Isolation of a Tenascin-R Binding Protein from Mouse Brain Membranes

A PHOSPHACAN-RELATED CHONDROITIN SULFATE PROTEOGLYCAN*

Zhi-Cheng Xiao‡‡, Udo Bartsch‡, Renée K. Margolis§, Genevieve Rougon, Dirk Montag‡, and Melitta Schachner‡‡‡‡

From the ‡Department of Neurobiology, Swiss Federal Institute of Technology, Hönggerberg, CH-8093 Zürich, Switzerland, the **Zentrum für Molekulare Neurobiologie, Universität Hamburg, Martinistrasse 52, D 20246 Hamburg, Federal Republic of Germany, the ‡Department of Pharmacology, State University of New York, Health Science Center, Brooklyn, New York, New York 11203, and the ‡Laboratoire de Génétique et Physiologie du Développement, CNRS 9943, Faculté des Sciences de Luminy-Case 907, F-13288 Marseille Cedex 9, France

We have isolated a chondroitin sulfate proteoglycan from mouse brain by affinity chromatography with a fragment of the extracellular matrix glycoprotein tenascin-R (TN-R) that comprises the amino-terminal cysteine-rich stretch and the 4.5 epidermal growth factor-like repeats. The isolated chondroitin sulfate proteoglycan has a molecular mass of 500–600 kDa and carries the HNK-1 carbohydrate epitope. Treatment with chondroitinase ABC reveals a major band of approximately 400 kDa and two minor bands at 200 and 150 kDa. Immunoblot analysis relates the molecule to phosphacan but not to the chondroitin sulfate proteoglycans neurocan and versican. Binding of the phosphacan-related molecule to the epidermal growth factor-like repeats of TN-R is Ca²⁺-dependent. Co-localization of the molecule with TN-R in the retina and optic nerve by immunocytochemistry suggests a functional relationship between the two molecules in vivo. Inhibition of neurite outgrowth from hippocampal neurons by the phosphacan-related molecule in vitro is neutralized by TN-R when coated as a uniform substrate. Furthermore, the phosphacan-related molecule neutralizes growth cone repulsion induced by TN-R coated as a sharp substrate boundary with or without prior treatment with chondroitinase ABC. These observations indicate that TN-R can interact with a phosphacan-related molecule and thereby modulate its inhibitory influence on neuritogenesis.

Extracellular matrix molecules play important roles in cell interactions in the developing nervous system, such as neuronal migration and neuritogenesis. Chondroitin sulfate proteoglycans (CSPGs) constitute the major population of proteoglycans (PGs) in the central nervous system (1), but up to now only a few of them have been well characterized. Known CSPGs include versican (2), NG2 (3), neurocan (4), versican/PG-M (2, 5), phosphacan (6), brevican (7), astrochondrin (8), and DSD-1-PG (9). CSPGs may have diverse effects on neurons. Some stimulate axon growth and support the survival of retinal neurons (10). Recent observations indicate that DSD-1-PG promotes neurite outgrowth of embryonic day 14 mesencephalic and embryonic day 18 hippocampal neurons from rat (9). However, other CSPGs inhibit neurite outgrowth, such as NG-2, an integral membrane CSPG expressed on the surface of glial progenitor cells. NG-2 inhibits growth of cerebellar neurons, even in the presence of laminin or LI, two potent promoters of axonal extension. The NG2 proteoglycan remains inhibitory after digestion with chondroitinase ABC, indicating that the inhibitory activity is a property of the core protein and not the covalently attached chondroitin sulfate glycosaminoglycan chains (11). The neurocan and phosphacan core proteins also inhibit neurite outgrowth (12, 13). Astrochondrin is also involved in neurite outgrowth and cerebellar granule cell migration (8).

Phosphacan, a 500–600-kDa CSPG with a 400-kDa protein core, may play a role in inhibition of neuronal migration (14). For instance, phosphacan has been shown to interact heterophilically with N-CAM, Ng-CAM, and tenasin-C (TN-C) (6, 13, 15, 16), suggesting that it may regulate neuronal adhesion. Interestingly, phosphacan has recently been shown to be a member of receptor tyrosine phosphatase β/β (RPTPβ/β) subfamily, which lacks phosphatase domains (6). The subfamily of membrane-bound protein-tyrosine phosphatases, including RPTPβ/β and RPTPγ, is composed of tandem catalytic domains in their cytoplasmic regions, a single transmembrane domain, and variable extracellular domains consisting of an amino-terminal carbonic anhydrase-like domain followed by a fibronectin type III repeat and by a cysteine-free domain of variable length (17). These structural features of their extracellular domains with homology to cell adhesion molecules suggest that RPTPs play a role in either cell-cell or cell-matrix interaction. The presence of catalytic domains in their intracellular segment implicate that RPTPs could be direct signal transducers of cell contact phenomena (18).

Chondroitin sulfate proteoglycans have been implicated in interactions with other extracellular matrix glycoproteins, such as tenasin-C (TN-C) (19, 20). The extracellular matrix

* This work was supported by a grant from Association Française contre les Myopathies to G. R.
+ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
†‡ To whom correspondence should be addressed. Tel.: 41-1-6333396 or 49-40-4717 6246; Fax.: 41-1-6331046 or 49-40-4717 6248.
* The abbreviations used are: CSPG, chondroitin sulfate proteoglycan; PGs, proteoglycans; CMF-HBSS, Ca²⁺-, Mg²⁺-free Hank’s balanced salt solution; EGF-S, epidermal growth factor-like repeats; EGF-L, fragment containing the cysteine-rich stretch and the epidermal growth factor-like repeats; RPTP, receptor tyrosine phosphatase; BSA, bovine serum albumin; MDCK, Madin-Darby canine kidney cells; PAGE, polyacrylamide gel electrophoresis; FG, fibrinogen knob; FN, fibronectin.
glycoprotein TN-R, a member of the tenascin family, has a modular structure with a cysteine-rich amino-terminal region followed by epidermal growth factor (EGF)-like repeats, fibronectin-type III (FN III) domains, and a fibrinogen-like (FG) domain at the carboxyl-terminal end (21–30). TN-R is predominantly expressed by oligodendrocytes during the onset and early phases of myelin formation and remains expressed by some oligodendrocytes in the adult. TN-R is also expressed by some neurons in the spinal cord, retina, cerebellum, olfactory bulb, and hippocampus (25, 31–34). TN-R is a multi-functional molecule that promotes neurite outgrowth when presented as a sharp substrate boundary, and induces axonal defasciculation in vitro (35–38). Taking advantage of the availability of different recombinant TN-R domains, distinct but also overlapping functions for the different TN-R domains have recently been identified (39). These findings support the cell culture observations on the diverse properties of TN-R and suggest the existence of multiple neuronal TN-R receptors and binding proteins.

To study further the multiple functions of TN-R during development, we have used a biochemical approach to identify a new binding protein, and we further characterize the interaction by immunohistochemistry and functional in vitro assays. We report here that the new functional binding protein of TN-R is a CSPG related to phosphacan. Immunohistochemical studies demonstrate a striking co-localization of the phosphacan-related molecule and TN-R in the retina and optic nerve of adult mice. Furthermore, the phosphacan-related molecule by itself inhibits neurite outgrowth from hippocampal neurons but blocks the inhibitory effects of TN-R on neurites and growth cones.

EXPERIMENTAL PROCEDURES

Animals—ICR mice and Sprague-Dawley rat embryos were obtained from the animal facilities at the University of Zurich.

Materials—CNBr-activated Sepharose 4B was from Pharmacia-LKB, Uppsala, Sweden. Enhanced chemiluminescence assay kit was purchased from Amersham Corp. (Amersham, UK). Laminin from Engelbreth-Holm-Swarm sarcoma and poly-l-lysine (PLL) were purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies to rabbit, rat, or mouse IgG and IgM were purchased from Dianova (Hamburg, FRG). Chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4. from Proteus vulgaris, protease-free) and heparinase were from Boehringer Mannheim.

Antibodies—Monoclonal antibody 473-HD, a rat IgM against a chondroitin-deaminated sulfate epitope on mouse brain proteoglycans, has been described (9). Rabbit polyclonal antibodies were generated against a peptide derived from human versican (40) and recombinantly expressed human versican (kind gifts of Drs. A. Aspberg, R. Le Baron, E. Rooslahti, and D. Zimmermann). Monoclonal L2 antibody 473 from rat (41) has been described (42). Polyclonal antibodies against glutathione S-transferase neurocan fusion proteins and polyclonal antibodies against phosphacan have also been described (13, 43).

Purification of TN-R, Phosphacan, Neurocan, and TN-R Fusion Proteins—Purification of TN-R from adult mouse brains by immunoaffinity chromatography was performed as described (31). Generation and purification of the recombinant fragments of TN-R as fusion proteins with glutathione S-transferase were performed as described (39).

For the preparation of membrane extracts, about 0.2 to 1 g of 5–20-day-old mouse brains, cerebellum, hippocampus, muscle cells, and MDCK cells were homogenized in 10 ml of buffer A (0.4 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, and 0.02% sodium azide, pH 7.5) and incubated overnight. After centrifugation at 100,000 × g for 1 h at 4 °C, the supernatant was diluted with 10 volumes of buffer B (10 mM Tris-HCl, 0.02% sodium azide, 2% Triton X-100, pH 7.5) and applied to a column containing EGF-L or FG for affinity chromatography. EGF-L or FG (3 μg each) was covalently coupled to CNBr-activated Sepharose 4B according to the manufacturer’s instructions. Unbound material was washed from the columns with 20 bed volumes of buffer F (1 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, and 0.02% sodium azide, pH 7.5). The CSPG was eluted from the EGF-L column with buffer G (0.1 M diethylamine, 0.1 M NaCl, 1 mM EDTA, and 1 mM EGTA, pH 11). The eluted fractions were quickly neutralized with 1 M Tris-HCl, pH 6.8, dialyzed against PBS with 0.1% Triton X-100, and stored at −70 °C in aliquots.

Enzymatic Treatment with Chondroitinase ABC and Heparinase—CSPGs were treated with chondroitinase ABC (0.02 units/ml) in 50 mM Tris acetate, pH 8.0, at 37 °C for 2.5 h in the presence of protease inhibitors (5 mM benzamidine, 1 mM iodoacetamide, and 5 mM p-tosyl-l-lysine chloromethyl ketone, sodium salt). Treatment with heparinase was according to the manufacturer’s instructions.

Iodination of TN-R Fragments and Overlay Assay—Recombinant fragments of the cysteine-rich stretch and the EGF-like domains (EGF-L), the EGF-like domains (EGF-S), the fibronectin type III (FN) repeats 5–8 (FN8–8), and the fibrinogen knob (FG) (40 μg each) were iodinated in 20 μl of 0.5 mM phosphate buffer, pH 7.4, with 2 μCi of 125I (Amersham Corp., IMS-30) using IODO-GEN as described (39). After 15 min at 4 °C the reaction was stopped by adding an excess of tyrosine in 20 μl of 50 mM phosphate buffer, pH 7.4. Free 125I-tyrosine was separated from the iodinated fusion proteins by chromatography on a Sephadex G25 column (PD-10, Pharmacia Biotech Inc.) with PBS as eluant. Radioactive peptides were recovered in the void volume. The fractions with highest radioactivity were pooled and immediately diluted with PBS containing BSA (1 mg/ml) and stored frozen at −20 °C in 1-ml aliquots (50 × 106 cpn/ml) until use. The specific radioactivity was estimated to be approximately 1.5 Ci/μmol of fusion proteins.

After SDS-PAGE (see previous section), proteins were transferred to nitrocellulose filters (Amersham Corp.) that were then incubated for 4 h at 37 °C in PBS containing 10% fetal calf serum, 4% defatted milk powder (fatul), and 4 μg/ml (blocking buffer). Membranes were incubated for 2 h at room temperature with the iodinated fusion proteins at 5 × 106 cpn/ml in blocking buffer containing 3 μM CaCl2, washed three times in PBS for 3–5 min, once in PBS containing 0.1% Tween 20 for 3–5 min, and once in PBS containing BSA (4 mg/ml) for 5 min, all at 4 °C. Bound fusion proteins were detected by autoradiography by exposing to Kodak film at −80 °C. In competition experiments, membranes were incubated with 10 μg/ml unlabeled fusion proteins for 2 h before addition of iodinated TN-R fragments.

Immunohistochemical Analysis—The distribution of TN-R and phosphacan immunoreactivity was studied in tissue sections from adult mouse retina and optic nerve. Preparation of tissue and cryostat sections and immunostaining of sections was performed as described in detail elsewhere (47). The monoclonal anti-TN-R antibody 619 recognizing the 160- and 180-kDa components of TN-R (48) and polyclonal rabbit anti-phosphacan (13) antibodies were used as primary antibodies. Fluorescein isothiocyanate-conjugated goat anti-mouse (Dynatech) or fluorescein isothiocyanate-conjugated goat anti-rabbit (Dakopatts) antibodies were used to visualize the primary antibodies. For negative controls, incubation of sections with primary antibodies was omitted.

Assays for Substrate Properties of the Phosphacan-related Molecule—Tissue culture dishes (Becton Dickinson) with a diameter of 3.5 cm were coated with methanol-solubilized nitrocellulose according to Lagena and Lemmon (49) and air-dried under a sterile hood.

For assays addressing the effect of the phosphacan-related molecule on the growth cone repulsion by TN-R, nitrocellulose-coated dishes were incubated with PBS containing 0.01% PLL at 37 °C for 1 h. The dishes were washed three times with PBS and air-dried under a sterile hood. 2.5–μl spots of the different test proteins (the phosphacan-related molecule, the phosphacan-related molecule plus TN-R, and BSA plus TN-R), each at a concentration of 125 ng, except for chondroitinase ABC which was at 0.02 units/ml, were then applied as single spots in duplicate to the dishes and incubated overnight at 37 °C in a humidified
atmosphere. Shortly before plating the cells, the dishes were washed with Ca\textsuperscript{2+} - and Mg\textsuperscript{2+}-free Hank’s balanced solution (CMF-HBSS). Explants were prepared from cerebella of 6–7-day-old mice and maintained in a chemically defined medium (50). Explants were allowed to grow neurites for 72 h and then fixed with glutaraldehyde in PBS at a final concentration of 2.5%.

After fixation, cultures were stained with 0.5% toluidine blue (Sigma) in 2.5% sodium carbonate, washed five times with water, and air-dried. All experiments were performed at least three times.

**Assay for Neurite Outgrowth—** Hippocampal neurons from 18–19-day-old rat embryos were prepared as described (51, 52). For the assays on neurite outgrowth, hippocampal neurons were maintained in chemically defined medium (35, 39, 53). In brief, 96-well multiwell plates (Nunc) were pretreated with 0.01% PLL for 1–2 h at 37°C, washed twice with water, and air-dried. Proteins were coated at a concentration of 125 μg onto the dried surfaces overnight at 37°C in a humidified atmosphere. Substrate coating efficiency was determined as described (48). The plates were washed three times with CMF-HBSS, and hippocampal neurons were plated at a density of 3,000 cells per well in 100 μl of chemically defined medium. After 12 h, cells were fixed without a preceding washing step by gentle addition of 25% glutaraldehyde to a final concentration of 2.5%. After fixation, cultures were stained with toluidine blue, and morphological parameters were quantified with an IBAS image analysis system (Kontron, Milan, Italy). For morphometric analysis, randomly cells without contact to other cells were evaluated. Neurites were defined as those processes with a length of at least one cell body diameter. To determine the total neurite length per cell, 50 cells in each of two wells were analyzed per experiment. To determine the number of cells with neurites, 100 neurons in each of two wells were counted per experiment. Raw data from at least three independent experiments were analyzed by analysis of variance and then Newman-Keuls test with p < 0.05 and p < 0.01 being considered significant or highly significant, respectively.

**RESULTS**

The EGF-L Domain of TN-R Binds to a Component from Central Nervous System Membranes—In our initial experiments, we used in overlay assays several \textsuperscript{125}I-iodinated TN-R domains to look for TN-R binding proteins in brain and other tissues. The fragments of TN-R tested were bacterially expressed recombinant protein fragments comprising the EGF-L (the region consisting of a cysteine-rich amino-terminal part and epidermal growth factor-like repeats), EGF-S (the region consisting of epidermal growth factor-like repeats), FN6–8 (the region consisting of 6–8 fibronectin type III homologous repeats), and FG (the fibrinogen-like domain) domains (39). Detergent extracts of crude membrane were prepared from 5–20-day-old mouse brain, hippocampus, cerebellum, muscle, and MDCK cells. The membrane proteins were separated by 6% SDS-PAGE (with 0.5% bisacrylamide for low cross-linking) under reducing conditions and transferred to nitrocellulose membranes. The membranes were then incubated with 5 × 10\textsuperscript{4} cpm/ml different \textsuperscript{125}I-labeled TN-R domains. Of the four iodinated peptides, only the EGF-L domain bound to a single component of the crude membrane preparations from brain but not to membranes from muscle or MDCK (Fig. 1A). The binding component appeared as a broad band of approximately 500–600 kDa. This binding was Ca\textsuperscript{2+} -dependent (Fig. 1A) and not detectable after addition of EDTA or in the absence of Ca\textsuperscript{2+} in the blocking buffer (not shown). This binding was strongly reduced by pre-blocking with 1 μg/ml unlabeled EGF-L (not shown). Under the same conditions, the other fragments, including EGF-S, FN6–8, and FG, did not show any detectable binding to the crude membrane preparations (not shown). Thus, the binding observed with EGF-L to the 500–600-kDa component from membranes of hippocampus, cerebellum, as well as whole brain is specific.

**Affinity Purification of the EGF-L Binding Component from Brain Membrane Extracts Is Ca\textsuperscript{2+} -dependent**—To support the assignment of the 500–600-kDa component as a TN-R binding protein and to confirm that ligand binding to this component is Ca\textsuperscript{2+} -dependent, we performed two types of affinity chromatography using EGF-L and FG of TN-R. EGF-L or FG were covalently coupled to CNBr-activated Sepharose 4B, and detergent extracts of 5–20-day-old mouse brains, prepared as described under “Experimental Procedures,” were passed over the columns. After extensive washing, a 500–600-kDa component was eluted from the EGF-L affinity column. This component bound to EGF-L in a Ca\textsuperscript{2+} -dependent manner (Fig. 1B) since, in the absence of Ca\textsuperscript{2+} in the isolation steps, there was no detectable binding to the EGF-L column (Fig. 1B). The presence of 0.1% Triton X-100 in the extraction buffer was necessary to keep the 500–600-kDa component soluble. Under the same assay conditions, the 500–600-kDa component did not bind to the FG affinity column (not shown).

To determine whether the binding between EGF-L and the 500–600-kDa component was direct or mediated by a co-purifying molecule, we tested directly binding of EGF-L to the 500–600-kDa component in an overlay assay. Three other TN-R domains, EGF-S, FN6–8, and FG, were tested as controls. The affinity-purified 500–600-kDa component was only bound by \textsuperscript{125}I-EGF-L, and binding was Ca\textsuperscript{2+} -dependent (Fig. 1C). Binding was reduced by addition of 1 μg/ml unlabeled EGF-L (Fig. 1C) and by depletion of Ca\textsuperscript{2+} from the blocking buffer (not shown). Under the same assay conditions, the other three domains did not show any detectable binding by overlay assays (not shown). Taken together, these results indicate that the 500–600-kDa component is a TN-R binding protein expressed in brain tissue, which can directly bind to EFG-L of TN-R in a Ca\textsuperscript{2+} -dependent manner.

**The Affinity-purified 500–600-kDa Component Is a Chondroitin Sulfate Proteoglycan Carrying the HNK-1 Glycan**—The large apparent size of the affinity-purified EGF-L binding component, together with the recent reports of interactions occurring between TN-R and CSPGs (20, 54), led us test if the 500–600-kDa component was a proteoglycan (PG). We treated the affinity-purified 500–600-kDa component with chondroitinase ABC and analyzed the resulting products by SDS-PAGE and Western blot analysis. Treatment with chondroitinase ABC altered the mobility of this component and resulted in the

**FIG. 1. Identification and purification of a protein binding to EGF-L of TN-R.** A, interaction of TN-R with detergent extracts of membranes from different tissues using \textsuperscript{125}I-labeled EGF-L in an overlay assay. Lane 1, 5–20-day-old mouse brain; lane 2, hippocampus; lane 3, cerebellum; lane 4, muscle; lane 5, MDCK. B, a 500–600-kDa component purified by affinity chromatography on EGF-L. Mouse brain membranes (5–20-day-old) were detergent-solubilized, and the EGF-L binding protein was separated on an EGF-L column in the presence or absence of 3 mM Ca\textsuperscript{2+}. Eluates were visualized by silver staining of a 6% SDS gel. Lane 1, brain membrane fraction; lane 2, eluate in absence of Ca\textsuperscript{2+}; lane 3, elute in presence of Ca\textsuperscript{2+}. C, overlay assay to detect the interaction between EGF-L and the EGF-L affinity-purified 500–600-kDa component (lanes 1 and 2) using \textsuperscript{125}I-labeled EGF-L in the absence (lane 1) and presence of excess unlabeled EGF-L (lane 2). The position and molecular mass in kDa of marker proteins run on the gels are indicated.
Several CSPGs have been reported to appear as a more compact major smear migrating at approximately 400 kDa and a minor band at 150 kDa (Fig. 2A). Western blot analysis was performed with detergent extracts of brain membranes, the affinity-purified 500–600-kDa component, and the affinity-purified 500–600-kDa component treated with heparinase and chondroitinase ABC using 473-HD monoclonal antibody against a dermatan-chondroitin sulfate epitope. This antibody recognized bands at approximately 400 kDa and a minor band at 150 kDa (Fig. 2A). These experiments indicate that the EGF-L binding component is a CSPG.

We also investigated whether the EGF-L affinity-purified CSPG was HNK-1-positive. Immunoblots of brain membranes and the EGF-L affinity-purified CSPG were performed and probed with the 412 monoclonal antibody against the HNK-1 carbohydrate epitope (41). The 412 antibody reacted both with components of brain membranes and the EGF-L affinity-purified CSPG (Fig. 2C).

**The EGF-L Affinity-purified CSPG Is Immunochemically Related to Phosphacan**—Several CSPGs have been reported to bind to TN-C and TN-R (15, 20, 54). We investigated further whether the EGF-L affinity-purified CSPG could be one of these CSPGs. SDS-PAGE on 5% gels was performed under reducing conditions with the affinity-purified CSPG, versican, neurocan, phosphacan, and brain membrane extracts. The proteins were then transferred to nitrocellulose membranes. The membranes were probed with two polyclonal antibodies against versican, a polyclonal antibody against neurocan, and a polyclonal antibody against phosphacan, all of which are known to react with the mouse homologs. Only the polyclonal antibody against phosphacan recognized the EGF-L affinity-purified CSPG at about 500–600 kDa, which is the apparent molecular weight of phosphacan (Fig. 3A). The polyclonal antibodies against versican did not show detectable bindings to the CSPG (not shown), and the polyclonal antibody against neurocan only showed a very weak binding to the EGF-L affinity-purified CSPG (not shown).

To confirm further that the EGF-L affinity-purified CSPG is a CSPG related to phosphacan, the EGF-L affinity-purified CSPG, phosphacan, and neurocan were treated with chondroitinase ABC. After separation by SDS-PAGE on 5 or 6% gels under reducing conditions, these proteins were transferred to nitrocellulose membranes and detected with the phosphacan or neurocan monoclonal antibodies. The phosphacan polyclonal antibody recognized the major broad band at 400 kDa resulting from treatment of the EGF-L affinity-purified CSPG with chondroitinase ABC (Fig. 3B), also detected by silver staining as shown in Fig. 2A, and a major broad band at 400 kDa of phosphacan after treatment with chondroitinase ABC. Neurocan and chondroitinase ABC were not immunoreactive with the phosphacan polyclonal antibody (Fig. 3B). Polyclonal antibody against neurocan did not react with the EGF-L affinity-purified CSPG, phosphacan, or chondroitinase ABC (not shown). Thus, the reactivity with the polyclonal antibody against phosphacan revealed that the EGF-L affinity-purified CSPG is a phosphacan-related molecule.

**Immunohistochemical Analysis of the Expression of the EGF-L Affinity-purified CSPG in the Retina and Optic Nerve**—To analyze whether the EGF-L affinity-purified CSPG and TN-R are co-localized in vivo, the localization of both molecules was studied immunohistochemically in tissue sections from adult mouse optic nerve and retina. TN-R immunoreactivity was present in the retinal nerve fiber layer and the inner and outer plexiform layer (Fig. 4a). In the optic nerve, TN-R immunoreactivity was restricted to the myelinated part of the nerve and barely detectable in the unmyelinated region of the nerve near the retina (Fig. 4a). Spots of increased TN-R posi-
tivity in the myelinated part of the optic nerve (Fig. 4c) correspond to an accumulation of the molecule at nodes of Ranvier (32). Interestingly, anti-phosphacan immunoreactivity revealed a distribution in the adult mouse retina and optic nerve very similar to that described for TN-R (Fig. 4, b and d). In the retina, phosphacan immunoreactivity was detectable in the nerve fiber layer, and the inner and outer plexiform layer (Fig. 4b). Similarly to TN-R, the strongest expression of phosphacan was observed in the outer plexiform layer (compare Fig. 4, a and b). The unmyelinated proximal part of the optic nerve was weakly stained by anti-phosphacan antibodies, whereas the myelinated distal part of the nerve was strongly immunoreactive (Fig. 4b). Remarkably, spots of increased phosphacan immunoreactivity were visible in the myelinated part of the optic nerve, suggesting that phosphacan, like TN-R, is accumulated at nodes of Ranvier (compare Fig. 4, c and d). As a negative control, no labeling was observed when primary antibodies were omitted (not shown). It remains to be determined if this immunoreactivity with anti-phosphacan antibodies reflects the localization of phosphacan or a newly identified phosphacan-related molecule.

The EGF-L Affinity-purified Phosphacan-related Molecule Exerts Inhibitory Effects on Neurite Outgrowth of Hippocampal Neurons in Vitro—According to several lines of evidence, CSPGs can act as either positive and negative modulators of axonal growth (9–11, 13, 40, 55–59). As shown in Fig. 1A, the EGF-L affinity-purified CSPG was detectable in hippocampus, and we tested its effects on neurite outgrowth of hippocampal neurons. The EGF-L affinity-purified CSPG was coated into 96-well multiwell dishes. Hippocampal neurons were plated at low density (36). On substrate-coated PLL neurites grew poorly, as described previously (39) (Figs. 5F and 7). Interestingly, the EGF-L affinity-purified CSPG strongly inhibited neurite outgrowth (Fig. 5A). On this substrate, only about 32% of all cells extended neurites (Fig. 6), and most cell bodies with concentrically extending lamellipodia were seen (Fig. 5A). To further support that the EGF-L affinity-purified CSPG is an inhibitory substrate and does not contain neurotoxic components that are heat-stable, we heated the EGF-L affinity-purified CSPG at 100 °C for 10 min and then coated it on PLL-treated plates. On this substrate, neurite extension was better than on the EGF-L affinity-purified CSPG (Figs. 5B and 7) and the numbers of cells with neurites increased to about 61% (Fig. 6). These results indicate that the EGF-L affinity-purified CSPG exerts inhibitory effects on the neurite outgrowth of hippocampal neurons.

Interaction between EGF-L Affinity-purified Phosphacan-related Molecule and TN-R Promotes Neurite Outgrowth of Hippocampal Neurons in Vitro—We investigated co-effects of TN-R and the EGF-L affinity-purified CSPG on neurite outgrowth from hippocampal neurons in cultures. For these assays, a mixture of the EGF-L affinity-purified CSPG and TN-R was coated into 96-well dishes, and hippocampal neurons were then plated. TN-R has been shown to modulate neurite extension of hippocampal neurons by promoting neurite outgrowth and inducing polarization (36). On the control substrates laminin, TN-R, and L1 (Fig. 5, C–E, Fig. 6; Table I), neurites grew well, whereas on PLL alone, neurites grew poorly (Fig. 6; Table I), and as mentioned above, the EGF-L affinity-purified CSPG was inhibitory for neurite outgrowth (Fig. 5, A and B; Figs. 6 and 7; Table I). Conversely, well developed morphology of neurites was seen on mixed TN-R/EGF-L affinity-purified CSPG substrates as determined by microscopy (Fig. 5G) and morphology (Fig. 6 and 7; Table I). Interestingly, this mixture even increased the length of both the longest neurite and the total neurites by 39 and 23%, respectively, compared with the TN-R substrate (Fig. 7; Table I). The number of neurites per cell was not significantly different from that of TN-R alone (Table I). Thus, the complex between the EGF-L affinity-purified CSPG and TN-R exhibits the best activity for promotion of neurite outgrowth, comparing with TN-R or CSPG, when presented as a uniform substrate.

The EGF-L Affinity-purified Phosphacan-related Molecule Neutralizes Growth Cone Repulsion Induced by TN-R—To support further that the EGF-L affinity-purified CSPG is a functional partner of TN-R, we investigated the co-effects of these two molecules on growth cone repulsion from cerebellar microexplant cultures. For these experiments, molecules and mixtures of molecules were coated onto an adhesive substrate, and their ability to modify the adhesivity of this substrate was assessed (39, 52, 60, 61). The different molecules and mixtures were coated as spots in tissue culture dishes coated with PLL on translucent nitrocellulose. Freshly dissociated cerebellar microexplants were plated onto these substrates. Under these conditions, the perimeters of the substrate spots represent a substrate border for neurites extending from explants located...
between the spots on the permissive substrate (Fig. 8). As shown previously (37–39), TN-R has repulsive properties for growth cones approaching from a permissive substrate to the border of an area coated with TN-R (Fig. 8A). The EGF-L affinity-purified CSPG alone was not repellent for growth cones or neurites (Fig. 8B). The mixture of the EGF-L affinity-purified CSPG/TN-R allowed the neurites to cross the border (Fig. 8C). The blocking effect of the EGF-L affinity-purified CSPG was dose-dependent (not shown). Strong repulsive effects were seen with the mixtures of the heat-treated EGF-L affinity-purified CSPG/TN-R and laminin/TN-R (Fig. 7, E–G). These observations together with the recent findings on the role of Ca\(^{2+}\)-binding EGF domain in protein-protein interaction indicate that Ca\(^{2+}\) binding to EGF repeats maintains the amino-terminal region in a conformation able to mediate protein-protein contacts (62). We observed, using EGF-L affinity chromatography and overlay binding assays, that binding of the EGF-L affinity-purified CSPG with EGF-L domain was Ca\(^{2+}\)-dependent (Fig. 1). To test further whether the interaction of TN-R with the EGF-L affinity-purified CSPG occurred as a protein-protein interaction, we investigated whether the glycoprotein core of the EGF-L affinity-purified CSPG also had the ability to block the repulsive effects induced by TN-R on growth cones. The EGF-L affinity-purified CSPG was treated with chondroitinase ABC and then coated as a mixture with TN-R as mentioned above. The mixture of TN-R/glycoprotein core of the EGF-L affinity-purified CSPG was not repellent for growth cones or neurites (Fig. 7D). Strong repulsive effects similar to those on TN-R substrate were seen with the mixtures of the heat-treated phosphacan-related molecule/TN-R, laminin/TN-R, and chondroitinase ABC/TN-R (Fig. 7, A, E–G). These observations together with the recent findings on the role of Ca\(^{2+}\)-binding EGF domain in protein-protein interaction indicate that Ca\(^{2+}\) binding to EGF repeats maintains the amino-terminal region in a conformation able to mediate protein-protein contacts (62).
Phosphacan: a Tenascin-R Binding Protein

FIG. 6. Effects of the EGF-L affinity-purified phosphacan-related molecule on neurite outgrowth of hippocampal neurons.

Hippocampal neurons were plated as single cell suspensions on the EGF-L affinity-purified CSPG (CSPG) and other substrates applied to PLL-treated tissue culture plastic. Cells were maintained for 12 h before fixation and staining with toluidine blue. Coating concentration was 125 nm for all proteins. Bars represent percentages of cells with neurites (mean ± S.D.) from at least three independent experiments. Bars marked by * are significantly (p < 0.05) different from the control (CSPG after heating at 100 °C).

TABLE I

| Substrate | Length of total neurites | Number of neurites/cell | Number of measured cells |
|-----------|-------------------------|-------------------------|-------------------------|
| % PLL     |                         |                         |                         |
| CSPG      | 130                     | 192                     | 172                     | 302                     |
| CSPG 100  | 183                     | 273                     | 172                     | 301                     |
| L1        | 303                     | 327                     | 167                     | 300                     |
| Laminin   | 247                     | 308                     | 182                     | 302                     |
| TN-R      | 217                     | 323                     | 241                     | 300                     |
| PLL       | 100                     | 100                     | 100                     | 300                     |
| CSPG + TN-R| 217                   | 323                     | 241                     | 300                     |
| BSA + TN-R| 144                     | 200                     | 176                     | 300                     |

The phosphacan-related molecule by itself had an overall inhibitory effect on neurite outgrowth of hippocampal neurons. Interestingly, the complex of the phosphacan-related molecule/TN-R promoted neurite outgrowth of these neurons when coated as a uniform substrate. The phosphacan-related molecule also inhibited growth cone repulsion induced by a sharp substrate border of TN-R. The interaction of TN-R with the phosphacan-related molecule is likely not dependent on the glycosaminoglycan moiety. These results agree with the view that TN-R provides multiple recognition sites that are able to interact in a complex manner with other recognition molecules in certain microenvironments and that these interactions influence axonogenesis (29, 39).

The EGF-L Affinity-purified Phosphacan-related Molecule Is a Member of the Receptor Protein-Tyrosine Phosphatase Subfamily Related to Phosphacan—Recent findings demonstrated that restrictin (a TN-R homolog in chick) co-purifies with two brain CSPGs including neurocan and phosphacan (20) and that the versican C-type lectin domain is able to bind to TN-R (54). We have used four soluble TN-R fragments, including EGF-L, EGF-S, FN6–8, and FG domains, to probe TN-R-binding components from brain membranes. A single TN-R binding protein was identified with the EGF-L domain of TN-R, an aminoterminal fragment. Under the same assay conditions, the other three fragments have not shown detectable binding to the molecule. We have further succeeded in isolating the same molecule with EGF-L affinity chromatography, and the interaction is specific for EGF-L because the 500–600-kDa CSPG was not retained by a column containing EGF-S. The binding of EGF-L to the 500–600-kDa CSPG in crude extracts of brain membranes and the EGF-L affinity-purified molecule was specific, as shown by competition of radiolabeled EGF-L with unlabeled EGF-L. The epitopes of the EGF-L affinity-purified molecule recognized by 473-HD antibody were sensitive to chondroitinase ABC in support of the fact this molecule is a CSPG. Furthermore, the recognition of the protein core by polyclonal antibodies to phosphacan, but not by a neurocan...
antibody and two versican antibodies, suggests that this CSPG is a member of the RPTPz/b subfamily related to phosphacan (6). The existence of other receptors cannot be ruled out. For example, F3, a glycosylphosphatidylinositol-linked membrane protein as a TN-R receptor has been shown to bind to EGF-L domain (39). However, it is known that F3 is not soluble in the presence of Triton X-100 (63) used here to solubilize the CSPG. Taken together, the above findings implicate the existence of interactions between TN-R and a phosphacan-related molecule and indicate that the EGF-L of TN-R is a ligand for this molecule.

Binding of EGF-L to the Glycoprotein Core of the Phosphacan-related Molecule—Because of the widespread distribution of EGF-like repeats among extracellular matrix molecules, there has been considerable interest in their biological role. Results from mutagenesis studies (64–66) and recent crystal structure investigations (62) strongly suggest that EGF-like repeats are involved in mediating protein-protein interactions via the binding of Ca$^{2+}$ to EGF repeats to maintain the conformation of the amino-terminal region (67). A subset of EGF-like domains contains five amino acids that constitute a Ca$^{2+}$-binding consensus sequence: Asp/Asn, Asp/Asn, Gln/Glu, Asp/Asn, and Tyr/Phe. The role of binding of Ca$^{2+}$ to EGF-like repeats is to stabilize protein-protein contacts. In agreement with this view, both binding of EGF-L domain to the phosphacan-related molecule and purification of this protein on EGF-L affinity columns depend on the presence of Ca$^{2+}$. On the other hand, the observations in functional assays that 1) chondroitinase-treated phosphacan-related molecule is even more potent than the intact molecule in neutralizing TN-R-induced growth cone repulsion, and 2) that the heat-treated phosphacan-related molecule loses its functional properties toward neurite outgrowth favor the view that the interaction between the two molecules is not dependent on glycosaminoglycans or other carbohydrates. The interaction between the phosphacan-related molecule and EGF-L is sufficient to mask the inhibitory epitopes in EGF-L. Interestingly, EGF-S comprising the EGF-like repeats without the cysteine-rich stretch did not show detectable binding to the phosphacan-related molecule, suggesting that the cysteine-rich stretch may influence the conformation of the EGF-like repeats.

The Phosphacan-related Molecule Exerts Inhibitory Effects on Neurite Outgrowth—Several CSPGs have been implicated as negative or positive modulators of axonal growth (10, 13, 55, 56). For instance, neurocan co-coated with Ng-CAM as a uniform substrate has shown inhibitory properties for neurite outgrowth from chick embryo neurons (12). CSPGs are found up-regulated in central nervous system lesions, and it has been
proposed that their inhibitory properties might influence axonal regeneration in the lesioned adult central nervous system (57–59). RPTPζ/β is expressed on radial glial cells in the developing central nervous system, and it has been suggested that phosphacan has inhibitory effects on neuronal migration (14). Similar to these CSPGs, the substrate-coated phosphacan-related molecule prevented neurite outgrowth of 70% of all hippocampal neurons in culture. These inhibitory effects were reduced by heat treatment, indicating that the inhibitory effects of the phosphacan-related molecule is due to its protein core. It remains to be investigated how the inhibitory effects are related to central nervous system development and regeneration in the adult.

The Phosphacan-related Molecule Neutralizes the Inhibitory Effects Induced by TN-R—It has been shown that CSPGs can interact with recognition molecules to modulate neuronal adhesion and signal transduction. For instance, neurocan and phosphacan inhibit homophilic interactions of Ng-CAM and N-CAM and the binding of neurons to these cell adhesion molecules (68). Neurocan exhibits binding properties for the neuronal cell adhesion molecules Ng-CAM and N-CAM and the extracellular matrix glycoprotein TN-C (12, 15). Extending these studies, we found that interactions of the phosphacan-related molecule with TN-R, a neuronal growth inhibitory molecule, when offered as a sharp substrate boundary modulate neurite outgrowth. Interestingly, the phosphacan-related molecule by itself exerts inhibitory effects on neurite extension from hippocampal neurons, whereas the complex of the phosphacan-related molecule and TN-R not only promotes neurite outgrowth of hippocampal neurons, once neurite outgrowth is initiated, but also neutralizes the repulsion of growth cones induced by TN-R from cerebellar microexplants. One possibility is that the binding between the phosphacan-related molecule and EGF-L functionally neutralizes or sterically blocks the inhibitory sites of both molecules. Alternatively, the interaction of TN-R with the phosphacan-related molecule could directly trigger intracellular signaling cascades since the phosphacan-related molecule is a member of the RPTPζ/β subfamily that comprises transmembrane molecules with two phosphatase domains in their intracellular segments. TN-R can exert repulsive effects on cells and its interaction with RPTPζ/β, which may favor dephosphorylation of tyrosyl residues, would offer a counterpoint force to adhesion effects related to protein-tyrosine kinases, such as focal adhesion kinase (18). Although the mechanisms of the interactions of TN-R with the phosphacan-related molecule have yet to be elucidated, the results here strongly implicate the interactions of TN-R with the phosphacan-related molecule in important regulatory roles in axonogenesis. It remains to be investigated whether the phosphacan-related molecule, like TN-R and other multi-functional molecules, is composed of neurite outgrowth inhibitory and promoting domains. The next steps are to unravel the signal transduction pathways initiated by TN-R binding to this protein. Because TN-R has multiple cell surface receptors, such as F3 and MAG (38, 39), it will be of particular interest to obtain more information about the interplay among these molecules and the functional consequences of their interactions with each other and their ligands.

Acknowledgments—We are grateful to Gui-Xia Yu for excellent technical assistance; Drs. Anders Aspberg, Richard Le Baron, Erkki Ruoslahti, and Dieter R. Zimmermann for the kind gifts of antibodies to versican; and Dr. Richard Margolis for comments on the manuscript.

REFERENCES
1. Margolis, R. K., and Margolis, R. U. (1993) *Experientia* (Basel) 49, 429–446
2. Zimmermann, D. R., and Ruoslahti, E. (1989) *EMBO J.* 8, 2975–2981

2 Z.-C. Xiao et al., manuscript in preparation.
55. Snow, D., Lemmon, V., Carrino, D., Caplan, A., and Silver, J. (1990) *Exp. Neurol.* **109**, 11–130
56. Britte, P. A., Canning, D. R., and Silver, J. (1992) *Science* **255**, 733–736
57. McKeon, R. J., Schreiber, R. C., Rudge, J. S., and Silver, J. (1991) *J. Neurosci.* **11**, 3398–3411
58. Bovolenta, P., Wandosell, F., and Nieto-Sampedro, M. (1993) *Eur. J. Neurosci.* **5**, 454–465
59. Pindzola, R. R., Deller, C., and Silver, J. (1993) *Dev. Biol.* **156**, 24–48
60. Faissner, A., Kruse, J., Kühn, K., and Schachner, M. (1990) *J. Neurochem.* **54**, 1004–1015
61. Prieto, A. L., Andersson-Fisone, C., and Crossin, K. L. (1992) *J. Cell Biol.* **119**, 663–678
62. Rao, Z. H., Handford, P., Mayhew, M., Knott, V., Brownlee, G. G., and Stuart, D. (1995) *Cell* **82**, 131–141
63. Olive, S., Dubois, C., Schachner, M., and Rougon, G. (1995) *J. Neurochem.* **65**, 2307–2317
64. Winship, P. R., and Dragon, A. C. (1991) *Br. J. Haematol.* **77**, 102–109
65. Dietz, H. C., McIntosh, I., Sakai, L. Y., Corson, G. M., Chalberg, B. C., Pyeritz, R. E., and Francomano, C. A. (1993) *Genomics* **17**, 468–475
66. Hewett, D. R., Lynch, J. R., Smith, R., and Sykes, B. C. (1993) *Hum. Mol. Genet.* **2**, 475–477
67. Campbell, I. D., and Bork, P. (1993) *Curr. Opin. Struct. Biol.* **3**, 385–392
68. Grunet, M., Placeu, A., and Margelis, R. U. (1993) *J. Cell Biol.* **120**, 815–824
69. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254