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Synthesis of Truncated Amino-Terminal Trimers of Thrombospondin†

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ABSTRACT: Thrombospondin (TSP) is a 450-kDa glycoprotein that is comprised of three identical disulfide-bonded subunits (1152 amino acids) held together near the heparin-binding amino-terminal globular domains. TSP truncated at residue 277 (TSP-277) or 381 (TSP-381) consisted largely of disulfide-bonded trimers when expressed in COS cells or insect cells. In addition, TSP-381 formed heterotrimers with endogenous COS cell TSP. Cleavage of TSP and the truncated mutants in the proteolytically sensitive region between residues 220 and 237 yielded monomeric amino-terminal fragments. Cys-252 and Cys-256 are the only cysteines between residues 238 and 277 and therefore must bridge among subunits. TSP-381 in which Cys-252 and Cys-256 were changed to glycine was secreted efficiently by COS cells but with only a minor portion of the protein in the form of disulfide-bonded trimers. The sequence of TSP between residues 258 and 283 is predicted to form an amphiphatic α-helix. We suggest that assembly of TSP trimers involves formation of an α-helical coiled-coil structure which is stabilized by formation of disulfides.

Thrombospondin (TSP) is a 450-kDa glycoprotein that is found in platelet α-granules (Baenzinger et al., 1971, 1972) and is synthesized and secreted by a variety of cells in culture (Raugi et al., 1982; Mosher et al., 1982; Jaffe et al., 1983; Asch et al., 1986). TSP interacts with a number of macromolecules including collagen type V, fibronectin, histidine-rich glycoprotein, plasminogen, fibrinogen, proteoglycans, heparin, and sulfated glycolipids (Lawler, 1986; Frazier, 1987; Mosher, 1990). TSP has been implicated in platelet aggregation (Leung, 1984; Silverstein, 1986) and also plays a role in the growth (Majack et al., 1986, 1988; Phan et al., 1989) and

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A

B

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1    NRIPESGGDNSVFDIFELTGAARKGSGRRLVKGPDPSSPAFRIEDANLIPPVPDDKFQDL   60
61   VDAVRTEKGFLLLASLRQMKKTRGTLLALERKDHSGQVFSVVSNGKAGTLDLSLTVQGKQ  120
121  HVVSVEEALLATGQWKSITLFVQEDRAQLYIDCEKMENAELDVPIQSVFTRDLASIARLR  180
181  IAKGGVNDNFQGVLQNVRFVFGTTPEDILRNKGCSSSTSVLLTLDNNVVNGSSPAIRTNY  240
241  IGHKTKDLQAICGISCDELSSMVLELRGLRTIVTTLQDSIRKVTEENKELANELRRPPLC  300
301  YHNGVQYRNNEEWTVDSCTECHCQNSVTICKKVSCPIMPCSNATVPDGECCPRCWPSDSA  360
361  DDGWSPWSEWTSCSTSCGNGIQQRGRSCDSLNNRCEGSSV                      400
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FIGURE 1: (A) Restriction enzyme sites used in construction of TSP-277 are indicated. Vector sequences from pGEM are shown as wavy lines. Numbers in parenthenses show base pairs in the TSP cDNA sequence (Lawler & Hynes, 1986). The A in ATG is at position 76. (B) Amino acid sequence of the first 400 amino acids of TSP (Lawler & Hynes, 1986; Kobayashi et al., 1986). The last amino acids encoded by TSP-277 and TSP-381 are indicated by the large arrowheads. The last amino acids encoded by the constructs of Prochownik et al. (1989) are shown by the small arrowheads. Cysteine residues are underlined. The N-linked glycosylation site is indicated by the triangle. Trypsin and thrombin probably cleave after Arg-237; chymotrypsin and thermolysin cleave before Asn-230.

adhesion (Roberts et al., 1987; Lawler et al., 1988; Kaesberg et al., 1989; Murphy-Ullrich & Hook, 1989) of cells in culture. The amino-terimnal heparin-binding domain (HBD) of TSP is involved in a number of these processes (Roberts et al., 1987; Kaesberg et al., 1989; Murphy-Ullrich & Hook, 1989). The HBD also mediates the binding and degradation of TSP by several cell types (McKeown-Longo et al., 1984; Murphy-Ullrich et al., 1987, 1988).

TSP is a trimer of three identical subunits which are held together by disulfide bonds located near the HBD (Lawler & Hynes, 1986). Digestion of TSP with a number of proteases including thrombin and trypsin results in release of a 25–30-kDa amino-terminal fragment which retains the ability to bind heparin (Lawler & Slater, 1981; Dixit et al., 1984; Raugi et al., 1984; Lawler et al., 1985). This HBD lacks the region involved in interchain disulfide bond formation. To identify which cysteines are involved in interchain disulfide bonding, and to gain insight into structural determinants that allow efficient trimerization, amino-terminal fragments of TSP were expressed using baculovirus and COS cell expression systems. These studies show that in truncated TSP molecules, Cys-252 and/or Cys-256 stabilize trimerization.

Materials and Methods

Baculovirus Vector Construction

TSP-277. A 1.27-kb TSP cDNA clone (M10S) in pGEM2 (Promega, Madison, WI) was obtained from J. Lawler (Harvard Medical School, Boston, MA; Lawler & Hynes, 1986). This clone contains 75 bp of 5′ untranslated DNA, and the first 1165 bp from the coding region of TSP. A 999 bp *Pst*I fragment was generated by partial digestion with *Pst*I and was subcloned into pGEM5Zf (see Figure 1A). This cDNA contains 40 bp from the multiple cloning site of pGEM2, 75 bp from the 5′ untranslated region, and the first 884 bp from the coding region of TSP. A clone in the negative orientation was isolated, linearized with *Spe*I, and blunt-ended using standard procedures (Maniatis et al., 1982). Following ligation to *Xba* linkers (Stratagene, La Jolla, CA) containing stop codons in all three frames, the DNA was gel-purified and then digested with *Bam*HI to generate a 957 bp TSP fragment. The digested fragments were ligated to *Bam*HI linkers (Stratagene) to create a *Bam*HI site at the 3′ end of the TSP DNA. The 957 bp fragment containing 29 bp from the 5′-untranslated region of TSP, and the first 884 bp from the coding region of TSP, was cloned into the *Bam*HI cloning site of the baculovirus replacement vector pAcYMI (Matsuura et al., 1987). This DNA encodes an 18 amino acid signal peptide, and the first 277 amino acids of mature TSP. Sequences present in the *Xba* linkers and in the multiple cloning site of pGEM5Zf created eight amino acids at the 3′ end of the TSP coding sequence, Ala-Ala-Ala-Leu-Ala-Ser-Leu-Asn.

TSP-381. M10S was excised from pGEM2, blunt-ended, and then ligated to a universal termination terminator (GCTTAATTAATTAAGC; Pharmacia, Piscataway, NJ), to generate a stop codon at the 3′ end of the DNA. After addition of *Hin*dIII linkers (Stratagene), the DNA was subcloned into pGEM7Zf. Clones in the positive orientation were selected, digested with *Bam*HI, and cloned into pAcYMI as above. This *Bam* fragment contains 29 bp from the 5′-untranslated region and the first 1192 bp from the coding region of TSP. Addition of linkers added an Ala to the 3′ end of the TSP coding sequence.

Cells and Virus. The insect cell line IPLB-SF-21 (Vaughn et al., 1977) was a gift from P. Friesen (University of Wisconsin, Madison). SF-21 cells were cultured in TC100 medium (Gibco, Grand Island, NY) containing 10% fetal calf serum, supplemented with 0.6 μg/mL amphotericin B (Gibco), 150 μg/mL streptomycin–sulfate (Sigma), and 99.7 units/mL penicillin (Sigma). Cells were grown to confluence [(0.8–1.0 × 10^6 cells/mL] in monolayer culture at 27 °C. Nonrecombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) was a gift from P. Friesen. Viral DNA was prepared as described (Miller et al., 1986).

Production of Recombinant Virus. pAcYMI containing TSP-277 or TSP-381 cDNA was cotransfected with AcNPV DNA into SF-21 cells with lipofectin (BRL, Gaithersburg, MD) according to manufacturer's instruction. Recombinant viruses (TSP-277/AcNPV and TSP-381/AcNPV) were selected and plaque-purified 3 times as described (Miller et al., 1986; Summers & Smith, 1987). SF-21 cells (10^7 cells/

100-mm dish) were infected with recombinant virus at a multiplicity of infection of 10–20 in serum-containing medium. After 24 h, the medium was removed, the cells were gently washed 3 times with serum-free TC100, and then incubated with 5 mL/dish serum-free TC100 for 48 h at 27 °C. The conditioned medium was collected and centrifuged at 2500 rpm for 10 min at room temperature. The supernatant was decanted and stored frozen until use. Conditioned medium from infected cells was analyzed by a competitive enzyme-linked immunosorbant assay (Jaffe et al., 1983) using monoclonal antibody A2.5 (a gift from W. Frazier, Washington University, St. Louis, MO), which recognizes the HBD of platelet TSP (Dixit et al., 1985). These data showed that recombinant HBD (rHBD) was secreted and that maximal amounts of protein were present in medium collected 24–72 h after infection. Analysis of cell extracts indicated that <1% of the total rHBD produced by insect cells was located intracellularly.

Purification and Characterization of TSP and Truncated TSP

Purification of Platelet TSP. TSP was purified from platelet releasate as described (Mosher et al., 1982).

Heparin–Sepharose Chromatography. Conditioned medium from cells infected with TSP-277/AcNPV was applied to a heparin–Sepharose (Pharmacia) column (2 mL) at a flow rate of 1 mL/min. Following a wash with 0.15 M NaCl/20 mM Tris, pH 7.4 (TBS), bound proteins were eluted either with 0.65 M NaCl/20 mM Tris, pH 7.4, or with a gradient from 0.15 to 0.65 M NaCl in 20 mM Tris, pH 7.4. Fractions (1 mL) were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (see below). Western blot analysis (see below) showed that there was no detectable rHBD in the heparin–Sepharose unbound fraction.

A2.5–Agarose Chromatography. A2.5 (4 mg) was linked to cyanogen bromide activated agarose (1.75 mL) according to manufacturer's instructions (Pharmacia). Conditioned medium was applied to the A2.5 column at a flow rate of 1 mL/min. The column was washed with 2 M NaCl/20 mM Tris, pH 7.4, prior to elution with 2 M $MgCl_2$/20 mM phosphate, pH 7.2. Fractions containing rHBD were dialyzed against TBS.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. SDS–PAGE was performed according to Laemmli (1970). Proteins were mixed with sample buffer (final concentration: 50 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 0.02% bromphenol blue) and run on 8 or 10% slab gels. Proteins were visualized by staining with 0.05% Coomassie blue. Molecular weight standards were fibronectin (240K), phosphorylase B (92K), bovine serum albumin (68K), ovalbumin (46K), chymotrypsinogen (24.5K), and hemoglobin (16.5K). ^{14}C-labeled molecular weight standards (Amersham, Arlington Heights, IL) were myosin (200K), phosphorylase B (92.5K), bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K), and lysozyme (14.3K).

Two-Dimensional Nonreduced/Reduced Gels. Proteins purified on an A2.5 column were run under nonreducing conditions in the first dimension on a 10% SDS–polyacrylamide gel. After electrophoresis, a gel track was removed, soaked in sample buffer containing 10% β-mercaptoethanol for >1 h, and then electrophoresed on a second 10% gel. Proteins were visualized by silver staining (Wray et al., 1981).

Western Blot Analysis. Conditioned medium proteins from infected cells were concentrated by heparin–Sepharose or A2.5–agarose chromatography, separated by SDS–PAGE, and transferred onto nitrocellulose paper (Schleicher & Schuell, Keene, NH) (Towbin et al., 1979). Blots were incubated overnight with a polyclonal antibody to TSP that was generated in this laboratory. Blots were washed extensively with TBS, and with TBS containing 0.5% Nonidet-P40, and then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Cappel, West Chester, PA) at 1:1000 or 1:2000 for 1 h. Blots were developed with either 4-chloro-1-naphthol/H_2O_2 (Kirkegaard and Perry, Gaithersburg, MD) or with diaminobenzidine (Sigma). Western blots of cell extracts from infected cells showed that rHBD trimers, dimers, and multimers were found in the same relative proportions in intracellular and secreted pools.

Protease Digestion. rHBD trimers produced by TSP-277/AcNPV-infected cells were isolated from conditioned medium by heparin–Sepharose chromatography. rHBD trimers comprised >90% of the total protein as assessed by SDS–PAGE. rHBD was diluted to 0.15 M NaCl with 20 mM Tris, pH 7.4, and then concentrated by ultrafiltration to approximately 340 μg/mL. rHBD (15 μg) and platelet TSP (15 μg) were digested with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin (Cooper Biomedical, Malvern, PA) at a final concentration of 20 μg/mL for 1 min at room temperature (Lawler & Slater, 1981). Soybean trypsin inhibitor (Sigma) (50 μg/mL) was added to a final concentration of 50 μg/mL. Chymotrypsin (1:200 w/w) and thermolysin (1:100 w/w) (Sigma) digestions were performed for 1 h at room temperature (Dixit et al., 1984; Lawler et al., 1986a,b). Reactions were stopped by adding an equal volume of 2× sample buffer with or without β-mercaptoethanol. Samples were heated to 100 °C for 4 min and then analyzed by SDS–PAGE.

COS Cell Expression and Mutagenesis. TSP-277 and TSP-381 cDNAs were cloned into the *Bam*HI cloning site of the COS cell expression vector pSVL (Pharmacia, Piscataway, NJ). DNA was transfected into COS-1 cells (a gift from D. Greenspan, University of Wisconsin, Madison) using DEAE-dextran (Pharmacia) as described (Sambrook et al., 1989). Cultures were labeled with Tran ^{35}S-label (35–50 μCi/mL; ICN, Irvine, CA) and [^3H]leucine (100 μCi/mL; NEN, Wilmington, DE) 40–48 h after transfection in medium containing 1% fetal bovine serum. Conditioned medium proteins were harvested after 24 h and stored frozen until use.

Conditioned medium proteins were immunoprecipitated with monoclonal antibody A2.5. Conditioned medium (400–500 μL) was incubated with immunoprecipitation buffer (0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, and 1% deoxycholate in 50 mM phosphate buffer, pH 7.4) and 1.4 μg of A2.5 for 1 h on ice. Rabbit anti-mouse IgG (3 μg) was added, and the mixture was incubated for 1 h on ice. This mixture was then incubated with protein A–agarose (Calbiochem, La Jolla, CA) for 1 h at room temperature. After the mixture was washed 5 times with immunoprecipitation buffer, proteins were eluted from the protein A–agarose with sample buffer. The same relative proportions of rHBD trimers, dimers, and multimers were found when detergent extracts of cells were immunoprecipitated.

Site-Directed Mutagenesis. Oligonucleotide-directed mutagenesis was performed according to the method of Kunkel (Kunkel et al., 1987). TSP-381 cDNA cloned into M13mp18 was used as a template for mutagenesis experiments. A 34-base oligonucleotide (GCAAGCCATCGGCGGCATCT-CCGGTGATGAGCTG) was synthesized by using an Applied Biosystems automated DNA synthesizer. The underlined bases represent substitutions of the wild-type sequence designed to replace codons for Cys-252 and Cys-256 with glycine codons. Phage were screened by hybridization to ^{32}P-labeled mutant

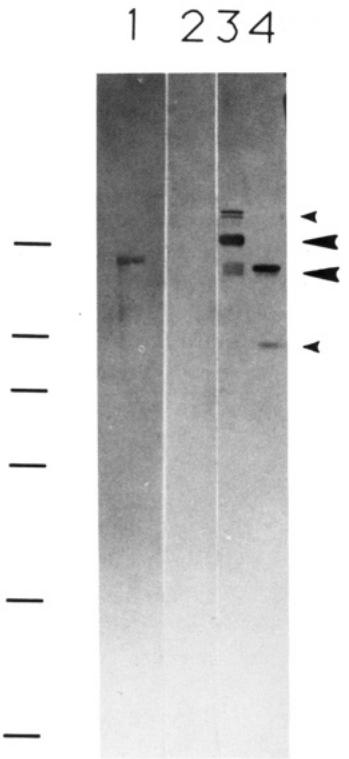

FIGURE 2: Western blot of heparin-binding proteins from insect cells infected with wild-type and recombinant viruses. Conditioned medium (500 μL) from cells infected with wild-type virus (lane 2), TSP-381/AcNPV (lane 3), and TSP-277/AcNPV (lane 4) was incubated with 100 μL of heparin–agarose for 1 h at room temperature. After the mixture was washed with TBS, proteins were solubilized by the addition of 50 μL of nonreducing sample buffer. Platelet TSP (1.5 μg) was run under reducing conditions (lane 1). Proteins were separated on an SDS–polyacrylamide gel, then transferred to nitrocellulose paper, and incubated with a polyclonal antibody to TSP. The positions of trimeric TSP-277 (lane 4) and TSP-381 (lane 3) are indicated by the large arrowheads. The positions of high molecular weight multimers of TSP-381 (lane 3) and TSP-277 dimers (lane 4) are indicated by the small arrowheads. Molecular weight standards were visualized by staining with Ponceau S prior to primary antibody incubation, and are indicated by the dashes: 240K, 92K, 68K, 43K, 24.5K, and 16.5K.

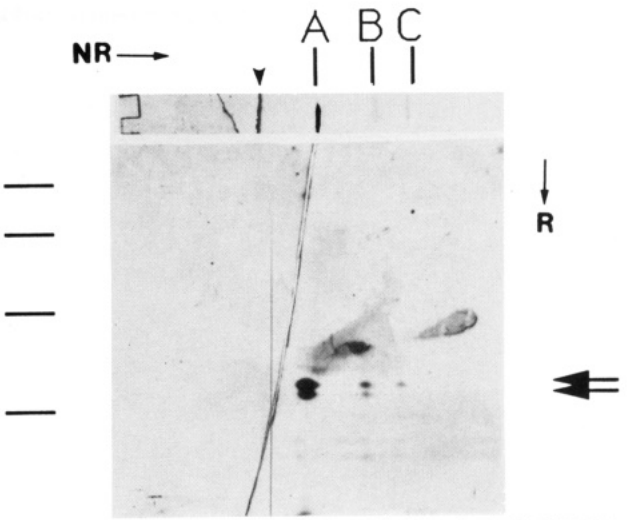

FIGURE 3: Two-dimensional nonreduced/reduced gel of TSP-277. TSP-277 was purified by A2.5 affinity chromatography and then concentrated by ultrafiltration. Proteins were run under nonreducing conditions in the first dimension (top; NR) and under reducing (R) conditions in the second dimension. Following electrophoresis, the gel was silver-stained. A, B, and C mark the positions of the three proteins with apparent molecular weights of 110K, 71K, and 60K that copurify by A2.5 chromatography. Molecular weight standards, 92K, 68K, 43K, and 24.5K, are indicated by the dashes. Arrows show the position of reduced TSP-277. The arrowhead marks the beginning of the resolving gel in the nonreduced gel.

oligonucleotide under permissive and nonpermissive temperatures as described (Zoller et al., 1989). The mutations were confirmed by DNA sequencing (Sanger et al., 1977). The mutant DNA, TSP-381 C→G, was subcloned into the COS cell expression vector pSVL.

Results

Expression of TSP-277 and TSP-381 in Insect Cells. To determine which cysteines are responsible for interchain disulfide bond formation in TSP, we expressed two amino-terminal TSP fragments using baculovirus and COS cell expression systems. The longer construct (TSP-381) encodes TSP amino acid residues through Ile-381, contains 16 cysteines, and ends in the middle of the first type I domain (Lawler & Hynes, 1986; Frazier et al., 1987; see Figure 1B). The shorter construct (TSP-277) encodes residues through Gln-277, contains only four cysteines, and ends before the start of the procollagen domain (Lawler & Hynes, 1986; Kobayashi et al., 1986; Frazier, 1987).

Medium isolated from insect cells infected by both constructs contained proteins which bound to heparin–Sepharose and cross-reacted with a polyclonal antibody to TSP (Figure 2). The rHBD encoded by the TSP-277 construct migrated with an apparent molecular weight of 110K (lane 4), while the rHBD encoded by the TSP-381 construct migrated with an apparent molecular weight of approximately 150K (lane 3). Medium isolated from cells infected with wild-type virus did not cross-react with the TSP antibody (lane 2). Without carbohydrate, the predicted size of rHBD encoded by TSP-381 is approximately 125 kDa, while that encoded by TSP-277 is 99 kDa. The high molecular weight (>200K) immunoreactive proteins produced by TSP-381-infected cells probably represent high molecular weight oligomers of rHBD, while the lower molecular weight proteins may represent dimers (see below). Dimers were also produced by TSP-277-infected cells (lane 4; see below). rHBD trimers, dimers, and multimers were also detected in cell extracts in the same relative proportion (not shown), indicating that secreted and intracellular rHBDs are similar.

To determine whether truncated TSP produced in insect cells retains its ability to interact with A2.5, an antibody that recognizes unreduced amino-terminal heparin-binding fragment (Dixit et al., 1985), conditioned medium from TSP-277-infected cells was fractionated on an A2.5–agarose column. Three proteins with apparent molecular weights of 110K, 71K, and 60K were retained by the antibody column (Figure 3, NR). To determine whether these three proteins represent processing variants of HBD, TSP-277 isolated from an A2.5 column was analyzed by two-dimensional nonreduced/reduced gel electrophoresis. Figure 3 shows that the three proteins migrated identically as closely spaced doublets of approximately 29–28 kDa in the reduced dimension (R). The lower band of this doublet may represent underglycosylated rHBD (see below). These data indicate that the three proteins are all multimers of HBD. We suspect, based on size, that the 110-kDa protein is a trimer, whereas the 71- and 60-kDa proteins are both dimers. The disulfide-bonding patterns within the dimers may be different, thus causing a difference in shape in the nonreduced state.

Comparison of Proteolytic Fragments of rHBD and Platelet TSP. TSP-277 contains only four cysteine residues. Residues 153 and 214 are involved in an intrachain disulfide bond and are present in the 25–30-kDa monomer that results when TSP

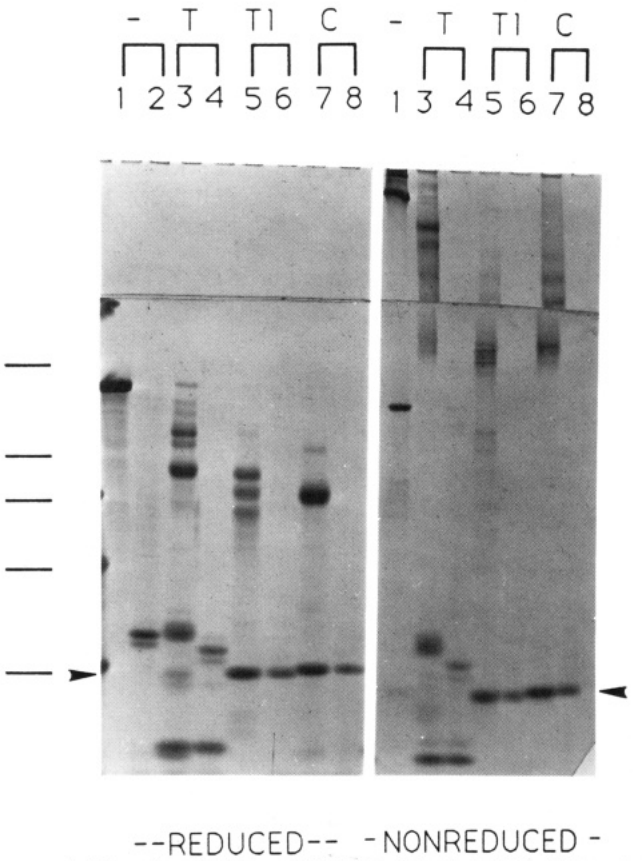

FIGURE 4: Digestion of TSP and TSP-277. Platelet TSP (odd lanes) and TSP-277 (even lanes) were undigested (–, lanes 1 and 2) or treated with trypsin (T, lanes 3 and 4), thermolysin (Tl, lanes 5 and 6), or chymotrypsin (C, lanes 7 and 8). Proteins were run under reducing or nonreducing conditions on a 10% gel. Nonreduced TSP and TSP-277 were loaded in the same track (lane 1), but only TSP-277 entered the separating gel. The positions of the 25-kDa heparin-binding fragment resulting from chymotrypsin and thermolysin digestions are shown by arrowheads. Molecular weight standards, 240K, 92K, 68K, 43K, and 24.5K, are shown by dashes.

is digested with proteases (Lawler & Slater, 1981; Dixit et al., 1984). Our finding that TSP-277 is secreted largely as a disulfide-bonded trimer implies that Cys-252 and Cys-256 participate in interchain disulfide bonds. To test this conclusion, TSP-277 was digested with proteases which cleave between Cys-214 and Cys-252. Limited trypsinization of TSP-277 produced a fragment that migrated further by SDS–PAGE than the 30-kDa fragment produced by digestion of platelet TSP (Figure 4, lanes 3 and 4). Digestion with thrombin gave similar results (data not shown). In contrast, fragments produced by chymotrypsin and thermolysin digestion of platelet TSP and rHBD comigrated (Figure 4, lanes 5–8). These results suggest that the difference in migration of the platelet and recombinant HBD fragments is due to a difference in glycosylation at Asn-230, because thermolysin and chymotrypsin cleave before Asn-230 to form a HBD which lacks the N-glycosylation site that is present in the 30-kDa tryptic HBD (Lawler et al., 1986a,b). rHBD treated with chymotrypsin and thermolysin migrated as one band, whereas a doublet was seen in the tryptic digest. The lower band in this doublet may represent nonglycosylated or underglycosylated rHBD, since insect cells are known to process sugar chains differently than mammalian cells (Miller, 1988; Jarvis & Summers, 1989). Evidence that Cys-153 and Cys-214 are involved in an intrachain disulfide bond in TSP-277 was shown by the shift in the apparent size of the proteolyzed HBD upon reduction.

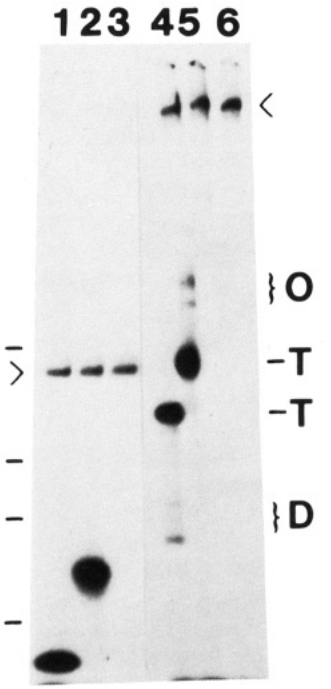

FIGURE 5: Truncated TSP produced in COS cells. COS cells were transfected with TSP-277 (lanes 1 and 4), TSP-381 (lanes 2 and 5), or vector alone (lanes 3 and 6) DNA. Conditioned medium proteins were immunoprecipitated with monoclonal antibody A2.5. Proteins were run under reducing (lanes 1–3) or nonreducing conditions (lanes 4–6). Molecular weight standards, 200K, 92.5K, 69K, and 46K, are indicated by the dashes. Arrowheads indicate the position of COS cell TSP. The positions of proposed high molecular weight oligomers (O), trimers (T), dimers (D), and monomers (M) are indicated.

Analysis of Mutant and Nonmutant rHBD in COS Cells. The truncated TSPs were also expressed in COS cells which should glycosylate more normally than insect cells, and also synthesize full-length TSP endogenously. Truncated subunits isolated from the culture medium of COS cells transfected with TSP-277 and TSP-381 DNA were slightly larger than the subunits produced by insect cells (Figure 5). Treatment with tunicamycin caused an approximately 3-kDa reduction in the apparant molecular weight of COS cell molecules (data not shown).

Mutant TSP-381 was expressed in which Cys-252 and Cys-256 were mutated to glycine residues (TSP-381 C→G). Figure 6A compares the patterns of mutant and nonmutant TSP-381 produced by COS cells under nonreducing conditions on SDS–PAGE. TSP-381 C→G migrated as dimers under nonreducing conditions; smaller amounts of trimers, high molecular weight oligomers, and monomeric HBD were also present. Different oligomerization forms of nonmutant and mutant proteins all comigrated in the reduced dimension when run on a two-dimensional nonreduced/reduced gel (Figure 6B). In addition, some COS cell TSP comigrated with TSP-381 under nonreducing conditions, indicating the presence of heterotrimers (Figure 6B). No evidence for heterotrimer formation was found with the TSP-381 C→G mutant (Figure 6B).

Discussion

TSP is a multifunctional protein which has been implicated in cell adhesion, growth, and migration (Lawler, 1986; Silverstein et al., 1986; Frazier, 1987; Mosher, 1990). Many of these interactions are mediated by the amino-terminal HBD. The HBD has been isolated by proteolysis of TSP by trypsin, thrombin, chymotrypsin, and thermolysin (Lawler & Slater,

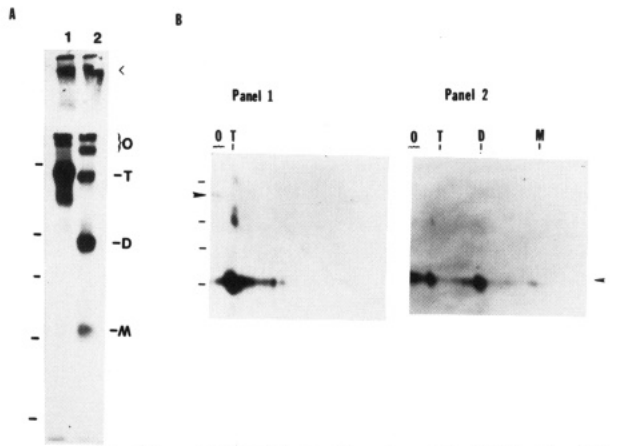

FIGURE 6: TSP-381 and TSP-381 C→S produced in COS cells. (A) One-dimensional gel without reduction. COS cells were tranfected with TSP-381 (lane A1 and panel B1) or TSP-381 C→G (lane A2 and panel B2) DNA. Conditioned medium proteins were immunoprecipitated with monoclonal antibody A2.5. The positions of high molecular weight oligomers (O), homotrimers (T), dimers (D), and monomers (M) seen without reduction are indicated. Similar results were obtained when cell extracts were immunoprecipitated. Molecular weight standards, 200K, 92.5K, 69K, 46K, and 30K, are denoted by dashes. The arrowhead in (A) indicates the position of COS cell TSP. (B) Two-dimensional nonreduced/reduced gels. In (B), proteins were run under nonreducing conditions in the horizontal dimension and under reducing conditions in the vertical dimension. The positions of reduced COS cell TSP (large arrowhead) and TSP-381 (small arrowhead) are indicated. The homotrimers of COS cell TSP did not enter the resolving gel in the first dimension, and thus are not detected. The protein migrating near the 93-kDa marker is a nonspecifically immunoprecipitated protein that did not appear in other reduced gels. The large arrowhead in panel B1 points to subunits of TSP in presumed heterotrimers of TSP and TSP-381.

1981; Dixit et al., 1984; Raugi et al., 1984; Galvin et al., 1985; Lawler et al., 1985). Digestion with these enzymes results in the formation of a 25–30-kDa monomeric heparin-binding fragment.

Each mature TSP chain contains 69 cysteine residues. The HBD produced by trypsin or chymotrypsin digestion contains two cysteines, Cys-153 and Cys-214 (see Figure 1B). These cysteines are thought to be disulfide-bonded to each other (Lawler & Slater, 1981; Figure 4). Frazier has proposed that Cys-252 and Cys-256 are involved in trimerization of TSP, by analogy with the cysteines involved in the interchain disulfide bonds which mediate trimerization of fibrinogen (Frazier, 1987). Our data are compatible with Frazier's proposal in that truncated TSP molecules containing only four cysteine residues (TSP-277) are capable of trimerization and gave rise to the expected monomeric HBD upon limited proteolysis (Figure 4). Our data are also compatible with the results of Prochownick et al. (1989). These investigators found that a truncated TSP ending with Thr-218 did not trimerize, while a protein ending with Val-333, which contains nine cysteine residues, did trimerize.

Formation of disulfide-bonded trimers by TSP-277 was not totally efficient, as evidenced by the presence of disulfide-bonded dimers (Figure 3). The presumptive dimers migrate as two bands of different mobility, which may represent disulfides in parallel (i.e., Cys-252–Cys-252 and Cys-256–Cys-256) and antiparallel (i.e., Cys-252–Cys-256) configurations. TSP-381 formed larger oligomers in addition to the homotrimer. Analysis of the oligomers produced in COS cells on two-dimensional nonreduced/reduced gels suggests that some of these oligomers represent heterotrimers of endogenous COS cell full-length TSP and rHBD (Figure 6B); other probably contain only TSP-381. Higher molecular weight

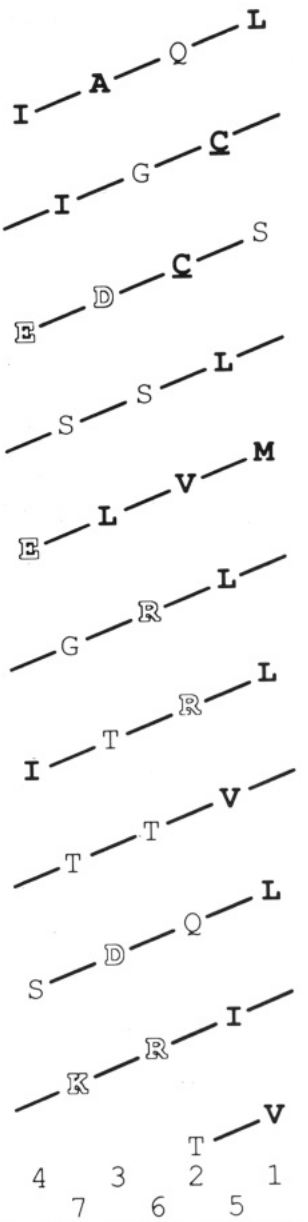

FIGURE 7: Depiction of the sequence of TSP about the interchain disulfides as an α-helical surface. The sequence from Leu-248 (upper right) to Thr-284 (lower left) is shown. Hydrophobic residues are in boldface type. Charged residues are outlined. Cys-252 and Cys-256 are underlined. Note the hydrophobic surface on the right-hand side of the surface below the cysteines.

oligomers were not detected with TSP-277 (Figures 2 and 5), indicating that sequences C-terminal to Gln-277 are necessary for association with full-length TSP.

In the TSP-381 C→G mutant, oligomers larger than trimers were present at relatively high levels. Analysis of these oligomers on two-dimensional nonreduced/reduced gels failed to demonstrate any association of COS cell TSP chains with mutant rHBD. TSP-381 C→G would have to use cysteines other than Cys-252 and Cys-256 to form interchain disulfide bonds. TSP-381 ends in the middle of the first type I domain. Although the disulfide bonding pattern of the type I domains of TSP is not known, it is likely that the usual cystine pairs can not form. As a result, novel interchain disulfide bonding may occur, which could lead to the formation of oligomers. The interchain disulfide bonding pattern appeared to be unstable in the TSP C→G mutant, as the level of dimers was greatly increased by freezing and thawing of the protein. This increase in dimer levels was prevented by pretreatment with

the sulfhydryl-modifying agent *N*-ethylmaleimide (data not shown).

Inspection of the amino acid sequence around Cys-252 and Cys-256 gives additional insight into the forces that govern the trimerization of TSP. The Chou–Fasman and Garnier algorithims (Chou & Fasman, 1978; Garnier et al., 1978; Devereux et al., 1984) both predict that residues 246 through 296 form an α-helix. The sequence between residues 258 and 283 contains regular spaced bulky hydrophobic residues so that the helix is amphipathic (Figure 7); this periodicity is detected by hydrophobic moment analysis as per Eisenberg et al. (Eisenberg et al., 1984; Devereux et al., 1984). It is likely that, as in fibrinogen (Doolittle, 1978), three amphipathic helices assemble in a coiled-coil arrangement stabilized by intersubunit disulfides.

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Registry No. Cys, 52-90-4.

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