Expression and Analysis of COOH-Terminal Deletions of the Human Thrombospondin Molecule

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Abstract. Thrombospondin (TSP) is a homotrimeric extracellular glycoprotein with a subunit molecular mass of 140 kD. The subunits have a modular or domain-like structure and are held together by interchain disulphide bonds. A number of domains have been identified including those for the binding of collagen, fibrinogen, and heparin. Due to the trimeric form of the TSP molecule, the various domains are trivalent in nature and this contributes to the ability of TSP to mediate cell–substrate interactions. Indeed, TSP has recently been shown not only to promote cell adhesion but also to be intimately involved in cell growth and migration. The adhesive function of TSP is attributable to the "solid-phase" or matrix-bound form of the molecule. There is some evidence that the heparin-binding domain mediates incorporation of soluble TSP into the insoluble matrix form. The heparin-binding domain of TSP is a compact globular amino-terminal moiety that contains two clusters of basic amino acids and a single intrachain disulphide bond. To delineate the role of the heparin-binding domain in matrix assembly and to define further the precise region of interchain disulphide bonding that re-

sults in trimer formation, we have expressed deleted forms of the cDNA encoding TSP in SV-40–transformed, African green monkey kidney cells. The proteins synthesized from the various deleted TSP cDNAs were examined for (*a*) secretion into the culture medium and incorporation into the extracellular matrix; (*b*) binding to heparin–Sepharose; (*c*) immunoprecipitability by a conformation-specific monoclonal antibody; and (*d*) ability to form trimers. This analysis allowed us to draw the following conclusions. (*a*) A 218 amino acid NH₂-terminal protein that preserves the intrachain disulphide bridge of the heparin-binding domain is capable of binding to heparin-Sepharose and incorporating into the extracellular matrix. (*b*) A shorter 164 amino acid NH₂-terminal peptide that does not contain the intrachain disulphide bridge of the heparin-binding domain is neither able to bind to heparin–Sepharose nor able to incorporate into the extracellular matrix. (*c*) The region of interchain disulphide bridging necessary for trimer assembly resides within a cluster of seven cysteine residues immediately adjacent to the heparin-binding domain.

THROMBOSPONDIN (TSP)[1] was originally described as a component of platelet alpha granules (1), where it plays a key role in mediating the secretion-dependent or secondary phase of platelet aggregation (5, 22). Interest in TSP has been stimulated by the finding that it is synthesized by a number of normal and transformed cells including endothelial cells, smooth muscle cells, keratinocytes, fibroblasts, glial cells, macrophages, squamous carcinoma cells, and type II pneumocytes. The newly synthesized TSP is secreted and incorporated into the extracellular matrix. Using immunofluorescence localization in tissue sections, TSP had been identified in a variety of cellular locations including muscle, skin, kidney, glandular epithelium, and blood vessels (see reference 9 for review).

TSP has been shown to be a potent attachment factor for a variety of cells (33, 35, 36) and has also been found to be

an autocrine growth "facilitator" for aortic smooth muscle cells where an increase in TSP gene transcription occurs as an "immediate early" response to the mitogenic actions of platelet-derived growth factor (23, 26). Conversely, agents such as heparin or anti–TSP antibodies which inhibit incorporation of TSP onto the cell surface serve as growth inhibitors (25). Heparin has also been shown to inhibit the TSP-induced enhancement of mitogenic response to epidermal growth factor (24).

TSP can bind to other constituents of the extracellular matrix, to circulating polypeptides and to intact cells. Bound substances include fibrinogen, fibronectin, plasminogen, plasminogen activator, type V collagen, and glycosaminoglycans (see reference 20 for review). These interactions are mediated by various protease-resistant regions or domains that compose the TSP molecule. One such domain is the amino-terminal heparin-binding domain (HBD) that contains a high affinity site for heparin. This domain has been identified by subjecting proteolytic fragments of TSP to hepa-

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1. *Abbreviations used in this paper:* HBD, heparin-binding domain; TSP, thrombospondin.

rin–Sepharose affinity chromatography. The isolated HBD is a monomeric peptide whose exact size (M_r 25–30 kD) depends upon the specific protease used. Since the amino-terminal amino acid sequence of the HBD is identical to that of intact TSP, the HBD occupies the amino terminus of TSP (3). The amino acid sequence of the HBD, as predicted from the nucleotide sequence of TSP cDNA, reveals it to be hydrophobic in character and to contain two clusters of basic amino acids that probably contribute to the binding of anionic heparin (6, 18, 21). There are only two cysteines within the HBD that form an intrachain disulphide bond which may help maintain the native conformation of this domain (3, 6).

A number of findings suggest that the HBD is of major physiologic relevance. Heparin, presumably by binding to the HBD, inhibits the incorporation of radioiodinated TSP into isolated fibroblast cell matrices and also inhibits the incorporation of newly synthesized TSP into the matrix of aortic smooth muscle cells (23, 28). This is thought to be an integral part of the mechanism by which heparin exerts its growth inhibitory effect on aortic smooth muscle cells (23). Recent evidence suggests that the cell surface receptor for TSP is a heparan sulphate proteoglycan and that heparin, by competing for the binding of TSP to its receptor, is capable of inhibiting cell surface association (30, 31). Electron microscopy of rotary-shadowed, intact TSP demonstrates the HBD to be a highly compact globular structure at one end of the molecule which is connected to the opposite globular end by a thin connecting stalk (10). The three TSP subunits appear to be interconnected via the stalks at a point just distal to the carboxy terminus of the HBD. The stalk-like connecting region also contains a binding site for collagen (11, 29) and thus may contribute to the binding of secreted TSP to the cell layer. To directly ascertain the regions of the TSP molecule required for matrix incorporation and trimer assembly and to further explore structure–function relationships in the biologically important HBD, we undertook the expression and analysis of carboxy-terminal deletions of the TSP molecule.

Materials and Methods

Isolation of TSP cDNA

To isolate a full length-coding TSP cDNA, we screened 10^5 colonies of a human fibroblast cDNA library using a previously characterized 5' 1.8-kb TSP cDNA (6). Details regarding cDNA library construction, screening, subcloning, and DNA sequencing were the same as previously described (6).

Cell Culture and DNA Transfections

COS-1 cells (SV-40-transformed, African green monkey kidney) (12) were grown in DME supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. DNA transfections were performed by the calcium phosphate coprecipitation method as previously described (32).

Labeling and Immunoprecipitation

COS-1 cells were metabolically labeled 48–60 h after transfection. Cells were rinsed with DME lacking cysteine and methionine. Labeling was performed in 2 ml of the above medium supplemented with 0.5% bovine serum albumin and 100 μCi/ml each of [^{35}S]methionine and [^{35}S]cysteine (New England Nuclear, Boston, MA). After 4 h, 1 ml of complete DME with albumin was added for an additional 4-h incubation, the medium was collected, centrifuged to remove cell debris, and protease inhibitors were added to a final concentration of 1 mM phenylmethyl sulfonyl fluoride, 1 mM

leupeptin, and 20 μg/ml pepstatin A (Sigma Chemical Co., St. Louis, MO). The cell layer was solubilized in a detergent solution composed of 0.5% sodium dodecyl sulphate and the above mentioned protease inhibitor cocktail. In some cases, isolated extracellular matrices were prepared by the method of Gospodarowicz and Ill (13). Briefly, after removal of media, the cell layer was washed with phosphate-buffered saline and then treated for 30 min to 1 h with buffered saline containing 0.5% Triton X-100. The cultures were washed repeatedly with phosphate-buffered saline until only a few cytoskeletons and nuclei could be observed associated with the intact extracellular matrix. The matrix so prepared was dissolved in 0.5% sodium dodecyl sulphate and protease inhibitor cocktail.

For immunoprecipitation studies, aliquots of radiolabeled samples (either media, cell layer, or extracellular matrix) were clarified by centrifugation and diluted fivefold in a buffer containing 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1% Na-deoxycholate (Sigma Chemical Co.). This dilution step reduced the final concentration of sodium dodecyl sulphate to 0.1%. After dilution, the samples were subjected to immunoprecipitation with either an anti-TSP polyclonal antiserum or an anti-TSP monoclonal antibody (A2.5). In some instances the immunoprecipitated material was resuspended in 100 μl of 10 mM Tris (pH 7.6) with 1% sodium dodecyl sulphate, heated to 90°C for 10 min, spun, and the supernatant reimmunoprecipitated. The control antibody used in cases of immunoprecipitation with polyclonal anti-TSP antibody was preimmune serum while the control antibody used in cases of immunoprecipitation with the anti-TSP monoclonal antibody, A2.5, was purified mouse IgG (Cooper Biomedical, Inc., Malvern, PA). The specificity of the anti-TSP antibodies and the procedure for immunoprecipitation has been described previously (2, 5, 36). The antifibronectin polyclonal antiserum was a kind gift from Dr. J. McDonald (Washington University, St. Louis, MO). Immunoprecipitated material was resolved by SDS-PAGE on 10% acrylamide slab gels or on 4–10% acrylamide gradient gels using the buffer system of Laemmli as described previously (3). After electrophoresis, gels were fixed, treated with EnHance (New England Nuclear), dried, and exposed to XAR film (Eastman Kodak Co., Rochester, NY) at −70°C.

Heparin–Sepharose Affinity Chromatography

Media from metabolically labeled, transfected COS-1 cells was clarified by centrifugation, adjusted to a salt strength of 0.18 M NaCl, and applied to a 500-μl heparin–Sepharose column that had been preequilibrated in 0.02 M Tris, pH 7.6, 0.18 M NaCl. The column was step eluted with 0.35 and 0.5 M NaCl and 500-μl fractions collected. Each fraction was subjected to immunoprecipitation with an anti-TSP polyclonal antibody as described above. The immunoprecipitates were resolved by SDS-PAGE on a 5–15% gradient gel and visualized by subsequent autoradiography.

Construction of TSP Expression Vectors

The cDNA, cloned in pUC18, was linearized at the unique Bgl II site located at position 1,693. 10 μg of linearized DNA was subjected to digestion with nuclease Bal 31 according to conditions recommended by the supplier (New England Biolabs, Beverley, MA). Aliquots were removed at various intervals and the reactions terminated by dilution in 10 mM EDTA. After blunt-ended repair with the Klenow fragment of Escherichia coli DNA polymerase, 300 ng of a palindromic synthetic DNA linker was ligated to the free ends. The linker sequence was 5'-pTAATAGATCTATTA-3' and contained a translation termination codon and a Bgl II site. After digestion with excess Bgl II, plasmid DNAs were self-ligated and transformed into E. coli strain
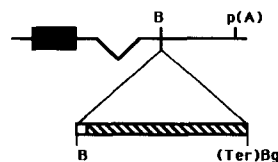
Figure 1. TSP expression vector. The expression vector pSVL (Pharmacia Fine Chemicals) contains an SV-40 viral late promoter (dark box) and VP-1 splice site (ᴠ) upstream of a polylinker sequence. The SV-40 late polyadenylation site [p(A)] is also noted. Appropriately deleted TSP mutants containing a synthetic ochre termination codon and Bgl II site [(Ter)Bg] were digested with Bam HI and Bgl II. The Bam HI site is within the TSP 5' untranslated region. The coding region is represented by the cross-hatched area. The Bam HI-Bgl II fragment was cloned into the Bam HI site (B) of the pSVL vector.

MC1061 (6). The sizes of individual deletions were estimated by agarose gel electrophoresis of Sst I–Bgl II-digested bacterial miniprep samples. To verify that the termination codon was in frame with the TSP-coding sequence, individual Sst I–Bgl II fragments from relevant plasmids were subcloned into ml3mpl8 and subjected to dideoxy sequencing. We identified four clones terminating at amino acids 449, 333, 218, and 164, respectively. Each of the four deletion mutants as well as the full-length TSP cDNA was then cloned into the eukaryotic expression vector pSVL (Pharmacia Fine Chemicals, Piscataway, NJ). This vector contains a SV-40 viral late promoter and VP-1 splice site located upstream of a polylinker (Fig. 1). TSP deletions were digested with Bam HI and Bgl II and ligated into the Bam HI site of the polylinker. Correct orientations were checked by appropriate restriction digests.

Results and Discussion

Cloning of TSP cDNA and Construction of Expression Vectors

To study the structure–function relationships in the TSP molecule we cloned a cDNA for TSP that contained the entire coding sequence. This was accomplished by screening a fibroblast cDNA library with a previously characterized 1.8-kb partial cDNA for TSP that encoded the amino-terminal HBD (6).

Several clones containing various size cDNAs for TSP were isolated. The largest cDNA (designated F2) was 4.6 kb in size. To confirm the authenticity of this cDNA, we sequenced it on both strands by the method of Maxam and Gilbert (27).

The DNA sequence for F2 was essentially identical to the sequence for endothelial cell TSP previously reported by Lawler and Hynes (21), except for nucleotide position 1,805 (T instead of C). This results in the alteration of amino acid 559 (leucine instead of proline). Due to the conservative nature of this change, it probably represents a polymorphism, although a DNA sequencing error cannot be ruled out. The fibroblast-derived cDNA sequenced by us is slightly larger than the endothelial cell cDNA characterized previously, being 45 bp more extended in the 5' direction and 99 bp longer in the 3' direction. Though both the fibroblast TSP cDNA (4.6 kb in size) and the endothelial TSP cDNA (4.5 kb in size) contain the entire coding sequence, they are not "full length", as the size of the mRNA for TSP is ~6.0 kb (18, 26). Since neither cDNA contains the canonical polyadenylation signal AATAAA, it is probable that they lack 3' untranslated sequences.

All TSP mutations were constructed by nuclease Bal 31 digestion as described in Materials and Methods and confirmed by sequence analysis (Fig. 2) before transfection and expression in COS-1 cells. The positions of insertion of the ochre termination codons, which should result in the biosynthesis of truncated molecules, are shown in Fig. 3. All the mutations are referred to by the identity of the amino acid preceding the termination codon. The shortest deletion, Pro164 is thus composed of 164 amino acids starting from the amino terminus of the mature protein whereas the largest deletion, Pro449, is composed of 449 amino acids from the amino terminus. Also shown in Fig. 3 are the positions of the various cysteine residues and the single intrachain disulphide bond present in the HBD. It is evident that the amino acid sequence at the carboxy terminus of the HBD is extremely cysteine rich. It is in this region that the residues involved in interchain disulphide linkage are thought to reside (10, 21).
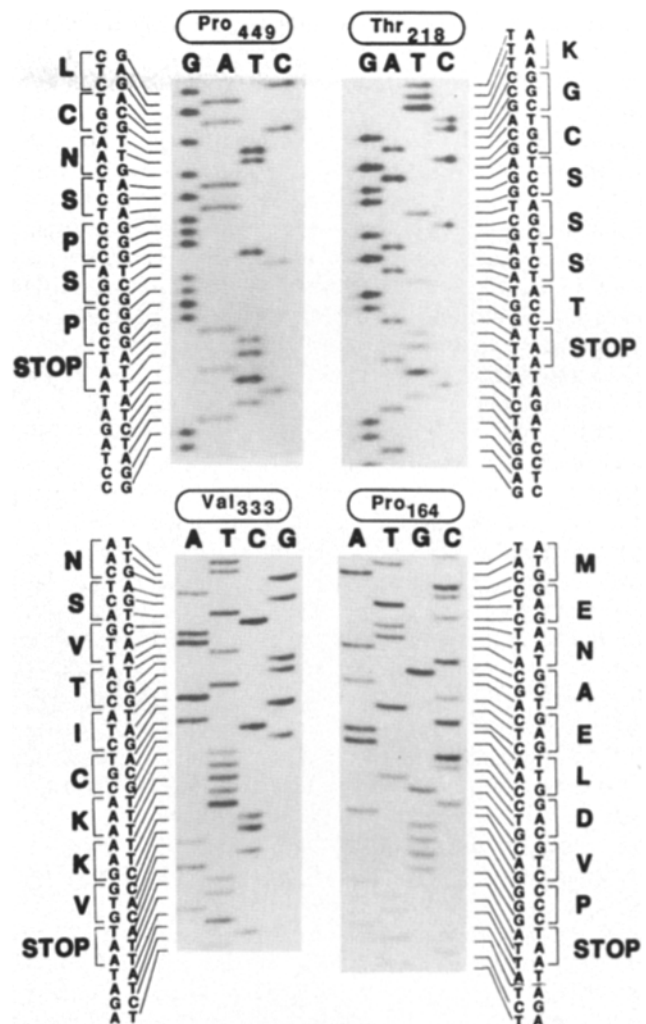

Figure 2. Nucleotide sequences of truncated TSP cDNAs. Selected Bal 31 deletion mutagenesis of a full length–coding TSP cDNA was carried out as described in Materials and Methods. Appropriate restriction fragments containing the mutagenized sequences were cloned into M13 phage vectors, single-stranded DNA was isolated, and dideoxynucleotide sequencing was performed. Shown are the actual nucleotide sequences at and across the site of insertion of the stop codon. The various mutations are identified by the identity of the amino acid residue preceding the ochre-inserted termination codon. For instance, the deletion mutation Pro449 encodes a protein composed of 449 amino acids where the carboxy-terminal residue is a proline. The amino acids are shown adjacent to the nucleotide sequence using the single letter code.

### Expression of Deleted TSP cDNAs in COS-1 Cells

Each of the pSVL-TSP expression vectors was transfected into COS-1 cells by the calcium phosphate method (32). 50–64 h after transfection, the cells were metabolically labeled with [35S]cysteine and [35S]methionine for 4 h followed by a 4-h chase. Media and cell layer (cells plus extracellular matrix) were harvested separately and immunoprecipitated with either a control or anti–TSP polyclonal antiserum. The results of such an analysis are shown in Figs. 4 and 5. In all instances we detected a truncated TSP protein of the predicted size both in the medium and cell layer of the transfected cells. In untransfected control cells, we detected
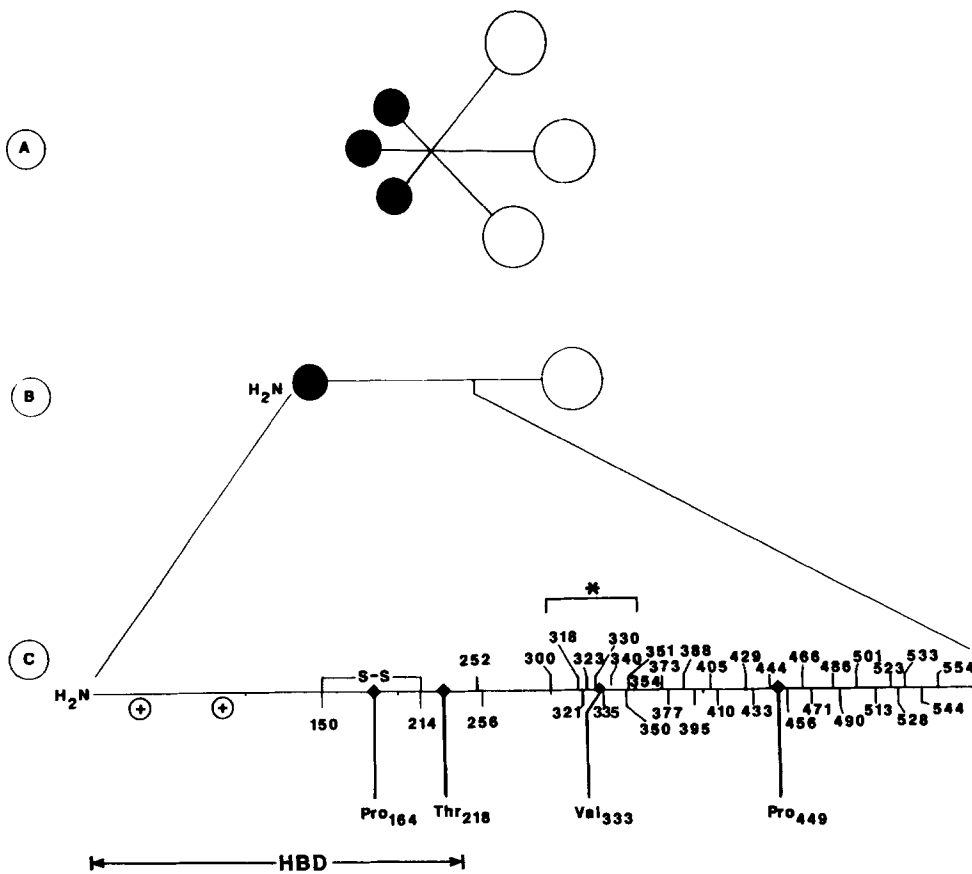
Figure 3. Diagrammatic representation of the TSP molecule and the sites of insertion of the ochre termination codon. (*A*) Schematic diagram of the trimeric TSP molecule based on rotary-shadowed electron micrographic images, epitope mapping, and domain structure studies (9, 20). The amino-terminal HBD is shaded. (*B*) Single TSP subunit showing the amino-terminal HBD (shaded) connected via a thin stalk to the carboxy-terminal domain (not shaded). (*C*) Line representation of the amino-terminal half of the TSP molecule. The position of the various cysteine residues is indicated by their numbered location in the amino acid sequence. Also shown is the single intrachain disulphide bond present in the HBD between cysteine residues 150 and 214. The cluster of cysteine residues that has marked homology to a cysteine-rich domain in von Willebrand factor and the alpha 1 chain of type I procollagen is indicated by an asterisk. Clusters of positively charged basic amino acid residues in the HBD are indicated (+).
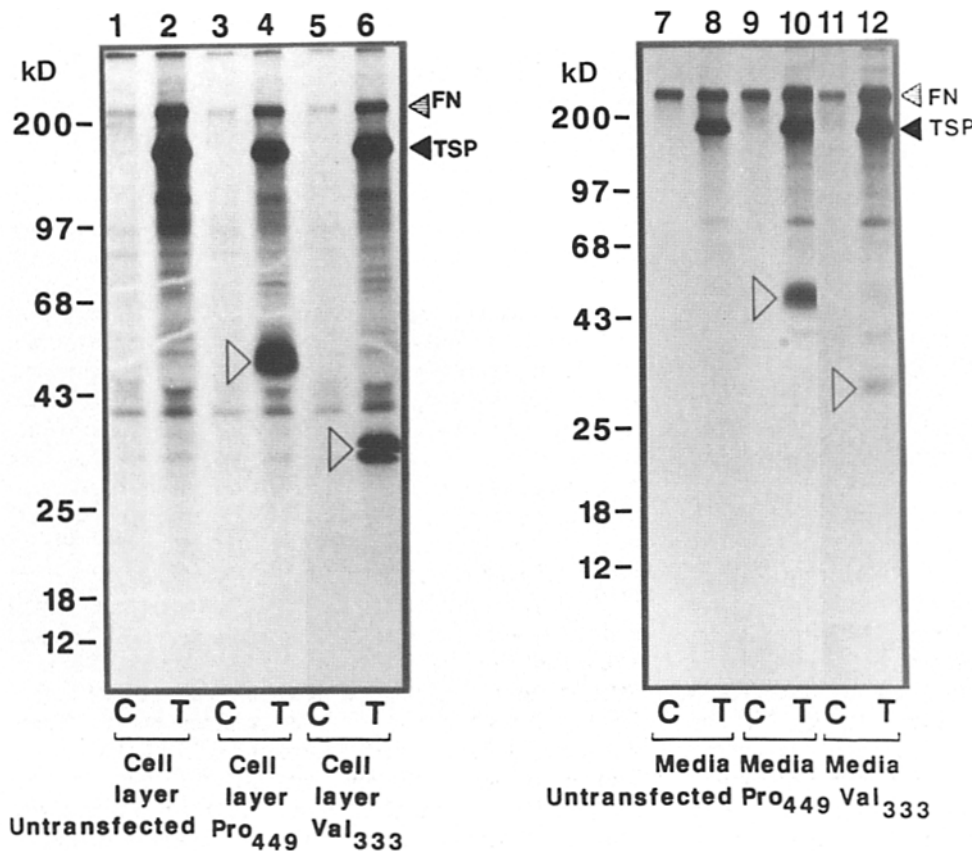
Figure 4. Synthesis of truncated TSP proteins (Pro$_{449}$ and Val$_{333}$) by transfected COS-1 cells. COS-1 cells were transfected with expression vectors encoding the Pro$_{449}$ and Val$_{333}$ deletion mutants. Untransfected controls and transfected cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, media was separated from cell layers, and each was subjected to immunoprecipitation with anti-TSP antibody (*T*; lanes *2, 4, 6, 8, 10,* and *12*) or control antibody (*C*; lanes *1, 3, 5, 7, 9,* and *11*). The Val$_{333}$ band in lane 6 is interrupted by a crack in the fluorograph. To the right of the gels, arrowheads indicate the position of migration of endogenous TSP and fibronectin (*FN*). Open triangles in the gels show the presence of truncated proteins Pro$_{449}$ and Val$_{333}$ both in the cell layer and medium of the transfected cells. Molecular mass standards in kilodaltons are shown to the left.
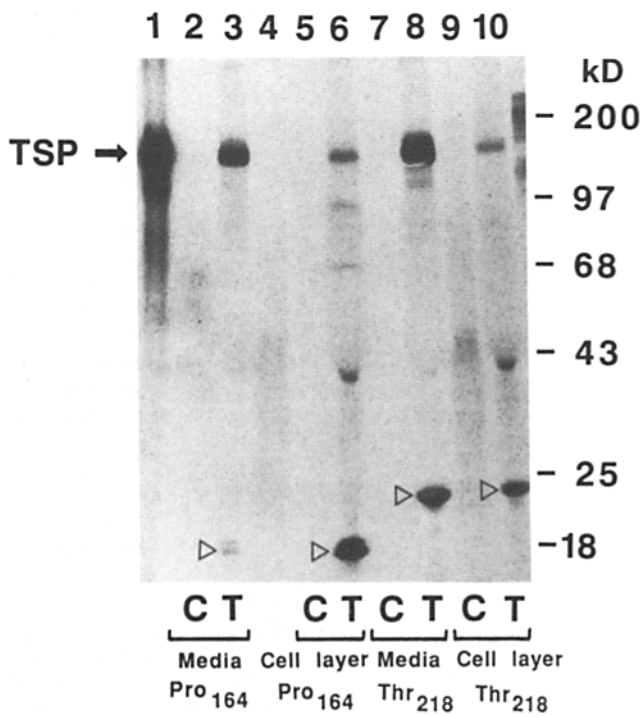
Figure 5. Synthesis of truncated TSP proteins (Thr_{218} and Pro_{164}) by transfected COS-1 cells. On immunoprecipitation with anti–TSP antibody (T; lanes $3, 6, 8,$ and 10) but not control antibody (C; lanes $2, 5, 7,$ and 9) the presence of truncated protein was detectable both in the medium and cell layer (*open arrowheads*). Lane 1 contains iodinated platelet TSP as a marker. Samples were subjected to two rounds of immunoprecipitation to reduce background (see Materials and Methods). This resulted in loss of fibronectin from the immunoprecipitates and is thus not seen on autoradiography. The position of migration of endogenous TSP is shown to the left. Molecular mass standards in kilodaltons are shown to the right.

only endogenous TSP (Fig. 4, lanes $1, 2, 7,$ and 8). Transfection of COS-1 cells with the pSVL-TSP-Pro_{449} expression vector resulted in the synthesis of a 48-kD protein present both in the medium and cell layer that was immunoprecipitated by an anti–TSP antibody but not by a control antibody (Fig. 4, lanes $3, 4, 9,$ and 10). The diffuse nature of this band is probably due to glycosylation. Pro_{449} contains two potential NH_2-linked glycosylation sites (Asn-X-Ser/Thr) at amino acid residues 230 and 342 (21). Also immunoprecipitated by the anti–TSP antibody was endogenous COS-1 cell TSP and a high molecular mass protein of 220 kD. This protein was immunoprecipitated by a monospecific antifibronectin polyclonal antibody (data not shown) and its presence in the immunoprecipitates reflects the ability of the TSP and fibronectin to bind to each other (18a, 19). Expression of the deletion mutant pSVL-TSP-Val_{333} resulted in the biosynthesis of a 35-kD protein present both in the cell layer and media of transfected COS-1 cells (Fig. 4, lanes $5, 6, 11,$ and 12). Expression of sequences that encode the HBD with the intrachain disulphide bridge intact (Thr_{218}) resulted in the biosynthesis of a 24-kD protein (Fig. 5, lanes 8 and 10). The final deletion mutant protein (Pro_{164}), besides being smaller than Thr_{218}, possesses a single cysteine. This precludes normal intrachain disulfide bond formation and might be expected to grossly alter the HBD conformation. Since there is a con-

siderable body of work to suggest that the transport of a malfolded protein through the secretory pathway may be inhibited (7, 8), it was rather surprising to detect the synthesis of a truncated protein of M_r 19 kD by COS-1 cells after transfection (Fig. 5, lanes 3 and 6).

Conformation and Heparin-binding Properties of the Truncated TSP Proteins

To examine whether the deleted TSP proteins assumed a conformation similar to that in the native TSP molecule, we immunoprecipitated the proteins with a conformation-specific monoclonal antibody (A2.5). In previous studies (4), we had shown that the monoclonal antibody A2.5 is directed against the active site of the HBD of TSP. This conclusion was drawn from the fact that while the monoclonal antibody A2.5 immunoprecipitated the HBD of TSP, heparin successfully inhibited the binding of the antibody, presumably by competing for the binding site (4). Given the ability of heparin to compete for this antibody binding site, it was concluded that this site is sterically very close to or is the actual heparin-binding active site (4). Further, the antibody A2.5 will recognize the HBD only in its native conformation and not when it has been denatured (4).

The conformation-specific monoclonal antibody A2.5 was able to recognize and immunoprecipitate three of the truncated proteins (Pro_{449}, Val_{333}, and Thr_{218}) but was unable to identify the shortest truncated protein (Pro_{164}) (Fig. 6, lanes $4, 6, 8,$ and 10). Since the Pro_{164} protein represents the HBD without the intrachain disulphide bond, it is likely that disruption of this bond leads to a conformational change that it is no longer recognizable by the antibody A2.5. In untransfected control cells, the monoclonal antibody A2.5 immunoprecipitated endogenous TSP only (Fig. 6, lane 2).

All four truncated proteins retain the two clusters of positively charged amino acids between Arg_{23} and Lys_{32} and between Arg_{77} and Arg_{83} that are important in the binding of anionic heparin (21). We next examined the degree to which the truncated proteins bound heparin–Sepharose. We reasoned that quantitating the strength of binding to anionic heparin by the various truncated proteins would allow us to determine factors other than charge that may be important in maintaining the configuration of the heparin-binding active site.

Medium containing the various labeled truncated proteins was allowed to bind to a heparin–Sepharose column which was then sequentially eluted with a step-wise salt gradient. Eluted column fractions were immunoprecipitated with anti–TSP polyclonal antisera, resolved by PAGE, and visualized by autoradiography. Fig. 7 shows the elution profile with increasing salt strength of intact TSP, fibronectin, and truncated proteins from the affinity column. As seen in Fig. 7 A, three of the truncated proteins (Pro_{449}, Val_{333}, and Thr_{218}) bound to heparin–Sepharose with affinities resembling that of the native protein. The larger truncated proteins Pro_{449} and Val_{333} were eluted from the column at the highest salt strength (0.5 M NaCl). The next smallest truncated protein (Thr_{218}) was eluted by the 0.35 M salt wash but the higher 0.5 M salt wash was required for complete elution. There was some elution of intact TSP in the 0.35 M NaCl wash. This presumably represents a proteolyzed form of TSP that has lost one or more of the three HBDs that are normally present in the intact molecule and results in a lowered affinity
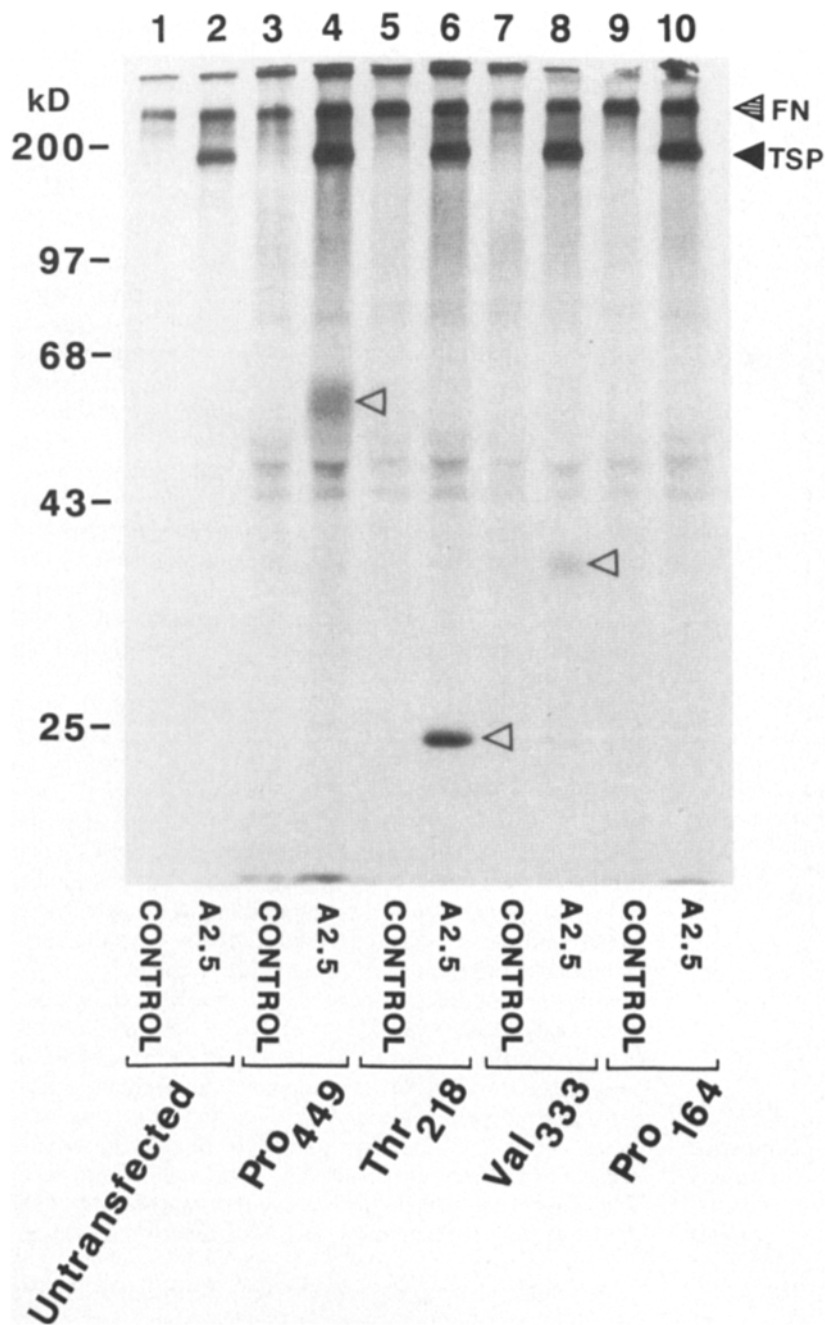
Figure 6. Immunoprecipitation of truncated TSP proteins with a conformation-specific anti–TSP monoclonal antibody (A2.5). Metabolically labeled media from untransfected COS-1 cells and cells transfected with expression vectors encoding the various deletion mutants were immunoprecipitated with the conformation-specific monoclonal antibody (A2.5). The immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography. Immunoprecipitation with monoclonal antibody A2.5 (*A2.5*; lanes *2, 4, 6, 8,* and *10*) but not control antibody (*CONTROL*; lanes *1, 3, 5, 7,* and *9*) led to the detection of three truncated TSP proteins in the media (*open arrowheads*; Pro_{449}, Thr_{218}, and Val_{333}). The position of migration of endogenously synthesized TSP and fibronectin (*FN*) is shown to the right of the figure. Molecular mass standards in kilodaltons are shown to the left.

for heparin–Sepharose. The smallest truncated protein Pro_{164} did not bind to the heparin–Sepharose column at all and was quantitatively recovered in the flow through (Fig. 7 *B*, lane *1*). There thus seemed to be an affinity gradient for the binding of the various truncated proteins to anionic heparin. The two larger truncated proteins (Pro_{449} and Val_{333}) had a binding affinity to heparin that resembled that of native TSP. The next, shorter truncation, Thr_{218}, which maintained both the cluster of positively charged amino acids and the intrachain disulphide bond of the HBD, seemed to have a somewhat lower affinity as the 0.35 M salt wash was able to displace some of the truncated protein from the column (Fig. 7 *A*, lane *6*). The increased affinity of truncated pro-

teins Pro_{449} and Val_{333} relative to Thr_{218} may be related to their trivalent structure (see below). The truncated protein unable to bind heparin was the shortest deletion mutant (Pro_{164}) which, while retaining the cluster of positively charged amino acids, no longer had the single intrachain disulphide bond normally present in the HBD. It can thus be argued that, in this shortest truncated protein, the alteration in conformation resulting from loss of the single intrachain disulphide bond so alters the active site that the clusters of positively charged amino acids that compose it are no longer accessible to bind to heparin. Media from untransfected control cells was also subjected to heparin–Sepharose affinity chromatography and as expected (6) most of the endogenous
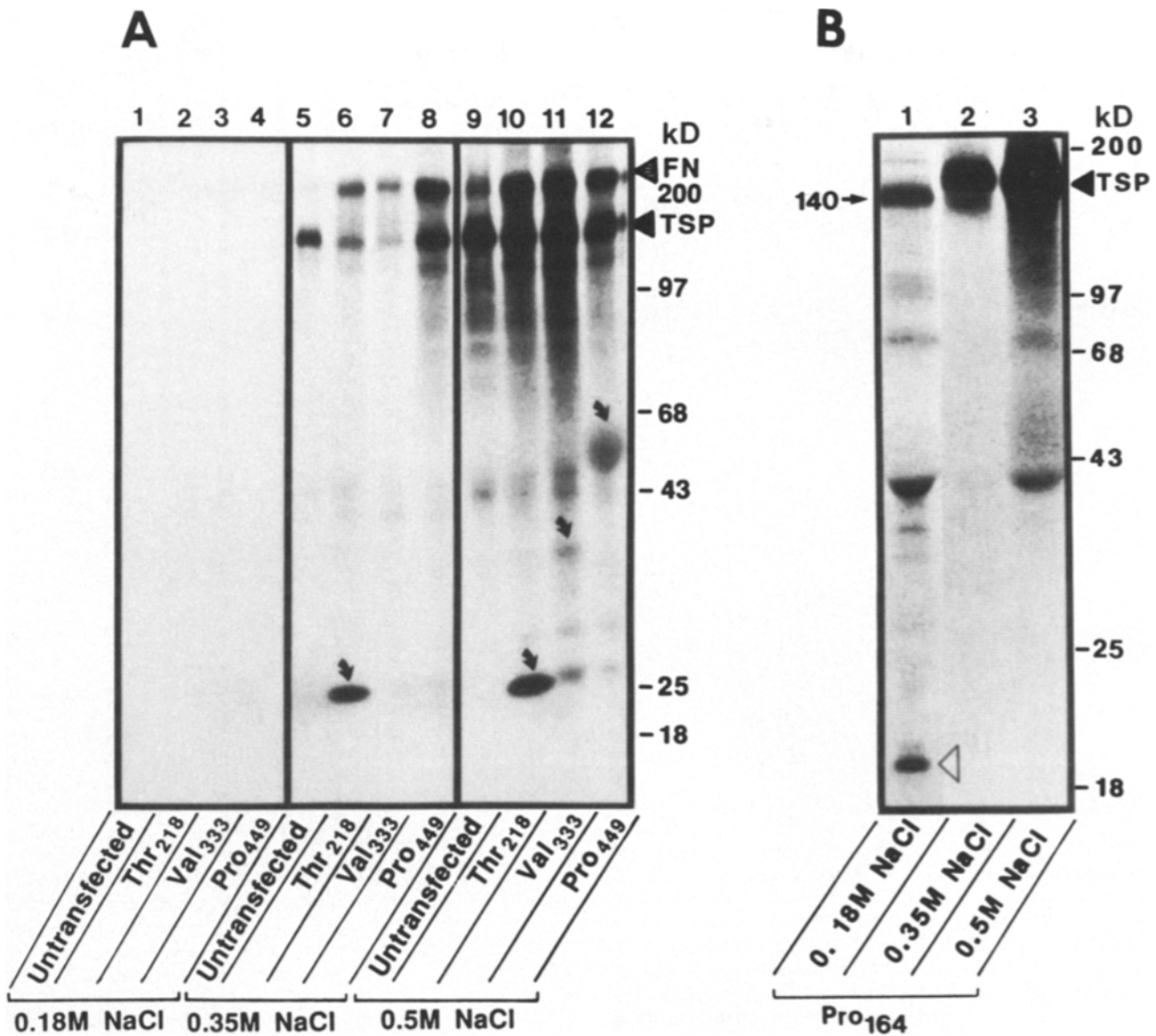
Figure 7. Heparin–Sepharose affinity chromatography of truncated TSP proteins. Metabolically labeled media from untransfected COS-1 cells and cells transfected with the various expression vectors was made up to 0.18 M with respect to NaCl and applied to a heparin–Sepharose column. The column was eluted step-wise with 0.35 and 0.5 M NaCl. Each fraction was immunoprecipitated with an anti–TSP antibody. (*A*) The elution profile with increasing salt strength of the truncated proteins Thr_{218}, Val_{333}, and Pro_{449}. The truncated TSP proteins are indicated by arrows. The position of migration of endogenous fibronectin (*FN*) and TSP is shown on the upper right. There was appreciable loss of truncated proteins, especially Val_{333} and Pro_{449}, during affinity chromatography due to nonspecific binding to column and plastic tubing. This necessitated longer exposures of the autoradiogram contributing to increased background. (*B*) Elution profile with increasing salt strength of truncated protein Pro_{164}. The position of migration of the truncated protein Pro_{164} is shown by an open arrowhead. Also shown are the positions of migration of endogenous TSP (migrates on SDS-PAGE as a 180-kD protein) and its proteolyzed form which has lost the HBD and migrates on SDS-PAGE as a 140-kD protein. Molecular mass markers in kilodaltons are shown to the right of the gels.

TSP was eluted in the high 0.5 M salt wash (Fig. 7 *A*, lanes *1*, *5*, and *9*).

Studies on Trimer Assembly

The trivalent nature of the TSP molecule enables it to effectively participate in cross-linking functions such as cell–cell and cell–substrate interactions (14, 15). It is remarkable that proteins like TSP which are composed of two or more subunits are capable of accurate self assembly. It is necessary for such subunits to correctly identify their appropriate partner subunit in a milieu of other proteins in the endoplasmic reticulum, align themselves appropriately, and enter into legitimate disulphide linkage via correct cysteine residues. It is not unreasonable to expect that a process as complex as this may depend upon self-recognition elements that allow for subunit identification, alignment, and oligomer assembly via intersubunit disulphide bonding. The trimeric nature of TSP, coupled with the availability of conformation-specific antibodies and functional assays makes it a good system in
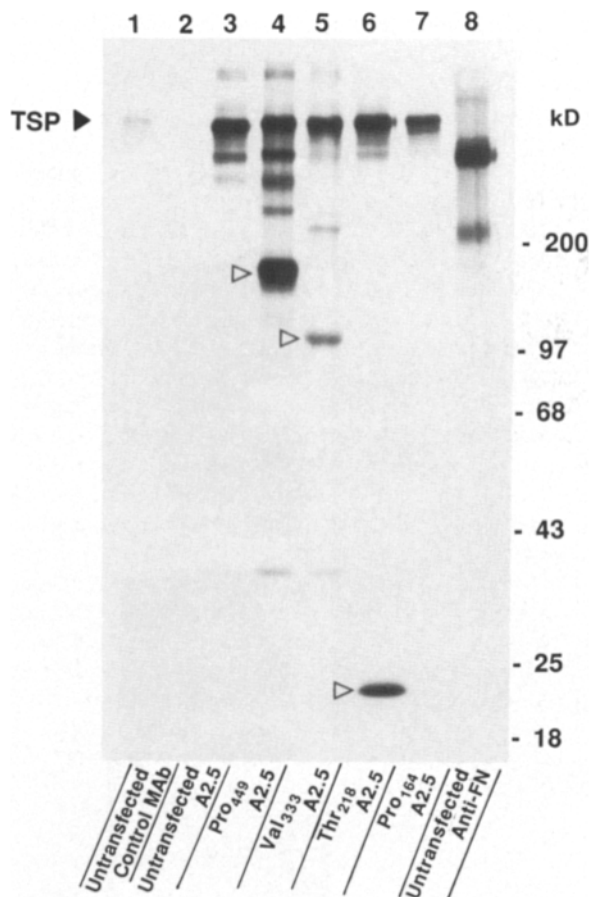
Figure 8. Studies on trimer assembly of the truncated proteins Pro$_{449}$, Val$_{333}$, and Thr$_{218}$. Metabolically labeled medium from COS-1 cells transfected with expression vectors encoding the deletion mutants was subjected to immunoprecipitation with the anti–TSP monoclonal antibody A2.5. The immunoprecipitates were resolved on SDS-PAGE in the absence of reductant and visualized by autoradiography. The presence of truncated TSP proteins is indicated by open arrowheads. Lane *1* contains iodinated platelet TSP as a marker. Metabolically labeled media from untransfected cells also subjected to immunoprecipitation with control monoclonal antibody (*Control MAb*; lane *2*), anti–TSP monoclonal antibody (*A2.5*; lane *3*), and antifibronectin antibody (*Anti–FN*; lane *8*). Molecular mass markers in kilodaltons are shown to the right.

which to explore the "signals" that dictate oligomer assembly. To see if the various truncated proteins could enter into trimer assembly, the immunoprecipitated complexes were resolved by SDS-PAGE in the absence of reductant and visualized by subsequent autoradiography. If the truncated proteins do indeed form trimers then a tripling in their apparent molecular mass in the absence of reductant would be expected. Fig. 8 shows such an analysis. Purified platelet TSP was iodinated and electrophoresed in unreduced form as a marker and it migrated as a trimer of ∼540 kD (Fig. 8, lane *1*). When medium from untransfected cells was immunoprecipitated with an antifibronectin antibody, two high molecular mass proteins are visualized whose migratory behavior is consistent with their being different forms of fibronectin (Fig. 8, lane *8*). More specifically, the larger, slower migrating form is probably the unreduced dimeric; while the smaller, faster migrating form is one in which partial proteol-
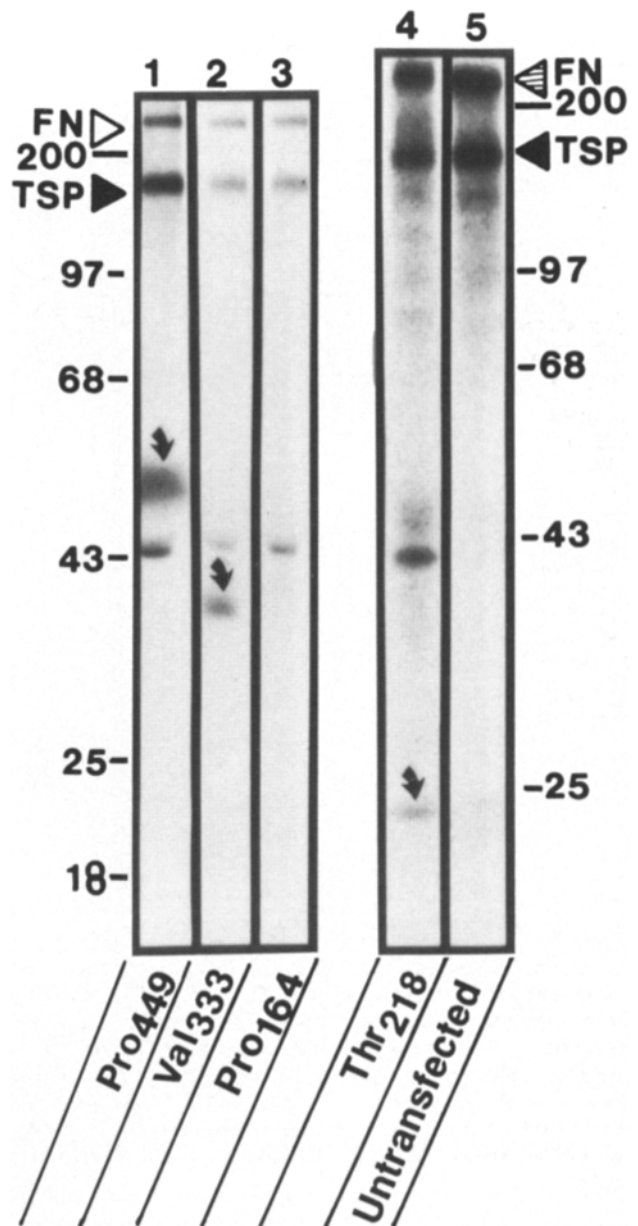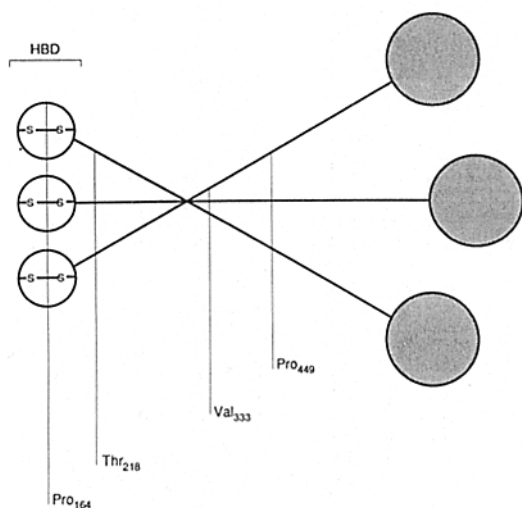
Figure 9. Incorporation of truncated TSP proteins into Triton X-100–insoluble matrix. COS-1 cells were transfected with expression vectors encoding the various TSP truncated proteins. After transfection the cells were metabolically labeled and Triton X-100–insoluble matrices prepared as described in Materials and Methods. The matrices were dissolved in SDS, immunoprecipitated with an anti–TSP antibody, the precipitated material resolved by SDS-PAGE, and visualized by autoradiography (lanes *1–4*). The presence of truncated TSP proteins is indicated by means of a curved arrow. Also shown is the position of migration of TSP (*arrowhead*) and fibronectin (*FN*). Triton X-100–insoluble matrix from untransfected cells was also subjected to immunoprecipitation by anti–TSP antibody (lane *5*). A nonspecific protein of M_r 43 kD was found to be present in immunoprecipitates of transfected cells. Molecular mass markers in kilodaltons are indicated at the margins of the figure.

ysis has removed the interchain disulphide bridge present at the carboxy-terminal end of fibronectin (17). When medium from untransfected cells was immunoprecipitated with an anti–TSP monoclonal antibody (A2.5), we detected a protein

	Heparin Binding	Heparin Affinity	Trimer Assembly	Matrix Incorporation
INTACT	+	+++	+	+
Pro$_{449}$	+	+++	+	+
Val$_{333}$	+	+++	+	+
Thr$_{218}$	+	++	-	+
Pro$_{164}$	-	-	-	-

Figure 10. Tabular summary and diagrammatic representation of the trimeric TSP molecule including the sites of ochre codon insertion resulting in synthesis of truncated TSP proteins. The table summarizes the behavior of intact endogenous TSP and the truncated TSP proteins. The diagram below the table shows the trimeric nature of the TSP molecule including the single intrachain disulphide bond in the HBD and the sites of ochre codon insertion resulting in the synthesis of the four truncated TSP proteins (Pro$_{164}$, Thr$_{218}$, Val$_{333}$, and Pro$_{449}$). The carboxy-terminal globular domain is shaded.

of higher molecular mass that comigrated with the iodinated TSP standard (Fig. 8, lane *1*) and thus presumably represents the endogenous trimeric form of TSP (Fig. 8, lane *3*).

Truncated TSP proteins Pro$_{449}$ and Val$_{333}$ migrated with approximate molecular masses of 180 and 110 kD, respectively, which is roughly three times their molecular mass in the presence of reductant (Fig. 8, lanes *4* and *5*). There are several faint bands between homotrimeric intact TSP at 540 kD and homotrimeric Pro$_{449}$ (at 180 kD) and Val$_{333}$ (at 110 kD) in lanes *4* and *5* of Fig. 8. These presumably represent heterotrimeric forms between endogenous TSP subunits and the expressed truncated TSP proteins. The truncated protein Thr$_{218}$ which contains only two cysteine residues in intrachain disulphide linkage did not appreciably alter its molecular mass in the nonreduced form (Fig. 8, lane *6*). Therefore, Thr$_{218}$ must not be capable of trimerizing as this requires at least two additional cysteines to participate in interchain disulphide linkage. The cysteine residues responsi-

ble for trimer assembly must therefore be two of the seven that lie between Val$_{333}$ and Thr$_{218}$ (see Fig. 3). As discussed previously, the truncated protein Pro$_{164}$ is not recognized by the conformation-specific monoclonal antibody A2.5 and is thus not immunoprecipitated (Fig. 8, lane *7*).

What are the signals for TSP self-recognition, alignment, and oligomerization? They may be sequences or conformations within the HBD that display a high but probably transient affinity for one another during the process of trimer assembly. Whatever their nature (conformation or sequence) they must lie within the amino-terminal 333 amino acids as it is possible to delete 2/3 of the TSP molecule from the carboxy terminus and yet still retain the information required for subunit identification, alignment, and oligomerization.

Interestingly, the deletion mutant Val$_{333}$ disrupts a cysteine-rich domain that is present in two other oligomeric proteins, namely the alpha 1 chain of type 1 procollagen and the von Willebrand factor (16). In these three proteins, the homologous domains are similar in length and have nine invariant cysteines, thus implying the existence of a common conformation. It has been postulated that the presence of this common cysteine-rich domain adjacent to the region of interchain disulphide linkage may in some way facilitate the process of oligomer assembly. Even though the deletion mutant Val$_{333}$ disrupts the cysteine-rich domain trimerization still occurs, suggesting that this highly conserved motif mediates a function other than trimer assembly.

Incorporation of Truncated TSP Peptides Into the Extracellular Matrix

Previous studies have shown that matrix-bound TSP is resistant to extraction with nonionic detergents (28). Given this, we asked whether the secreted truncated proteins were incorporated into a Triton X-100–insoluble matrix. The procedure for preparation of extracellular matrix from the transfected COS-1 cells was essentially identical to that described by previous workers (13). Isolated matrices from transfected cells were solubilized in SDS and immunoprecipitated with polyclonal anti-TSP antiserum. In untransfected cells both TSP and fibronectin were detectable in the matrix (Fig. 9, lane *5*). Truncated TSP proteins Pro$_{449}$ and Val$_{333}$ were also found in the matrix (Fig. 9, lanes *1* and *2*). The truncated protein Thr$_{218}$, which is univalent but capable of binding to heparin, is also incorporated into the matrix but to a lesser extent (Fig. 9, lane *4*). The smallest truncated TSP protein (Pro$_{164}$) that is univalent but unable to bind heparin was not detectable in the matrix even on prolonged exposure of the autoradiogram (Fig. 9, lane *3*). It can thus be postulated that for entry of TSP into the extracellular matrix, a functioning HBD is necessary. It is likely that TSP initially binds via its HBD to heparan sulphate proteoglycans in the extracellular matrix. This is supported by the finding that heparin is able to competitively inhibit the binding of iodinated intact TSP to isolated extracellular matrices (28).

In summary (Fig. 10), the above studies allow us to conclude that an intact HBD is required for heparin binding and matrix incorporation. Further, the region of interchain disulphide linkage lies adjacent to the HBD. It should now be possible to define more accurately by site-directed mutagenesis the signals involved in TSP subunit recognition, alignment, binding, and oligomerization.

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