REVIEW ARTICLE

Glycosaminoglycans and the regulation of blood coagulation

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INTRODUCTION

Blood coagulation involves the sequential activation of a series of serine proteinases, which culminates in the generation of thrombin and subsequent thrombin-catalysed conversion of fibrinogen into insoluble fibrin (Furie and Furie, 1988). Inhibitory modulation of this process, of paramount physiological importance, is primarily achieved by two principally different mechanisms (Figure 1). The enzymes may be inactivated by serine proteinase inhibitors (known as 'serpins'), which act by formation of stable 1:1 molar complexes with their target enzymes (Travis and Salvesen, 1983). Alternatively, the so-called protein C pathway leads to inactivation of auxiliary coagulation proteins (factors V_a and $VIII_a$) by cleavage at distinct sites (Esmon, 1989; Dahlbäck, 1991). The prime site of regulation is the surface of vascular endothelial cells, which have been known to possess anticoagulant properties (Colburn and Buonassisi, 1982). These properties are particularly conspicuous in the microcirculation, with its high wall surface to blood volume ratio (Busch, 1984).

The ability of certain sulphated polysaccharides, glycosaminoglycans, to interfere with blood coagulation has a long-standing record, as illustrated by the extensive clinical use of heparin as an antithrombotic agent (see Rodén, 1989). The main effect of heparin (and of its relative, heparan sulphate) is to accelerate the inactivation of coagulation enzymes by the serpin antithrombin (Rosenberg, 1977; Björk and Lindahl, 1982). A more complex picture emerged with the finding of an additional serpin, heparin cofactor II, which is 'activated' not only by heparin, but also by another glycosaminoglycan, dermatan sulphate, and which selectively inactivates thrombin (Tollefsen et al., 1982; Tollefsen, 1989). Remarkably, also the other major regulatory mechanism, the protein C pathway, involves a glycosaminoglycan-containing molecular species, since the protein C activation cofactor, thrombomodulin, turned out to be a proteoglycan with a functionally important, covalently bound glycosaminoglycan chain (Bourin and Lindahl, 1990; Bourin et al., 1990). In this Review we attempt to summarize our current understanding of glycosaminoglycan involvement in the regulation of blood coagulation.

THE GLYCOSAMINOGLYCANS

The proteoglycans comprise a heterogeneous group of macromolecular glycoconjugates that are composed of sulphated glycosaminoglycan chains covalently linked to a protein core. They are widely distributed in animal tissues and appear to be synthesized by virtually all types of cells. All glycosaminoglycans identified, except the nonsulphated polysaccharide hyaluronan, which occurs as free glycosaminoglycan chains, are synthesized in proteoglycan form. The large (and growing) number of core proteins identified, the variable extent of substitution with glycosaminoglycan chains and the variability in glycosaminoglycan structure contribute to the overall structural diversity of the proteoglycans. A detailed consideration of these features is beyond the scope of this Review, in which the structural aspects will be restricted to a brief presentation of relevant glycosaminoglycan sequences. For more comprehensive overviews on proteoglycan biochemistry the reader is referred to reviews by Fransson (1985, 1987), Hassell et al. (1986), Poole (1986), Ruoslahti (1988, 1989), Gallagher (1989) and Kjellén and Lindahl (1991).

Classification of glycosaminoglycans takes note of the basic structure of the glycan backbone, which may be composed of (1) $(HexA\text{-}GalN)_n$, (2) $(HexA\text{-}GlcN)_n$, or (3) $(Gal\text{-}GlcN)_n$ type disaccharide units. The type-3 disaccharide unit occurs in keratan sulphate only, which has so far not been implicated with blood coagulation and will not be considered further in this Review. Types 1 and 2 may be further subdivided, type 1 into chondroitin sulphate and dermatan sulphate, type 2 into heparan sulphate and heparin (Figure 2). The type-2 saccharides include also hyaluronan, which differs from the heparin/heparan sulphate family with regard to position of glycosidic linkages and by lacking sulphate substituents; again, this glycosaminoglycan species does not seem to be directly involved in blood coagulation. The definition of subspecies within each class of glucosamino- or galactosamino-glycans is complicated by extensive microheterogeneity of the glycan structures, which is best understood through a short discourse of glycosaminoglycan biosynthesis.

The biosynthesis of heparin/heparan sulphate (Lindahl, 1989; Lindahl and Kjellén, 1991) is initiated by formation of a polysaccharide chain with the structure $(\text{-GlcA}\beta1,4\text{-GlcNAc}\ \alpha1,4\text{-})_n$. This polymer is N-deacetylated/N-sulphated and subsequently undergoes, in the order mentioned, C-5 epimerization of GlcA to IdoA units, 2-O-sulphation of IdoA, and 6-O-sulphation of GlcN units. Additional O-sulphate substituents may be incorporated at C-3 of GlcN units (Kusche et al., 1988) and at C-2 (or C-3) of GlcA units (Bienkowski and Conrad, 1985; Kusche and Lindahl, 1990). Due to the stepwise nature of the process, and the substrate specificities of the enzymes involved, the product of any given reaction will be the substrate for the subsequent reaction. Polymer modification is incomplete in the sense that the enzymes generally act upon only a fraction of the potential substrate residues; hence, the structural complexity and heterogeneity of the polysaccharide chain under modification increase throughout the process. Following the initial N-deacetylation/N-sulphation reaction all subsequent modifications will depend on N-sulphate groups for substrate recognition. Therefore, the IdoA units and O-sulphate groups of the final products are accumulated in the N-sulphated regions of the glycosaminoglycan chains, while the N-acetylated sequences retain GlcA units and remain largely nonsulphated. Heparin is extensively N-sulphated, and therefore rich in IdoA and O-sulphate groups,

Abbreviations used: EGF, epidermal growth factor; HexA, unspecified hexuronic acid; GlcN, glucosamine; GalN, galactosamine; Gal, galactose; GlcA, D-glucuronic acid; IdoA, L-iduronic acid.

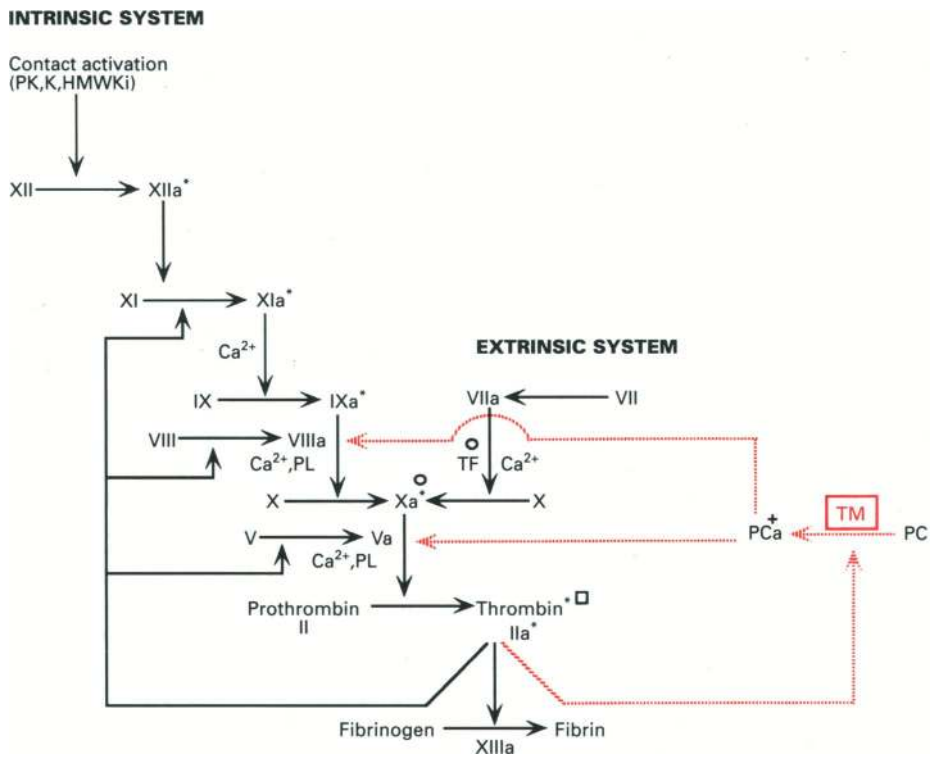

Figure 1 Overview of blood coagulation

The coagulation factors are designated with Roman numerals and the suffix a indicates a proteolytically activated factor. The solid arrows indicate pathways resulting in acceleration of blood clotting, whereas broken arrows (red) represent inhibitory mechanisms. The symbols associated with certain coagulation factors or reactions indicate target sites for various proteinase inhibitors (*, antithrombin; □, heparin cofactor II; ○, tissue factor pathway inhibitor; +, protein C inhibitor). Other abbreviations: PK, prekallikrein, K, kallikrein, HMWKi, high-molecular-weight kininogen; PL, phospholipid; TF, tissue factor. Note the protein C activation cofactor function of thrombomodulin. The factor XI-activating role of thrombin was recently postulated by Gailani and Broze (1991).

whereas heparan sulphate contains more N-acetylated, unmodified, regions (Höök et al., 1974; Gallagher and Walker, 1985; Lindahl and Kjellén, 1991). However, mixed-type, 'irregular' regions may occur in both heparin and heparan sulphate and, furthermore, may be of functional importance, as illustrated by the antithrombin-binding region (Figure 3). This pentasaccharide sequence is composed of three GlcN units, one of which is preferentially N-acetylated, one GlcA unit, and one IdoA unit, with O-sulphate groups in various positions.

The galactosaminoglycans, chondroitin sulphate and dermatan sulphate, are generated by principally similar modifications of an initial polymerization product, which has the structure $(GlcA\beta1,3\text{-}GalNAc\beta1,4\text{-})_n$ (Rodén, 1980; Fransson, 1985). The structural diversity is less pronounced than for the heparin-related glycosaminoglycans, since the GalNAc residues remain exclusively N-acetylated. Nevertheless, owing to the variable location of (O-)sulphate groups and the presence of GlcA as well as IdoA units, as many as nine different HexA-GalNAc disaccharide units have been identified (Seldin et al., 1984). By definition, chondroitin sulphate contains GlcA as the only HexA component, whereas any galactosaminoglycan with detectable amounts of IdoA will be referred to as a dermatan sulphate. The sulphate content is usually ~1/disaccharide unit, thus less variable than in the heparin/heparan sulphate family, although 'oversulphated' species have been described.

Most of the biological activities known to be associated with proteoglycans are due to interactions between the negatively charged glycosaminoglycan chains and various proteins (Jackson et al., 1991; Kjellén and Lindahl, 1991). In general, IdoA-containing glycosaminoglycans interact more avidly than do those containing GlcA only (Casu et al., 1988). With regard to the proteins involved in blood coagulation and its regulation the interactions with glycosaminoglycans vary from specific, 'lock-and-key' type binding to relatively nonspecific, co-operative electrostatic association.

EFFECTS OF GLYCOSAMINOGLYCANS ON PROTEINASE INHIBITORS

The major inhibitors of serine proteinases involved in blood coagulation are antithrombin and heparin cofactor II. Their mechanisms of action are profoundly influenced by glycosaminoglycans, which accelerate the rates of inhibition by binding to the inhibitors and, albeit with some exceptions, to their target enzymes. Other inhibitors, such as protein C inhibitor, protease nexin-1, and tissue factor pathway inhibitor are also affected by glycosaminoglycans; however, these interactions have not been elucidated to the same extent and will therefore be considered in less detail.

Antithrombin

Antithrombin is an α_2-glycoprotein of M_r ~58000 that is synthesized in the liver and occurs in human blood at ~2.7 μM concentration (for review, see Björk and Danielsson, 1986). Antithrombin is the major inhibitor of thrombin in plasma, but also inactivates the other serine proteinases of the intrinsic pathway, factors IXa, Xa, XIa and XIIa (Figure 1).

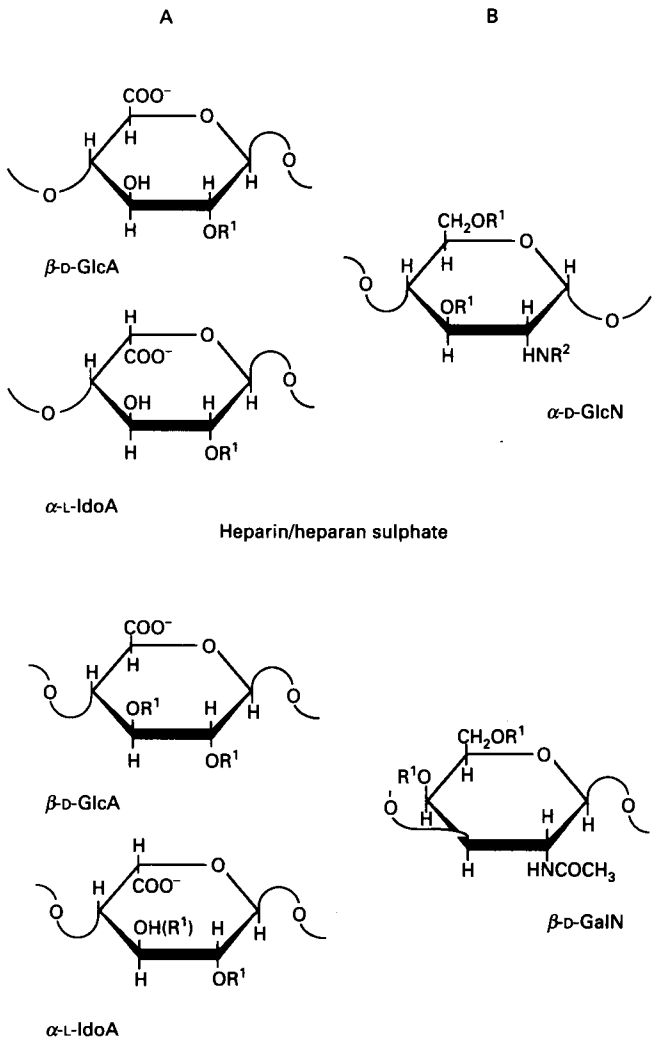

Figure 2 Structures of glycosaminoglycans

The sulphated glycosaminoglycans are linear polymers of alternating A and B units, where A is a HexA residue (GlcA or its C-5 epimer, IdoA), and B is either GlcN (in the glucosaminoglycans, heparin and heparan sulphate) or GalN (in the galactosaminoglycans, chondroitin sulphate and dermatan sulphate). Additional species such as hyaluronan and keratan sulphate, without any apparent relation to blood coagulation, are not included. R^1 = -H or $-SO_3^-$; R^2 = $-SO_3^-$ or $-COCH_3$. The assignment of positions for the sulphate groups on the GlcA units remains somewhat tentative. For additional information see the text.

Antithrombin inhibits serine proteinases by forming tight, equimolar complexes through interaction between a specific reactive bond of the inhibitor and the active site of the enzyme (Rosenberg and Damus, 1973; Björk et al., 1989a; Olson and Björk, 1992). The stability of these complexes has suggested that they represent acyl-intermediates formed during cleavage of the reactive bond as in reaction with a normal substrate. That the proteinase may cleave the reactive bond in the inhibitor instead of forming a stable complex is indicated by the observation that small amounts of free inhibitor, cleaved at the reactive site, are produced during the reaction of antithrombin with proteinases (Björk and Fish, 1982; Olson, 1985). The reactive bond of antithrombin has been identified as the Arg-393–Ser-394 bond near the C-terminus of the inhibitor (see Björk et al., 1989*a*; Olson and Björk, 1991a). A peptide sequence 8–12 amino-acid residues N-terminal to the reactive bond (designated residues P8–P12) appears to be of critical importance for antithrombin to function as an inhibitor of proteinases. Natural antithrombin variants in which amino acids within this region are mutated (Devraj-Kizuk et al., 1988; Molho-Sabatier et al., 1989), were shown to be inactive as inhibitors, but are instead excellent substrates of their target enzymes, which efficiently cleave antithrombin at the reactive bond (Caso et al., 1991; Ireland et al., 1991). Moreover, Asakura et al. (1990) showed that a monoclonal antibody that binds to the P8–P12 region also transforms antithrombin from an inhibitor to a substrate of thrombin. A more detailed understanding of this inhibitor–substrate transition has emerged from X-ray crystallographic studies of homologous serpins (Huber and Carrell, 1989; Mourey et al., 1990), including the noninhibitory serpin, ovalbumin (Stein et al., 1990), and from molecular dynamics simulations (Engh et al., 1990). Cleavage of the reactive bond induces a drastic conformational change of the P1–P16 region, an exposed peptide loop in the native state, such that it becomes inserted into the major β-sheet of the protein. These findings suggest that a partial insertion of the P1–P16 loop might be involved in trapping a proteinase in a stable complex (Skriver et al., 1991). Mutations in the P8–P12 region of the loop would interfere with such insertion and thereby allow the exposed reactive bond to be cleaved as a normal substrate. In support of this hypothesis, addition of a synthetic, competing P1–P14 peptide, blocking the insertion site for the reactive loop, resulted in a loss of the ability of antithrombin to inhibit thrombin, and in concomitant cleavage by the enzyme of the reactive bond of the inhibitor (Björk et al., 1992). Recent X-ray diffraction studies on human plasminogen activator inhibitor-1 support the general concept of a conformationally flexible reactive loop adjacent to the scissile bond in serpins (Mottonen et al., 1992).

While it had been known since the work of Howell in the 1920s that heparin requires a plasma cofactor for its anticoagulant action, it was not until 1968 that Abildgaard (1968) established the identity of this cofactor with antithrombin. The purified protein was found to account for both the 'progressive antithrombin activity' (slow inhibition of thrombin in the absence of heparin) and 'heparin cofactor activity' (rapid inhibition of thrombin in the presence of heparin). Rosenberg and Damus (1973) then suggested that heparin binds to antithrombin and effects a conformational change which results in a greatly accelerated reaction with thrombin. Following complex formation with thrombin, antithrombin loses its high affinity for heparin, which will be released and ready to 'activate' another antithrombin molecule (Björk and Nordenman, 1976; Olson and Shore, 1986; Peterson and Blackburn, 1987a). Heparin thus acts as a catalyst.

The accelerating effect of heparin on antithrombin–proteinase reactions depends on the presence of a unique antithrombin-binding pentasaccharide sequence in the glycosaminoglycan chain (Lindahl et al., 1980, 1984; Casu et al., 1981; Thunberg et al., 1982; Atha et al., 1984, 1985). This region is composed of one GlcA unit, one IdoA unit and three GlcN units, two of which are invariably N-sulphated whereas the remaining one may be either N-acetylated or N-sulphated (Figure 3). The structure/function relationships pertaining to this sequence have been elucidated in detail and have been confirmed by chemical synthesis (Choay et al., 1983; Petitou et al., 1988a,b; Petitou, 1989; Grootenhuis and van Boeckel, 1991). Both N-sulphate groups (Riesenfeld et al., 1981), the nonreducing-terminal 6-O-sulphate group (Lindahl et al., 1983) and the 3-O-sulphate group on the internal GlcN unit

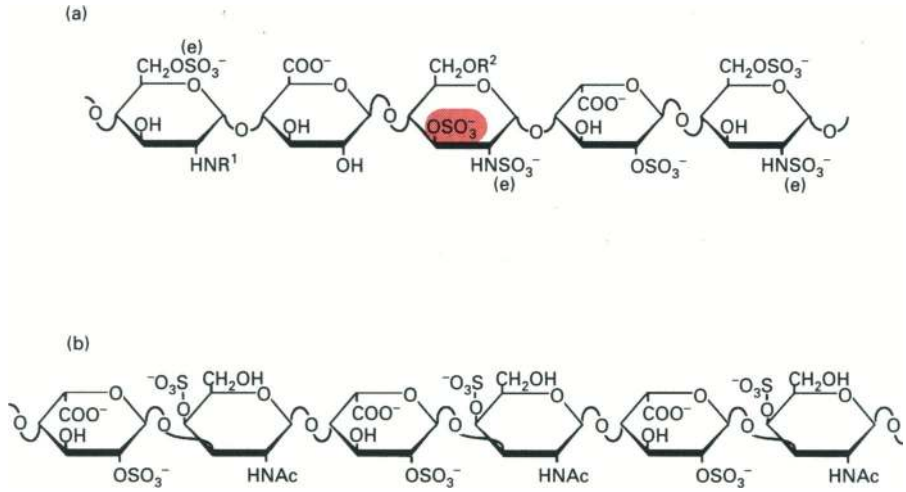

Figure 3 Serpin-binding regions in glycosaminoglycans

The structures shown represent (**a**) the antithrombin-binding region in heparin (heparan sulphate), and (**b**) the heparin cofactor II-binding region in dermatan sulphate. (**a**) Structural variants in the antithrombin-binding region are indicated by R^1 (-SO_3^- or -$COCH_3$) and R^2 (-H or -SO_3^-). The 3-*O*-sulphate group highlighted in red is a marker group for the antithrombin-binding region, and is essential for the high-affinity binding of the polysaccharide to antithrombin. The three sulphate groups marked (e) are also highly important to this interaction. (**b**) The heparin cofactor II-binding sequence (of highest affinity) in dermatan sulphate is composed of three consecutive -IdoA(2-OSO_3)-GalNAc(4-OSO_3)- disaccharide units that create a region of high negative charge density. For further information see the text.

(Atha et al., 1985; Petitou et al., 1988a) are essential for the biological activity. The latter residue is a distinguishing structural feature of the antithrombin-binding sequence, and thus, by and large, serves to indicate anticoagulant activity, although it has also been detected in other regions of heparin (Kusche et al., 1990) and heparan sulphate (Pejler et al., 1987a; Edge and Spiro, 1990; Kojima et al., 1992a) chains. The occurrence of 3-*O*-sulphate groups in only 30–40 % of the molecules in commercially available heparin preparations (Kusche et al., 1990) explains the previous, at the time highly unexpected, finding that only a fraction of such preparations exhibited high affinity for antithrombin (Andersson et al., 1976; Höök et al., 1976; Lam et al., 1976). Heparin (Horner, 1986; Horner et al., 1988) as well as heparan sulphate (Hovingh et al., 1986; Lane et al., 1986; Marcum et al., 1986; Pejler and David, 1987; Pejler et al., 1987b; Horner, 1990; Lindblom et al., 1991; Kojima et al., 1992a) preparations from various sources vary with regard to the proportion of molecules having high affinity for antithrombin. Of particular interest is the demonstration of proteoglycans with antithrombin-binding heparan sulphate chains, synthesized by vascular endothelial cells (Marcum et al., 1986, Kojima et al., 1992a). Such proteoglycans showed no apparent correlation between the proportion of antithrombin-binding heparan sulphate chains and core protein structure (Kojima et al., 1992a,b).

The heparin-binding region in antithrombin appears to be a composite site involving peptide sequences from different parts, largely in the N-terminal domain, of the protein. Identification of individual amino-acid residues contributing to binding has been based on studies of antithrombin variants or of chemically modified derivatives with decreased or abolished heparin binding (Brennan et al., 1988; Chang, 1989; Björk et al., 1989a; Borg et al., 1992; Gandrille et al., 1990; Sun and Chang, 1990; Olson and Björk, 1992), but also on n.m.r. spectroscopy (Gettins and Wooten, 1987). Two separate regions are implicated, one involving His-1, Ile-7, Arg-24, Pro-41 (Chang and Tran, 1986), Arg-47 (Koide et al., 1984) and Trp-49 (Blackburn et al., 1984), and the other, further upstream in the sequence, Leu-99 (Olds et al., 1992), Lys-107, Lys-114, Lys-125, Arg-129, Asn-135, Lys-136, and Arg-145. A disulphide bond connecting these two regions also seems to be essential for heparin binding (Sun and Chang, 1989). These amino-acid residues may either directly contribute to heparin binding by participating in ionic interactions with the polysaccharide, or they may be essential by maintaining the structural integrity of the binding site (Shah et al., 1990). The two peptide regions implicated in heparin binding map to the A and D α-helices on the surface of a three-dimensional model of antithrombin, such that the basic residues were noted to form a band of positive charge, of appropriate size for interaction with the antithrombin-binding pentasaccharide sequence in heparin (Huber and Carrell, 1989). Interestingly, a monoclonal antibody that recognizes one of these peptide blocks (residues 104–251) not only inhibited binding of heparin to antithrombin but actually induced an increase in the rate of antithrombin–thrombin complex formation (Smith et al., 1990). More recent data suggest that also mutants having single amino-acid substitutions within the C-terminal sequence 402–407 show defective heparin binding (D. A. Lane, personal communication). By contrast, an adjacent mutation (Arg-393 His/Pro), involving the reactive site of the inhibitor, was found to actually increase heparin affinity (Owen et al., 1991).

There is now ample evidence that the interaction between antithrombin and heparin chains that contain the specific antithrombin-binding pentasaccharide sequence is accompanied by a conformational change in the inhibitor (see Björk et al., 1989a; Olson and Björk, 1992). In a comparative study Shore et al. (1989) found that full-length, high-affinity heparin and synthetic, antithrombin-binding pentasaccharide induced highly similar conformational changes in antithrombin, as evidenced by spectroscopic methods. Rapid kinetic experiments showed that full-length heparin and the pentasaccharide both bind antithrombin in a two-step process, comprised by an initial weak interaction that is essentially identical for the two saccharides, followed by

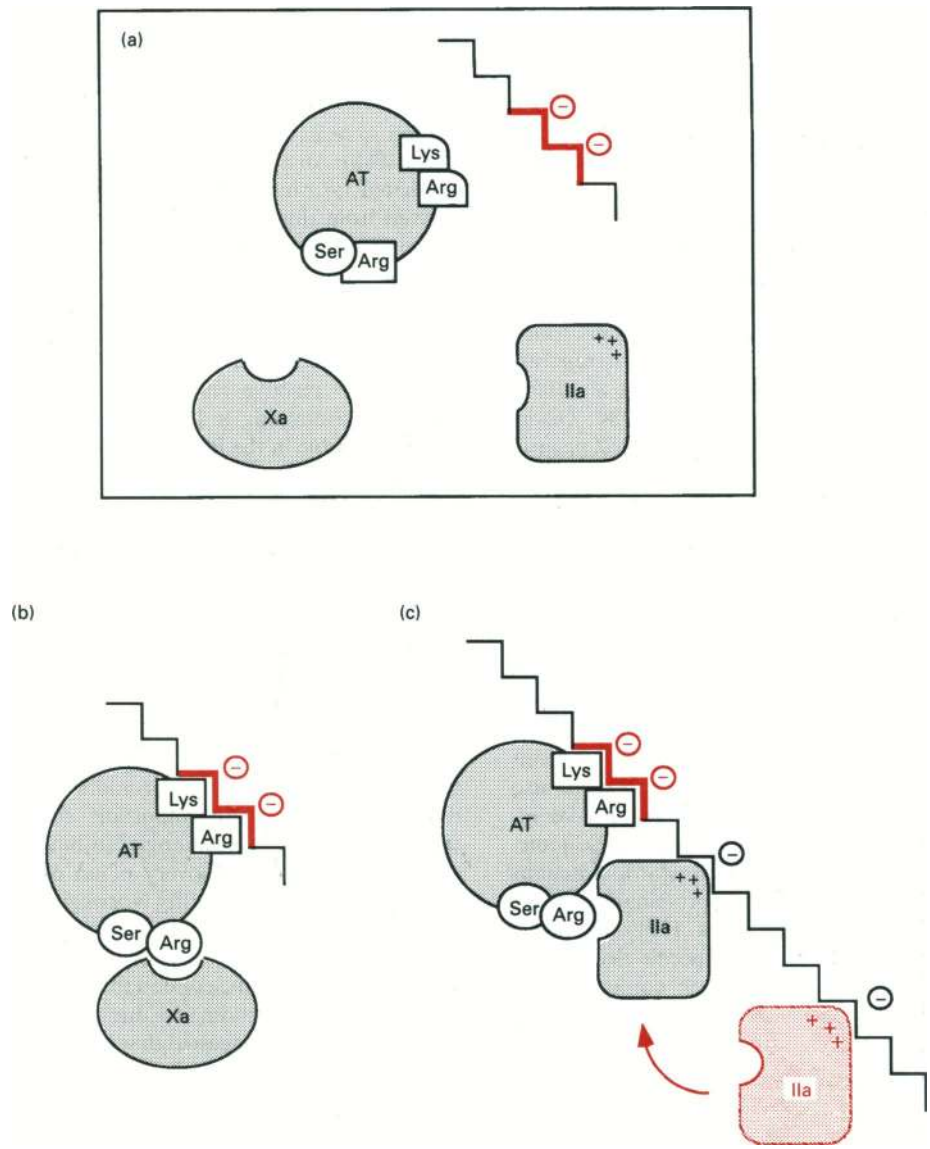

Figure 4 Effects of heparin on the reactions between antithrombin and coagulation enzymes

The scheme is specially designed to illustrate the importance of glycosaminoglycan chain length in the various antithrombin–proteinase reactions. (**a**) Schematic display of the interacting species. The adjacent Ser and Arg residues in antithrombin (AT) represent the reactive site of the inhibitor, normally in a conformation not conducive to interaction with proteinases. The Lys/Arg designation illustrates the heparin-binding site of antithrombin. (**b**) Binding to heparin induces a conformational change in the antithrombin that will facilitate its reaction with factor Xa. This mechanism works also with short heparin oligosaccharides, provided that they contain the specific antithrombin-binding pentasaccharide sequence (indicated by red segments). (**c**) The effect of heparin on the reaction between antithrombin and thrombin (IIa) involves binding of both the enzyme and the inhibitor to the heparin chain, which thus needs to contain a saccharide segment of certain length in addition to the antithrombin-binding region. The thrombin molecule will bind in a nonspecific fashion, through positive surface charges (Heuck et al., 1985; Beresford et al., 1990), to any site along the glycosaminoglycan chain, and will then move along the chain until it encounters the bound antithrombin ('template' or 'surface approximation' mechanism).

a conformational change that is responsible for generating the high-affinity binding (see also Olson et al., 1981; Peterson and Blackburn, 1987b). While this conformational change appears to be stabilized to a somewhat greater extent by the full-length heparin than by the pentasaccharide, binding studies indicated that the major portion (>90%) of the binding energy of the full-length heparin interaction is due to the pentasaccharide.

The mechanism behind the heparin-induced acceleration of antithrombin-proteinase reactions has been an issue of controversy. The conformational change of the antithrombin molecule, largely induced by the specific antithrombin-binding pentasaccharide sequence was assumed to be an important contributor to the rate enhancement (Rosenberg and Damus, 1973; Jordan et al., 1980; Carrell et al., 1987), presumably by causing the reactive bond of the inhibitor to be more accessible to the active-site of the proteinases (Figure 4b). This conclusion appeared to be supported by the findings that the reactions of antithrombin with proteinases such as factor Xa, factor XIIa and plasma kallikrein were potentiated in approximately similar fashion by full-length heparin and by the antithrombin-binding pentasaccharide, or small oligosaccharides containing this specific sequence. On the other hand, the reactions of antithrombin with other proteinases such as thrombin, factor IXa and factor XIa were negligibly influenced by small-sized, high-affinity oligosaccharides; instead, a minimum chain length of ~18 saccharides was found to be required to significantly enhance the rates of inhibition (Laurent et al., 1978; Holmer et al., 1980, 1981; Oosta et al., 1981; Choay et al., 1983; Lane et al., 1984; Shore et al.,

1989). These findings were better explained by an alternative mechanism, which predicted that heparin was acting as a surface or bridge to approximate antithrombin and the enzyme by the binding of both proteins to the same heparin chain (Figure 4c; Laurent et al., 1978; Machovich and Arányi, 1978; Pomeranz and Owen, 1978; Holmer et al., 1979). This proposal was supported by chemical modification studies in which the rate-enhancing effect of heparin could be selectively abolished by modification of basic residues of the proteinase assumed to be involved in heparin binding (Pomeranz and Owen, 1978; Machovich et al., 1978). Further, kinetic experiments showed inhibition of the rate enhancement at high heparin concentrations which correlated with the binding of inhibitor and proteinase to separate heparin chains (Jordan et al., 1979, 1980; Oosta et al., 1981; Griffith, 1982; Nesheim, 1983; Hoylaerts et al., 1984; Olson, 1988). Finally, binding studies indicated that the smallest heparin fragment capable of significantly accelerating the antithrombin–thrombin reaction corresponded to the smallest saccharide that could bind both antithrombin and the active-site blocked proteinase (Danielsson et al., 1986).

While a substantial body of evidence thus supports the importance of the surface approximation or bridging mechanism for the acceleration of the antithrombin–thrombin reaction by heparin, some investigators maintain that this mechanism plays only a secondary role and that activation of antithrombin through the conformational change would be the primary basis for the effects of heparin on all antithrombin–proteinase reactions (Huber and Carrell, 1989; Beresford and Owen, 1990; see also Björk et al., 1989a; Olson and Björk, 1992). Additional information was obtained through detailed studies of the ionic-strength dependence of the various macromolecular interactions involved in heparin-mediated antithrombin–proteinase complex formation (Shore et al., 1989; Olson and Björk, 1991). Thrombin binding to heparin essentially involves a nonspecific electrostatic association of the proteinase with any three contiguous disaccharide units of the polysaccharide chain (Olson et al., 1991). Quantitatively similar electrostatic contributions were found for the (specific) antithrombin–heparin and (nonspecific) thrombin–heparin interactions, contrary to the predominantly non-ionic mode of antithrombin–thrombin interaction. Moreover, the salt-dependence of the accelerating effect of heparin on the thrombin–antithrombin complex formation was found to be indistinguishable from the salt dependence of thrombin binding to heparin. Heparin-dependent approximation of antithrombin and thrombin bound to the polysaccharide thus quantitatively accounts for the rate-enhancing effect on the antithrombin–thrombin reaction, whereas the antithrombin conformational change contributes to a minor degree only. It therefore seems reasonable to conclude that whereas both the antithrombin conformational change and surface approximation mechanisms contribute to the accelerating effect of heparin on antithrombin–proteinase reactions, the relative contribution of each mechanism varies with the target proteinase. Additional complexity, and further support for the bridging mechanism, emerged from studies of the rate-enhancing effect of heparin on the reactions of antithrombin with the proteinases plasma kallikrein and factor XIa (Olson, 1989; Shore et al., 1989; Björk et al., 1989b). This (rather weak) effect is reinforced by a protein cofactor, high-molecular-weight kininogen, which apparently acts by promoting the interactions between the heparin chain and the target proteinases.

Heparin cofactor II

Heparin cofactor II, another serpin that occurs in plasma at micromolar concentration (for review, see Tollefsen, 1989), is identical (or closely related) to human Leuserpin 2 (hLS2; Ragg, 1986; Ragg and Preibisch, 1988). It consists of a single polypeptide chain composed of 480 amino acid residues, as deduced from cDNA analysis, and the gene has been localized to chromosome 22 (Ragg, 1986; Inhorn and Tollefsen, 1986; Blinder et al., 1988). An acidic domain near the N-terminus contains two sulphated tyrosine residues (Hortin et al., 1986), and peptides cleaved from this portion of heparin cofactor II by neutrophil proteinases have potent chemotactic activity (Pratt et al., 1990). Heparin cofactor II inactivates thrombin (Figure 1) by formation of a stable 1:1 complex, but does not react with factor Xa (Tollefsen et al., 1982; Parker and Tollefsen, 1985; Griffith et al., 1985a). It functions as a pseudosubstrate for thrombin, the Leu-444–Ser-445 reactive site peptide bond, located near the C-terminus, containing a leucine rather than the more typical arginine residue in the P1 position (Griffith et al., 1985b). While hereditary heparin cofactor II deficiency has been documented in a few patients suffering from thrombosis (Tran et al., 1985; Sié et al., 1985; Weisdorf and Edson, 1991), the role of heparin cofactor II in the prevention of thrombosis remains unclear (Bertina et al., 1987; Tollefsen, 1990; Toulon et al., 1991).

Because of the P1 leucine, the inhibition of thrombin is very slow in the absence of a glycosaminoglycan. Both dermatan sulphate and heparin increase the rate of inhibition of thrombin by heparin cofactor II more than 1000-fold, whereas chondroitin 4- or 6-sulphate have no effect (Tollefsen et al., 1983). According to Scully et al. (1986) chondroitin 4,6-disulphate (also known as CS-E) has some ability to promote the heparin cofactor II–thrombin reaction. An important step toward the characterization of the glycosaminoglycan-binding domain of heparin cofactor II was the discovery of an inhibitor variant ('HCII Oslo', isolated from a healthy Norwegian blood donor), capable of binding heparin but unable to interact with dermatan sulphate (Andersson et al., 1987). The aberrant properties of HCII Oslo could be traced to a nucleotide substitution in the codon for Arg-189, resulting in a histidine residue at this position (Blinder et al., 1989). This finding suggested that the positive charge on Arg-189 may be involved in the binding of dermatan sulphate but not of heparin, and provided the first evidence that the two glycosaminoglycans are bound to different sites. The further characterization of these sites relied essentially on site-directed mutagenesis in the normal heparin cofactor II cDNA, followed by analysis of the resulting expressed recombinant proteins. Mutations of Arg-189, Arg-192, and Arg-193, with loss of the corresponding positive charges, were found to specifically interfere with dermatan sulphate binding but did not affect the interaction with heparin. Conversely, mutation of Lys-173 decreased binding to heparin but not to dermatan sulphate. Finally, mutations of Arg-184 or Lys-185 affected the interactions of heparin cofactor II with both glycosaminoglycans. The binding sites for heparin and dermatan sulphate in heparin cofactor II thus appear to be overlapping but not identical (Blinder et al., 1989; Church et al., 1989; Blinder and Tollefsen, 1990; Ragg et al., 1990a,b; Whinna et al., 1991).

The effect of heparin on the inhibition of thrombin by heparin cofactor II appears to be governed mainly by overall charge (Hurst et al., 1983), without requirement for any specific oligosaccharide sequence akin to that involved in antithrombin binding (Griffith, 1983; Petitou et al., 1988c; Sié et al., 1988; Kim and Linhardt, 1989; Tollefsen et al., 1990). Moreover, the catalytic efficiency of heparin saccharides increased continuously with the molecular size of the chain, up to >20 monosaccharide residues (Maimone and Tollefsen, 1988; Sié et al., 1988; Bray et al., 1989; Tollefsen et al., 1990). The heparin cofactor II-mediated anticoagulant activities of dermatan sulphate preparations cor-

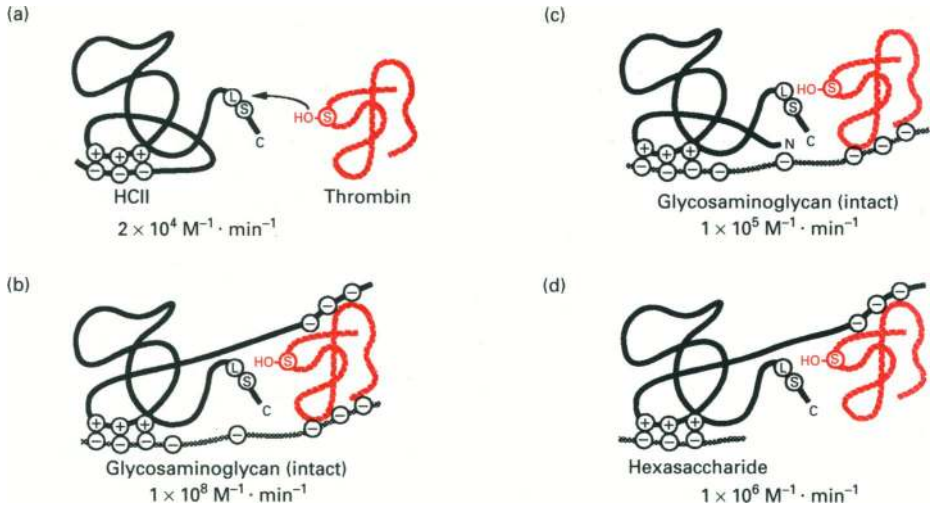

Figure 5 Model for the effects of glycosaminoglycans on the inhibition of thrombin by heparin cofactor II

The figure shows interactions of native or truncated heparin cofactor II with thrombin (red), in the presence or absence of glycosaminoglycan chains/oligosaccharides, and the corresponding approximative second-order rate constants for thrombin inhibition. (**a**) Native heparin cofactor II and thrombin in the absence of glycosaminoglycan; (**b**) native heparin cofactor II and thrombin in the presence of full-sized glycosaminoglycan chain; (**c**) heparin cofactor II, lacking the N-terminal domain, and thrombin in the presence of full-sized glycosaminoglycan chain; (**d**) native heparin cofactor II and thrombin in the presence of dermatan sulphate hexasaccharide. For further information see the text. (From Van Deerlin and Tollefsen, 1991, with permission from the authors.)

related with the degrees of sulphation (Akiyama et al., 1982; Munakata et al., 1987; Scully et al., 1988) and appeared to be promoted by the occurrence of disulphated -IdoA(2-OSO_3)-GalNAc(4-OSO_3)- disaccharide units (Scully et al., 1988). Among the oligosaccharides produced by partial chemical depolymerization of dermatan sulphate, the smallest fragment displaying both appreciable anticoagulant activity and affinity for heparin cofactor II was a dodecasaccharide (Tollefsen et al., 1986), suggesting that binding of dermatan sulphate to both heparin cofactor II and the thrombin target molecule was important for efficient proteinase inhibition. The smallest heparin cofactor II-binding high-affinity oligosaccharide, a hexasaccharide, generated in a similar fashion and isolated by affinity chromatography, was found to consist of three consecutive disulphated -IdoA(2-OSO_3)-GalNAc(4-OSO_3)- units (Maimone and Tollefsen, 1990; Figure 3b). Such clusters of disulphated disaccharide units are generally rare in dermatan sulphate, which thus seems to bind heparin cofactor II in a more selective manner than does heparin.

More detailed information regarding the role of glycosaminoglycans in heparin cofactor II-mediated thrombin inhibition was obtained through analysis of recombinant inhibitor mutants (Ragg et al., 1990a,b; VanDeerlin and Tollefsen, 1991). The N-terminal region contains two repeated acidic domains that are homologous to thrombin-binding domains in the C-terminal portion of hirudin and have been implicated as a site of interaction between heparin cofactor II and thrombin (Hortin et al., 1989). Deletion of this region did not affect the rate of inactivation of thrombin by heparin cofactor II in the absence of glycosaminoglycan but dramatically (by about three orders of magnitude) impeded the increase in reaction rate normally obtained in the presence of heparin or dermatan sulphate. This and other observations have been interpreted in terms of the model illustrated in Figure 5, which shows the effects of glycosaminoglycans on the interactions of native or truncated heparin cofactor II with thrombin. In the absence of glycosaminoglycan, the N-terminal, hirudin-like acidic domain of heparin cofactor II binds intramolecularly to a glycosaminoglycan-binding site, and covalent complex formation with thrombin occurs at the basal rate (Figure 5a). Glycosaminoglycans displace the acidic region from the internal binding site, thereby enabling it to interact with the hirudin-binding site of thrombin (referred to as the 'anion-binding exosite' by Fenton, 1986); simultaneous binding of the glycosaminoglycan chain also to a glycosaminoglycan-binding site of thrombin results in maximal acceleration of the rate of thrombin inhibition (Figure 5b). Heparin cofactor II lacking the N-terminal region is unable to interact with the hirudin-binding site of thrombin, even in the presence of glycosaminoglycan, the resulting modest increase in reaction rate presumably being due to approximation of the two proteins bound to the glycosaminoglycan chain (Figure 5c). Oligosaccharides too short to simultaneously accommodate both proteins may nevertheless promote heparin cofactor II–thrombin complex formation (van Deerlin and Tollefsen, 1991; see also Bray et al., 1989) by displacing the acidic N-terminal region, which may then interact with thrombin (Figure 5d). Accordingly, deletion of the acidic region reduces thrombin inhibition to the basal rate in the presence of small oligosaccharides. Results in accord with these conclusions were obtained in studies on the inhibition of proteolytically modified thrombin by heparin cofactor II in the presence of heparin (Rogers et al., 1992). Ultimate confirmation of the model will require crystallization of heparin cofactor II and its complexes with saccharides and thrombin.

Protein C inhibitor

Activated protein C is a serine proteinase with anticoagulant properties (see below and Figures 1 and 6), not inhibitable by antithrombin (Suzuki et al., 1983). A 55 kDa inhibitor of protein Ca has been isolated from plasma (Marlar and Griffin, 1980; Canfield and Kisiel, 1982; Suzuki et al., 1983, 1984; Laurell and Stenflo, 1989). The rather low rate of protein C inactivation by this inhibitor was found to be increased ~30-fold in the presence

of heparin (Suzuki et al., 1984). A non-heparin dependent inhibitor of protein C was identified as α1-antitrypsin (Heeb and Griffin, 1988). Further, proteins with protein C inhibitory activity, unaffected by heparin, may be released from platelets (Fay and Owen, 1989; Jane et al., 1989). A novel heparin-dependent inhibitor of protein C, $M_r \sim 50000$, was isolated from human urine (Geiger et al., 1988) and found to be immunologically distinct from other known proteinase inhibitors, including antithrombin and plasminogen activator inhibitor-1. This inhibitor was considered to be the urinary counterpart of the previously described plasma protein C inhibitor (Marlar and Griffin, 1980; Marlar et al., 1982; Suzuki et al., 1983, 1984) and to be functionally related to urinary urokinase inhibitor (Stump et al., 1986) which also shows heparin-dependence. In the absence of heparin, the urinary inhibitor inhibits both protein C and urokinase at similar rates, whereas in the presence of heparin the inhibitory activity is preferentially increased toward protein C (Geiger et al., 1988). The heparin-dependent plasma and urinary protein C inhibitors were immunologically identical and, moreover, closely related to the plasminogen activator inhibitor-3 (Heeb et al., 1987).

The heparin-dependent protein C inhibitor/plasminogen activator inhibitor-3 belongs to the serpin family and shows some sequence similarity to other members of the family (Suzuki et al., 1987a). The effect of heparin is strongly dependent on charge interactions and can be mimicked by dextran sulphate, the inhibitory activity increasing with both the molecular size and the degree of sulphation of the polymer (Suzuki, 1985; Kazama et al., 1987). These observations are in accord with a 'template' model by which binding of inhibitor and target enzyme to the same glycan chain are required for efficient protein C inhibition.

A three-dimensional model for protein C inhibitor, based on the structural homology with α1-antitrypsin, implicated a two-helix motif in glycosaminoglycan binding (Kuhn et al., 1990). The N-terminal A+ helix along with the internal H helix expose altogether 12 positive charges, sufficient to accommodate an oligosaccharide of 8–10 residues [a corresponding saccharide sequence in heparin will contain 12–16 negative charges (Casu et al., 1988)]. Monoclonal antibodies directed against the positively charged N-terminal peptide sequence (amino acid residues 5–19) prevented the interaction between protein C inhibitor and heparin as well as the stimulatory effect of heparin on protein C inactivation by the inhibitor, in agreement with the postulated role of the A+ helix (Meijers et al., 1988). The model showed no positive surface associated with the D helix, which is implicated in heparin binding to antithrombin (Carrell et al., 1987). Recent findings by Pratt and Church (1992) suggest that the ability of glycosaminoglycans to accelerate proteinase inhibition by protein C inhibitor depends on the formation of ternary inhibitor–glycosaminoglycan–enzyme complexes.

Other proteinase inhibitors

Infusion of heparin *in vivo* releases an anticoagulant protein into the blood from binding sites, presumably involving glycosaminoglycan structures, at the endothelial cell surface (Sandset et al., 1988). This protein, currently known as tissue factor pathway inhibitor (formerly extrinsic pathway inhibitor or lipoprotein-associated coagulation inhibitor) is a multivalent proteinase inhibitor that directly inhibits factor Xa and, in a factor Xa-dependent fashion, the factor VIIa/tissue factor catalytic complex (Broze et al., 1988; see Figure 1). The different activities have been ascribed to two separate Kunitz-type proteinase inhibitor domains that are arranged in tandem along with a third, similar domain with unknown function (Wun et al., 1988; Girard et al., 1989). The inhibitor binds to heparin–agarose and heparin reportedly promotes the inhibition of factor Xa by tissue factor pathway inhibitor (Broze et al., 1990). The interaction between heparin and this inhibitor is not entirely elucidated, but appears to involve a basic sequence close to the C-terminus of the molecule. Studies of full-length and truncated forms of recombinant tissue factor pathway inhibitor indicate that the basic N-terminal sequence is essential for the inhibitory potency (Broze et al., 1992).

Protease nexin-1 is a 43 kDa proteinase inhibitor which shares ~30% sequence identity with antithrombin (MacGrogan et al., 1988). It inactivates certain serine proteinases, such as thrombin, plasmin and urokinase, by forming a complex with their catalytic site serine residues. The complexes bind back to the cells, via a receptor for the protease nexin-1 moiety, and are rapidly internalized and degraded. This process provides a mechanism for inhibiting and clearing proteinases from the extracellular environment (Low et al., 1981). Protease nexin-1 is synthesized and released by a variety of cultured cells including fibroblasts, smooth muscle cells, astrocytes and to a lesser extent, neurons (Baker et al., 1980; Laug et al., 1989; Rosenblatt et al., 1987; Wagner et al., 1991). Heparin binds to protease nexin-1 and increases the rate of thrombin inhibition by protease nexin-1 about 200-fold (Baker et al., 1980; Scott et al., 1985). Binding of protease nexin-1 to the extracellular matrix of fibroblasts, in particular to heparan sulphate chains, accelerates its reaction with thrombin, and at the same time modulates its target proteinase specificity such that it no longer inhibits urokinase or plasmin (Farrell and Cunningham, 1986, 1987; Farrell et al., 1988; Wagner et al., 1989; Cunningham et al., 1992). These observations suggest that thrombin is a likely physiological target of protease nexin-1 in the extracellular environment. Interestingly, protease nexin-1 shows neurite outgrowth-promoting activity, which has been ascribed to blocking of the thrombin-induced retraction of neurites (Gurwitz and Cunningham, 1988, 1990).

Physiological aspects

Although heparin displays potent anticoagulant properties, the extravascular location of the connective-tissue type mast cells that harbour this polysaccharide argues against a role as a natural anticoagulant/antithrombotic agent. Alternative functions proposed for anticoagulant mast-cell heparin include modulation of inflammatory reactions involving macrophage procoagulant activities (Lindahl et al., 1989). However, vascular glycosaminoglycans, primarily heparan sulphate, are believed to bind thrombin and antithrombin and to catalyse the antithrombin–thrombin reaction (Lollar and Owen, 1980; Busch and Owen, 1982; Marcum et al., 1984; Marcum and Rosenberg, 1985; Stern et al., 1985). Binding of thrombin and antithrombin to endothelial heparan sulphate would permit the control of haemostasis at the blood-cell interface where the coagulation enzymes are generated. According to Stern et al. (1985) heparan sulphate provides $\sim 50 \times 10^3$ antithrombin-binding sites per endothelial cell. As noted in the section on antithrombin, heparan sulphate derived from vascular endothelial cells has been shown to contain the specific antithrombin-binding region. On the other hand, the alleged catalytic effect of heparan sulphate on the antithrombin–thrombin reaction could not be confirmed in recirculating rabbit Langendorff heart preparations (Lollar et al., 1984). Moreover, attempts to localize the antithrombin-binding sites at the cell surface indicated that the high-affinity heparan sulphate proteoglycans expressed in cell culture, or

occurring in aorta, were preferentially facing the extracellular matrix compartment, thus not in direct contact with the blood (de Agostini et al., 1990). The anticoagulant/antithrombotic functions ascribed to heparan sulphate at the vascular cell surface remain to be conclusively established.

Despite the uncertainty concerning the details of the inhibitory process it seems clear that antithrombin has a major functional role in the normal control of haemostasis. Individuals with decreased levels of antithrombin in plasma are at risk of developing thrombosis (Egeberg, 1965). Other thrombin-inhibitory serpins, such as heparin cofactor II (Tollefsen et al., 1983; Church et al., 1991) or protease nexin-1 (Cunningham et al., 1992), are believed to have primarily extravascular functions, modulating the various activities (mitogenic, inflammatory, chemotactic, anti-neurite forming etc.) ascribed to thrombin in the extracellular matrix.

Interactions of intravascular glycosaminoglycans may influence the coagulation process in a number of ways. Formation of a ternary complex with fibrin and heparin (regardless of affinity for antithrombin) modulates the susceptibility of thrombin to antithrombin inhibition (Hogg and Jackson, 1990a,b). Fibrin monomers thus will protect thrombin from inhibition by antithrombin–heparin (Hogg and Jackson, 1989) but not from inhibition by heparin cofactor II in the presence of dermatan sulphate (Okwusidi et al., 1991). A number of protein ligands, including platelet factor 4, histidine-rich glycoprotein, vitronectin (S-protein) (Lane, 1989; Lane et al., 1984, 1986, 1987; Preissner and Jenne, 1991) and the enzymes lipoprotein lipase (Olivecrona and Bengtsson-Olivecrona, 1989) and extracellular superoxide dismutase C (Karlsson et al., 1988) may potentially sequester endothelial heparan sulphate from interaction with antithrombin. In fact, such blocking might explain the unexpected promoting effect of low-affinity heparin on the antithrombotic activity of heparin oligosaccharides having high affinity for antithrombin (Barrowcliffe et al., 1984); the low-affinity heparin would bind the endogenous protein ligands (except antithrombin) and thus clear the heparan sulphate chains. Heparin is also believed to release the tissue factor pathway inhibitor from endothelial cells (Sandset et al., 1988) and to promote its inhibition of the factor VII/tissue factor complex (see above). It has been proposed that tissue factor pathway inhibitor may account for a significant proportion of the anticoagulant/antithrombotic action of heparin *in vivo*, in spite of the fact that its concentration in plasma is only approximately one-thousandth that of antithrombin (Abildgaard, 1992). Finally, interactions of glycosaminoglycans that do not directly affect the actual coagulation process may instead modulate important associated systems. For instance, Ehrlich et al. (1991) found that heparin promotes the reaction between thrombin and plasminogen activator inhibitor 1, a serpin with regulatory function in the fibrinolytic pathway, thus leading to depletion of this inhibitor and increased fibrinolysis.

THROMBOMODULIN: A NOVEL PROTEOGLYCAN

Thrombomodulin is an integral membrane protein that provides high-affinity binding sites for thrombin at the luminal surface of the vascular endothelium (Esmon and Owen, 1981; Esmon et al., 1982a). Thrombomodulin forms a 1:1 molar complex with thrombin (Esmon et al., 1982b) which thus loses its procoagulant properties and instead acquires specific and complex anticoagulant activities (Figure 6). Thrombomodulin has a widespread distribution in the mammalian organism. It has been isolated from rabbit lung (Esmon et al., 1982b), bovine lung (Jakubowski et al., 1986; Suzuki et al., 1986), human placenta (Salem et al., 1984b; Kurosawa and Aoki, 1985) and human platelets (Suzuki et al., 1988). Immunochemical staining demonstrated the presence of thrombomodulin antigen on the vascular endothelium of both large and small vessels, including lymphatics, on syncytiotrophoblasts of human placenta (Maruyama et al., 1985a; DeBault et al., 1986), and in certain extravascular compartments (Boffa et al., 1987). Smooth muscle cells in culture were shown to express thrombomodulin (Soff et al., 1991). While initial reports (Maruyama et al., 1985a; Ishii et al., 1986; Wen et al., 1987) suggested that thrombomodulin is absent from brain, a more recent study provided immunochemical evidence for the occurrence of thrombomodulin in cryosections of brain capillaries (Wong et al., 1991).

Thrombomodulin is an acidic protein with an isoelectric point around 4 (human species; Kurosawa and Aoki, 1985). It consists of a single polypeptide chain, with an apparent M_r of (68–78) × 10^3 before reduction and (74–105) × 10^3 after reduction. It has a marked tendency to form multimers (Winnard et al., 1989), and is heavily glycosylated with both N- and O-linked sugar substituents. The structure of thrombomodulin (Figure 7) was elucidated by cloning of the (intron-less) genes for the human, bovine, and murine proteins (Jackman et al., 1986, 1987; Wen et al., 1987; Suzuki et al., 1987a,b; Dittman et al., 1988). An N-terminal domain with structural homology to animal lectins (Patthy, 1988) is followed by six tandem-organized regions homologous to epidermal growth factor (EGF repeats), a serine/threonine-rich domain, a transmembrane hydrophobic domain and a cytoplasmic tail. The M_r calculated from thrombomodulin cDNA sequences is ~60 × 10^3. The domain containing the EGF-like repeats appears to be the most conserved (~75% identity) between species. Indeed, this region was implicated as the 'template' for the activation of protein C (see below) (Kurosawa et al., 1987, 1988; Stearns et al., 1989; Suzuki et al., 1989; Zushi et al., 1989). The thrombin-binding site, located within the fifth and sixth EGF-like repeats, involves in particular the amino acid sequence (Glu-408–Glu-426) of the fifth repeat, whereas protein C binds to thrombomodulin through the third and fourth EGF-like repeats, with a specific Ca^{2+}-dependent binding site within the latter region (Hayashi et al., 1990). The minimal unit of thrombomodulin capable of binding thrombin and accelerating protein C activation appears to be comprised by the fourth, fifth and sixth EGF-like repeats.

Effects of thrombomodulin on specific functions of thrombin

Binding of thrombin to (rabbit) thrombomodulin results in profound changes in the mode of action of the enzyme (Figure 6), especially in relation to macromolecular substrates (Esmon et al., 1982a; Hofsteenge et al., 1986; Bourin et al., 1988). Thrombin bound to thrombomodulin no longer cleaves fibrinogen (this inhibitory action will in the following be referred to as the direct anticoagulant activity of thrombomodulin; reaction 2 in Figure 6), nor is it able to activate factor V or platelets (Esmon et al., 1982a, 1983; Murata et al., 1988). Instead, thrombomodulin dramatically (~20000-fold) accelerates the rate by which thrombin activates protein C (protein C activation cofactor activity; reaction 1 in Figure 6). Moreover, thrombomodulin accelerates the rate by which thrombin is inhibited by antithrombin (antithrombin-dependent anticoagulant activity; reaction 3 in Figure 6) (Bourin et al., 1986; Hofsteenge et al., 1986; Preissner et al., 1987).

Bourin et al. (1986) noted that a freshly isolated preparation of rabbit thrombomodulin, characterized by its protein C activation cofactor activity, bound to an anion-exchange resin and thus displayed acidic properties. During storage this preparation

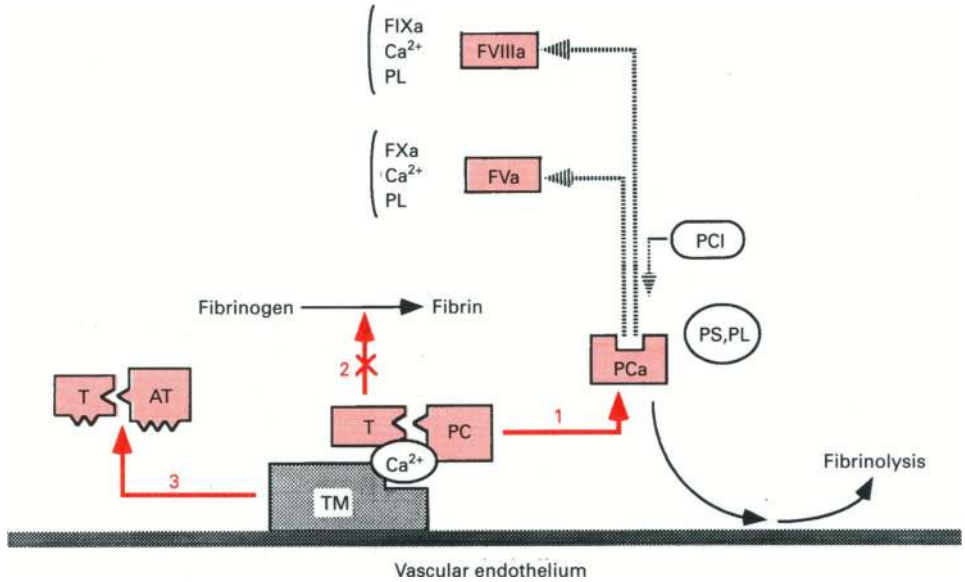

Figure 6 Anticoagulant pathways associated with thrombomodulin

Binding of thrombin (T) to thrombomodulin (TM) will influence the biological activities of thrombin, as indicated by arrows 1–3. (1) Acceleration of the activation of protein C by thrombin (referred to in the text as protein C activation cofactor activity of thrombomodulin). Activated protein C, a serine proteinase, specifically inactivates (broken arrows) the coagulation cofactors Va (involved in factor Xa-catalysed activation of prothrombin) and VIIIa (involved in factor IXa-catalysed activation of factor X). Inactivation of factors Va and VIIIa by activated protein C requires protein S (PS) and phospholipids (PL) as cofactors. According to Sakata et al. (1985) activated protein C may also promote fibrinolyis. (2) Inhibition of thrombin-catalysed cleavage of fibrinogen (direct anticoagulant activity of thrombomodulin). (3) Accelerated inactivation of thrombin by antithrombin (antithrombin-dependent anticoagulant activity of thrombomodulin).

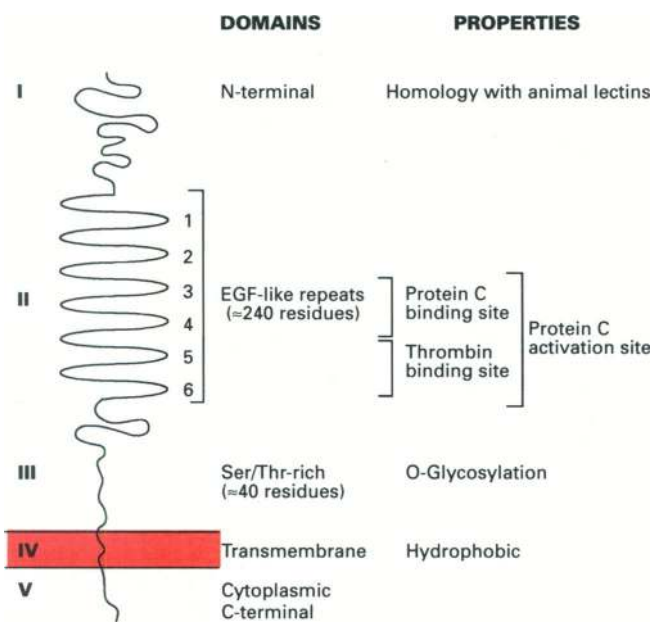

Figure 7 The molecular organization of thrombomodulin

The schematic representation is based on data relating to human thrombomodulin (Wen et al., 1987; Suzuki et al., 1987b). The molecule is composed of (I) an N-terminal domain, (II) a domain comprised of six EGF-like repeats, (III) a domain rich in serine and threonine residues, (IV) a trans-membrane hydrophobic region, and (V) a cytoplasmic C-terminal domain. The EGF-like regions 3–4 and 5–6 provide the major binding sites for protein C and thrombin, respectively.

gradually lost its acidic properties, apparently due to proteolytic cleavage. While the acidic form of thrombomodulin expressed all the three anticoagulant activities indicated above, the resulting non-acidic form showed protein C activation cofactor activity but no direct nor antithrombin-dependent anticoagulant activity. The two latter activities were tentatively ascribed to the presence of an acidic domain, containing a 'heparin-like' component, separated from the protein C activation site of thrombomodulin by a proteinase-sensitive region (see Figure 8). Before discussing the properties of this acidic domain the characteristics of the various biological activities 1–3 will be outlined.

Protein C activation cofactor activity

Protein C is a plasma protein with vitamin K-dependent anticoagulant properties that are expressed following activation by limited proteolytic cleavage, catalysed by thrombin. Activated protein C, a serine proteinase, inactivates the auxiliary coagulation proteins, factors Va and VIIIa, hence preventing the generation of factor Xa and thrombin (Stenflo, 1976; Kisiel, 1979; Kisiel et al., 1977; Walker et al., 1979; Dahlbäck and Stenflo, 1980; Owen and Esmon, 1981; Stenflo and Fernlund, 1982; Marlar et al., 1982; Esmon, 1987). Activated protein C requires protein S, another vitamin K-dependent protein, as a cofactor in order to inactivate factors Va and VIIIa (Walker, 1980; Walker et al., 1987). The anticoagulant protein C pathway is outlined in Figure 6. A crucial physiological role of this pathway is indicated by the strong correlation between congenital deficiencies of protein C or protein S and recurring thromboembolic episodes (High, 1988). Thrombomodulin has a key role in promoting the activation of protein C by thrombin. Accordingly, injection of recombinant human thrombomodulin into mice was found to prevent thrombin-induced thrombo-

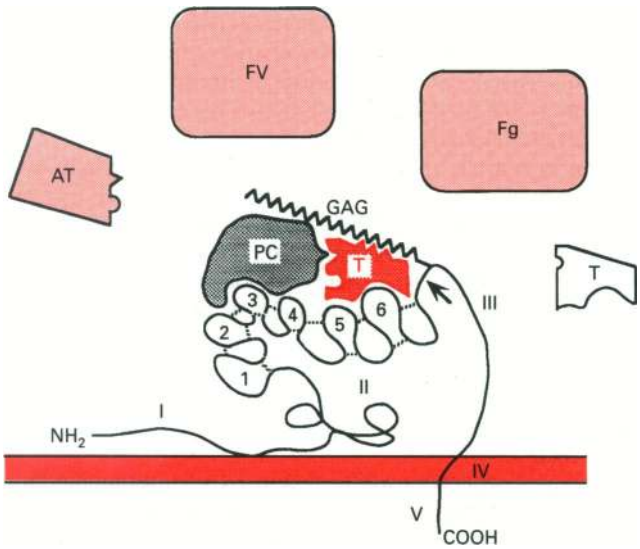

Figure 8 Model of the rabbit thrombomodulin proteoglycan

The depicted mode of interaction of thrombomodulin with the endothelial cell surface shows the thrombin molecule to be bound 'on top of' thrombomodulin (Lu et al., 1989). The EGF-like structures are stabilized by intrachain disulphide bridges that appear to be essential for expression of all the various anticoagulant activities of thrombomodulin. The glycosaminoglycan (GAG) chain is shown to be inserted in domain III which provides potential O-glycosylation sites (Figure 7; see also Figure 9 for identification of potential glycosaminoglycan-attachment sites). The arrow indicates a site of proteolytic cleavage that will generate a nonacidic form of thrombomodulin (Bourin et al., 1986). The model is intended to illustrate that the polysaccharide component of thrombomodulin is not essential for the activation of protein C (PC) by thrombin, whereas it is required to promote the inhibition of thrombin (T) by antithrombin (AT) and to prevent the cleavage of fibrinogen (Fg) as well as the activation of factor V (FV) by thrombin. Binding of thrombin to thrombomodulin will induce a conformational change in the catalytic centre of the enzyme (Musci et al., 1988) and unmask a site capable of interaction with the glycosaminoglycan chain. This secondary interaction with the glycosaminoglycan chain in turn induces a further conformational change of the thrombin molecule, which facilitates its reaction with antithrombin. For further information see the text. (Modified from Bourin and Lindahl, 1990.)

embolism (Gomi et al., 1990). No clinical condition associated with thrombomodulin deficiency has yet been described.

Direct anticoagulant activity

The major, direct procoagulant function of thrombin is to catalyse the cleavage of fibrinogen to form the fibrin clot. Addition of rabbit thrombomodulin to thrombin, in amounts equal to or slightly exceeding molar equivalency, was found to eliminate the clotting ability of thrombin (Esmon et al., 1982a). Thrombomodulin isolated from other species varied with regard to direct anticoagulant activity (Jakubowski et al., 1986; Maruyama et al., 1985). As mentioned above, this activity was associated with the occurrence of an acidic domain in the thrombomodulin molecule. Lack of activity thus could be due to the absence of such a component, possibly a result of proteolytic cleavage (see above), or to the presence of a 'neutralizing' basic protein (see below).

Antithrombin-dependent anticoagulant activity

Rabbit thrombomodulin induced a 4–8-fold acceleration of the rate of inhibition of thrombin by antithrombin (Bourin et al., 1986; Hofsteenge et al., 1986; Preissner et al., 1987). The process was saturable with regard to thrombomodulin (Hofsteenge et al., 1986; Bourin and Lindahl, 1990), irrespective of antithrombin concentration, the maximal rate of thrombin inhibition being achieved at a molar ratio of thrombomodulin/thrombin ≈ 1. Similar conditions were required to completely inhibit the thrombin-catalysed cleavage of fibrinogen (Esmon et al., 1982a) and to maximally stimulate protein C activation (Esmon et al., 1982b). Once inactivated, the bound thrombin is released from thrombomodulin, which is then ready to accommodate another thrombin molecule (Bourin et al., 1988). Albeit less efficient than heparin, thrombomodulin thus acts as a catalyst for the inhibition of thrombin by antithrombin. Hirahara et al. (1990) described the formation of complexes between either rabbit or human thrombomodulin and antithrombin. On the other hand, thrombomodulin failed to bind to an antithrombin–Sepharose matrix (Hofsteenge et al., 1986; Preissner et al., 1987).

Contrary to rabbit thrombomodulin, neither bovine lung thrombomodulin (Jakubowski et al., 1986; Suzuki et al., 1986) nor human placenta thrombomodulin (Hirahara et al., 1990) were found to accelerate the inhibition of thrombin by antithrombin. However, Preissner et al. (1990) observed that preparations of human thrombomodulin were contaminated by vitronectin (S-protein), one of the so-called heparin-neutralizing proteins that would be likely to interfere with any glycosaminoglycan-dependent phenomenon such as the antithrombin-dependent anticoagulant activity (see below). Another seemingly contradictory feature was noted in studies on the effects of exogenous heparin on the thrombomodulin–antithrombin–thrombin system (Bourin, 1989). Whereas thrombomodulin promoted the inactivation of thrombin by antithrombin, it protected the bound thrombin against the rapid reaction with antithrombin normally induced by exogenous heparin.

The effects of thrombomodulin on the inactivation of thrombin by heparin cofactor II were again inconsistent. Rabbit thrombomodulin did not accelerate the reaction but, in fact, protected the thrombin from inactivation by heparin cofactor II (Koyama et al., 1991). Human recombinant thrombomodulin, on the other hand, promoted both the antithrombin–thrombin and the heparin cofactor II–thrombin reactions (Koyama et al., 1991). Moreover, the recombinant thrombomodulin protected thrombin against fast inhibition by heparin–antithrombin as well as by dermatan sulphate–heparin cofactor II complexes (Koyama et al., 1991).

The thrombomodulin proteoglycan

Functional domains of thrombomodulin

Experiments to be described below indicate that the acidic properties associated with some of the modulatory activities of thrombomodulin are due to a (presumably single) chondroitin sulphate (or dermatan sulphate) chain. The mode of interaction of this glycosaminoglycan component with thrombomodulin-bound thrombin is depicted by the model shown in Figure 8, which suggests that the diverse activities of thrombomodulin all relate to the same thrombin molecule, bound at the protein C activation site (Bourin et al., 1988). This proposal was based in particular on the effects of monoclonal antibodies that were found to abrogate the protein C activation cofactor activity, and at the same time reverse the antithrombin-dependent as well as the direct anticoagulant activities. Binding of the glycosaminoglycan chain to the thrombin appeared to be prerequisite to the two latter activities, since these were eliminated in the presence of a synthetic polyamine, Polybrene (Bourin et al., 1986), or of proteins such as platelet factor 4 and S-protein (vitronectin)

Figure 9 Potential glycosaminoglycan-attachment sites in thrombomodulin from various species

Alignment of amino acid (represented by the one-letter code) sequences from domain III of thrombomodulin from different species, as deduced from the corresponding cDNA structures. The -Ser-Gly- sequences representing potential glycosaminoglycan-attachment sites are shown in red. Ser-490 (marked by an asterisk) in human thrombomodulin is part of the 'conventional' -Ser-Gly-Xaa-Gly- sequence implicated as recognition structure for the xylosyltransferase that initiates formation of glycosaminoglycan chains (Bourdon et al., 1987). Alternatively, Ser-492 (arrow) which occurs within the conserved -Gly-Ser-Gly-Glu- sequence might provide a glycosaminoglycan-attachment site common to the three different thrombomodulin species. The numbering of the amino acid residues 481–516 of human thrombomodulin is according to Wen et al. (1987). The sequence alignments are according to Suzuki et al. (1987b) for human and bovine thrombomodulin and according to Dittman and Majerus (1989) for human and murine thrombomodulin.

which are known to interact with glycosaminoglycan chains (Bourin et al., 1988; see also Preissner et al., 1990; Koyama et al., 1991). None of these compounds had any apparent effect on the protein C activation cofactor activity. These findings are in accord with the initial observation (see above) of an acidic form of thrombomodulin which displayed all three different anticoagulant activities and a nonacidic form that promoted protein C activation but lacked the antithrombin-dependent and direct anticoagulant activities (Bourin et al., 1986). Moreover, nonacidic fragments of rabbit thrombomodulin generated by proteolysis or cyanogen bromide treatment showed protein C activation cofactor activity but only minimal direct anticoagulant activity (Kurosawa et al., 1987; Stearns et al., 1989). These fragments contained region II, composed of the EGF-like repeats, but lacked region III which contains potential O-glycosylation sites (see Figures 7 and 8).

Characterization of the glycosaminoglycan component

Conclusive evidence as to the nature of the acidic domain of rabbit thrombomodulin was obtained by digestion with the bacterial eliminase, chondroitinase ABC (Bourin, 1989; Bourin et al., 1988; Bourin and Lindahl, 1990). Such treatment reduced the apparent M_r of unreduced thrombomodulin on SDS/PAGE from ~90000 to ~74000, along with loss of the direct and antithrombin-dependent anticoagulant activities (see below). A total of ~180 μg of polysaccharide was recovered by ion-exchange chromatography, following release by alkaline β-elimination from 6 mg of rabbit thrombomodulin (Bourin et al., 1990). The isolated product was subjected to partial N-deacetylation by hydrazinolysis and was then re-N-[^{3}H]acetylated by treatment with [^{3}H]acetic anhydride. The resulting ^{3}H-labelled product showed an M_r of (10–12) $\times 10^3$ on gel chromatography and was susceptible to digestion by chondroitinase ABC or AC and by testicular hyaluronidase (Bourin et al., 1990). Analysis of digestion products by use of the radiolabel enabled a fairly detailed structural characterization of the molecule. Most of the internal region of the glycosaminoglycan chain consisted of monosulphated disaccharide units, the sulphate groups being located at C-4 or C-6 of the *N*-acetylgalactosamine residues. By contrast, the nonreducing end of the chain showed an unusual structure, with a terminal tetrasulphated [GalNAc(4,6-di-OSO_3)-GlcA-GalNAc(4,6-di-OSO_3)-] trisaccharide sequence (Bourin et al., 1990). This local accumulation of negative charges may contribute to the functional properties of the thrombomodulin-bound glycosaminoglycan component.

The proteoglycan nature of rabbit thrombomodulin was confirmed by the isolation of ^{35}S-labelled thrombomodulin from rabbit heart endothelial cells that had been cultured in the presence of $Na_2{}^{35}SO_4$ (Bourin et al., 1990). Chondroitinase digestion indicated that virtually all of the label had been incorporated into a galactosaminoglycan chain, composed of essentially 4/6-monosulphated disaccharide units and about 8% disulphated disaccharide units. Analysis of the glycosaminoglycan component of human recombinant thrombomodulin indicated mainly 4-mono-O-sulphated disaccharide units (Nawa et al., 1990).

Amino-acid sequences within region III of thrombomodulin from different species were compared with regard to potential glycosaminoglycan attachment sites (Figure 9). The tetrapeptide structure -Ser-Gly-Xaa-Gly- (where Xaa may be any amino acid), previously postulated to constitute a consensus sequence for glycosaminoglycan attachment (Bourdon et al., 1987), was identified within this domain (residues 490–493), Ser-490 providing a likely potential acceptor site for the xylosyltransferase. However, this sequence was not present in bovine or murine thrombomodulin. Alternatively, -Ser-Gly- structures adjacent to acidic amino acid residues may be substituted by glycosaminoglycan chains (Rodén et al., 1985; see also Fransson, 1989; Gallagher, 1989). This requirement is fulfilled by the sequence -Gly-Ser-Gly-Glu-, residues 491–494 in human thrombomodulin, which is conserved in all three thrombomodulin species. The constituent serine unit (residue 492 in human thrombomodulin) thus may provide a glycosaminoglycan attachment site that is common to all three thrombomodulin species (see also Parkinson et al., 1992a).

Functional role of the glycosaminoglycan component

Digestion of rabbit thrombomodulin with chondroitinase ABC had no apparent effect on the protein C activation cofactor activity (Figure 10a; however, see recombinant thrombomodulin below) but virtually eliminated both the direct (Figure 10b) and the antithrombin-dependent (Figure 10c) anticoagulant activities (Bourin, 1989; Bourin et al., 1988; Bourin and Lindahl, 1990; Preissner et al., 1990). Moreover, chondroitinase-digested thrombomodulin had lost the ability of the native compound (Esmon et al., 1982a) to prevent the activation of factor V by thrombin (Bourin and Lindahl, 1990). Similarly, the glycosaminoglycan component of thrombomodulin was implicated in the inhibition of other thrombin-dependent phenomena, including activation of platelets and endothelial cells (Parkinson et al., 1991a,b). Finally, the presence or absence of the glycosaminoglycan component was found to profoundly influence the effects of added polysaccharides (Bourin, 1989; Koyama et al., 1991). In contrast to accelerating the slow thrombin inhibition by antithrombin and heparin cofactor II in the absence of exogenous glycosaminoglycans, the chondroitin (dermatan) sulphate component of thrombomodulin was found to prevent the rapid thrombin inhibition by antithrombin in the presence of added heparin and by heparin cofactor II in the presence of dermatan sulphate. Presumably, the interaction between the thrombomodulin-bound glycosaminoglycan chain and thrombin will preclude formation of the ternary serpin–glycosaminoglycan–thrombin complexes that are required to mediate the effects of

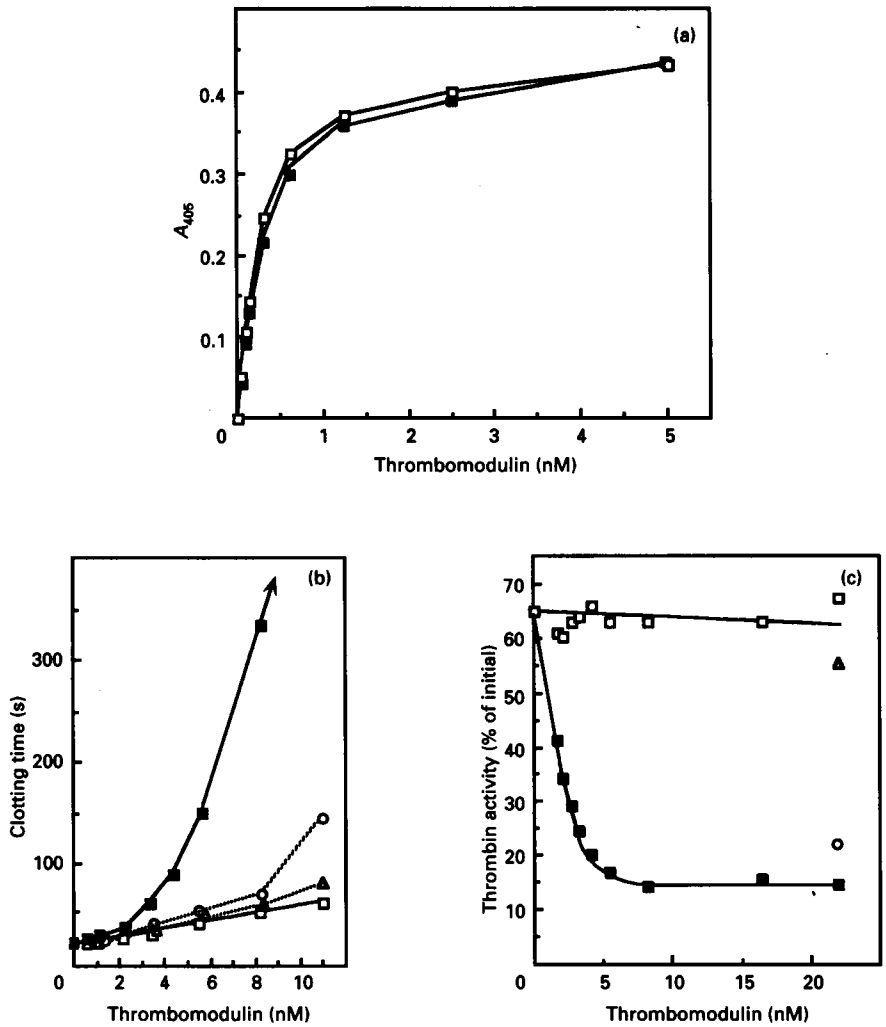

Figure 10 Effects of glycosaminoglycan-degrading enzymes on the various anticoagulant activities of rabbit thrombomodulin

Thrombomodulin was analysed for (**a**) protein C activation cofactor activity (determination of activated protein C formed, as measured using a chromogenic substrate), (**b**) direct anticoagulant activity (ability to prolong the clotting of fibrinogen; the arrow indicates that samples remained non-coagulable for >10 min); (**c**) antithrombin-dependent anticoagulant activity (determination of residual thrombin activity following incubation with antithrombin). The thrombomodulin was tested at the concentrations indicated, either in native form (■; thrombomodulin incubated with chondroitinase buffer but without enzyme), or after digestion with chondroitinase ABC (□), chondroitinase AC (○) or testicular hyaluronidase (△). The less pronounced effect of chondroitinase AC, as compared with chondroitinase ABC, might suggest the occurrence of some IdoA (in addition to GlcA) units, but could also reflect the more pronounced exo-enzyme character of the former enzyme (Fransson, 1985). (From Bourin and Lindahl, 1990.)

the exogenous glycosaminoglycans (Bourin, 1989; Parkinson et al., 1992b).

More detailed information regarding the influence of the thrombomodulin-bound chondroitin sulphate chain on thrombin function was obtained through studies of recombinant thrombomodulin (Parkinson et al., 1990a, 1992a,b; Nawa et al., 1990). Distinct soluble 'glycoforms' of deletion mutants of recombinant human thrombomodulin were found to differ with regard to the presence or absence of the single glycosaminoglycan chain. The major anticoagulant activities of the two forms differed as predicted from previous studies on native and chondroitinase-digested rabbit thrombomodulin; moreover, the functional properties of the glycosaminoglycan-substituted form could be converted into those of the unsubstituted form by such digestion. In addition, the two forms differed significantly with regard to affinity for thrombin, maximal rates of protein C activation by the thrombin–thrombomodulin complex and optimal Ca^{2+} ion concentration for protein C activation (Parkinson et al., 1990a, 1992a,b). The glycosylation state of thrombomodulin *in vivo*, particularly with regard to the occurrence of chondroitin sulphate, is thus likely to have profound functional effects. In accord with this view, recent studies with cultured human and bovine endothelial cells showed altered thrombin affinity and kinetics of protein C activation following treatment of the cells with a β-D-xyloside, known to inhibit glycosaminoglycan initiation on proteoglycan core proteins (Parkinson et al., 1990b). Further studies are required to define the mechanisms that control the posttranslational modifications in thrombomodulin biosynthesis, in particular the formation and elaboration of glycosaminoglycan structures.

The studies outlined above suggest that the properties of thrombomodulin as a modulator of thrombin function rely heavily on its ability to efficiently compete with various ligands, such as fibrinogen and certain thrombin receptors, for binding to thrombin (Figure 8). A glycosaminoglycan chain in the O-glycosylation region appears to be critically involved in the

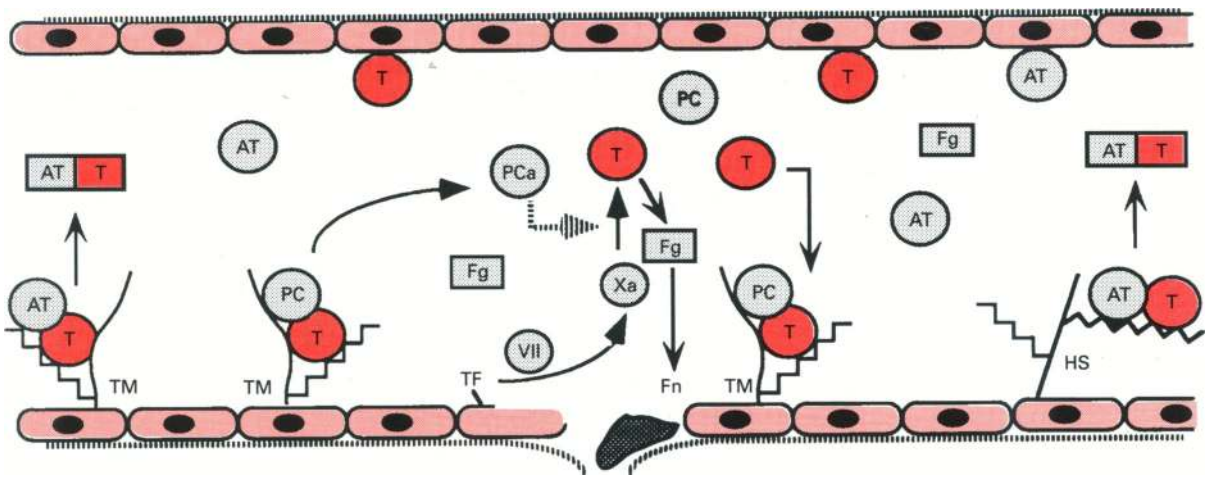

Figure 11 Regulation of blood coagulation at the vascular endothelial surface

Damage to the endothelial surface (exposure of subendothelial structures) leads to expression of tissue factor (TF), activation of the coagulation cascade and finally to fibrin (Fn) deposition. Mechanisms are activated to ensure that clot formation be restricted to the area of vascular damage. Surplus thrombin (T) is either directly inactivated by antithrombin (AT) bound to heparan sulphate (HS) proteoglycans or bound to thrombomodulin (TM), another endothelial proteoglycan. Due to the shielding effect of the constituent glycosaminoglycan chain, the thrombomodulin-bound thrombin is unable to clot fibrinogen (Fg) and to amplify coagulation by positive feed-back mechanisms. Instead, it activates the anticoagulant protein C (PC) pathway, thus leading to down-regulation of the coagulation cascade. The glycosaminoglycan component of thrombomodulin also promotes the continuous inactivation of bound thrombin, which is subsequently released in complex with antithrombin, and replaced by new thrombin molecules. This mechanism will ensure that activation of protein C is readily terminated once the excess thrombin has been cleared.

inhibition of the interactions of thrombin with procoagulant substrates and cellular receptors. The precise mechanism behind these effects is still unclear. A number of thrombin ligands, including thrombomodulin, fibrinogen (Wu et al., 1991), hirudin (Hofsteenge and Stone, 1987; Rydel et al., 1990) and the platelet/endothelial cell receptor for thrombin (Vu et al., 1991) seem to interact with a positively charged surface region located some distance from the thrombin active site, the so-called anion-binding exosite of the proteinase (see Fenton, 1986). Parkinson et al. (1992b) proposed that the acidic glycosaminoglycan component of thrombomodulin interferes with these interactions by binding to and thus blocking the exosite. While this possibility appears attractive, there are reports suggesting that other (EGF-like peptide) regions of the thrombomodulin molecule may bind to the thrombin exosite (see e.g. Hortin and Benutto, 1990). On the other hand, thrombin apparently contains more than one such site (Rogers et al., 1992). The precise nature of the glycosaminoglycan–thrombin interaction remains to be defined. If, in fact, such interaction serves to increase the overall affinity of thrombin for thrombomodulin then some of the anticoagulant effects ascribed to the glycosaminoglycan component could simply be due to shifting the equilibrium of free versus thrombomodulin-bound thrombin.

The complex interactions between thrombomodulin and thrombin become particularly intriguing in relation to the antithrombin-dependent anticoagulant activity. This activity depends on the presence of the thrombomodulin-bound glycosaminoglycan chain. The isolated labelled polysaccharide showed no appreciable affinity for either immobilized thrombin nor antithrombin (Bourin et al., 1990). Although it expressed weak antithrombin-dependent thrombin inhibition, approximately equal to that observed with the 'oversulphated' chondroitin sulphate-E (see Scully et al., 1986), this activity was about 20-fold lower, on a molar basis, than that of the corresponding thrombomodulin-bound glycosaminoglycan chain (Bourin et al., 1990). Possible reasons for this discrepancy have been considered in constructing the model shown in Figure 8. It is proposed that thrombin first binds to the core protein of the thrombomodulin proteoglycan. The resulting conformational change of the thrombin provides for efficient protein C activation, but also promotes binding of the glycosaminoglycan chain to the thrombin molecule (possibly at an anion-binding exosite; see above). The dual effects of binding to both the protein and the glycosaminoglycan components induces a further conformational change of the thrombin which thereby becomes more amenable to inhibition by antithrombin (see Figure 8). The antithrombin-dependent anticoagulant activity of thrombomodulin seems to present an unprecedented case of functional co-operation between the carbohydrate and protein moieties of a glycoconjugate.

AN INTEGRATED VIEW

An integrated view on the role(s) of glycosaminoglycans in the regulation of blood coagulation is presented in Figure 11. By necessity, the concepts are simplified and a number of potentially significant aspects have been deliberately ignored. The scheme is focused on the major roles of endogenous, intravascular proteoglycans and does not account for effects of exogenous glycosaminoglycans such as heparin or dermatan sulphate, used as drugs. Moreover, heparin cofactor II is considered to have mainly an extravascular function and thus will not be considered.

Efficient inhibition of a 'cascade' mechanism, such as that involved in blood coagulation, should theoretically aim for 'upstream' targets early in the sequence. In fact, however, inhibition of thrombin, the last proteinase to be activated in the process, appears to be most critical for control of haemostasis (Ofosu et al., 1985, 1987, 1990; Béguin et al., 1988). The probable reason for this seeming anomaly is that thrombin, in addition to clotting fibrinogen, also catalyses two reactions that lead to amplification of the coagulation cascade, i.e. activation of factors V and VIII (Figure 1). In addition, Gailani and Broze (1991) recently proposed that thrombin is a major activator of factor XI (Figure 1), thus initiating the intrinsic pathway of coagulation. Suppression of these thrombin-dependent amplification reactions

delays prothrombin activation and is believed to explain, in large part, the antithrombotic effect of heparin (Ofosu et al., 1989).

The occurrence at the endothelial cell surface of proteoglycans containing heparan sulphate chains with high affinity for antithrombin (see Kojima et al., 1992a,b) provides a means of inactivating not only thrombin, but also the other serine proteinases of the coagulation cascade, by complex formation with antithrombin (Figure 11). The potential of this system depends in particular on the regulation, still poorly understood (Kusche et al., 1990; Rosenberg and de Agostini, 1992), of the biosynthetic machinery involved in generating the specific antithrombin-binding pentasaccharide sequence. Furthermore, preferential inactivation of certain proteinase species may conceivably be achieved by modulating the size and charge properties of the individual heparan sulphate chains (see above).

A more complex mechanism, also dependent on glycosaminoglycan involvement, for inhibition of coagulation is utilized by the thrombomodulin system (Figure 11), which is specifically designed to modulate thrombin function. Once bound to thrombomodulin, virtually all known procoagulant functions of thrombin are switched off, the catalytic power of the enzyme instead being channelled into generation of the anticoagulant protein Ca. Most of the thrombin formed, at least in the microcirculation, is believed to be bound to thrombomodulin (Busch and Owen, 1982) and McIntosh and Owen (1987) proposed that complex formation of thrombin with thrombomodulin represents the major anticoagulant mechanism normally in operation at the intact endothelial surface (see Figure 11). The chondroitin sulphate component of thrombomodulin contributes to the anticoagulant function by shielding thrombin from procoagulant interactions with various macromolecular substrates (see detailed discussion above). In addition, given the large proportion of thrombomodulin-bound thrombin, inactivation of such thrombin molecules by antithrombin, promoted by the chondroitin sulphate chain (Figure 11), may well constitute the functionally predominant pathway for elimination of thrombin from the circulation. On the other hand, this effect of the glycosaminoglycan chain may seem paradoxical, since it will also terminate the anticoagulant action (protein C activation) of the target thrombin. However, once inactivated, the thrombin, complexed with antithrombin, will be released from the thrombomodulin and give way for another thrombin molecule (Bourin et al., 1988). The functional purpose of this process conceivably relates to the overall regulation of thrombomodulin action. Activation of protein C should continue only as long as excess thrombin is being generated. The antithrombin-dependent anticoagulant action thus may be perceived as a mechanism to ascertain that protein C activation is terminated without undue delay.

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