Functional role of the polysaccharide component of rabbit thrombomodulin proteoglycan

Effects on inactivation of thrombin by antithrombin, cleavage of fibrinogen by thrombin and thrombin-catalysed activation of Factor V

Marie-Claude BOURIN and Ulf LINDAHL*

Department of Veterinary Medical Chemistry, The Swedish University of Agricultural Sciences, The Biomedical Center, S-751 23 Uppsala, Sweden

Thrombomodulin (TM), a major anticoagulant protein at the vessel wall, serves as a potent cofactor for the activation of Protein C by thrombin. Previous work has indicated that (rabbit) TM is a proteoglycan that contains a single polysaccharide chain, tentatively identified as a sulphated galactosaminoglycan, and furthermore suggested that this component may be functionally related to additional anticoagulant activities expressed by the TM molecule [Bourin, Öhlin, Lane, Stenflo & Lindahl (1988) *J. Biol. Chem.* **263**, 8044–8052]. Results of the present study establish that (enzymic) removal of the polysaccharide chain abolishes the inhibitory effect of TM on thrombin-induced fibrinogen clotting as well as the promoting effect of TM on the inactivation of thrombin by antithrombin, but does not affect the ability of TM to serve as a cofactor in the activation of Protein C. Studies of yet another biological activity of rabbit TM, namely the ability to prevent the activation of Factor V by thrombin [Esmon, Esmon & Harris (1982) *J. Biol. Chem.* **257**, 7944–7947], confirmed that TM markedly delays the conversion of the native 330 kDa Factor V precursor into polypeptide intermediates, and further into the 96 kDa heavy chain and 71–74 kDa light-chain components of activated Factor Va. In contrast, the activation kinetics of a similar sample of Factor V incubated with thrombin in the presence of chondroitinase ABC-digested TM did not differ from that observed in the absence of TM. It is concluded that the inhibitory effect of TM on Factor V activation also depends on the presence of the polysaccharide component on the TM molecule.

INTRODUCTION

Thrombomodulin (TM) is an endothelial cell-surface protein with high affinity for thrombin and important regulatory functions in haemostasis (for review see Esmon, 1989). Free thrombin converts fibrinogen into fibrin, but also has other procoagulant effects, such as activation of platelets and of the coagulation Factors V, VIII and XIII. Upon binding to TM, thrombin loses its ability to activate platelets (Esmon *et al.*, 1983), Factor V (C. T. Esmon *et al.*, 1982) and Factor XIII (Polgar *et al.*, 1986), and furthermore no longer induces clotting of fibrinogen (C. T. Esmon *et al.*, 1982). Instead, TM-bound thrombin shows potent anticoagulant properties, specifically expressed in the activation of Protein C (Owen & Esmon, 1981), a vitamin K-dependent serine proteinase (Stenflo, 1976). Activated Protein C in turn inactivates Factors Va and VIIIa by limited proteolysis, and thus serves to regulate thrombin formation through negative-feedback mechanisms. Finally, rabbit TM was found to promote the inactivation of thrombin by antithrombin (Bourin *et al.*, 1986; Hofsteenge *et al.*, 1986; Preissner *et al.*, 1987).

Studies on the structure–function relationships of rabbit TM revealed the presence of a strongly acidic domain, initially believed to comprise a heparin-like polysaccharide but later identified as a sulphated galactosaminoglycan (Bourin *et al.*, 1986, 1988). The (presumably single) polysaccharide chain had no detectable influence on the activation of Protein C, but appeared to be essential for the ability of TM to accelerate the inactivation of thrombin by antithrombin (Bourin *et al.*, 1988;

Bourin, 1989). The present investigation extends these studies and defines the role of the polysaccharide component in relation to two additional anticoagulant properties of TM, namely the inhibitory effects on thrombin-induced fibrinogen clotting and on the activation of Factor V by thrombin.

MATERIALS AND METHODS

Reagents

Thrombomodulin was purified from frozen rabbit lungs as described previously (Bourin *et al.*, 1988). Briefly, the procedure involved an initial ion-exchange chromatography of tissue extract (Salem *et al.*, 1984), two consecutive affinity chromatographies on immobilized di-isopropylphosphorothrombin (N. L. Esmon *et al.*, 1982), a second ion-exchange chromatography at low pH (Bourin *et al.*, 1986) and finally immunoaffinity chromatography using monoclonal antibodies raised against rabbit TM (Bourin *et al.*, 1988). Bovine Protein C (Stenflo, 1976) was a gift from Dr. J. Stenflo (Department of Clinical Chemistry, Malmö General Hospital, Malmö, Sweden). Bovine thrombin (2900 units/mg) purified by the method of Lundblad *et al.* (1975) and bovine antithrombin purified by the method of Miller-Andersson *et al.* (1974) were given by Dr. I. Björk of this department. Purified bovine Factor V (Dahlbäck, 1980) was provided by Dr. B. Dahlbäck (Department of Clinical Chemistry, Malmö General Hospital, Malmö, Sweden). The purified protein (6 mg/ml) was stored at -20°C in 50 mM-Tris/HCl/0.1 M-NH₄Cl/10 mM-CaCl₂/10 mM-benzamidine/50 % (v/v) glycerol, pH 7.5. Bac-

Abbreviations used: TM, thrombomodulin; EGF, epidermal growth factor.

* To whom correspondence should be addressed.

terial chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4), chondroitinase AC (chondroitin AC lyase, EC 4.2.2.5) and bovine testicular hyaluronidase (EC 3.2.1.35) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Digestion of TM with galactosaminoglycan-degrading enzymes

The polysaccharide component of TM was eliminated by digestion with bacterial eliminases in 50 mM-Tris/HCl/0.1 M-NaCl/30 μ M-sodium acetate/0.2% Nonidet P40, pH 8.0, in the presence of proteinase inhibitors [2.5 mM-*o*-phenanthroline (Merck, Darmstadt, Germany), 10 μ g of pepstatin/ml and 20 μ g of leupeptin/ml (Calbiochem, Behring Diagnostics, La Jolla, CA, U.S.A.), and 1 mM-benzamidine (Sigma Chemical Co.)]. Digests containing, per ml of incubation mixture, 8 μ g of TM and either 20 munits of chondroitinase ABC or 100 munits of chondroitinase AC were incubated overnight at 37 °C. For some experiments (see the legends to Figures) the conditions were modified such that 30 μ g of TM per ml of buffer was incubated with 50 munits of chondroitinase ABC for 1 h. Under these incubation conditions no contaminating proteinase activity was detectable in the chondroitinase preparation (see Bourin, 1989). Alternatively, degradation of the polysaccharide component of TM was achieved by digestion with testicular hyaluronidase. Samples containing 8 μ g of TM and 20 units of hyaluronidase per ml of 0.15 M-sodium acetate/0.2 M-NaCl, pH 5.5, were incubated overnight at 37 °C. Control incubations, with enzymes omitted, were performed in parallel. All incubations were terminated by heating at 90 °C for 1 min. Samples of the appropriately diluted incubation mixtures were used in various experiments to provide the indicated amounts of native or polysaccharide-depleted TM.

Anticoagulant activities of TM

The effects of TM on the activation of Protein C by thrombin (referred to as the Protein C activation cofactor activity), on the inactivation of thrombin by antithrombin (antithrombin-dependent anticoagulant activity) and on the thrombin-induced coagulation of fibrinogen (direct anticoagulant activity) were measured as described in detail previously (Bourin *et al.*, 1988). Briefly, the Protein C activation cofactor activity of TM was assessed by determining the rate of hydrolysis of the chromogenic substrate S-2266, catalysed by Protein Ca that had been generated during previous incubation of Protein C with thrombin in the presence of TM. The antithrombin-dependent anticoagulant activity was determined from the decrease in thrombin activity (measured by the hydrolysis of the chromogenic substrate S-2238) following incubation of thrombin with antithrombin and TM. The direct anticoagulant activity of TM was indicated by the prolongation of clotting time, recorded by a thrombometer, induced by adding TM to a mixture of thrombin and fibrinogen. For additional experimental details see the legend to Fig. 1.

The effect of TM on the activation of Factor V by thrombin was studied by using gel electrophoresis to identify the various polypeptide products generated in the process. Factor V (250 μ g, final concn. 3 μ M) was incubated in 250 μ l of 50 mM-Tris/HCl/0.1 M-NaCl/2 mM-CaCl₂, pH 7.4, with 0.1 μ g of thrombin (11 nM) and 1 μ g of TM (40 nM). The thrombin was preincubated with either native or chondroitinase-digested TM for 1 min at 37 °C before the addition of Factor V. Control incubations lacked TM altogether. After various periods of incubation at 37 °C 25 μ l portions were withdrawn, immediately transferred to test tubes containing 50 μ l of 2% SDS/0.3% dithiothreitol and heated at 90 °C for 5 min. The incubations were analysed by SDS/PAGE on slab gels with the dimensions 1 mm $\times$ 25 cm $\times$ 30 cm, performed according to the procedure of Blobel & Dobberstein (1975), with 4% acrylamide in the stacking

gel and 5–15% acrylamide in the running gel. Protein bands were stained with Coomassie Brilliant Blue. Stained gels were scanned at 633 nm with an LKB Ultrosan XL instrument equipped with an He/Ne laser source, and the resulting curves were integrated by using an Olivetti M 280 personal computer and the LKB software 2400 program (version 1–2). The relative amount of dye associated with each protein band was expressed as a percentage of the total dye recorded for all the bands of a particular sample.

RESULTS

In previous reports enzymic digestion of the polysaccharide component of rabbit TM was found to abolish the antithrombin-dependent anticoagulant activity of this molecule, whereas it did not affect the Protein C activation cofactor properties (Bourin *et al.*, 1988; Bourin, 1989). The present study corroborates these findings and extends them with regard to the role of the polysaccharide chain in relation to the former activity. Moreover, the polysaccharide component is implicated in two additional effects of TM, namely inhibition of thrombin-induced clotting of fibrinogen (direct anticoagulant activity of TM) and inhibition of thrombin-induced activation of Factor V.

Protein C activation cofactor, direct anticoagulant and antithrombin-dependent anticoagulant activities of TM

Isolation of TM from rabbit lung in the absence of added proteinase inhibitors was shown to generate two protein fractions capable of promoting the activation of Protein C by thrombin, an acidic species containing a sulphated polysaccharide and a non-acidic species lacking this component (Bourin *et al.*, 1986; Bourin, 1989). The conclusion from this finding, that the polysaccharide moiety is of no importance to the activation of Protein C, was confirmed by digesting TM with chondroitinase ABC; this treatment eliminated the polysaccharide without affecting the Protein C activation cofactor properties. Fig. 1(a) shows that the addition of TM, at increasing concentrations to incubations of Protein C and thrombin, induced the generation of similar amounts of Protein Ca regardless of whether or not the TM had been subjected to chondroitinase digestion. Yet the two forms of TM could be separated by anion-exchange chromatography and detected by their ability to promote Protein C activation [pattern similar to Fig. 1(b) in Bourin *et al.* (1986)]. These findings agree with the observation by Bourin (1989) that native and chondroitinase-digested rabbit TM bind thrombin with equal affinity, as determined by a Protein C activation assay.

C. T. Esmon *et al.* (1982) showed that rabbit TM will prevent thrombin from clotting fibrinogen, virtually complete inhibition occurring at a TM/thrombin molar ratio of approx. 1:1. Experiments based on the use of polyanion-binding agents such as Polybrene or platelet factor 4 suggested that the polysaccharide component of TM is somehow involved in the inhibition (Bourin *et al.*, 1986, 1988). This notion was strongly supported by the finding that digestion of TM with chondroitinase ABC greatly diminished the inhibitory effect on fibrinogen clotting; whereas the native TM rendered the sample incoagulable at a molar ratio to thrombin of about 1:1, the chondroitinase ABC-treated TM only increased the clotting time from 20 to about 50 s (corresponding to inhibition of about half of the thrombin added; Fig. 1b). TM digested with testicular hyaluronidase was similarly ineffective, whereas chondroitinase AC-digested TM was slightly inhibitory at high concentrations.

At low TM concentrations the antithrombin-dependent anticoagulant activity was found to be proportional to the amount of native TM added. Approaching molar equivalence to throm-

Fig. 1. Elimination of the polysaccharide component of TM; effects on the (a) Protein C activation cofactor, (b) direct anticoagulant and (c and d) antithrombin-dependent anticoagulant activities

TM was tested at the concentrations indicated with regard to the various anticoagulant activities, either in native form (■; TM incubated with chondroitinase buffer, but without enzyme) or after digestion with chondroitinase ABC (□; overnight digestion, see the Materials and methods section), chondroitinase AC (○) or testicular hyaluronidase (△). The assays for anticoagulant activities were performed as described in the Materials and methods section, the final concentrations for the various reagents being (a) 10 nM-thrombin and 0.5 μ M-Protein C, (b) 9 nM-thrombin and 2 mg of bovine fibrinogen/ml, (c) 13 nM-thrombin and 135 nM-antithrombin and (d) 16 nM-thrombin and 43 nM-antithrombin (upper graph), 86 nM-antithrombin (middle graph) or 172 nM-antithrombin (lower graph). The arrow in panel (c) indicates that samples containing > 8 nM-TM remained non-coagulable for at least 10 min.

bin, the effect of TM levelled off, and at molar excess over thrombin TM did not further promote thrombin inactivation (Fig. 1c). At all TM concentrations tested substitution of chondroitinase ABC-digested for native TM resulted in loss of the antithrombin-dependent anticoagulant activity attributed to TM. Again, digestion with testicular hyaluronidase was about equally effective, whereas the effect of chondroitinase AC was marginal (Fig. 1c).

Testing the effect of native TM at different concentrations of antithrombin gave the results shown in Fig. 1(d). The plateau levels of residual thrombin activity at which additional amounts of TM did not further promote the inactivation of thrombin were inversely related to antithrombin concentration, such that at the lowest antithrombin concentration tested (43 nM) 60% of the thrombin initially present resisted the effects of TM whereas at the highest antithrombin concentration (172 nM) only about 10% of the thrombin remained active after the 5 min incubation period. The high plateau levels obtained at low antithrombin concentrations could be decreased by prolonging the time of incubation of TM, antithrombin and thrombin (results not shown). The plateau levels were achieved at approximately the same, equimolar, TM/thrombin ratio, regardless of antithrombin concentration, in accord with the view (Bourin *et al.*, 1988) that the target thrombin molecules are quantitatively bound to TM.

In fact, the three curves were largely superimposable, given the different starting points recorded in the absence of TM. The differences between the curves thus are essentially due to the progressive action of antithrombin, which entails a more rapid thrombin inactivation at higher antithrombin concentration but does not depend on any participation of the TM molecule.

Inhibition of thrombin-catalysed activation of Factor V

The polypeptide pattern generated during the activation of bovine Factor V by thrombin, and revealed by SDS/PAGE (Fig. 2a), was similar to that described by Nesheim & Mann (1979). The nomenclature introduced by those authors is adopted to define the various components. Factor V is a high-molecular-mass (330 kDa) single-chain polypeptide (component A in Fig. 2a) that circulates in plasma as the precursor form of activated Factor Va. This component was the only polypeptide detectable in significant amounts in the unmodified starting material (zero-time sample), and it remained unchanged during a 30 min control incubation performed in the absence of thrombin (results not shown). Addition of thrombin resulted in the formation of two activation intermediates, 205 kDa (component B) and 155 kDa (component C), which were subsequently converted into the 96 kDa heavy chain (component D) and 71–74 kDa light chain (components E/F) end products. Most of the intact Factor V

Fig. 2. Factor V activation in the presence or in the absence of TM

Samples of Factor V and thrombin (omitted in the zero-time control) were incubated (a) in the absence of TM, (b) in the presence of native rabbit TM and (c) in the presence of chondroitinase ABC-digested (1 h digestion; see the Materials and methods section) TM, under the conditions described in detail in the Materials and methods section. After the incubation periods indicated (minutes), 25 μ l portions of the incubation mixtures were withdrawn, and the reactions were interrupted by boiling with SDS/dithiothreitol (see the Materials and methods section). The samples were then subjected to SDS/PAGE and subsequently stained with Coomassie Brilliant Blue. The migration positions of molecular-mass markers are indicated at the margin of one of the gels (the gels were run separately; hence a slight difference in overall migration distances can be seen). The designation of the protein bands A–F is in accordance with Nesheim & Mann (1979).

(component A) had disappeared already after 0.5 min of incubation with thrombin, at which time the two intermediates, components B and C, were clearly seen. Whereas component C was rapidly subjected to further cleavage, yielding the Factor Va heavy chain (component D), band B decreased relatively slowly in intensity during the 30 min of the reaction. The appearance of light chain (components E/F) seemed to parallel the disappearance of component B.

The changes in the relative amounts of the various polypeptides during the Factor V activation process were quantified by gel scanning. Fig. 3 illustrates the changes with time in the proportions of intact Factor V (component A; Fig. 3a), one of the intermediates (component C; Fig. 3b) and Factor Va light chain (components E/F; Fig. 3c). The rapid disappearance of intact Factor V in the presence of thrombin alone, more than 80% being eliminated after 0.5 min, was reflected by the generation of component C, which reached its maximal value within the same period of time. Also within the first minute of incubation, significant amounts of components E/F, corresponding to 10–20% of the total polypeptide, or about one-third of the final amount of Factor Va light chain, were formed. The final concentration of light chain was attained after approx. 10 min.

The addition of TM had a profound effect on the activation process. Under the conditions of the experiment (11 nM-thrombin and 40 nM-TM) all the thrombin present would be expected to be bound to TM (see C. T. Esmon *et al.*, 1982). This assumption was corroborated by the finding (see Fig. 1b) that even at a lower molar ratio to thrombin TM was able to inhibit completely the

clotting of fibrinogen. Although Factor V was also activated by thrombin in the presence of TM, the process was markedly delayed, such that intact component A remained detectable in the activation mixtures even after 10 min of incubation (Figs. 2b and 3a). Moreover, the formation and disappearance of the intermediate component C was similarly delayed (Figs. 2b and 3b), the peak concentration of this component being attained after about 5 min of incubation and significant amounts remaining after 30 min. For comparison, in the absence of TM component C reached its maximal concentration after about 0.5 min and was essentially gone after 5 min (Fig. 3b). The apparent rate of conversion of component C in the presence of TM was less than 10% of that observed in the absence of TM. Finally, the generation of Factor Va light chain (components E/F) was also affected by the presence of TM (Figs. 2b and 3c). The process was delayed, compared with a control incubation lacking TM; the final amounts of light chain formed were smaller, and in particular there was a conspicuous lag phase of approx. 3 min. during which no light chain could be detected.

In order to evaluate the role of the polysaccharide component of TM in relation to Factor V activation, incubations of Factor V and thrombin were repeated in the presence of TM that had been previously digested with chondroitinase ABC. Control experiments based on the inhibitory effect of native TM on fibrinogen clotting by thrombin indicated that the polysaccharide chain had been eliminated by this treatment. No contaminating proteolytic activity was detected in the chondroitinase preparations used in the present study (see Bourin, 1989), and it was therefore assumed (lacking sequence information for native

Fig. 3. Time course of Factor V activation

The stained gels shown in Fig. 1 were analysed by quantitative scanning as described in the Materials and methods section, and the relative amounts of protein in each band were estimated. The results obtained for component A (a), component C (b) and components E/F (c) were plotted against the time of incubation. The activation of Factor V was performed in the presence of thrombin alone (●), thrombin and native TM (○) or thrombin and chondroitinase-digested TM (△).

and digested TM) that the polypeptide portion remained unaffected by the digestion. Since rabbit TM molecules with and without the polysaccharide chain bind thrombin with apparently equal avidity (Bourin, 1989), it may also be assumed that the thrombin was quantitatively bound to native as well as to chondroitinase-digested TM during the incubation with Factor V. Yet virtually all the effects observed with native TM on Factor V activation were abolished by pretreatment with chondroitinase ABC (Figs. 2 and 3). Analysis by SDS/PAGE thus showed that, in contrast with native TM, the chondroitinase-digested TM did not significantly delay the generation of polypeptide intermediates,

or the final products, from Factor V during incubation with thrombin. This conclusion is particularly clearly borne out by the quantitative gel scans shown in Fig. 3; the curves illustrating the disappearance of native Factor V (Fig. 3a), the transient formation of the intermediate component C (Fig. 3b) and the generation of light chain (Fig. 3c) are virtually indistinguishable for the samples incubated in the absence of TM and in the presence of chondroitinase-digested TM. These findings indicate that the inhibitory effects of rabbit TM on the activation of Factor V by thrombin are due to the polysaccharide component of the TM molecule.

DISCUSSION

Rabbit TM may be depicted as a proteoglycan in which both the protein and the polysaccharide constituents contribute to the functional properties of the molecule (Fig. 4). Binding of thrombin to a primary site, located within the fifth and sixth EGF-like regions of the protein moiety (Kurosawa *et al.*, 1988), is a prerequisite to the rapid activation of Protein C. As a result of this binding thrombin also loses some of its ability to clot fibrinogen (Stearns *et al.*, 1989). However, more important to

Fig. 4. Model of the rabbit TM proteoglycan

The schematic representation of domain structures is based on the molecular organization of the human TM species (Wen *et al.*, 1987; Suzuki *et al.*, 1987). The TM molecule is composed of (I) an N-terminal domain, which contains a few hydrophobic amino acid residues, (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 numbered 2-6 provide the binding sites for thrombin and Protein C required for efficient activation of the latter component (Stearns *et al.*, 1989). The depicted mode of interaction of TM with the endothelial cell surface allows the thrombin to be bound 'on top' of the TM molecule (Lu *et al.*, 1989). The chondroitin sulphate chain is shown to be inserted in domain III, which provides potential O-glycosylation sites. The construction of the model is intended to illustrate that the polysaccharide component of TM does not influence the activation of Protein C by thrombin, whereas it will potentiate the inhibition of thrombin by antithrombin and prevent the cleavage of fibrinogen and the activation of Factor V by thrombin. The EGF-like structures are stabilized by intrachain disulphide bridges that are probably essential for expression of all the various anticoagulant activities of TM. For additional information see the text.

this latter effect is a secondary interaction involving thrombin and the polysaccharide component of TM, as demonstrated by the results shown in Fig. 1(b). The polysaccharide chain is also essential for the ability of TM to promote the inactivation of thrombin by antithrombin (Fig. 1c), but does not seem to contribute to the activation of Protein C (Fig. 1a; Bourin *et al.*, 1988; Bourin, 1989). Finally, the present study provides evidence that the inhibition by TM of thrombin-catalysed Factor V activation depends entirely on the presence of the polysaccharide component.

The activation of Factor V by thrombin comprises two fast reactions (cleavage into the intermediates B and C, and conversion of the intermediate C into the end product D) and a somewhat slower process (cleavage of the intermediate B) (Nesheim & Mann, 1979). The results of the present study confirm the finding by C. T. Esmon *et al.* (1982) that rabbit TM prevents the activation of Factor V by thrombin. The presence of native TM thus resulted in appreciable retardation of both the initial proteolytic cleavage of Factor V and the further processing of the intermediates. In particular, the disappearance of the intermediate C, i.e. the generation of Factor Va heavy chain, was dramatically delayed compared with control incubations with thrombin in the absence of TM. The effects of TM on the activation of Factor V seemed to be restricted to the rate of the process, and did not invoke any apparent qualitative changes of either the intermediates or the end products. The galactosaminoglycan chain of TM is directly involved in the inhibition of Factor V activation. Upon digestion with chondroitinase ABC, TM thus lost its inhibitory properties, such that both the initial cleavage reaction and the subsequent modification processes occurred at the same rate as in the absence of TM.

The mechanisms by which the polysaccharide component influences anticoagulant activities of TM are yet unclear. Recent structural analysis indicates that the polysaccharide chain is a chondroitin sulphate, of molecular mass approx. 10 kDa, that carries an oversulphated region at the non-reducing terminus (M.-C. Bourin & U. Lindahl, unpublished work). This finding agrees with the observation that the functional properties ascribed to the polysaccharide are abolished by digestion with chondroitinase ABC and/or testicular hyaluronidase. The somewhat lower efficiency of chondroitinase AC might suggest the occurrence of some L-iduronic acid (in addition to D-glucuronic acid) units, but could also reflect the more pronounced exoenzyme character of this enzyme (Fransson, 1985). The model shown in Fig. 4 implies that the functional role of the polysaccharide may be explained in terms of steric factors as well as more specific interactions. It appears likely that the polysaccharide chain will sterically hinder the TM-bound thrombin from interacting with bulky molecules such as fibrinogen or Factor V. As regards the antithrombin-dependent anticoagulant activity the polysaccharide chain has a positive, and probably more complex, role in promoting the interaction between antithrombin and thrombin. After release from TM by alkaline β -elimination, the antithrombin-dependent anticoagulant activity of the free polysaccharide chain is no more than 5% of that of the TM-bound polysaccharide (results not shown). Moreover, the activity of TM is abolished by reduction and carboxymethylation of the protein component (Preissner *et al.*, 1987). These findings, together with the data shown in Fig. 1(d) of the present paper, indicate that the target thrombin molecule must be bound to TM (presumably at the Protein C activation site) that also contains the covalently bound polysaccharide chain. Interaction of this chain with the thrombin molecule might render the thrombin more susceptible to inactivation by antithrombin, as outlined in Fig. 4. The alternative possibility,

that the polysaccharide facilitates the thrombin-antithrombin reaction by capturing the inhibitor, cannot be ruled out, but appears less likely since the free radiolabelled polysaccharide was found to lack appreciable affinity for antithrombin-Sepharose at physiological ionic strength (M.-C. Bourin & U. Lindahl, unpublished work). Regardless of the precise details of the mechanism, the antithrombin-dependent anticoagulant activity of TM seems to present an unprecedented case of functional co-operation between the carbohydrate and protein moieties of a glycoconjugate.

Factor Va is an essential component of the prothrombinase complex (comprising Factor Xa, Factor Va, acidic phospholipids and Ca^{2+} ions) that converts prothrombin into thrombin. Both platelets (Tracy *et al.*, 1981) and endothelial cells (Rodgers & Shuman, 1983) provide a suitable surface for the activation of prothrombin by Factor Xa. TM occurs both on the vascular endothelium and in platelets (Suzuki *et al.*, 1988), and it impedes the function of the prothrombinase complex by promoting the generation of Protein C α_2 , which inactivates Factor Va (Walker *et al.*, 1979). This anticoagulant effect, exerted by the protein moiety of TM, is enforced by the polysaccharide component, which prevents the generation of Factor Va. The polysaccharide is involved in further modulation of thrombin activity, interfering with cleavage of fibrinogen and promoting the inactivation of thrombin by antithrombin. The highly complex functional properties of the rabbit TM galactosaminoglycan thus differ considerably from those of the heparin-like polysaccharides, which are also present on the endothelial cell surface (Marcum *et al.*, 1986) and which accelerate the inactivation of thrombin by means of their specific antithrombin-binding pentasaccharide sequence (Lindahl *et al.*, 1984). It is not clear at present whether the proteoglycan properties of rabbit TM are shared also by TM derived from other species. Studies of human TM (Kurosawa & Aoki, 1985; Maruyama *et al.*, 1985) and bovine TM (Jakubowski *et al.*, 1986; Suzuki *et al.*, 1986) seem to indicate less pronounced, or even lack of, antithrombin-dependent and direct anticoagulant activities, suggesting that these species may lack the galactosaminoglycan component. On the other hand, our previous work with rabbit TM showed that unless the appropriate proteinase inhibitors are added the polysaccharide chain is readily lost as a result of proteolytic cleavage during isolation, leaving a truncated protein that retains the Protein C activation cofactor but none of the other anticoagulant activities (Bourin *et al.*, 1986; Bourin, 1989).

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