Isolation and Characterization of the Glycosaminoglycan Component of Rabbit Thrombomodulin Proteoglycan*

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Previous studies on rabbit thrombomodulin (TM) revealed that certain anticoagulant activities expressed by TM depend on the presence of an acidic domain tentatively identified as a sulfated galactosaminoglycan (Bourin, M.-C., Ohlin, A.-K., Lane, D., Stenflo, J., and Lindahl, U. (1988) J. Biol. Chem. 263, 8044-8052). The glycan was released by alkaline β-elimination, isolated by ion-exchange chromatography, and radiolabeled by partial N-deacetylation (hydrazinolysis) followed by re-N-[3H]acetylation. The labeled radiolabeled by partial N-deacetylation (hydrazinolysis) followed by re-N-[3H]acetylation. The labeled product behaved like standard chondroitin sulfate on ion-exchange chromatography, exhibited a $M_\text{r}$ of $10^8$ on gel chromatography, and was susceptible to degradation by chondroitinase and testicular hyaluronidase. The major labeled degradation products following digestion of the glycosaminoglycan with chondroitinase were identified, depending on the incubation conditions, either as 4/6-mono-O-sulfated, 4,5-ununsaturated disaccharides (ΔHexA-GalNAc(S)) and N-acetylgalactosamine 4,6-di-O-sulfate (GalNAc(diS)), the latter component accounting for ~25% of the total label, or as a major fraction of labeled triasaccharide, with the predominant structure GalNAc(diS)-GlcA-GalNAc(diS). The terminal GalNAc(diS) unit (not substituted at C3) was shown to be more susceptible to N-deacetylation during hydrazinolysis than were the internal GalNAc units (substituted at C3), and thus was more extensively labeled, resulting in over-representation of this unit. It is concluded that rabbit TM is a chondroitin sulfate proteoglycan, which carries a single glycan side chain characterized by an unusual accumulation of sulfate groups at the nonreducing terminus. Metabolically $^{35}$S-labeled TM was isolated from cultured rabbit heart endothelial cells and characterized as a chondroitin sulfate proteoglycan which accounted for 1–2% of the total $^{35}$S-labeled cell-associated macromolecules. The isolated chondroitin sulfate showed weaker antithrombin-dependent anticoagulant activity, on a molar basis, than the intact TM proteoglycan. The anticoagulant action of TM thus depends on a unique form of functional collaboration between the different constituents of a glycoconjugate.

Thrombomodulin (TM) is an integral glycoprotein of the endothelial cell surface, with high affinity for thrombin, and an essential role in maintaining the nonthrombogenic properties of the endothelium (Maruyama et al., 1985; DeBault et al., 1986). Upon binding to TM the properties of activation are changed, such that the procoagulant activity is lost and, in fact, the formation of the enzyme itself is precluded. TM acts as a cofactor (Esmun et al., 1982b) in the thrombin-catalyzed activation of Protein C (Stenflo, 1976; Kisiel et al., 1970) which is thereby converted into an active protease. The activated Protein C, inhibits further thrombin generation by inactivating the clotting Factors V, and VIII, through limited proteolysis (Walker et al., 1979; Marlar et al., 1982). Moreover, studies on rabbit TM showed that it will inhibit thrombin-induced cleavage of fibrinogen and activation of Factor V (Esmun et al., 1982a), but promote the inactivation of thrombin by the protease inhibitor antithrombin (Bourin et al., 1986; Hofsteenge et al., 1986; Preisser et al., 1987). Clones of cDNA encoding for bovine (Jackman et al., 1986), human (Suzuki et al., 1987; Wen et al., 1987; Jackman et al., 1987), and murine (Dittman et al., 1988) TM have been obtained and the corresponding amino acid sequences have been deduced. TM is a multidomain protein consisting of a NH₂-terminal portion, a domain composed of 6 epidermal growth factor-like structures which contains the Protein C-activation site (Steurns et al., 1989; Zushi et al., 1989), a domain rich in serine and threonine residues which provides potential O-glycosylation sites, a hydrophobic transmembrane domain, and a cytoplasmic COOH tail.

Two of the biological activities of rabbit TM, i.e. the ability to inhibit thrombin-induced fibrinogen clotting and the accelerating effect on the inactivation of thrombin by antithrombin, were found to depend on the presence of an additional, acidic domain (Bourin et al., 1986, 1988). More recent studies showed that this component also provides the inhibitory effect of TM on the activation of Factor V by thrombin (Bourin and Lindahl, 1990). On the other hand, it did not seem to be involved in the activation of Protein C (Bourin et al., 1986, 1988; Bourin 1989). The acidic properties of rabbit TM were lost on digestion with chondroitin lyase ABC (EC 4.2.2.4) (chondroitinase ABC) or testicular hyaluronidase, along with all biological activities, except the ability to serve as a cofactor in the activation of Protein C (Bourin et al., 1986; Bourin and Lindahl, 1990). Moreover, as a result of such digestion, the apparent $M_\text{r}$ on sodium dodecyl sulfate-
polyacrylamide gel electrophoresis dropped from ~90,000 to ~74,000 (Bourin, 1989). These findings strongly suggested that the acidic domain of rabbit TM is comprised of a (presumably single) chain of sulfated galactosaminoglycan. In the present report we describe the isolation and characterization of this component.

**EXPERIMENTAL PROCEDURES**

**Materials**

TM was purified from a detergent extract of rabbit lungs according to a procedure involving an initial ion-exchange chromatography step (Salem et al., 1984), two successive affinity chromatographies on immobilized deoxyribonuclease (DNase I) and hydrazine, and degradation to sulfated \( \text{AHexA-GalNAc} \) disaccharides, isolated from pig second ion-exchange chromatography, at low pH (Bourin et al., 1986), and finally, an immunoaffinity chromatography on immobilized monoclonal anti-TM antibodies (Bourin et al., 1988). The procedure employed has been described in detail by Bourin et al. (1988).

Rabbit heart endothelial cells were isolated by retrograde perfusion, using digestion with bacterial collagenase (Worthington Biochemical Corp.) essentially as previously described for the isolation of human endothelial cells from umbilical vein (Wall et al., 1970). The cells obtained from one heart were plated in a 25-cm² culture flask (Falcon 3013, Becton Dickinson, Lincoln Park, NJ) that had been precoated with 2% gelatin in phosphate-buffered saline (PBS) and were cultured in M199 medium with Earle's salts (Gibco, Grand Island, NY) containing 18% fetal calf serum (Whittaker M. A. Bioproducts, Walkersville, MD), in Hepes buffer (Gibco). The medium was changed every 3–4 days. After 2–4 weeks, when the cells were confluent, they were scraped off, suspended in 12 ml of culture medium, and passed into 3 gelatin-coated 25-cm² flasks. The endothelial origin of the cells obtained at this stage was ascertained by their ability to endocytose acetylated low density lipoprotein labeled with \( ^3 \text{H} \)acetylated by treatment with \( ^3 \text{H} \)acetate in Tris buffer. The yield of immunopurified labeled macromolecules corresponded to approximately 1500 cpm of \( ^35 \text{S} \) per 25-cm² flask.

**Isolation of the Glycosaminoglycan Component of TM**

Immunopurified TM (2 or 4 mg, 120 mg/ml) was adjusted to 0.5 M with respect to NaOH and kept at 4 °C for 15 h in order to release O-linked saccharides from the protein. After neutralization followed by dialysis against 0.1 M NaCl, 0.05 M Tris-HCl, 0.2% Nonidet P-40 the samples were applied to columns (1 ml) of DEAE-cellulose which were eluted in a stepwise fashion with 3 × 0.5 ml of each of 0.1, 0.25, 0.5, 0.75, and 1.2 M NaCl in 0.05 M sodium acetate, pH 4.0. The effluent fractions were analyzed for hexuronic acid by microadaptation of the carbazole reaction (Ditter and Muir, 1962). The positive fractions were pooled, dialyzed extensively against water, and lyophilized. The amounts of glycosaminoglycan isolated were estimated from the carbazole analysis, assuming that the hexuronic acid component accounted for 30% of the weight.

**Radiolabeling of the Glycosaminoglycan Component of TM**

**Chemical Labeling—Isolated glycosaminoglycan (30 μg obtained from 1 mg of TM) was dissolved in 0.25 ml of hydrazine, containing 2.5 mg of hydrazine sulfate, and heated at 100 °C in a sealed tube for 30 min. After repeated evaporation of the hydrazine in the presence of NaOH, the glycosaminoglycan was dried to a brown mass by lyophilization and dissolved in hydrazine (from Eastman Kodak Co. (Rochester, NY), hydrazine sulfate and mercuric acetate from Merck (Darmstadt, Federal Republic of Germany), \( ^3 \text{H} \)acetic anhydride (500 mCi/mmol) from The Radiochemical Center (Amersham, Buckinghamshire, United Kingdom), and Na\(_2\)SO\(_4\) from Du Pont New England Nuclear.)

**Immunopurified TM (2 or 4 mg, 120 mg/ml) was adjusted to 0.5 M with respect to NaOH and kept at 4 °C for 15 h in order to release O-linked saccharides from the protein. After neutralization followed by dialysis against 0.1 M NaCl, 0.05 M Tris-HCl, 0.2% Nonidet P-40 the samples were applied to columns (1 ml) of DEAE-cellulose which were eluted in a stepwise fashion with 3 × 0.5 ml of each of 0.1, 0.25, 0.5, 0.75, and 1.2 M NaCl in 0.05 M sodium acetate, pH 4.0. The effluent fractions were analyzed for hexuronic acid by microadaptation of the carbazole reaction (Ditter and Muir, 1962). The positive fractions were pooled, dialyzed extensively against water, and lyophilized. The amounts of glycosaminoglycan isolated were estimated from the carbazole analysis, assuming that the hexuronic acid component accounted for 30% of the weight.**
Glycosaminoglycan Component of Thrombomodulin

Degradation of Saccharides

Digestion of \(^{3}H\)-labeled TM glycosaminoglycan with chondroitinase ABC was done in 30 \(\mu\)M sodium acetate, 0.05 M Tris-\(\mathrm{HCl}\), pH 6.9, under conditions found to result in either partial (400 milliunits of enzyme/ml) or essentially complete (100 or 200 milliunits of enzyme/ml) degradation of the substrate. The incubations were maintained at 37°C for 18 h, in the presence of 1 mg of carrier chondroitin sulfate A. Digestion with chondroitinase AC was done in the same buffer using 400 milliunits/ml. Digestion with testicular hyaluronidase was performed under the conditions reported by Bourin et al. (1988).

Cleavage of \(N\)-sulfated glycosaminoglycans by treatment with HNO\(_3\) at pH 1.5 was performed according to Shively and Conrad (1976).

Analytical Methods

Anion-exchange chromatography of \(^{3}H\)-labeled glycosaminoglycan, isolated from TM, was performed using a 3-ml column of DEAE-cellulose (DE-52), which was eluted using a linear gradient (total volume, 160 ml) extending from 0.05 to 1.5 M LiCl. Hyaluronan (0.5 mg), chondroitin sulfate A (2 mg), and heparin (2 mg) were used as internal standards. Effluent fractions of 2 ml were analyzed for radioactivity by scintillation spectrometry, and for hexuronic acid using the carbazole reaction (Bitter and Muir, 1962).

Gel chromatography of intact TM glycosaminoglycan was performed using a column (1 \(\times\) 150 cm) of Sephadex G-100, which was eluted with 0.2 M NH\(_4\)HCO\(_3\), at a flow rate of 7.5 ml/h. The column was calibrated with chondroitin sulfate standards of known molecular weight (M, 14,100 and 8,800), which were detected by the carbazole reaction.

Products of TM glycosaminoglycan obtained by digestion with chondroitinase AC or ABC were analyzed on a column (1 \(\times\) 200 cm) of Sephadex G-25 (superfine grade), which was similarly eluted with 0.2 M NH\(_4\)HCO\(_3\). High voltage paper electrophoresis was conducted on Whatman 3MM paper in 1.6 M formic acid (pH 1.7, 30 V/cm, 80 min). Paper chromatography was performed on similar paper, developed with acetic acid, 1-butanol, 1\% NH\(_4\)OH, 3/2/l, for 29 h. Radioactive components were detected after drying the paper strips, by cutting 1-cm segments which were extracted with 1 ml of water, mixed with 4 ml of scintillation mixture (Beckman "Ready Safe"), and counted in a Beckman LS 3800 scintillation spectrometer.

The amino sugar composition of TM glycosaminoglycan was determined using an amino acid analyzer, following hydrolysis of the glycans with 2 M HCl at 110°C for 16 h.

Glycosaminoglycans were analyzed for anthr流insulfonic-dependent anticoagulant activity essentially as described by Bourin et al. (1988) with slight modifications. Briefly, samples were incubated with anthrombin I (136 nM) and thrombin (16 nM), and after 10 min of incubation the residual thrombin activity was determined from the rate of hydrolysis of the chromogenic substrate S-2238 (D-Phe-pipecolyl-Arg-p-nitroanilide; KabiVitrum, Stockholm, Sweden).

RESULTS

Isolation and Chemical Radiolabeling of TM Glycosaminoglycan—Preparations of purified TM were subjected to alkaline β-elimination, and the released glycosaminoglycan was isolated by chromatography on DEAE-cellulose, as described under "Experimental Procedures." Hexuronic acid was detected (carbazole reaction) in the fractions eluted with 0.75 M NaCl, which were pooled, dialyzed, and concentrated. Hexuronic acid analysis of the final products indicated yields of ~60 and 120 \(\mu\)g of glycosaminoglycan from 2 and 4 mg of TM starting material, respectively. Previous data (Bourin, 1989) suggested that the glycosaminoglycan component accounts for ~10% of the TM molecule. In view of the small overall amounts of material, and the extent of manipulation involved in the isolation, these yields are not considered to be unduly low. The molar ratio of hexosamine/hexuronic acid was ~0.9/1, galactosamine constituting 92% of the total amino sugar. These findings are in accord with our previous proposal that the glycosaminoglycan component of rabbit TM is a sulfated galactosaminoglycan (Bourin et al., 1988; Bourin, 1989).

The amounts of glycosaminoglycan isolated were insufficient for structural analysis by conventional techniques, and samples were instead subjected to radiolabeling using the chemical N-[\(^{3}H\)]acetylated labeling procedure devised by Höök et al. (1982). The material was first subjected to hydratrinolysis under conditions expected to bring about the release of 10–15% of the total N-acetyl groups (see Höök et al., 1982). The resulting free amino groups were then substituted with \(^{3}H\)-acetyl residues, yielding a product with a specific activity of ~60,000 cpm/\(\mu\)g. Analysis of the labeled product by anion-exchange chromatography on DEAE-cellulose showed a single, distinct peak of radioactivity which emerged at the elution position of standard chondroitin sulfate (Fig. 1). Gel chromatography on Sephadex G-100 revealed a somewhat polydisperse material with a peak elution volume corresponding to an \(M\), of 10,000–12,000 (Fig. 2), indicating a glycosaminoglycan chain composed of ~20 disaccharide units.

Structural Analysis of Chemically Radiolabeled TM Glycosaminoglycan—Preliminary degradation experiments, involving chromatography of the products on Sephadex G-50, indicated that the \(^{3}H\)-labeled TM glycosaminoglycan was susceptible to digestion by chondroitinase ABC, chondroitinase AC, and testicular hyaluronidase, but resisted degradation by nitrous acid (data not shown). These observations, compatible with the properties of a chondroitin sulfate, were pursued in more detailed studies.

Exhaustive digestion of the TM glycosaminoglycan with chondroitinase ABC, followed by gel chromatography of the products on Sephadex G-25, yielded a major peak of heterogeneous labeled material that emerged partly retarded in relation to the elution position of standard ADi(S) disaccharide (Fig. 3A).

Further separation of this material by high voltage paper electrophoresis at pH 1.7 yielded two major components (Fig 4A) which had migrated like a monosulfated ADi(S) disaccharide (55% of the total label) and a faster migrating, disulfated GalNAc(diS) monosaccharide (37% of the label). In addition, minor components of intermediate mobility were observed.
paper electrophoresis of Fraction II showed monosulfated
sulfation I in Fig. 3B), which was barely noticeable after more
standard on high voltage electrophoresis at pH 1.7 (Fig. 4C),
will in the following be referred to as Fraction I. On repeated
this component, isolated by preparative paper electrophoresis,
was degraded further as shown by the gel chromatogram in
incubation with either chondroitinase ABC or AC, Fraction I
sively from the nonreducing terminus of the intact glycosa-
glycosaminoglycan. Arbitrarily decreasing the amount of
minoglycan chain.
chondroitinase ABC added to the incubations (from 200 to 40
GalNAc unit was obtained by analyzing the products gener-
ated through partial eliminase digestion of the labeled TM
Sigma; data not shown) were virtually completely degraded
chondroitin sulfate, as well as a N-deacetylated, re-N-['H-
pattern of the labeled digestion products on gel chromatog-
raphy (Fig. 3B; it may be noted that the unlabeled carrier
maximally retarded material (designated Fraction II in Fig.
3B) thus was smaller than that obtained at the higher enzyme
concentration; in addition, it was less heterogeneous, appear-
ing at the elution position of ADi disaccharide and lacking
the smaller sized components noted in Fig. 3A. Accordingly,
paper electrophoresis of Fraction II showed monosulfated
disaccharide as the only significant component present (Fig.
4B). In addition to Fraction II, the gel chromatogram showed
appreciable amounts of a less retarded, distinct fraction (Fra-
cion I in Fig. 3B), which was barely noticeable after more
complete degradation. Fraction I contained a major compo-
ment that migrated faster than the disulfated disaccharide
standard on high voltage electrophoresis at pH 1.7 (Fig. 4C),
and would thus represent a highly sulfated oligosaccharide;
this component, isolated by preparative paper electrophoresis,
will in the following be referred to as Fraction I. On repeated
incubation with either chondroitinase ABC or AC, Fraction I
was degraded further as shown by the gel chromatogram in

Fig. 2. Gel chromatography of N-['H]acetyl-labeled TM
glycosaminoglycan. A sample (5000 cpm) of labeled glycosami-
glycan was applied, along with reference samples of chondroitin
sulfate (0.5 mg of each) of known molecular weight (M, 8,800 and
14,100), to a column (1 x 150 cm) of Sephadex G-100, equilibrated
with 0.2 M NH4HCO3. Effluent fractions of 2 ml were collected and
analyzed for radioactivity and hexuronic acid. The peak elution
positions of the standards are indicated by arrows.

one of which (6% of the label) appeared identical to the
disulfated disaccharide, ADi(diS). The [DI(S) fraction was
isolated by preparative paper electrophoresis and separated
further by paper chromatography, which revealed 4- and 6-
sulfated isomers in about equal proportions (data not shown).
While 4,5-unsaturated disaccharides will constitute the major
degradation products of any galactosaminoglycan prepara-
tion, the large amounts of GalNAc(diS) monosaccharide were
unexpected, since such a component would be derived exclu-
sively from the nonreducing terminus of the intact glycosa-
maminoglycan chain.

Further information as to the origin of the disulfated
GalNAc unit was obtained by analyzing the products gener-
ated through partial eliminase digestion of the labeled TM
glycosaminoglycan. Arbitrarily decreasing the amount of
chondroitinase ABC added to the incubations (from 200 to 40
milliunits/ml) resulted in a marked change in the elution
pattern of the labeled digestion products on gel chromatog-
raphy (Fig. 3B; it may be noted that the unlabeled carrier
maximally retarded material (designated Fraction II in Fig.
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concentration; in addition, it was less heterogeneous, appear-
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the smaller sized components noted in Fig. 3A. Accordingly,
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4B). In addition to Fraction II, the gel chromatogram showed
appreciable amounts of a less retarded, distinct fraction (Fra-
cion I in Fig. 3B), which was barely noticeable after more
complete degradation. Fraction I contained a major compo-
ment that migrated faster than the disulfated disaccharide
standard on high voltage electrophoresis at pH 1.7 (Fig. 4C),
and would thus represent a highly sulfated oligosaccharide;
this component, isolated by preparative paper electrophoresis,
will in the following be referred to as Fraction I. On repeated
incubation with either chondroitinase ABC or AC, Fraction I
was degraded further as shown by the gel chromatogram in

Fig. 3. Gel chromatography of products obtained on diges-
tion of labeled TM glycosaminoglycan with bacterial elimi-
nases. Samples, mixed with 1 mg of chondroitin sulfate A standard
were incubated with chondroitinase ABC or AC as described under
"Experimental Procedures" and the products applied to a column (1 x
200 cm) of Sephadex G-25, equilibrated with 0.2 M NH4HCO3.
Effluent fractions of 2 ml were collected at a rate of 6 ml/h and were
analyzed for ['H (●, ●) or 35S (▲) radioactivity. The elution position
of the unsulfated disaccharides (ΔDi(S)) produced by digestion of
the carrier (detected by the carbazole reaction) is indicated by an
arrow in Panel A. A, exhaustive digestion of N-['H]acetyl-labeled TM
glycosaminoglycan (0.5 x 106 cpm) with chondroitinase ABC (100
milliunits; 500 µl of incubation volume; open symbols); superimposed
is a chromatogram of an undigested control (closed symbols). B, partial
digestion of N-['H]acetyl-labeled TM glycosaminoglycan (2 x 106
cpm) with chondroitinase ABC (100 milliunits; 250 µl of incubation
volume). C, digestion of fraction I (200 x 106 cpm) from the chro-
matography shown in Panel B with chondroitinase AC (50 milliunits;
250 µl of incubation volume). D, digestion of 35S-labeled TM (15,000
cpm) from rabbit heart endothelial cells with chondroitinase ABC
(100 milliunits; 500 µl of incubation volume). Effluent fractions pooled
as indicated by the horizontal bars were analyzed further as described
in the text.

Fig. 3C. Essentially similar results were obtained with both
enzymes; however, chondroitinase AC gave a somewhat higher
yield of degradation products. The elution pattern showed
two major peaks, one of which emerged at the same elution
volume as the original Fraction I and apparently represented
unchanged material. The other major peak (designated Ib in
Fig. 3C) was clearly retarded in relation to the ΔDi(S) disac-
charide standard. In addition to these major fractions, a
smaller amount of labeled material of intermediary size (Fra-
cion Ia) was observed. High voltage paper electrophoresis of
Fractions Ib and Ia revealed GalNAc(diS) (Fig. 5A) and
disulfated disaccharide (Fig. 5B) as the only labeled compo-
nents present in significant amounts. The amounts of
GalNAc(diS) formed on chondroitinase digestion of Fraction
Glycosaminoglycan Component of Thrombomodulin

500

0

-5 0 5 10 15 20 25 30 35 40

Migration distance (cm)

A

B

C

I corresponded approximately to those initially present in the retarded fraction appearing after exhaustive digestion of the TM glycosaminoglycan (Fig. 3A).

Apart from one conspicuous feature, i.e. the very high yield of labeled GalNAc(diS) (which will be elaborated on below), the results described above are readily rationalized in terms of a galactosaminoglycan chain with a heavily sulfated non-reducing terminal region (see the model in Fig. 6). Exhaustive digestion with chondroitinase will lead to cleavage of both the terminal linkage (marked ω in Fig. 6) and the penultimate galactosaminidic linkage (ω-1), and thus to the release of GalNAc(diS) and ΔDi(diS), in addition to the ΔDi(S) derived from the more internal portions of the glycosaminoglycan chain. All of these components were found on paper electrophoresis (Fig. 4A) of the mono-/disaccharide fraction recovered (Fig. 3A) after digestion. The ω-1 linkage is more susceptible to eliminase cleavage than is the terminal ω-linkage, and partial digestion will therefore preferentially release the intact terminal trisaccharide (Fraction I in Fig. 3B), again along with the monosulfated ΔDi(S) disaccharide (Fraction II in Fig. 3B). The trisaccharide can be degraded further into disulfated disaccharide (Fraction Ia) and GalNAc(diS) (Fraction Ib), as shown in Fig. 3C.

In a uniformly N-[3H]acetyl-labeled chondroitin sulfate chain composed of ~20 disaccharide units, a nonreducing terminal GalNAc residue would account for ~5% of the total radioactivity. However, calculations based on the data shown in Figs. 3A and 4A indicate that the GalNAc(diS) released, presumably from the nonreducing terminus, by digestion with chondroitinase ABC, corresponded to ~25% of the total label. These relations suggest that the nonreducing terminal GalNAc unit had become preferentially labeled during the N-deacetylation/re-N-[3H]acetylation labeling procedure employed. Moreover, such unequal distribution of label is clearly indicated by the markedly different yields of GalNAc(diS)
monosaccharide and \( \Delta \text{Di(diS)} \) disaccharide products obtained on further cleavage of the trisaccharide in Fraction I (Fig. 3C). It is recalled that the initial N-deacetylation reaction was designed to release only 10–15% of the total N-acetyl groups (see “Experimental Procedures”). If it is assumed that a nonreducing terminal GalNAc unit is more susceptible to hydrazinolysis than are the internal GalNAc units, a higher proportion of the terminal units would be deacetylated and thus available for re-N-[\( ^3 \text{H} \)]acetylation. In fact, Erbing et al. (1976) showed that the reactivity of N-acetylated hexosamines toward hydrazine was greatly decreased by the presence of a substituent at C3 (as will apply for internal but not for terminal GalNAc units in a chondroitin sulfate chain). Since this argument is of vital importance to the interpretation of the results of the present study, additional experiments were specifically designed to elucidate whether the terminal and internal GalNAc residues differed with regard to the rate of N-deacetylation during hydrazinolysis.

A sample of N-[\( ^3 \text{H} \)]acetyl-labeled TM glycosaminoglycan was subjected to hydrazinolysis under the conditions described under “Experimental Procedures,” and samples were withdrawn after 5, 10, 20, and 45 min. The partially deacetylated glycans were reisolated by gel chromatography, and the residual N-[\( ^3 \text{H} \)]acetyl label was determined. A plot of such label versus the time of hydrazinolysis (see inset in Fig. 7D) revealed a markedly biphasic reaction course with a rapid initial release of \( ^3 \text{H} \), almost 40% of the original label being lost within the first 5 min of treatment. After re-N-acetylation with unlabeled acetic anhydride, each sample was digested with chondroitinase ABC (exhaustive digestion in the presence of 0.1 mg of chondroitin sulfate A carrier), and the products were subjected to high voltage paper electrophoresis (Fig. 7). The fully labeled starting material yielded two major peaks of radioactivity, corresponding to \( \Delta \text{Di(S)} \) disaccharide and GalNAc(diS) monosaccharide (Fig. 7A), in accord with the previous structural analysis of this material. Progressive hydrazine treatment resulted in selective loss of the latter component, which had virtually disappeared after 45 min of reaction (Fig. 7, B–D). These findings, which are reflected by an approximately 10-fold increase in the ratio of \( \Delta \text{Di(S)}/ \text{GalNAc(diS)} \) over the 45-min reaction period (Table I), confirms the postulated propensity of the terminal GalNAc unit towards N-deacetylation during hydrazinolysis.

**Metabolic Labeling of TM Glycosaminoglycan**—Cultured endothelial cells from rabbit heart were incubated with Na\(^{35}\text{SO}_4\), and were subsequently extracted in the presence of detergent as described under “Experimental Procedures.” The extract was fractionated according to the scheme adopted for TM, involving successive chromatographies on DEAE-cellulose, immobilized disopropyl phosphothrombin, and immobilized anti-TM monoclonal antibodies (see “Experimental Procedures”). The recoveries of \( ^3 \text{S} \)-labeled macromolecules in the three steps were 85–90, 30–35, and \( \approx 1\% \), respectively, based on nondialyzable radioactivity in the cell extract. The labeled chondroitin \( ^{[35]} \)Sulfate (see below) in the final immunoaffinity purified fraction was quantitatively retained by the affinity matrix on repeated application to the immobilized antibodies (data not shown).

Purified \( ^3 \text{S} \)-labeled TM was incubated with chondroitinase ABC and the digest was analyzed by gel chromatography (Fig. 3D). Most of the digestion products emerged at a retarded elution position similar to that of disaccharides. Further separation of this fraction by paper chromatography showed 4-sulfated \( \Delta \text{Di(S)} \), 6-sulfated \( \Delta \text{Di(S)} \), and \( \Delta \text{Di(diS)} \), amounting to 70, 22, and 8%, respectively, of the total radioactivity. Similar, although less pronounced, degradation was obtained...

**FIG. 7. Effects of hydrazinolysis on N-[\( ^3 \text{H} \)]acetyl-labeled TM glycosaminoglycan.** \( ^{3} \text{H} \)-Labeled TM glycosaminoglycan was treated with hydrazine/hydrazine sulfate at 100 °C as described under “Experimental Procedures.” Samples of \( \approx 30,000 \) cpm were withdrawn after 0 (A), 5 (B), 20 (C), and 45 (D) min, and were recovered by evaporation followed by passage through Sephadex G-15. After re-N-acetylation using unlabeled acetic anhydride each sample was digested with chondroitinase ABC (200 milliunits/ml), and the digests were directly spotted onto Whatman 3MM paper and subjected to high voltage electrophoresis. The standards shown below the electrophorograms were as indicated. The insert in panel D shows the amounts of residual radioactivity associated with the glycosaminoglycan after different times of hydrazinolysis.

**TABLE I**

<table>
<thead>
<tr>
<th>Time of hydrazinolysis</th>
<th>( \Delta \text{Di(S)} )</th>
<th>GalNAc(diS)</th>
<th>( \Delta \text{Di(S)}/\text{GalNAc(diS)} )</th>
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Glycosaminoglycan Component of Thrombomodulin

Fig. 8. Antithrombin-dependent anticoagulant activity of rabbit TM and various glycosaminoglycan preparations. Samples containing different amounts of TM or free glycosaminoglycans were incubated with thrombin and antithrombin as described under “Experimental Procedures,” and residual thrombin activities were determined. The amounts of thrombin inactivated due to the so-called progressive action of antithrombin, obtained in the absence of glycosaminoglycan, have been subtracted. The free glycans tested were chondroitin sulfate A (A), chondroitin sulfate E (B), dermatan sulfate (Δ), and heparin (C; see insert), and isolated TM glycosaminoglycan (D); the amounts of glycosaminoglycan indicated were based on carboxyldrazine analysis, assuming an arbitrary hexuronic acid content of 30%. Samples of intact TM (C) are also referred to by their calculated contents of glycosaminoglycan, assuming that chondroitin sulfate accounts for 10% of the TM proteoglycan.

by digestion with chondroitinase AC (data not shown). Gel chromatography on Sephadex G-100 of the intact 35S-labeled glycosaminoglycan, following release by alkaline β-elimination, indicated an M, of ≈14,000, i.e. approximately similar to that of the corresponding chemically N-[3H]acetyl-labeled TM component (data not shown).

Antithrombin-dependent Anticoagulant Activity of TM Glycosaminoglycan—Intact TM, or the isolated TM glycosaminoglycan, were tested with regard to the ability to promote the inactivation of thrombin by antithrombin (Fig. 8). Under the conditions of the assay, 50% inhibition of the thrombin was achieved in the presence of either 600 ng of free glycosaminoglycan, or 200 ng of intact TM (which would contain 15–20 ng of bound glycosaminoglycan). The TM-bound glycosaminoglycan thus is a much more efficient accelerator of the thrombin-antithrombin reaction than is the corresponding released component. This finding is in agreement with our previous conclusion, that the target thrombin molecule is preferentially bound to the Protein C activation site of TM (Bourin et al., 1988). Nevertheless, it is apparent that also in free form the TM glycosaminoglycan exerted significant, albeit weak, antithrombin-dependent anticoagulant activity. This activity was comparable to that of the oversulfated chondroitin sulfate E, of which 900 ng was required to achieve 50% thrombin inactivation (Fig. 8). For comparison, similar inactivation was obtained with only 0.3 mg (0.05 milliunit) of heparin (Fig. 8, inset). By contrast, neither standard chondroitin sulfate A nor dermatan sulfate had any appreciable effect on thrombin inactivation, even at high concentration (4 μg/assay).

DISCUSSION

The various anticoagulant activities associated with rabbit TM (see Introduction) all depend on the high affinity binding of thrombin to a site which appears to be located within the 5th and 6th epidermal growth factor-like repeats of the polypeptide (Kurosawa et al., 1988). Moreover, the expression of these properties, with the exception of the Protein C activation cofactor activity, invariably requires the presence of an acidic domain identified as a sulfated glycosaminoglycan. Attempts to further define this component, by determining the effects of specific glycanases on the anticoagulant activities of rabbit TM (Bourin et al., 1988; Bourin and Lindahl, 1990), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of native and chondroitinase-digested TM (Bourin, 1989), pointed to the presence of a single chondroitin sulfate chain. The results of the present study confirm this proposal, and provide more detailed information concerning the structure of the glycosaminoglycan component. They were obtained by direct analysis of the isolated glycan, following radiolabeling by chemical or metabolic techniques.

A conspicuous property of the N-[3H]acetyl-labeled glycosaminoglycan was the over-representation of labeled GalNAc(diS) residues. Once recognized as a characteristic of the nonreducing terminal unit, this anomaly could be used to specifically probe the terminal region and, in fact, highlighted what appears to be the outstanding structural feature of the TM glycosaminoglycan chain. A major fraction of such chains is terminated on a GalNAc(diS)-Glca-GalNAc(diS) trisaccharide sequence, whereas the internal portions have a conventional structure with predominantly monosulfated disaccharide units. The occurrence of nonreducing terminal GalNAc(diS) units has been reported previously, in studies of chondroitin sulfate from rat and chick embryo cartilage (Otsubo et al., 1985). A sulfotransferase which incorporates a sulfate group at C6 of terminal GalNAc 4-sulfate units was purified from quail oviduct (Nakanishi et al., 1981), and a specific role for this enzyme in the regulation of biosynthetic chain elongation (“capping” phenomenon) was proposed. It is questionable whether the TM glycosaminoglycan chain conforms to this concept, since not only the terminal but also the penultimate GalNAc residue carries two sulfate groups (Fig. 6). Such terminal accumulation of multiple sulfate groups appears to be a unique feature of the rabbit TM chondroitin sulfate. It is noted that no appreciable amounts of terminal GalNAc(diS) units were observed on analysis of similarly N-[3H]acetyl-labeled chondroitin sulfate C from shark cartilage (Sigma standard; data not shown).

Analysis of metabolically 35S-labeled TM isolated from rabbit heart endothelial cells confirmed the conclusion that this macromolecule is a chondroitin sulfate proteoglycan. Due to the relatively low overall yield of labeled TM, and the presumably uniform distribution of label among all available sulfate groups, no attempt was made to specifically define the structure at the nonreducing terminus. The identification of A4,5-unsaturated, mono-[35S]sulfated disaccharides as the major labeled products obtained after digestion with chondroitinase ABC is in agreement with the structure deduced for the N-[3H]acetyl-labeled TM glycosaminoglycan. It is noteworthy that the chondroitin [35S]sulfate associated with TM corresponded to only 1–2% of the total cell-associated sulfated glycosaminoglycans (based on the recovery of labeled macromolecules from the DEAE-cellulose chromatography step); TM thus appears to account for only a minor fraction of the total proteoglycans synthesized by the endothelial cells.

The structure-function relationships bearing on the glycosaminoglycan domain of the TM proteoglycan are only partly understood. It appears likely that some of the effects ascribed to this component, e.g., the ability to prevent thrombin-induced fibrinogen cleavage or Factor V activation (Bourin et al., 1988; Bourin and Lindahl, 1990), are primarily due to...
steric factors that render the bound thrombin inaccessible to interaction with certain proteins. With regard to the antithrombin-dependent anticoagulant activity of TM the effect of the glycosaminoglycan chain seems to be just the opposite; it promotes the interaction between the bound thrombin and antithrombin. The difference in molecular size between fibrinogen (M, 340,000) and Factor V (M, 330,000) on one hand, and antithrombin (M, 68,000) on the other, is probably of importance in this context. However, more specific factors must also be involved, since the glycosaminoglycan probably serves to approximate antithrombin towards the bound thrombin. Some antithrombin-dependent anticoagulant ("heparin-like") activity was expressed by the free glycan chain after release from the TM protein component by alkaline β-elimination. Free TM glycosaminoglycan thus differed from the standard chondroitin sulfate A and dermatan sulfate tested, neither of which showed any significant activity (Fig. 6), but was similar to chondroitin sulfate E which is known to be a weak anticoagulant (Scully et al., 1966). These findings suggest that the occurrence of L-iduronic acid units is less conducive to the particular anticoagulant activity studied than is the presence of disulfated GalNAc(diS) monosaccharide units. In chondroitin sulfate E, which has a higher overall sulfate density than the TM chondroitin sulfate, such units must occur in the internal portions of the glycosaminoglycan chain (Razin et al., 1982), whereas in the TM molecule they seem to be restricted to, or at least markedly over-represented at, the nonreducing terminus. The nature of the association between this strongly negatively charged "tail" and anticoagulant activity is unclear; in the appropriate affinity chromatography systems, the N-[3H]acetylethanolamide-labeled TM glycosaminoglycan failed to show any appreciable affinity for either antithrombin or disasopropyl phosphothrombin (data not shown). Nevertheless, it seems reasonable to assume that the distinctive distribution of sulfate groups in the TM glycosaminoglycan contributes somehow also to the antithrombin-dependent anticoagulant activity of the intact TM proteoglycan. This latter activity, much stronger than that of the isolated glycan chain, results from the concerted action of the glycosaminoglycan and protein components of the proteoglycan. It is essentially conceivable that binding to the Protein C activation site induces a conformational change in the thrombin molecule which increases its affinity for the chondroitin sulfate chain.

In recent years, a number of macromolecules, previously recognized simply as proteins, have been identified as proteoglycans and some of these species carry chondroitin sulfate chains. Type IX collagen is unique among the collagens in glycosaminoglycans and some of these species carry chondroitin sulfate chains. This latter activity, much stronger than that of the isolated glycan chain, results from the concerted action of the glycosaminoglycan and protein components of the proteoglycan. It is essentially conceivable that binding to the Protein C activation site induces a conformational change in the thrombin molecule which increases its affinity for the chondroitin sulfate chain.
Isolation and characterization of the glycosaminoglycan component of rabbit thrombomodulin proteoglycan.
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