

Spatiotemporal Expression Patterns of 6B4 Proteoglycan/Phosphacan in the Developing Rat Retina

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PURPOSE. To investigate expression of 6B4 proteoglycan/phosphacan, the major constituent of chondroitin sulfate proteoglycan and a possible modulator of neural network formation in the developing central nervous system, in developing rat retina.

METHODS. Changes in expression and localization of 6B4 proteoglycan in developing rat retina were investigated by reverse transcription–initiated polymerase chain reaction (RT-PCR), immunohistochemistry, and immunoblot analysis.

RESULTS. Semiquantitative RT-PCR revealed that mRNA expression of 6B4 proteoglycan in retinas peaked at postnatal day 14 (P14) and then decreased at P42. Immunohistochemical analyses using MAb 6B4, a monoclonal antibody against 6B4 proteoglycan, revealed faint immunoreactivity in the inner aspects of the retina at embryonal day 16 (E16). At birth, weak immunoreactivity was present in the nerve fiber layer (NFL) and inner plexiform layer (IPL). At P7 and P14, the NFL, IPL, and outer plexiform layer (OPL) stained intensely, but the ganglion cell layer (GCL) remained unstained. Between P21 and P42, immunoreactivity in the NFL and IPL weakened slightly. Immunoblot analyses showed a MAb 6B4 immunopositive band in the retinal soluble fraction treated with chondroitinase ABC. The amount of the immunopositive band increased rapidly as retinal development proceeded. Surprisingly, a significant amount of the immunopositive band was present in the retina even before digestion with chondroitinase ABC, indicating that at least part of 6B4 proteoglycan in rat retina exists in a non-proteoglycan form.

CONCLUSIONS. The existence of 6B4 proteoglycan/phosphacan was thus demonstrated in rat retina, although some biochemical parameters were different from those of the 6B4 proteoglycan seen in brain. (*Invest Ophthalmol Vis Sci.* 2000;41:1990–1997)

Proteoglycans play pivotal roles in developmental processes such as neurite outgrowth, neural cell adhesion, and differentiation of the mammalian central nervous system.^{1,2} Biochemical studies have revealed that the expression of a diverse set of proteoglycans is regulated precisely during brain development.^{3,4} Two proteoglycans, neurocan and phosphacan, are the major constituents of chondroitin sulfate proteoglycan in the rat brain,⁵ and both are thought to play a major role in formation of the neural network because they are expressed in a spatiotemporally regulated manner during development of the central nervous system.^{6–11} In our previous study,¹² we demonstrated that the expression of neurocan is regulated temporally and

spatially in developing neural retina, which suggests a major role for this proteoglycan in retinal neural network formation. However, to date, knowledge about the role of another major neural proteoglycan, phosphacan, in developing retina is limited.^{13–15}

In our previous study,⁶ a monoclonal antibody, MAb 6B4, recognized a central nervous system–specific chondroitin sulfate proteoglycan. This proteoglycan was designated as 6B4 proteoglycan based on the name of the antibody and was eventually revealed to be identical to phosphacan.¹⁶ In addition, molecular cloning studies demonstrated that 6B4 proteoglycan/phosphacan is an alternatively spliced product of the receptor-type protein tyrosine phosphatase (RPTP ζ/β),^{17–19} which lacks the transmembrane and intracellular regions. 6B4 proteoglycan/phosphacan has been shown to modulate neurite extension^{8,10,11} and to alter cellular behavior via binding to neural cell adhesion molecules and tenascin.^{7–9} Moreover, some heterophilic interactions of 6B4 proteoglycan/phosphacan are implicated in neural cell migration in brain development,^{7–9} and it is possible that 6B4 proteoglycan/phosphacan may also be involved in retinal development. Accordingly, in an effort to elucidate the role of 6B4 proteoglycan in retinal development, we conducted molecular biochemical investigations. Herein we report the existence of 6B4 proteoglycan/phosphacan in the retina and show changes in its temporal and spatial expression during retinal development.

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METHODS

Semiquantitative Reverse Transcription–Initiated Polymerase Chain Reaction and Subsequent Southern Blot Analysis

All animals were given water and food ad libitum. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Wistar rats at various stages of development from embryonal day 16 (E16) to postnatal day 42 (P42) were killed by intraperitoneal overdose injection of pentobarbital. After enucleation of the eyes, neural retinas were removed with scissors and forceps under an operating microscope. Retinal total RNA extracted by the acid guanidium thiocyanate–phenol chloroform extraction method was used to synthesize template cDNAs for subsequent reverse transcription–initiated polymerase chain reaction (RT–PCR) experiments with the use of reverse transcriptase (First-Strand cDNA Synthesis Kit; Amersham Pharmacia Biotech, Uppsala, Sweden), as described previously.²⁰ Before PCR experiments for 6B4 proteoglycan, each cDNA concentration was normalized to β -actin gene expression in a manner similar to that described previously.²⁰ The sequences for specific primers to β -actin were AGCTGAGAGGGAAATCGTGC (sense) and ACCAGACAGCACTGTGTTGG (antisense).²¹ For PCR experiments, the following conditions were used: denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds, and polymerization at 72°C for 1 minute for 19 cycles (β -actin primers) or 27 cycles (6B4 proteoglycan primers). The sequences of the sense and the antisense primers for 6B4 proteoglycan were TATGCTACCCAGAAGCACA and TCTGCTGGTGACCAGAATT, respectively.¹⁶ The PCR products were separated by 2% agarose gel electrophoresis and then transferred to a membrane, Hybond-N+ (Amersham Pharmacia Biotech) by the capillary transfer method with 20 \times SSC. For Southern blot analysis, the synthesized internal oligonucleotide probe (sequence: CTGATGGTGTCTGGAAGCA) was labeled by ECL 3'-oligolabeling and detection systems (Amersham Pharmacia Biotech) to exclude the nonspecific bands. Optical densities of the hybridizing bands were measured by a Power Macintosh G3 computer (Apple Computer, Cupertino, CA) and NIH Image 1.59. A standard curve was generated from optical densities of the hybridizing bands from serial dilutions of template cDNAs, and linearity of the created standard curve among the selected concentrations was confirmed. The relative levels of mRNA expression were calculated as a ratio to adult (P42) rat retinas (number of retinal samples, $n = 3$ at each developmental stage).

Immunohistochemistry

Enucleated eyes from both embryonal and postnatal Wistar rats were fixed for 2 hours at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) with gentle shaking, washed for 5 minutes in PBS, and then gently shaken overnight at 4°C in 30% sucrose/PBS before freezing on powdered dry ice. Sections (16- μ m-thick) were cut using a cryostat and then collected onto silanized slides (DAKO JAPAN, Kyoto, Japan). After the retinal sections were rinsed twice for 3 minutes each time in PBS, the sections were incubated in cold methanol (–20°C) for 15 minutes, followed by washing three times for 3 minutes each in PBS. Sections were then covered for 15 min-

utes with 50 mM glycine in PBS and rinsed for 3 minutes in PBS before being covered for 1 hour with blocking solution (2% bovine serum albumin [BSA]/2% horse normal serum/2% goat normal serum in PBS). After being washed for 3 minutes in PBS, the sections were incubated overnight at 4°C in a humidified chamber with the first antibody, MAb 6B4. Sections were rinsed three times for 3 minutes each in PBS to remove the first antibody, then incubated for 1 hour at room temperature with the second antibody, fluorescein-conjugated goat anti-mouse IgM (Vector Laboratories, Burlingame, CA), and washed six times for 3 minutes each in PBS. Sections were mounted in Vectashield (Vector Laboratories), and the slides examined under a confocal microscope (model LSM410; Carl Zeiss, Oberkochen, Germany). The immunohistochemical results were confirmed from five experiments in at least 4 different eyes at each developmental stage.

Preparation of the S₁ Fractions

Preparation of the retinal soluble fraction (S₁ fraction) was carried out by a modification of the procedure developed by Maeda et al.²² In brief, 60 eyes were enucleated from 30 postnatal rats (P14), and the retinal tissue was collected in Hanks' balanced salt solution. Wet weight of the total collected retinal tissue was approximately 1 gram. Retinal tissue was homogenized with a tight-fitting glass-polytetrafluoroethylene Potter homogenizer in 5 ml of 0.32 M sucrose, 5 mM EDTA, 1 mM benzamidine, 50 mM Tris-HCl (pH 7.5) containing 100 μ M phenylmethylsulfonyl fluoride (PMSF), 10 μ M leupeptin, and 10 μ M pepstatin as protease inhibitors. The homogenized solution was centrifuged at 1000g for 5 minutes at 4°C, and the supernatant (SUP-I) was stored. The pellet was homogenized in 2.5 ml of the same solution and the homogenate again subjected to centrifugation. The resultant supernatant (SUP-II) was added to the previously prepared supernatant (SUP-I), and the combined solution was subjected to ultracentrifugation at 105,000g for 60 minutes at 4°C. The final supernatant (S₁ fraction) obtained by the ultracentrifugation contains the soluble proteoglycans²² and was used for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Chondroitinase ABC Digestion

Protease-free chondroitinase ABC (EC 4.2.2.4; Seikagaku, Tokyo, Japan) was used to digest the chondroitin sulfate side chains linked to core proteins. The protein concentration of the S₁ fraction was measured by Bio-Rad DC protein assay (Bio-Rad Laboratories, Tokyo, Japan). Proteins (200 μ g) were precipitated from an aliquot of the S₁ fraction by adding 3 volumes of 95% ethanol/1.3% potassium acetate and were suspended in 119 μ l of distilled water. To this suspension, 15 μ l of 1 M Tris-HCl buffer (pH 7.5), including 0.3 M sodium acetate, 4 μ l of inhibitor mixture-I (20 mM PMSF/7.2 mM pepstatin), 8 μ l of inhibitor mixture-II (0.1 M EDTA/0.1 M N-ethylmaleimide [NEM]), and 4 μ l of the enzyme solution (0.01 U/ μ l) were added. The mixture (total 150 μ l) was incubated at 37°C for 2 hours. The enzyme reaction was stopped by adding 450 μ l of 95% ethanol containing 1.3% potassium acetate on ice, the mixture was then centrifuged at 15,000g for 15 minutes at 4°C, and the precipitated material then was used as the sample for immunoblot analysis.

Glycosidase Digestion of Proteins in the S₁ Fraction

The S₁ fraction was digested with some glycosidase enzymes in an attempt to remove oligosaccharide side chains of glycoproteins. The precipitated S₁ fraction (200 μg protein) was suspended in 75 μl of a solution containing 5 mM EDTA, 5 mM NEM, 1 mM PMSF, 0.1 mM pepstatin, 50 mM sodium acetate (pH 5), and 20 mU neuraminidase (EC 3.2.1.18; Seikagaku). The solution was then incubated at 37°C for 120 minutes. The same volume of a solution containing 5 mM EDTA, 5 mM NEM, 1 mM PMSF, 0.1 mM pepstatin, 15 mM sodium acetate, and 50 mM Tris-HCl (pH 7.4) was added to the sample solution, after which the mixture was incubated at 37°C for an additional 120 minutes in the presence of 40 mU keratanase (EC 3.2.1.103; Seikagaku). Proteins were precipitated from the mixture with ethanol and denatured by boiling for 2 minutes in 13 μl of a solution containing 1% SDS and 10 mM sodium phosphate (pH 7.2). The sample solution was diluted with 137 μl of a solution containing 1% N-octyl-β-D-glucoside (Wako, Osaka, Japan), 5 mM EDTA, 5 mM NEM, 1 mM PMSF, 0.1 mM pepstatin, 10 mM sodium phosphate (pH 7.2), 1 mU of O-glycanase (EC 3.2.1.97; Boehringer Mannheim, Tokyo, Japan), and/or 5 U of N-glycanase (EC 3.2.2.18; Boehringer Mannheim), and the reaction mixture was incubated at 37°C for 120 minutes.

Partial Purification of Soluble Proteoglycans

Soluble proteoglycans of the retina were purified from the S₁ fraction by DEAE-Sephacel column chromatography, as described previously,³ with slight modification as follows: S₁ fraction (5 ml) was dialyzed against 2 M urea containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM NEM, and 0.2 mM PMSF. After removing insoluble materials by centrifugation at 27,000g for 30 minutes at 4°C, the supernatant was applied to a DEAE-Sephacel column (1.0 × 4 cm) (Amersham Pharmacia Biotech). The column was then washed with 15 ml of the same urea buffer and with 15 ml of 0.35 M NaCl-containing urea buffer. Elution of the proteoglycans was carried out with 0.7 M NaCl-containing urea buffer. The eluted materials were precipitated with 95% ethanol containing 1.3% potassium acetate and were then treated with chondroitinase ABC as described above.

Immunoblot Analysis of Glycosidase-Treated Samples

The samples were electrophoresed by SDS-PAGE on a 3% stacking gel and a 4.5% or 6% separating gel. The proteins separated by electrophoresis were then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was washed with PBS for 5 minutes, incubated in a blocking solution (2% BSA/2% normal horse serum/2% normal goat serum in PBS) for 1 hour at room temperature and treated sequentially with MAb 6B4 at room temperature for 2 hours and with biotinylated anti-mouse IgM for 30 minutes at room temperature. Immunoreactive materials on the membrane were detected using a Vectastain elite ABC kit (Vector Laboratories).

Immunoblot Analysis of Retinal Tissue Homogenates

Rat retinal tissue (30 mg wet weight) at various developmental stages from E16 to P42 was homogenized in 50 μl ice-cold PBS

containing 10 mM NEM, 20 mM EDTA, and 2 mM PMSF. The homogenate was mixed with 200 μl of 20 mM Tris-HCl buffer (pH 7.5) containing 2% SDS, 10 mM NEM, 20 mM EDTA, and 2 mM PMSF, and the mixture was boiled for 5 minutes. The protein concentration of the boiled solution was measured by Bio-Rad DC protein assay. Proteins (200 μg), which were precipitated from the solution by adding 3 volumes of 95% ethanol/1.3% potassium acetate, were dissolved in a mixture of 20 μl distilled water, 20 μl sample buffer (0.1 M Tris-HCl, pH 7.5/4% SDS/20% glycerol/0.002% bromophenol blue), and 20 μl sample buffer containing 1.5% dithiothreitol. A volume of 15 μl of each sample was subjected to immunoblot analysis as described above. The optical densities of immunoreactive bands were measured by a Power Macintosh G3 computer (Apple Computer) and NIH Image 1.59 (number of retinal samples, *n* = 3 at each developmental stage).

RESULTS

Semiquantitative RT-PCR and Subsequent Southern Blot Analysis

RT-PCR using primers specific to 6B4 proteoglycan showed that cDNA fragments of the expected length (420 bp) were amplified in cDNAs from the rat retina. Southern blot analysis using an internal oligonucleotide showed that the amplified PCR products of the expected length hybridized with internal probes, indicating that they were derived from the expected sequence of rat 6B4 proteoglycan core protein gene. To quantify relative levels of mRNA expression of 6B4 proteoglycan gene during retinal development, we carried out semiquantitative RT-PCR experiments and subsequent Southern blot analysis after normalization to β-actin (Fig. 1). The semiquantitative analyses demonstrated that the mean level (±SE) of gene expression for 6B4 proteoglycan at E16 was 0.3 ± 0.3-fold that of the adult (P42) retinas. At early postnatal stages (P0), mRNA expression for 6B4 proteoglycan was increased, reached a peak on P14 (5.0 ± 2.3-fold), and then decreased in adult (P42) retinas.

Immunohistochemical Studies

MAb 6B4 immunoreactivities in rat retinas were faint at E16, when homogeneous retinal (neuroblast) cells were present throughout the retina (Fig. 2A), only faint immunoreactivities were observed in the inner aspects and extended radially in these embryonal rat retinas. At birth (P0), as the ganglion cell layer (GCL) and inner plexiform layer (IPL) developed, weak immunoreactivities were present in the nerve fiber layer (NFL) and IPL. At P7 and P14, as the IPL became well differentiated and the outer plexiform layer (OPL) was clearly observed, those retinal layers composed of neuronal axons (such as the NFL, IPL, and OPL) were stained intensely. Interestingly, the GCL was only barely stained at any time after birth. As the retinal layers became mature (between P21 and P42), staining of the NFL and IPL weakened slightly, although there was no significant difference in staining of the OPL between early postnatal stages (P7-P14) and late postnatal stages (P21-P42). Moreover, the optic nerve was also stained at P21 (Fig. 2B). No MAb 6B4 immunoreactivities were detected in other ocular tissues, including the cornea, iris, ciliary body, lens, and sclera.

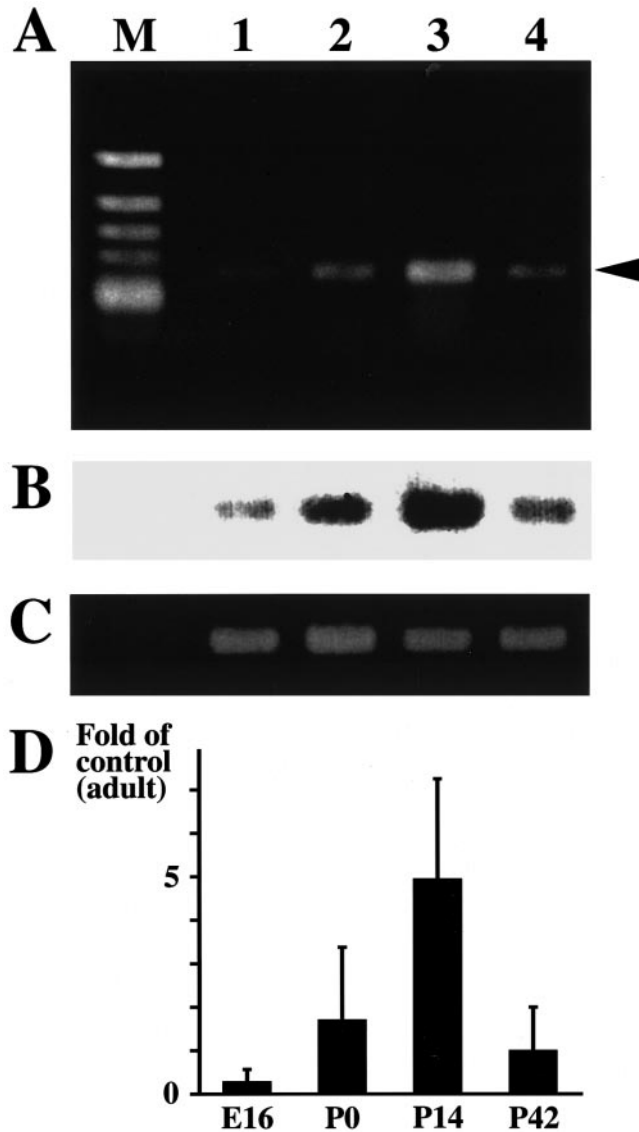


FIGURE 1. Semiquantitative RT-PCR experiments and Southern blot analyses of 6B4 proteoglycan gene expression during retinal development. (A) PCR experiments on 6B4 proteoglycan. Lane 1, E16; lane 2, P0; lane 3, P14; and lane 4, P42 (adult rat). Lane M, a marker (*HincII*-digested ϕ X174 DNA). An arrowhead indicates the bands of 420 bp. (B) Southern blot analysis of 6B4 proteoglycan. (C) PCR experiments on β -actin. (D) Southern blot analysis and subsequent densitometric analysis of 6B4 proteoglycan gene expression. The relative level (mean \pm SEM) at each developmental stage was evaluated as a ratio of adult rat retina on P42 ($n = 3$). Error bars represent standard error.

Immunoblot Analysis of 6B4 Proteoglycan

Mab 6B4 has been shown to recognize 6B4 proteoglycan in the brain.¹⁰ In soluble fractions derived from early postnatal rat brains, immunoblot analysis using this antibody showed a single specific band with a molecular mass of 300 kDa. In an attempt to further characterize 6B4 proteoglycan in developing neural tissue, we carried out immunoblot analysis on rat retinas. S₁ fraction of P14 rat retina was subjected to immunoblot analysis before and after chondroitinase ABC treatment. As

described previously,¹⁰ the S₁ fraction from P14 rat brain showed an immunoreactive band of approximately 300 kDa after treatment with chondroitinase ABC (Fig. 3; lane 1), whereas the broad band greater than 300 kDa was detected without digestion by chondroitinase ABC (lane 2). On the

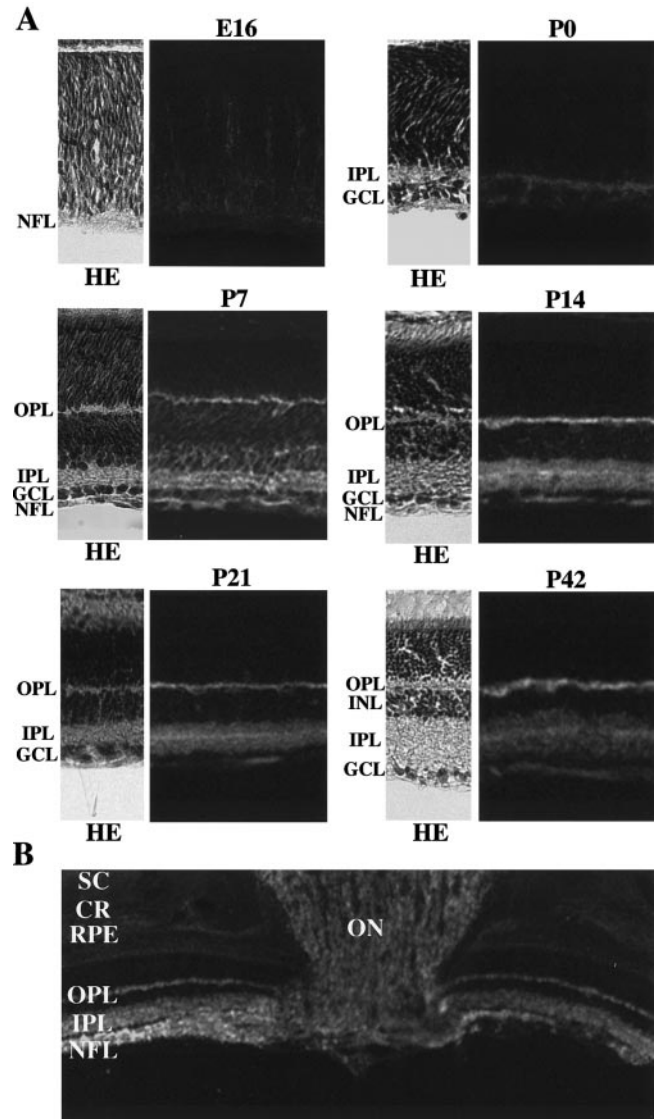


FIGURE 2. Immunohistochemistry for 6B4 proteoglycan during retinal development. The confocal images were stained with Mab 6B4. (A) Retinal sections during development (E16–P42) were used for immunohistochemistry. Arranged micrographs show sections stained with hematoxylin-eosin (HE). Faint immunoreactivity was observed in the inner aspects of the retinas, including the NFL, at E16. Additionally, some faint radial immunoreactivity was also observed. At birth (postnatal day 0; P0), weak immunoreactivity was present in the NFL and the inner IPL. At P7 and P14, the NFL, IPL, and OPL stained intensely, although the GCL remained unstained. As the retinal layers matured (between P21 and P42), staining of the NFL and IPL weakened slightly, although staining of the OPL was basically unchanged. Magnifications, $\times 200$. (B) Immunohistochemical image of the optic nerve at P21. The optic nerve was stained as well as the NFL, IPL, and OPL. Note no immunoreactivity in either the choroidal tissue or sclera. Magnification, $\times 100$. RPE, retinal pigment epithelium; ON, optic nerve; CR, choroidal tissue; SC, sclera.

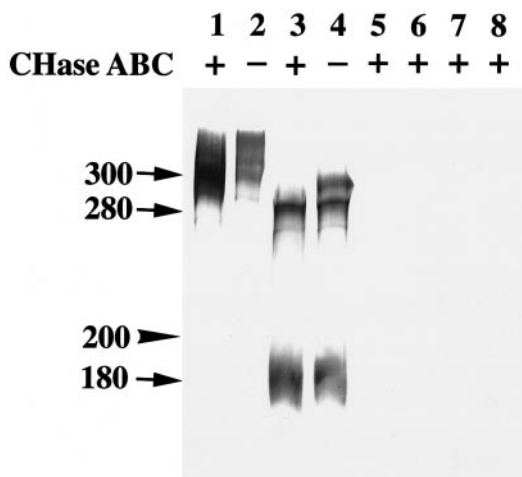


FIGURE 3. Immunoblot analyses of S_1 fractions. S_1 fractions prepared from P14 rat cerebrum,¹ retina,³ kidney,⁵ lung,⁶ heart⁷ and liver⁸ were digested with chondroitinase ABC (CHase ABC) and then applied to 4.5% SDS-PAGE. In the cerebral S_1 fraction, the immunopositive band was observed around 300 kDa after the treatment with chondroitinase ABC,¹ whereas the broad band of over 300 kDa was detected without digestion by chondroitinase ABC.² In the retinal S_1 fraction, the 280-kDa band was observed both after treatment with³ and without⁴ chondroitinase ABC. Note that an additional broad band was observed at approximately 300 kDa without treatment by chondroitinase ABC. Another band was detected at approximately 180 kDa in the retinal S_1 fraction. In S_1 fractions from the kidney,⁵ lung,⁶ heart,⁷ and liver,⁸ no immunoreactive bands were detected. The positions of the molecular mass markers are indicated in kilodaltons.

other hand, in the S_1 fraction from P14 rat retinas, a 280-kDa band was observed after treatment with chondroitinase ABC (lane 3), and, unexpectedly, a significant portion of the 280-kDa band was detected in the sample even before chondroitinase ABC treatment (lane 4), in addition to the chondroitinase-sensitive band of approximately 300 kDa. This indicates that a significant amount of 6B4 proteoglycan/phosphacan exists in the retina in a non-proteoglycan form without chondroitin sulfate side chains. Another chondroitinase-resistant band was detected at approximately 180 kDa in the retinal S_1 fraction. As controls, we analyzed S_1 fractions from kidney (lane 5), lung (lane 6), heart (lane 7), and liver (lane 8) treated with chondroitinase ABC; no immunoreactive bands were detected.

Glycosidase Digestion of S_1 Fraction

To characterize oligosaccharides linked to the MAb 6B4-recognizing proteins, we digested the S_1 fraction sequentially with various glycosidases. After chondroitinase ABC treatment of the S_1 fraction, the immunopositive band became more distinct (Fig. 4, lane 2) than the band from the intact S_1 fraction (lane 1). Subsequent neuraminidase treatment of the chondroitinase ABC-digested S_1 fraction resulted in increased mobility of the immunopositive band on SDS-PAGE (lane 3). Subsequent digestion with keratanase did not appear to affect mobility (lane 4), but *O*-glycanase and/or *N*-glycanase digestion studies showed that mobility of the immunopositive band was slightly increased (lanes 5, 6, and 7). In contrast, in the S_1 fraction of intact brain, an immunopositive band was detected as a broad band greater than 300 kDa (lane 8). After chondroitinase ABC

digestion, the molecular mass of the band decreased to 300 kDa (lane 9). After digestion by all the glycosidases listed above (lane 10), the immunopositive band became sharper, and the molecular mass was close to that of the band of retinal S_1 fraction digested by these glycosidases, which indicates that the retinal 6B4 proteoglycan has fewer oligosaccharide side chains than does the brain 6B4 proteoglycan.

To further examine whether the retinal 6B4 proteoglycan lacks chondroitin sulfate side chains, we concentrated soluble proteoglycans from the retinal S_1 fraction by DEAE-Sephacel column chromatography. In immunoblot experiments using MAb 6B4 (Fig. 5), a single band of approximately 300 kDa (6B4 proteoglycan/phosphacan) was shown in the cerebral sample treated with chondroitinase ABC (lane 3), which is consistent with a previous report.¹⁰ The 6B4 proteoglycan without chondroitinase ABC treatment barely penetrated the 6% polyacrylamide gel (lane 4), which is also consistent with the previous result, because the molecular mass of the intact molecule is 600 to 1000 kDa.⁶ Interestingly, a major immunoreactive band was found in the retinal sample treated with chondroitinase ABC treatment (lane 1), whereas only a faint band was detected at the top of the separating gel in the sample not treated with chondroitinase ABC (lane 2). The detected bands in the retinal S_1 fraction eluted from the DEAE-Sephacel column were a major band and another faint band at approximately 230 kDa. Because only glycosaminoglycan side chains are bound to the DEAE-Sephacel column on chromatography under the experimental conditions used, non-proteoglycan materials cannot bind to the column. In the present study, no immunoreactive bands were detected within the separating gel in the retinal

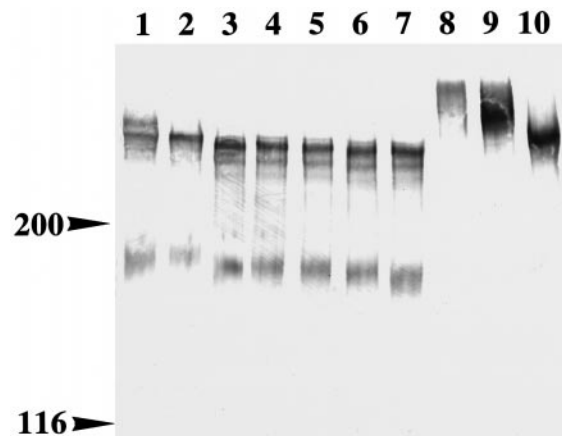


FIGURE 4. Effects of glycosidase digestion on the electrophoretic mobilities of MAb 6B4-immunopositive bands in S_1 fractions. Retinal S_1 fraction was digested sequentially with chondroitinase ABC,² neuraminidase,³ keratanase,⁴ *O*-glycanase,⁵ *N*-glycanase,⁶ and a mixture of *O*-glycanase and *N*-glycanase,⁷ and the digested samples were applied to 4.5% SDS-PAGE. An untreated sample was applied to lane 1 of the gel. The mobilities of these bands were affected only slightly by digestion with a series of glycosidases. The brain S_1 fractions were also digested sequentially with chondroitinase ABC,⁹ neuraminidase, keratanase, and a mixture of *O*-glycanase and *N*-glycanase.¹⁰ An untreated brain sample was applied to lane 8 of the gel. After digestion by various glycosidases, the molecular mass of the immunopositive band was close to that of the band of retinal S_1 fraction digested by these glycosidases. The positions of molecular mass markers are indicated in kilodaltons.

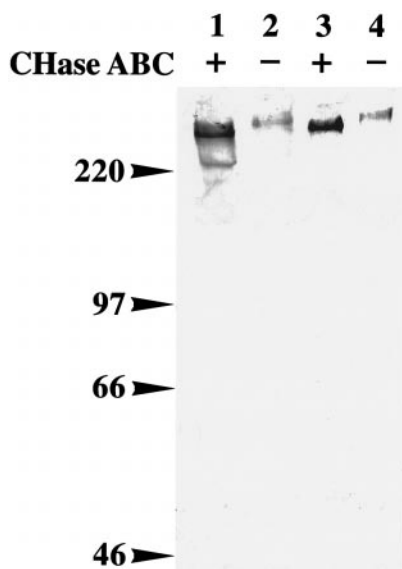


FIGURE 5. Immunoblot analyses for 6B4 proteoglycan concentrated from the retina (*lanes 1 and 2*) and cerebrum (*lanes 3 and 4*) of a P14 rat by DEAE-Sephacel column chromatography. A single band of 300 kDa (6B4 proteoglycan) was shown in the cerebral sample treated with chondroitinase ABC (CHase ABC; *lane 3*). The 6B4 proteoglycan without chondroitinase ABC treatment barely penetrated the 6% polyacrylamide gel (*lane 4*). A major immunopositive band was found in the retinal sample treated with chondroitinase ABC (*lane 1*), whereas only a faint band was detected at the top of the separating gel in the sample without chondroitinase ABC treatment (*lane 2*). The positions of molecular mass markers are indicated in kilodaltons.

sample not treated with chondroitinase ABC, indicating that no MAb 6B4-immunoreactive non-proteoglycan-type materials were contaminated in the partially purified retinal proteoglycan fraction. These findings, taken together, indicate that there does exist 6B4 proteoglycan-bearing chondroitin sulfate chains in rat retinas, although the major form is expressed as a non-proteoglycan type.

Developmental Change in the Amounts of 6B4 Proteoglycan in the Retina

To elucidate the temporal pattern of expression of 6B4 proteoglycan in the retina, retinal homogenates at various developmental stages from E16 to P42 were subjected to immunoblot analysis (Fig. 6A). These experiments showed that no immunopositive bands were detected in E16 retinas; rather, the 6B4 proteoglycan band was first detected at E18, and intensity of the band increased rapidly as development proceeded. Intensity reached a maximum on P14, and then decreased gradually, reaching adult levels at P42. Additionally, another band at around 180 kDa was detected in P14 retinas, and its expression level remained significant through P42.

The intensities of the immunolabeled bands of 6B4 proteoglycan were semiquantified by a densitometric analysis, and relative levels were calculated as the percentage of the maximum level (P14). The amount (\pm SE) of the band at E18 was $1.7\% \pm 1.7\%$ of the peak level at P14 (defined as 100%). The

amount increased rapidly to a peak level at P14 and then decreased gradually to the adult level ($32.1\% \pm 14.7\%$; Fig. 6B).

DISCUSSION

Our present study has revealed the expression of mRNAs and core proteins of 6B4 proteoglycan/phosphacan during retinal development of the rat. The mRNAs and core proteins of 6B4 proteoglycan are highly expressed in developing rat retinas (Figs. 1 and 6). Immunohistochemical studies showed that the immunoreactivity for 6B4 proteoglycan is intensely observed in NFL, IPL, and OPL (Fig. 2), where retinal neurites are localized, during postnatal development (between P7 and P14), when the retinal neurites undergo extension and when synapse formation occurs. 6B4 proteoglycan/phosphacan is one of the major proteoglycans in the central nervous system, and it

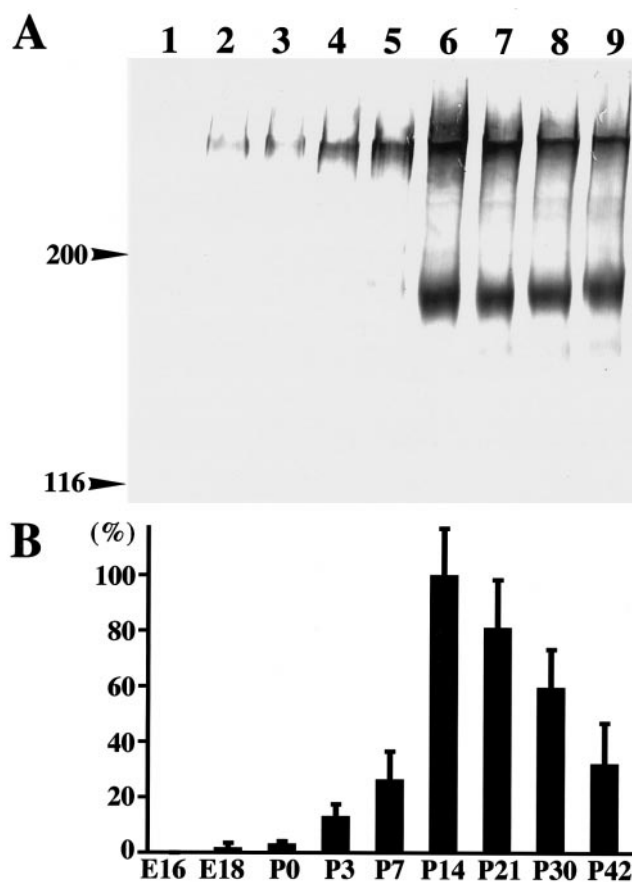


FIGURE 6. Immunoblot analysis for 6B4 proteoglycan during retinal development. (A) Retinal homogenates (50 μ g protein) were prepared from the retinas of rats at various ages. Immunoblot experiments showed that the intensities of the immunoreactive bands increased rapidly as development proceeded. Additionally, another band at \sim 180 kDa was detected in P14 retinas, and its expression level remained significant through P42. The samples were from E16 (*lane 1*), E18 (*lane 2*), P0 (*lane 3*), P3 (*lane 4*), P7 (*lane 5*), P14 (*lane 6*), P21 (*lane 7*), P30 (*lane 8*), and P42 (*lane 9*) animals. The positions of molecular mass markers are indicated in kilodaltons. (B) Densitometric analysis of the intensity of 6B4 proteoglycan bands. The relative levels were calculated as the percentage of the peak level on P14 ($n = 3$). Error bars represent standard errors.

has been shown to modulate neurite extension.^{8,10,11} This proteoglycan can alter cellular behaviors via binding to neural cell adhesion molecules and tenascin.⁷⁻⁹ The antibody used, MAb 6B4, has been reported to specifically recognize 6B4 proteoglycan/phosphacan in brain.¹⁰ The work described herein demonstrates that the expression of 6B4 proteoglycan increases in early postnatal periods, reaches a peak at P14, and then decreases in mature retinas. Thus, the predominant localization in developing synaptic layers and the temporal expression pattern suggest that 6B4 proteoglycan may play a role in neurite extension and/or synaptic formation in the retina and brain.

Our immunoblot experiments detected a MAb 6B4-immunopositive band in the retinal S₁ fraction after treatment with chondroitinase ABC at 280 kDa on SDS-PAGE. This molecular mass is different from that (300 kDa) known for the core glycoprotein of 6B4 proteoglycan in rat brain.¹⁰ The immunopositive band is also detected clearly in the retinal sample without chondroitinase ABC digestion. Further immunoblot analysis after sequential treatments with various glycosidases showed that the molecular mass of the immunoreactive bands derived from the retinal S₁ fraction became similar to that of the cerebral S₁ fraction on SDS-PAGE. It is thought that the core protein of retinal 6B4 proteoglycan could be less glycosylated, although we cannot exclude the possibility that the difference may be caused by the presence of splice variants. Also, even after sequential treatments with glycosidases, the molecular mass of the retinal immunoreactive bands did not completely coincide with those of the cerebrum on SDS-PAGE. It is well known that it is quite difficult to remove all the oligosaccharides linked to highly glycosylated core proteins including brain 6B4 proteoglycan. The reason may be because of the difference in glycosylation of the core proteins.

After chondroitinase ABC treatment, the immunopositive band of 280 kDa in the retinal S₁ fraction became more intense than before enzymatic treatment (Fig. 3). To confirm that 6B4 proteoglycan-bearing chondroitin sulfate chains are expressed in the retina, we performed additional studies with ion-exchange chromatography on a DEAE-Sephacel column. The MAb 6B4-immunopositive band was produced by digestion with chondroitinase ABC of the concentrated soluble proteoglycans from the retinal S₁ fraction (Fig. 5). These findings show that, although the non-proteoglycan form is expressed as the major form of 6B4 proteoglycan in the retina, some are actually present in a proteoglycan form.

Some proteoglycans are expressed as non-proteoglycan forms in certain situations. These proteoglycans are sometimes referred to as part-time proteoglycans. For example, appican, a chondroitin sulfate proteoglycan, is derived from an amyloid precursor protein (APP) mRNA that lacks exon 15. Splicing out of this exon creates a new consensus sequence for the attachment of a chondroitin sulfate chain in the resultant APP product.^{23,24} Moreover, Chinese hamster ovary and COS-7 cells express a non-proteoglycan form of thrombomodulin, whereas primary arterial endothelial cells and lung carcinoma cells express a proteoglycan form with chondroitin sulfate and a non-proteoglycan form.²⁵ In the human retina, a sialoprotein associated with cones and rods (SPACR), which is abundantly present in the interphotoreceptor matrix, is a non-proteoglycan form of interphotoreceptor matrix proteoglycan-1 (IMP1), a chondroitin sulfate proteoglycan.²⁶ Our findings,

together with these reports in the literature, suggest that the major retinal 6B4 proteoglycan is a non-proteoglycan form of 6B4 proteoglycan. So far, there have been no reports on the expression of the non-proteoglycan form of 6B4 proteoglycan/phosphacan as the major form in neural tissues. Thus, our study for the first time revealed that neural retinal tissues have an interesting feature in regards to the expression of 6B4 proteoglycan/phosphacan. In addition, another faint band of 230 kDa was detected in the S1 fraction eluted from the DEAE column (Fig. 5; lane 1). However, we were unable to detect this faint band in other immunoblot experiments using retinal homogenates. It implies that the possible proteoglycan is contained at a very low level in the retina or that the 230-kDa band is caused by artificial degeneration products of the DEAE column and subsequent elution treatments.

In rat ocular tissues, our immunohistochemical analysis clearly demonstrated that the immunoreactivities for 6B4 proteoglycan/phosphacan are limited to neural retinas and their axons. There are some previous immunohistochemical studies of retinal sections using anti-phosphacan (6B4 proteoglycan) antibodies. Meyer-Puttlitz et al.¹³ and Milev et al.¹⁴ reported that an anti-phosphacan polyclonal antibody is reactive with the NFL at E16 and with the IPL and OPL at P21. Using the same polyclonal antibody, Xiao et al.¹⁵ showed that the NFL, IPL, and OPL in adult mouse retina are also immunopositive. The immunoreactive patterns coincide with the results of our immunohistochemical study of developing retina using MAb 6B4. However, no conclusive data on the spatiotemporal expression patterns of phosphacan during retinal development have been reported because of a lack of detailed biochemical, molecular biological, and immunohistochemical studies for phosphacan of the retina. This is the first report to reveal detailed information on spatiotemporal expression patterns of 6B4 proteoglycan/phosphacan in the developing retina and its non-proteoglycan form. In rat retinas at early postnatal developmental stages (P7-P14), the differentiation of retinal cells and formation of neural networks among the differentiated retinal neuronal cells occur. Thus, the developmentally regulated expression patterns of 6B4 proteoglycan suggest that they are involved in the formation of neural networks during retinal development.

In conclusion, we have demonstrated the expression of mRNAs and core proteins for 6B4 proteoglycan/phosphacan in the rat retina. The core protein is less glycosylated than that of 6B4 proteoglycan in the brain. A significant portion of 6B4 proteoglycan/phosphacan in the retina exists in a non-proteoglycan form without chondroitin sulfate side chains. Moreover, our results show that, in developing rat retina, the expression of 6B4 proteoglycan is regulated both temporally and spatially, suggesting that it may play an important role in the differentiation and neural network formation in the mammalian retina.

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