C-Mannosylation and O-Fucosylation of the Thrombospondin Type 1 Module*

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Thrombospondin-1 (TSP-1) is a multidomain protein that has been implicated in cell adhesion, motility, and growth. Some of these functions have been localized to the three thrombospondin type 1 repeats (TSRs), modules of ~60 amino acids in length with conserved Cys and Trp residues. The Trp residues occur in WXW patterns, which are the recognition motifs for protein C-mannosylation. This modification involves the attachment of an α-mannosyl residue to the C-2 atom of the first tryptophan. Analysis of human platelet TSP-1 revealed that Trp-368, -420, -423, and -480 are C-mannosylated. Mannosylation also occurred in recombinant, baculovirally expressed TSR modules from Sf9 and “High Five” cells, contradictory to earlier reports that such cells do not carry out this reaction. In the course of these studies it was appreciated that the TSRs in TSP-1 undergo a second form of unusual glycosylation. By using a novel mass spectrometric approach, it was found that Ser-377, Thr-432, and Thr-489 in the motif CSX(ST)CG carry the O-linked disaccharide Glc-Fuc-O-Ser/Thr. This is the first protein in which such a disaccharide has been identified, although protein O-fucosylation is well described in epidermal growth factor-like modules. Both C- and O-glycosylations take place on residues that have been implicated in the interaction of TSP-1 with glycosaminoglycans or other cellular receptors.

Thrombospondin (TSP)1-1 is a large homotrimeric glycoprotein present in the α-granules of platelets (1). The 150-kDa monomer has a modular structure, with (from the N to C terminus) a heparin-binding domain, oligomerization domain, procollagen module, three type 1 repeats (TSRs, also called propeptide modules), three EGF-like repeats, a calcium binding domain, and a globular C-terminal domain (2). It belongs to a protein family, which contains at least four other members (3). Upon activation with thrombin, for example, TSP-1 is secreted and binds to the surface of activated platelets, thereby promoting their aggregation (4). In addition, TSP-1 is incorporated into the fibrin network of the clot, where it influences the structure of the fibrin filaments (5, 6) and possibly their cleavage during fibrinolysis (7). TSP-1 is also secreted by growing cells and can interact with cellular surfaces, extracellular matrix, and soluble macromolecules. Roles for TSP-1 in a variety of processes, such as embryonic development, response to injury, tumor growth, and metastasis, angiogenesis, inflammation, growth factor activation, and neurite outgrowth, have been postulated (for reviews see Refs. 8–11).

The three TSR modules, which are found in TSP-1 and -2 but not in the other TSPs, are of particular importance to the present study. TSRs are ~60 amino acids in length and are characterized by conserved Cys, Trp, Ser, and Arg residues (9). Molecules that have been proposed to bind these modules in TSP-1 include fibrinogen/fibrin, CD36, heparan sulfate, other glycosaminoglycans and sulfatide, fibronectin, TGF-β, laminin, and collagen VII (see Ref. 9 for a review). By using synthetic peptides, the amino acid sequence WSXW, which is present in all three TSRs, has been proposed to be involved in the binding of heparin as well as TGF-β (12, 13). However, in studies using proteolytic fragments obtained from platelet TSP-1 (14), or recombinant TSRs from Sf9 cells (15), no heparin binding was observed at physiologic ionic strength. The amino acid sequence CSVTCG has been suggested to be a cell-binding site in the second and third TSR of TSP-1 (16–18). Although direct binding between the peptide YCSVTCG and Jurkat cells bearing CD36 has been reported (18), no increased binding of TSP-1 to COS cells expressing the receptor could be observed (19). At present the reasons for the discrepancies between different approaches remain unclear.

Post-translational modifications in TSP-1 could complicate the use of synthetic peptides or recombinant proteins. The sequence WXXW has recently been shown to be the recognition motif for a glycosyltransferase that attaches an α-mannopyranosyl residue to the C-2 position of the first Trp (20). We have shown that other proteins that contain TSRs, i.e. C6, C7, C8a, C8β, C9, and propep, are C-mannosylated on multiple Trp residues (21, 22). This form of protein glycosylation appears to be widespread in biology, since the C-mannosyltransferase activity has been found in mammals, birds, amphibians, and fish (23, 24), and tryptophan hexosylation has been found in insect cells (25).

Here we demonstrate that human platelet TSP-1 and recombinant TSRs expressed in various insect cells are C-mannosyl-
Unusual Glycosylation of Thrombospondin

**EXPERIMENTAL PROCEDURES**

**Materials—**Monosaccharide standards for HPAE-PAD analysis were obtained from Sigma. Human platelet TSP-1 was purified from the supernatant of thrombin-stimulated platelets as described previously (2). Recombinant TSR3 (see Fig. 1) and recombinant mature proteins and TSR123 were expressed in Sf9 cells (Invitrogen, Carlsbad, CA). The recombinant proteins included the vector-derived amino acids 366–530 of the mature human TSP-1 was used (2). Tryptic peptides have been numbered according to their occurrence in the mature polypeptide and have been labeled with the prefix “T.” The following affixes were used to indicate subfragments: chymotrypsin (Ch), endoproteinase AspN (D), aminopeptidase (AmP), carboxypeptidase (CPase). Different subfragments have been numbered with Arabic numbers according to their order in the parent peptide. In case of heterogeneity in a peptide, subforms have been indicated with Latin letters (e.g. T55a-Ch-1).

**Isolation of Peptides—**Proteins were reduced and carboxamidomethylated as described (22) and cleaved with proteases at 37 °C. TSP-1 (0.4 or 5 mg) was digested twice with trypsin (1% w/v) in 50 mM NH4HCO3, pH 7.8, for 2 and 18 h, and relevant tryptic peptides were subfragmented with chymotrypsin (1% w/v) for 7 h. Recombinant TSR3 from Sf9 cells (70 μg in 400 μl of 50 mM Tris-HCl, pH 8.0) was digested twice with trypsin (1.5% w/v) for 2 and 4 h and subsequently with endoproteinase AspN (1.5% w/v) for 4 h. Peptides from recombinant TSR3 from High-Five cells were prepared in the same way, except that the tryptic peptide TSR3-H5-T55 was purified before cleavage with endoproteinase AspN. Recombinant protein TSR123 from Drosophila S2 cells (100 μg in 150 μl of 50 mM Tris-HCl, pH 8.0) and TSR123 from High-Five cells (130 μg or 2 mg in the same buffer) were cleaved twice with trypsin (1.5% w/v) for 2 h. Digests were fractionated by HPLC or LC-ESIMS using Vydac C8 and C18 columns essentially as described previously (22). If necessary, final purification of peptides was achieved by HPLC using 25 mM NH4 acetate, pH 6.0, containing 2 or 80% CH3CN as buffer A and B, respectively.
Analysis of C-Mannosylated Peptides—Purified C-mannosylated peptides were examined by low energy CID tandem-MS experiments of the [M + 2H]2+ ions, using a Sciex API 300 mass spectrometer. The resolution of the first quadrupole was set to 1300 for the selected mass, whereas the third quadrupole was set at isotopic resolution over the entire mass range. Solid phase Edman degradation and identification of PTH-(C2-Man-)Trp were performed as described previously (22).

Peptides T42-Ch-2, T49-Ch-2, and T55a-Ch-2 from platelet TSP-1 were subfractionated as follows. Digestions were performed at 37 °C with enzymes obtained from Roche Molecular Biochemicals. Where indicated, purification was achieved by LC-MS. The amounts of peptide were estimated from peak heights in HPLC experiments. The following buffers were used: 50 mM NH4HCO3, pH 7.3, for aminopeptidase M, the same buffer at pH 7.8 for CPase B, 50 mM NH4 acetate, pH 5.0, for CPase Y, 10 mM Tri-HCl, pH 8.0, for endoproteinase AspN, and 5 mM NH4HCO3 for subtilisin. Peptide T42-Ch-2 (200 pmol in 10 μl) was cleaved with 0.03 units of aminopeptidase M for 17 h, and the product was isolated. In a second experiment the purified product of the aminopeptidase digestion was further digested in 5 μl with CPase B (0.7 μg) for 2 h, dried, and cleaved with CPase Y (1 μg) for 44 h. Peptide T49-Ch-2 (100 pmol in 10 μl) was treated with aminopeptidase M in the same way. A second sample (200 pmol in 5 μl) was cleaved with endoproteinase AspN (220 ng) for 2 h. The purified product was then digested in 5 μl with 1 μg of CPase Y for 26 h. Peptide T55a-Ch-2 (200 pmol in 10 μl) was cleaved with subtilisin (200 ng) for 18 h. Purified fragment product in 10 μl was further cleaved with aminopeptidase M, as above. The cleavage of another 200 pmol in 5 μl with CPase B and Y was carried out as described for peptide T42-Ch-2.

The final products of these cleavages were analyzed in a nano-ESIMS experiment, monitoring neutral loss of 308 or 146 Da from either the [M + H]+ and/or [M + 2H]2+ ion, using the API 300 mass spectrometer. Their identity was verified in a low energy CID tandem-MS experiment, using the same apparatus. Methods for the MS analyses have previously been described (28). The corresponding peptides from recombinant TSR3 from SF9 and High-Five cells were treated and analyzed in the same way.

RESULTS

Platelet TSP-1 Is C-Mannosylated on Trp-368, -420, -423, and -480—Counting from the N terminus of mature human
platelet TSP-1, the tryptic peptides T42, T49, and T55, originating from TSR-1, -2 and -3 respectively, contain potential C-mannosylation sites (Fig. 1). Reduced and carboxamidomethylated TSP-1 was cleaved with trypsin and fractionated by reversed phase LC-ESIMS (Fig. 2A). The MS data were extracted for the theoretical mass of each of these peptides, taking into account the possible presence of 0–3 hexosyl residues. This allowed the detection of modified peptides T42, T49, and T55a, as well as a minor amount of unmodified T55b (Fig. 2A).

Nanospray ESIMSMS analysis of the impure peptides revealed that in all three peptides the third Trp residue in the WXXW motif was unmodified (data not shown). This allowed the use of chymotrypsin to generate the N-terminal fragments of these peptides (affixed “Ch-1”), which contain the C-mannosylation sites, and their C-terminal fragments (affixed “Ch-2”) (Fig. 2, B–D). Peptides T42-Ch-1, T49-Ch-1, and T55a-Ch-1 were purified to apparent homogeneity, as judged from the single amino acid sequence observed by Edman degradation.

Peptide T42-Ch-1, originating from the first TSR module, had a mass of 2230.0 Da, which was consistent with residues 355–371 containing one hexosyl residue. The substituent could be present at the Trp residue at position 2, 11, or 14 of the peptide or it could be O-glycosidically linked to one of the hydroxy amino acids (Fig. 1). The latter could be excluded, because no 162-Da losses in MS or MSMS experiments, indicating O-linked hexose, were observed. It should be noted that under the MS conditions used, such phenomena were indeed observed in other peptides (see below). The ESIMSMS spectrum of peptide T42-Ch-1 is shown in Fig. 3A. The m/z values of ions b2–b8 all showed the Trp at position 2 of the peptide (Trp-355) to be unmodified. Modification of the Trp at positions 11 or 14 of the peptide (Trp-365 and -368, respectively) would result in MSMS spectra that differed in the ions b11–13 and y4–6. Five of the six ions that were expected for Trp-368 containing the 162-Da substituent were observed (Fig. 3A), but none of those indicating modification of Trp-365 were detected. In low energy CID tandem-MS, ions containing a (C2-Man-)Trp showed a characteristic loss of 120 Da (22, 29, 30). The spectrum of T42-Ch-1 (Fig. 3A) exhibited this phenomenon for the parent, y5, y6, and b14 ions, thus providing evidence for the C-glycosidic linkage.

Edman degradation yielded PTH-Trp in cycles 2 and 11, and in cycle 14 a PTH-derivative was observed, which comigrated exactly with PTH-(C2-Man-)Trp obtained from residues 5–10 of human RNase2 (Table I).

Peptide T49-Ch-1 (1666.8 Da), from the second TSR module, was 324 Da heavier than expected from the cDNA sequence, suggesting the presence of two hexosyl residues. Fig. 3B shows its nanospray ESIMSMS. No losses indicating O-linked hexosyl residues were observed. The presence of the ions y5, y6, b6, and b8 strongly indicated that each of the Trp residues at positions 5 and 8 of the peptide (Trp-420 and -423, respectively) was carrying a hexosyl residue. Ions that would indicate that one of these residues was unmodified and the other modified with a
324-Da disaccharide were not detected. The characteristic 120-Da loss from the parent and b9 ions occurred twice, in agreement with two C-hexosylated residues in this peptide. Edman degradation of peptide T49-Ch-1 yielded PTH-(C-Man-)Trp in cycles 5 and 8 (Table I).

The mass of peptide T55a-Ch-1 (1861.8 Da) suggested that it contained one hexosyl residue, which could be located on Trp residues at positions 9 or 12 of the peptide (Trp-477 and -480, respectively) or on Ser-481. However, no indication of an O-linked sugar was obtained. The two possible modifications of the Trp residues would yield different b9–b11 and y4–y6 ions. Five of the six ions that located the hexosyl residue to Trp-480 were observed (Fig. 3C), whereas only the ion at m/z 1287.7 would indicate that Trp-477 was modified. However, a more likely explanation for the latter would be loss of H2O from the (C-hexosylated) y10 ion (m/z 1305.7), since it was part of a continuous series of losses of H2O (* in Fig. 3C). The presence of a C–C linkage was concluded from the 120-Da losses from the parent, b12–b13, y5, y7–y10, and y12 ions. Edman degradation yielded PTH-Trp in cycle 9 and PTH-(C2-Man-)Trp in cycle 12 (Table I).

The minor amount of unmodified peptide T55b (3062 Da) was analyzed by nanospray ESIMS without further purification. The results (not shown) confirmed its identity and the absence of modification of its Trp residues.

The stoichiometry of C-mannosylation of the TSR modules of platelet TSP-1 was high. T42 and T49 were found in the C-mannosylated form only. The degree of modification of Trp-480 in peptide T55 was ~90%. Since both the modified and unmodified peptides comigrated with other peptides, this percentage could only be estimated from the area under the peak of [M + nH]2+ ions for n = 2 and 3, in the total ion current trace associated with the LC-ESIMS separation shown in Fig. 2A. A summary of these results is given in Fig. 4.

Platelet TSP-1 Contains an O-Linked Disaccharide on Ser-377, Thr-432, and Thr-489—The C-terminal, chymotryptic fragments of peptides T42, T49, and T55a were isolated in pure form (T42-Ch-2, T-49-Ch-2 and T55a-Ch-2; Fig. 2, B–D). The spectra of these peptides yielded masses that were 308 Da higher than predicted from the sequence. Characteristically, the theoretically predicted mass was observed as well (Fig. 2, B–D; insets). This was reminiscent of the behavior of O-linked saccharides, for which facile dissociation in MS and low energy CID tandem-MS experiments have been well documented (31, 32). Furthermore, the mass of 308 Da exactly fitted the sum of the masses of a deoxyhexosyl (146 Da) and a hexosyl residue (162 Da). These findings strongly suggested the presence of O-linked sugars in these peptides.

Peptide T42-Ch-2 contained five hydroxyamino acids that potentially could be O-glycosylated (Fig. 1). Four of these could be removed by digestion with aminopeptidase M. The resulting peptide, T42-Ch-2-AMP, still contained the 308-Da substituent (Table I). The Q-TOF tandem-MS of the doubly charged molecular ion of the peptide at m/z = 564.29 is shown in Fig. 5A. The observation of the B fragment ions of the HexdHex disaccharide at m/z = 309.12, deoxyhexose at m/z = 147.08, and hexose at m/z = 163.07, as well as the ions corresponding to their neutral loss of water provided evidence for the presence of the disaccharide moiety. The signals at m/z = 822.87 [M dHex + 2H]2+, 897.36 [b6 dHexHex]2+, and 1153.44 [b8 dHexHex]2+
strongly indicated that the disaccharide HexdHex is attached to Ser-377 through the dHex residue.

Independent evidence for the site of modification was obtained by treating this peptide with CPase B followed by CPase Y. This yielded a peptide with a mass of 629.9 Da, having the sequence S(Cam)CG 1308 Da (Table I). Since the cysteine

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**FIG. 3—continued**

**TABLE I**

Characterization of C-mannosylated (C-Man) and O-glycosylated peptides from TSP-1

| Peptidea | Mass (Da)b | Identityc | Modified residue | PTH-derivative d |
|----------|------------|-----------|------------------|------------------|
| TSP-T42-Ch-1 | 2068.1 | 2230.3 | CWSDSADDGWSPW#SEW | Trp-368 | 18.39 | 18.43 |
| TSP-T49-Ch-1 | 1341.6 | 1666.7 | QDGGW#SHW#SPW | Trp-420 | 16.75 | 16.80 |
| TSP-T49-Ch-2 | 1699.9 | 1862.0 | DACPINGGWSPW# | Trp-480 | 18.36 | 18.30 |
| TSP-T55a-Ch-2 | 1380.6 | 1688.9 | DICSVT#CGGGVQK | Thr-489 | + 308 Da |
| TSP-T55a-Ch-2-AMP | 805.4 | 1113.5 | TCGGVQK | + 308 Da |
| TSP-T55a-Ch-2-CPase | 853.3 | 1161.4 | DICSVTC | + 308 Da |

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a Peptides have been numbered as described under “Experimental Procedures”; cleavages with protease have been indicated as follows: T, trypsin; Ch, chymotrypsin; AMP, aminopeptidase M; D, endoproteinase AspN; CPase, carboxypeptidase; Subt, subtilisin.

b Monoisotopic masses have been used for values <1500 Da, whereas average masses have been used >1500 Da.

c W# indicates (C2-Man-)Trp; S# or T#, Ser or Thr with O-linked Hex-dHex. Cysteines have been carboxamidomethylated. The identity of the peptides was verified by ESIMS analysis.

d The elution time of the PTH-derivative of a modified Trp obtained by Edman degradation was compared with that of (C2-Man-)Trp from the peptide F-T-(C2-Man-)WAQW from RNase 2 (29), which was sequenced immediately before or after the sample. The differences in elution time between samples are due to aging of the column and to adjustments in buffer composition to optimize the separation of the other PTH-derivatives.

e Mass calculated from the deduced amino acid sequence.
residue had been carboxymethylated, it could not carry the substituent, which therefore had to be present on the Ser residue (Table I).

In peptide T49-Ch-2 five potential glycosylation sites, i.e. Ser-427, Ser-428, Ser-430, Thr-432, and Thr-439 (Fig. 1), are present. The Q-TOF tandem-MS of the doubly charged molecules of peptide T49-Ch-2 (Fig. 5) shows the presence of oligosaccharides at m/z 510.22 and 1153.44, which correspond to the fragments of the glycoproteins.

**FIG. 5.** Low energy CID nano-ESI Q-TOF tandem MS sequencing of the glycopeptides T42-Ch-2-AMP (A), T49-Ch-2 (B), and T55-Ch-2 (C). For experimental conditions and assignment see the text.
ular ion of the peptide at \( m/z = 903.88 \) is shown in Fig. 5B. The observation of the B fragment ions of the HexdHex disaccharide at \( m/z = 309.12 \), deoxyhexose at \( m/z = 147.06 \), and hexose \( m/z = 163.05 \), as well as the ions corresponding to their respective neutral losses of water, provided evidence for the presence of the disaccharide moiety. Its attachment to Thr-432 could be concluded from the observation of three singly charged glycosylated fragments: \( m/z = 1286.59 \) \([y_9 \text{HexdHex}]^+\), \( m/z = 1472.54 \) \([y_{11} \text{HexdHex}]^+\), and \( m/z = 1632.69 \) \([y_{12} \text{HexdHex}]^+\). The signal at \( m/z = 822.87 \) \([\text{MdHex} + 2\text{H}]^{2+}\) presented direct evidence for the HexdHex disaccharide attachment to Thr-432 through the dHex residue. No ions were detected that would indicate that either dHex, Hex, or the disaccharide Hex-dHex was attached to any of the remaining four potential glycosylation sites.

Aminopeptidase M treatment of peptide T49-Ch-2 yielded the peptide T(Cam-)CGDGVITR \( 1308 \text{ Da} \), whereas digestion with endoproteinase AspN followed by CPase B and Y, in a separate experiment, resulted in the peptide SS(Cam-)CSVT(Cam-)C \( 1308 \text{ Da} \) (Table I). The two peptides only had the sequence -T(Cam-)C- in common, confirming that the substituent was present on the Thr residue.

In peptide T55-Ch-2 two possible O-glycosylation sites, i.e. Ser-487 and Thr-489, are present (Fig. 1). The Q-TOF tandem MS of the doubly charged ion of this peptide \( m/z = 845.01 \) is shown in Fig. 5C. The observation of the B fragment ions of the HexdHex disaccharide \( m/z = 309.31 \) and the hexose \( m/z = 163.24 \), as well as the ions corresponding to the neutral loss of water, provided evidence for the disaccharide. Its attachment to Thr-489 could be concluded from the observation of two out of four glycosylated peptide fragments, \( m/z = 952.50 \) \([y_8 \text{dHex}]^+\) and \( m/z = 1114.57 \) \([y_8 \text{HexdHex}]^+\). Furthermore, the signal at \( m/z = 822.87 \) \([\text{MdHex} + 2\text{H}]^{2+}\) presented direct evidence for the HexdHex disaccharide attachment to Thr-489 through the dHex residue. It is important to note that no ions were detected, which would indicate that either dHex, Hex, or the disaccharide HexdHex was attached to Ser-487.

Peptide T55-Ch-2 yielded the peptide T(Cam-)CGGGQVK \( 1308 \text{ Da} \) (1113.2 Da) upon digestion with subtilisin followed by aminopeptidase M, and in a second experiment the peptide DI(Cam-)SVT(Cam-)C + 308 Da (1161.5 Da) was obtained after consecutive CPase B and Y digestion (Table I). The two fragments only had the sequence -T(Cam-)C- in common, confirming that the modification was present on Thr-489.

**Fucose and Glucose Are the Major Constituents of O-Linked Glycans on TSP-1**—Results of HPAE-PAD analysis of monosaccharides released from O-glycosylated peptides of TSP-1 are summarized in Table II. Deoxyhexoses (Fuc and Rha) and hexoses (Glc, Gal, and Man), commonly occurring in O-linked oligosaccharides, were used as standards for instrument calibration. In all three peptides, very intense fucose and glucose peaks were observed (Fig. 6), which led to the conclusion that fucose and glucose are the major constituents of O-linked glycans on TSP-1. The glucose peak was sometimes followed by a much smaller peak with a retention time roughly the same as that of the mannose standard. Although the difference in retention time (30 s) and the shape of the peak suggested that it could not be assigned to mannose, additional analysis should be carried out. A summary of the analyses of the O-linked glycans is given in Fig. 4.

**Recombinant TSR3 from Sf9 or High-Five Cells Are C-Mannosylated on Trp-480 and O-Glycosylated on Thr-489**—Sf9
cells infected with recombinant baculovirus carrying the construct encoding TSR3 (TSR3-Sf9) yielded protein that was heterogeneous with respect to its mass. A small amount of a species with the mass expected from the cDNA sequence (7250 Da, 5% of the total) was detected by LC-ESIMS. In addition, four other species that had masses of 7396, 7412, 7558, and 7720 Da were found. Because the mass increments closely corresponded to that of integral numbers of dHex and Hex, the recombinant protein seemed to be glycosylated at multiple sites. A tryptic/endoproteinase AspN digest was fractionated by LC-ESIMS (Fig. 7), yielding multiple forms of both the N-terminal peptide, TSR3-Sf9-T55-D-1, as well as the C-terminal one, TSR3-Sf9-T55-D-2. All other observed peptides (Fig. 7, fractions 1, 2, 4, and 6) had masses as predicted from the cDNA sequence. Peptide TSR3-Sf9-T55-D-1a had a mass of 1758.8 Da, in agreement with the presence of one hexosyl residue. ESIMSMs and Edman degradation showed Trp-480 to be C-mannosylated, whereas the other Trp residues were unmodified (Table III). Peptide TSR3-Sf9-T55-D-1b had a mass of 1796.0 Da, in agreement with the theoretical value (Table III) and thus was unmodified. By integrating the areas under the relevant peaks, it could be estimated that Trp-480 was 55% C-mannosylated. Both peptides contain the sequence Asn-Gly, which is sensitive to deamidation at basic pH values. The deamidated forms of these peptides eluted 0.4 min after the main peak (fractions 7a and 8a; Fig. 7) and had a mass that was 1 Da higher.

Two major forms of the C-terminal peptide were isolated as follows: TSR3-Sf9-T55-D-2a, which was 308 Da heavier than expected from the cDNA sequence (Table III), and peptide TSR3-Sf9-T55-D-2b, which was 146 Da heavier. A small amount of a peptide with the mass expected from the cDNA sequence was detected in fraction 4, together with another peptide. Peptides TSR3-Sf9-T55-D-2a and -2b were examined by nanospray Q-TOF MS as described above for the peptides from platelet TSP-1. In addition, exopeptidase fragments were analyzed. The data (Table III) demonstrated that a disaccharide of the form Hex-dHex- was O-linked to Thr-489 in peptide TSR3-Sf9-T55-D-2a. A single dHex residue was present at the same position in peptide TSR3-Sf9-T55-D-2b. Integration of the peaks of these peptides (Fig. 7) showed that Thr-489 in TSR3 from Sf9 cells was 95% O-glycosylated. The ratio between mono- and disaccharide was 1:1 (Fig. 4). In a separate experiment, tryptic peptides from this protein were analyzed without previous cleavage with endoproteinase AspN. This yielded six different TSR3-Sf9-T55 peptides, representing all possible combinations between C- and O-glycosylation at Trp-480 and

| Peptide          | Fuc | Rha | Glc | Gal | Man |
|------------------|-----|-----|-----|-----|-----|
| TSP1-T42-Ch-2-AMP| 1   |     | 1.2 |     | 22  |
| TSP1-T49-Ch-2    |     | 1.1 |     | 22  |
| TSP1-T55-Ch-2    | 1   |     | 1.1 |     |     |
| TSR3-Sf9-T55-D-2a| 1   |     | 1.1 |     |     |
| TSR3-Sf9-T55-D-2b|     |     | 1   |     | 22  |

* Some of the peptides contained a minor peak with a retention time roughly the same as that of the mannose standard (see “Results”).
* No clear-cut data were obtained, probably due to the presence of contaminations.
Thr-489, respectively (data not shown). Taken together, these results explained the five forms of TSR3-Sf9, i.e. 7250 Da (no modifications), 7396 Da [(dHex-)Thr], 7412 Da [(C2-Man-)Trp], 7558 Da [(C2-Man-)Trp and (dHex-)Thr], and 7720 Da [(C-Man-)Trp and (HexdHex-)Thr]. Monosaccharide analysis (Table II) showed that only fucose could be positively identified in TSR3-Sf9-T55-D2a, whereas fucose and glucose were detected in TSR3-Sf9-D2a.

The same experiments, except for the monosaccharide analyses, were repeated with recombinant TSR3 from High-Five cells (TSR3-H5) (data not shown). The results revealed the same modifications, although at lower stoichiometries (Fig. 4).

Recombinant Modules TSR123 from S2 Cells Are Not C-Mannosylated but Are O-Glycosylated on Ser-377, Thr-432, and Thr-489—Fig. 8A shows the part of the LC-ESIMS chromatogram containing the peptides with the C-mannosylation motifs and the O-glycosylations. All other observed major peptides had masses as predicted from the cDNA sequence. The MS data were extracted for the theoretical masses of TSR123-S2-T42, -T49, and -T55, including the C- and O-glycosylations described above. Only two species were detected for each of the three tryptic peptides, the unmodified form and one, which was 146 Da heavier (Table IV). No evidence for C-glycosylation was observed at all. To verify this, each of the tryptic peptides was cleaved with chymotrypsin. The results of an analytical LC-ESIMS experiments for each of the peptides is shown in Fig. 8, B–D. The peptides produced from the part of TSR123-S2-T42, -T49, and T55 containing the C-mannosylation motifs all had
masses in agreement with those calculated from the DNA sequence (data not shown), and thus were unmodified. The C-terminal portions of these peptides, however, occurred in two forms, one having a mass that agreed with the cDNA sequence and one that was 146 Da heavier. The heavier peptides showed facile dissociation of the substituent in the LC-ESIMS experiment (Fig. 8, B–D), as was observed with the O-glycosylated peptides described above. The primary structure of these peptides was verified by nanospray ESIMSMS (Table IV), and the ratio of modified/unmodified peptides was estimated by integrating the area under their peaks in Fig. 8, B–D. Given the location of the O-linked sugars in platelet TSP-1 and recombinant TSR3-Sf9 and TSR3-H5, we assume that the dHex residues are located on Ser-377, Thr-432, and Thr-489. These residues have been assigned to this sequence, including cell adhesion functions. In fact out of 88 TSRs examined, 63 had a Ser or Thr at the position preceding the Cys. Numerous functions have been assigned to this sequence, including cell adhesion (16, 33) and binding to the cell surface receptor CD36, an 88-kDa membrane protein that is expressed on many cells (18). This receptor mediates the in vitro inhibition of basic fibroblast growth factor-induced endothelial cell migration by TSP-1 (34) and is part of the signaling pathway utilized by TSP-1 in the inhibition of neovascularization in vivo (35). Direct binding between native TSRs and CD36 remains to be demonstrated, as does any effect of the O-linked disaccharide.

O-Linked fucose has been found in the EGF-like modules of a number of proteins (Table V) and has been assumed to be exclusive for this type of structure (36). The presence of the disaccharide Glc-Fuc-O-Ser/Thr in human platelet TSP-1 (34) and the O-fucosylation of the proteinase inhibitor PMP-C from Locusta migratoria (37) show that this modification is more general. TSP-1 is the first identified protein that carries the O-linked disaccharide Glc-Fuc-O-Ser/Thr. Labeling experiments had provided evidence that Glc-β1,3-Fuc-a1-O-Ser/Thr occurs in unidentified proteins from Chinese hamster ovary cells (38). In addition, it had been isolated as the amino acid fucoside from human urine (39, 40).

The results presented in this paper show that two sequences that have been implicated in several activities of human platelet TSP-1 do not occur as such but are covalently modified.

O-Glycosylation—By using a novel, potent mass spectrometric approach that will be described in detail elsewhere, we demonstrated that the three TSRs in platelet TSP-1 carry the O-linked disaccharide Glc-Fuc-O-Ser/Thr in the sequence CSX(S/T)CG. This sequence is common to many TSR-containing proteins. In fact out of 88 TSRs examined, 63 had a Ser or Thr at the position preceding the Cys. Numerous functions have been assigned to this sequence, including cell adhesion (16, 33) and binding to the cell surface receptor CD36, an 88-kDa membrane protein that is expressed on many cells (18). This receptor mediates the in vitro inhibition of basic fibroblast growth factor-induced endothelial cell migration by TSP-1 (34) and is part of the signaling pathway utilized by TSP-1 in the inhibition of neovascularization in vivo (35). Direct binding between native TSRs and CD36 remains to be demonstrated, as does any effect of the O-linked disaccharide.

TABLE III
Characterization of C-mannosylated (C-Man) and O-glycosylated peptides from recombinant module TSR3 from Sf9 cells

| Peptide  | Mass (Da) | Identity | Modified residue | PTH-derivative |
|----------|-----------|----------|------------------|---------------|
| TSR3-Sf9-T55-D1a | 1595.7 | 1758.9; n - 1 | 1758.8 | ADPGINGGWGPWMSPW | Trp-480 | 18.84 | 18.81 |
| TSR3-Sf9-T55-D1b | 1595.7 | 1596.0 | | ADPGINGGWGPWSRW | None |
| TSR3-Sf9-T55-D2a | 1380.6 | 1688.9 | 1688.3 | DICSVTCGCGGQK | Thr-489 |
| TSR3-Sf9-T55-D2a-Subt-AMP | 805.4 | 1113.5 | 1113.2 | TCGGGQK | +308Da |
| TSR3-Sf9-T55-D2a-CPase B/Y | 853.3 | 1161.4 | 1161.5 | DICSVT | +308Da |

| TSR3-Sf9-T55-D2b | 1380.6 | 1525.7 | 1526.0 | DICSVT*CGGGQK | Thr-489 |
| TSR3-Sf9-T55-D2b-Subt-AMP | 805.4 | 425.2 | 424.9 | TC | +146Da |
| TSR3-Sf9-T55-D2b-CPase B/Y | 853.3 | 999.4 | 999.7 | DICSVT | +146Da |

a The names of the peptides refer to Fig. 7.
b Monoisotopic masses have been used for values <1500 Da, whereas average masses have been used >1500 Da.
c W*, indicates (C-Man)Trp; S* or T*, Ser or Thr with O-linked dHex. Cysteines have been carboxamidomethylated. The identity of the peptides was verified by ESIMSMS analysis.
d The elution time of the PTH-derivative of a modified Trp obtained by Edman degradation was compared with that of (C-Man)Trp from the peptide F-T-(C2-Man-)WAQW from RNase 2 (29), which was sequenced immediately before or after the sample. The differences in elution time between samples are due to aging of the column and to adjustments in buffer composition to optimize the separation of the other PTH-derivatives.
e Mass calculated from the deduced amino acid sequence.
f Tr*: Thr with O-linked dHex.

DISCUSSION
The results presented in this paper show that two sequences that have been implicated in several activities of human platelet TSP-1 do not occur as such but are covalently modified.

O-Glycosylation—By using a novel, potent mass spectrometric approach that will be described in detail elsewhere, we demonstrated that the three TSRs in platelet TSP-1 carry the O-linked disaccharide Glc-Fuc-O-Ser/Thr in the sequence CSX(S/T)CG. This sequence is common to many TSR-containing proteins. In fact out of 88 TSRs examined, 63 had a Ser or Thr at the position preceding the Cys. Numerous functions have been assigned to this sequence, including cell adhesion (16, 33) and binding to the cell surface receptor CD36, an 88-kDa membrane protein that is expressed on many cells (18). This receptor mediates the in vitro inhibition of basic fibroblast growth factor-induced endothelial cell migration by TSP-1 (34) and is part of the signaling pathway utilized by TSP-1 in the inhibition of neovascularization in vivo (35). Direct binding between native TSRs and CD36 remains to be demonstrated, as does any effect of the O-linked disaccharide.

O-Linked fucose has been found in the EGF-like modules of a number of proteins (Table V) and has been assumed to be exclusive for this type of structure (36). The presence of the disaccharide Glc-Fuc-O-Ser/Thr in human platelet TSP-1 (34) and the O-fucosylation of the proteinase inhibitor PMP-C from Locusta migratoria (37) show that this modification is more general. TSP-1 is the first identified protein that carries the O-linked disaccharide Glc-Fuc-O-Ser/Thr. Labeling experiments had provided evidence that Glc-β1,3-Fuc-a1-O-Ser/Thr occurs in unidentified proteins from Chinese hamster ovary cells (38). In addition, it had been isolated as the amino acid fucoside from human urine (39, 40). O-Fucosylation of EGF-like modules occurs most likely in the Golgi (41), and the fucosyltransferase has been isolated (42). Based on primary structure comparisons, a consensus sequence for this modification in EGF-like modules (CXXGG(S/T)C) has been proposed (43). However, the O-fucosylation sites in TSP do not conform to this sequence (Table V). This raises the question as to whether the same
fucosyltransferase is involved or whether more than one transferase exists. O-Linked fucose monosaccharide can be elongated into a di- or a tetrasaccharide in a protein-specific manner (Table V) (38). Supposedly, the elongating transferases recognize the fucose and as yet unidentified signals in the protein. Until now, the tetrasaccharide has exclusively been found in the EGF-like modules of factor IX and Notch (36, 44), whereas disaccharide has only been observed in TSRs (this paper).³ This raises the possibility that structural features specific for each of these modules lead to elongation of O-fucose into disaccharide and tetrasaccharide, respectively. Whether this is determined by the primary or the tertiary structure, as for other elongating glycosyltransferases (45), remains to be

³ S. Hartmann and J. Hofsteenge, unpublished results.
determined. In this context it should be noted that proper folding of the EGF-like module is required for fucosylation (42), strongly indicating that elongation also must take place after attainment of the three-dimensional structure.

The importance of the O-linked, fucose-based tetrasaccharide has recently been shown in the Notch receptor system. Drosophila Notch plays a role in intercellular signaling through interactions with ligands like Delta and Serrate/Jagged. These interactions are positively or negatively regulated, respectively, by the secreted molecules, do not bind heparin (see Introduction). It is difficult to reconcile these results at present, but purification of the various glycoforms of recombinant TSRs identified in this paper and comparison with the heparin-binding properties may resolve some of these discrepancies.

The peptide SHWSPWSS has been found to inhibit TSP-1 binding to heparin (13), and peptides containing this sequence in addition to conserved basic residues display high affinity heparin binding (52). Importantly, the two Trp residues are essential for this interaction. On the one hand, these data are supported by the observation that heparin binding to midkine, a protein that is now recognized to be a TSR homologue (53), affects the NMR resonances of the Trp residue corresponding to the second Trp in the above-mentioned peptide (54). On the other hand, recombinant TSRs, or a proteolytic fragment from TSP-1, which are both folded in the native TSRs from TSP-1 bind to these molecules. Therefore, any effect that C-mannosylation has on these interactions is unknown. The situation concerning heparin binding to these sequences is inconsistent. The peptide SHWSPWSS has been found to inhibit TSP-1 binding to heparin (13), and peptides containing this sequence in addition to conserved basic residues display high affinity heparin binding (52). Importantly, the two Trp residues are essential for this interaction. On the one hand, these data are supported by the observation that heparin binding to midkine, a protein that is now recognized to be a TSR homologue (53), affects the NMR resonances of the Trp residue corresponding to the second Trp in the above-mentioned peptide (54). On the other hand, recombinant TSRs, or a proteolytic fragment from TSP-1, which are both folded molecules, do not bind heparin (see Introduction). It is difficult to reconcile these results at present, but purification of the various glycoforms of recombinant TSRs identified in this paper and comparing their heparin-binding properties may resolve some of these discrepancies.

C-Mannosylation of Trp has only been documented for vertebrate tissues and cells, although the earliest evidence for heparosylation of Trp was found in a neuropeptide from stick insect (25). Previously, it has been reported that Spodoptera frugiperda cells (Sf9) or Drosophila melanogaster Schneider 2 (S2)

| Peptide* | Mass (Da)b | Identityc |
|----------|------------|-----------|
| TSR123-S2-T42-Ch-3a | 1555.7 | TS/Ca(36)/STS/Ca(36)/CGNGIQQR |
| TSR123-S2-T42-Ch-3b | 1701.8 | TS/Ca(36)/STS/Ca(36)/CGNGIQQR + 146 Da |
| TSR123-S2-T49-Ch-4a | 1497.7 | SS/Ca(36)/STS/Ca(36)/CGDGVITR |
| TSR123-S2-T49-Ch-4b | 1644.3 | SS/Ca(36)/STS/Ca(36)/CGDGVITR + 146 Da |
| TSR123-S2-T55-Ch-3a | 1379.6 | DI/Ca(36)/STS/Ca(36)/CGGGVQK |
| TSR123-S2-T55-Ch-3b | 1526.7 | DI/Ca(36)/STS/Ca(36)/CGGGVQK + 146 Da |

a The names of the peptides refer to Fig. 8, following the nomenclature given under “Experimental Procedures.”
b Monoisotopic masses have been used for values <1500 Da, whereas average masses have been used >1500 Da.
c Cysteines have been carboxamidomethylated. The identity of the peptides was determined by ESIMS.
d Mass calculated from the deduced amino acid sequence.

| Protein | O-Glycosylation | Ref. |
|---------|-----------------|-----|
| Human platelet TSP-1 | Glc-Fuc-O | This paper |
| Urokinase | CXXGG/T/Si-C | (58) |
| Tissue-type plasminogen activator | Fuc-O | (59) |
| Factor VII | | (60) |
| Factor XII | | (61) |
| Amino acid fucoside from urine | (T/S) | (40) |
| | Glc-β1,3-Fuc-O | (39) |
| Factor IX | CXXGG/T/Si-C | (44) |
| | Sia-α2-6-Gal-β1,4-GlcNac-β1,3-Fuc-O | (43) |
| Notch | | (36) |
| PMP-C, L. migratoria | CXXGTXXXX-C | (37) |
| | Fuc-O | |

The importance of the O-linked, fucose-based tetrasaccharide has recently been shown in the Notch receptor system. Drosophila Notch plays a role in intercellular signaling through interactions with ligands like Delta and Serrate/Jagged. These interactions are positively or negatively regulated, respectively, by the secreted protein Fringe. Moloney et al. (46) and Brückner et al. (47) have recently demonstrated that Drosophila and mammalian Fringe proteins are O-fucose-specific β1,3N-acetylglucosaminyltransferases that perform the first step in the synthesis of the tetrasaccharide. Importantly, this modification modulates the ligand-receptor interaction in vivo. Because TSP-1 also functions in protein-protein interactions at the cell surface (9), it may be worthwhile to search for proteins that modulate these, analogous to Fringe in the Notch system. An enzyme activity that elongates α-fucose to the disaccharide has been identified in Chinese hamster ovary cells (48). The enzyme is widespread in biology, suggesting that the disaccharide has an important biological function (36, 48).

TSP-1 has three EGF-like modules, none of which has the recognition sequence for fucose-specific β1,3N-acetylglucosaminyltransferases that perform the first step in the synthesis of the tetrasaccharide. Importantly, this modification modulates the ligand-receptor interaction. On the one hand, these data are supported by the observation that heparin binding to midkine, a protein that is now recognized to be a TSR homologue (53), affects the NMR resonances of the Trp residue corresponding to the second Trp in the above-mentioned peptide (54). On the other hand, recombinant TSRs, or a proteolytic fragment from TSP-1, which are both folded molecules, do not bind heparin (see Introduction). It is difficult to reconcile these results at present, but purification of the various glycoforms of recombinant TSRs identified in this paper and comparing their heparin-binding properties may resolve some of these discrepancies.

C-Mannosylation of Trp has only been documented for vertebrate tissues and cells, although the earliest evidence for heparosylation of Trp was found in a neuropeptide from stick insect (25). Previously, it has been reported that Spodoptera frugiperda cells (Sf9) or Drosophila melanogaster Schneider 2 (S2)
cells do not C-mannosylate RNase 2 (23). For S2 cells, these findings were confirmed by the results obtained with TSR123. However, in SF9 cells, and in addition Trichoplusia ni cells (High-Five), did C-mannosylate TSRs (Fig. 4). The reason for this difference remains unclear. The only relevant distinction between the two studies may be the use of different secretion signals. Nevertheless, our results show conclusively that insects are capable of C-mannosylation and that the modification is widespread in the animal kingdom.

Not all TSRs have the same C-mannosylation pattern. In TSP-1, the first Trp in the sequence WXWXXXWXXC is only modified in TSR2 (Fig. 4). Furthermore, the third Trp in this propeptide, and the terminal components of complement are modified in 8 of 9 cases (21, 22). This led to the hypothesis that an additional signal, different from the sequence WXW, is present in these proteins (22). In contrast, in TSP-1 the third Trp was never C-mannosylated (Fig. 4). Either the TSRs in these proteins differ structurally or megakaryocytes, the site of synthesis of platelet TSP-1 (Fig. 4). Modification of Trp-XX- mannosyltransferase or dolichyl-P-

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