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Expression and initial characterization of recombinant mouse thrombospondin 1 and thrombospondin 3

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Abstract To analyze the function of TSP family members, we have expressed and purified mouse TSP1 and TSP3 encoded by recombinant baculoviruses in *Spodoptera frugiperda* cells and compared these TSPs to mouse TSP2 prepared in a similar way. Yields of purified TSP1 and TSP3 were 5–15 and 2–4 μg, respectively, per ml of conditioned medium. Mature, secreted mouse TSP1 and TSP3 had the previously predicted NH_2-terminal sequences of DHVKDTSFDLFSI, and SQDLQVIDLLT, respectively. Analysis by SDS-PAGE and rotary shadowing electron microscopy indicated that TSP1 and TSP2 are disulfide bonded trimers whereas TSP3 is a disulfide-bonded pentamer. Binding assays with $^{45}Ca^{2+}$ as ligand and immobilized TSP1, TSP2 and TSP3 demonstrated that all three TSPs are calcium-binding proteins. These results are consistent with previous studies of TSP structure and demonstrate that *Spodoptera* cells process and secrete TSPs having the same subunit organizations and structure as the native vertebrate molecules.

Key words: Thrombospondin; Extracellular matrix; Baculovirus; Recombinant protein

1. Introduction

Thrombospondins (TSPs) are a family of structurally related modular glycoproteins [4]. To date, five thrombospondins have been identified in vertebrates. Thrombospondins are divided into two subgroups based on their primary structures, with TSP1 and TSP2 in one group, and TSP3–TSP5 in the other (review [4]). TSP5 is also known as cartilage oligomeric matrix protein (COMP) [33,36].

Among TSPs, human platelet TSP1 is the most well characterized. TSP1 is a trimeric, secreted glycoprotein. Each TSP1 subunit contains an NH_2-terminal globular domain (the proline- and arginine-rich [PARP] module [3]), a domain that mediates trimerization, a procollagen module, three type 1 (properdin) modules, three type 2 (EGF-like) modules, a series of type 3 (Ca^{2+}-binding) repeats, and a COOH-terminal globular domain (review [4]). TSP1 and TSP2 have a similar domain organization [22,23]. Conceptual translational products of the cDNAs of TSPs 3–5 reveal a different domain arrangement as compared to TSPs 1 and 2 [5,28,29,33,36,38]. In TSPs 3–5, the procollagen module and the type 1 modules are missing, and four, instead of three, type 2 modules are found. The NH_2-terminal PARP module is present in TSPs 1–4. The Ca^{2+}-binding repeats and the COOH-terminal globular region characteristic of the TSP family are well conserved in all TSPs.

The patterns of expression of TSP1, TSP2 and TSP3 mRNAs are distinct in tissues of embryonic and developed mice. Overall, TSP1 expression is predominant over the other TSPs, except for the vasculature in early mouse embryonic development where TSP2 is prominently transcribed from days 10–18 [20]. Thereafter, constant expression of TSP1 and TSP2 is observed in many organs and connective tissues. TSP3 expression is turned on later than that of TSP1 or TSP2 (from day 15 onward), and is mostly refined to gut, cartilage, lung and central nervous system [20,38]. Both TSP1 and TSP2 expression increase when mouse embryonal carcinoma cells are induced to differentiate into neurons, glial cells and fibroblasts, but TSP1 protein displays a fibrillar distribution pattern in the extracellular matrix whereas TSP2 is cell associated [30]. The different spatial and temporal expression patterns of TSP1, TSP2 and TSP3 suggest that the three TSPs may have unique tissue-specific functions.

We have reported the expression of mouse TSP2 encoded by recombinant baculovirus [8]. To understand more about the structure and function of different TSPs, we have also expressed mouse TSPs 1 and 3 using the baculovirus system and compared the properties of the recombinant proteins to those determined for proteins purified from natural sources or inferred from conceptual sequences of cloned cDNAs.

2. Materials and methods

2.1. Production of recombinant viruses

Procedures for generation of recombinant baculoviruses encoding mouse TSP2 [8] and mouse TSP1 [9] have been previously described.

A cDNA clone of mouse TSP3 (phage 10 clone) was a kind gift from Dr. Paul Bornstein, University of Washington, Seattle, WA. The TSP3 sequence encoded by this clone starts 41 bases 3′ to the start codon and spans the rest of the coding region [5]. Two complementary oligonucleotides with terminal *Nco*I (5′) and *Bsa*AI (3′) restriction sites were synthesized to fill in the missing 5′ coding region. The annealed oligonucleotides along with a restriction fragment of TSP3 that spans the sequence from the *Bsa*AI site at bp 237 to the *Eco*RI site of bp 1624 were cloned into the *Nco*I and *Eco*RI sites of plasmid pAcSG2 (Pharmingen, San Diego, CA). The resulting construct was subjected to *Eco*RI and partial *Nco*I digestion yielding a fragment encoding the 5′-end of TSP3, i.e. from the initiator ATG to the *Eco*RI site at bp 1624. A restriction fragment that covered the 3′-end of the coding sequence, i.e. bps 1625–2935, was obtained by digestion of the original phage 10 clone with *Eco*RI and *Bsa*BI. The 5′ and 3′ TSP3 fragments were finally cloned into the *Nco*I and *Sma*I sites of pAcSG2.

Recombinant virus encoding mouse TSP3 was generated using the BaculoGold transfection system (Pharmingen) with lipofectin (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instruc-

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Abbreviations: COMP, cartilage oligomeric matrix protein; EGF, epidermal growth factor; FN, fibronectin; PAGE, polyacrylamide gel electrophoresis; PARP, proline- and arginine-rich protein; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TSP, thrombospondin

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tions. After transfection, recombinant viruses were plaque purified once and then a high titer pass three virus pool was produced in serum-free SF900 II medium (Gibco BRL).

2.2. Production and purification of mouse TSP1, TSP2 and TSP3

SF9 cells in serum-free SF 900 II medium at a density of 1.5×10^7 cells/ml in a 500 ml spinner flask were infected with the respective recombinant viruses at a multiplicity of infection of 10. After 1 h at 27°C with occasional agitation, cells were diluted to a final density of 2.5×10^6 cells/ml with medium containing 0.2% bovine serum albumin (Sigma, St. Louis, MO). The culture was incubated for approx. 48 h at 27°C before conditioned medium was harvested. The medium was made 2 mM in phenylmethylsulfonyl fluoride and cleared of cells and cellular debris by centrifugation at $1000\times g$ for 15 min at 22°C. The cell-free conditioned medium was collected and used for purification within the same day.

Human TSP1 was purified from platelets [42]. Mouse TSP2 was purified by affinity chromatography on heparin-agarose as described [8]. Mouse TSP1 and TSP3 were purified on heparin-agarose at room temperature as described for mouse TSP2. For TSP1, proteins were eluted with a step gradient of 300 mM, 450 mM and 2 M NaCl in 10 mM Tris, pH 7.4, 0.3 mM $CaCl_2$, with the bulk of TSP1 eluting at 450 mM NaCl. For TSP3, proteins were eluted with a step gradient of 275, 400 and 600 mM NaCl in 10 mM Tris, pH 7.4, 0.3 mM $CaCl_2$, with the majority of TSP3 molecules being present in the 275 and 400 mM NaCl fractions. All buffer solutions used in the purification of TSPs 1–3 contained 0.3 mM $CaCl_2$.

2.3. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) and immunoblotting

Discontinuous SDS-PAGE was performed according to the method of Laemmli as described [42]. Proteins were detected by staining with Coomassie brilliant blue. For immunoblotting, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) and detected by overnight incubation with specific antibodies. Polyclonal antibodies that had been raised against a gene *10* fusion protein (pET expression vector) containing residues 88–200 of mouse TSP2, against a similar fusion protein containing residues 92–205 of mouse TSP1 [23,35], and polyclonal antibodies to human TSP3 [38] were kindly provided by Dr. Vishva Dixit, University of Michigan, Ann Arbor, MI. Antisera were diluted 1:500 in 10 mM Tris, pH 7.4, 150 mM NaCl, containing 0.05% Tween 20 and 1% bovine serum albumin. Bound antibodies were subsequently detected with horseradish peroxidase conjugated goat anti-rabbit IgG (Cappel, West Chester, PA).

2.4. Amino acid sequencing

Purified mouse TSP1 (50 μg) was absorbed onto the polyvinylidene difluoride (PVDF) membrane of a ProSpin sample preparation cartridge (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. TSP3 (75 μg) was separated by SDS-PAGE and transferred electrophoretically to a PVDF membrane. Protein was visualized by staining with Ponceau S, and the band corresponding to TSP3 was cut out, rinsed with H_2O, and dried under nitrogen. The NH_2-terminal protein sequences were determined by Edman degradation performed at the Protein Sequence and Peptide Synthesis Facility at the Biotechnology Center of the University of Wisconsin using an automated Model 477A Liquid Pulse Sequencer and Model 475A Gas Phase Sequencer with on-line Model 120A PTH Analyzer and Model 610A Data Analysis System (Applied Biosystems).

2.5. Rotary shadowing electron microscopy

Purified TSP1–TSP3 were diluted in 10 mM Tris, pH 7.4, 0.3 mM $CaCl_2$ to obtain a final NaCl concentration of 150 mM and loaded onto 0.6 ml heparin agarose columns. Columns were washed with 0.1 M ammonium bicarbonate and bound TSP molecules eluted with 1 M ammonium bicarbonate. Rotary shadowing was perfomed in a Balzers BAF400K system as described [14]. Briefly, equal volumes of the protein samples and 80% glycerol were mixed and sprayed onto freshly cleaved mica pieces. Rotary shadowing with platinum was performed at an angle of 7° to a thickness of 2.5 nm. Replicas of the TSP molecules were examined in a transmission electron microscope at an accelerating voltage of 80 kV and pictures were taken at a magnification of 73 000×.

2.6. Dot-blot calcium-binding assay

Purified TSPs were diluted to approx. 20 μg/ml in TBS (10 mM Tris, pH 7.4, 150 mM NaCl) containing 1 mM $CaCl_2$ and 500, 250 or 100 μl/well blotted onto a nitrocellulose membrane by filtration using a Minifold dot-blot apparatus (Schleicher and Schuell). The membrane was subsequently washed three times with TBS containing 1 mM $CaCl_2$ and then overlaid with TBS containing 1 mM $CaCl_2$ and 1 μCi/ml $^{45}CaCl_2$ for 1 h at room temperature. After incubation, the membrane was washed twice for 2.5 min each with 50% ethanol, and air dried. Bound radioactivity was quantified by phosphorimaging. After collecting data on bound radioactivity, protein adsorbed to the nitrocellulose membrane was stained with Amido black. The membrane was made transparent with decahydronaphthalene (Sigma) and the amount of protein bound in each well was quantified by scanning with a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer, San Francisco, CA) and normalized to a concentration series of human TSP1 adsorbed onto the same membrane.

3. Results

Spodoptera frugiperda cells grown in serum-free medium were used to express full length mouse TSP1 and TSP3. In the construction of the replacement vectors, excess 5′ untranslated sequences were removed from the TSP cDNAs because it has been suggested that these sequences might interfere with protein expression [34]. Polyclonal antibodies against the heparin-binding domain of mouse TSP1 specifically recognized on immunoblots a band migrating in SDS-PAGE at approximately the size expected for TSP1 when conditioned medium from cells expressing mouse TSP1 was analyzed (data not shown). Anti-TSP3 antibodies specifically detected a ~150 kDa band on immunoblots after SDS-PAGE separation of conditioned medium of cells infected with recombinant mouse TSP3 virus under reducing conditions (data not shown). The size of about 150 kDa is consistent with that previously reported for mouse TSP3 expressed in 293 cells [38]. Purification of recombinant TSPs by affinity chromatography on heparin (Fig. 1) yielded 5–15 and 2–4 μg/ml conditioned medium for mouse TSP1 and TSP3, respectively.

Occasionally, the TSPs produced seemed to be heterogeneous, as indicated by the presence of a weakly staining smear under the major TSP band on immunoblots (data not shown). The heterogeneity represented only a small portion of the purified TSP molecules as judged from protein staining of SDS-PAGE gels (not detectable, see Fig. 1) and rotary shadowing electron microscopy (see below). Because no heterogeneity could be detected with antibodies to the NH_2-terminal PARP domain in immunoblots of trypsin digests of recombinant mouse TSP2 [8], we believe that the heterogeneity results from a random termination of polypeptide elongation. Alternatively, the type 1–3 repeat regions may randomly misfold and become targets for endo- and exo-proteinases. Such events could be due to the shutdown of the cellular quality control machinery of insect cells at a very late stage of virus infection [34].

NH_2-terminal amino acid sequence analysis of purified mouse TSP1 gave the sequence NRIPESGGXNGVFDIFELI, indicating that processed, mature mouse TSP1 starts with Asn at position 19 [23]. This is in agreement with the NH_2-terminal amino acid sequence of human platelet TSP1 [15,40], and Von Heijne's prediction of a signal sequence cleavage site [43]. Mouse TSP3 had an NH_2-terminal sequence of XQDLQVIXLXT. This sequence conforms to the site of predicted signal peptide cleavage between Gly-21 and Ser-22

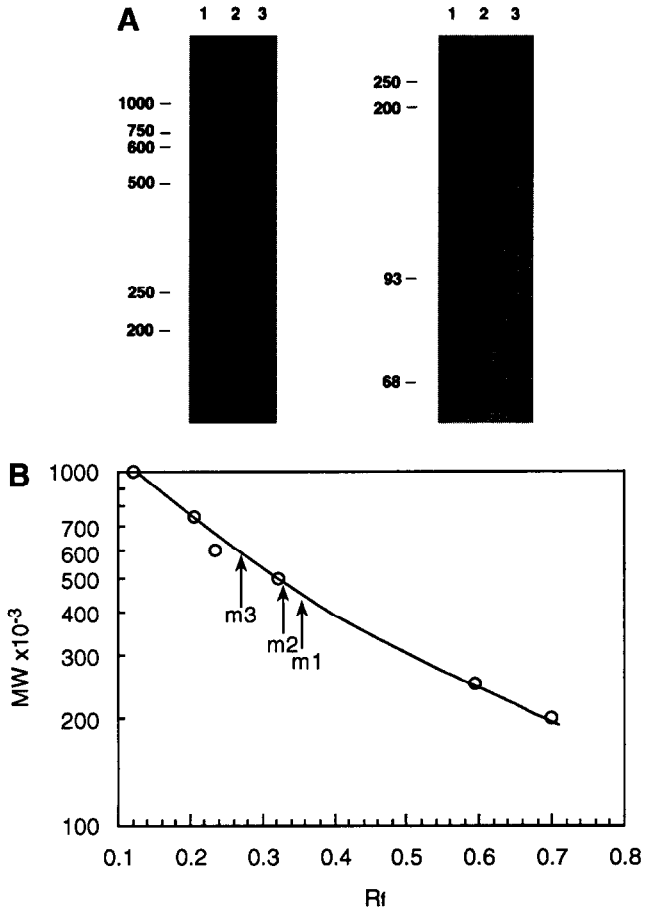
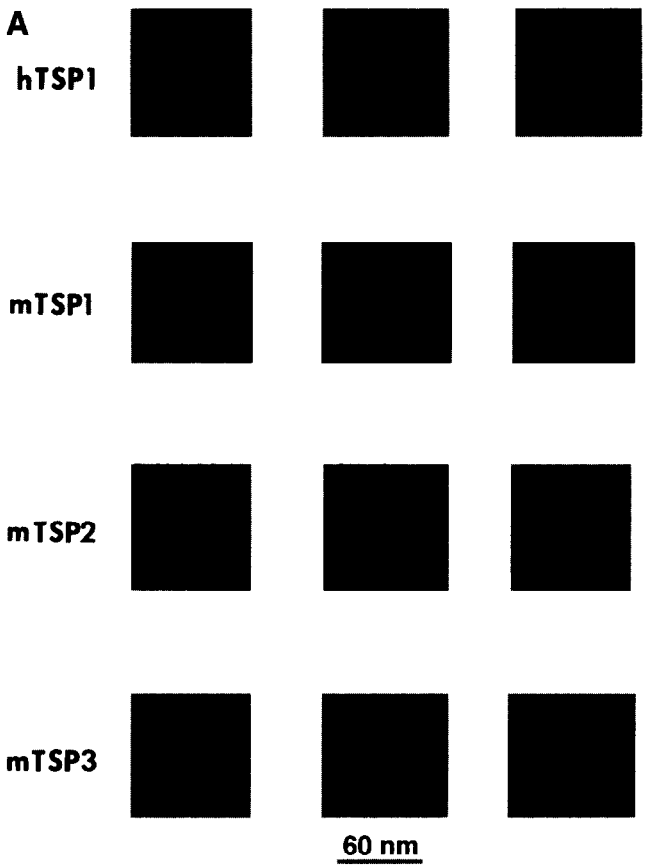


Fig. 1. SDS-PAGE analysis of recombinant mouse TSPs. (A) Purified TSP1 (1), TSP2 (2) and TSP3 (3) were separated on a 3.5% non-reducing (NR) or a 7% reducing (R) SDS-polyacrylamide separating gel. The molecular mass markers are indicated on the left and are: For non-reducing conditions (NR): fibronectin ladder [32] generated by cross-linking fibronectin monomers with factor XIIIa (250 kDa, monomer; 500 kDa, dimer; 750 kDa, trimer; and 1000 kDa, tetramer); 600 kDa, skeletal muscle nebulin; 200 kDa, myosin heavy chain. For reducing conditions (R): 250 kDa, fibronectin; 93 kDa, phosphorylase A; 68 kDa, serum albumin; and 43 kDa, ovalbumin. (B) The sizes of recombinant mouse TSPs were estimated from SDS-PAGE under non-reducing conditions. Molecular weights (MW) of size markers (open circles) were plotted versus their mobility (see A, NR). The mobility of TSP1 (m1), TSP2 (m2) and TSP3 (m3) is indicated (arrows).

[5,38,44] to yield the NH_2-terminal sequence SQDLQVIDLLT in the mature protein.

SDS-PAGE analysis in 3.5 or 7% separating gels was used to assess the sizes of non-reduced and reduced mouse TSPs, respectively. Under reducing conditions, the subunit of TSP1 migrated faster on SDS-PAGE than the subunit of TSP2 (Fig. 1A), with an apparent size of 160 and 185 kDa, respectively. A similar difference in size has been observed between mouse TSP1 and TSP2 secreted by transiently transfected human 293 cells [35]. The size of reduced TSP3 monomer was 145 kDa (Fig. 1A). Plotting the logarithm of the molecular weights of standard proteins (see figure legend) versus mobility allowed us to estimate the sizes of non-reduced mouse TSP1–TSP3 molecules to be approx. 420, 480 and 590 kDa, respectively (Fig. 1B). The ratios of sizes for the non-reduced over reduced forms of both TSP1 and TSP2 are 2.6, suggesting that the mature molecules are homotrimers when considering that both TSP1 and TSP2 are rich in intrachain disulfide bonds and are expected to be in relatively more compact form with faster mobility under non-reducing than under reducing conditions. For TSP3, the size ratio of the non-reduced to reduced form is 4.1, suggesting a subunit composition larger than a trimer, presumably a pentamer.

To explore further the quaternary structure and subunit composition of recombinant mouse TSP1, TSP2 and TSP3, rotary shadowing electron microscopy was performed. Well-



B

	length of rod-like connecting domain (nm) mean+/-S.D.	diameter of COOH-terminal globe (nm) mean+/-S.D.	number of resolved central globules	number of resolved peripheral globules
hTSP1	31.2 ± 2.3	13.2 ± 2.3	1 (3)	3
mTSP1	26.9 ± 1.2	12.4 ± 1.7	1 (3)	3
mTSP2	30.0 ± 1.3	13.6 ± 0.9	1 (3)	3
mTSP3	14.6 ± 1.7	11.1 ± 0.9	1	5

Fig. 2. Rotary shadowing electron microscopy of human TSP1 purified from platelets and recombinant mouse TSP1, TSP2 and TSP3 expressed in insect cells. (A) A selection of images of representative particles in each protein preparation is shown. (B) Structural domains of TSP molecules (at least five molecules of each TSP gene product) were measured and the results are given as mean ± standard deviation. For TSP1 and TSP2, the central globule could be resolved into three small globular domains in some molecules.

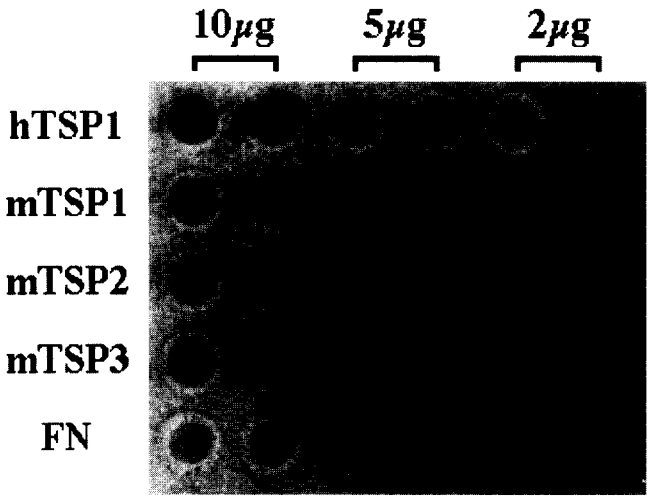


Fig. 3. Ca^{2+} binding of human TSP1 purified from platelets and recombinant mouse TSP1, TSP2 and TSP3 expressed in insect cells. 10, 5, or 2 μg of human platelet TSP1 (hTSP1), recombinant mouse TSP 1–3 (mTSP1–3) or fibronectin (FN) was dot blotted in duplicate onto a nitrocellulose membrane and incubated with $^{45}Ca^{2+}$. Ca^{2+} binding was visualized (shown result) and quantified by phosphorimaging. The amount of protein bound to the membrane was subsequently determined as described in Section 2. When the ratio of bound $^{45}Ca^{2+}$ to protein adsorbed to the membrane was normalized to human TSP1 in two separate experiments, the following average numbers were derived: mouse TSP1, 0.38; mouse TSP2, 0.54; mouse TSP3, 0.27; and fibronectin, <0.10.

defined molecules consisting of globular- and rod-like domains were visible (Fig. 2). Molecules of mouse TSP1 and TSP2 displayed a similar structure that is also consistent with images of human platelet TSP1 reported previously [11,26] and replicated by us (Fig. 2A). Each molecule had three thin flexible regions, connected at one end, where each rod-like domain is attached to a small globular domain. The three small globular domains are close together, and on some of the replicas of the molecules, appeared as one big globule (Fig. 2A). The connecting strands on the more extended molecules had a length of 31.2 ± 0.9, 26.9 ± 1.2 and 30.0 ± 1.3 nm for human TSP1, mouse TSP1 and mouse TSP2, respectively (Fig. 2B) (measurements are expressed as mean ± S.D. and are not corrected for the thickness of the metal coat since the globular domains might be more heavily decorated than the thinner connecting regions). At the other end of each rod-like domain, a large globule can be seen with a size of about 12 nm. Images of mouse TSP3 were distinctly different from those of TSP1 or TSP2. Five globular domains appeared to be each connected by a thin connecting region to a central globule (Fig. 2A). The rod-like connecting domains had a length of 14.6 ± 1.7 nm, and were shorter than those of TSP1 or TSP2 (Fig. 2B), compatible with the fact that TSP3 is missing the procollagen and all three type 1 modules, which form part of the connecting strand.

The ability of the recombinant TSPs to bind Ca^{2+} was examined. In a ligand blot experiment where purified TSPs immobilized on a nitrocellulose membrane were incubated with $^{45}Ca^{2+}$, all TSPs, including human TSP1 purified from platelets, and recombinant mouse TSP1, TSP2 and TSP3, bound Ca^{2+} (Fig. 3). When Ca^{2+} binding of the recombinant mouse proteins was compared by phosphorimaging to human TSP1, which is known from titration studies to contain approx. 11 Ca^{2+}-binding sites per subunit [31], the signals of bound $^{45}Ca^{2+}$ for mouse TSPs 1, 2 or 3 were 25–55% as intense as that detected with an equivalent amount of human TSP1. Only background levels of radioactivity were associated with an equivalent amount of immobilized fibronectin.

4. Discussion

In this paper, we report the expression and characterization of mouse TSP1 and TSP3 and compare these TSPs to the previously expressed mouse TSP2 [8]. Insect cells were able to synthesize and then assemble and secrete TSPs appropriately as trimers (TSP1 and TSP2) or pentamers (TSP3). The capability to produce large quantities of different TSPs, all expressed in the same system, should enable well-controlled comparative structural and functional studies of these molecules. The system should also be well-suited for expression of TSP5 and thus facilitate the study of the pathogenesis of pseudo-chondrodysplasia [6,18].

The fact that TSPs 1–3 bind to heparin agarose provided a convenient and consistent method for purification. Heparin binding was expected for mouse TSP1 because of its sequence homology to human TSP1, which is known to bind heparin through its NH_2-terminal PARP module [12,24,40]. Mouse TSP2 has previously been shown to bind heparin [8,35]. Despite the fact that the TSP3 NH_2-terminal PARP module lacks the known heparin binding sequences [27], and that TSP3 also lacks the type 1 modules that possess heparin binding sequences [7,16], mouse TSP3 bound to heparin-agarose. This result is consistent with reports by other laboratories showing that both TSP3 and TSP4 bind heparin [17,29,39] while TSP5, which lacks the full PARP module, does not [17,19]. It is not yet clear what sequences in TSP3 or TSP4 are responsible for the heparin binding property. However, the fact that NH_2-terminally truncated TSP4 molecules, missing the PARP module, isolated from bovine tendon do not bind heparin suggests that heparin binding is mediated by the PARP module also in TSP4 [17]. Given that fact that some of the cell adhesion properties of TSP1 and TSP2 are mediated via the heparin binding modules, it will be interesting to test if

A:

```
          a  d  a  d  a  d  a  d  a  d  a  d
mTSP1:    TKDLQAICGLSCDELSSMVLELKGLRTIVTTLQDSIRKVTEE  304
            :|  :|   ||:||| |: || ||  :|  |   : :|: :
mTSP2:    VEKAQEVCTHSCEELSNMMNELSGLHVMVNQLSKNLERVSSD  300
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B:

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          a  d  a  d  a  d  a  d  a  d  a  d
mTSP3:    TKALVTQLTLFNQILVELRDDIRDQVKEMSLIRNTIMECQVC  269
            ::  :   | :| :::: :| |||| ::::||::|| :|
hTSP4:    NRQFLGQMTQLNQLLGEVKDLLRQQVKETSFLRNTIAECQAC  261
            ::  :   | :| :::: :| |||| ::::||::|| :|
rTSP5:    APQMLRELQETNAALQDVRELLRQQVKEITFLKNTVMECDAC  71
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Fig. 4. Sequence alignment of the coiled-coil regions of TSPs. Sequences of mouse TSP1 and mouse TSP2 (A), and mouse TSP3, human TSP4 and rat TSP5/COMP (B) are aligned, with the a and d positions of the presumptive heptad repeats indicated. Identical residues are indicated by vertical lines and conservative replacements by dots.

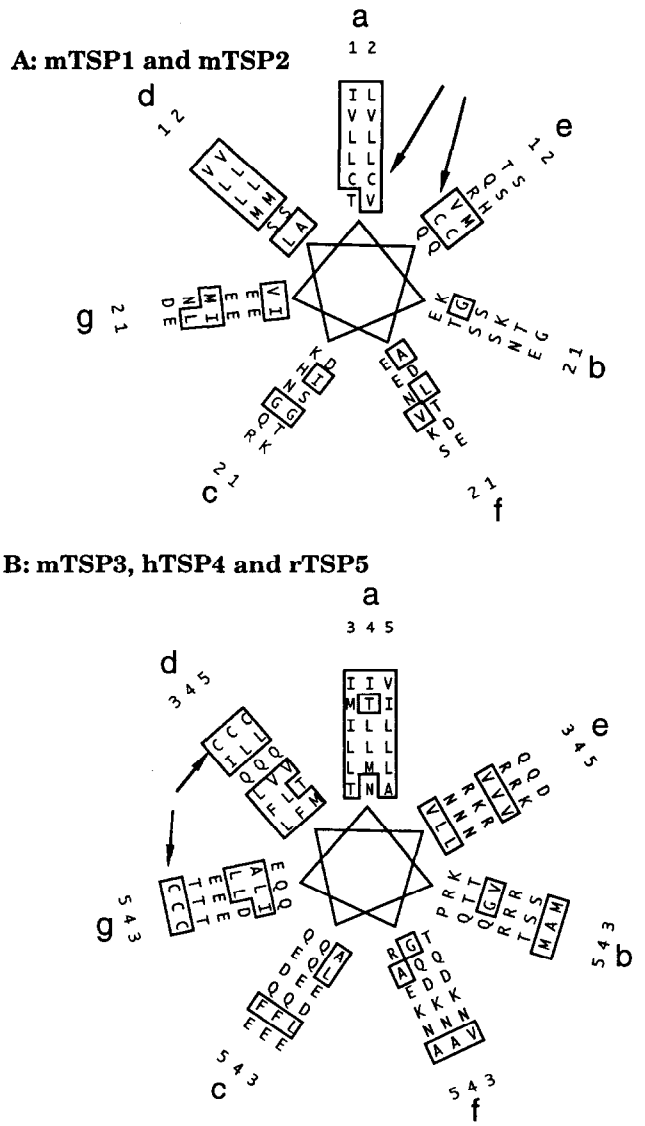

Fig. 5. Helical wheel presentation of the coiled-coil structure of one subunit of TSPs. Sequences of the presumptive coiled-coil regions of mouse TSP1 (1) and mouse TSP2 (2) (A), or mouse TSP3 (3), human TSP4 (4) and rat TSP5/COMP (5) (B) are depicted in helical wheel forms, with the heptad repeat positions indicated (a–g). Residues from different TSPs appearing at the same position of the same α-helix turn (compare to Fig. 4) are placed side by side with each other. NH_2-termini of the sequences are placed at the inside of the wheels. Apolar residues are boxed. Arrows point to the cysteines forming interchain disulfide bonds. In the coiled-coil structure, three subunits (for TSP1 and TSP2) or five subunits (for TSPs 3–5) will be close to each other with position a from one helix interacting with position d from the next one, thereby forming the hydrophobic core.

TSP3 can mediate cell adhesion, and, if it can, whether adhesion is mediated through heparan sulfate proteoglycans as for human TSP1 [21] and mouse TSP2 [8].

When comparing TSP gene products, the sequence identity increases from the NH_2-terminus to the COOH-terminus [4]. All TSP family members identified to date have a series of Ca^{2+}-binding loops formed by the type 3 repeats located next to the COOH-terminal globe. TSP1 purified from human platelets binds about 11 Ca^{2+} ions per subunit with an affinity of 20–120 mM [25,31]. CISP (the presumed bovine form of TSP2) purified from adrenocortical cells, also binds Ca^{2+} [37]. We demonstrate that recombinant mouse TSP1, TSP2, and TSP3 expressed in insect cells are all Ca^{2+}-binding proteins. The semi-quantitative method used indicates that comparable amounts of Ca^{2+} are bound by the recombinant mouse proteins, a result consistent with the high conservation of the multiple aspartate-rich Ca^{2+}-binding motifs.

Synthesis, assembly, and secretion of large multi-subunit proteins present a special challenge to cells. Given the efficiency of assembly of mouse TSPs in heterologous insect cells, there must be a strong force behind the multimerization process. The sequences around the proposed multimerization region in mouse TSP1 and TSP2 (Fig. 4A), and mouse TSP3 in comparison to human TSP4 and rat COMP/TSP5 (Fig. 4B) are shown. These alignments show a striking pattern of heptad repeats that are the building units of coiled-coil structures, with positions a and d being apolar residues and other positions polar or charged residues [10]. A drawing in a helical wheel form shows that when arranged in an α-helix, the a and d residues are mostly hydrophobic residues facing one side of the helix (Fig. 5). Hydrophobic interactions between residues a and d are suggested to be a prerequisite for coiled-coil formation [2,10]. The Cys residues involved in the interchain disulfides are located on either a (for TSPs 1 and 2) or d (TSPs 3–5) positions and the residue next to it, i.e. residue e (TSPs 1 and 2) or g (TSPs 3–5). These Cys residues are located at either the start (for TSPs 1 and 2) or the end (TSPs 3–5) of the coils. As with laminins [1], this arrangement could help to lock and strengthen the coiled-coil structure. Charged side arms are proposed to interact with each other through ionic interactions and help to determine the specificity and subunit composition [2,10]. When comparing the residues immediately next to the hydrophobic center of the coiled-coil, position g is enriched in negatively charged residues for both groups. Compared to the residues in position e of the TSPs 1 and 2, TSPs 3–5 are enriched in highly positively charged residues. These may participate in ionic interactions that favor the pentameric arrangement of TSPs 3–5.

The scenario for TSPs, then, is one in which the NH_2-terminal portions through the end of the heptad repeat regions are assembled co-translationally into trimers or pentamers, followed by sequential folding of the more COOH-terminal modules. Evidence for human TSP1 that trimerization is mediated through the coiled-coil structure that in turn can be stabilized by the interchain disulfide bonds [41] comes from studies of TSP1 truncated at residue 381, TSP381. TSP381 formed heterotrimers with endogenously produced TSP when transfected into COS cells [41]. Mutated TSP381 in which the cysteines forming interchain disulfides (Cys-270 and Cys-274) were changed to glycines were secreted from COS cells, suggesting appropriate folding by non-covalent interaction of the coiled-coil region [41]. TSP4 purified from tendon [17] or expressed in NIH 3T3 cells [29] and TSP3 expressed in mammalian 293 cells [39] are pentamers. Similar to TSP381, when Cys-245 and Cys-248 in the multimerization region of TSP3 were mutated to serines, TSP3 was secreted but migrated as a monomer on SDS-PAGE without reduction [39]. A recombinant fragment representing the homologous multimerization region of TSP5/COMP was shown to form a pentameric coiled-coil structure by crystallographic analysis [13], although the refined structure has not yet been reported. The ability of the oxidized and reduced recombinant TSP5/

COMP fragment to form pentamers is strong evidence that the heptad repeat region contains the information needed for correct multimer formation.

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