

Thrombospondin-4, an Extracellular Matrix Protein Expressed in the Developing and Adult Nervous System Promotes Neurite Outgrowth

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Abstract. Extracellular matrix (ECM) molecules are involved in multiple aspects of cell-to-cell signaling during development and in the adult. In nervous system development, specific recognition processes, e.g., during axonal pathfinding and synaptogenesis involve modulation and signaling by ECM components. Much less is known about their presence and possible roles in the adult nervous system. We now report that thrombospondin-4 (TSP-4), a recently discovered member of the TSP gene family is expressed by neurons, promotes neurite outgrowth, and accumulates at the neuromuscular junction and at certain synapse-rich structures in the adult.

To search for muscle genes that may be involved in neuromuscular signaling, we isolated cDNAs induced in adult skeletal muscle by denervation. One of these cDNAs coded for the rat homologue of TSP-4. In skeletal muscle, it was expressed by muscle interstitial cells. The transcript was further detected in heart and in the

developing and adult nervous system, where it was expressed by a wide range of neurons. An antiserum to the unique carboxyl-terminal end of the protein allowed to specifically detect TSP-4 in transfected cells in vitro and on cryostat sections in situ. TSP-4 associated with ECM structures in vitro and in vivo. In the adult, it accumulated at the neuromuscular junction and at synapse-rich structures in the cerebellum and retina. To analyze possible activities of TSP-4 towards neurons, we carried out coculture experiments with stably transfected COS cells and motor, sensory, or retina neurons. These experiments revealed that TSP-4 was a preferred substrate for these neurons, and promoted neurite outgrowth. The results establish TSP-4 as a neuronal ECM protein associated with certain synapse-rich structures in the adult. Its activity towards embryonic neurons in vitro and its distribution in vivo suggest that it may be involved in local signaling in the developing and adult nervous system.

COMPONENTS of the (ECM)¹ play major roles in cell-to-cell communication during development and in the adult. These are mediated by direct and indirect signaling mechanisms. Binding to specific cellular integrin receptors by ECM components directly activates intracellular signal transduction pathways. Potentiation of growth factor-mediated signaling through stabilization and/or direct coactivation mechanisms produces local modulation of growth factor activity. Finally, synergistic interactions between integrin- and peptide growth factor-initiated signal transduction pathways are likely to greatly enhance the efficacy and specificity of local cell interactions (Hynes, 1992; Adams and Watt, 1993).

In the nervous system, local interactions mediated by

ECM components are involved in neurite outgrowth, axonal pathfinding, and synapse formation (Sanes, 1989; Venstrom and Reichardt, 1993; Letourneau et al., 1994). This has been particularly well documented for the development and regeneration of neuromuscular connections. In this system, synaptic basal lamina contains information sufficient to direct the differentiation of the pre- and postsynaptic apparatus during reinnervation (Hall and Sanes, 1993). This is reflected by the existence of ECM components that either accumulate or are excluded from the synaptic cleft at the neuromuscular junction (Sanes, 1989; Hall and Sanes, 1993). Of particular significance among the synaptic components are agrin, ARIA, and s-laminin (McMahan et al., 1992; Hall and Sanes, 1993). Agrin is an ECM molecule that induces clustering of postsynaptic acetylcholine receptors and is therefore thought to play a major role in synapse formation (McMahan et al., 1992). Some of the actions of agrin may be mediated by dystroglycan, a proteoglycan that interacts with both, certain ECM components like laminin and agrin, and with a specific transmembrane complex thought to provide

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; TSP, thrombospondin; TSP-4-COS, thrombospondin-expressing COS.

a physical link between the ECM and the cortical cytoskeleton (see e.g., Sealock and Froehner, 1994 for a review). While additional ECM-mediated interactions are likely to be involved, it is already clear that local interactions involving specific ECM components play a crucial role in synapse formation and maturation.

Thrombospondins are a family of highly related ECM glycoproteins with well documented roles in cell signaling (Bornstein, 1992; Adams and Lawler, 1993; Lawler et al., 1993b). Thrombospondin was originally discovered as a prominent secretory component of platelet α -granules involved in plasmin activation and platelet aggregation. This protein, which is now designated thrombospondin-1 (TSP-1), remains the best characterized of the five presently known TSPs. It consists of an heparin-binding amino-terminal domain, a linker with homology to procollagen, three TSP-type-1 (properdin) repeats, three TSP-type-2 (EGF) repeats, seven TSP-type-3 (calcium-binding) repeats, and a cell-binding carboxyl-terminal domain. Multiple functions have been assigned to the various domains. TSP-1 affects a wide range of cellular activities, including cell migration, adhesion, spreading, and neurite outgrowth (Taraboletti et al., 1987; O'Shea et al., 1990, 1991; Neugebauer et al., 1991; Osterhout et al., 1992; Adams and Lawler, 1993; Venstrom and Reichardt, 1993). It is involved in angiogenesis and metastasis (Tolsma et al., 1993; Nicosia and Tuszynski, 1994). Unique to TSP, and of particular interest to cell signaling is the fact that it binds and modulates extracellular proteases and TGF β (Lawler et al., 1986; Schultz-Cherry et al., 1993). TSP-1 is expressed by many cell and tissue types during development, and it is a serum-responsive gene in fibroblasts. TSP-2 is highly related to TSP-1, but it is encoded by a separate gene, and its expression is not regulated by serum (Bornstein, 1992; Laherty et al., 1992). TSP-3 (Vos et al., 1992; Qabar et al., 1994), TSP-4 (Lawler et al., 1993a), and the cartilage protein COMP (Oldberg et al., 1992) are related to TSP-1, but have distinct amino-terminal domains, lack the procollagen homology linker and the TSP-type-1 repeats, and display restricted expression patterns. Several important activities of TSP-1 have been assigned to the TSP-type-1 repeats. These include its angiogenic activity, binding and activation of TGF β , several interactions with ECM components, and a neurite promoting activity (Adams and Lawler, 1993; Tolsma et al., 1993; Schultz-Cherry et al., 1994; Stewart, B. D., A. N. Qabar, M. Hartsch, V. M. Dixit, and K. S. O'Shea. 1994. *Mol. Biol. Cell.* 5:176A). In addition, high-affinity binding of TSP-1 and -2 to proteoglycans involves the unique NH₂-terminal heparin binding domain. Little is presently known about the functions of the other members of the TSP gene family, but structural considerations clearly indicate that they cannot be extrapolated from those of TSP-1 and -2.

Thrombospondin(s) have been detected immunologically in the developing and adult nervous system (Corless et al., 1992; Hoffman et al., 1994). However, the high degree of relatedness among the five known members of the TSP gene family has been a hindrance in determining which members of this family are specifically associated with neural systems. Therefore, while the neurite-promoting activity of TSP-1 in vitro is well documented, the assignment of any potential role of TSPs in neural functions

depends on the identification and functional characterization of the TSPs expressed by neurons and glia.

We have set out to identify muscle components involved in signaling between muscle and nerve at the neuromuscular junction. To this end, we screened a subtracted cDNA library enriched in genes induced in adult skeletal muscle 7 d after denervation. At this time, denervated muscle has become competent to form synapses with incoming nerves, and certain muscle genes expressed during synapse development are reinduced (Hall and Sanes, 1993). We report that one cDNA isolated in our screen codes for the rat homologue of TSP-4. Its unique carboxyl-terminal sequence allowed us to generate a specific antiserum to map its distribution without interference from previously known members of the TSP family. In denervated muscle, TSP-4 is strongly induced in muscle interstitial cells, presumably fibroblasts. It is detected in the developing optic nerve and cerebellar cortex. In the adult nervous system, it is expressed by certain neurons, and accumulates at the neuromuscular junction and at certain synapse-rich layers. Outside the nervous system, TSP-4 is expressed in skeletal muscle and in the heart (Lawler et al., 1993a). Experiments with stably transfected cells indicate that TSP-4 binds to cell surfaces, can be incorporated into extracellular matrix, and has potent adhesion and neurite promoting activity towards motor, sensory, cerebellar, and retina neurons. The distribution of TSP-4 expression and immunoreactivity in the nervous system suggests that it may be a major neural TSP in the adult. Its neurite promoting activity and association with certain synapse-rich layers suggest that it may be involved in signaling in the developing and adult nervous system.

Materials and Methods

Reagents

A partial clone coding for rat TSP-4 was isolated from a subtracted plasmid cDNA library enriched in transcripts induced in adult diaphragm muscle 7 d after denervation. The construction of the library and the screening procedure were as described (Arber et al., 1994). Subsequently, several additional clones, including a 3.1-kb clone coding for the entire amino acid sequence of TSP-4 were isolated through rescreening of the same library. A 2.2-kb cDNA clone coding for *Xenopus laevis* TSP-4 was a kind gift of J. Lawler (Brigham and Women's Hospital, Boston, MA). The rabbit antibody against the carboxyl-terminal peptide CQEFQIQTFDRLDN was produced by coupling the peptide to keyhole limpet hemocyanin (Sigma Chem. Co., St. Louis, MO) via an amino-terminal Cys. Further antibodies were from the following sources: tenascin (rat monoclonal antibody; kind gift of R. Chiquet-Ehrismann, Friedrich Miescher Institute, Basel), fibronectin (monoclonal antibody; Sigma Chem. Co.), and chick GAP-43 (antiserum to carboxyl-terminal peptide [Aigner and Caroni, 1993]). Rhodamine- α -bungarotoxin, rhodamine-phalloidin, and biotin-, rhodamine-, and lucifer yellow-conjugated secondary reagents were from Molecular Probes, Inc. (Eugene, OR). All cell lines (mouse 3T3 fibroblasts; mouse C2C12 myoblasts; rat L6 myoblasts; monkey kidney epithelial cells COS-1) were from American Type Cell Culture Collection (Rockville, MD).

Nucleic Acid Hybridization

RNA isolation and Northern blot analysis with digoxigenin-labeled riboprobes (reagents from Boehringer Mannheim Corp., Indianapolis, IN) were carried out as described in a previous study (Arber et al., 1994). For Northern blots, equal amounts of total RNA were loaded on a formaldehyde gel, as verified by staining with methylene blue. Hybridization was performed at high stringency (68°C), as described by the manufacturer. In

situ hybridization with digoxigenin-labeled cRNA probes was carried out according to a published protocol (Schaeren-Wiemers and Gerfin-Moser, 1993). Briefly, fresh tissues were quickly frozen in mounting medium, 12- μ m cryostat sections were collected, and then fixed with 4% paraformaldehyde. High-stringency hybridization was carried out over night at 72°C. After extensive washing, sections were incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer Mannheim), and the color reaction was allowed to proceed for 1 d. In control experiments corresponding sense probes yielded no detectable hybridization pattern.

Tissue Culture Experiments

3T3 and COS-1 cells were cultured in DMEM supplemented with 10% FCS. For transfections, a CMV promoter-based eukaryotic expression vector (pcDNA3; Invitrogen, San Diego, CA) containing a neomycin resistance gene for selection of stable clones was used. Transfections were carried out with the lipofectamine reagent (GIBCO BRL, Gaithersburg, MD), as recommended by the manufacturer. The COS-TSP-4 clone was selected for its particularly efficient expression of TSP-4, as determined by immunocytochemistry. Primary cultures of chick motoneurons, dorsal root ganglia and retina neurons, and mouse cerebellar neurons were established as described (Doherty et al., 1990; Neugebauer et al., 1991; Aigner and Caroni, 1993; Camu et al., 1993). Motoneurons were cultured in the presence of 2% horse serum, 2 ng/ml CNTF, and 10 ng/ml bFGF (Camu et al., 1993). The primary neurons were plated onto 1-d cultures of control or TSP-4-expressing COS cells. In the absence of serum, COS cells preplated at ~30% confluency onto uncoated glass or tissue culture plastic usually formed small aggregates, allowing for the analysis of substrate preference by the primary neurons. Confluent monolayers of COS or COS-TSP-4 cells were produced by preplating the cells at 80–90% confluency in the presence of serum. Cocultures were analyzed after 24 h. Neurite lengths were determined after labeling of the neuritic processes for the neural cortical cytoskeleton-associated protein GAP-43, as described (Aigner and Caroni, 1993).

Immunocytochemistry

Cells were fixed for 30 min with prewarmed (37°C) 4% paraformaldehyde in phosphate buffer (0.1 M sodium phosphate, pH 7, 50 mM sucrose, 0.4 mM CaCl₂). For GAP-43 stainings, antibody incubations were performed at room temperature in PBS with 0.1% BSA, 1% FCS, 50 mM glycine, 0.02% NaN₃, and 0.2% saponin, as described (Aigner and Caroni, 1993). The antiserum to TSP-4 was used at a dilution of 1:500. For TSP-4 stainings, cell lines were fixed as described above, permeabilized for 5 min in PBS with 0.5% Triton-X100, and all further incubations were carried out in the absence of detergent. Fresh tissues were mounted, frozen, and then 12- μ m cryostat sections were cut. These were immediately placed at 4°C, preincubated for 30 min at 4°C in PBS with 5% BSA, and then incubated in the same medium with primary antibody for 16–18h at 4°C. Subsequent steps were performed at room temperature in PBS with 5% BSA. For TSP-4 immunohistochemistry, paraformaldehyde-fixed tissues yielded similar, but less reproducible results. In addition, overall signal intensity was significantly reduced, the relative intensity of neurite-associated signal was slightly increased, and cell body-associated labeling was selectively reduced. The use of detergents led to an almost complete loss of signal. Double-labeling immunocytochemistry was performed with biotinylated second antibodies, followed by lucifer yellow-conjugated streptavidin for the first channel, and rhodamine-coupled phalloidin, - α -bungarotoxin, or second antibodies for the second epifluorescence channel. Reagent concentrations were as described (Aigner and Caroni, 1993).

Results

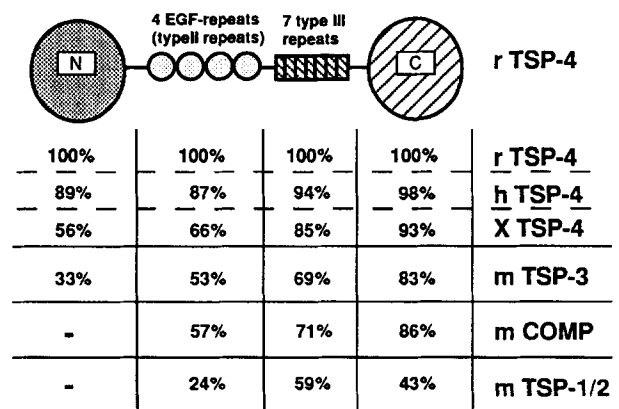
Rat TSP-4 Is Expressed in Striated Muscle and in the Nervous System, and Is Induced in Skeletal Muscle by Denervation

To identify muscle proteins that may be involved in activity-sensitive signaling and/or synapse formation at the neuromuscular junction, we screened a subtracted cDNA library enriched in transcripts induced in adult rat diaphragm

muscle by denervation. One clone of interest coded for a member of the TSP gene family. Sequencing of full-length cDNA clones indicated that it was highly homologous to TSP-4 (Lawler et al., 1993a) (Fig. 1a). A similar close relation to TSP-4 was obtained when sequences were compared at the nucleotide level. RNA blots probed with either rat or a 2.2-kb *Xenopus laevis* TSP-4 cDNA (Lawler et al., 1993a) yielded identical results. The size and tissue distribution of all RNA species that hybridized with either of the probes were indistinguishable (data not shown). Based on these criteria, we concluded that we had isolated the rat homologue of *Xenopus* and human TSP-4.

The expression of TSP-4 is restricted to a small number of tissues in the adult rat (Fig. 2). In skeletal muscle, where it is highest, expression declines at the end of the first post-natal week, and is strongly induced by denervation. Significant expression levels were further detected in heart and brain, and very weak signals were detected in skin. No TSP-4 transcripts were detected in a variety of tissues, including lung, liver, and placenta. Since lung has the highest contents of TSP-3 transcripts, and since most negative tissues express significant amounts of TSP-1 and -2, the data indicate that these hybridization conditions are specific for TSP-4. Furthermore, the indistinguishable tissue distribution of the three hybridizing species detected on the North-

a



b

m TSP-1 MVFFSDMKYECRDS.
 m TSP-2 MVYFSDLKYECRDA.
 m TSP-3 DFEFFRRQLLQGRV.
 r COMP TIPEDYERHRLRRA.
 r TSP-4 **FQEFQIQTFDRLDN**

Figure 1. Schematic representation of structural domain homologies between rat (*r*) TSP-4, its human (*h*) and *Xenopus laevis* (*X*) homologues, and the other members of the TSP gene family (mouse sequences; m). (a) Degree of identity at the amino acid level. TSP-4 is most homologous to TSP-3. When compared to TSP-1 and -2, TSP-4, -3, and COMP lack the type-I repeats, have four type-II repeats instead of three, and lack any homology in the amino-terminal domain. (b) Carboxyl-terminal sequences of TSPs. Note unique sequence of TSP-4. The boxed residues correspond to the synthetic peptide that was used for antibody production. These sequence data are available from EMBL/Genbank/DDBJ under accession number X8996.

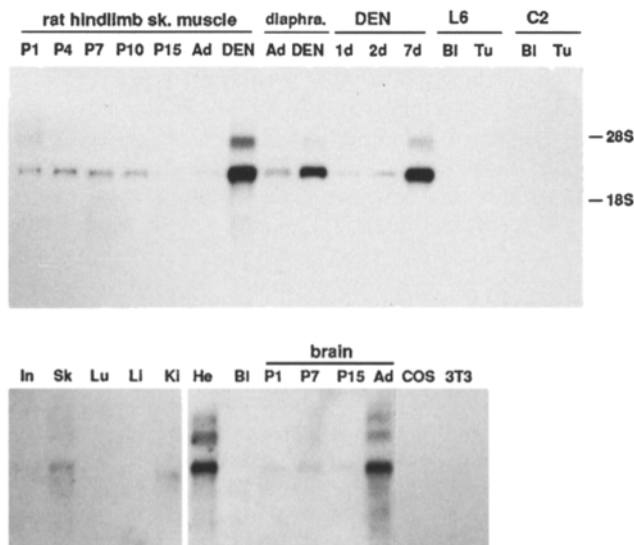


Figure 2. Distribution of TSP-4 mRNA in rat tissues and in selected cell lines. (*Top*) TSP-4 mRNA in skeletal muscle and myogenic cell lines. In gluteus muscle (*rat hindlimb*), transcript contents decreased around P10 and were strongly upregulated 8 d after denervation. A similar upregulation 8 d after denervation was detected in diaphragm muscle (*diaphra.*). A time course of the upregulation process is shown for the gastrocnemius muscle (*DEN*, days after denervation). Note that low levels of TSP-4 mRNA were also detectable in adult innervated skeletal muscle. No transcript was detected in the myogenic cell lines L6 (*rat*) and C2 (C2C12, mouse) at the myoblast (*Bl*) or myotube (*TU*, 4 d after induction of differentiation) stage. (*Bottom*) transcript contents in adult intestine (*In*), skin (*Sk*), lung (*Lu*), liver (*Li*), kidney (*Ki*), heart (*He*), blood (*Bl*), and brain (*P1*, *P7*, *P15*, adult). Cell lines: 3T3 (mouse fibroblasts), COS (monkey kidney epithelial cells). Note that in addition to skeletal muscle, only heart and brain contained substantial amounts of transcript. The hybridizing species in kidney has a smaller size and may not correspond to TSP-4 mRNA. Equal amounts of total RNA were loaded for all samples. Exposure times: *top*, 5 min; *bottom*, 30 min.

ern blots suggests that the two larger RNAs are derived from the TSP-4 gene. They may represent processing intermediates of TSP-4 mRNA. In contrast, the weakly hybridizing species detected in kidney appeared to be smaller, and its identity is not clear. As mentioned above, the same hybridization pattern was detected with a probe derived from *Xenopus* TSP-4 cDNA, indicating that the data of Fig. 2 do reflect the distribution of rat TSP-4 mRNA.

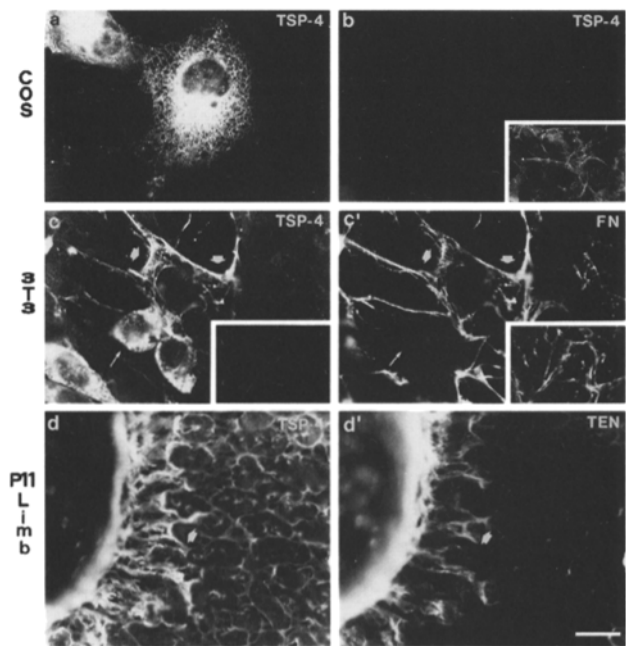
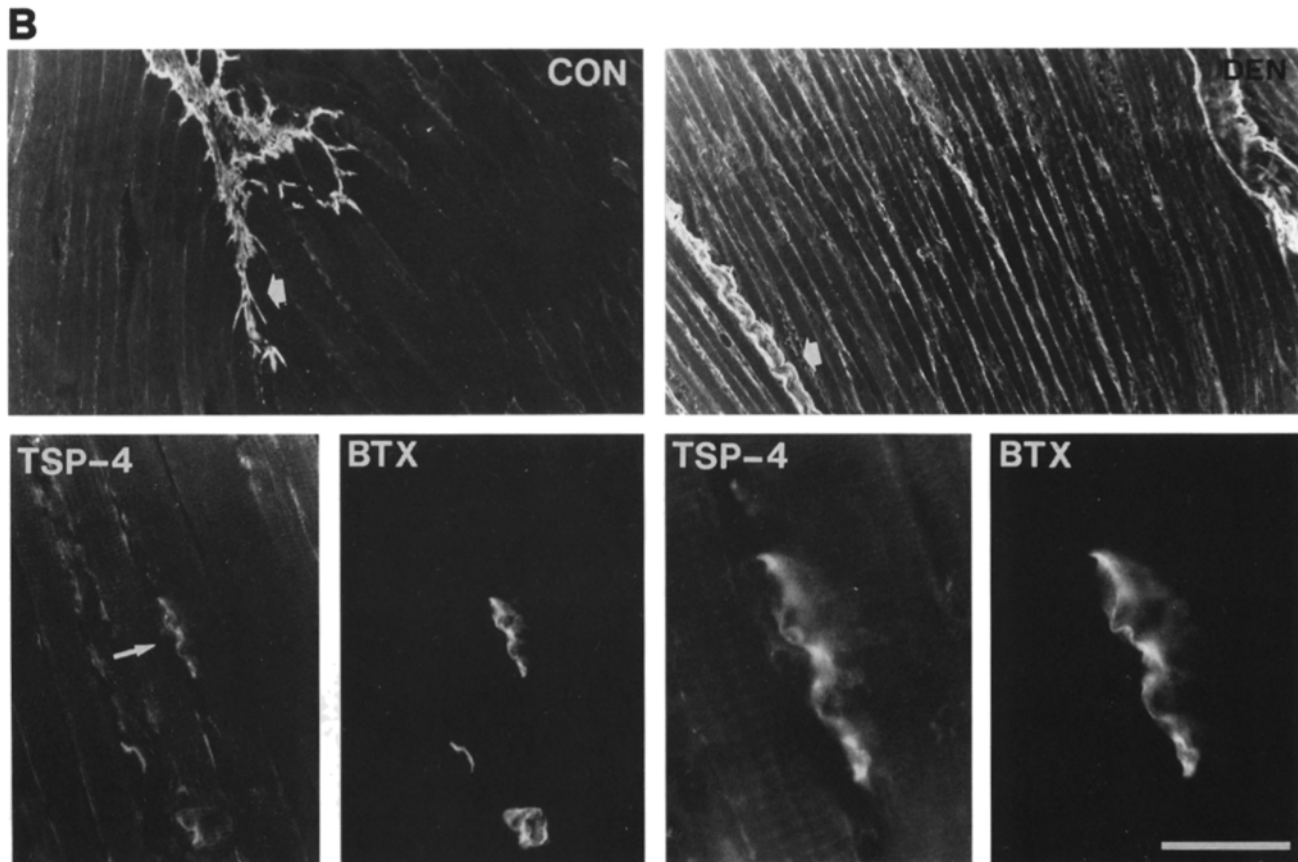
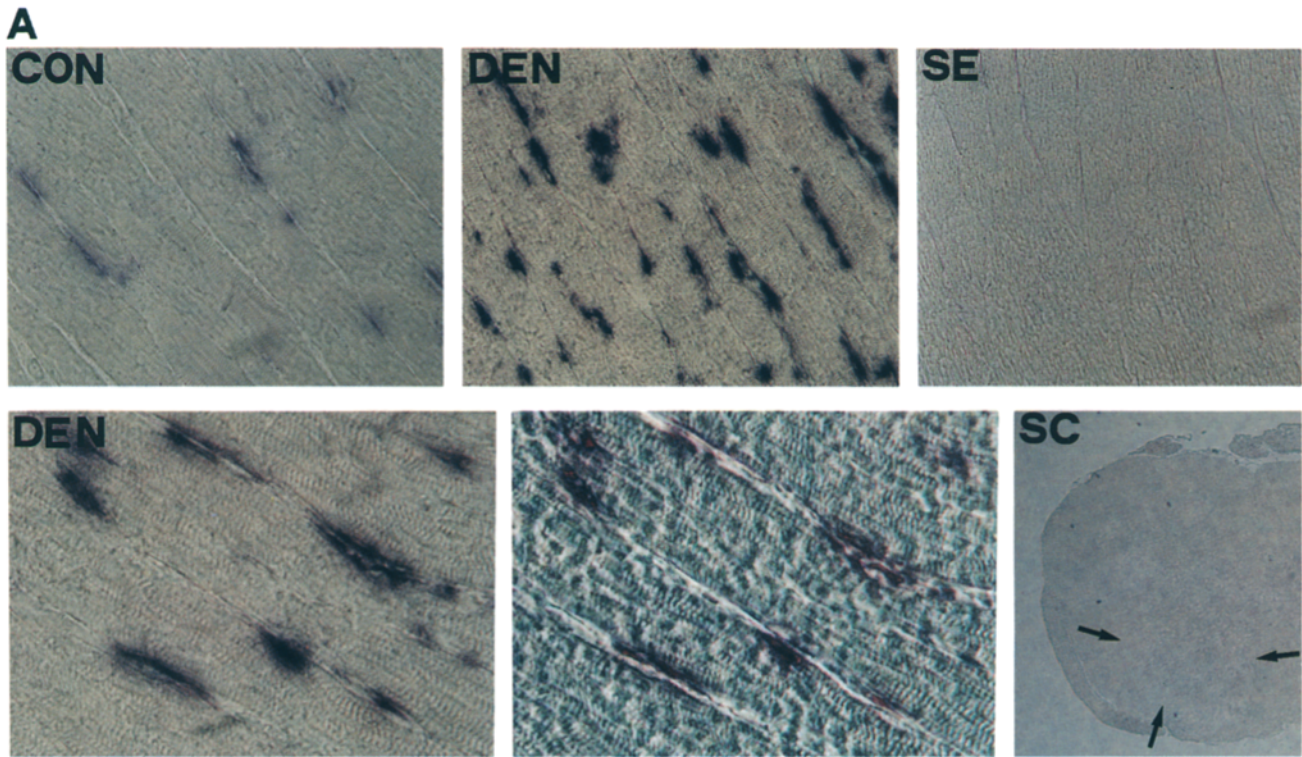


Figure 3. Detection of TSP-4 with an antiserum to its unique carboxyl-terminal sequence, and incorporation of the protein into ECM in vitro and in vivo. (*a* and *b*): Upon transfection with a TSP-4 expression construct (*a*), COS cells displayed prominent ER and Golgi labeling characteristic of a secretory protein. COS cells transfected with a control construct (*b*) were not labeled with the anti-TSP-4 antiserum (the inset in *b* shows the corresponding double-labeling for f-actin (rhodamine-phalloidin), to visualize the position of the COS cells). (*c* and *c'*) When TSP-4 was expressed in 3T3 fibroblasts it was incorporated into ECM, as demonstrated by its codistribution with fibronectin (*c'*) in the double-labeling experiment. The inserts show corresponding double-labeling experiments of an insert-free plasmid transfection; no endogenous antigen was detected by the TSP-4 antiserum in this fibroblastic cell line. Note that in these cells TSP-4 localized in a matrix- (*large arrows*) and cell-associated (*small arrow*) pattern. (*d* and *d'*) Association of TSP-4 with ECM in vivo. Double-labeling of P11 mouse hindlimb for TSP-4 (*d*) and the ECM protein tenascin (*d'*). The section includes the interface between bone and muscle, where both antigens colocalized (*arrow*). At this early postnatal time, substantial amounts of TSP-4 were also detected in the extracellular space around muscle fibers, where tenascin immunoreactivity was essentially absent. Bar: (*a-c*) 16 μm ; (*d*) 30 μm .

Figure 4. In skeletal muscle, TSP-4 is expressed by interstitial cells and accumulates at the neuromuscular junction. (*A*) Detection of TSP-4 mRNA in control (*CON*) and denervated (*DEN*; 8 d) adult mouse gastrocnemius muscle, and absence of a corresponding signal in lumbar spinal motoneurons (*SC*). In situ hybridization (*ISH*) with digoxigenin-alkaline phosphatase method (all samples processed in the same way). In a control experiment, hybridization of control muscle with corresponding sense probe (*SE*) yielded no signal. Note occasional labeled cells in control muscle, and large numbers of labeled cells in denervated muscle. The second row shows TSP-4 *ISH* of denervated muscle at higher magnification (*left*, bright field; *center*, corresponding phase contrast optics). The distribution of TSP-4 expressing cells is characteristic of interstitial cells, and no label was detected in skeletal muscle fibers. The photograph to the right (*SC*) shows TSP-4 *ISH* of corresponding lumbar spinal cord (cross-section; left half shown in the figure; arrows indicate the outer reach of the ventral horn, where spinal motoneurons are located). (*B*) Detection of TSP-4 on cryostat sections of adult mouse gastrocnemius muscle (*CON*), and of corresponding muscle 8 d after denervation (*DEN*). Note that most antigen in the control muscle accumulated in the connective tissue rich perimysium (*arrow*), whereas a strong increase in TSP-4 immunoreactivity was detected around muscle fibers after denervation. The weak labeling between muscle fibers in the control muscle reflects low levels of TSP-4, since in parallel experiments TSP-4-devoid tissues such as lung and liver were not labeled by the anti-TSP-4 antibody (data not shown). (*Bottom*) Double-



labeling of adult innervated mouse gastrocnemius muscle for TSP-4 and α -bungarotoxin (*BTX*). The toxin specifically binds to nicotinic acetylcholine receptors on muscle fibers, thus visualizing neuromuscular junctions. The panels to the right show a higher magnification of the neuromuscular junction indicated by the arrow (*left panel*). Note accumulation of TSP-4 at the innervated neuromuscular junction. In a control experiment, the TSP-4 signal could be abolished by preadsorption of the antiserum with excess synthetic peptide (data not shown). Bar: (*A, top*) 125 μm ; (*A, bottom, DEN and center*) 60 μm ; (*A, bottom, SC*) 1,000 μm ; (*B, top*) 200 μm ; (*B, bottom, left*) 75 μm ; (*B, bottom, right*) 25 μm .

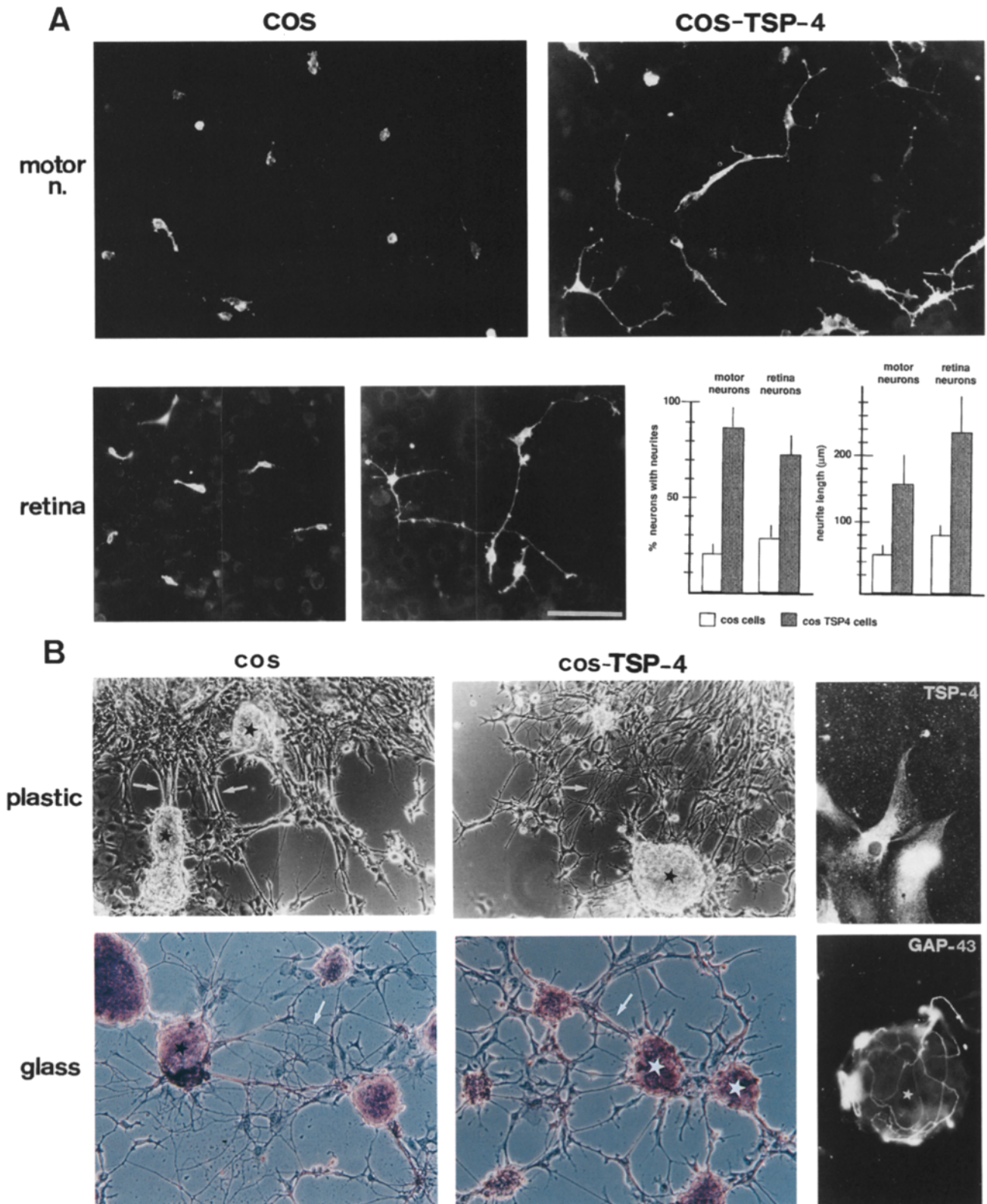


Figure 5. TSP-4 promotes neurite adhesion and outgrowth. Chick neurons were cultured for 24 h in the presence of stably transfected COS cells. These carried either TSP-4 expression vector (*COS-TSP-4*) or insert-free vector (*COS*). (**A**) Dissociated E5.5 motoneurons or E6 retina neurons grown on confluent monolayers of *COS* (left) or *COS-TSP-4* cells. Neurons were visualized with an antibody to GAP-43. A quantitative analysis of such experiments is also shown in the figure (neurites measured at least one cell diameter; neurite length: mean total length of neurites (only neurons with neurites were included); $n = 500$). (**B**) E7 chick sensory neurons (top, explants; bottom, dissociated cells) grown in the presence of *COS* cell aggregates. Upper row: When *COS* cells were precultured on tissue culture plastic (aggregates indicated by stars), TSP-4 adhered to the substratum (right, *COS-TSP-4* culture labeled with TSP-4 antiserum). Neurites leaving the explant (arrows) defasciculated to extend on the substratum in the presence of TSP-4, whereas they grew in tight bun-

TSP-4 Is a Secreted Protein That Can Be Incorporated into ECM

As shown in Fig. 1 *b*, the last 14 amino acids at the carboxyl-terminal end of TSP-4 show no homology with the corresponding residues of other known members of the TSP gene family. Therefore, to generate a reagent specific for TSP-4 we produced an antiserum to the synthetic peptide CQEFQIOTFDRLDN. As demonstrated by the data shown in Fig. 3, this antiserum specifically detected TSP-4 in immunocytochemistry experiments. Thus upon transfection with TSP-4 cDNA, monkey kidney epithelial cells COS-1 displayed robust labeling of the secretory pathway with the TSP-4 antiserum (Fig. 3 *a*). Preimmune serum gave no signal, the signal was abolished by preincubation of the antiserum with excess synthetic peptide (data not shown), and the TSP-4 antiserum detected no antigen in cells transfected with an unrelated construct (Fig. 3 *b*). This was expected as no TSP-4 mRNA was detected in COS cells (Fig. 2). Secreted TSP-4 was detected in a diffuse pattern on the surface of the transfected COS cells, and on tissue culture plastic (see Fig. 5).

To determine whether TSP-4 can be incorporated into ECM we transfected 3T3 mouse fibroblasts, a cell line that efficiently produces ECM. These cells express TSP-1, but, like COS-1 cells, they do not express TSP-4 mRNA (Fig. 2). As shown in Fig. 3 *c*, TSP-4 immunoreactivity was detected in transfected, but not in control cultures. In addition, secreted TSP-4 colocalized with fibronectin in fibrillar structures characteristic of ECM (Fig. 3 *c'*), indicating that TSP-4 can be efficiently incorporated into ECM *in vitro*. Finally, tenascin and TSP-4 colocalized at certain ECM-rich structures *in vivo*, including the myotendinous junction (Fig. 3 *d*) and the sclera of the eye (see Fig. 7), indicating that a strong interaction between TSP-4 and ECM also can take place *in vivo*.

In Skeletal Muscle, TSP-4 Is Expressed by Interstitial Cells, and Accumulates at the Neuromuscular Junction

A comparison of main tissues in adult rat yielded highest TSP-4 mRNA contents in skeletal muscle (Fig. 2). *In situ* hybridization analysis revealed that the transcript was present in muscle interstitial cells, but not in skeletal muscle fibers (Fig. 4 *A*). Denervation induced both a clear increase in signal intensity at single muscle interstitial cells, and an increase in the contents of TSP-4 expressing cells in the muscle (Fig. 4 *A*; top row, compare left and central panel). This is consistent with the known proliferation of muscle interstitial cells in denervated muscle (Murray and Robbins, 1982). Consistent with the pattern of proliferation of muscle interstitial cells (Connor and McMahan, 1987; Gatchalian et al., 1989), analysis of serial sections processed either for *in situ* hybridization or for the presence of RITC- α -bungarotoxin binding sites revealed that, TSP-4-positive cells in denervated muscle tended to be more

abundant in the vicinity of neuromuscular junctions (data not shown). That no muscle fiber-associated signal was detected in the *in situ* hybridization experiments is consistent with the observation that no TSP-4 transcript was detected in two distinct myogenic cell lines, irrespective of their differentiation status (Fig. 2). Muscle interstitial cells expressing TSP-4 are probably fibroblasts (see also Connor and McMahan, 1987; Gatchalian et al., 1989). Interestingly, however, not all fibroblasts express this member of the TSP gene family in the adult (see also Northern blot data of Fig. 2). It therefore appears that one distinguishing property of muscle associated fibroblasts is the expression of TSP-4.

Immunocytochemical analysis of developing and denervated muscle revealed that most TSP-4 immunoreactivity was distributed in an homogeneous manner in the extracellular space between skeletal muscle fibers (Fig. 4 *B*). Like that detected on transfected cells, this signal could be abolished when the antiserum was preadsorbed with excess synthetic peptide (data not shown). As expected from the Northern blot and *in situ* hybridization data, TSP-4 immunoreactivity was much weaker in adult innervated muscle, where the signal was most prominent in the ECM-rich spaces between muscle fiber groups (Fig. 4 *B*, upper row). Interestingly, in adult innervated muscle a distinct accumulation of TSP-4 was detected at the neuromuscular junction (Fig. 4 *B*, bottom row). Synaptic TSP-4 immunoreactivity was abolished by preadsorption of the antiserum with excess synthetic peptide, and was less prominent 8 d after denervation, when the signal was distributed more homogeneously in muscle interstitial spaces (data not shown). The cellular origin of the TSP-4 at the neuromuscular junction is not certain. However, due to the apparent absence of detectable TSP-4 mRNA in spinal motoneurons (Fig. 4 *A*, second row, right) and the presence of TSP-4 transcript in interstitial cells in adult innervated muscle (Fig. 4 *A*, top row, left; see also Fig. 2 for Northern blots), it seems likely that TSP-4 secreted by muscle interstitial cells accumulates at the neuromuscular junction.

TSP-4 Promotes Neurite Outgrowth In Vitro

TSP-4 is induced in denervated muscle, is expressed in the developing and adult nervous system, but it lacks the type-I repeats that have been linked to the neurite promoting activity of TSP-1 (Stewart, B. D., A. N. Qabar, M. Hartsch, V. M. Dixit, and K. S. O'Shea. 1994. *Mol. Biol. Cell.* 5:176A). It was therefore of major interest to determine whether TSP-4 may promote neurite adhesion and/or growth. To address this question we analyzed cocultures of embryonic neurons and naive (COS) or TSP-4-expressing COS cells (TSP-4-COS). Cocultures were selected because they are well suited to visualize both substrate selection and neurite elongation by neurons. An advantage of using COS cells for this type of experiments was that nontransfected

dles in its absence (*left*). (*Bottom*) When COS cells (aggregates indicated by stars) were precultured on glass, TSP-4 did not adhere to the substrate. In the presence of COS-TSP-4 cells, neurons and their neurites (visualized with an antibody to GAP-43; brown color reaction) selectively grew on the TSP-4-expressing cells (*arrow*). In the absence of TSP-4 (*left*), neurites did not discriminate between COS cells and the glass surface. The right panel shows a COS-TSP-4 aggregate labeled for GAP-43 to visualize sensory neurites that had exclusively grown within the aggregate. Bar: (*A*, top) 120 μ m; (*A*, bottom) 70 μ m; (*B*, top, right) 200 μ m; (*B*, bottom, right) 170 μ m.

cells did not appear to affect neurite outgrowth to any obvious extent.

In a first experimental approach, we analyzed neurite outgrowth from motor, retina, cerebellar, or sensory neurons on confluent monolayers of COS or TSP-4-COS cells. As shown in Fig. 5 A, motor and retina neurons extended substantially more and longer neurites on the TSP-4 expressing cells. Similar results were obtained with two additional TSP-4-COS clones (data not shown). The figure shows the data for E5.5 chick spinal cord-derived motoneurons and E7 chick retina neurons, but similar findings were obtained for granule cells from P6 mouse cerebellum and dissociated E7-to-E16 chick sensory neurons.

In the second type of experiment, COS cells were plated on tissue culture plastic at low density and in the absence of serum, favoring the formation of small aggregates. On the next day, chick sensory neurons were plated in the form of explants. Under these conditions, significant amounts of secreted TSP-4 bound to the substratum (Fig. 5 B, upper row, right panel). This was particularly obvious in 3-d low-density TSP-4-COS cultures, where TSP-4 deposits highlighted COS cell migration paths (data not shown). In the cocultures, a marked increase in substratum-associated neurite outgrowth was detected in the presence of TSP-4-COS cells (Fig. 5 B, upper row). This was reflected in a significant reduction of neurite fasciculation in the TSP-4 containing cultures. Therefore, under conditions where secreted TSP-4 adheres to the substratum, sensory neurites show preferential adhesion to the substratum.

Finally, COS or TSP-4-COS cells were plated under the same conditions as described above, but on uncoated glass. Dissociated sensory neurons were added the next day. In this experiment, secreted TSP-4 binds to the surface of the COS cells, but we could detect no TSP-4 immunoreactivity in association with the glass surface. As shown in Fig. 5 B (lower row), in the presence of naive COS cells sensory neurons bound and extended neurites without preference between glass and COS cells. In contrast, in the presence of TSP-4-COS cells, all cell bodies and neurites were confined to TSP-4-expressing cells. Therefore, under these experimental conditions, sensory neurons display a marked preference for growing on TSP-4-expressing cells. Combined with the findings described above these results indicate that TSP-4 is an attractive substrate for sensory neurites and has neurite promoting activity for a variety of neurons in vitro.

In the Adult Nervous System, TSP-4 Is Expressed by Neurons and Accumulates at Locations Rich in Synapses

In the adult central nervous system TSP-4 expression was mainly detected in neurons, where it was restricted to discrete neuronal populations, mainly large long-projection neurons. In the following, we focus our analysis to three systems with particularly obvious structural organization, the cerebellum, the retina, and the hippocampus.

During the formation of the cerebellar cortex, TSP-4 was detected in a homogeneous extracellular pattern in the external and internal granule cell layers, and in the forming molecular layer (Fig. 6, a and b). In addition, ac-

cumulation of TSP-4 immunoreactivity was detected on Purkinje cells. This distribution was different from that of tenascin (Fig. 6, a' and b'), a prominent ECM protein in the developing cerebellum that has been involved in granule cell migration (Chuong et al., 1987; Husmann et al., 1992). In the adult cerebellar cortex, TSP-4 accumulated on the cell bodies of Purkinje cells, and at the glomeruli in the granule cell layer (Fig. 6 c). The latter contain the synapses between granule cells, mossy fibers and Golgi cells. Like during development, tenascin had a distribution distinct from that of TSP-4, and accumulated in the molecular layer (Fig. 6 c'). In situ hybridization analysis revealed that in the adult cerebellum TSP-4 is mainly expressed by granule cells (Fig. 6 d).

In the adult hippocampus, prominent TSP-4 immunoreactivity was detected on granule cell neurons of the dentate gyrus and on pyramidal neurons of CA1, CA2, and CA3 (Fig. 6 e). The corresponding in situ hybridization revealed highest transcript levels on dentate gyrus granule cells (Fig. 6 f). The selective association of TSP-4 immunoreactivity with these neuronal cell bodies is striking, and a similar distribution of the label was detected on certain cortical pyramidal neurons (data not shown). The pattern of the signal associated with these neurons suggests that it mostly reflects intracellular TSP-4.

During early postnatal development, high levels of TSP-4 accumulated at the ganglion cell layer and in the optic nerve (Fig. 7 b). In the adult retina, TSP-4 accumulated at the outer limiting membrane and at the internal and external plexiform layers, i.e., at synapse-rich layers (Fig. 7 a). In addition, it accumulated at the sclera, i.e., an ECM-rich structure. In the adult retina tenascin immunoreactivity was only detectable at the sclera (data not shown). In situ hybridization analysis revealed TSP-4 expression in most neurons in the adult retina, with lowest levels in retinal ganglion cells (data not shown).

Discussion

We have characterized the expression pattern, localization, and some functional properties of TSP-4, a recently discovered member of the TSP gene family. Like TSP-3 and COMP, this TSP has a unique NH₂-terminal domain, and lacks the TSP-type-I (properdin) repeats that have been linked to some of the major known activities of TSP-1 and -2. This raises the question of what type of activities can be assigned to TSP-4, TSP-3, and COMP. In further analogy to these latter TSPs, TSP-4 displays a very restricted and unique expression pattern, raising the possibility that it may have acquired specialized functions.

We now show that the expression of TSP-4 in rodents is essentially restricted to skeletal and heart muscle, and to the nervous system. In skeletal muscle TSP-4 is expressed by muscle interstitial cells, but not by muscle fibers, and its expression is strongly upregulated upon denervation. In the postnatal and adult nervous system, it is expressed by certain types of neurons. TSP-4 binds to ECM in vitro and associates with ECM in vivo. In addition, it binds to cell surfaces in vitro and accumulates at the neuromuscular junction and at certain synapse-rich structures in vivo. Coculture experiments with stably transfected COS cells es-

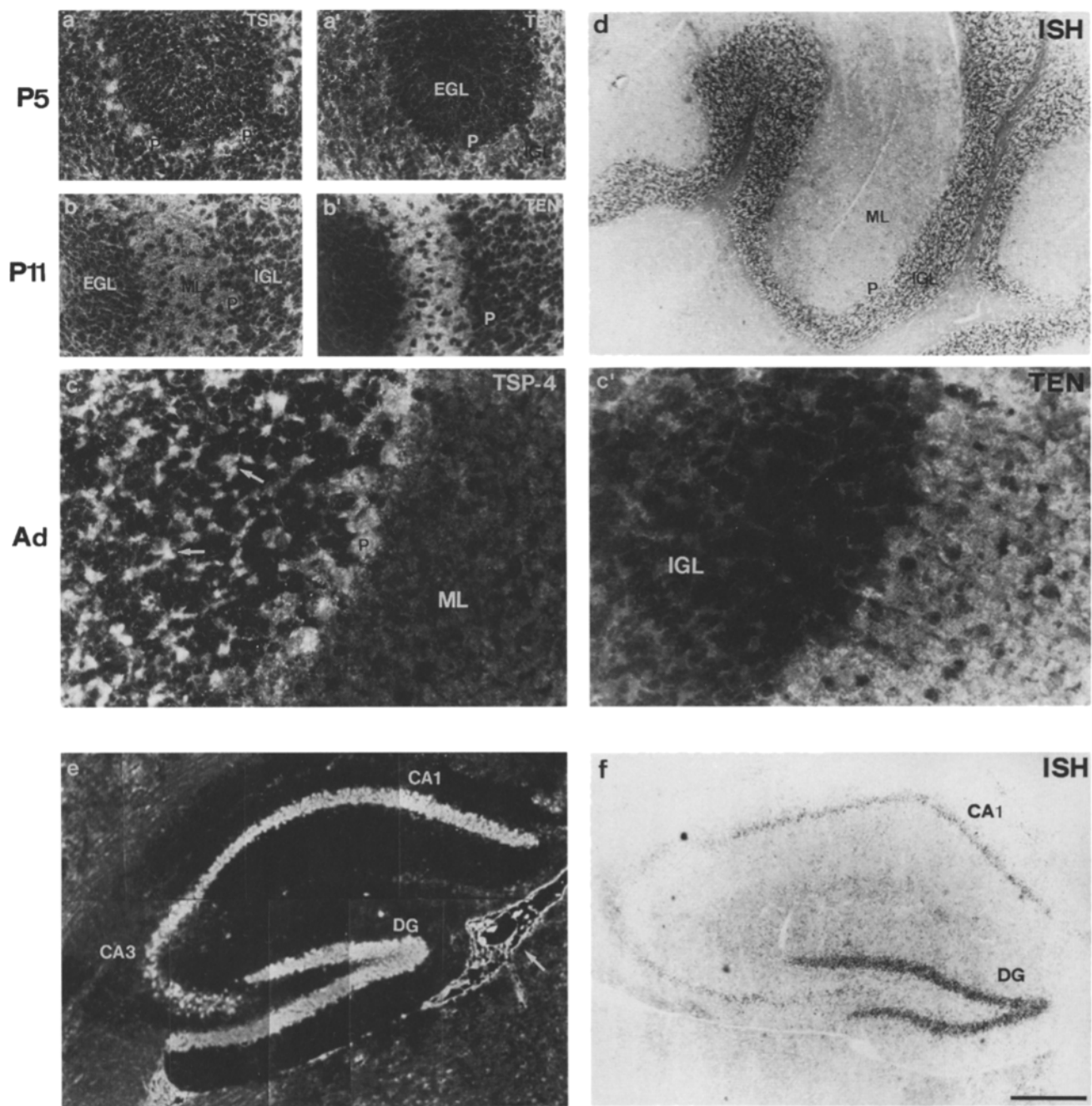


Figure 6. Expression and distribution of TSP-4 in the mouse cerebellum and hippocampus; expression by neurons and association with neuronal cell bodies and synapse-rich structures. (a–d) Cerebellar cortex; (e and f) hippocampus. The localization of TSP-4 in the developing (P5 and P11) and adult cerebellum was compared to that of the ECM protein tenascin (TEN, double-labeling immunocytochemistry; TSP-4 on the left). Both proteins accumulate in the developing molecular layer (ML and P11), through which granule cells migrate from the external granule cell layer (EGL) to reach the internal granule cell layer (IGL). In the adult, TSP-4 accumulates at the cell bodies of Purkinje cells (P), and at the glomeruli in the IGL (arrows, in parallel experiments these synapse-rich structures displayed prominent labeling with an antibody to the synaptic vesicle antigen SV2 (data not shown)). In the adult cerebellum tenascin accumulated in the ML, but not in the IGL. In situ hybridization (ISH) of adult cerebellum with a TSP-4 probe revealed prominent expression in granule cells in the IGL, and weaker expression by Purkinje cells (d). In the adult hippocampus TSP-4 accumulated at neuronal cell bodies in the dentate gyrus (DG) and the hippocampal structure, including CA1 and CA3 (e). It also associated with ECM in the ventricles (e, arrow). TSP-4 mRNA was detected in the corresponding granule cell (DG) and pyramidal neuron cell bodies in CA1, CA2, and CA3 (f). Bar: (a and b) 70 μm ; (c) 30 μm ; (d–f) 250 μm .

established that TSP-4 is an adhesive and preferred substrate for neurons and their neurites, and that it promotes neurite outgrowth. The results establish TSP-4 as a prominent nervous system TSP affecting neuronal functions. Its

expression pattern and localization in the adult nervous system are unusual for an ECM protein, and assign it to the restricted group of extracellular proteins that may affect neuronal signaling in the adult.

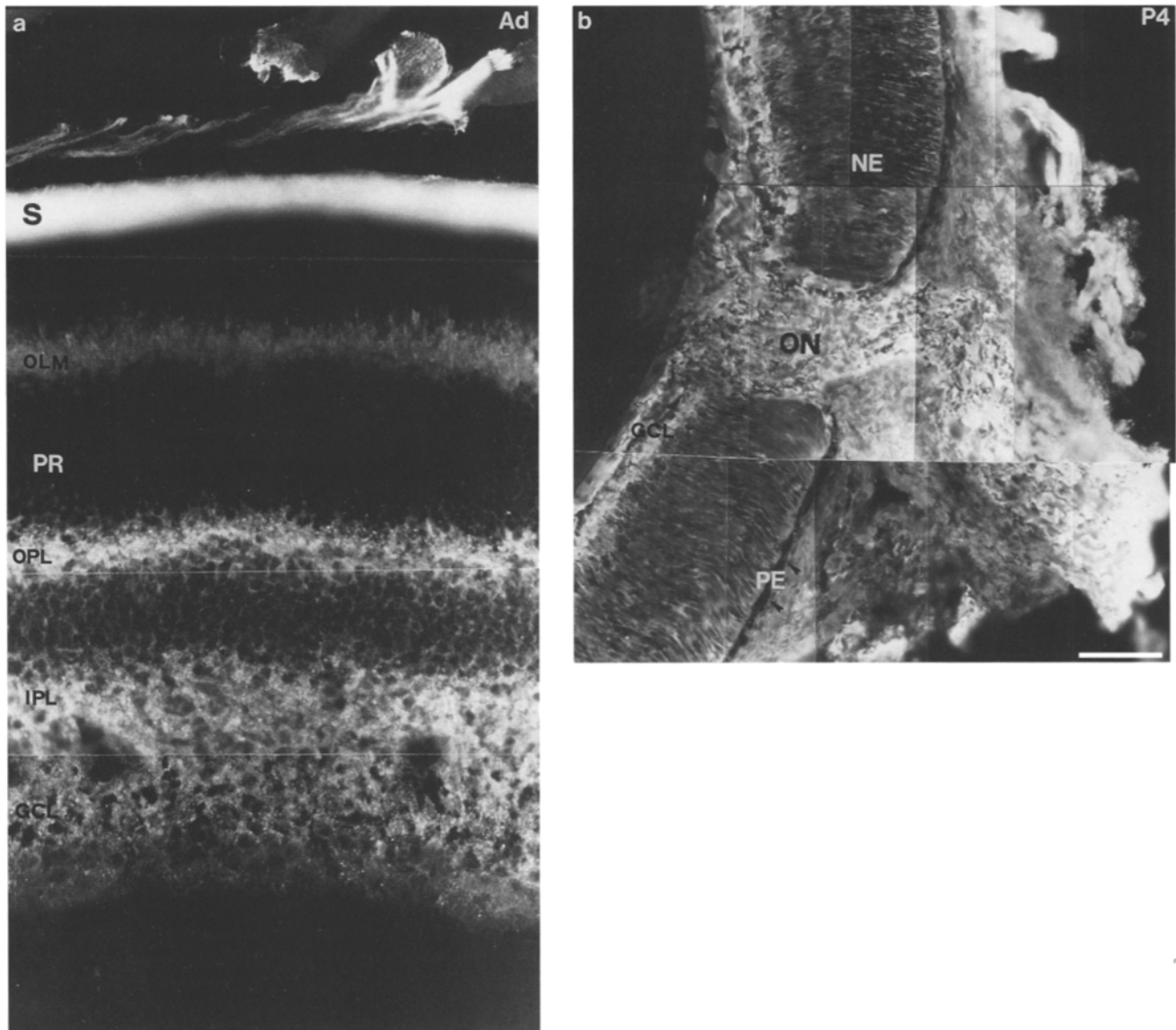


Figure 7. Distribution of TSP-4 in the developing and adult mouse retina. In the adult (a), TSP-4 accumulates in a spotted pattern at synapse-rich layers in the inner (IPL) and outer (OPL) plexiform layers, and at the outer limiting membrane (OLM). In addition, it is detected at the retinal ganglion cell layer (GCL), and at ECM-rich structures at the sclera (S) and at muscle attachment sites (top). No signal was detected at the level of the photoreceptors (PR). (b) At P4, prominent labeling of the GCL and of the optic nerve (ON) was detected. Antigen was also detected in the differentiating neural epithelium (NE). For orientation, the position of the pigment epithelium (PE) is also indicated (arrows). Bar: (a) 40 μm ; (b) 64 μm .

TSP-4 Is a Prominent Nervous System TSP

Because of the high degree of homology among the TSPs, and the unexpected expression pattern of TSP-4 in the nervous system, it was particularly important to establish the specificity of our reagents and methods for the detection of TSP-4. The antiserum to the carboxyl-terminal peptide of TSP-4 failed to bind to any specific protein on immunoblots. For that reason we had to determine its immunocytochemical specificity through a series of unambiguous positive and negative controls. We first demonstrated that it specifically bound to a secretory and ECM-binding protein only when cell lines devoid of any endogenous TSP-4 mRNA were transfected with an expression construct coding for TSP-4. We then showed that the distribu-

tion and regulation of the corresponding antigen(s) on cryostat sections was entirely consistent with the results from Northern blot and in situ hybridization data. Finally, we excluded any cross-reactivity with other known members of the TSP gene family by selecting a unique peptide sequence for antibody production, and by verifying that tissues devoid of TSP-4, such as lung failed to bind the anti-TSP-4 antibodies. Based on these criteria we conclude that we specifically visualized the distribution of TSP-4 mRNA and protein.

The expression of TSP-4 in striated muscle is consistent with the findings from a previous report (Lawler et al., 1993a). A TSP, presumably TSP-1 was detected in developing skeletal muscle and may be expressed by skeletal muscle fibers (Hantai et al., 1991). In addition, TSP immu-

noreactivity was increased in patients with amyotrophic lateral sclerosis (Rao et al., 1992). Since this motoneuron disease is associated with extensive denervation of skeletal muscle fibers, it is possible that some of the signal detected in that study may have reflected elevated TSP-4.

The expression of TSP-4 in neurons and the striking association with certain synapse-rich layers in the adult nervous system are unique for TSP-4. Although other TSPs have been detected in the developing nervous system by Northern blot analysis, these transcripts were dramatically downregulated during the late phases of development (O'Shea and Dixit, 1988; O'Shea et al., 1990; Lawler et al., 1993a; Qabar et al., 1994). In contrast, TSP-4 mRNA contents were higher in adult than in early postnatal brain. Early immunocytochemistry studies revealed substantial contents of thrombospondin in the developing and adult nervous system (O'Shea and Dixit, 1988; see also Hoffman et al., 1994). The antibodies used in these studies had, however been raised against platelet thrombospondin (TSP-1), and it must therefore be assumed that they may have cross-reacted with other TSPs. More recent studies have analyzed the distribution of TSP-1 in mouse embryos with a specific monoclonal antibody (Corless et al., 1992). Antigen was detected in fibrillar structures in the neuropil, and in the cytosol of differentiating neurons. This expression pattern is substantially different from that detected in this study, and it will be interesting to carry out a comparison of TSP-1 and -4 expression in the nervous system both at the transcript and protein level. A recent study applied an antiserum to a unique synthetic peptide to determine the distribution of TSP-3 in the embryonic mouse (Qabar et al., 1994). In contrast to platelet thrombospondin and TSP-4, this TSP apparently did not associate with major ECM structures. In addition, this study reported substantial levels of TSP-3 in sensory ganglia and peripheral nerves, and suggested that this TSP may be expressed by Schwann cells. In similar experiments, we found no TSP-4 in these structures, neither during development, nor in the adult, nor upon lesion (data not shown). It is, however possible that TSP-3 and -4 may accumulate at similar locations in other parts of the developing nervous system (Arber, S., and P. Caroni, unpublished results).

The specific concentration of TSP-4 immunoreactivity at some neuronal cell bodies, e.g., granule cell and pyramidal neurons in the hippocampus, Purkinje cells in the cerebellum, and subpopulations of pyramidal neurons in the cerebral cortex is striking. A similar accumulation of TSP immunoreactivity at certain neuronal cell bodies, especially those in the hippocampus, was reported in previous studies (Corless et al., 1992; Hoffman et al., 1994). In our study, comparison with the localization of TSP-4 mRNA and close examination of the immunocytochemical signal suggest that it mainly reflects intracellular TSP-4 synthesized by the same population of neurons. This is particularly surprising, as localization of TSP-4 immunoreactivity in the neuropil and at certain synapse-rich layers is detected in other nervous system structures. One possible explanation for these findings is that some nervous system regions, e.g., the neuropil in the hippocampus lack high-affinity binding sites for TSP-4. As a consequence, TSP-4 may either fail to accumulate at these locations, or may dissociate from them during the staining procedure. The

latter possibility should not be disregarded, since in our hands, with the exception of the antigen associated with prominent ECM structures, the use of fixatives precluded the reliable detection of TSP-4 on cryostat sections. Similar observations were reported in previous studies for platelet thrombospondin and TSP-3, suggesting that this may be a general problem for the detection of TSPs outside major ECM-containing regions (Hoffman et al., 1994; Qabar et al., 1994). These observations also imply that it is probably not possible with these antibodies to analyze the distribution of TSP-4 at the electron microscopy level. This would have allowed to define with more precision the localization of TSP-4 at cell bodies and in synapse-rich layers.

Possible Functions of TSP-4

Most presently known biological activities of TSP-1 have been assigned to the NH₂-terminal linkage region and the type-I repeats. These include its angiogenic activity, and binding and activation of TGF β . Because these domains are absent in TSP-3, COMP, and TSP-4, the functions of these TSPs cannot be extrapolated from previous studies with platelet TSP. In fact, very little is known about possible functions of TSP-3, and the only functional information about TSP-4 is that a fusion protein containing its carboxyl-terminal domain promotes myoblast adhesion (Adams and Lawler, 1994). We now demonstrate that TSP-4 associates with cell surfaces, can incorporate into ECM, is a preferred substrate for neurons, and promotes neurite outgrowth. The association of TSP-4 with ECM and its neurite-promoting activity distinguish it from the related protein TSP-3. Thus TSP-3 binds heparin, but apparently does not associate to a significant extent with ECM in vivo (Qabar et al., 1994). In addition, it promotes cell attachment but not neurite outgrowth in vitro (Stewart, B. D., A. N. Qabar, M. Hartsch, V. M. Dixit, and K. S. O'Shea. 1994. *Mol. Biol. Cell.* 5:176A). The activity of TSP-4 towards neurons and its expression and distribution in the nervous system suggest that it may play a role in neural signaling during late development and in the adult. It is however clear that, like for other potentially multifunctional ECM molecules, its actual functions in situ will be difficult to define and may require targeted disruption and ectopic expression experiments in vivo.

It has been reported that platelet TSP has both adhesive and anti-adhesive properties, and that it probably plays a role in cell migration (see e.g., Tarabozetti et al., 1987). Antibodies to platelet TSP inhibit cerebellar granule cell migration in explant cultures (O'Shea et al., 1990). Because the same antibodies revealed the presence of substantial TSP-immunoreactive material within the migration path of these cells in the molecular and internal granule cell layer, it seems likely that a TSP plays a role in this process. Our data now suggest that some of that material may be TSP-4, raising the possibility that this TSP may be involved in granule cell migration. Likewise, dramatically elevated contents of TSP-4 in the interstitial spaces of denervated muscle may promote tissue remodeling.

The association of TSP-4 with the neuromuscular junction and with certain synapse-rich regions in the adult central nervous system is striking. At the neuromuscular junction, synaptic accumulation of TSP-4 was not detectable

before the second postnatal week (data not shown). In adult innervated muscle some TSP-4 mRNA was detected in interstitial cells, whereas no corresponding signal was detected in spinal motoneurons. The source of TSP-4 at the neuromuscular junction is therefore likely to be muscle interstitial cells (Fig. 5 A). We detected no obvious correlation between the distribution of TSP-4 mRNA and the position of synapses in innervated muscle. A possible explanation for these observations may be that interstitial cell-derived TSP-4 accumulates at the adult neuromuscular junction due to a high density of corresponding binding sites. A further possibility may be that at the neuromuscular junction TSP-4 is protected against degradation. Several synaptic ECM components have been identified at the neuromuscular junction (Sanes, 1989; Hall and Sanes, 1993), and among them agrin is also expressed in the central nervous system (McMahan et al., 1992). In the adult nervous system, processes affected by TSP-4 may include the modulation of extracellular proteolytic activities and signaling through its cell surface receptors. In addition, TSP-4 may associate and interact with further ECM components to control local signaling.

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Note Added in Proof: The accession number for the rat TSP-4 sequence reported in this paper is: X89963.

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