Expression and Mutagenesis of Thrombospondin†

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ABSTRACT: Thrombospondin is a 420 000-dalton adhesive glycoprotein that is composed of three subunits of equivalent molecular weight. When the cDNA for the complete coding region of the human endothelial cell thrombospondin subunit is expressed in mouse NIH 3T3 cells, a 420 000-dalton protein is synthesized and secreted. The expressed protein comigrates with human platelet thrombospondin both in the presence and in the absence of a reducing agent. The expressed protein binds to a monoclonal anti-thrombospondin antibody, heparin, and calcium. In addition to the 420 000-dalton protein, the transfected cell lines also express a variable amount of a 140 000-dalton polypeptide. When the culture supernatants that are produced by cells that are expressing thrombospondin are applied to heparin–Sepharose, the 420 000-dalton and the 140 000-dalton proteins are bound to the column and are eluted with buffer containing 0.55 and 0.3 M NaCl, respectively. The 140 000-dalton protein only binds to heparin–Sepharose in the presence of calcium. Deletion of the region of homology with procollagen results in defective assembly of the trimer. Deletion of the type 1 or type 2 repeats results in decreased stability of the subunit with the predominant polypeptides that are expressed having molecular weights of 127 000 and 130 000, respectively. These polypeptides retain low-affinity heparin-binding activity. High-affinity heparin binding is markedly diminished by mutations in either of two sequence motifs that include clusters of lysines and arginines. Decreased high-affinity heparin binding is observed when (1) K(80) and K(81) are mutated to Q(80) and N(81), (2) R(23) and K(24) are mutated to Q(23) and N(24), or (3) R(28) and R(29) are mutated to N(28) and Q(29). These results indicate that both of these regions of positively charged amino acids are required for high-affinity heparin binding.

Thrombospondin is a 420 000-dalton adhesive glycoprotein that is composed of three subunits of equivalent molecular weight (Galvin et al., 1985; Lawler et al., 1985). Recently, a second form of thrombospondin (thrombospondin 2) has been identified in the mouse and the chicken (Bronstein et al., 1991; Lawler et al., 1991a). The work described here pertains to the initially described form of thrombospondin (thrombospondin 1). Globular structures are present at the NH_2- and COOH-terminals of each subunit (Galvin et al., 1985; Lawler et al., 1985). These regions do not show strong homology with other proteins and do not contain multiple copies of sequence motifs (Lawler & Hynes, 1986). The NH_2-terminal globular domain has been shown to contain a heparin-binding site (Lawler & Slayter, 1981; Dixit et al., 1984; Raugi et al., 1984; Aiken et al., 1986). Thrombospondin binds to syndican, which is a cell-surface proteoglycan that contains both heparin sulfate and condroitin sulfate side chains (Sun et al., 1989; Bernfield & Sanderson, 1990). In addition, thrombospondin and syndican colocalize in many tissues of the developing mouse embryo.[1] The high-affinity heparin-binding site has been shown to be included in the first 218 amino acids of the mature thrombospondin peptide (Prochownik et al., 1989; Yabkowitz et al., 1989). Two regions of amino acid sequence of thrombospondin are similar to other heparin-binding proteins (Lawler & Hynes, 1986; Cardin & Weintraub, 1989). These regions are the peptides ARKGSGRR (residues 22–29) and MKKTRG (residues 79–84). Both of these regions have a high number of basic amino acids and may form α helices on the basis of secondary structure predictions (Lawler, 1981; Lawler & Hynes, 1987).

The central portion of each subunit is composed of regions of homology with other proteins and multiple copies of amino acid sequence motifs (Lawler & Hynes, 1986). A region of homology with procollagen is present between residues 263 and 361. This region is included in the amino propeptide portions of types I and III collagen and appears to be involved in the initial phase of fibrilogenesis, after which it is removed by proteolysis (Fleischmajer et al., 1985). In thrombospondin, this region is adjacent to the two cysteines which form the interchain disulfide bonds (Sottile et al., 1991). The three type 1 repeats are homologous to the complement components C6 to C9 and properdin (Lawler & Hynes, 1986; Holt et al., 1990). Two or three of the type 1 repeats contain the VTCG cell binding sequence in human thrombospondin 1 and chicken thrombospondin 2, respectively (Rich et al., 1990; Lawler et al., 1991a). These sequences have been proposed to mediate the interaction of thrombospondin with cell surfaces (Asch et al., 1990, 1991; Prater et al., 1991). Thrombospondin also contains three type 2 or EGF-like repeats (Lawler & Hynes, 1986). EGF-like repeats are also found in the adhesive glycoproteins fibronectin, laminin, and tenascin (Kornblihtt & Gotman, 1988; Engel, 1989).

Recently, a 140 000-dalton polypeptide, that includes the carboxyl-terminal two-thirds of the thrombospondin subunit, has been identified in the culture supernatants of BHK cells

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[1] C. Corless, A. Mendoza, T. Collins, and J. Lawler, submitted for publication.

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Table I: Thrombospondin Mutations

construct	mutation	oligonucleotide (5′ → 3′)[a]
pTSPΔp	deletes residues 284–357	CAGCCATCGTC-CGCAGAGTCCA-CTTTGCGGATG-CTGTCCT
pTSPΔt1	deletes residues 361–530	GGATTGGACAG-GCATCCATCCG-CAGAGTCGCTG-GGCCAAC
pTSPΔt2	deletes residues 531–673	CAGCCATCCAG-GTCTGTGTCA-ATTGGACAGT-CCTGCTTGT
pTSPhep(23,24)	R(23)K(24) → Q(23)N(24)	CCCAGACCCATT-CTGGCGGCA-CCGGTGAGTTC
pTSPhep(28,29)	R(28)R(29) → N(28)Q(29)	CCCTTCACCAG-TTGGTTCCC-AGACCCC
pTSPhep(80,81)	K(80)K(81) → Q(80)N(81)	GCGTGCCCCGT-GTATTCTGCA-TCTGCCTC

[a] The underline identifies the bases that differ from the normal thrombospondin sequence.

(Rastinejad et al., 1989; Good et al., 1990). Production of this band is regulated by expression of a tumor suppressor gene (Rastinejad et al., 1989; Good et al., 1990). This polypeptide and the intact thrombospondin molecule block neovascularization in the rat cornea (Good et al., 1990). In addition, antibodies to thrombospondin promote tube formation by endothelial cells in culture (Iruela-Arispe et al., 1991). Thrombospondin exhibits contrasting effects on the growth of endothelial cells and smooth muscle cells. Endothelial cell proliferation is markedly inhibited by thrombospondin (Bagavandoss & Wilks, 1990). Taraboletti et al. (1990) have shown that thrombospondin inhibits the mitogenic response of endothelial cells to serum and bFGF. By contrast, thrombospondin enhances the proliferative response of rat aortic smooth muscle cells to epidermal growth factor (Majack et al., 1986; Majack & Bornstein, 1987).

In this paper, we report on the expression of a full-length cDNA clone for human endothelial cell thrombospondin in mouse 3T3 cells. The same construct also produces a 140 000-dalton polypeptide that contains a low-affinity heparin-binding site. In addition, we have constructed and expressed mutants that have defective heparin binding, defective subunit assembly, or defective subunit stability.

Materials and Methods

Construction of Expression Vectors and Mutagenesis. A full-length cDNA clone that includes the entire coding region of endothelial cell thrombospondin was constructed by a four-part ligation of the *Bam*HI to *Eco*RI fragment of clone M10, the *Eco*RI to *Bgl*I fragment of clone M10, the *Bgl*I to *Pvu*II fragment of clone M6, and the plasmid pGEM-2 (Promega Biotec, Madison, WI) (Lawler & Hynes, 1986). The construct was then digested with *Sac*I and blunted, and *Sal*I linkers were ligated to it. The excess linkers were removed by precipitation, and the construct was digested with *Sal*I and ligated into the *Sal*I site of the retrovirus pLJ (Schwarzbauer et al., 1987). Whereas the retroviral-mediated system was effective, the level of expression for this construct was low. To obtain higher levels of expression, and to prepare a template for oligonucleotide-directed mutagenesis, the full-length construct was cloned into the *Sal*I site of M13mp8. Mutations were produced in the thrombospondin sequence using the oligonucleotide-directed in vitro mutagenesis system (Amersham, Arlington Heights, IL). Deletion mutants were produced that delete the region that is homologous to procollagen (pTSPΔP), the type 1 repeats (pTSPΔt1), or the type 2 repeats (pTSPΔt2) (Figure 1). Oligonucleotides (40-mers) were synthesized that included 20 bp of sequence that flanked each side of the region to be deleted (Table I). Mutagenesis of the two putative heparin-binding sequences involved the mutation of two adjacent lysine or arginine residues (Table I, Figure 1). These residues were mutated to either asparagine or glutamine because these substitutions occur with relatively high frequency in proteins in general and, therefore, may be expected to be less likely to perturb protein structure (Schulz & Schirmer, 1979). The oligonucleotides that were used to produce pTSPhep(23,24) and pTSPhep(80,81) include a substitution that does not change the amino acid sequence. This substitution removes an *Nci*I site from the mutant strand. As a result, when the sample is cut with *Nci*I after the mutant strand is synthesized, only the normal strand is nicked (Nakamaye & Eckstein, 1986). The mutations pTSPhep(23,24) or pTSPhep(28,29) were combined with the mutation pTSPhep(80,81) using the *Apa*I sites that flank the mutated nucleic acids of the pTSPhep(80,81) mutation. Constructs that contained the desired mutations and no other nucleotide substitutions were identified by nucleotide sequencing using Sequenase (United States Biochemical Corp., Cleveland, OH), following the manufacturer's protocols. *Bam*HI and *Eco*RI sites were used to combine mutant and normal regions of DNA to decrease the amount of sequencing that was required to check each construct.

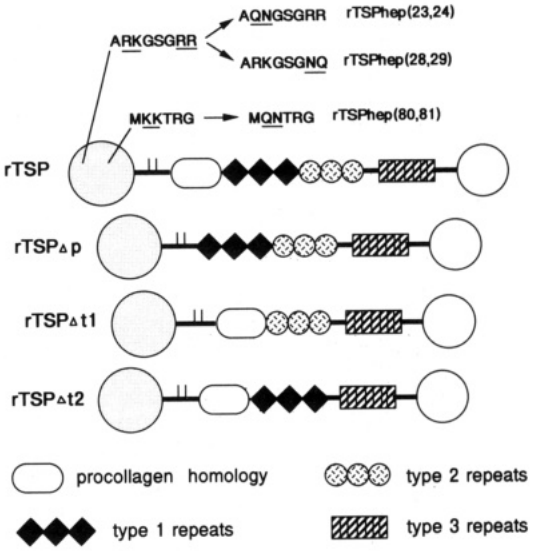

FIGURE 1: Schematic representation of the thrombospondin mutants. The subunit of the thrombospondin molecule is depicted as being composed of globular regions at the NH_2- and COOH-terminals (large circles) with the region of homology with procollagen, the type 1, type 2, and type 3 repeats comprising the center of the molecule. The two vertical lines indicate the position of the two interchain disulfide bonds. Constructs have been made that delete the region of homology with procollagen (rTSPΔP), the type 1 repeats (rTSPΔt1), and the type 2 repeats (rTSPΔt2). Shown at the top are the constructs that have been made within the two sequences that conform to heparin-binding motifs within the NH_2-terminal globular domain.

Transfection and Tissue Culture. The final form of each construct was moved from M13mp8 to the mammalian expression vector pLEN-PT using *Xba*I sites. This vector was constructed by Drs. Paul Johnson and Richard Hynes by cloning the polylinker from the pECE vector into the *Bam*HI site of pLEN (California Biotechnology Inc., Mountain View, CA) (Ellis et al., 1986). Expression of the inserted DNA is driven by the human metallothionein II promoter. A mixture

of the construct (5–10 μg) and pSV2neo (0.5–1.0 μg) was transfected into NIH 3T3 cells using the Lipofectin (Bethesda Research Laboratories, Gaithersburg, MD) protocol. The cells were grown in 100-mm dishes until they were approximately 50% confluent. The cells were washed once with 3 mL of OptiMEMI reduced serum medium (Gibco Laboratories, Gaithersburg, MD) containing no serum, and then 3 mL of the same medium was placed in the dish. The DNA–Lipofectin mixture was added to the dishes with continuous swirling. After 24 h, the medium was changed to DME containing 10% FBS. After 48 h, the cells were trypsinized and replated in DME containing 10% FBS and 1 mg/mL Geneticin (G418, Gibco Laboratories). After approximately 10 days, individual G418-resistant colonies were subcloned, or the cells were allowed to grow and were handled as pools of G418-resistant clones. To produce culture supernatants for analysis, the cells were grown to confluence in four T75 flasks. Fresh medium was placed on the cells, and the cells were grown for 48 h. The conditioned medium was removed, and DFP was added to 1 mM and PMSF was added to 5 mM. After several hours at 0 °C, the culture supernatants were frozen and stored at −20 °C.

Heparin–Sepharose Chromatography. Culture supernatants were fractionated on columns (2–6 mL) of heparin–Sepharose (Pharmacia, Piscataway, NJ). After application of the sample, the columns were eluted stepwise with 20 mM Tris-HCl (pH 7.6) containing 0.15, 0.3, or 0.55 M NaCl and 0.02% NaN_3. Either 2 mM $CaCl_2$ or 5 mM EDTA was included in all of the buffers. When the experiment was performed in the presence of EDTA, the initial culture supernatant was adjusted to 5 mM EDTA prior to application to the column. To maximize the activity of the low-affiity heparin-binding site, some culture supernatants were mixed with an equal volume of 1 mM $CaCl_2$ prior to application to the column. In these cases, the column was eluted stepwise with 20 mM Tris-HCl (pH 7.6), 2 mM $CaCl_2$, and 0.02% NaN_3 containing 0.075, 0.3, and 0.55 M NaCl.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). One- and two-dimensional SDS–PAGE was carried out using the discontinuous system of Laemmli (1970) as described previously (Lawler et al., 1985). The electrophoresed proteins were transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, CA) or Immobilon-P (Millipore, Bedford, MA) membranes and probed with the monoclonal antibody MA-I as described previously (Lawler et al., 1985). The bound MA-I was detected with horseradish peroxidase conjugated goat anti-mouse IgG (Cooper Biomedical Inc., West Chester, PA) or with ^{125}I-labeled goat anti-mouse IgG (New England Nuclear, Boston, MA).

Results

Expression of Thrombospondin. When the vector pTSP is transfected, the cells synthesize and secrete a 420 000-dalton protein that comigrates with human platelet thrombospondin (Figure 2). A single band with a molecular weight of 185 000 is observed when the sample is electrophoresed in the presence of a reducing agent. The expressed thrombospondin is detected in the culture supernatants with the monoclonal antibody MA-I (Lawler et al., 1985). This antibody reacts with human thrombospondin but does not bind bovine thrombospondin that is present as a component of the serum in the culture media (data not shown). This monoclonal antibody does not stain a band in culture supernatants that are harvested from cells that are transfected with the pLEN-PT vector alone, indicating that it does not recognize murine thrombospondin either (data not shown). This observation is consistent with the fact that

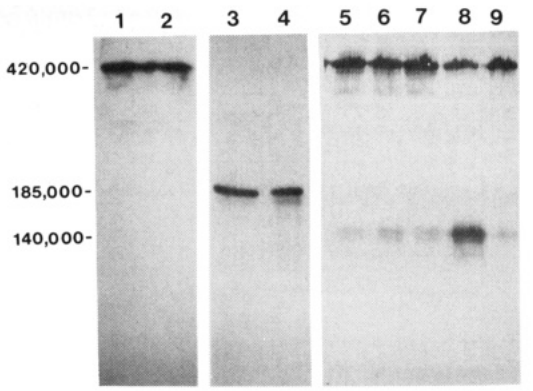

FIGURE 2: Immunoblot of recombinant thrombospondin. Purified human platelet thrombospondin (lanes 1 and 4) and partially purified recombinant thrombospondin (lanes 2 and 3) were electrophoresed in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of reducing agent. Recombinant thrombospondin was partially purified from the culture supernatant of cells that were transfected with pTSP by heparin–Sepharose affinity chromatography. The proteins that were eluted with 0.55 M NaCl were electrophoresed in lanes 2 and 3. The culture supernatant from individual clones of cells that were transfected with pTSP were electrophoresed in the absence of reducing agent (lanes 5–9). After electrophoresis, the proteins were transferred to Immobilon-P and probed with MA-I followed by ^{125}I-labeled goat anti-mouse antibodies.

MA-I has been produced in mouse and that the murine sequence differs from the human sequence in the epitope for MA-I.

In addition to the 420 000-dalton band, a 140 000-dalton band is observed in the culture supernatants from the pTSP-transfected cells (Figure 2). The quantity of the 140 000-dalton band varies in the G418-selected clones with some lines expressing low levels, whereas others produce significantly higher levels (Figure 2). The electrophoretic mobility of the 140 000-dalton band is approximately the same in the presence and absence of a reducing agent, indicating that it is a single polypeptide. Cysteines-252 and -256 have been shown to form the only interchain disulfide bonds in the molecule (Sottile et al., 1991). These observations suggest that the 140 000-dalton polypeptide is derived from the middle and COOH-terminal portions of each chain. This is consistent with the fact that the epitope for MA-I is between residues 877 and 1009 (Lawler & Hynes, 1986).

When the culture supernatants from cells expressing pTSP are applied to a column of heparin–Sepharose, both the 420 000-dalton and the 140 000-dalton proteins are retained by the column (Figure 3). The majority of the 140 000-dalton band is eluted from the column with buffer containing 0.25 M NaCl, whereas the 420 000-dalton band is eluted with a buffer containing 0.55 M NaCl (Figure 3). To establish that the 140 000-dalton band is binding to heparin and not retained by noncovalent association with other portions of the thrombospondin molecule, the isolated 140 000-dalton polypeptide has been dialyzed to reduce the NaCl concentration and reapplied to the column. The 140 000-dalton polypeptide retains the ability to bind heparin (Figure 3). If the culture media are treated with 5 mM EDTA prior to heparin–Sepharose affinity chromatography, the 140 000-dalton polypeptide is eluted with the material that flows through the column without binding and in the buffer containing 0.15 M NaCl (see Figure 7, bottom panel). Platelet thrombospondin has been shown to undergo a calcium-dependent conformational change that can be detected by limited tryptic digestion (Lawler & Simons, 1983). To establish that the expressed material undergoes a calcium-dependent conformational change, we have digested the material eluted from heparin–Sepharose with 0.55 M NaCl

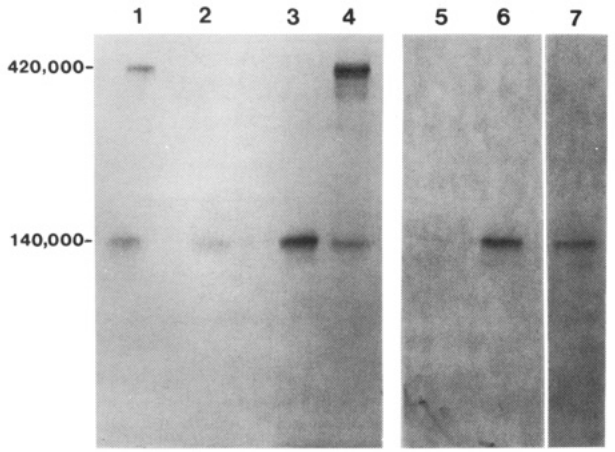

FIGURE 3: Heparin–Sepharose affinity chromatography of recombinant thrombospondin. The culture supernatant produced by cells that were transfected with pTSP was applied to a column of heparin–Sepharose. The original sample (lane 1) and the proteins eluted with buffer containing 0.15 M NaCl (lane 2), 0.25 M NaCl (lane 3), and 0.55 M NaCl (lane 4) were electrophoresed in the absence of reducing agent. The fractions that contained the proteins that were eluted with 0.25 M NaCl were pooled and dialyzed against 20 mM Tris-HCl (pH 7.6), 0.14 M NaCl, 2 mM $CaCl_2$, and 0.02% NaN_3. After dialysis, the sample was reapplied to the heparin–Sepharose column, and the column was eluted with buffer containing 0.15 M NaCl (lane 5), 0.3 M NaCl (lane 6), and 0.55 M NaCl (lane 7). All samples were electrophoresed in the absence of reducing agent, transferred to nitrocellulose, and probed with MA-I followed by peroxidase-conjugated goat anti-mouse antibodies.

in the presence of 2 mM $CaCl_2$ or after treatment with 5 mM EDTA. Distinct tryptic peptide maps were observed in the presence and absence of calcium (data not shown).

Deletion Mutagenesis of the Region of Homology with Procollagen. Portions of the thrombospondin molecule are homologous with other proteins and are composed of repeated structural motifs. To begin to evaluate the role of these structures in thrombospondin function, deletion mutants have been constructed in which the region of homology with procollagen (pTSPΔP), the type 1 repeats (pTSPΔt1), and type 2 repeats (pTSPΔt2) have been deleted (Figure 1). Cells that have been transfected with pTSPΔP synthesize and secrete a protein with an apparent reduced molecular weight of 170 000 as the principle band that binds MA-I (Figure 4). A variable amount of a 130 000-dalton band is also observed. When the samples are electrophoresed in the absence of a reducing agent, bands are observed with apparent molecular weights of 400 000, 320 000, and 170 000 (Figure 4). Two-dimensional nonreduced/reduced gel electrophoresis reveals that all of these bands are composed of the 170 000-dalton polypeptide (Figure 4). This observation suggests that the 320 000-dalton band represents a dimer of the 170 000-dalton band and that the 170 000-dalton band represents monomeric thrombospondin subunits. These species are not observed when the vector pTSP is expressed (Figure 4). The 400 000-dalton band is, in general, a minor species in the culture supernatants of cells that are expressing pTSPΔP. The relative quantities of the 320 000- and 170 000-dalton bands are variable in these samples.

Deletion of the Type 1 Repeats. The type 1 repeats of thrombospondin are adjacent to the region of homology with procollagen. Expression of the construct (pTSPΔt1) that lacks this region results in the synthesis and secretion of a polypeptide that binds MA-I and has an apparent molecular weight of 98 000 in the absence of a reducing agent (Figure 5). When these samples are reduced, a polypeptide with a molecular weight of 127 000 is consistently observed (Figure 5). In addition, variable quantities of polypeptides with molecular weights of 140 000 and 155 000 in the presence of a reducing agent are observed. Similar patterns are observed when individual subclones or pools of G418-selected cells are analyzed. When the culture supernatants from cells that are expressing pTSPΔt1 are applied to a column of heparin–Sepharose, the 127 000-dalton polypeptide is retained by the column and eluted with 0.3 M NaCl (Figure 6A).

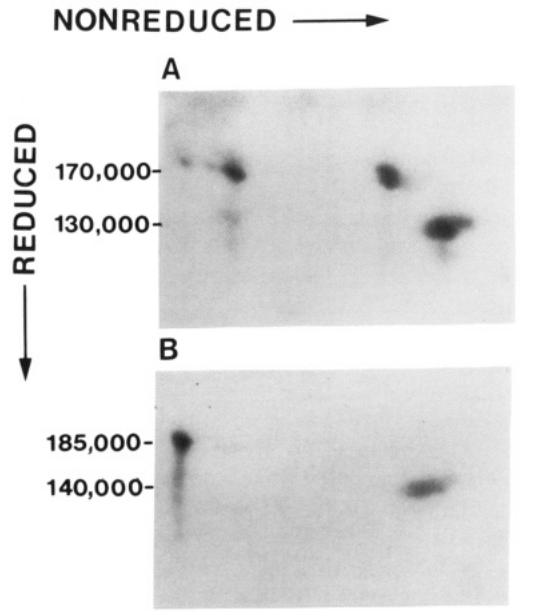

FIGURE 4: Immunoblot of two-dimensional nonreduced/reduced SDS–PAGE of the culture supernatant from cells that were transfected with pTSPΔP (A) and pTSP (B). After electrophoresis, the proteins were transferred to Immobilon-P and probed with MA-I followed by ^{125}I-labeled goat anti-mouse antibodies.

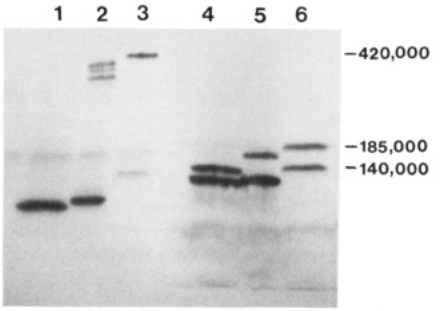

FIGURE 5: Immunoblot of variant forms of thrombospondin. The culture supernatants from cells that were expressing pTSPΔt1 (lanes 1 and 4), pTSPΔt2 (lanes 2 and 5), or pTSP (lanes 3 and 6) were electrophoresed in the absence (lanes 1–3) or presence (lanes 4–6) of reducing agent. After electrophoresis, the proteins were transferred to Immobilon-P and probed with MA-I followed by ^{125}I-labeled goat anti-mouse antibodies.

Deletion of the Type 2 Repeats. The type 2 repeats of thrombospondin are deleted from the pTSPΔt2 construct. Expression of this construct results in the production of polypeptides with molecular weights of 130 000 and 165 000 in the presence of a reducing agent that bind MA-I (Figure 5). The quantity of these polypeptides relative to each other is variable. A polypeptide with a molecular weight of 105 000 is the principal species observed in the absence of a reducing agent (Figure 5). Variable bands with molecular weights of 355 000, 375 000, and 400 000 are also observed in the absence of reducing agent (Figure 5).

When the culture supernatants from cells that are expressing pTSPΔt2 are applied to a column of heparin–Sepharose, both the 130 000- and 165 000-dalton polypeptides are retained by

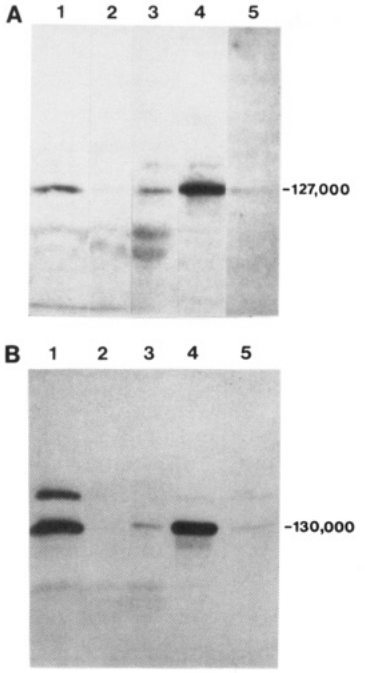

FIGURE 6: Heparin–Sepharose affinity chromatography of the culture supernatants from cells that are expressing pTSPΔt1 (A) or pTSPΔt2 (B). The samples were mixed with an equal volume of 1 mM $CaCl_2$ prior to application to the column. The original samples (lane 1), the flow-through (lane 2), and the proteins that were eluted with buffer containing 0.075 M NaCl (lane 3), 0.3 M NaCl (lane 4), or 0.55 M NaCl (lane 5) were pooled and electrophoresed on 3–10% polyacrylamide gradient gels. After electrophoresis, the proteins were transferred to Immobilon-P and probed with MA-I followed by ^{125}I-labeled goat anti-mouse antibodies. Note that the original sample for the cells transfected with pTSPΔt2 (lane B1) was taken prior to the dilution with 1 mM $CaCl_2$.

the column. The majority of the 130 000-dalton polypeptide is eluted from the column with 0.3 M NaCl (Figure 6B). The 165 000-dalton polypeptide is present in the peaks that are produced by elution with 0.3 and 0.55 M NaCl.

Mutation of the High-Affinity Heparin-Binding Site. Five constructs were produced that included amino acid substitutions in the two sequences that conform to heparin-binding motifs in other proteins (residues 22–29 and 79–84). These include three constructs, pTSPhep(23,24), pTSPhep(27,28), and pTSPhep(80,81), (Table I), in which two adjacent basic amino acids are mutagenized and two constructs, pTSPhep-(23,24+80,81) and pTSPhep(27,28+80,81), that combine the mutations in the two heparin-binding motifs. When culture supernatants from pools of cells that are expressing the normal construct (pTSP) are applied to heparin–Sepharose, the expressed human thrombospondin is observed in the 0.55 M peak (Figure 7). When the proteins expressed by pTSPhep-(23,24+80,81) or pTSPhep(27,28+80,81) are applied to a column of heparin–Sepharose, there is a marked decrease in the quantity of expressed protein that is eluted from the column with 0.55 M NaCl (Figure 7). The expressed thrombospondin is eluted in the unbound flow-through and in the pools that are eluted with 0.15 and 0.3 M NaCl (Figure 7). These results support the hypothesis that resiudes 22–29 and/or 79–84 contain the high-affinity heparin-binding site. When the proteins expressed by pTSPhep(23,24), pTSPhep(27,28), or pTSPhep(80,81) are applied to a column of heparin–Sepharose, the quantity of expressed protein that is eluted with 0.55 M NaCl is again markedly reduced (Figure 7). The expressed thrombospondin is eluted in the unbound flow-through fractions from the column and in the pools that are eluted with buffer containing 0.15 NaCl and 0.3 M NaCl.

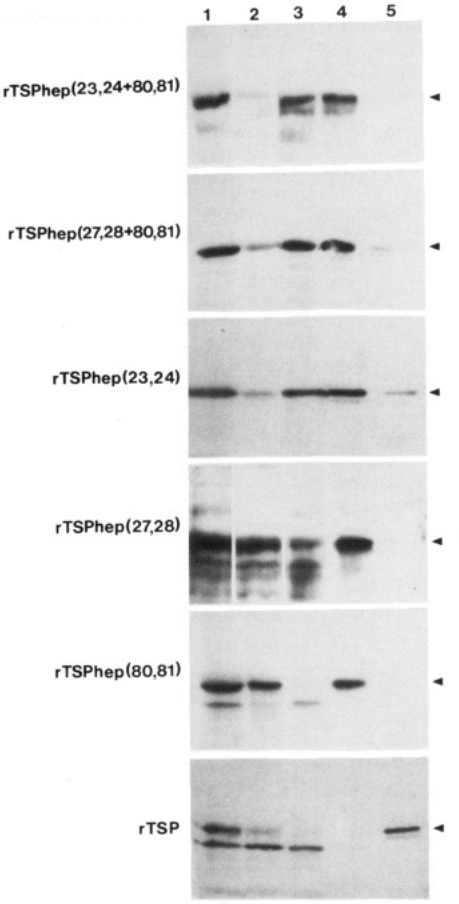

FIGURE 7: Heparin–Sepharose affinity chromatography of the culture supernatants from cells that are expressing thrombospondin with mutations in the high-affinity heparin-binding domain. The heparin mutation is indicated on the left. The control recombinant thrombospondin (rTSP) is shown in the bottom panel. The samples were adjusted to 5 mM EDTA prior to heparin–Sepharose affinity chromatography. The original sample (lane 1), the nonaffinity peak (lane 2), and the fractions that were eluted with buffer containing 0.15 M NaCl (lane 3), 0.3 M NaCl (lane 4), or 0.55 M NaCl (lane 5) were pooled and electrophoresed on 3–10% polyacrylamide gradient gels. After electrophoresis, the proteins were transferred to Immobilon-P and probed with MA-I followed by ^{125}I-labeled goat anti-mouse antibodies. The position of the 185 000-dalton thrombospondin subunit is indicated by the arrow.

DISCUSSION

Expression of the normal human endothelial cell cDNA construct, pTSP, results in the production of a 420 000-dalton protein that comigrates with platelet thrombospondin. After reduction, a 185 000-dalton band is observed that comigrates with reduced platelet thrombospondin. This protein also binds the monoclonal antibody MA-I and heparin. Heparin–Sepharose chromatography suggests that the affinity of the expressed protein for heparin is comparable to that of platelet thrombospondin. In addition, this protein exhibits a calcium-dependent conformational change as judged by limited tryptic digestion in the presence and absence of calcium. These results indicate that the expressed protein is structurally and functionally similar to normal platelet thrombospondin. It should be noted that the expressed thrombospondin molecules may represent a mixture of human and mouse subunits. It has been reported that the level of thrombospondin synthesis by fibroblasts in culture is inversely proportional to cell density (Mumby et al., 1984). In this study, the culture supernatants were harvested from cells that had formed a confluent monolayer in an effort to decrease synthesis of murine thrombospondin subunits and thus increase the relative proportion

of subunits that are synthesized from the construct. The fact that heparin binding is almost completely abolished in the mutants that have been constructed here suggests that the expressed form is the predominant species. If there was a significant synthesis of normal murine subunits, then they might provide high-affinity heparin binding to those molecules into which they were assembled. This conclusion is based on the assumptions (1) that the murine and human subunits can be assembled into the same molecule and (2) that the inclusion of one or two subunits with a normal high-affinity heparin-binding site is sufficient to support high-affinity heparin binding for the intact molecule. The former assumption is supported by the high degree of identity between the human and mouse amino acid sequences (Bornstein et al., 1990; Lawler et al., 1991b). The latter assumption is supported by the observation that isolated heparin-binding domains display comparable affinity for heparin–Sepharose as does the intact molecule (Lawler & Slayter, 1981).

Expression of the full-length cDNA results in the production of a 140 000-dalton polypeptide. The molecular properties of this band are identical to a thrombospondin-derived peptide found in the supernatant of transformed BHK cells (Rastinejad et al., 1989; Good et al., 1990). The level of this peptide is higher in the supernatant of the nontumorgenic cells than in the supernatants of their tumorgenic counterparts. This peptide and the intact thrombospondin molecule were found to inhibit angiogenesis (Good et al., 1990). In this study, a 140 000-dalton polypeptide is observed when the cDNA for the coding region of thrombospondin is transfected into the cells. The structural and immunological properties of the expressed 140 000-dalton polypeptide indicate that it is similar to or identical with the 140 000-dalton polypeptide produced by transformed cells. The 140 000-dalton expressed polypeptide comigrates with the 140 000-dalton polypeptide that is synthesized by the 3T3 cells when the cells are grown in media containing reduced levels of serum (unpublished data). The production of this peptide by cells that have been transfected with a full-length cDNA construct for thrombospondin suggests that this polypeptide is not produced as a result of alternative splicing. It is possible that the message is reduced in size by an endonuclease and methionine(262) is then used for initiation (Kozak, 1989). However, it would seem more likely that this peptide is produced by proteolysis after secretion.

The fact that the 140 000-dalton expressed polypeptide binds heparin is consistent with the observations of others (Haverstick et al., 1984; Dardik & Lahav, 1987; Prater et al., 1991). Haverstick et al. (1984) have shown that two distinct thermolytic fragments of thrombospondin could bind to heparin–Sepharose and could neutralize the inhibitory activity of heparin in a hemagglutination assay. Proteolytic removal of 20 000-dalton fragments from the COOH-terminal of the molecule inhibited the ability of the large fragment to bind to heparin–Sepharose. By contrast, Dardik and Lahav (1987) mapped a low-affinity heparin-binding site to a 70 000-dalton polypeptide that comprises the central portion of each subunit. The interchain disulfides, the region of homology with procollagen, and the type 1 and type 2 repeats are included within the 70 000-dalton region. Prater et al. (1991) have proposed that the type 1 repeats contain a glycoconjugate-binding site. In the present study, we have observed that polypeptides that lack the type 1 or type 2 repeats retain the low-affinity heparin-binding site. These data may indicate that heparin-binding sites are present in both the middle portion and the COOH-terminus of the molecule. Further experiments will be required to specifically map the sites for these interactions. We also plan to more quantitatively determine the affinity of this site for heparin and other proteoglycans. It is possible that this site will have a higher affinity for proteoglycans other than heparin. The presence of additional proteoglycan-binding sites indicates that the effect of heparin on the interaction of thrombospondin with cell surfaces should be analyzed with caution. It is frequently assumed that heparin inhibits interactions that are mediated by the high-affinity site. In fact, these interactions may involve low-affinity sites.

When the region of thrombospondin that is homologous to procollagen is deleted, forms of the molecule that apparently represent monomers and dimers are observed in the culture supernatants. The absence of trimeric structures may be due to either defective subunit assembly or a failure to transport this form. The presence of aberrant monomers and dimers would support the possibility that subunit assembly is defective. This observation indicates that the residues between T(284) and S(357) are involved in the self-association of the three subunits. This region is adjacent to the cysteine residues that form the interchain disulfide bonds [Cys(252) and Cys(256); Sottile et al., 1991]. A shortened form of the thrombospondin polypeptide (residues 1–333) has been shown to form trimers (Prochownik et al., 1989), suggesting that an important sequence for trimer assembly is in the region between residues 284 and 333.

Deletion of the type 1 or the type 2 repeats from the central portion of the thrombospondin molecule results in the production of the low molecular weight, single-chain polypeptides that are detectable with MA-I. The molecular properties of these species suggest that they represent shortened forms of the 140 000-dalton polypeptide. The very low levels of higher molecular weight species would suggest that deletion of these regions has made the subunits more labile to proteolysis.

The results presented here indicate that mutagenesis of lysine or arginine residues between positions 23 and 29 or lysine residues at positions 80 and 81 decreases the affinity of thrombospondin for heparin. These observations indicate that both clusters of positively charged residues interact directly with heparin and participate in forming the high-affinity heparin-binding site. In antithrombin III, two separate domains form a single heparin-binding site. Naturally occurring mutations of R(47) result in the loss of heparin-binding activity (Owen et al., 1987; Borg et al., 1988). Studies on the heparin-binding activity of proteolytic fragments of antithrombin III and on the effects of antibodies to antithrombin III peptides indicate that residues 124–125 are also involved in the interaction with heparin (Rosenfeld & Danishefsky, 1986; Smith & Knauer, 1987; Vaughn et al., 1988). Presumably, folding of the polypeptide brings these two sites into close proximity to form a single heparin-binding site. Our data would indicate that the high-affinity heparin-binding site of thrombospondin is similar. We cannot rule out the possibility that the mutations in one or both of these regions are disrupting the overall structure of the NH_2-terminal region of the molecule and thus affecting heparin binding in a less specific manner. It has been reported that a truncated form of the molecule (residues 1–164) that lacks the intrachain disulfide bond does not bind heparin (Prochownik et al., 1989). We have not observed a decrease in the stability of the polypeptides that are expressed by the heparin-binding mutants as we have when the type 1 and type 2 repeats are deleted. We have tried to choose amino acid substitutions so as to minimize the probability of inducing gross conformational changes. Determinations of relative substitution frequencies during evolution indicates that as-

paragine and glutamine residues replace lysine and arginine residues with the highest frequency, excluding the frequency of lysine and arginine residues replacing each other (Schulz & Schirmer, 1979). Asparagine is found in one of the positions usually occupied by a basic amino acid in the heparin-binding motifs of vitronectin and lipoprotein lipase (Cardin & Weintraub, 1989). Glutamine is found in one of the positions usually occupied by a basic amino acid in the heparin-binding motif of histidine-rich glycoprotein (Cardin & Weintraub, 1989). In addition, asparagine or glutamine is found in the variable positions between the conserved basic amino acids in the heparin-binding motifs of vitronectin, apolipoprotein B-100, lipoprotein lipase, glial-derived nexin, histidine-rich glycoprotein, endothelial cell growth factor, antithrombin III, fibronectin, lipocortin, and thrombin (Cardin & Weintraub, 1989). Thus, in general asparagine and glutamine residues would appear to be compatible with structures in and around heparin-binding sites. The present study was intended to identify which region of positively charged resiudes is important. Since both appear to be important, we are in the process of constructing additional mutants in both of these regions. In some of our constructs, a small amount of residual heparin-binding activity is observed. Heparin–Sepharose affinity chromatography does not provide a sufficiently quantitative approach to characterize these interactions. We are currently working on purifying sufficient quantities of the various mutants to be able to perform quantitative binding assays with soluble heparin. These assays will enable us to better quantify the affinity of the individual mutant as well as these combinations.

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Substitution of Glutamic Acid 109 by Aspartic Acid Alters the Substrate Specificity and Catalytic Activity of the β-Subunit in the Tryptophan Synthase Bienzyme Complex from *Salmonella typhimurium*†

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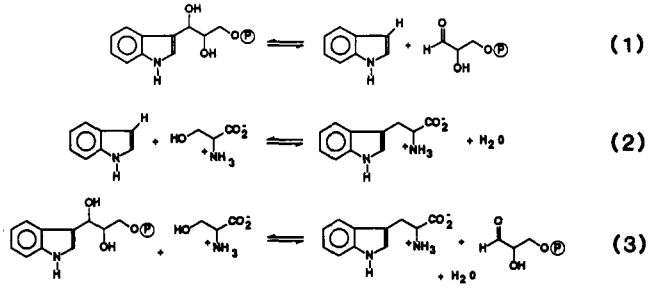
ABSTRACT: In an effort to understand the catalytic mechanism of the tryptophan synthase β-subunit from *Salmonella typhimurium*, possible functional active site residues have been identified (on the basis of the 3-D crystal structure of the bienzyme complex) and targeted for analysis utilizing site-directed mutagenesis. The chromophoric properties of the pyridoxal 5′-phosphate cofactor provide a particularly convenient and sensitive spectral probe to directly investigate changes in catalytic events which occur upon modification of the β-subunit. Substitution of Asp for Glu 109 in the β-subunit was found to alter both the catalytic activity and the substrate specificity of the β-reaction. Steady-state kinetic data reveal that the β-reaction catalyzed by the βE109D $\alpha_2\beta_2$ mutant enzyme complex is reduced 27-fold compared to the wild-type enzyme. Rapid-scanning stopped-flow (RSSF) UV–visible spectroscopy shows that the mutation does not seriously affect the pre-steady-state reaction of the βE109D mutant with L-serine to form the α-aminoacrylate intermediate, E(A-A). Binding of the α-subunit specific ligand, α-glycerol phosphate (GP) to the $\alpha_2\beta_2$ complex exerts the same allosteric effects on the β-subunit as observed with the wild-type enzyme. However, the pre-steady-state spectral changes for the reaction of indole with E(A-A) show that the formation of the L-tryptophan quinonoid, $E(Q_3)$, is drastically altered. Discrimination against $E(Q_3)$ formation is also observed for the binding of L-tryptophan to the mutant $\alpha_2\beta_2$ complex in the reverse reaction. In contrast, substitution of Asp for Glu 109 increases the apparent affinity of the βE109D α-aminoacrylate complex for the indole analogue indoline and results in the increased rate of synthesis of the amino acid product dihydroiso-L-tryptophan. Thus, the mutation affects the covalent bond forming addition reactions and the nucleophile specificity of the β-reaction catalyzed by the bienzyme complex.

The bacterial $\alpha_2\beta_2$ tryptophan synthase bienzyme complex catalyzes the final two reactions in the biosynthesis of L-tryptophan (Yanofsky & Crawford, 1972; Miles 1979, 1991). The α-subunit catalyzes the reversible aldolytic cleavage of 3-indole-D-glycerol 3′-phosphate (IGP) to D-glyceraldehyde 3-phosphate (G3P)[1] and indole (α-reaction). The β-subunit catalyzes the essentially irreversible condensation of indole with L-serine (L-Ser) to form L-tryptophan (L-Trp) (β-reaction). The physiological αβ-reaction is the sum of the individual α- and β-reactions linked via the common intermediate indole.



Crystallographic studies of the $\alpha_2\beta_2$ complex from *Salmonella typhimurium* have revealed that the α and β active sites are separated by nearly 30 Å but are directly connected by a tunnel of sufficient size to accommodate indole (Hyde et al., 1988; Hyde & Miles, 1990). Therefore, indole may be directly channeled between catalytic centers. Rapid kinetic studies have confirmed that the tunnel is the preferred route of entry for indole into the β active site in the β-reaction (Dunn et al., 1987b, 1990) and indole appears to be channeled between the α- and β-sites in the αβ-reaction (Lane & Kirschner, 1991; Anderson et al., 1991).

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[1] Abbreviations: PLP, pyridoxal phosphate; L-Ser, L-serine; L-Trp, L-tryptophan; DIT, dihydroiso-L-tryptophan; IGP, 3-indole-D-glycerol 3′-phosphate; IPP, 3-indolylpropanol 3-phosphate; G3P, D-glyceraldehyde 3-phosphate; GP, α-glycerol phosphate; 3-Cl-Ala, 3-chloro-L-alanine; BZI, benzimidazole; βE109D, $\alpha_2\beta_2$ complex of tryptophan synthase in which glutamate 109 in the β-subunit has been replaced by aspartate; RSSF, rapid scanning stopped flow; SWSF, single wavelength stopped flow; NMR, nuclear magnetic resonance; GPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactic dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate-*2,2,3,3-d*$_4$; $\alpha_2\beta_2$, native tryptophan synthase from *S. typhimurium*; E(A-A), enzyme-bound Schiff base of α-aminoacrylate; $E(Q_1)$, $E(Q_2)$, or $E(Q_3)$, quinonoidal intermediates formed in the conversion of L-Ser and indole to L-Trp; $E(A_{ex})$, aldimine intermediates formed between the substrate amino acids and the PLP cofactor; E(GD), geminal diamine intermediate formed between the PLP cofactor, the amino group of the substrate, and the ε-amino group of Lys 87.

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