Recombinant human thrombomodulin

Regulation of cofactor activity and anticoagulant function by a glycosaminoglycan side chain

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Two glycoforms of a secretable human thrombomodulin mutant [TMD1-105 and TMD1-75; Parkinson, Grinnell, Moore, Hoskins, Vlahos & Bang (1990) *J. Biol. Chem.* **265**, 12602–12610] were expressed in human 293 cells and used to study the role of glycosylation in the functions of this endothelial-cell thrombin receptor. Carbohydrate content analysis and intrinsic labelling with [³H]glucosamine and [³⁵S]sulphate showed that TMD1-105 contained a chondroitin sulphate whereas TMD1-75 did not. Other than chondroitin sulphate, the carbohydrate contents of the two glycoforms were identical, indicating similar glycosylation patterns at other *O*-linked and *N*-linked sites in the two glycoforms. The properties of TMD1-105 were converted into those of TMD1-75 by chondroitin ABC lyase digestion. Trypsin digestion of labelled TMD1-105 permitted isolation of two overlapping peptides that contained chondroitin sulphate, spanned the entire *O*-glycosylation domain and had *O*-glycosylation sites at Ser-492, Ser-498, Thr-500, Thr-504 and Thr-506. The chondroitin sulphate-attachment site was assigned to Ser-492 as this residue is conserved in mouse and bovine

thrombomodulin and lies within a sequence Ser-Gly-Ser-Gly-Glu-Pro, which has strong similarity to chondroitin sulphate attachment sites in other proteoglycans. Five peptides with *N*-linked carbohydrate were also isolated and contained glycosylation sites in the lectin-like domain (Asn-47, Asn-115, Asn-116) and in the fourth (Asn-382) and fifth (Asn-409) epidermal growth factor domains. The role of *N*-linked and simple *O*-linked carbohydrates in the functions of human thrombomodulin remain unclear. The present studies demonstrate, however, that the presence of chondroitin sulphate in human thrombomodulin has profound effects on all of the anticoagulant properties of this important anticoagulant thrombin receptor.

INTRODUCTION

Thrombomodulin (TM) plays a pivotal role in haemostasis by regulating the macromolecular specificity of thrombin at the endothelial-cell surface (Esmon, 1989). Formation of a high-affinity thrombin–TM complex accelerates the rate of thrombin-dependent formation of activated protein C and also directly inhibits thrombin's procoagulant activities (Esmon & Owen, 1981; Esmon *et al.*, 1983).

Expression of a secretable deletion mutant of human TM in human 293 and Syrian-hamster AV12 cells was recently reported (Parkinson *et al.*, 1990a). This mutant contained all of the extracellular domains of TM and was secreted as two discrete glycoforms of approx. 105 kDa and approx. 75 kDa (TMD1-105 and TMD1-75). The functional properties of TMD1-105 and TMD1-75 differed with respect to cofactor activity for protein C activation, Ca²⁺-dependence and inhibition of thrombin's procoagulant activities. These observations were the first report of a critical role for glycosylation in the functions of human TM. Preliminary evidence indicated that TMD1-105 contained a chondroitin sulphate glycosaminoglycan (CSGAG) whereas TMD1-75 did not. Results similar to those described above were confirmed by an independent study that showed the presence of chondroitin 4-sulphate in a secretable mutant of human TM expressed in Chinese-hamster ovary cells (Nawa *et al.*, 1990). A series of elegant studies with rabbit TM had previously shown a significant role for CSGAG in inhibiting thrombin's procoagulant activities and in accelerating the rate of thrombin inhibition by antithrombin III (Bourin, 1989; Bourin & Lindahl, 1990; Bourin *et al.*, 1988, 1990; Preissner *et al.*, 1987, 1990).

The present studies investigated the relationship between

TMD1-105 and TMD1-75 at the carbohydrate level in more detail. This was done in order to identify carbohydrate structures other than CSGAG that could account for the functional differences between the two glycoforms. The only difference in glycosylation between the two glycoforms that was identified was the presence of CSGAG in TMD1-105 and the absence of CSGAG from TMD1-75. The site of CSGAG attachment in TMD1-105 was localized to the *O*-glycosylation domain immediately *C*-terminal to the primary thrombin-binding site in epidermal growth factor domains 5 and 6. The results indicate that the presence of CSGAG in the *O*-glycosylation domain of human TM can regulate all the anticoagulant functions of this important thrombin receptor.

MATERIALS AND METHODS

Materials

Recombinant TMD1-105 and TMD1-75 expressed in human 293 cells were purified as described previously (Parkinson *et al.*, 1990a). Bovine thrombin (2400 N.I.H. units/mg) was from Enzyme Research Laboratories (South Bend, IN, U.S.A.). Recombinant human protein C was purified as previously described (Grinnell *et al.*, 1987). Superose-6 HR 10/30 and Mono-Q HR 5/5 columns were from Pharmacia/LKB (Piscataway, NH, U.S.A.). [³H]Glucosamine (30–60 Ci/mmol) and H₂³⁵SO₄ (1050–1600 Ci/mmol) were from DuPont NEN Research Products (Boston, MA, U.S.A.). Dulbecco's modified Eagle's medium and gentamicin were from Gibco Laboratories (Grand Island, NY, U.S.A.). Fetal-calf serum was from HyClone Laboratories. Chondroitin ABC lyase was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hygromycin B was from Eli Lilly and

Abbreviations used: TM, thrombomodulin; CSGAG, chondroitin sulphate glycosaminoglycan.

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Co. (Indianapolis, IN, U.S.A.). Unless otherwise stated, all other reagents were of highest purity commercially available.

Metabolic labelling of TMD1

A stable human 293 cell transformant expressing recombinant TMD1 was maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal-calf serum, 50 µg of gentamicin/ml and 200 µg of hygromycin/ml. The line was grown to confluence in a roller bottle and 100 ml serum-free medium collections were obtained daily as described previously (Parkinson *et al.*, 1990a). Serum-free medium contained 5 µCi of [³H]glucosamine/ml and 10 µCi of [³⁵S]sulphate/ml as precursors for *N*-/*O*-linked carbohydrate and sulphated glycosaminoglycans respectively. Labelled recombinant TMD1-105 and TMD1-75 were purified from 1 litre of serum-free medium as described previously (Parkinson *et al.*, 1990a).

Reduction and carboxymethylation

Samples of TMD1-105 or TMD1-75 in 0.5 ml of 6 M-guanidinium hydrochloride/20 mM-Tris/HCl buffer, pH 8.5, were reduced by incubation with 5% (v/v) 2-mercaptoethanol for 2 h at room temperature. Samples were then treated with 50 µl of vinylpyridine for 1.5 h in the dark, acidified with 200 µl of 70% (v/v) formic acid and buffer-exchanged with the use of Centricon-30 microconcentrators (Amicon, Lexington, MA, U.S.A.).

Carbohydrate analysis

Reduced and carboxymethylated samples of TMD1-105 and TMD1-75 were desalted into 50 mM-ammonium bicarbonate, freeze-dried and resuspended in water. Sialic acid (*N*-acetylneuraminic or *N*-acetylglucosylneuraminic acid) released by neuraminidase and neutral/basic monosaccharides released by trifluoroacetic acid hydrolysis were analysed by high-pH anion-exchange h.p.l.c. with the use of pulsed amperometric detection as described previously (Yan *et al.*, 1990). Sialic acid yields from 2 h and 4 h digests were within 5% of each other and the average for the two experiments was taken. Yields of other monosaccharides were taken as the average of duplicates after hydrolysis with 2 M-trifluoroacetic acid for 6 h. Glycosyl content was determined as mol/mol of protein by using a calculated molecular mass of 52 kDa for both TMD1-105 and TMD1-75. Previous studies showed that both glycoforms had the same sequence and composition, consistent with a polypeptide comprising residues Ala-19-Ser-515 (Parkinson *et al.*, 1990a).

Chondroitin lyase digestions

For SDS/PAGE and cofactor activity studies, samples of TMD1-105 and TMD1-75 (75 µg in 1 ml) were digested with or without 200 milliunits of chondroitin ABC lyase as described previously (Parkinson *et al.*, 1990a). For gel-filtration analysis, samples of labelled TMD1-105 (1×10^6 total c.p.m.) and TMD1-75 (0.34×10^6 total c.p.m.) were incubated with and without 50 milliunits of chondroitin ABC lyase in a total volume of 0.5 ml. Superose-6 gel-filtration analyses of 0.2 ml portions from each incubation were performed as described previously (Parkinson *et al.*, 1990a). Fractions (0.5 ml) were collected and portions were removed for scintillation counting to determine ³H and ³⁵S content.

Trypsin digestion and peptide mapping

Labelled TMD1-105 was mixed with unlabelled TMD1-105 at a ratio of 1:20, reduced and carboxymethylated as described above, and buffer-exchanged into 0.1 M-sodium phosphate buffer, pH 7.5. Tos-Phe-CH₂Cl-treated trypsin (type XIII; Sigma Chemical Co.) was added at a 1:50 trypsin/TMD1-105 weight

ratio and the sample was incubated at 37 °C for 18 h. For isolation of CSGAG-containing peptides, Mono-Q anion-exchange chromatography was used (see the Results section). Other tryptic-digest peptides were separated by reverse-phase h.p.l.c. on a BakerBond Widepore 5 µm-particle-size column (4.6 mm × 250 mm) with a linear gradient of 5–50% (v/v) acetonitrile in 0.1% trifluoroacetic acid over 45 min (1 ml/min). Effluent was monitored simultaneously for u.v. absorbance at 220 nm with a Waters 485 u.v. detector and for radioactivity with a Radiomatic Flow-1-Beta on-line radioactivity detector to monitor both ³H and ³⁵S; a Flow-scint IV (Radiomatic, Meriden, CT, U.S.A.)/h.p.l.c. effluent ratio of 4:1 (v/v) was used. A stream-splitter valve diverted 25% of the effluent into the radioactivity detector, while the remaining 75% was collected in 1 min fractions. Data were processed by using the PE-Nelson Turbochrome 2700 software.

Cofactor activity assays

The Ca²⁺-dependence of TMD1-105 and TMD1-75 cofactor activity was determined by a two-stage amidolytic assay with the use of thrombin and recombinant human protein C as described previously (Parkinson *et al.*, 1990a). Cofactor activity (thrombin-dependent formation of activated protein C) was determined 2 min after the addition of chromogenic substrate S-2366 (Kabi Vitrum, Franklin, OH, U.S.A.) and expressed as *A*₄₀₅ units.

Scintillation counting of radioactivity

The ³H and ³⁵S contents of labelled proteins were determined with a Beckman LS5000 TD scintillation counter, with Scintisol (Isolab, Akron, OH, U.S.A.) as scintillant.

Electrophoresis

SDS/PAGE analysis of chondroitin lyase digests of TMD1-105 and TMD1-75 were performed under reducing conditions with the use of silver-stained 10–15% gradient Phastgels (Pharmacia/LKB) as described previously (Parkinson *et al.*, 1990a).

Protein assays

Protein assays were performed with the bichinonic acid assay reagent kit (Pierce Chemical Co., Rockford, IL, U.S.A.), with BSA as standard.

RESULTS

Carbohydrate composition of TMD1-105 and TMD1-75

Table 1 summarizes the glycosyl contents of TMD1-105 and TMD1-75. The results confirmed that the extracellular domains of TM were extensively glycosylated. Except in the case of galactosamine, the glycosyl contents of the two glycoforms were essentially identical. The presence of galactose and sialic acid in both glycoforms is indicative of complex-type *N*-linked carbohydrates.

The high yield of galactosamine from TMD1-105 compared with that of TMD1-75 (29.1 and 4.9 mol/mol of protein respectively) is consistent with the presence in TMD1-105 of a galactosaminoglycan that is absent from TMD1-75. However, some caution is needed in the interpretation of these findings. The repeating disaccharide unit of chondroitin sulphate dermatan sulphate polymers comprises *N*-acetylgalactosamine linked to either glucuronic acid or iduronic acid (Conrad, 1989). These polymers are relatively acid-resistant and the stability and yields of their trifluoroacetic acid hydrolysis products are poorly characterized. It was possible, nevertheless, to detect a monosaccharide with elution characteristics identical with those of standard glucuronic acid in trifluoroacetic acid hydrolysates of

Table 1. Monosaccharide content of TMD1-105 and TMD1-75 expressed in 293 cells

Reduced and carboxymethylated samples of TMD1-105 and TMD1-75 were analysed for sialic acid and neutral/basic monosaccharide content as described in the Materials and methods section.

Monosaccharide	Glycosyl content (mol/mol of protein)	
	TMD1-105	TMD1-75
Fucose	7.0	7.7
Galactose	20.0	20.8
Glucose	1.1	1.2
Mannose	14.2	14.8
Glucosamine	21.8	22.1
Galactosamine	29.1	4.9
Glucuronic acid	30.5	< 1.0
Sialic acid	8.2	8.9

TMD1-105 (30.5 mol/mol of protein). No glucuronic acid could be detected in TMD1-75 (< 1.0 mol/mol of protein). The similar yields of galactosamine and glucuronic acid from TMD1-105 strongly support the presence of CSGAG in this glycoform. The absence of glucuronic acid from TMD1-75 suggests that galactosamine present in this glycoform may derive from carbohydrates distinct from CSGAG. These could be *O*-linked structures having *N*-acetylgalactosamine as the linkage sugar to serine/threonine of the core protein, or could also be *N*-linked. *N*-Acetylgalactosamine in *N*-linked carbohydrate is unusual, but has been found in human protein C expressed in the human 293 cells that were used for the current study (Yan *et al.*, 1990). The similarity of the glycosyl contents of the other sugars in TMD1-105 and TMD1-75 suggests that the galactosamine observed in TMD1-75 derives from carbohydrates that are also present in TMD1-105.

Metabolic labelling of TMD1-105 and TMD1-75

The ³H and ³⁵S contents of TMD1-105 and TMD1-75 are summarized in Table 2. In both samples over 95% of total radioactivity was precipitable with 10% (w/v) trichloroacetic acid. Incorporation of ³H into TMD1-105 (51 700 c.p.m./μg) was 3-fold higher than for TMD1-75 (16900 c.p.m./μg). More notably, the incorporation of ³⁵S into TMD1-105 (10800 c.p.m./μg) was approx. 27-fold higher than for TMD1-75 (403 c.p.m./μg). The high ³⁵S contents in TMD1-105 support the proposal that TMD1-105 is post-translationally modified by the addition of a sulphated glycosaminoglycan, in agreement with the glycosyl content analyses described above.

Chondroitin lyase digestion of TMD1-105 and TMD1-75

The presence of chondroitin sulphate in TMD1-105 and TMD1-75 was also studied by chondroitin ABC lyase digestion and SDS/PAGE. The results are shown in Fig. 1. The electrophoretic mobility of digested TMD1-105 (lane 3) was identical with that of TMD1-75 (lane 4) and with that of TMD1-75 digested with chondroitin lyase (lane 5). This band is marked with an asterisk in the Figure to distinguish it from other bands in the digests, which derive from the chondroitin lyase preparation (lane 6). These findings could be confirmed by Western blotting with anti-TMD1 polyclonal antibodies (results not shown).

The relationship between TMD1-105 and TMD1-75 was also studied by digestion of the intrinsically labelled proteins with chondroitin ABC lyase followed by analysis of the digestion products by Superose-6 gel-filtration chromatography. The results are shown in Fig. 2. TMD1-75 was resistant to digestion

Table 2. Incorporation of [³H]glucosamine and [³⁵S]sulphate into TMD1-105 and TMD1-75

Samples of purified TMD1-105 and TMD1-75 that had been metabolically labelled with [³H]glucosamine and [³⁵S]sulphate as described in the Materials and methods section were assayed for protein content, and ³H and ³⁵S contents were determined by scintillation counting of radioactivity.

Glycoform	³ H content	³⁵ S content
	(c.p.m./μg of protein)	(c.p.m./μg of protein)
TMD1-105	51 700	10800
TMD1-75	16900	403

Fig. 1. SDS/PAGE of chondroitin lyase digests of TMD1-105 and TMD1-75

Samples of TMD1-105 (lanes 2 and 3) and TMD1-75 (lanes 4 and 5) were incubated with (lanes 3 and 5) and without (lanes 2 and 4) 200 munits of chondroitin ABC lyase as described in the Materials and methods section. The Figure shows a silver-stained 10–15% gradient phast gel of reduced portions of the digests (approx. 100 ng/lane). Molecular-mass markers are indicated (lanes 1 and 7). The band at approx. 75 kDa (lanes 3–5) is marked with an asterisk to distinguish it from proteins in the chondroitin lyase preparation that was run as a control (lane 6).

with chondroitin lyase: all the ³H and ³⁵S radioactivity was recovered in the same fractions as undigested material (see panels *a* and *b*). ³⁵S in TMD1-75 was not released by chondroitin lyase, suggesting the presence of sulphated sugars distinct from CSGAG. The absence of low-molecular-mass products from the digest of TMD1-75 suggested that the chondroitin ABC lyase preparation used was not contaminated with non-specific glycosidases and that the digest conditions used did not lead to proteolysis. For TMD1-105, approx. 60% of total ³H and ³⁵S radioactivity was recovered as low-molecular-mass products (presumably chondroitin sulphate disaccharides) after chondroitin lyase digestion (see panel *c*). The protein fraction from the digest of TMD1-105 was eluted in the same fractions as that from TMD1-75, and contained about 30% of the total ³H and ³⁵S radioactivity. A small fraction of TMD1-105 (< 10% of total radioactivity) remained undigested. When considering ³⁵S radioactivity only, chondroitin lyase digestion resulted in the release of approx. 90% of the radiolabel from TMD1-105 as low-molecular-mass products (see panel *d*). These results indicate that all of the increased ³H and ³⁵S content of TMD1-105 as compared with TMD1-75 (see above) can be accounted for by the presence of CSGAG. The CSGAG moiety thus represents

Fig. 2. Chondroitin lyase digests of labelled TMD1-75 and TMD1-105

The Figures shows Superose-6 gel-filtration chromatograms of labelled TMD1-75 and TMD1-105 that were incubated with (●) and without (○) chondroitin ABC lyase. Fractions (0.5 ml) were collected and ^3H and ^{35}S contents were determined in 0.2 ml portions. Panels (a) and (c) show total ^3H and ^{35}S radioactivities for TMD1-75 and TMD1-105 respectively. Panels (b) and (d) show ^{35}S radioactivities for TMD1-75 and TMD1-105 respectively. Exclusion (V_0) and total (V_t) volumes of the column are indicated by arrows. The details of the experiments are described in the Materials and methods section.

about two-thirds of the total carbohydrate present in TMD1-105.

Role of CSGAG in protein C activation by TMD1-105 and TMD1-75

Previous studies showed that the Ca^{2+} -dependences of TMD1-105 and TMD1-75 are markedly different (Parkinson *et al.*, 1990a). Fig. 3 shows the effects of chondroitin lyase digestion on the Ca^{2+} -dependence of TMD1-105 and TMD1-75 cofactor activity. The Ca^{2+} -dependence of TMD1-75 was unaffected by chondroitin lyase digestion. In contrast, a dramatic change in the Ca^{2+} -dependence of TMD1-105 was seen after chondroitin lyase digestion. Digested TMD1-105 had a Ca^{2+} -dependence virtually identical with that of TMD1-75.

Glycosaminoglycan-attachment site in TMD1-105

Given the critical role of CSGAG in the functions of human TM, attempts were made to identify the site of CSGAG attachment. Labelled TMD1-105 was digested with Tos-Phe- CH_2Cl trypsin, and Mono-Q anion-exchange chromatography was used to separate acidic CSGAG-containing peptides from unglycosylated peptides and peptides containing other carbohydrate structures. This chromatogram is shown in Fig. 4. Two peaks, one in the breakthrough and one eluted at approx. 0.5 M-NaCl, contained ^3H but no ^{35}S . A third peak was eluted by stepping the gradient from 1 M- to 2 M-NaCl, and this contained over 95% of the ^{35}S radioactivity and most of the ^3H radioactivity. The high recovery of ^3H radioactivity in this peak suggested that the isolated CSGAG-containing peptides contained carbohydrates additional to CSGAG.

The isolated CSGAG-containing peptides were desalted,

Fig. 3. Effects of chondroitin lyase on Ca^{2+} -dependence of TMD1-105 and TMD1-75 cofactor activity

Samples of TMD1-105 (● and ○) and TMD1-75 (■ and □) were treated with (● and ■) and without (○ and □) chondroitin ABC lyase as described in the Materials and methods section. Cofactor activity for thrombin-dependent formation of activated protein C (A_{405} units) was determined at the indicated Ca^{2+} concentrations as described in the Materials and methods section.

freeze-dried and sequenced. The data obtained are summarized in Table 3. Two overlapping peptides were observed, both of which started at the C-terminal side of expected trypsin-cleavage

Fig. 4. Isolation of peptides containing chondroitin sulphate from a trypsin digest of labelled TMD1-105

Labelled TMD1-105 was reduced, carboxymethylated and digested with Tos-Phe-CH₂Cl-treated trypsin as described in the Materials and methods section. The digest was chromatographed on a Mono-Q HR 5/5 anion-exchange column at a flow rate of 1 ml/min with the indicated gradient (broken line) from 0 M to 2 M-NaCl in 20 mM-Tris/HCl buffer, pH 8.0. Fractions (1 ml) were collected and 20 µl portions were removed for scintillation counting to determine ³H (○) and ³⁵S (●) content. Fractions 16–20, which contained over 90 % of the ³⁵S radioactivity, were collected for sequence analysis.

Table 3. Amino acid sequences of chondroitin sulphate-containing peptides isolated from TMD1-105

Chondroitin sulphate-containing peptides isolated by anion-exchange chromatography (see Fig. 4) were desalted and subjected to N-terminal sequence analysis. The quantity of amino acid obtained at each cycle is shown. Peptide 1 and peptide 2 correspond to residues Val-485-His-514 and His-475-Pro-496 of the human TM sequence.

Cycle no.	Peptide 1		Peptide 2	
	Amino acid	Amount (pmol)	Amino acid	Amount (pmol)
1	Val	81	His	8
2	Asp	—	Ile	47
3	Gly	—	Gly	—
4	Gly	—	Thr	—
5	Asp	14	Asp	14
6	Ser	20	Cys	—
7	Gly	34	Asp	10
8	Ser	8	Ser	8
9	Gly	27	Gly	27
10	Glu	46	Lys	30
11	Pro	36	Val	18
12	Pro	56	Asp	25
13	Pro	56	Gly	16
14	Ser	—	Gly	—
15	Pro	26	Asp	16
16	Thr	—	Ser	7
17	Pro	31	Gly	14
18	Gly	17	Ser	—
19	Ser	5	Gly	—
20	Thr	—	Glu	8
21	Leu	16	Pro	6
22	Thr	—	Pro	8
23	Pro	14		
24	Pro	—		
25	Ala	12		
26	Val	6		
27	Gly	5		
28	Leu	6		
29	Val	4		
30	His	3		

Ser-492 in peptide 2 was a blank cycle, indicating that this residue was glycosylated.

Identification of N-linked glycosylation sites in TMD1-105

An h.p.l.c. tryptic peptide map of labelled TMD1-105 is shown in Fig. 5. In addition to the breakthrough peak, which contained most of the ³H radioactivity (essentially all the ³⁵S radioactivity was also recovered in this peak), five ³H-containing peptides were identified. These peptides were sequenced and identified as follows: peptide 1, Ala-372–Asn-382 (N-linked sugar at Asn-382); peptide 2, Gly-106–Tyr-119 (N-linked sugar at either Asn-115 or Asn-116); peptide 3, Ala372–Ala392 (N-linked sugar at Asn-382); peptide 4, Ala-37–Ile-51 (N-linked sugar at Asn-47); peptide 5, Cys-404–Ala-423 (N-linked sugar at Asn-409). In peptides 1, 3, 4 and 5, blank cycles were seen for the N-linked asparagine residues. In the case of peptide 2, asparagine was present at low amounts in the sequencing cycles corresponding to residues Asn-115 and Asn-116, suggesting that TMD1-105 was produced as a mixture of proteins partially glycosylated at both these sites. Peptides 1 and 3 were most probably the results of differential C-terminal tryptic cleavage. Peptide 2 resulted from tryptic cleavage of a Phe–Ala bond. Such a cleavage is unusual, but has been observed previously (Kriauciunas *et al.*, 1991; C. J. Vlahos, unpublished work).

Fig. 5. Tryptic peptide map of labelled TMD1-105

Peptides derived from a Tos-Phe-CH₂Cl-treated trypsin digest of labelled TMD1-105 were subjected to reverse-phase h.p.l.c. as described in the Materials and methods section. Fractions were monitored for u.v. absorbance at 220 nm (panel a) and for ³H content (panel b). Peptides 1–5 were sequenced to identify N-glycosylation sites (see the Results section).

sites, indicating that the trypsin digest was not complete. The corresponding sequences in human TM were Val-485–His-514 for peptide 1 and His-475–Pro-496 for peptide 2. The peptides span the entire O-glycosylation domain of human TM and contain several serine and threonine residues, which are potential glycosylation sites (Wen *et al.*, 1987). The low yield of amino acids made the identification of O-glycosylated sites difficult. Blank cycles were seen for Ser-498, Thr-500, Thr-504 and Thr-506 in peptide 1, indicating these to be sites of O-glycosylation. In both peptides, Ser-490 did not appear to be glycosylated. The glycosylation of Ser-492 in peptide 1 could not be assigned owing to overlap with Ser-482 at the same cycle in peptide 2. However,

DISCUSSION

An extensive study of the functional properties of TMD1-105 and TMD1-75 was previously reported (Parkinson *et al.*, 1990a). The two glycoforms differed with respect to K_d for thrombin, k_{cat} for thrombin-dependent protein C activation and Ca^{2+} -dependence for protein C activation. They also differed in their ability to directly inhibit thrombin's procoagulant activities, e.g. fibrinogen cleavage and platelet activation. This latter activity was considerably higher for TMD1-105 than for TMD1-75, and only TMD1-105 was sensitive to digestion with chondroitin ABC lyase.

In the present studies, attempts to identify carbohydrate structures other than CSGAG that could account for the functional differences between the two glycoforms were unsuccessful. The only difference in glycosylation between TMD1-105 and TMD1-75 that could be identified was the presence of CSGAG in TMD1-105. This was corroborated by both glycosyl content analysis and by chondroitin lyase digestion of labelled proteins. The results also showed that the biochemical and functional properties of TMD1-105 were converted into those of TMD1-75 simply by digestion with chondroitin ABC lyase. This forces the conclusion that all of the functional differences between the two glycoforms can be accounted for by CSGAG. Studies with rabbit TM showed that CSGAG was involved in inhibition of thrombin's procoagulant activities and acceleration of thrombin inhibition by antithrombin III, but not in protein C activation by the thrombin-TM complex (Bourin *et al.*, 1988, 1990; Bourin, 1989). These studies did not examine, however, the Ca^{2+} -dependence of protein C activation as is described here. Removal of CSGAG from TMD1-105 clearly converted the Ca^{2+} -dependence of protein C activation into that of TMD1-75. This regulatory role of CSGAG in protein C activation by the thrombin-TM complex has previously been overlooked and suggests a much wider role for CSGAG in regulation of the functions of the thrombin-TM complex than had previously been appreciated.

Subtraction of the glucosamine content of TMD1-75 from that of TMD1-105 gives a conservative estimate of an average CSGAG chain length of 25 disaccharide units/molecule of TMD1-105. It is not surprising that a highly anionic sulphated polysaccharide of this size would have major effects on the functions of the thrombin-TM complex. Electrostatic interactions in a ternary complex of thrombin, TM and protein C would be expected to be profoundly affected by such a structure.

X-ray crystallography of thrombin has shown several surface clusters of positively charged residues along an extended groove, distant from the active site (Bode *et al.*, 1989; Rydel *et al.*, 1990). This groove, often referred to as the 'anion-binding exosite', is a determinant of thrombin's substrate-specificity. Electrostatic interaction between positively charged surface loops in the exosite (or possibly other sites) of thrombin and the negatively charged CSGAG chain of TMD1-105 may account for the higher thrombin affinity of TMD1-105 (K_d approx. 3 nM) than TMD1-75 (K_d approx. 16 nM). By binding to the exosite region of thrombin, CSGAG would act as both a steric and an electrostatic impediment to interaction of thrombin with its procoagulant substrates and platelet/endothelial receptors. Absence of CSGAG, as in TMD1-75, would result in a lower-affinity thrombin-TM complex and allow for interaction of thrombin's substrates or receptors with the exosite. This would explain the marked difference in the abilities of TMD1-105 and TMD1-75 to inhibit thrombin's procoagulant activities and to inhibit thrombin-stimulated platelet activation (Parkinson *et al.*, 1990a).

Interaction between the γ -carboxyglutamic acid-containing (Gla) domain of protein C and the epidermal growth factor

repeats of TM via a Ca^{2+} bridge has been suggested (Kurosawa *et al.*, 1987). CSGAG present in the *O*-glycosylation domain of TMD1-105 may cause charge repulsion of the negatively charged Gla domain of protein C. The 10-fold higher Ca^{2+} optimum of TMD1-105 (3 mM) compared with TMD1-75 (0.3 mM) may reflect additional Ca^{2+} ions required to neutralize this charge repulsion effect of CSGAG and to allow the formation of the Ca^{2+} bridge between the two proteins. Such a mechanism is consistent with the effects of chondroitin lyase on the Ca^{2+} -dependency of TMD1-105, as discussed above.

Because of the complexity of glycosylation in the *O*-glycosylation domain of TMD1-105, the location of CSGAG attachment could not be unequivocally identified. CSGAG attachment in proteoglycans typically occurs via a xylose *O*-linked to serine in sequences containing Ser-Gly repeats (Bourdon *et al.*, 1987). The tetrapeptide sequence Ser-490-Gly-493 was proposed as a potential site of CSGAG attachment in human TM (Bourin *et al.*, 1988), and was present in both CSGAG-containing peptides isolated in this study. The data obtained for these peptides indicated that Ser-492 was glycosylated but Ser-490 was not. It is noteworthy that Ser-492 is conserved in the human, bovine and mouse TM genes, whereas Ser-490 is not (Wen *et al.*, 1987; Jackman *et al.*, 1986; Dittman *et al.*, 1988). The site of CSGAG attachment in TMD1-105 can thus be conditionally assigned to Ser-492. More detailed peptide mapping studies and/or site-specific mutagenesis of the *O*-glycosylation sites in this domain will be required for confirmation of this assignment.

The present study identified *N*-glycosylation sites in TMD1-105 at Asn-47, Asn-382 and Asn-409. These sites all conform to the *N*-glycosylation consensus sequence and were predicted from earlier molecular-biology studies (Wen *et al.*, 1987). It was difficult to assign glycosylation at Asn-115 and Asn-116 because small quantities of asparagine were seen in the sequencing cycles corresponding to each of these residues rather than the expected blank cycles. The data suggest that TMD1-105 expressed in human 293 cells was partially glycosylated at both these sites. Further studies will be required to confirm this finding. The amino acid sequence in this region, Thr-Gly-Asp-Asn-Asn-Thr-Ser, does not appear to favour one asparagine residue over the other for *N*-glycosylation. The nature and function of the carbohydrate chains present at the sites described above were not studied. Asn-47, Asn-115 and Asn-116 all lie within the *N*-terminal domain of TM. The function of this domain is not known, but it has sequence similarity to lectin-like domains in other proteins (Patthy, 1988). The presence of TM at cell-cell interfaces in non-vascular tissues has led to the suggestion that the lectin domain may confer TM with carbohydrate-dependent adhesive properties involved in cell-cell interactions (Imada *et al.*, 1990). The function of the carbohydrate at Asn-409 is not known, but this site is conserved and also glycosylated in the fifth epidermal growth factor domain of rabbit TM and is thus at or near the primary thrombin-binding site (Stearns *et al.*, 1988). Removal of this carbohydrate by digestion with *N*-Glycanase, however, did not appear to effect thrombin binding. The function of this carbohydrate and that at Asn-382 in the fourth epidermal growth factor domain are thus not known.

The present studies clearly demonstrate the presence and dramatic functional significance of CSGAG in the extracellular domains of human TM. The presence of CSGAG in native human endothelial-cell TM, either in culture or *in vivo*, remains controversial (Dittman & Majerus, 1990). Human TM purified from placenta had a molecular mass of approx. 105 kDa and did not appear as a diffuse band on SDS/PAGE, as would be expected for a protein modified with heterogeneous CSGAG (Salem *et al.*, 1984). Moreover, human placental TM was a poor inhibitor of thrombin's procoagulant activities (Maruyama *et*

al., 1985), and in this regard resembled TMD1-75 and not TMD1-105. There is thus no evidence that native human TM is modified by CSGAG. In these earlier studies, however, the placental TM was eluted from anion-exchange columns at low ionic strength (below 0.5 M-NaCl), and in fact 80 % of cofactor activity was lost in this step. Recombinant human TM that is modified with CSGAG is eluted from anion-exchange resins at high anionic strength (> 1.0 M-NaCl; Parkinson *et al.*, 1990a). The high losses of cofactor activity described in the earlier studies could be explained by placental TM modified with CSGAG not having been eluted from the anion-exchange columns with the low-ionic-strength gradients used. Indirect evidence for the presence of CSGAG on human endothelial-cell TM has been obtained (Parkinson *et al.*, 1990b). Cell-surface TM showed decreased affinity for thrombin following treatment of human endothelial-cell cultures with β -D-xyloside, an inhibitor of CSGAG attachment to proteoglycans, suggesting that cultured human endothelial cells do possess TM that is modified by CSGAG.

The studies described above and those presented here suggest that the presence of CSGAG in human TM *in vivo* should be re-investigated since it is clear that the presence of this carbohydrate has profound effects on TM function. The analytical techniques described in the present studies, particularly metabolic labelling, have permitted identification of all of the glycosylation sites in the extracellular domains of human TM and could be used to resolve this issue. Finally, the present studies indicate that human TM should be considered as a 'part-time' proteoglycan, raising the possibility that tissue-specific regulation of TM glycosylation (e.g. addition of CSGAG) may be a mechanism for regulation of the protein C anticoagulant pathway and the anticoagulant properties of the endothelial-cell surface.

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