

Identification of the predominant glycosaminoglycan-attachment site in soluble recombinant human thrombomodulin: potential regulation of functionality by glycosyltransferase competition for serine⁴⁷⁴

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Thrombomodulin (TM) is an endothelial cell thrombin receptor that converts thrombin from a procoagulant to an anticoagulant enzyme. It has previously been shown that TM is expressed in both a high-*M_r* form containing chondroitin sulphate and a low-*M_r* form lacking this modification. Site-directed mutagenesis of a soluble human TM derivative (TMD1) was employed to determine the attachment site(s) of this functionally important oligosaccharide on the core protein. Although there are four serine residues within the Ser/Thr-rich domain of TMD1 that might support glycosaminoglycan assembly, our analysis demonstrates that the primary site of attachment is at Ser⁴⁷⁴, and evidence is presented for low levels of attachment at Ser⁴⁷². It was possible to improve the overall degree of attachment by mutating

Ser⁴⁷² to glutamic acid (so as to conform Ser⁴⁷⁴ to the xylosyltransferase acceptor consensus acidic-Gly-Ser-Gly-acidic); however, a significant proportion (approx. 35%) of the total TM still lacked a glycosaminoglycan moiety. Mutants that possess a substitution for Ser⁴⁷⁴ show an increased mobility of their low-*M_r* form on SDS/PAGE compared with native TMD1. Isolation and sequencing of a C-terminal peptide demonstrated that this serine is modified in the low-*M_r* form of native TMD1. An apparent 'acceptor consensus overlap' at Ser⁴⁷⁴ suggests that the mechanism behind the glycosaminoglycan split of TM may involve a competition for substrate between xylosyltransferase and *N*-acetylgalactosaminyltransferase.

INTRODUCTION

Thrombomodulin (TM) is an integral membrane protein of vascular endothelium which serves as a key regulatory molecule of the coagulation process (for review, see refs. [1–4]). The anticoagulant effects of TM on thrombin are manifested: (a) directly, by interfering with cleavage of fibrinogen by thrombin [5,6], activation of platelets [7] and feedback amplification of the clotting cascade by factor V [5] and factor VIII [8–10] activation; (b) indirectly, by facilitating the association of the inhibitor antithrombin III with thrombin [11,12]; and (c) by its role as a cofactor for thrombin-dependent activation of protein C, increasing the activation rate more than a thousandfold compared with free α -thrombin [13,14]. Once activated, protein C down-regulates both the intrinsic and extrinsic coagulation pathways by proteolytically inactivating clotting factors V₁ and VIII₁ [9,15–19]. Thus TM effectively converts thrombin from a procoagulant into an anticoagulant enzyme.

Sequence analysis of human [20,21], bovine [22] and mouse [23] cDNAs has elucidated the following structure for TM: a large N-terminal domain, a series of six repeats similar to epidermal growth factor (EGF), a Ser/Thr-rich *O*-glycosylation domain, a transmembrane domain and a cytoplasmic C-terminal domain (Figure 1a). Recently, significant advances have been made in understanding the structural basis for interactions between TM and thrombin by the use of biochemical and recombinant DNA techniques. A primary binding site for thrombin has been localized to the fifth and sixth EGF-like repeats of TM [26–29], with the Ser/Thr-rich domain serving as

an important spacer to position this site correctly above the membrane surface [30,31]. In addition to this 'scaffold' function, the Ser/Thr-rich domain is also the probable attachment site for a glycosaminoglycan side chain(s) which may serve as a secondary binding site for thrombin [32,33]. This highly charged oligosaccharide probably contributes to the anticoagulant activity of TM by binding and directing antithrombin III to complexed thrombin [11,12,32–34], by altering the conformation of the active site of thrombin such that it is more susceptible to inhibition [33,35], and by increasing its cofactor affinity for activation of protein C by thrombin [32,33,36–38].

The fact that the glycosylation state of TM affects its anticoagulant potential is especially intriguing in the light of observations that TM can be expressed both with and without glycosaminoglycan modification. The earliest suggestion of this glycoform split came during the ion-exchange purification of rabbit lung TM by Bourin et al. [11], in which it was noted that thrombin cofactor activity was separated into non-acidic (unretarded) and acidic (retarded) fractions, with the acidic (glycosaminoglycan-modified) fraction representing approx. 50% of the total material. For human TM, the *in vivo* degree of glycosaminoglycan modification remains unclear. Early attempts to purify human endothelial-cell TM [3,39] yielded material that apparently lacked glycosaminoglycans. These purification schemes, however, employed elution from anion-exchange columns at low ionic strength which would have favoured recovery of primarily non-proteoglycan forms of TM [36,40] and indeed, approx. 80% of cofactor activity was lost in this step of the purification. More recent studies [37] have, in fact, provided

Abbreviations used: APTT, activated partial thromboplastin time; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; GalNAc transferase, *N*-acetylgalactosaminyltransferase; TM, thrombomodulin; MAb, monoclonal antibody; ECL, enhanced chemiluminescence.

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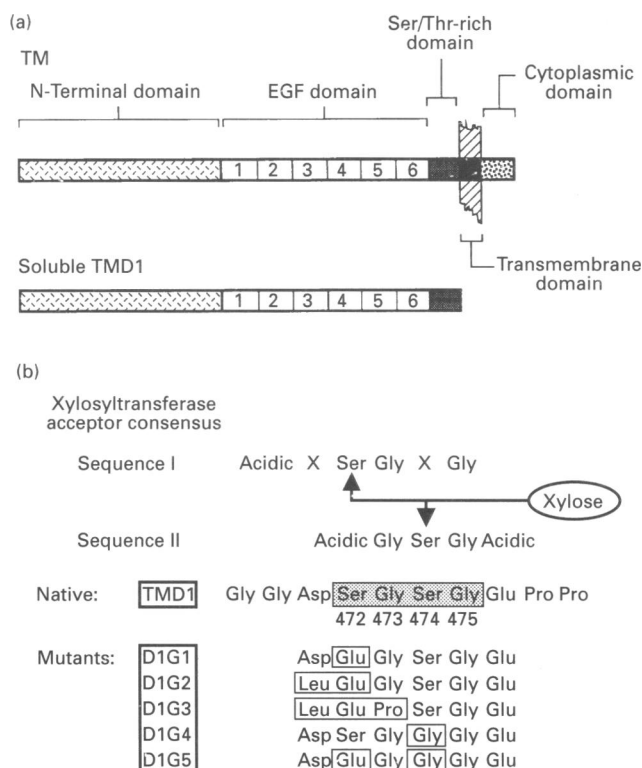


Figure 1 Schematic of TM, soluble TMD1 and TMD1 derivatives

(a) Human TM consists of an extracellular N-terminal domain, an EGF domain comprised of six repeats similar to EGF, a Ser/Thr-rich domain, a transmembrane domain and C-terminal cytoplasmic domain. TM mutant TMD1, by virtue of the deletion of coding sequence for the transmembrane and cytoplasmic domains, is secreted into cell culture medium. (b) Glycosaminoglycan side chain synthesis is initiated by the attachment of xylose to the appropriate serine residue of the core protein. The region surrounding the Ser⁴⁷²-Gly⁴⁷⁴-Gly tetrapeptide (native TMD1, boxed/shaded residues) of the Ser/Thr-rich domain of TM is depicted, along with an optimal alignment of the two proposed xylosyltransferase acceptor sequences. Sequence I is the xylosyltransferase acceptor consensus sequence proposed by Bourdon et al. [24], and sequence II is that of Zimmermann and Ruoslahti [25]. Also indicated are amino acid changes in this region for TMD1 derivatives obtained by site-directed mutagenesis (mutants D1G1–5, boxed residues). Numbering of amino acids is based on the processed/mature N-terminus of TM as described by Suzuki et al. [20].

evidence for the presence of glycosaminoglycans on human endothelial-cell TM based on the inhibition of thrombin cofactor activity after treatment of cells with β -D-xyloside, an inhibitor of glycosaminoglycan attachment. At the very least, it appears that *in vivo* human TM is also expressed both with and without glycosaminoglycan modification.

Full-length recombinant human TM and soluble C-terminal deletion mutants (lacking the transmembrane and cytoplasmic domains) have been expressed in several different mammalian cell lines [36,38,41]. Both membrane-bound and soluble derivatives of recombinant human TM exhibit a split between non-acidic low- M_r and acidic high- M_r forms. Treatment of the high- M_r form with the glycan-degrading enzyme, chondroitinase, yielded a molecule similar to the low- M_r form in terms of both its anticoagulant activity and mobility on SDS/PAGE [36,38,42]. Moreover, the glycosaminoglycans from both rabbit lung TM [43] and recombinant human TM [38] have been isolated and identified as chondroitin sulphate.

Although there are four serine residues in the Ser/Thr-rich domain of TM that could be potential sites of glycosaminoglycan

attachment, a number of groups [1,32,34,37] have noted that only Ser⁴⁷² (Figure 1b; numbering of amino acids based on the processed/mature N-terminus of TM as described in ref. [20]) exhibits significant homology with the proposed xylosyltransferase acceptor consensus acidic-X-Ser-Gly-X-Gly [24]. With the exception of the positioning of Asp⁴⁷¹, there is a strong correlation for Ser⁴⁷² being a substrate of xylosyltransferase (the initiating enzyme in glycosaminoglycan synthesis). Amino acid sequencing of partially purified peptides encompassing this region, although not conclusive, has suggested that Ser⁴⁷⁴ may also be a site of glycosaminoglycan attachment [40]. The amino acid sequence surrounding Ser⁴⁷⁴ is similar to the other proposed xylosyltransferase acceptor consensus of acidic-Gly-Ser-Gly-acidic [25].

In the present report, potential sites of glycosaminoglycan attachment to human TM were analysed using soluble derivatives to overcome the inherent difficulties in extracting membrane-bound molecules. We have identified a primary glycosaminoglycan site at Ser⁴⁷⁴ and a secondary site at Ser⁴⁷². The region surrounding the primary acceptor site shows a significant conservation of amino acid sequence across three species, confirming the functional and evolutionary importance of TM glycosaminoglycan. Alteration of amino acids surrounding Ser⁴⁷⁴, so as to conform to its corresponding xylosyltransferase acceptor consensus sequence, resulted in an increase in the degree of glycosaminoglycan assembly. Further, we have observed that Ser⁴⁷⁴ is also modified in the low- M_r form of TM. Potential mechanisms for differential glycosaminoglycan assembly on TM are discussed.

MATERIALS AND METHODS

Materials

Molecular biology techniques utilized the following: restriction endonucleases, T4 polynucleotide kinase and T4 DNA ligase from either New England Biolabs or Boehringer Mannheim; Muta-Gene phagemid *in vitro* mutagenesis kit (170-3581) from Bio-Rad; and ampicillin (A-9518) from Sigma. Mammalian cell culture used: Dulbecco's modified Eagle's medium (DMEM, 320-1965AK and 430-3000EH), Ham's F12 medium (78-5114EH) and gentamicin (600-5750AD) from GIBCO-BRL; Hepes (16926) from United States Biochemical; fetal bovine serum (A-1111-L) from Hyclone; methotrexate (A-6770) from Sigma; Pentax human transferrin (82-316) from Miles Scientific; and bovine insulin from Eli Lilly and Company. Concentration of TM-containing media employed: benzamidine hydrochloride (B-6506) from Sigma; fast flow Q Sepharose (17-0510-01) from Pharmacia; and Ultrafree-CL 10000 nominal molecular mass limit filtration units (UFC3-LGC-00) from Millipore. Western-blot analysis utilized: biotinylated goat anti-(rabbit IgG) (BA-1000), biotinylated horse anti-(mouse IgG) (BA-2000) and Vectastain ABC kit (PK-4000) from Vector; enhanced chemiluminescence (ECL) Western-blotting detection system (RPN-2106), streptavidin-horseradish peroxidase (RPN-1231), Rainbow M_r protein markers (RPN-756) and Hyperfilm-ECL (RPN-2103) from Amersham; monoclonal anti-(human TM) IgG (2379) from American Diagnostica; and 4-chloro-1-naphthol (170-6534) from Bio-Rad. The TM cofactor activity assay (amidolytic) included: Pentax fatty acid-free BSA (82-002) from Miles Scientific; human thrombin (T-6759) and hirudin (H-7016) from Sigma; and chromogenic substrate S-2366 (82-10-90) from Chromogenix. Chondroitinase AC analysis used chondroitinase AC (C-2262), leupeptin (L-2884) and pepstatin (P-4265) from Sigma. Functional cofactor activity assay utilized assayed reference plasma (5185) and the activated partial thromboplastin time (APTT) reagent kit (5383) from Helena Laboratories. The

purified recombinant proteins, soluble human TM [36] and human protein C [44], were prepared as described previously. The generous gift of rabbit anti-(human TM) polyclonal antibody was from Ms. Schwu Fan Ma of Indiana University at Indianapolis. Unless otherwise stated, all other chemicals used were of the highest purity commercially available.

Site-directed mutagenesis of TM

Changes in the putative glycosaminoglycan-attachment region of human TM were made by site-directed mutagenesis using the soluble derivative TMD1. A 193 bp *KpnI/HindIII* fragment corresponding to a region from approximately the middle of the sixth EGF domain to the Ser/Thr-rich domain was isolated from the plasmid pUC18-TMD1 [36] and cloned into the phagemid vector PTZ19U. This clone, designated PTZ19-TMD1, was ultimately used as the source of single-stranded DNA template for the generation of all glycosylation mutants. Oligonucleotide primers were made with an Applied Biosystems model 380 DNA synthesizer and had the following sequences (nucleotide substitutions underlined): Pm-D1G1, 5'-GGCAAGGTGGACGGTG-GCGACGAGGGATCCGGCGAGCCCCGCCCAGC-3'; Pm-D1G2, 5'-GGCAAGGTGGACGGTGGCCTCGAGGGA-TCCGGCGAGCCCCGCCCAGC-3'; Pm-D1G3, 5'-GGCA-AGGTGGACGGTGGCCTCGAGCCCTCCGGCGAGCC-CCGCCAGC-3'; Pm-D1G4, 5'-CAAGGTGGACGGTGG-CGACTCAGGCGGCGGCGAGCCCCGCCCAGC-3'; and Pm-D1G5, 5'-CTGTGACTCCGGCAAGGTGCGACGGTG-GCGACGAAGGCGGCGGCGAGCCCCGCCCAGC-3'. These primers were designed not only to change the coding sequences as outlined in Figure 1b, but also to introduce silent mutations facilitating restriction endonuclease screening before actual DNA sequencing. Introduced restriction sites were *Bam*HI (D1G1), *Bam*HI and *Xho*I (D1G2), *Xho*I (D1G3), *Dde*I (D1G4) and *Sal*I (D1G5). Mutagenesis was performed by the method originally described by Kunkel [45] with modifications to the protocol in the Bio-Rad phagemid *in vitro* mutagenesis kit. Approx. 200 ng of template was annealed to 8 pmol of phosphorylated primer in 10 μ l of buffer containing 20 mM Tris/HCl (pH 7.4), 2 mM MgCl₂ and 50 mM NaCl at 85 °C, allowed to slow-cool to room temperature over a period of approx. 2 h, and then placed on ice. The primer extension reaction was modified as follows: initial incubation was for 15 min at 4 °C, 15 min at 25 °C, and 90 min at 37 °C, followed by the addition of 5 μ l of deionized/distilled water, 1 μ l of ligation buffer (10 $\times$ as described by New England Biolabs) and 1 μ l of T4 DNA ligase (400 units/ μ l) for a final volume of 20 μ l. Incubation was continued overnight at 16 °C, and then 80 μ l of deionized/distilled water was added as diluent, and the reaction mixture was frozen briefly at -20 °C. Competent MV1190 (*dur⁺ ung⁺*) cells were transformed with 7.5 μ l of the above reaction, and individual resultant ampicillin-resistant colonies were picked for analysis. Preliminary screening of colonies was by DNA miniprep procedure and restriction endonuclease digestion. Candidate positives were then verified by dideoxynucleotide sequencing using an ABI 370A automated fluorescent sequencer. After sequence confirmation of the appropriate mutation, double-stranded phagemid DNA was isolated from bacterial cells, and the TM insert was isolated. Reconstitution of the complete soluble TM coding sequence was accomplished via ligation of the 193 bp *KpnI/HindIII* fragment (from PTZ19-D1G1, PTZ19-D1G2, PTZ19-D1G3, PTZ19-D1G4 or PTZ19-D1G5) with the 2828 bp *KpnI/HindIII* fragment from pUC18-TMD1. DNA from the resultant clones (designated

pUC18-D1G1, pUC18-D1G2, pUC18-D1G3, pUC18-D1G4 and pUC18-D1G5) and pUC18-TMD1 was used to transform *Escherichia coli* strain GM48 (*dam⁻*) to facilitate removal of the entire soluble TM coding sequence on a *Bcl*I fragment. The University of Wisconsin GCG sequence analysis software package version 7.0 [46] was used extensively in the design of oligonucleotide primers and identification of convenient restriction sites.

Expression of recombinant TM

The 1561 bp *Bcl*I fragment of each of the pUC18 clones above was excised and inserted into the mammalian cell expression vector pGT-d at its unique *Bcl*I site. The completed expression plasmids were designated pGTD-TMD1, pGTD-D1G1, pGTD-D1G2, pGTD-D1G3, pGTD-D1G4 and pGTD-D1G5. The vector pGT-d contains the following DNA sequences beginning at the *Eco*RI site in pBR322 and proceeding counterclockwise: the *Eco*RI to *Acc*I restriction fragment of pBR322 containing ampicillin resistance and the origin of replication (pBR322 nucleotides 4363–2297); the *Acc*I to *Stu*I restriction fragment of BK virus P2 [47] (BKV-P2 nucleotides 4339–5122); the poly(GT)₂₀ element (a synthetic linker comprised of 20 GT repeats and *Bam*HI ends that were treated with the Klenow fragment of *E. coli* DNA polymerase I and ligated to the *Stu*I of BKV-P2); the *Stu*I to Klenow-treated *Avr*II restriction fragment of BKV-P2 which contains the BKV-P2 enhancer (BKV-P2 nucleotides 5122–5175 and 1–366); the adenovirus 2 major late promoter optimally spaced to the BKV-P2 enhancer for the most efficient transcription (adenovirus 2 nucleotides 5931–6701); a synthetic linker consisting of the spliced sequence of the adenovirus tripartite leader; a *Bcl*I linker; the 610 bp *Mbo*I fragment of simian virus 40 containing the small t antigen splice site and the polyadenylation signal; the blunt-ended *Bam*HI restriction fragment of pSV2-dhfr (ATCC 37146) containing the murine dihydrofolate reductase coding sequence and the simian virus early promoter/enhancer, small t antigen splice site and polyadenylation signal. Each of the TM mutant expression plasmids was purified on a CsCl density gradient, linearized with *Fsp*I, and used to transfect AV12 cells.

Cell culture and isolation of stable transformants

The adenovirus-transformed syrian hamster AV12 cell line (ATCC CRL 9595) was maintained in DMEM supplemented with 10% (v/v) fetal bovine serum and 50 μ g/ml gentamicin. On the day before transfection, cells were plated at a density of 2 $\times$ 10⁵ cells/55 cm². Each plate of AV12 cells was transfected for 4 h at 37 °C with 30 μ g of carrier-free plasmid DNA using the calcium phosphate-DNA precipitate technique [48]. Growth medium was replaced approx. 2 days after the transfection with selection medium containing 500 nM methotrexate. Approx. 2–3 weeks after transfection, a limited number of well isolated methotrexate-resistant colonies were picked for screening and grown to confluency in 24-well tissue culture plates. The remaining colonies from the original transfection were pooled. Cultures were washed once with Hanks' balanced salt solution to remove serum proteins and incubated with serum-free medium (3 parts DMEM, 1 part Ham's F12 medium, 20 mM Hepes, pH 7.5) supplemented with 50 μ g/ml gentamicin, 1 μ g/ml human transferrin and 1 μ g/ml bovine insulin. Conditioned medium was collected and assayed directly for TM antigen production. For each TM mutant, individual clones at a range of production levels were retained for analysis.

Concentration of TM-containing medium

For both individual and pooled clones, 150 ml of conditioned serum-free medium was collected and centrifuged at 5000 *g* for 30 min to remove insoluble material. After addition of EDTA and benzamidine hydrochloride to 5 mM each, medium was stored briefly at -20°C . Conditioned medium was thawed and loaded at 4°C on to a 3 ml column of fast-flow Q Sepharose which had been equilibrated previously with buffer A (100 mM NaCl, 20 mM Tris/HCl, 5 mM EDTA, 5 mM benzamidine hydrochloride, pH 7.4). After being washed with 10 column vol. of buffer A, bound TM was recovered quantitatively by step elution with buffer A containing 1 M NaCl. The eluate was then dialysed extensively at 4°C with buffer B (100 mM NaCl, 50 mM Tris/HCl, 30 μM sodium acetate, pH 8.0). Further concentration was achieved by centrifugation at 2500 *g* in Millipore Ultrafree-CL 10000 NMWL filtration units (low-binding cellulose). Conditioned medium from pooled clones was also prepared by omitting the fast-flow Q Sepharose step and material concentrated/dialysed directly by Ultrafree-CL filtration.

Quantification of TM

Samples of unknown concentration, along with dilutions of highly purified low- M_r TMD1 as a standard curve, were immobilized on nitrocellulose using a Schleicher & Schuell Minifold 96-well filtration manifold. TM antigen was detected as outlined below for Western-blot analysis. After chromogenic development, the membrane was rinsed with PBS, completely dried in air and placed on top of a layer of mineral oil in an inverted microplate lid. A_{595} and concentrations relative to the standard curve were measured in a ThermoMax kinetic microtitre plate reader (Molecular Devices, applications bulletin 005-A). Confirmation of antigen-based quantification was obtained by utilizing a cofactor activity assay which was a modification of the two-stage amidolytic assay described previously [49]. This assay was performed at a Ca^{2+} concentration (1 mM) at which the high- and low- M_r forms of soluble TM were previously shown to possess similar cofactor activity [36]. Samples were serially diluted in activation buffer (100 mM NaCl, 1 mg/ml BSA, 20 mM Tris/HCl, pH 7.4) containing 1 mM CaCl_2 and incubated in a 100 μl volume with 2 nM human thrombin (0.25 NIH unit/ml) and 400 nM recombinant human protein C (25 $\mu\text{g}/\text{ml}$) at 37°C for 25 min. A 20-fold excess of hirudin (100 μl of 5 NIH units/ml in activation buffer containing 5 mM CaCl_2) was then added to inhibit thrombin. Chromogenic substrate S-2366 was added at a final concentration of 0.4 mM and the amidolytic activity was measured as the change in absorbance units/min at 405 nm in a ThermoMax kinetic microtitre plate reader. Values obtained were compared with a standard curve based on serial dilutions of a known concentration of purified low- M_r TMD1.

SDS/PAGE and Western-blot analysis

Samples of soluble TM derivatives were subjected to SDS/PAGE [50] on 8% polyacrylamide gels (acrylamide/bisacrylamide, 30:0.8, w/v) under reducing and non-reducing conditions. Proteins were either stained directly with Coomassie Blue or electroblotted on to nitrocellulose. After electrophoretic transfer, the membrane was blocked for 1 h in 5% (w/v) non-fat dry milk in PBS, pH 7.4, and then incubated with a rabbit anti-(human TM) polyclonal antibody followed by a biotinylated goat anti-(rabbit IgG). The blot was developed using the Vector avidin-biotin complex kit and 4-chloro-1-naphthol. Alternatively, detection

was accomplished by incubation with monoclonal anti-(human TM) IgG (American Diagnostica, MAb 2379 at a 1:5000 dilution), followed by biotinylated horse anti-(mouse IgG) then streptavidin-horseradish peroxidase. The blot was then developed using the Amersham ECL Western-blotting system and exposed to Hyperfilm-ECL (typical exposures ranged from 5 s to 2 min). Analysis of blots/developed film was performed on a Pharmacia LKB Ultrascan XL laser densitometer.

Chondroitinase AC digestions

Samples of each TM derivative (200 ng) were incubated overnight at 37°C with and without 400 munits of chondroitinase AC in buffer consisting of 100 mM NaCl, 50 mM Tris/HCl and 30 μM sodium acetate, pH 8.0, in the presence of 5 mM benzamidine hydrochloride, 10 $\mu\text{g}/\text{ml}$ pepstatin and 20 $\mu\text{g}/\text{ml}$ leupeptin (110 μl total volume).

Thrombin cofactor activity

The thrombin cofactor activity was determined at two different Ca^{2+} concentrations: 0.3 mM CaCl_2 , the optimum Ca^{2+} concentration for activation by the low- M_r form of soluble TM; and 3.0 mM CaCl_2 , the optimum Ca^{2+} concentration for activation by the high- M_r form of soluble TM [36]. Reactions (100 μl volume) were carried out in activation buffer (100 mM NaCl, 1 mg/ml BSA, 20 mM Tris/HCl, pH 7.4) containing the above concentrations of CaCl_2 , 400 nM recombinant human protein C (25 $\mu\text{g}/\text{ml}$), 2 nM human thrombin (0.25 NIH unit/ml) and 0.5 nM recombinant soluble TM (40 ng/ml); incubation was at 37°C for 25 min. A 20-fold excess of hirudin (100 μl at 5 NIH units/ml) in activation buffer containing either 5.7 mM CaCl_2 (low- M_r optimum reaction) or 3.0 mM CaCl_2 (high- M_r optimum reaction) was then added to inhibit thrombin. Chromogenic substrate S-2366 was added at a final concentration of 0.4 mM and the amidolytic activity was measured as the change in absorbance units/min at 405 nm in a ThermoMax kinetic microtitre plate reader.

Anticoagulant activity assay

The APTTs were determined by preincubating 10 μl of each TM derivative at the indicated concentrations (see Figure 4b) with 50 μl of assayed reference plasma and 50 μl of APTT reagent for 5 min at 37°C in a 96-well microtitre plate. Reactions were initiated by the addition of 50 μl of 25 mM CaCl_2 (prewarmed at 37°C), and A_{595} was monitored using a ThermoMax kinetic microtitre plate reader, with clotting times determined as time to V_{max} . Results obtained by this method were equivalent to those obtained in assays utilizing a fibrometer.

Peptide isolation and amino acid sequencing

Purified recombinant soluble human TM (low- M_r TMD1, 340 μg) was incubated in 500 mM Tris/HCl, pH 8.5, containing 6 M guanidinium chloride, 10 mM EDTA and 10 mg/ml dithiothreitol (800 μl total volume) under argon for 2 h at room temperature. Portions (200 μl) of the protein solution were then injected on to a 5 μm Vydac C_4 h.p.l.c. column (4.6 mm $\times$ 150 mm); the column was washed for 5 min with 5% acetonitrile in 0.1% trifluoroacetic acid (v/v), and peptides were eluted with a linear gradient of 5–95% acetonitrile in 0.1% trifluoroacetic acid (v/v) over 45 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected and pooled after successive injections. Edman degradation of TM-derived peptides was

carried out on the PI 2090E integrated microsequencing system. Analysis of phenylthiohydantoin amino acids was performed on the modified HP 1090 h.p.l.c. A Porton peptide support disc was used for sample support.

RESULTS

Modification of putative glycosaminoglycan site

The domain organization of TM is depicted schematically in Figure 1(a). Deletion of coding sequence for the transmembrane and cytoplasmic domains was used to generate the soluble mutant TMD1 [36], which allows for the isolation of protein without the inherent difficulty of extracting membrane-bound molecules. Retained in TMD1 is the Ser/Thr-rich domain which probably serves as the site of attachment of one or more glycosaminoglycan side chains to TM. To obtain a definitive assignment of where glycosaminoglycans are attached to recombinant soluble human TM, site-directed mutagenesis was used to generate a number of derivatives with substitutions as depicted in Figure 1(b). Each of these mutants was expressed and prepared as described in the Materials and methods section.

Immunoblot analysis of TM glycoforms

Western-blot analysis utilizing a polyclonal antibody demonstrates the glycosaminoglycan split described previously for recombinant soluble TM (Figure 2, lane 1, TMD1) [36]. The high- M_r form, migrating as a diffuse band from M_r 105000 to 180000, represents a soluble TM containing glycosaminoglycan chain(s) of various lengths, whereas the lower- M_r (75000) form apparently lacks this modification. Interestingly, this partial glycosylation has been observed (a) in three different cell lines in which soluble TM has been expressed, CHO [38], 293 and AV12 [36], and (b) over a roughly 100-fold range in expression level (results not shown). This suggests that differential glycosylation

is an inherent property of the TM core protein. The overall glycosylation pattern with mutant D1G1 and D1G2 was similar to that of native TMD1, whereas mutants D1G3–5 were predominantly low- M_r forms. The slight smear below the low- M_r form (Figure 2, lanes 1–6), which probably represents heterogeneity in N-linked and/or more 'typical' O-linked glycosylation, was essentially unchanged by mutation of the putative glycosaminoglycan-attachment region.

To assess the effect of each mutation on glycosylation more definitively, we quantified the relative amounts of each form by densitometry. Owing to the inherent difficulty in comparing scanning data derived from a diffuse high- M_r band, chondroitinase treatment was used to collapse the high- M_r form into the low- M_r form, thus allowing an accurate determination of the relative proportion of glycosaminoglycan in TMD1 (Figure 3a, lanes 5 and 6). Comparison of the degree of glycosaminoglycan attachment in the mutants (Figure 3b) was made by scanning the glycoforms and directly comparing with standard curves derived from dilutions of TMD1 on the same blot. Densitometric scanning did not allow for accurate quantification of < 5% high- M_r form. Although immunoblots of material from high-producing individual clones yielded lower backgrounds and allowed direct visualization of low levels (< 5% total antigen) of high- M_r glycoform, interpretation of the results is complicated by the possibility of clonal variation. Thus quantitative analysis of glycoform distribution was performed with material prepared from pooled clones. The absence of high- M_r form from D1G5 (Figure 3b), which possesses substitutions for both Ser⁴⁷² and Ser⁴⁷⁴, was also observed in immunoblots of material from high-producing individual clones (results not shown). This suggests that glycosaminoglycan side chain attachment is limited to one or both of these serine residues in native TMD1. The glycoform distribution observed for D1G4 (Figure 3b), in which the only substitution is a glycine for Ser⁴⁷⁴, suggests that this serine is the predominant glycosaminoglycan-attachment site. Interestingly, a small amount of high- M_r form could be visualized over background in D1G4 on immunoblots of material derived from high-producing individual clones (results not shown). This suggests that Ser⁴⁷² may represent a secondary site for initiation of glycosaminoglycan synthesis. Mutants D1G1 and D1G2 both possess a high/low glycoform split, confirming the assignment of Ser⁴⁷⁴ as the primary glycosaminoglycan-attachment site. As shown in Figure 3(b), the substitution of Glu for Ser⁴⁷² in D1G1 and D1G2 increases the relative amount of high- M_r form by 36% and 55% respectively compared with native TMD1. This substitution places Ser⁴⁷⁴ in precise accordance with the xylosyltransferase consensus acidic-Gly-Ser-Gly-acidic as proposed by Zimmermann and Ruoslahti [25]. It appears, at least in the context of sequences presented here, that an additional acidic residue (Asp⁴⁷¹ in D1G1) adjacent to the core consensus of Glu-Gly-Ser-Gly-Glu does not enhance acceptor potential, as the amount of high- M_r form in D1G2 (Leu substitution for Asp⁴⁷¹) is actually higher than in D1G1. The mutant D1G3 (Figure 3b), which is identical with D1G2 except for the substitution of Pro for Gly⁴⁷³ to disrupt the acceptor sequence surrounding Ser⁴⁷⁴, appears as predominantly the low- M_r form. As with mutant D1G4, we were able to detect a small amount of high- M_r form in D1G3 on immunoblots of material derived from high-producing individual clones (results not shown).

Therefore the above results indicate that human TM contains a primary glycosaminoglycan-attachment site at Ser⁴⁷⁴. In addition, it was possible to improve the overall degree of attachment by mutating Ser⁴⁷² to Glu (so as to conform Ser⁴⁷⁴ to the xylosyltransferase acceptor consensus acidic-Gly-Ser-Gly-acidic); however, a significant proportion (approx. 35%) of the

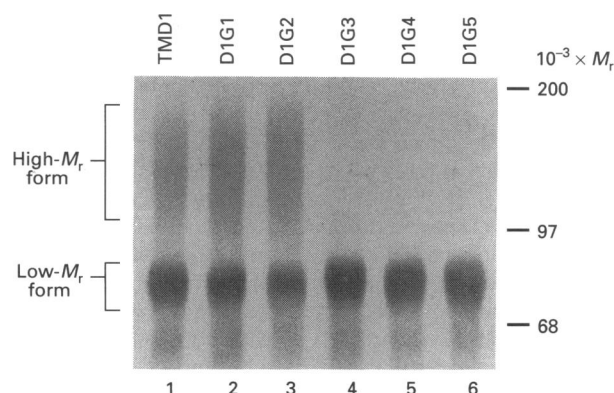


Figure 2 Western-blot analysis of glycoform distribution for recombinant TMD1 and derivatives

Conditioned medium concentrate containing soluble recombinant TM was subjected to SDS/PAGE (8%, reducing conditions, 4 μ g of soluble TM per lane), protein transferred to nitrocellulose, and TM antigen detected by polyclonal antibody. The apparent 'glycosaminoglycan split' for the native sequence (TMD1) consists of a diffusely migrating high- M_r band (TM containing glycosaminoglycan chains of various length) and a fairly discrete lower- M_r band (TM lacking glycosaminoglycan). Migrations of M_r standards are indicated on the right.

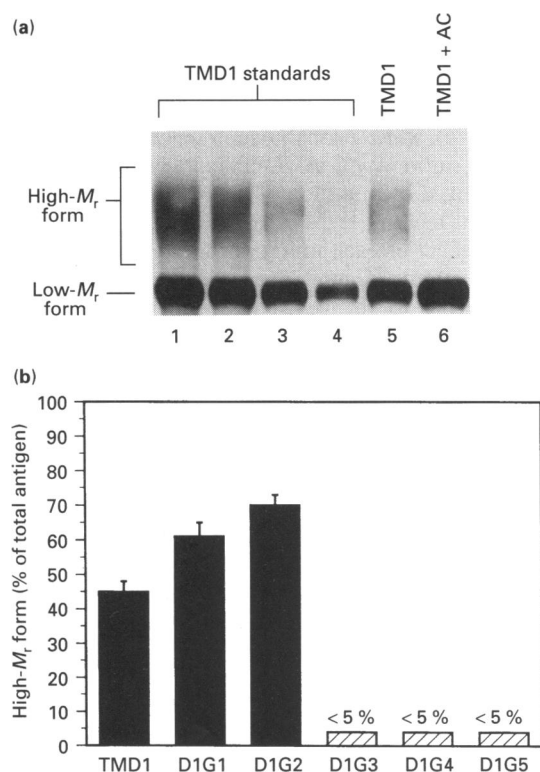


Figure 3 Degree of glycosaminoglycan modification in TMD1 and derivatives

(a) Conditioned medium concentrate containing soluble recombinant TMD1 (200 ng) was incubated without (lane 5) or with (lane 6) chondroitinase AC, then subjected to SDS/PAGE (8%, reducing conditions) and Western-blot development with monoclonal antibody as described in the Materials and methods section. Densitometer scanning of the low-M_r form of TMD1 in lanes 5 and 6 was compared with a standard curve derived from scanning of the low-M_r forms in TMD1 standards (lanes 1–4 represent 400, 300, 200 and 100 ng of untreated TMD1 respectively). (b) Comparison of the amount of high-M_r form was by densitometry as described in the Materials and methods section. Results are the means $\pm$ S.E.M. of four independent determinations. Densitometric scanning did not allow for accurate quantification of < 5% high-M_r form.

total TM still lacked a glycosaminoglycan moiety. For those samples containing significant proportions of high-M_r form (D1G1 and D1G2), digestion with chondroitinase AC reduced essentially all of the high-M_r glycoform to the low-M_r form (results not shown), confirming that the local sequence alterations of TMD1 derivatives did not alter the type of glycosaminoglycan assembled (chondroitin sulphate). For derivative D1G4, in which the only glycosaminoglycan-attachment site is the secondary site of native TM (Ser⁴⁷²), chondroitinase digestion also appeared to abolish its faint high-M_r form. These observations suggest that chondroitin sulphate is probably the major type of oligosaccharide assembled at both Ser⁴⁷² and Ser⁴⁷⁴ attachment sites.

Functional properties

The activities of TMD1 and its derivatives were determined by direct thrombin cofactor activity towards activation of human protein C and by anticoagulant activity in the APTT assay. As previously described, the low- and high-M_r forms of TMD1 have different optimal Ca²⁺ concentrations for cofactor activity of 0.3

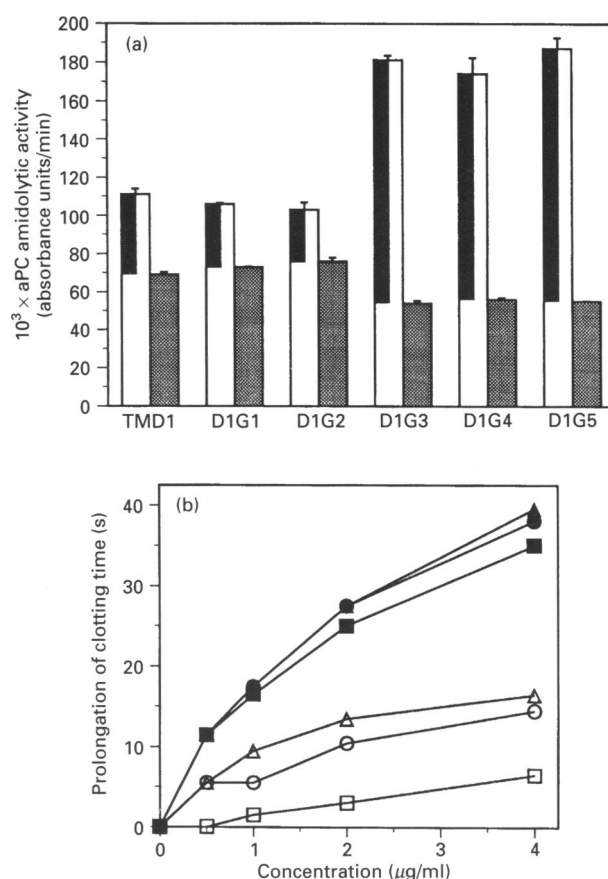


Figure 4 Thrombin cofactor and anticoagulant activities of TMD1 and derivatives

(a) The thrombin cofactor activities of each derivative were determined at 0.3 mM (open bars) and 3 mM Ca²⁺ (shaded bars) in an assay measuring the thrombin-catalysed activation of zymogen protein C to its active form (aPC), as described in the Materials and methods section. The black bar represents the difference in the activities of the two Ca²⁺ concentrations. (b) The anticoagulant activity (prolongation of clotting time) of TMD1 and derivatives, at various concentrations, was determined in an APTT assay as described in the Materials and methods section. □, D1G5; ○, D1G4; △, D1G3; ■, TMD1; ●, D1G2; ▲, D1G1.

and 3.0 mM respectively. As our derivatives contained a mixture of both forms, we assayed cofactor activity at both Ca²⁺ concentrations as shown in Figure 4(a). The difference between the activity at 0.3 and 3.0 mM Ca²⁺ (black bar) should indicate the relative level of glycosaminoglycan attachment. As shown, D1G1 and D1G2 showed decreased activity at 0.3 mM Ca²⁺ and increased activity at 3.0 mM Ca²⁺, consistent with the increased degree of glycosaminoglycan attachment determined by densitometry (Figure 3b). The difference in activities at 0.3 and 3.0 mM Ca²⁺ observed with D1G5, with both Ser⁴⁷² and Ser⁴⁷⁴ residues substituted, exhibits the maximal activity difference with low-M_r form. The slight reduction in the difference with D1G3 and D1G4 suggests the presence of low levels of high-M_r form.

An assessment of activity in a clotting assay is shown (Figure 3b) for various concentrations of each soluble TM. The degree of prolongation of clotting time corresponded to the amount of high-M_r form present. Activities of TMD1, D1G1 and D1G2 were all significantly higher than D1G5 (entirely low-M_r form) and the activities of D1G1 and D1G2 were slightly higher than TMD1. The anticoagulant activities of D1G3 and D1G4 were slightly elevated over the D1G5 baseline, again indicating the

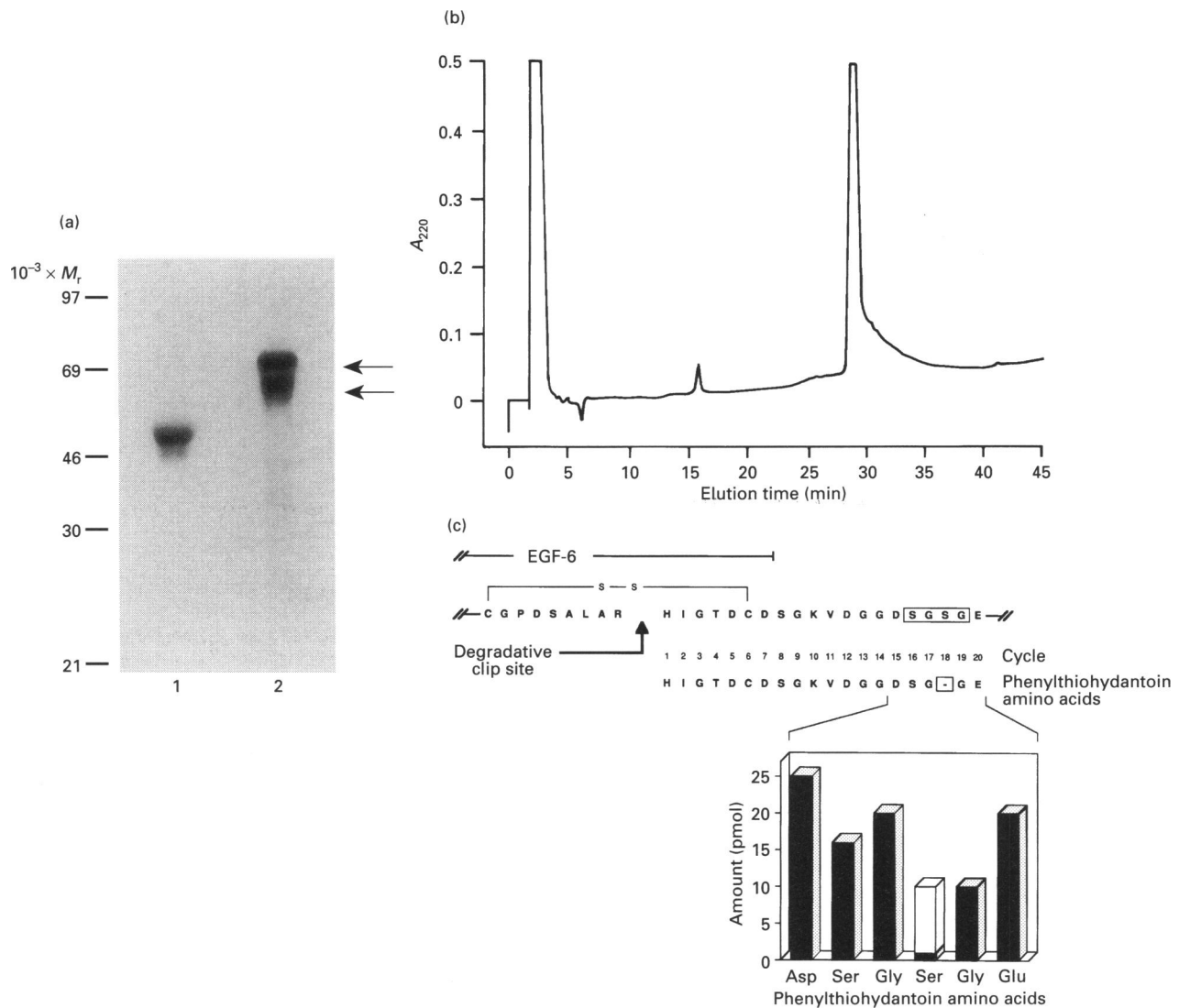


Figure 5 Isolation and amino acid sequencing of C-terminal peptide from low- M_r TMD1

(a) Purified low- M_r TMD1 (non-reducing and reducing conditions) was subjected to SDS/PAGE (10%) and visualized with Coomassie Blue stain. Migrations of M_r standards are indicated on the left. (b) Purified low- M_r TMD1 was reduced and subjected to reversed-phase h.p.l.c. as described in the Materials and methods section. The peak eluted at 30 min represented both intact low- M_r TMD1 and the large N-terminal fragment of 'clipped' material. The peptide eluted at 15.9 min was isolated for more thorough analysis. (c) Schematic of the clip site within TMD1 and amino acid sequence of the isolated peptide as determined in the Materials and methods section. □, Expected; ■, observed.

presence of a limited amount of high- M_r form. The roughly equivalent activity of D1G4 (high- M_r form by glycosaminoglycan assembly at Ser⁴⁷²) with D1G3 (high- M_r form by glycosaminoglycan assembly at Ser⁴⁷⁴) suggests that the glycosaminoglycan moiety on either serine is functional. Thus all mutants appeared to behave according to their degree of glycosaminoglycan attachment for both direct inhibition of thrombin procoagulant activities and cofactor activity for protein C activation.

Characterization of low- M_r C-terminal peptide

Typically, in order to visualize the diffusely migrating high- M_r form of TM by SDS/PAGE, samples were overloaded with respect to the low- M_r form. However, it was noted that, when amounts that yielded a discrete band for the low- M_r form were loaded, there was a subtle increase (M_r , approx. 2000) in the mobility of D1G4 and D1G5 low- M_r forms compared with the

low- M_r form of TMD1 and the other derivatives (results not shown). Although it was possible that this was simply an inherent property of the amino acid changes made, the fact that two different mutants with substitutions for Ser⁴⁷⁴ showed identical mobility shifts led to speculation that this serine residue was modified in the low- M_r form of native TMD1.

To explore this possibility further, we exploited a fortuitous degradative clip that occurred in purified low- M_r TMD1 during extended storage (approx. 1 year) at 4 °C. This material, when subjected to SDS/PAGE, appeared intact under non-reducing conditions (Figure 5a, lane 1). However, under reducing conditions (Figure 5a, lane 2), a distinct doublet was observed. The upper band corresponds to intact low- M_r form and the lower band represents clipped material. Amino acid sequencing of the intact/clipped mixture indicated that clipped low- M_r TMD1 would be useful in isolating a C-terminal peptide containing the glycosaminoglycan-attachment region. Therefore purified low-

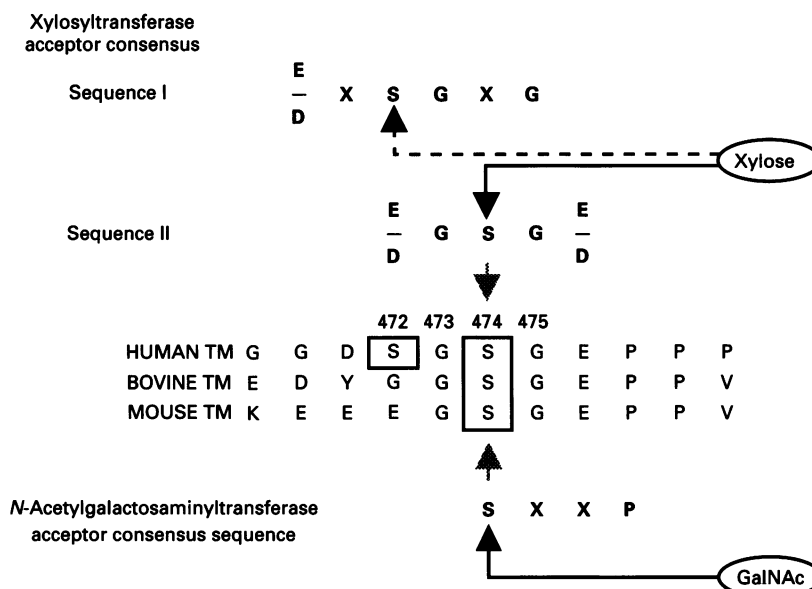


Figure 6 Sequence alignment of putative glycosaminoglycan-attachment region in human, bovine and mouse TM

Amino acid sequences of bovine [22] and mouse [23] TM were aligned with that of the putative human [20,21] TM glycosaminoglycan-attachment region. Also indicated are the proposed acceptor consensus sequences for xylosyltransferase (initiating glycosaminoglycan synthesis) [24,25] and the proposed acceptor consensus sequence for GalNAc transferase (initiating simple *O*-glycosylation) [54,55].

M_r TMD1 was reduced, subjected to reversed-phase h.p.l.c. and the two major peaks observed in the chromatograph (Figure 5b) were isolated and subjected to N-terminal sequence analysis. The large peak eluted at 30 min represented the N-terminus of intact TMD1 (as well as the large N-terminal fragment of clipped material), and the peak eluted at 15.9 min yielded an N-terminus starting at His⁴⁵⁷ within EGF-6. More complete sequence analysis of this C-terminal peptide (Figure 5c) revealed only background levels of serine at cycle 18, the expected position of Ser⁴⁷⁴. Therefore, in addition to its modification in the high- M_r form of TM, Ser⁴⁷⁴ is also modified in the low- M_r form.

DISCUSSION

Glycosaminoglycans are a unique class of O-linked carbohydrates possessing the general structure serine/threonine-xylose-galactose-galactose-(disaccharide)_n (reviewed in refs. [51]–[53]). Unlike N-linked glycosylation, core protein sequences directing the attachment of O-linked sugars are relatively undefined. Initiation of simple O-linked polysaccharides occurs via the attachment of an *N*-acetylgalactosamine to the hydroxy group of a serine or threonine. Although an absolute acceptor consensus for *N*-acetylgalactosaminyltransferase (GalNAc transferase) has not been established, a bias towards both Pro-Ser/Thr and Ser/Thr-X-X-Pro has been noted [54,55]. Even less defined are the core protein sequences directing O-linked glycosaminoglycan initiation by xylosyltransferase. The most general observation is the association of modified serine residues with an adjacent glycine. Additional sequence and/or structural requirements must exist, however, as numerous proteins containing Ser-Gly dipeptides are not substrates for xylosyltransferase [52].

The definitive assignment of glycosaminoglycan-attachment sites has been obtained for only a limited number of proteoglycans, e.g. type IX collagen proteoglycan [56] and fibroblast proteoglycan PG40 [57]. In the current study, site-directed

mutagenesis was utilized to identify the sites of glycosaminoglycan attachment to soluble derivatives of human TM. The Ser/Thr-rich domain of human TM contains one Ser⁴⁶⁴-Gly dipeptide, a Ser⁴⁷²-Gly-Ser⁴⁷⁴-Gly tetrapeptide and a Gly-Ser⁴⁸⁵ dipeptide sequence. Although any one of these four serine residues might serve as a potential site of glycosaminoglycan attachment, our analysis indicates that the majority of glycosaminoglycan side chains on soluble recombinant human TM occur at Ser⁴⁷⁴. Although it was not possible to accurately quantify high- M_r material of less than 5% of the total by scanning, our functional data suggested the presence of small amounts of high- M_r material in both D1G3 and D1G4. In addition, in individual high-producing clones, we could visually detect small amounts of high- M_r material in both D1G3 and D1G4, but not in D1G5, and this material disappeared on chondroitinase treatment. The results suggest that Ser⁴⁷² can act as a secondary attachment site. If such were the case, then the exact position of the glycosaminoglycan on the core protein is not essential for its contribution to functionality. It is conceivable, however, that glycosaminoglycan positioning on the core protein might define a minimal chain length necessary for interactions with thrombin.

In human TM (Figures 1 and 6), the sequence surrounding Ser⁴⁷² shows significant homology with the acidic-X-Ser-Gly-X-Gly consensus [24], whereas that of Ser⁴⁷⁴ is most homologous with the acidic-Gly-Ser-Gly-acidic consensus [25]. On the basis of the virtual overlap of these two initiating sequences, it is probable that structural processing determinants, beyond those responsible for xylose addition, are shared by these two serines. Thus the apparent 10-fold preference of xylosyltransferase for Ser⁴⁷⁴ over Ser⁴⁷² might suggest a hierarchy in acceptor potential of the two proposed consensus sequences. It should be noted, however, that both serines do not conform precisely to their respective consensus sequences. Attachment at Ser⁴⁷⁴ in human TM may be suboptimal in that it lacks the initial acidic residue

of the consensus acidic-Gly-Ser-Gly-acidic. This observation was confirmed by mutants that supply this acidic residue (D1G1 and D1G2; Figure 1b) and show an increased degree of attachment at this site (Figures 2 and 3b). For Ser⁴⁷², it is also possible that sequences directing attachment are suboptimal with regard to the acidic-X-Ser-Gly-X-Gly consensus. The acidic residue Asp⁴⁷¹, rather than being separated by an intervening amino acid, is directly adjacent to Ser⁴⁷². Although Bourdon et al. [24] did not specifically address this issue in deriving their consensus, it may be that an intervening amino acid between the acidic residue and target serine is critical for optimal acceptor potential. The acceptor sequence of Ser⁴⁷² may also be suboptimal with regards to the number of acidic residues, as the proteoglycans analysed by Bourdon et al. [24] contained two or three acidic residues upstream of the attachment site.

There must be other determinants, beyond those discussed above, that contribute to the proteoglycan/non-proteoglycan nature of TM. As demonstrated with mutant D1G1, even when the primary attachment site appears in the exact context of the acidic-Gly-Ser-Gly-acidic acceptor consensus, there is still a large proportion of TM molecules in the low- M_r form. Interestingly, it was noted that mutants that possess a substitution for Ser⁴⁷⁴ show an increased mobility of their low- M_r form on SDS/PAGE compared with native TMD1. This led us to speculate that the low- M_r form contains a small modification at Ser⁴⁷⁴ which contributes no more than 2000 to its apparent M_r . Isolation and sequencing of a C-terminal peptide confirmed that Ser⁴⁷⁴ is, in fact, modified in the low- M_r form. Although determination of the exact nature of this modification was prevented by a limited amount of peptide, our observations are consistent with the presence of a small carbohydrate moiety at Ser⁴⁷⁴ of the low- M_r form. One explanation is that the low- M_r form of TM simply reflects a rate-limiting step in glycosaminoglycan assembly, presumably occurring after initiation by xylosyltransferase. The origin of such a rate-limiting step might reside within structural determinants of the core protein itself and/or the cellular glycosylation machinery.

A more intriguing possibility is that the low- M_r form represents those TM molecules with a Ser⁴⁷⁴ modification incompatible with chondroitin sulphate glycosaminoglycan synthesis. In human TM (Figure 6), there is an apparent overlap at Ser⁴⁷⁴ between the acceptor consensus sequences of both xylosyltransferase (acidic-Gly-Ser-Gly-acidic) and GalNAc transferase (Ser-X-X-Pro). Several studies [58–64] have suggested that these two glycosyltransferases reside in the same subcellular compartment (*cis*-Golgi), and therefore it is possible that direct competition for substrate could occur. Attachment of GalNAc to Ser⁴⁷⁴, the first step in more simple *O*-glycosylation, would preclude the assembly of a chondroitin sulphate glycosaminoglycan. It is interesting to note the strict conservation, across three different species, of the hexapeptide motif Gly-Ser⁴⁷⁴-Gly-Glu-Pro. On the basis of the 'glycosyltransferase competition' model presented here, conservation of the first four residues reflects an important role in maintaining the potential for glycosaminoglycan assembly on TM, and the fifth residue, a proline, could be important for the regulation of the degree of such assembly.

The overlap of acceptor consensus sequences for GalNAc transferase and xylosyltransferase also occurs in a number of other integral membrane proteoglycans, including the transforming growth factor- β type III receptor [65,66] and the transferrin receptor [67,68]. Interestingly, like TM, the transferrin receptor has also been shown to be expressed both with and without glycosaminoglycan modification [69]. Perhaps the most striking example of this acceptor consensus overlap is found in

the large aggregating chondroitin sulphate/keratan sulphate proteoglycan of cartilage [70–73], in which approximately half of the large number of potential glycosaminoglycan-attachment sites could be subject to glycosyltransferase competition (80/148 Ser-Gly dipeptides in the human and 48/117 Ser-Gly dipeptides in the rat).

In conclusion, we have identified the primary glycosaminoglycan-attachment site in soluble recombinant human TM, Ser⁴⁷⁴, as well as a conserved hexapeptide motif that is probably critical for initiation of oligosaccharide assembly at this residue. Conservation of sequence surrounding this serine is particularly striking in that it occurs within the context of a domain which, compared with other domains of TM, exhibits the highest degree of interspecies divergence [21,23]. Although our data do not prove that the serine residues identified here are the same sites on non-recombinant/membrane-bound human TM, the strict conservation of the putative acceptor sequence at Ser⁴⁷⁴ would suggest that this is also the predominant chondroitin sulphate-attachment site *in vivo*. Furthermore, this primary glycosaminoglycan-attachment site, shared by an overlapping consensus sequence for GalNAc transferase, was also found to be modified in the low- M_r (non-glycosaminoglycan) form of soluble recombinant TM. A model involving glycosyltransferase competition for Ser⁴⁷⁴ is proposed to account for the expression of TM in its two distinct glycoforms. Interestingly, thrombin has been reported to alter endothelial cell glycosaminoglycan production at the level of polysaccharide assembly on proteoglycan core proteins [74,75]. Given the functional co-operativity between the TM core protein and its glycosaminoglycan, modulation of glycosyltransferase activities may represent an important means of regulating cell-surface anticoagulant potential.

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