparing the two structures, the difference in the molecular mechanisms developed in the construction of proteases belonging to different families is remarkable. This seems to be an indication of the multiplicity that we should take into account when studying the interactions between carbohydrates and proteins.

Complexity seems to be the path towards which nature has decided to evolve. Once enzymes succeeded in lowering the activation energy required for a given reaction to occur and speeded up the processes from an evolutionary satisfactory point of view, regulation of this catalysis probably started to play a much more important role than the reaction itself. Understanding the ways life regulates itself by controlling the molecular machinery is a necessary step to be taken as we intend to understand life at all.

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