A Second, Expressed Thrombospondin Gene (Thbs2)
Exists in the Mouse Genome*

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The diverse and occasionally conflicting properties described for the extracellular, cell surface-associated protein thrombospondin (TSP) have raised the possibility that functionally distinct forms of the protein exist in the same organism. We have isolated and characterized a partial cDNA clone for mouse TSP that is clearly homologous to, but distinct from, the coding sequence for mouse TSP deduced from a mouse genomic clone (Bornstein, P., Alfi, D., Devarayalu, L., Franson, P., and Li, P. (1990) J. Biol. Chem. 265, 16691-16698). This second TSP, which we term thrombospondin 2, is the product of a separate gene (Thbs2) and is expressed in a variety of mouse tissues in a pattern that differs from that for TSP1. Based on their translated amino acid sequences, it seems likely that TSP1 and TSP2 will be found to have both common and unique properties and that the functional consequences of TSP production will reflect the ratio of the levels of these two related proteins.

Thrombospondin (TSP) is a large, trimeric, secreted glycoprotein found in the α granules of platelets and produced by a variety of cells in culture (1, 2). While there is good evidence for a role for TSP in blood coagulation (3) the function of the protein extravascularly is not well understood. There is evidence that TSP could serve as an attachment factor for some cells (Refs. 1, 4, and 5; see Ref. 6 for additional references), but the protein might also reduce focal adhesions in endothelial cells and fibroblasts (6). TSP has been reported to facilitate the growth of smooth muscle cells (7, 8) and fibroblasts (9), to inhibit endothelial cell growth (10, 11), to stimulate chemotaxis of tumor cells (12) and neutrophils (13), and to inhibit angiogenesis in the rat cornea (14). Since TSP is present at the earliest cleavage stages of the mouse zygote (15) and has a unique distribution in the developing mouse embryo (16), the protein could also play an important role in mammalian embryogenesis and development.

The diverse and sometimes contradictory functions ascribed to TSP have raised the possibility that different forms of the protein may exist in the same organism. Thus far, evidence for heterogeneity at the protein level (17) can best be explained by post-translational changes. A lower molecular weight form of hamster TSP that lacks the NH2-terminal heparin-binding domain and interchain disulfide bonds has been described as an inhibitor of angiogenesis (14, 18), but this protein is most likely derived from TSP by limited proteolysis or by alternative splicing of TSP mRNA (19). Indeed, cDNA clones for human TSP were isolated independently by three laboratories from endothelial cell and fibroblast libraries (20-23) and were found to code for the same protein sequence. Subsequently, several human genomic clones were characterized (24-26). The coding sequences within these clones were concordant with previously established cDNA sequences. These findings, together with genomic Southern analysis (20), argue for a single locus for TSP in the human genome. Thus, the TSP gene has been mapped to human chromosome 15 (14, 27) and to mouse chromosome 2 (27) by in situ hybridization and, as well, to human chromosome 15 by Southern blot analysis of somatic cell hybrids (14, 26).

Recently, one of our laboratories reported the isolation and characterization of a mouse genomic TSP clone (28). The exon/intron structure and sequence of the first nine exons of the mouse gene were clearly similar to the previously described human gene; thus there was little doubt that homologous genes were being compared. However, when the coding sequences in the mouse gene were compared with the sequence of a mouse cDNA clone, isolated by screening an NIH 3T3 cell library with a full-length coding human cDNA clone, it became apparent that the mouse cDNA clone represented a product of a second mouse TSP gene which was related to, but distinct from, previously characterized human or mouse thrombospondins. We propose the name thrombospondin 2 for the translation product of this gene (Thbs2) and provide evidence in this paper for the existence and expression of mouse Thbs2.

EXPERIMENTAL PROCEDURES

Isolation of a TSP2 cDNA Clone—Poly(A)* RNA was isolated from quiescent NIH 3T3 cells treated for 4 h with 10% serum in the presence of 10 μg/ml cycloheximide. A cDNA library was constructed in the vector pBluescript II using 10 μg of poly(A)* RNA and modifications of the Gubler and Hoffman procedure (29). Ligation and packaging of the cDNA yielded a library of 3 × 108 independent recombinants. Approximately 2 × 106 plaques of this cDNA library were plated in Escherichia coli strain Y1000; duplicate nitrocellulose filters were lifted and hybridized with a 32P random primer-labeled 4.5-kilobase human TSP cDNA (30). Hybridization was performed in 30% formamide containing 5 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 3 × Denhardt's (31), 0.25% SDS, and 200 μg/ml salmon sperm DNA. Filters were washed in 2 × SSC at 50 °C and subjected to autoradiography. DNA was prepared from hybridizing plaques by using the large-scale liquid lysate method (31) and digested with EcoRI; the resulting fragments were resolved by agarose gel electrophoresis. The largest cDNA insert of approximately 2.4 kilobases was isolated by electroelution and subcloned into the plasmid pGEM-7Zf (Promega).

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1 The abbreviations used are: TSP, thrombospondin; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction.

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2 P. Bornstein, K. O'Rourke, K. Wikstrom, F. W. Wolf, R. Katz, P. Li, and V. M. Dixit, unpublished observations.
DNA Sequence Analysis—Plasmid DNA, prepared by cesium chloride banding, was sequenced on both strands by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.) and synthetic oligonucleotide primers. In some cases, additional DNA sequence information was obtained by using Applied Biosystems Model 373A DNA sequencer and a Taq dye primer cycle sequencing kit. DNA and amino acid sequences were analyzed using a Genetics Computer Group sequence analysis software package (version 6.2).

Polymerase Chain Reaction—The 5' end of TSP2 cDNA was amplified using the RACE protocol (anchoring PCR) (32). An antisense primer complementary to nucleotides 1237–1362 was annealed to 5 μg of preadipocyte 3T3L1 cell RNA and extended with reverse transcriptase. Excess primer was removed with a Centricon 100 spin filter (Amicon). The cDNAs were tailing using dATP and terminal deoxynucleotidyl transferase (GIBCO/Bethesda Research Laboratories) and amplified with a nested antisense primer complementary to nucleotides 460–479, a (dT)17-adapter primer, plus the same adapter primer lacking the poly(dT) sequence. Both adapter and gene-specific primers contained internal EcoRI sites. 35 cycles of amplification in 50 mM KCl, 2.5 mM MgCl2, 10 mM Tris, pH 8.3, 0.1% gelatin were performed using a step cycle file in a Perkin-Elmer Cetus Instruments thermal cycler with denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The products of the PCR reaction were digested with EcoRI and cloned into the EcoRI site of the phagemid vector pBSM13* for further analysis.

Southern Blot Analyses—Mouse genomic DNA was digested with several restriction endonucleases (see Fig. 2) under conditions recommended by the manufacturer. Digests (15 μg) were fractionated on agarose gels and transferred to nylon membranes. The membranes were baked, hybridized at 65°C with [32P]-labeled PCR-generated probes specific for TSP1 and TSP2, and washed to a stringency of 0.1 × SSC, 0.1% SDS at 57°C. The TSP1 probe spanned nucleotides 490–823 (28), and the TSP2 probe spanned nucleotides 485–812. After exposure to the first probe, the membranes were boiled in 0.1 × SSC, 0.5% SDS prior to hybridization with the second probe.

Analysis of TSP1 and TSP2 mRNA Levels in Mouse Tissues—RNA was extracted from various tissues of 4-week-old mice using modifications of the method of Chirgwin et al. (33), and RNA concentrations were determined by absorbance at 260 nm. The integrity of each RNA preparation was evaluated by ethidium bromide staining of ribosome bands in agarose gels. mRNA concentrations for TSP1 and TSP2 were determined by an RNase protection analysis (34, 35) using specific antisense riboprobes. A mouse genomic fragment that protected parts of exons 4 and 5 (28, 36) was used to assay for TSP1 mRNA, and a Gadl/Petl fragment (nucleotides 834–1348) of TSP2 cDNA was used to assay for TSP2 mRNA.

RESULTS AND DISCUSSION

We originally wished to isolate a mouse TSP cDNA clone in order to assess changes in levels of TSP mRNA during mouse embryo development (15, 16). Accordingly, an NIH 3T3 cell cDNA library was screened with a full-length human TSP cDNA. When the sequence of a cross-hybridizing clone was analyzed, it became apparent that the cDNA was the product of a gene that was related to but different from the gene coding for mouse TSP1. Since the 5' end of the sequence (which we identify as TSP2) was lacking from the clone isolated by a library screen, we extended the sequence to its 5' terminus (an additional 430 nucleotides) using the anchored PCR technique (32). The newly generated 5' sequence for TSP2 was judged to be correct since (a) an upstream primer made from the new sequence, in conjunction with a downstream primer, directed the synthesis of the predicted fragment by PCR of mouse RNA, (b) the 5' sequence coded for a signal peptide in the same reading frame as the remainder of TSP2 (see Fig. 1), and (c) the length of the newly generated 5' sequence was similar to that of TSP1. However, primer extension and RNase protection analysis will be required to determine the precise transcription start site for TSP2. By Northern analysis, the size of TSP2 mRNA is essentially the same as that of TSP1, i.e., about 6 kilobases. ¹

The sequence of 2837 nucleotides in TSP2 cDNA has been deposited in GenBank. ² The first 218 nucleotides are untranslated and contain an ATG (nucleotides 15–17) followed by two in-frame stop codons. The translation of nucleotides 219–2837 and the alignment of this amino acid sequence with that of TSP1, from mouse and human, is shown in Fig. 1. It is apparent, when mouse TSP1 and TSP2 are compared, that there is less conservation in sequence in the first 380 amino acids, which code for the signal peptide, heparin-binding domain, and procollagen homology, than in the subsequent 493 amino acids. Thus, the heparin-binding domain and the procollagen homology (amino acids 15–230 and 320–374, respectively, in TSP2) are highly conserved between human and mouse TSP1 (87 and 96% identity, respectively) but are poorly conserved between mouse TSP1 and TSP2 (38 and 42% identity, respectively). Nevertheless, all 10 cysteine residues are retained in identical positions in the procollagen homology in TSP1 and TSP2. Immediately downstream of the procollagen homology are three type I repeats of 52–57 amino acids (21) that resemble the heparin-binding domains of extracellular proteins, malarial TSP-related anonymous protein, properdin, and several complement components (see Refs. 26, 28, and 37 for a recent discussion and additional references). In both mouse and human TSP1 each repeat is encoded by a separate exon (26, 28). It is apparent from Fig. 1 that the degree of sequence identity of these repeats in TSP1 and TSP2 is far greater (65–75%) than in the N-terminal regions of the proteins. In comparison, these sequences are almost completely conserved (96–100% identity) between mouse and human TSP1.

Although we do not have coding sequence for mouse TSP1 beyond amino acid 548, a comparison of the remaining sequence in TSP2 with the corresponding sequence of human TSP1 (Fig. 1) suggests that the level of sequence identity between TSP1 and TSP2 is retained (72% identity between human TSPl and mouse TSP2). This pattern of a “gradient” of sequence conservation (less at the N-terminal regions of the proteins. In comparison, these sequences are almost completely conserved (96–100% identity) between mouse and human TSP1.

To ascertain whether TSP2 was encoded by a distinct genetic locus, restriction enzyme digests of mouse genomic DNA were fractionated on agarose gels, transferred to nylon membranes, and hybridized sequentially with probes that were specific for TSP1 and TSP2 (see “Experimental Procedures”). The results of these Southern blot analyses are shown in Fig. 2. The sizes of the restriction fragments detected with the TSP1 probe correspond to the restriction map of mouse TSP1 (28). It is apparent from the data in Fig. 2 that TSP1 and TSP2 are encoded by separate genes, probably present in single copies in the haploid mouse genome. The conclusion that a single locus for TSP exists in human and mouse (27)
resulted from the use of a probe that was specific for TSP1. The unlikely possibility that TSP2 represents a pseudogene is largely excluded by the open reading frame for the 883 amino acids shown in Fig. 1. Furthermore, we have recently isolated the Thbs2 gene and mapped its exon/intron boundaries, thus confirming the existence of the Thbs2 locus in the mouse.

That the Thbs2 gene is transcribed in the mouse was indicated by the ability to isolate the TSP2 cDNA clone and to amplify TSP2 sequences from 3T3 cell RNA. The pattern of expression of TSP2, in comparison with TSP1, in tissues of a 4-week-old mouse was determined by an RNase protection assay (Fig. 3). Since the two RNA probes are not very different in size and are of roughly the same specific activity, the intensity of the protected bands serves as an indication of mRNA concentration. It can be seen that both genes are widely expressed in mouse tissues but that expression is particularly prominent in connective tissues such as bone, tail, and skin. Relative to bone, expression of TSP1 was higher in lung and kidney than TSP2. Both genes were relatively poorly expressed in liver and thymus. The results presented in Fig. 3 are representative of analyses performed on three 4-week-old mice with excellent agreement among the determinations. It is likely, however, that the expression of both genes is developmentally regulated since Northern analyses indicate...
significant differences in mRNA levels in tissues of mouse embryos of different ages. We have also detected very substantial differences in mRNA levels for both TSP1 and TSP2 among different 3T3 cell lines. Our results provide strong evidence for the existence of at least two related, but distinct, thrombospondins in mouse tissues. This phenomenon is not limited to rodents since evidence has recently been obtained for the expression of TSP2 in human cells, and these studies, conducted in parallel with our own, have been mutually supportive. Based on the amino acid sequences established thus far for TSP1 and TSP2, the two thrombospondins are likely to have both common and distinct properties. It will therefore be necessary to reassess a number of studies previously conducted on both the distribution and function of this protein. For example, it is likely that antibodies specific for the heparin-binding domain of TSP1 will not cross-react with TSP2, whereas antibodies formed to epitopes located in the stalk or carboxy-terminal globular domain (1) might fail to distinguish between the two proteins. It is not difficult to imagine how such antigenic differences, coupled with differences in the level and specific cellular synthesis of the two proteins, could have complicated previous studies of the immunolocalization and level of production of TSP. It is also intriguing to consider the possibility that the two proteins might differ in some critical function. It seems possible, based on the conceptual translation product of TSP2, that this protein binds poorly or not at all to heparin, since the sequence of the heparin-binding domain seems to differ so greatly between TSP1 and TSP2. Thus, the ability of TSP2 to interact with heparan sulfate proteoglycans on the cell surface (38) may differ, at least quantitatively, from that of TSP1. Modulation of the level of the two thrombospondins could, therefore, alter the functional consequences of the interaction of the proteins with cell surfaces.

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