6B4 proteoglycan/phosphacan is a repulsive substratum but promotes morphological differentiation of cortical neurons

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SUMMARY

6B4 proteoglycan/phosphacan is one of the major phosphate-buffered saline-soluble chondroitin sulfate proteoglycans of the brain. Recently, this molecule has been demonstrated to be an extracellular variant of the proteoglycan-type protein tyrosine phosphatase, PTPζ (RPTPβ). The influence of the 6B4 proteoglycan, adsorbed onto the substratum, on cell adhesion and neurite outgrowth was studied using dissociated neurons from the cerebral cortex and thalamus. 6B4 proteoglycan adsorbed onto plastic tissue culture dishes did not support neuronal cell adhesion, but rather exerted repulsive effects on cortical and thalamic neurons. When neurons were densely seeded on patterned substrata consisting of a grid-like structure of alternating poly-L-lysine and 6B4 proteoglycan-coated poly-L-lysine domains, they were concentrated on the poly-L-lysine domains. However, 6B4 proteoglycan did not retard the differentiation of neurons but rather promoted neurite outgrowth and development of the dendrites of cortical neurons, when neurons were sparsely seeded on poly-L-lysine-conditioned coverslips continuously coated with 6B4 proteoglycan. This effect of 6B4 proteoglycan on the neurite extension of cortical neurons was apparent even on coverslips co-coated with fibronectin or tenascin. By contrast, the neurite extension of thalamic neurons was not modified by 6B4 proteoglycan. Chondroitinase ABC or keratanase digestion of 6B4 proteoglycan did not affect its neurite outgrowth promoting activity, but a polyclonal antibody against 6B4 proteoglycan completely suppressed this activity, suggesting that a protein moiety is responsible for the activity. 6B4 proteoglycan transiently promoted tyrosine phosphorylation of an 85×10^3 M_r protein in the cortical neurons, which correlated with the induction of neurite outgrowth. These results suggest that 6B4 proteoglycan/phosphacan modulates morphogenesis and differentiation of neurons dependent on its spatiotemporal distribution and the cell types in the brain.

Key words: 6B4 proteoglycan, phosphacan, proteoglycan, neurite outgrowth, tyrosine phosphorylation, rat

INTRODUCTION

Recently, it has been recognized that proteoglycans are important constituents of the extracellular matrix in the developing nervous system (Oohira et al., 1988; Herndon and Lander, 1990; Margolis and Margolis, 1993). Proteoglycans are composed of a core protein on which sulfated glycosaminoglycans are covalently attached. Sulfated glycosaminoglycans are classified into chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate and heparin. Glycosaminoglycans bind several extracellular matrix proteins and growth factors, and play important regulatory roles in processes such as cell adhesion, cell migration and differentiation (Ruoslahti, 1989; Ruoslahti and Yamaguchi, 1991). Several researchers have described glycosaminoglycans as repulsive substrata for neuronal adhesion and neurite outgrowth (Snow et al., 1990) and as a regulator of neuronal polarity (Lafont et al., 1992, 1994; Fernaud-Espinosa et al., 1994; Brittis and Silver, 1994). However, to elucidate the functional roles of brain proteoglycans, it is necessary to use native proteoglycans isolated from the brain.

We have recently cloned 6B4 proteoglycan (Maeda et al., 1994), one of the major phosphate-buffered saline (PBS)-soluble chondroitin sulfate proteoglycans of the brain, and established its purification method (Maeda et al., 1995). This proteoglycan was independently cloned by another group and called phosphacan (Mauel et al., 1994). 6B4 proteoglycan/phosphacan is an extracellular variant of a receptor-like protein tyrosine phosphatase, PTPζ (RPTPβ), which is also a membrane-bound chondroitin sulfate proteoglycan (Maeda et al., 1994; Barnea et al., 1994). This directly implies that chondroitin sulfate proteoglycans have a role in cell signaling.

6B4 proteoglycan/phosphacan is specifically expressed in the central nervous system and is developmentally regulated in various brain regions (Milev et al., 1994; Maeda et al., 1995). In the rat hindbrain, the expression of 6B4 proteoglycan is correlated with the development of the cerebellar mossy fiber system (Maeda et al., 1992). In the embryonal rat neocortex, 6B4 proteoglycan was detected along the radial glial fibers, a scaffold for neuronal migration (Maeda et al., 1995). In the
early postnatal rat neocortex, 6B4 proteoglycan is distributed uniformly throughout most of the cortical regions (Maeda et al., 1995). Immunohistochemical analysis with antisera against a RPTPβ peptide revealed a similar distribution in the rat neocortex (Canoll et al., 1993). These results suggest that 6B4 proteoglycan/phosphacan and PTPζ (RPTPβ) play important roles coordinately in neuronal cell migration, differentiation and circuit formation. Margolis and colleagues reported recently that phosphacan binds tenascin and Ng-CAM/L1, and inhibits neurite extension of chick brain neurons (Grumet et al., 1994; Miley et al., 1994). However, considering the complexity in the structure and expression pattern of 6B4 proteoglycan/phosphacan, it is premature to conclude that this molecule is an inhibitor of neurite outgrowth. We have demonstrated previously that purified 6B4 proteoglycan promotes neurite outgrowth of rat cortical neurons (Maeda et al., 1995).

In this study, we further analyzed the effects of 6B4 proteoglycan on rat cortical and thalamic neurons under various culture conditions. We found that 6B4 proteoglycan displays a repulsive effect on both cortical and thalamic neurons under conditions of high cell density in culture. Under low cell density culture conditions, in contrast, promotion of the morphological differentiation of cortical but not of thalamic neurons was clearly seen on 6B4 proteoglycan-coated substrata. Furthermore, 6B4 proteoglycan was found to promote the tyrosine phosphorylation of specific proteins, and the pattern of this phosphorylation was correlated with cellular susceptibility to 6B4 proteoglycan. These results suggest that 6B4 proteoglycan/phosphacan is an important regulator of the morphogenesis of neurons.

MATERIALS AND METHODS

Materials

6B4 proteoglycan was purified as described previously (Maeda et al., 1995). mAb 6B4 and polyclonal antisera against purified 6B4 proteoglycan (anti-6B4 proteoglycan) and antisera 31-5 (anti-31-5) were described previously (Maeda et al., 1992, 1994). Fibronectin was purchased from Nitta Gelatin. Tenascin purified from human glioma cell line u-251MG was from Chemicon International Inc. Dulbecco’s modified Eagle’s medium, F12 medium and B-27 supplement were purchased from Gibco. Protease-free chondroitinase ABC, keratanase and antikeratan sulfate monoclonal antibody 5-D-4 were from Seikagaku. CMF-HBSS was purchased from Nitta Gelatin. Tenascin purified from human glioma cell line u-251MG was from Chemicon International Inc. Dulbecco’s modified Eagle’s medium, F12 medium and B-27 supplement were purchased from Gibco. Protease-free chondroitinase ABC, keratanase and anti-keratan sulfate monoclonal antibody 5-D-4 were from Seikagaku Kogyo Co. Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc. A cocktail of monoclonal antibodies to phosphorylated neurofilaments, SMI 312, was obtained from Sternberger Monoclonals Inc. Antiserum against microtubule-associated protein 2 (MAP2) was a kind gift from Dr. M. Niinobe (Niinobe et al., 1988). HNK-1 monoclonal antibody was from Sorotec. FITC-conjugated anti-mouse IgG was from Jackson ImmunoResearch. Texas Red Avidin D and Vectastain ABC kit were from Vector Labs. Biotinylated anti-rabbit Ig, biotinylated anti-mouse Ig and streptavidin-conjugated alkaline phosphatase were from Amersham. PermaFluor was from Immunon. Poly-L-lysine (M₉ > 300 × 10⁵), rabbit IgG and mouse IgM (MOPC104E) were purchased from Sigma. ¹²⁵I-Bolton-Hunter reagent was from NEN. Micro BCA kit was from Pierce. HiTrap Protein G was from Pharmacia.

Preparation of dissociated neurons and astrocytes

Cerebra and thalami were dissected from embryonic day-16 (E16) Sprague-Dawley rats, and the meninges were removed. The tissues were incubated in Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution (CMF-HBSS) containing 0.1% trypsin for 15 minutes at 37°C. After three washes with CMF-HBSS, the tissues were triturated with Pasteur pipettes in CMF-HBSS containing 0.025% DNAase I, 0.4 mg/ml soybean trypsin inhibitor, 3 mg/ml bovine serum albumin and 12 mM MgSO₄. The cell suspension was centrifuged at 160 g for 5 minutes at 4°C, and the pelleted cells were washed once with CMF-HBSS. The cells were suspended in culture medium consisting of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F12 medium (DF medium) containing 2% B-27 supplement. Cell suspensions were seeded as described below. Astrocytes were prepared from E16 and E19 Sprague-Dawley rats according to the method described by Kato et al. (1979).

Cell culture

For cell adhesion experiments, 10 µg/ml 6B4 proteoglycan (as protein) diluted in PBS was spotted (30 µl) into each well of a 48-well Falcon tissue culture plates, which were then incubated overnight at 37°C. After three washes with CMF-HBSS, 150 µl of a 3×10⁶ cells/ml cell suspension was seeded into each well. After 2 hours of incubation at 37°C under 5% CO₂, cell layers were gently washed with PBS. For the preparation of 6B4 proteoglycan-coated substrata, glass coverslips (13 mm in diameter) were incubated in the solution containing 0.002% poly-L-lysine, washed five times with distilled water, and then air-dried. 6B4 proteoglycan diluted in PBS was spotted (10 µl) onto the poly-L-lysine-coated coverslips, which were then incubated overnight at 37°C. The coverslips were washed five times with CMF-HBSS, and used for cell culture. Poly-L-lysine-coated or uncoated coverslips were treated for 3 hours at room temperature with 10 µg/ml fibronectin or tenascin (10 µl/coverslip), washed three times with CMF-HBSS, and then coated with 6B4 proteoglycan (10 µg/ml, 10 µl) as described above. 40 µl of a 1×10⁴ cells/ml cell suspension were plated on the coverslips in a 24-well culture plate. After 60 minutes of incubation at 37°C under 5% CO₂, 0.5 ml of DF medium containing 2% B-27 supplement was added per well. The plating efficiency of cortical and thalamic neurons 3 hours after plating was more than 90%. Viability of cells as determined by trypan blue exclusion was more than 90% after 20 hours of culture, and more than 75% after 45 hours of culture. Cultures of both cortical and thalamic neurons were estimated to be 98% pure neurons judging from the immunohistochemical staining with anti-MAP2 and anti-neurofilament antibodies.

When 6B4 proteoglycan was applied in the soluble form, the poly-L-lysine-coated coverslips were first adsorbed with 0.25% bovine serum albumin and then a cell suspension (1×10⁵ cells/ml) was plated on the coverslips in a 24-well culture plate. After 60 minutes of incubation at 37°C under 5% CO₂, 0.5 ml per well of DF medium containing 10 µg/ml 6B4 proteoglycan and 2% B-27 supplement was added. Under these conditions, it was verified that 6B4 proteoglycan was not adsorbed to the substrata using ¹²⁵I-labeled proteoglycan (see below).

To prepare the patterned substrata, 6B4 proteoglycan-coated poly-L-lysine-conditioned coverslip surfaces were gently scraped with the tips of a Gilson pipetman. 20-µl aliquots of 3×10⁶ cells/ml cell suspension were seeded on the coverslips in 24-well culture plates, and 0.5 ml of DF medium containing 2% B-27 supplement was added per well after 60 minutes of incubation at 37°C under 5% CO₂. The plates were incubated at 37°C under 5% CO₂ for the indicated periods.

To test neutralizing activity of various antibodies (anti-6B4 proteoglycan antibody, anti-31-5 antibody, HNK-1 monoclonal antibody, IgG and IgM) on the neurite outgrowth-promoting activity, poly-L-lysine-conditioned coverslips coated with 2.5 µg/ml 6B4 proteoglycan were treated for 3 hours at 37°C with each antibody (100 µg/ml), washed three times with CMF-HBSS, and used for culture.

Morphological analysis

Neurite length and soma size of 100 neurons, selected randomly from three wells for each substratum condition, were measured. Neurites
were defined as processes extending from the cell body by more than the cell diameter. Neurite lengths were measured from the base to the tip. Soma surface areas were calculated from the long diameter of the cell somas, treating them as if they were circular. Each experiment was performed at least three times, and the results of typical experiments are shown.

**Enzyme digestion**

1 µg of 6B4 proteoglycan in 20 µl of 0.1 M Tris-HCl, pH 7.5, 30 mM sodium acetate was treated for 60 minutes at 37°C with 1 µl of keratanase and/or 0.7 µl of protease-free chondroitinase ABC. The samples were diluted with PBS and used for coating the poly-L-lysine-conditioned coverslips. Enzyme digestion was monitored by western blotting with polyclonal anti-6B4 proteoglycan and anti-keratan sulfate monoclonal antibody, 5-D-4. Complete removal of chondroitin sulfate and keratan sulfate was confirmed by dot blot analysis using 5-D-4 and anti-chondroitin sulfate monoclonal antibody (CS-56). Since dialysis of the samples to remove glycosaminoglycan digests had no effect on the activity, the enzyme-treated samples were used directly in the following experiments.

**125I-labeling of 6B4 proteoglycan**

8 µg of 6B4 proteoglycan were precipitated with 70% ethanol containing 1% sodium acetate, and the precipitate was washed three times with 70% ethanol. After dissolution in 15 µl of 0.1 M sodium phosphate buffer, pH 8.0, the protein was applied to the dried 125I-Bolton-Hunter reagent (35 µCi). The reaction mixture was incubated at 4°C for 3 hours, and then 15 µl of 1 M glycine was added to the solution. After incubation at 4°C for 1 hour, free 125I-Bolton-Hunter reagent was removed by ethanol precipitation, and the protein precipitate was dissolved in 150 µl of PBS. The specific radioactivity of the protein thus prepared was 1.2x10^6 cpm/µg.

**Immunocytochemistry**

 Cultures on the patterned substratum were washed once with PBS and incubated for 15 minutes in 4% paraformaldehyde/0.1 M sodium phosphate, pH 7.4. Fixed cells were washed three times with Tris-buffered saline (TBS), and incubated for 30 minutes in polyclonal anti-6B4 proteoglycan antiserum solution (1/1000). After washing three times with PBS, cells were incubated for 30 minutes in biotinylated anti-rabbit Ig solution (1/200), washed three times with PBS, and incubated for 30 minutes in Texas Red Avidin D solution (1/1000). After three washes with PBS, cells were mounted in PermaFluor and observed with a Zeiss fluorescence microscope.

Double-immunostaining was performed as follows. Cultures were washed once with PBS and then fixed with 4% paraformaldehyde/0.1 M sodium phosphate buffer, pH 7.4, for 15 minutes. Fixed cells were rinsed three times with TBS and blocked with 2% BSA/4% goat serum in PBS. Cells were then incubated for 2 hours with anti-MAP2 antiserum (1/2000) and anti-phosphorylated neurofilament antibodies, SMI 312 (1/500). After three washes with PBS, cells were incubated for 30 minutes with FITC-conjugated anti-mouse IgG (1/100) and biotinylated anti-rabbit Ig, followed by three washes with PBS, and then, finally incubated for 30 minutes with Texas Red Avidin D (1/1000). Cells were washed three times with PBS, mounted and observed as above. All solutions were diluted with PBS containing 0.05% BSA. Incubations were at room temperature.

**Immunohistochemistry**

After ether anesthesia, Sprague-Dawley rats were perfused with PBS, followed by a solution containing 4% paraformaldehyde and 0.1 M sodium phosphate buffer, pH 7.4, via the left ventricle, and washed out from the right atrium. The brains were then dissected out and embedded in paraffin after dehydration through a graded alcohol series. Paraffin-embedded samples were cut into sections 6 µm thick, which were then deparaffinized and equilibrated in PBS. The sections were incubated sequentially in the following solutions: (1) 2.5% H2O2/PBS for 60 minutes; (2) 1% BSA/4% goat serum/PBS for 60 minutes; (3) culture supernatant containing mAb 6B4 for 24 hours at 4°C; (4) biotinylated anti-mouse IgM solution for 60 minutes; (5) avidin-biotin-peroxidase complex solution for 30 minutes; (6) 0.1% diaminobenzidine/0.02% H2O2/PBS. A Vectastain ABC kit was used according to the supplier’s protocol.

**Detection of tyrosine-phosphorylated proteins**

Suspended cells (6x10^5 in 200 µl of DF medium) were seeded onto poly-L-lysine-coated coverslips (3 cm in diameter) untreated or treated with 10 µg/ml 6B4 proteoglycan. After incubation at 37°C under 5% CO2, cells were solubilized by adding 200 µl of 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 µM pepstatin A, 5 mM EDTA, 4 mM sodium vanadate, 100 mM NaF, 20 µM sodium pyrophosphate, 2% NP-40, 0.1% SDS, 100 mM Tris-HCl, pH 7.5. The solutions were mixed with three volumes of 95% ethanol containing 1.3% sodium acetate and incubated for 30 minutes at ~20°C. After centrifugation, the precipitated proteins were treated with SDS-PAGE sample buffer and separated by electrophoresis on 10% SDS-polyacrylamide gels according to the method of Laemmli (1970). Proteins were transferred to PVDF membranes, which were then blocked with 5% nonfat dried milk in PBS, incubated for 30 minutes with 4G10 monoclonal antibody (1/1000), and washed three times with PBS. The membranes were incubated for 30 minutes with biotinylated anti-mouse Ig (1/2000), washed three times with PBS, and incubated for 30 minutes with streptavidin-conjugated alkaline phosphatase (1/1000). After washing three times with PBS, the membranes were treated with 0.3 mg/ml nitroblue tetrazolium, 0.18 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 0.1 M NaCl, 50 mM MgCl2, 0.1 M Tris-HCl, pH 9.5. Antibodies and alkaline phosphatase were diluted with 0.05% BSA/PBS.

**Other methods**

Protein concentration was determined using a MicroBCA kit employing BSA as a standard. IgG fractions from anti-6B4 proteoglycan and anti-31-5 antisera were prepared with HiTrap Protein G according to the supplier’s protocol.

**RESULTS**

**6B4 proteoglycan/phosphacan does not support cell adhesion**

Previously, we purified 6B4 proteoglycan/phosphacan from 18-day-old rat whole brains using a dissociative purification procedure (Maeda et al., 1995). This highly purified preparation was used in the present study to analyze the effects of 6B4 proteoglycan/phosphacan on cortical and thalamic neurons.

Multiwell plastic tissue culture dishes were spotted with 10 µg/ml 6B4 proteoglycan, and then cortical neurons were seeded into each well. After 2 hours incubation, the cell layer was gently washed with PBS. As shown in Fig.1, neurons did not attach to the 6B4 proteoglycan-coated area but did attach to the surrounding region. This indicates that 6B4 proteoglycan does not support cell adhesion, and suggests the repulsive nature of this proteoglycan. To study whether 6B4 proteoglycan is a repulsive substratum, the proteoglycan was adsorbed next to poly-L-lysine-conditioned coverslips on which neurons were plated at high cell density. The amount of 6B4 proteoglycan immobilized on poly-L-lysine varied linearly with the concentration used for the treatment over a range of 0.3-30
treated 6B4 proteoglycan (at 100°C for 15 minutes) was still observed when heat molecule (Fig. 2E,F). The patterned growth in influence the repulsive effect of this ABC digestion of 6B4 proteoglycan did not substrata (data not shown). Chondroitinase responded similarly to the patterned proteoglycan-coated areas (Fig. 2C,D). Cells neurons avoided 6B4 proteoglycan areas, but also neurites did not invade the 6B4 proteoglycan-coated area in addition to the surrounding region (data not shown).

The repulsive property of 6B4 proteoglycan was further examined using grid-patterned substrata as described by Faisstner and Kruse (1990). The patterned substrata were prepared by coating poly-L-lysine-conditioned coverslips with 30 μg/ml 6B4 proteoglycan and subsequently removing the proteoglycan with plastic pipette tips. The poly-L-lysine layer underneath was not affected by this treatment. When cortical neurons were cultured on the patterned substrata, about 60% (7 of 12) of the coverslips had a grid-like arrangement of neurons on them after 20 hours. In this experiment, neurons were concentrated on the poly-L-lysine areas, where a fine meshwork of neurites was formed (Fig. 2A,B). Neurons seeded on the 6B4 proteoglycan-coated area assembled into clumps, or moved into the poly-L-lysine area nearby. Not only neurons avoided 6B4 proteoglycan areas, but also neurites did not invade the 6B4 proteoglycan-coated areas (Fig. 2C,D). Cells and neurites showed the same reactions for 3 days in culture. Thalamic neurons also responded similarly to the patterned substrata (data not shown). Chondroitinase ABC digestion of 6B4 proteoglycan did not influence the repulsive effect of this molecule (Fig. 2E,F). The patterned growth of neurons was still observed when heat treated 6B4 proteoglycan (at 100°C for 15 minutes) was used as the coating, or the 6B4 proteoglycan coating was treated with anti-6B4 proteoglycan antibody (100 μg/ml) (data not shown).

This effect of 6B4 proteoglycan was concentration-dependent, and at 10 μg/ml, neurons also attached to the proteoglycan-coated areas and formed a meshwork of neurites (Fig. 2G,H). When fibronectin was used instead of 6B4 proteoglycan, no such patterned growth of neurons was observed (data not shown). These results indicate that 6B4 proteoglycan is a repulsive substratum for cortical and thalamic neurons.

### 6B4 proteoglycan/phosphacan promotes morphological differentiation of cortical neurons but not thalamic neurons under low-density culture conditions

Previously, we demonstrated that 6B4 proteoglycan promoted neurite extension of cortical neurons (Maeda et al., 1995). Further experiments were performed to investigate the effects of 6B4 proteoglycan on cortical and thalamic neurons. To examine morphological differentiation of individual neurons, cells were cultured at low density on 6B4 proteoglycan-coated poly-L-lysine-conditioned coverslips. The morphology of neurons was examined after 20 hours in culture; 55% of cortical and 65% of thalamic neurons developed neurites on poly-L-lysine-coated coverslips (Fig. 3A,C). However, on the poly-L-lysine-conditioned coverslips coated with 30 μg/ml 6B4 proteoglycan, in contrast, more than 95% of cortical neurons and 85% of thalamic neurons developed neurites (Fig. 3B,D). As shown in Fig. 4A, 6B4 proteoglycan promoted neurite extension of cortical neurons in a dose-dependent manner. In contrast, the neurite length distribution of thalamic neurons was not affected by 6B4 proteoglycan, although the ratio of neurite-bearing cells was increased slightly (Fig. 4B). However, it is noteworthy that the growth cones of thalamic neurons on the 6B4 proteoglycan-coated substrata were small (Fig. 3F) and clearly different from those on the control coverslips which were large and well-developed (Fig. 3E). Culture on 6B4 proteoglycan-treated substratum did not affect the viability of neurons as far as it was checked after 20 and 45 hours in culture (data not shown).

Cell bodies of cortical and thalamic neurons spread well on the control coverslips, while on 6B4 proteoglycan, they

![Fig. 1. 6B4 proteoglycan did not support cell adhesion. Cortical neurons were plated on 6B4 proteoglycan (10 μg/ml as protein) spotted on tissue culture plastic plates. Cultures were gently washed with PBS after 2 hours of incubation, and monitored with phase-contrast microscopy (A,B). Neurons on the 6B4 proteoglycan-spotted areas were detached by washing. Cortical neurons were seeded onto poly-L-lysine-conditioned coverslips, uncoated (C) or coated (D) with 30 μg/ml 6B4 proteoglycan. Neurons formed large cell aggregates on 6B4 proteoglycan-coated coverslips after 20 hours in culture (D). Scale bars, (A) 50 μm; (B) 200 μm; (C,D) 100 μm.](image-url)
Effects of 6B4 proteoglycan on neurite outgrowth appeared small and rounded (Fig. 3). This is consistent with the finding that cortical and thalamic neurons showed less adhesiveness to the 6B4 proteoglycan-coated areas in the patterned substrata when plated densely. Reduction of soma surface area was also dependent on the 6B4 proteoglycan concentration (Fig. 5). When 6B4 proteoglycan was applied to the culture medium in soluble form, no effect on neurite outgrowth (data not shown) or soma surface area (Fig. 5) was observed.

Next, fibronectin and tenascin were tested instead of poly-L-lysine. We selected these substances as physiological substrata, because fibronectin and tenascin codistribute with 6B4 proteoglycan during development of the cortex (Sheppard et al., 1991; Maeda et al., 1995). 6B4 proteoglycan also promoted neurite outgrowth of cortical neurons on fibronectin-coated coverslips, and on fibronectin- or tenascin-coated poly-L-lysine-conditioned coverslips (Table 1). Since cortical neurons were not adhesive, substrata coated only with tenascin or 6B4 proteoglycan were not examined.

6B4 proteoglycan is a complex glycoconjugate bearing chondroitin sulfate, keratan sulfate and HNK-1 carbohydrates. To examine whether these carbohydrate moieties or the core protein portion is involved in the neurite-promoting activity of 6B4 proteoglycan, it was digested with keratanase and/or chondroitinase ABC prior to use as substratum. As shown in Fig. 6A, enzyme digestion did not affect the promotion of neurite outgrowth by cortical neurons. Coating efficiency of 6B4 proteoglycan was not changed after enzyme digestion (Fig. 6B), indicating that chondroitin sulfate and keratan sulfate moieties are not active for the promotion of neurite outgrowth. Next, 6B4 proteoglycan-coated coverslips were pretreated with several antibodies, and the influence on the neurite outgrowth was examined (Fig. 7). A polyclonal antibody against 6B4 proteoglycan (anti-6B4 proteoglycan) completely suppressed the neurite promoting activity of the proteoglycan, although a polyclonal antibody against the C-terminal portion of 6B4 proteoglycan (anti-31-5) did not.
was not effective (Fig. 7). HNK-1 monoclonal antibody treatment did not affect the activity. Interestingly, despite the suppression of neurite outgrowth by anti-6B4 proteoglycan, reduction of soma surface area of the cortical neurons on 6B4 proteoglycan substratum was still observed (data not shown).

6B4 proteoglycan promoted development of the dendrites of cortical neurons

We next examined the nature of the neurites of cortical neurons, the extention of which was promoted by 6B4 proteoglycan. Neurites are classified into dendrites and axons, which can be distinguished by antibodies specific for either dendrites (anti-microtubule associated protein 2, MAP2) or axons (anti-highly phosphorylated neurofilament, NFH). Initially, neurites of cultured neurons express both MAP2 and NFH, and then differentiate into axons and dendrites possessing either of the two proteins after 3 days in vitro (Lafont et al., 1992). Because the viability of the neurons rapidly decreased on the third day under our culture conditions, cells were fixed and double-stained with anti-MAP2 and anti-NFH after 48 hours in culture. In control cultures, neurons spread well bearing short processes, most of which were stained with both antibodies (Fig. 8C,D). In contrast, many of the neurons on the 6B4 proteoglycan-coated substrata extended several short processes and one long thin neurite. Most of the former were stained exclusively by anti-MAP2 and, therefore, identified as dendrites (Fig. 8A,B). Most of the latter were stained strongly by anti-NFH, but many of them were also weakly stained by anti-MAP2 (Fig. 8A,B). Although these neurites were axon-like, we could not conclude them as axons. Morphological analysis indicated that 6B4 proteoglycan increased the number and length of dendrites of cortical neurons cultured for 45 hours in vitro (Fig. 9).

Immunohistochemical analysis of cortex and thalamus

6B4 proteoglycan thus exerted contrasting effects on neurons, depending on the modes of interaction between neuron and substratum. When neurites encountered the proteoglycan-coated and uncoated boundary in vitro, they did not enter the coated area. On the uniformly coated substrata, 6B4 proteoglycan promoted differentiation of cortical neurons but not of thalamic neurons. It is tempting to speculate that this reflects the situation in vivo. Immunohistochemical analysis with a monoclonal antibody against 6B4 proteoglycan (mAb 6B4) indicated that there are clear boundaries of 6B4 proteoglycan distribution in the postnatal rat brain. In the primary somatosensory cortex, layer IV was devoid of staining (Fig. 10A). Such a distribution was apparent from postnatal day 5 (P5) to P16. After this period, the staining of the cortex became weak and the surroundings of a subset of neurons showed positive staining (Maeda et al., 1995). Fig. 10B shows the results of immunohistochemical analysis of the thalamus. The
Effects of 6B4 proteoglycan on neurite outgrowth

6B4 proteoglycan promotes tyrosine phosphorylation of 85x10^3 M_r protein

6B4 proteoglycan is the extracellular variant of a receptor-like protein tyrosine phosphatase, PTP_ζ (RPTPβ). Therefore, we examined the time course of changes in the tyrosine phosphorylation levels of the cellular proteins after cortical neurons were seeded on 6B4 proteoglycan-coated and uncoated substrata. Fig. 11A shows the results of western blotting with anti-phosphotyrosine antibody. 6B4 proteoglycan transiently promoted tyrosine phosphorylation of an 85x10^3 M_r protein in the cortical neurons. Tyrosine phosphorylation of a 120x10^3 M_r protein was gradually increased under both culture conditions, while that of other proteins was constant during the

Table 1. Analysis of neurite length on various substrata

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<tr>
<th>Substratum</th>
<th>Average neurite length (µm)</th>
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<tr>
<td></td>
<td>17 hours</td>
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<tr>
<td>Poly-L-lysine</td>
<td>19.4±2.2</td>
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<tr>
<td>Poly-L-lysine + 6B4 proteoglycan</td>
<td>82.1±2.4*</td>
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<tr>
<td>Fibronectin</td>
<td>53.8±3.2</td>
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<tr>
<td>Fibronectin + 6B4 proteoglycan</td>
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<td>Poly-L-lysine + fibronectin</td>
<td>26.6±2.4</td>
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<tr>
<td>Poly-L-lysine + fibronectin + 6B4 proteoglycan</td>
<td>48.1±2.8*</td>
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<tr>
<td>Poly-L-lysine + tenascin</td>
<td>43.7±4.8</td>
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<tr>
<td>Poly-L-lysine + tenascin + 6B4 proteoglycan</td>
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Coverslips or poly-L-lysine-conditioned coverslips were coated with 10 µg/ml fibronectin or tenascin, and then treated with 10 µg/ml 6B4 proteoglycan or PBS. Cortical neurons were seeded on these coverslips and average neurite length was measured after 17 or 45 hours in vitro. The significance of differences was estimated by the non-parametric Wilcoxon rank test. *P<0.05. The amounts of 6B4 proteoglycan (ng/cm²) adsorbed to the following substrata were: poly-L-lysine, 20±2; fibronectin, 9±2; poly-L-lysine + fibronectin, 16±1, and poly-L-lysine + tenascin, 16±1.

Fig. 4. Cumulative length distribution of neurites of cortical and thalamic neurons on 6B4 proteoglycan-coated substrata. Cortical (A) or thalamic (B) neurons were cultured on poly-L-lysine-conditioned coverslips coated with 0 (△), 3 (□), or 30 (○) µg/ml 6B4 proteoglycan. After 20 hours in vitro, neurons were analyzed morphometrically, and the neurite lengths were plotted in frequency distribution histograms. The graphs show the relative fractions of neurons (ordinate) with neurites longer than a given length (abscissa).

Fig. 5. 6B4 proteoglycan suppressed cell body spreading of neurons. Cortical (A) or thalamic (B) neurons were plated on poly-L-lysine-conditioned coverslips coated with various concentrations of 6B4 proteoglycan. After 20 hours in vitro, sizes of neuronal somata were measured. Substrate-bound 6B4 proteoglycan decreased the surface area of neuronal somata, but when the proteoglycan was applied to the culture medium in the soluble form, it showed no such effect (open bars).
culture period. On the fibronectin- or laminin-coated substrata, tyrosine phosphorylation of the \(85 \times 10^3\) Mr protein was the same as the control (data not shown). We next compared the tyrosine phosphorylation levels in cortical and thalamic neurons since they showed different sensitivities to 6B4 proteoglycan. The increase in tyrosine phosphorylation of the \(85 \times 10^3\) Mr protein was less obvious in thalamic neurons tested on the 6B4 proteoglycan-coated substrata (Fig. 11B).

**DISCUSSION**

In this study, we examined the effects of purified 6B4 proteoglycan on the cell adhesion and differentiation of cortical and thalamic neurons. 6B4 proteoglycan did not support cell attachment of cortical or thalamic neurons, in contrast to astrocytes, when used as a substratum. On 6B4 proteoglycan-coated coverslips preconditioned with poly-L-lysine as an adhesive substratum, neurons formed large aggregates with fasciculated neurites. This indicates the reduction of cell-substratum adhesion and the dominance of cell-cell adhesion forces under high cell density conditions. This repulsive nature of 6B4 proteoglycan was more clearly observed on the patterned substrata where cortical and thalamic neurons selectively attached to the 6B4 proteoglycan-free poly-L-lysine areas and the neurites did not invade the 6B4 proteoglycan-coated areas. Despite the repulsive nature of 6B4 proteoglycan, this molecule promotes neurite outgrowth of cortical neurons as revealed under low cell density conditions. This effect was cell-type specific, and neurite extension of thalamic neurons was not influenced by 6B4 proteoglycan. However, neither cortical nor thalamic neurons showed good spreading of the cell body on 6B4 proteoglycan/poly-L-lysine substrata, probably because of the reduction of cell-substratum adhesive forces. This situation is similar to that of J1/tenascin. Faissner and Kruse (1990) reported that central nervous system neurons had a grid-like growth pattern on patterned substrata of polyornithine alternating with J1/tenascin-coated polyornithine domains. Neurons were concentrated in polyornithine areas and avoided J1/tenascin areas. Under low cell density plating conditions, however, polyornithine coated with J1/tenascin promoted neurite outgrowth of hippocampal and mesencephalic neurons.
Effects of 6B4 proteoglycan on neurite outgrowth (Lochter et al., 1991). Reduction of cell spreading was also observed on the J1/tensascin-coated polyornithine substrata (Lochter et al., 1991). Despite these similarities, important differences should be noted. Not only the substratum-bound molecules but also soluble J1/tensascin inhibited neurite outgrowth and cell spreading on the polyornithine-coated substrata (Lochter et al., 1991). In contrast, when 6B4 proteoglycan was applied in soluble form, it did not affect either neurite outgrowth or cell spreading. This suggests that the mechanisms of action of 6B4 proteoglycan and J1/tensascin are different.

6B4 proteoglycan/phosphacan is a complex molecule bearing chondroitin sulfate, keratan sulfate and HNK-1 carbohydrate (Rauch et al., 1991; Maeda et al., 1992). The core protein is composed of an N-terminal carbonic anhydrase-like domain, fibronectin type III domain and the C-terminal serine, glycine-rich region (Krueger and Saito, 1992; Levy et al., 1993; Maeda et al., 1994; Mauel et al., 1994). Chondroitinase ABC and keratanase digestion of 6B4 proteoglycan did not affect the neurite promoting or anti-cell spreading activity of this molecule. Therefore, it is unlikely that the effects were caused by neutralization of the polycationic poly-L-lysine substrata. Furthermore, HNK-1 monoclonal antibody did not suppress the activities of 6B4 proteoglycan. In contrast, polyclonal antibodies against purified 6B4 proteoglycan (anti-6B4 proteoglycan) completely inhibited the neurite promoting activity of 6B4 proteoglycan, although anti-cell spreading activity was not suppressed. Polyclonal antibodies against the C-terminal region of 6B4 proteoglycan (anti-31-5) exerted no effect on its activities. These results suggest that the protein portion outside the C-terminal region is the active site for neurite promotion, and that the reduction of cell spreading is caused by a different site. As for this repulsive nature, it seems

![Fig. 8. Double immunostaining of cortical neurons cultured on poly-L-lysine-conditioned coverslips coated with or without 6B4 proteoglycan. Cortical neurons were cultured on poly-L-lysine-conditioned coverslips, uncoated (C,D) or coated (A,B) with 2 μg/ml 6B4 proteoglycan. After 2 days in vitro, the neurons were double-immunostained with anti-MAP2 (A,C) and anti-NFH (B,D). Arrowheads indicate an NFH-positive and MAP2-negative neurite. Arrows indicate an NFH- and MAP2-positive neurite. Scale bar, 50 μm.](image)

![Fig. 9. 6B4 proteoglycan promotes development of dendrites. Cortical neurons were cultured for 20 or 45 hours on poly-L-lysine-conditioned coverslips uncoated (black bars) or coated (white bars) with 2 μg/ml 6B4 proteoglycan, and double-immunostained with anti-MAP2 and anti-NFH. Number (A) and length (B) of neurites which were MAP2-positive and NFH-negative were measured.](image)
to be attributable to carbohydrate portions other than glycosaminoglycan of this proteoglycan because heat treatment does not abolish the activity. Consistently, 6B4 proteoglycan contains unidentified N- and O-linked oligosaccharides (Maeda et al., 1995). Milev et al. (1994) reported that phosphacan inhibits neurite outgrowth of neurons. However, they analyzed the behavior of chick brain cells on substrata coated with rat phosphacan. Considering the cell type-specificity of the 6B4 proteoglycan activity, this discrepancy may be derived from the difference in the origin of the cells. Alternatively, it may have been due to the difference in adhesive substratum, because they used Ng-CAM/L1-conditioned substrata coated with phosphacan.

Generally, chondroitin sulfate proteoglycans are regarded as barriers for neurite outgrowth (Snow et al., 1990; Oohira et al., 1991; Dou and Levine, 1994; Milev et al., 1994). However, recent studies indicated that some brain chondroitin sulfate proteoglycans and/or chondroitin sulfate chains promote neurite outgrowth (Iijima et al., 1991; Lafont et al., 1992; Faissner et al., 1994; Fernaud-Espinosa et al., 1994). Furthermore, immunohistochemical studies with monoclonal antibodies against chondroitin sulfate indicated that chondroitin sulfate proteoglycans cannot be regarded as a barrier to axonal outgrowth in the neocortex and retinal axons (Bicknese et al., 1994; McAdams and McLoon, 1995; Ring et al., 1995). It is important to note that repulsive effects of chondroitin sulfate proteoglycans were observed in vitro in the situation where neurites were confronted with a favorable substratum such as laminin on one side and proteoglycan-coated areas on the other (Snow et al., 1990). Patterned substratum experiments in this study indicated that the repulsive effect of 6B4 proteoglycan is concentration-dependent, and a fairly high concentration of proteoglycan was required to function as a repulsive substratum.

Immunohistochemical analysis with mAb 6B4 indicated that 6B4 proteoglycan is present relatively uniformly in the developing rat neocortex except for the layer IV of the somatosensory cortex during the limited period from P5 to P16. In the thalamus, however, 6B4 proteoglycan was present at the boundaries of the various nuclei. These results suggest that in the cerebral cortex, 6B4 proteoglycan promotes morphological differentiation of neurons, and in the thalamus contributes to the formation of molecular borders that restrict migration or neurite outgrowth of neurons from each thalamic nucleus. The loss of mAb 6B4 immunoreactivity in layer IV of the early postnatal rat primary somatosensory cortex may be related to the branch formation of thalamocortical axons restricted in this layer. Layer IV of the somatosensory cortex is organized into barrel structures, where the barrel centers are filled with the arborization of thalamocortical axons. Recently, expression of another major PBS-soluble chondroitin sulfate proteoglycan, neurocan, has been reported to be down-regulated in the barrel centers of the early postnatal rat somatosensory cortex (Oohira et al., 1994; Watanabe et al., 1995). J1/tenascin was observed surrounding the barrel hollows during the stage of barrel field formation in the mouse somatosensory cortex (Steindler et al., 1989; Crossin et al., 1989). Although no barrel-like distribu-
tion of 6B4 proteoglycan was seen clearly, neurocan, J1/tenascin and 6B4 proteoglycan/phosphacan may coordinate a role in the formation of the thalamocortical afferent system. In this context, it should be noted that phosphacan binds tenascin (Grumet et al., 1994; Milev et al., 1994). Geisert and Bidanset (1993) demonstrated that boundaries of the various thalamic nuclei were outlined with immunoreactivity to a keratan sulfate epitope. This keratan sulfate epitope may be attached to the 6B4 proteoglycan/phosphacan, because this proteoglycan is the major keratan sulfate-containing molecule in the brain (Rauch et al., 1991; Maeda et al., 1995).

6B4 proteoglycan exerts a neurite outgrowth promoting effect on neurons and, at the same time, a cell-type specific repulsive effect as a substratum. Such dual effects are also observed with a diffusible factor, Netrin-1 (Colamarino and Tessier-Lavigne, 1995) and a transmembrane member of the immunoglobulin superfAMILY, myelin-associated glycoprotein (MAG) (Mukhopadhyay et al., 1994). Netrin-1 functions as a chemoattractant for developing spinal commissural axons but is also a chemorepellent for trocheclor motor axons (Colamarino and Tessier-Lavigne, 1995). MAG promotes neurite outgrowth from newborn dorsal root ganglion (DRG) neurons, but strongly inhibits neurite outgrowth from developing cerebellar and adult DRG neurons (Mukhopadhyay, 1994). These studies suggest the presence of complex cell type-specific or developmentally regulated neuronal machineries to interpret various extracellular cues.

To investigate the possible participation of 6B4 proteoglycan in the cell signaling system, we analyzed the tyrosine phosphorylation pattern after cells were plated on proteoglycan-coated substrata. In cultures of cortical neurons, tyrosine phosphorylation of an 85×10^3 M_r protein was specifically promoted by 6B4 proteoglycan. At present, it is not clear whether this phenomenon is directly related to the neurite promoting activity of 6B4 proteoglycan. However, recent studies indicated that protein tyrosine phosphorylation is closely related to neurite extension (Atashi et al., 1992; Bixby and Jhabvala, 1993; Rogers et al., 1994). The mechanism by which 6B4 proteoglycan influences tyrosine phosphorylation is unknown, but it is conceivable that this proteoglycan competitively binds with ligands of PTP_\beta (RPTP_\beta) and as a result modulates its tyrosine phosphatase activity. Additionally, 6B4 proteoglycan may bind to cell adhesion molecules such as L1 and NCAM and modulate the signaling pathways via these molecules (Milege et al., 1994; Williams et al., 1994). Snow et al. (1994) reported that bovine nasal cartilage proteoglycan elevates the concentration of cytoplasmic calcium ions in dorsal root ganglion neurons. Further studies are required to reveal whether brain chondroitin sulfate proteoglycans such as 6B4 proteoglycan influence the intracellular calcium levels of brain cells.

Owing to the recent progress in the understanding of brain chondroitin sulfate proteoglycans, we cannot now consider these complex molecules to be a simple barrier to axonal outgrowth. Chondroitin sulfate proteoglycans should be regarded as modulators of neuronal differentiation and morphogenesis of the brain. Further studies are required to elucidate the interplay of brain chondroitin sulfate proteoglycans with protein tyrosine kinases and phosphatases, cell-surface receptors and cell adhesion molecules. Identification of the ligands of 6B4 proteoglycan and the intracellular targets of PTP_\beta (RPTP_\beta) will facilitate elucidation of novel aspects of the involvement of proteoglycans in brain development.

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