Carbohydrate Modification of 6B4 Proteoglycan, and Its Splicing Variant, Receptor-like Protein Tyrosine Phosphatase ζ in the Developing Chick Brain

Hiroki Hamanaka

Doctor of Science

Department of Molecular Biomechanics
School of Life Science
The Graduate University for Advanced Studies

1996

CONTENTS

PREFACE

The vertebrate brain is one of the most elaborative creature of biological evolution. It is composed of a large number of various kinds of neurons, connected to each other with proper synapses, and forming highly complicated neural networks. For instance, the human brain contains about 10^{11} neurons classifiable into as many as 10^4 different types, allowing us to live with highly intellectual faculties. Thus, it is a central theme of modern neuroscience to understand the brain function including perception, movement, learning, and thinking.

As well as the understanding of brain function, it is another important problem of neuroscience to unravel the mechanism underlying the construction of brain structure. The brain originates from the neural plate of the neurula embryo, which subsequently gives rise to the neural tube. The neural tube is further specialized to three distinct swellings at the rostral end of the tube. These swellings are called the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon), ultimately giving rise to the cerebral hemispheres, the adult midbrain, and the brain stem and cerebellum, respectively. During and after these gross patternings, many dynamic processes occur in the neural tube to build up the brain structure, such as neuroblast production, apototic cell death, neuroblast migration, neurite extension, synapse formation, and synapse stabilization and elimination. These developmental events involve a diversity of cell-cell and cell-environment interactions. Cell-environment interactions occur between the cell surface and the extracellular matrix (ECM), a complex association of extracellular glycoproteins organized into aggregates and polymers. In developing neural tissue, many types of ECM molecules are expressed with distinct spatial and temporal patterns. Developing brain contains diverse set of proteoglycans as a major ECM component whose expression is brain-specific and dynamically

regulated. This fact suggests that proteoglycans play imporant roles in the various events in neural development. Although several of neural proteoglycans have been identified and cloned, little is known about their specific functions.

In this thesis, I studied the feature of carbohydrate modification of 6B4 proteoglycan (phosphacan) and its splicing variant, protein tyrosine phosphatase ζ, (PTPζ), which are major chondroitin/keratan sulfate proteoglyans in the developing brain. The aim of this study is the assessment of the possibility that specific carbohydrate modifications of these molecules are involved in specific biological functions during neural development.

SUMMARY

The brain contains a diverse set of proteoglycans as the major component of the cell-surface glycoconjugates and of the extracellular matrix (ECM). 6B4 proteoglycan (6B4 PG) is one of the major soluble chondroitin sulfate proteoglycans in the brain, and has a structure corresponding to the extracellular domain of a proteoglycan-type protein tyrosine phosphatase, PTPζ (RPTPβ). In this study, I first identified chick 6B4 PG by purifying it from embryonic chick brain and determining the N-terminal amino acid sequence. The obtained sequence corresponded well to that of rat 6B4 PG. From the biochemical properties of the purified protein and its immunoreactivity with MAb 6B4, I concluded that MAb 6B4 (originally raised against rat 6B4 PG)-reactive proteoglycan in chick tissue is chick 6B4 PG.

Next, I prepared polyclonal antibodies which specifically recognize chick 6B4 PG and PTPζ, and analyzed the carbohydrate structures on the two molecules in the developing chick brain. Immunoprecipitation experiments using these antibodies revealed that almost all of the keratan sulfate and protein-bound Le^x carbohydrate structure in the brain were exclusively bound to 6B4 PG and PTPζ. The modifications with keratan sulfate and Le^x carbohydrate occurred on both of 6B4 PG and PTPζ. Furthermore, I found that the modifications of 6B4 PG and PTPζ with these carbohydrates are spatiotemporally regulated during development.

Addition of keratan sulfate to these proteoglycans markedly increased from embryonic day 11 (E11), in contrast to that of Le^x and HNK-1 carbohydrates which increased gradually during development in accordance with expression of the core proteins. This suggests that keratan sulfate plays some specific roles in development. In the developing tectum, in contrast to the uniform distribution of the core proteins during development, keratan sulfate was expressed only in the lateral region of the tectum at E10, and the

expression was extended toward the dorsal area until E13. Moreover, keratan sulfate modification occurs in several restricted regions of the early embryonic chick brain, especially at morphogenetic boundary regions such as the roof plate of the tectum, the zona limitans intrathalamica in the diencephalon, and the mes-metencephalic boundary.

At the mes-metencephalic boundary, keratan sulfate modification of these proteoglycans was specifically observed from E3 to E6 on a ring of cells encircling the neural tube. In this region, keratan sulfate staining was observed on the cell bodies and the radially oriented processes. These processes seem to be stained with anti-vimentin antibody but not with anti-neurofilament antibody. Therefore, I tentatively defined these processes as radial glial fibers that are considered to be the scaffold for migrating neurons. I also found that the neurofilament- and Ng-CAM-positive growing axons passed through the keratan sulfate-rich mes-metencephalic boundary region. The expression pattern of keratan sulfate in this region spatiotemporally corresponded well to the *Wnt-1* and *Fgf-8* expression and to the fovea isthmi formation, a groove separating the mes-metencephalon along the neural tube. I found that these locally expressed keratan sulfate is also bound to 6B4 PG and PTPζ. These results suggest that keratan sulfate on 6B4 PG and PTPζ plays important roles in brain morphogenesis by modulating the cell-cell and/or cell-substratum interactions mediated by these molecules.

Cell-surface carbohydrates play essential roles in various biological processes by mediating cell-cell interactions (Varki, 1993). In vertebrate neural development, carbohydrate expression is thought to be fine tuned to allow cells to respond to stimuli from environment or to influence other cells (Schachner and Martini, 1995). For instance, it has been well elucidated that polysialic acid modification on N-CAM regulates the patterns of muscle innervation (Rutishauser and Landmesser, 1991) and the migration of olfactory bulb interneuron precursors (Ono et al., 1994; Hu et al., 1996). Among the cell-suface and extracellular glycoconjugates, proteoglycans are major constituents in the developing brain. Proteoglycans are unique molecules which bear the long sulfated carbohydrate chains called glycosaminoglycan (GAG). The common GAGs include galactosaminoglycans (chondroitin sulfate and dermatan sulfate) and glucosaminoglycans (heparan sulfate, heparin, and keratan sulfate). In Table 1, repeating disaccharide units and the major positions of sulfation on each GAG are shown. Several researchers reported that GAGs act as inhibitory factor for neuronal cell attachment and neurite outgrowth (Carbonetto et al., 1983; Akeson and Warren, 1986; Snow et al., 1990a; Cole and McCabe, 1991; Geisert and Bidanset, 1993). However, these effects are still controversial, and it is premature to conclude that GAGs are simple inhibitor for neural cell adhesion and neurite outgrowth.

In an effort to understand a role of proteoglycan in neural develoment, we have focused our study on the structure and function of 6B4 proteoglycan (6B4 PG) (also called phosphacan by another group), which is one of the major soluble chondroitin sulfate proteoglycans in the developing brain (Maeda et al., 1992; Maurel et al., 1994). Molecular cloning of 6B4 PG revealed that it consists of an N-terminal carbonic anhydrase (CAH)-like domain, a fibronectin type III domain and the large cysteine-free, serine glycine-rich region. This

molecule corresponds to the extracellular region of a receptor-like protein tyrosine phosphatase, PTPζ (also called RPTPβ), which is also expressed in the form of chondroitin sulfate proteoglycan (Barnea et al., 1994; Maeda et al., 1994) (Fig. 1). 6B4 PG is thus an extracellular splicing variant of PTPζ. The expression of these proteoglycans are developmentally regulated in various brain regions, suggesting that they are involved in many developmental processes including neuronal cell migration, differentiation, and circuit formation (Maeda et al., 1992, 1995; Milev et al., 1994). It has been reported that 6B4 PG and PTPζ bind to tenascin, Ng-CAM/L1, N-CAM, and F3/contactin (Grumet et al., 1994; Milev et al., 1994; Peles et al., 1995). In addition to these molecules, we recently demonstrated that 6B4 PG binds to pleiotrophin (PTN), also called heparin-binding growth-associated molecule (HB-GAM) or heparin-binding neurite-promoting factor (HBNF), whose amino acid sequence shows ~ 50% homology to midkine (MK) (Maeda et al., in press). Several lines of evidence have revealed that PTN has neurite outgrowth promoting activity (Rauvala et al., 1989; Raulo et al., 1992; Kinnunen et al., 1996). The expression profiles of PTN in rat cerebral cortex are quite similar to those of 6B4 PG and PTPζ (Maeda et al ., 1995; Matsumoto et al., 1994). These features suggest that 6B4 PG and PTPζ are involved in the signal transduction evoked by several molecules in the environment and play important roles in the brain morphogenesis.

It has been reported that 6B4 PG is modified with several carbohydrate structures including chondroitin sulfate, keratan sulfate, HNK-1 epitope, and N- and O-linked oligosaccarides (Grumet et al., 1994; Maeda et al., 1995). The content of chondroitin sulfate on this molecule and its composition of 4-sulfated and 6-sulfated units are known to change dynamically during development (Rauch et al., 1991; Maeda et al. 1995). It has also been reported that only a portion of 6B4 PG is modified with keratan sulfate (Grumet et al., 1994; Maeda et al., 1995). I hypothesized that this may be explained by region-specific modification of 6B4 PG and PTPζ with keratan sulfate.

To assess this possibility, I examined the modification pattern of 6B4 PG and PTPζ with keratan sulfate in the chick brain during development. Almost all keratan sulfate in the developing brain was found to be exclusively bound to these two molecules. Moreover, addition of keratan sulfate to 6B4 PG and PTPζ occurred selectively at several boundary regions such as the roof plate of the tectum and the mes-metencephalic boundary. It has been proposed that keratan sulfate functions as a barrier for axonal outgrowth (Snow et al., 1990a; Geisert and Bidanset, 1993). However, I observed many axons passing through the keratan sulfate-rich mes-metencephalic boundary. The developmental changes in keratan sulfate expression in this region corresponded well to those of *Wnt-1* and *Fgf-8* expression and to formation of the fovea isthmi. Based on these findings, I propose that keratan sulfate expressed in the boundary regions influences patterning of the brain by modifying the signal transduction system composed of 6B4 PG and/or PTPζ, and possibly WNT1 and/or FGF8, rather than acting as a simple barrier of axonal outgrowth. In addition, I found that 6B4 PG and PTPζ are modified with Le^x carbohydrate, also known as SSEA-1 (stage-specific embryonic antigen-1), or CD15 in the brain. I discuss here the possible roles of these carbohydrates on the two molecules in neural development.

MATERIALS AND METHODS

Materials. Fertilized White Leghorn eggs were purchased from a local supplier. DEAE-Toyopearl and TSKgel G6000PW were from Tosoh Corporation. LiChrospher 4000DMAE was from Cica-MERCK. MAb 6B4 was described previously (Maeda et al., 1992). Anti-keratan sulfate monoclonal antibody 5D4 (Caterson et al., 1983), anti-Le^x monoclonal antibody (clone 73-30), protease-free chondroitinase ABC, endo-β-galactosidase, keratanase II, and heparitinases I and II were purchased from Seikagaku Kogyo Co. HNK-1 monoclonal antibody was from Serotec. Anti-vimentin monoclonal antibody (clone Vim3B4) was from Boehringer Mannheim Biochemica. Anti-chick neurofilament monoclonal antibody 29B8 was a kind gift from Dr. H. Fujisawa (Hatta et al., 1987). Anti-N-CAM monoclonal antibody 224-1A6 (Lemmon et al., 1982) and anti-NgCAM/L1 monoclonal antibody 8D9 (Lagenaur and Lemmon., 1987) were kind gifts from Dr. M.Yamagata. HRP-conjugated anti-mouse Ig, biotinylated anti-mouse Ig, biotinylated anti-rabbit Ig and streptavidin-conjugated alkaline phosphatase were from Amersham. Vectastain ABC kit and fluorescein avidin DCS were from Vector Labs. Rabbit anti-mouse IgM was from Zymed Laboratories Inc. Protein G Sepharose 4 Fast Flow and HiTrap Protein G were from Pharmacia.

Preparation of antibodies. 6B4 PG was purified from embryonic day 14 (E14) chick brain according to the method described previously for rat brain (Maeda et al., 1995). Briefly, 20 g of chick brain from E14 embryos was homogenized in 100 ml of a solution containing 5 mM EDTA, 5 mM N-ethylmaleimide (NEM), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin A, 0.15 M NaCl, and 10 mM sodium phosphate, pH 7.2, with eight strokes in a glass-Teflon Potter homogenizer. The homogenate was centrifuged at 30,000 x g for 30 min at 4°C, and the resultant pellet was homogenized and centrifuged again as above. The combined supernatant was

applied to a DEAE-Toyopearl column, and then the chondroitin sulfate proteoglycan fraction was purified by CsCl density gradient centrifugation. 6B4 PG was isolated by TSKgel G6000PW and LiChrospher 4000DMAE column chromatography.

Purified chick 6B4 PG (50 μg) was precipitated with ethanol and dissolved in 100 μl of a solution containing 30 mM sodium acetate, 1 mM PMSF, 0.1 mM pepstatin A, 10 mM EDTA, 10 mM NEM, and 0.1 M Tris-HCl, pH 7.5 (solution A), and digested with 50 milliunits (mU) of protease-free chondroitinase ABC for 1 hr at 37°C. After precipitation with ethanol, the pellet was dissolved in 100 μl of a solution containing 5 mM EDTA, 5 mM NEM, 1 mM PMSF, 0.1 mM pepstatin A, and 50 mM sodium acetate, pH 6.0, and digested with 10 mU of endo-β-galactosidase for 1 hr at 50°C. This sample treated with chondroitinase ABC and endo-β-galactosidase was separated by 5% SDS-PAGE and stained with Coomassie brilliant blue. The core protein band of 6B4 PG was excised out and fragmented using glass syringes attached to a double-ended Luer-Lock connector, and then injected into rabbits as an immunogen.

As chondroitinase ABC treatment yields unsaturated disaccharide stubs of chondroitin sulfate on the core protein, the obtained antiserum showed some reactivity to this unusual saccharide structure. To remove this reactivity, the IgG fraction was further purified as follows. The IgG fraction (500 μg) in 3 ml of a solution containing 20 mM NaCl and 10 mM sodium phosphate, pH 7.5 (solution B), was mixed with 1 mg of chondroitinase ABC-digested purified chick aggrecan. After incubation for 2 hr at room temperature, the mixture was applied to a column of DEAE-Toyopearl (2 ml) pre-equilibrated with solution B. Antibodies specific to 6B4 PG were eluted with solution B, while antibodies against unsaturated stubs on aggrecan core protein remained in the column. The anti-6B4 PG antibody thus obtained showed no reactivity to the unsaturated disaccharide stubs of chondroitin sulfate.

To generate polyclonal PTPζ-specific antibody (anti-PZ1), a peptide (CSNSSHESRIGLAEGLESEK) corresponding to amino acid residues 1597-1615 (Krueger and Saito, 1992) of human PTPζ with an additional cysteine at the N terminus was synthesized. This sequence is located at the extracellular juxtamembrane position of PTPζ, which is not present in 6B4 PG. The synthetic peptide was coupled to maleimide-activated keyhole limpet hemocyanin, and then injected into rabbits. IgG fractions from anti-6B4 PG and anti-PZ1 antisera were prepared with HiTrap Protein G according to the supplier's protocol.

Amino acid sequence analysis. Purified chick 6B4 PG (50 μg) was digested with chondroitinase ABC and endo-β-galactosidase as described above. The sample was separated by 5% SDS-PAGE in the buffer system of Laemmli (1970), and transferred onto a PVDF membrane according to the method of Towbin et al. (1979). The transferred core protein was stained with 0.5% Ponceau S in 1% acetic acid, and then destained with 1% acetic acid. The core protein band was excised out and analyzed on an automatic protein sequencer (PPSQ-10; Shimadzu).

Glycanase digestion of tissue homogenates. Tissues were homogenized in a solution containing 0.32 M sucrose, 1 mM EDTA, 0.1 mM PMSF, 10 μM pepstatin A, 10 μM leupeptin, 5 mM NEM, 1 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.4. For heparitinase digestion, the homogenates (10-30 μg protein) were treated with 1 mU of heparitinases I and II in a solution (15 μl) containing 5 mM calcium acetate, 1 mM PMSF, 0.1 mM pepstatin A, 10 mM NEM, and 0.1 M Tris-HCl, pH 7.5, for 2 hr at 37°C. For chondroitinase ABC digestion, the homogenates (10-300 μg protein) were treated with 10 mU of protease-free chondroitinase ABC in 40 μl of solution A for 1 hr at 37°C. For chondroitinase ABC + keratanase II digestion, the homogenates (100 μg protein) were treated with 10 mU of chondroitinase ABC and 5 mU of keratanase II in solution A for 1 hr at 37°C. For endo-β-galactosidase digestion, the homogenates (100 μg protein) were treated with 5 mU of endo-β-

galactosidase in a solution (40 µl) containing 5 mM EDTA, 5 mM NEM, 1 mM PMSF, 0.1 mM pepstatin A, and 0.1 M sodium acetate, pH 6.5, for 1 hr at 50°C. The digested samples were applied to 5% SDS-PAGE in the buffer system of Laemmli (1970), or analyzed by immunoprecipitation.

Immunoprecipitation. The chondroitinase ABC-treated tissue homogenates (40 µl) were mixed with 10 µl of 10% SDS and then boiled for 3 min. For immunoprecipitation experiments with anti-6B4 PG, enzyme-untreated homogenate was used. The boiled samples were diluted with 1 ml of phosphate-buffered saline (PBS) containing 5 mM EDTA, 5 mM NEM, 0.1 mM PMSF, 10 µM pepstatin A, 0.2% Triton X-100, and then incubated for 2 hr at 4°C with 10 µg of the appropriate antibody. Only for anti-Le^x Ab, 30 µg of rabbit anti-mouse IgM was added as the secondary antibody, and then samples were incubated for 1 hr at 4°C. The samples were mixed with 50 µl of a 70% (v/v) suspension of Protein G Sepharose 4 Fast Flow and incubated for 30 min at 4°C. The supernatant fractions of the immunoprecipitates were mixed with three volumes of 95% ethanol containing 1.3% sodium acetate and incubated for 30 min at -20°C. After centrifugation, the precipitated proteins were then solubilized with 50 µl of SDS-PAGE sample buffer and boiled for 5 min. The protein-bound Protein G Sepharose was washed three times with 1 ml of 0.5% Triton X-100/PBS, and then mixed with 50 µl of 2-fold concentrated SDS-PAGE sample buffer and boiled for 3 min. For immunoprecipitation with anti-6B4 PG, the protein-bound Protein G Sepharose was suspended in 30 µl of solution A, and treated with 10 mU of protease-free chondroitinase ABC for 1 hr at 37°C. Then, the sample was mixed with 10 µl of 4-fold concentrated SDS-PAGE sample buffer and boiled for 3 min. After centrifugation, the supernatants of the boiled samples were subjected to 5% SDS-PAGE.

Immunoblot analysis. After electrophoresis, the proteins were transferred onto PVDF membranes, which were then blocked with 3% gelatin in PBS for MAb 6B4 staining, or with 5% nonfat dried milk in PBS for the other

antibodies. The membranes were incubated for 1 hr with primary antibodies, and the signals were detected by processing with a Vectastain ABC kit or a biotinylated secondary antibody and alkaline phosphatase-conjugated streptavidin system according to the manufacturer's protocol.

Immunohistochemistry. For immunohistochemistry, embryos were collected in ice-cold PBS, fixed with 4% paraformaldehyde in PBS or Carnoy's fixative for 3-12 hr, 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 sequentially treated in the following order; (1) 2.5% H_2O_2/PBS for 10 min, (2) 1% BSA/4% goat serum/PBS for 1 hr, (3) a solution containing the primary antibodies diluted with 0.1% BSA/PBS or the tissue culture supernatant in the case of MAb 6B4 and anti-neurofilament staining, for 12-24 hr at 4°C, (4) biotinylated anti-mouse IgG or IgM solution for 1 hr, (5) avidin-biotin-peroxidase complex (ABC) solution for 1 hr, and (6) 0.1% diaminobenzidine/0.02% hydrogen peroxide/PBS. For N-CAM and Ng-CAM staining, cryostat sections of 10 μm thick were used.

Endo-β-galactosidase treatment of the tissue sections was performed as follows. After the sections were deparaffinized and immersed in 2.5% H_2O_2/PBS, they were equilibrated with a solution containing 5 mM EDTA, 5 mM NEM, 1 mM PMSF, 0.1 mM pepstatin A, 0.1 mg/ml BSA, and 0.1 M sodium acetate, pH 5.8 for 10 min. Endo-β-galactosidase was added at a concentration of 50 mU/ml and the samples were incubated for 1 hr at 50°C.

Whole-mount immunostaining. Whole-mount immunostaining was carried out as described by Shimamura and Takeichi (1992). Embryonic brains were dissected, freed from connective tissues in ice-cold PBS, and fixed with 4% paraformaldehyde in PBS for 3-12 hr. The samples were sequentially treated as follows: (1) methanol for 30 min at -20°C, (2) 3% H_2O_2/methanol for 30 min at -20°C, (3) 2% BSA/0.1% Triton X-100 for 3-12 hr at 4°C, (4) a

solution of primary antibody (MAb 5D4) diluted with 0.1% BSA/0.1% Triton X-100/PBS for 12 hr at 4°C, (5) HRP-conjugated anti-mouse Ig solution for 3-12 hr, and (6) 0.1% diaminobenzidine/0.02% hydrogen peroxide/PBS at 0°C. The samples were cleared through a series of graded glycerol.

Other methods. Fertilized eggs were incubated at 37.5°C in an atmosphere with constant humidity of 70%, and embryos were staged after Hamburger and Hamilton (1951). Chick aggrecan was purified from the cartilaginous portions of E17 tibias and femurs according to the method described by Heinegård (1972). Protein concentration was measured with a Bio-Rad protein assay kit with BSA as a standard.

Identification of chick 6B4 PG

6B4 PG (phosphacan) and PTPζ (RPTPβ) have been molecularly characterized in rat and human tissues. Monoclonal antibody 6B4 (MAb 6B4) was raised against the soluble chondroitin sulfate proteoglycan fraction from rat brain (Maeda et al., 1992). To deal with chick embryos for developmental analysis, I first identified chick homolog of 6B4 PG. On immunoblots of chondroitinase ABC-treated chick brain extract, MAb 6B4 reacted with a 300-kDa band as observed using rat brain extract (data not shown). To confirm that this MAb 6B4-reactive proteoglycan is the authentic chick homolog of 6B4 PG, I purified the proteoglycan reactive with MAb 6B4 from embryonic chick brain and analyzed its N-terminal amino acid sequence. Purification was carried out as described previously for rat 6B4 PG (Maeda et al., 1995). After chondroitinase ABC digestion, the purified proteoglycan showed a single core protein band of 300 kDa on SDS-PAGE as in the case of rat brain (Maeda et al., 1995) (Fig. 2*A*). This purified chick proteoglycan was recognized by MAb 6B4 (Fig. 2*B*, lane 1). Furthermore, the N-terminal amino acid sequence of the purified proteoglycan was YYXQXRKLTE, which corresponds well to that of rat 6B4 PG, YYRQQRKLVE (Maurel et al., 1994) except for the ninth position where valine was substituted by threonine; I could not assign amino acid residues at the positions marked with an X (Fig. 3). From these results, I concluded that MAb 6B4 recognizes the chick homolog of 6B4 PG.

To obtain antibodies for immunoprecipitation experiments and which selectively recognize PTPζ, I raised polyclonal antibodies against purified chick 6B4 PG (anti-6B4 PG) and against a peptide whose sequence is specific to PTPζ (anti-PZ1), respectively. Anti-6B4 PG recognized the purified chick 6B4 PG (Fig. 2*B*, lane 2). On immunoblots using anti-PZ1, embryonic day 14 (E14) chick brain homogenate gave a smear near the top of the gel (Fig. 4, lane 1). By

chondroitinase ABC digestion, a discrete band of the core protein also around 300 kDa was seen (Fig. 4, lane 2, arrowhead). In the previous study, rat PTPζ core protein was detected as a 380-kDa band which is different from 300-kDa band of 6B4 PG (Maeda et al., 1994). I could not distinguish the difference in the molecular size of chick 6B4 PG and PTPζ core proteins on immunoblots in this study. The peptide used for immunization blocked the binding of anti-PZ1 to PTPζ (Fig. 4, lane 3). Moreover, anti-PZ1 detected PTPζ but did not react with 6B4 PG, when it was tested using L cell transfectants with cDNA constructs expressing PTPζ or 6B4 PG (data not shown). From these results, I concluded that anti-PZ1 specifically recognizes PTPζ.

MAb 6B4 and anti-6B4 PG recognize both 6B4 PG and PTPζ , while anti-PZ1 reacts only with PTPζ. Using these antibodies, I analyzed in detail the expression of 6B4 PG and PTPζ during development of the chick brain. Although 6B4 PG is a secreted protein, it is present as a contaminant in the membrane fraction (Maeda et al., 1994). Therefore, separation of soluble and insoluble fractions is not sufficient to discriminate 6B4 PG from PTPζ. It is also difficult to distinguish 6B4 PG from PTPζ by their electrophoretic mobilities as described above. Thus, the immunoprecipitation with anti-PZ1 was used to discriminate between two molecules. Under my immunoprecipitation conditions, anti-PZ1 precipitated more than 90% of PTPζ in the tissue extract.

6B4 PG and PTPζ are the major keratan sulfate-containing molecules in the developing chick brain

It has been reported that rat 6B4 PG bears keratan sulfate chains (Grumet et al., 1994; Maeda et al., 1995). To determine how many molecules contain keratan sulfate in the developing chick brain, total protein extracts from the E14 brain were analyzed by immunoblotting using a monoclonal antibody to keratan sulfate, MAb 5D4. When intact or heparitinase-digested samples were analyzed, immunoreactivity was observed diffusely near the top of the gel (Fig.

5*A*, lanes 1 and 2). Following chondroitinase ABC digestion, this reactivity became a single band of 300 kDa (Fig. 5*A*, lane 3). The electrophoretic mobility of this band was not changed by additional digestion with heparitinase (Fig. 5*A*, lane 4). This result indicates that the chondroitin sulfate proteoglycan with a 300-kDa core glycoprotein is the major molecular species which contains keratan sulfate in the developing chick brain.

To characterize this proteoglycan, immunoprecipitation experiments were performed. When anti-6B4 PG was tested, this antibody precipitated almost all of the MAb 5D4-epitope from the chick brain extracts (Fig. 5*B*), indicating that 6B4 PG and/or PTPζ are the major keratan sulfate-containing proteoglycans in the developing chick brain. MAb 5D4 almost completely immunoprecipitated the keratan sulfate epitope in the brain extract (Fig. 5*C*, a), while a large proportion of the MAb 6B4-immunoreactive molecules remained in the supernatant (Fig. 5*C*, b, lane 2). This indicates that only a portion of 6B4 PG and/or PTPζ is modified with keratan sulfate in the developing brain.

Next, I examined whether keratan sulfate modification occurs on both 6B4 PG and PTPζ, or on only one of these molecules. I analyzed the keratan sulfate on PTPζ immunoprecipitated with anti-PZ1, and on 6B4 PG in the supernatant. The immunoprecipitated PTPζ was recognized not only with MAb 6B4 but also with MAb 5D4 (Fig. 6*A*, *B*, lane 1), indicating that PTPζ is modified with keratan sulfate. Moreover, large amounts of MAb 6B4- and 5D4-reactive substances remained in the supernatant fraction of the anti-PZ1 immunoprecipitation (Fig. 6*A*, *B*, lane 2). These results indicate that 6B4 PG is also modified with keratan sulfate.

6B4 PG and PTPζ are the major Le^X-containing proteins in the developing chick brain

It has been reported that Le^X carbohydrate, also known as CD15 or SSEA-1-epitope, is distributed in several regions in the central (Lagenaur et al., 1982;

Snow et al., 1990b; Oudega et al., 1992; Marcus et al., 1995) and the peripheral nervous system (Sieber-Blum, 1989). Le^X carbohydrate is considered to be involved in cell-cell recognition through homo- and heterophilic interactions. I examined whether 6B4 PG and PTPζ bear Le^X carbohydrate using purified chick 6B4 PG. By immunoblot analysis, 6B4 PG was detected with anti-Le^X Ab (Fig. 7, lane 1), and this reactivity disappeared following digestion with endo-β-galactosidase (Fig. 7, lane 2). Keratanase II, N-glycanase and O-glycanase digestion did not affect the reactivity (data not shown).

Next, I investigated how many glycoproteins have Le^X carbohydrate in the developing chick brain. Total protein extracts from E14 brain were analyzed by immunoblotting with anti-Le^X Ab. After digestion with chondroitinase ABC, a single discrete band of 300 kDa was observed (Fig. 8*A*). Furthermore, anti-6B4 PG precipitated almost all of the anti-Le^X epitope from the chick brain extracts (Fig. 8*B*), indicating that 6B4 PG and/or PTPζ are the major Le^X-containing proteins in the developing chick brain. Conversely, the core protein immunoprecipitated with anti-Le^X Ab was recognized with MAb 6B4 and anti-PZ1 (Fig. 8*C*), indicating that PTPζ is also modified with Le^X carbohydrate. The presence of Le^X carbohydrate on both 6B4 PG and PTPζ was further confirmed by immunoprecipitation experiments with anti-PZ1 (Fig. 8*D*).

Modification of 6B4 PG and PTPζ with keratan sulfate, Le^X, and HNK-1 carbohydrates are developmentally regulated in the chick brain

I examined the developmental changes in the modification pattern of 6B4 PG and PTPζ with several carbohydrates. The immunoprecipitates with anti-6B4 PG from brain extracts were analyzed at various stages by immunoblotting with antibodies which recognize carbohydrates. As shown in Figure 9*A*, the expression levels of the core proteins gradually increased from E5 to E18. On the other hand, keratan sulfate modification levels remained quite low until E7,

and then showed marked elevation at E11 (Fig. 9*B*). In contrast, the levels of Le^x and HNK-1 carbohydrates almost paralleled those of the core protein (Fig. 9*C*, *D*). The peculiar expression pattern of keratan sulfate suggests that the modification with this carbohydrate plays a significant role in the brain development. Therefore, I analyzed the expression patterns of 6B4 PG and keratan sulfate in the developing tectum.

In the developing chick tectum, keratan sulfate was distributed on fibrous structures in the intermediate zone. At E10, keratan sulfate staining was observed in the lateral region of the tectum (Fig. 10*A*). The positive region extended toward the dorsal area at E11 (Fig. 10*B*), and reached the dorsal tectum at E13 (Fig. 10*C*). In contrast, the core protein of 6B4 PG and PTPζ was distributed uniformly throughout the tectum at all stages examined (Fig. 10*D-F*). Taken together with the finding that 6B4 PG and PTPζ are the major keratan sulfate-containing proteoglycans in the developing chick brain, these results suggest that keratan sulfate modification of these proteoglycans is strictly regulated in a spatiotemporal manner in the developing tectum.

Keratan sulfate expression occurs at the boundary regions in the developing brain at early stages

In contrast to the broad distribution in the brain at stages later than E7 (Fig. 10*A-C*), keratan sulfate expression was observed in several restricted regions in the early stage (E3-E5). Whole-mount immunostaining of E5 embryos showed localized expression of keratan sulfate at the ventral side of the telencephalon, the zona limitans intrathalamica in the diencephalon, the pretectal area, the roof plate of the rostral part of the tectum, and the boundary of the mes-metencephalon (Fig. 11*A*). The expression in the telencephalon, probably in the prospective olfactory nervous system, was intense. Characteristic staining was observed in the neuromere boundary regions. In the diencephalon, keratan sulfate expression was observed at the zona limitans intrathalamica (arrowheads

in Fig. 11*A*), which represents the border between the ventral and dorsal thalamus and contains the primordial mammillothalamic tract. At the mes-metencephalic boundary (the isthmic region), staining was observed as a ring-like structure encircling the neural tube, although it was missing near the dorsal and ventral midlines (Fig. 11*B,D*). As shown in Figure 11*C*, the roof plate of the rostral part of the tectum was also intensely stained (arrowheads). In E13.0 mouse embryos, a ring-like staining pattern was seen at the mes-metencephalic boundary similar to that in E5 chick embryos (Fig. 11*E*).

Keratan sulfate expression at the mes-metencephalic boundary is developmentally regulated

In the mes-metencephalic region, keratan sulfate expression began at E3 (stage18) around the surface of neuroepithelial cells (Fig. 12*A*, arrowhead). The stained cells were radially oriented across the neural tube from the ventricle to the pial surface of the ventral mesencephalon. At this stage, it was impossible to anatomically define the mes-metencephalic boundary, because morphologically distinct structures were not present. At E4, a groove appeared on the basal plate of the neural tube (Fig. 12*B*, large arrowhead). This groove is known as the fovea isthmi, an anatomical landmark of the ventral mes–metencephalic boundary (Vaage, 1969; Kuhlenbeck, 1973). The region expressing keratan sulfate strictly corresponded to this groove at the ventricular side (Fig. 12*B*). At this stage, the staining became intense and was present not only on cell bodies but also on processes which extended radially toward the pial surface across the neural tube. Staining was also observed on the fibers running along the neural tube rostrocaudally and which make contact orthogonally with the keratan sulfate-positive radial processes (Fig. 12*B*, arrow). Staining was also observed in the dorsal region, in which the stained cells aligned from the ventricle to the dorsal pial surface where the neural tube established flexure (Fig. 12*B*, small arrowhead). This flexure is formed between the caudal tectum

and the rostral cerebellar anlage, which gives rise to the dorsal mes-metencephalic boundary. At E6, most staining on the cell bodies and fibrous structures disappeared except for the apical surfaces of the neuroepithelial cells (Fig. 12*C*, arrowhead). From this stage, the level of specific staining diminished from the mes-metencephalic boundary, although uniform staining was observed on the neuropil in the ventral mesencephalon (Fig. 12*D*).

To characterize the cells which express keratan sulfate at the mes-metencephalic boundary, serial sections of E5 brain were stained with MAb 5D4, anti-vimentin, and anti-neurofilament. As shown in Figure 13*A*, MAb 5D4-positive processes extended from the ventricular zone to the pial surface. These processes appeared to be stained with anti-vimentin (Fig. 13*B*, arrowhead), but not with anti-neurofilament (Fig. 13*C*). Thus, these processes were likely to be radial glial fibers. In addition, I stained serial sections with anti-N-CAM and anti-Ng-CAM/L1 (Fig. 13*E*, *F*). These cell adhesion molecules were expressed on neurofilament-positive fibers, passing through keratan sulfate positive-regions (Fig. 13*D*-*F*). Furthermore, in order to examine the correlation between the keratan sulfate expression and the mitotic state of cells, I double-stained a section with MAb 5D4 and hematoxylin. This showed that keratan sulfate is expressed on both the mitotic-active and post-mitotic migrating cells (Fig. 14).

To confirm that MAb 5D4-staining represents the distribution of keratan sulfate, I analyzed the effect of endo-β-galactosidase treatment, which degrades keratan sulfate, using serial sections. The immunoreactivity was reduced by the enzyme treatment (Fig. 15), indicating that MAb 5D4 specifically recognizes keratan sulfate.

6B4 PG and PTPζ are modified with keratan sulfate at the boundary regions in the embryonic chick brain

Keratan sulfate was expressed in highly restricted regions in the E5 brain (Fig. 16*A*), while the carrier protein 6B4 PG showed a wide distribution (Fig. 16*B*).

On immunoblots of proteins from several portions of E5 chick neural tissue, keratan sulfate was detected in the telencephalon, the mes-metencephalic boundary and the dorso-rostral portion of tectum including the roof plate, but not in the rhombencephalon (Fig. 17*A*), as expected from the results of tissue immunostaining (Figs. 11*A*, 16*A*). On the other hand, 6B4 PG and PTPζ were expressed in all regions including the telencephalon, the mes-metencephalic boundary, the dorso-rostral and caudal portion of tectum, and the rhombencephalon (Fig. 17*C*, *E*).

To determine whether this locally expressed keratan sulfate is attached to 6B4 PG and/or PTPζ, the immunoprecipitates obtained with MAb 5D4 were analyzed by immunoblotting with MAbs 5D4 and 6B4, and anti-PZ1. MAb 6B4 reacted with the MAb 5D4 immunoprecipitates from the telencephalon, the mes-metencephalic boundary and the rostral portion of tectum (Fig. 17*D*). Anti-PZ1 reacted with the precipitates from the telencephalon and the mes-metencephalic boundary (Fig. 17*F*). Furthermore, the immunoprecipitated PTPζ from E5 mes-metencephalic boundary was recognized by MAbs 6B4 and 5D4 (Fig. 17*G*, a,b, lane 1). I also observed immunoreactivities with MAbs 6B4 and 5D4 in the supernatant (Fig. 17*G*, a,b, lane 2). These results indicate that both 6B4 PG and PTPζ are modified with keratan sulfate in these restricted regions of the developing brain, and addition of keratan sulfate to PTPζ occurs in more restricted regions. The specificity of MAb 5D4 for keratan sulfate was confirmed by immunoblotting, since both keratanase II and endo-β-galactosidase treatment reduced the immunoreactivity with this MAb (Fig. 17*H*).

Keratan sulfate-containing proteoglycan in the developing brain

In the present study, I analyzed carbohydrate structures on 6B4 PG and its splicing variant PTPζ, and found that these are the two major keratan sulfate-containing proteoglycans in the developing chick brain. Krueger et al. (1992) reported that an HNK-1-reactive chondroitin sulfate proteoglycan with a 340-kDa core protein is substituted with keratan sulfate in embryonic chick brain. This proteoglycan was likely to be 6B4 PG, judging from its core protein size, and immunoreactivity profiles with regard to HNK-1 and MAb 5D4.

In this study, I used MAb 5D4, raised against human articular cartilage proteoglycan (Caterson et al., 1983), to detect keratan sulfate. This MAb has been shown to recognize highly sulfated sequences of at least hexasaccharide (Mehmet et al., 1986). MAb 5D4 staining revealed the localized expression of keratan sulfate in the early embryonic chick and mouse brain (Fig. 11). MAb 5D4 reactivity in the early embryonic chick brain completely disappeared after endo-β-galactosidase digestion (Fig. 15), indicating that this MAb specifically recognizes keratan sulfate. Furthermore, on immunoblots, MAb 5D4 definitely recognized keratan sulfate on 6B4 PG and PTPζ, which was sensitive to the enzyme digestion (Fig. 17*H*; Maeda et al., 1995). However, based on the results of immunohistochemical analysis, Meyer-Puttlitz et al. (1995) reported that keratan sulfate is not present generally in the embryonic rat brain and suggested that most of the immunoreactivity seen in embryonic brain with MAb 5D4 does not represent keratan sulfate-specific staining because the staining was not abolished by digestion with endo-β-galactosidase and keratanase. I do not know the reason for this discrepancy; however, this might have been due to differences in animal species studied or the methods used for preparation of tissue sections. It has been reported that MAb 5D4 does not detect all of the keratan sulfate structures (Mehmet et al., 1986). Actually, claustrin, a keratan

sulfate proteoglycan with 100- and 70-kDa core proteins in chick brain (Burg and Cole, 1994), is only weakly immunoreactive with MAb 5D4 (Cole and McCabe, 1991). In this context, I cannot rule out the possibility that there are other keratan sulfate-containing proteoglycans in the developing chick brain.

Spatiotemporally restricted modification of 6B4 PG and PTPζ with keratan sulfate in the early chick embryo

I described the expression pattern of keratan sulfate which is selectively added to 6B4 PG and PTPζ in the embryonic chick brain during development. At early stages (E3-E5), keratan sulfate was expressed in several restricted regions such as the ventral side of the telencephalon, the pretectal area, the roof plate of the rostral part of the tectum, the zona limitans intrathalamica in the diencephalon, and the mes-metencephalic boundary. 6B4 PG and PTPζ bind to several molecules including N-CAM, Ng-CAM, tenascin, contactin, and pleiotrophin. These interactions may modulate neuronal and glial adhesion, neurite outgrowth, and signal transduction across the membrane during brain development. It is likely that the regulated keratan sulfate modification of 6B4 PG and PTPζ mediates specific developmental processes at particular anatomical sites and stages of development.

In the mes-metencephalic boundary, keratan sulfate expression seems to be correlated with the formation of a groove called the fovea isthmi which is considered to be the anatomical landmark of this boundary (Vaage, 1969; Kuhlenbeck, 1973). The keratan sulfate expression began at E3 (Fig. 12*A* and 18), preceding groove formation. From E4 to E5, the groove became noticeable, and at these stages, intense staining was observed on the neuroepithelial cells located in this region (Fig. 12*B* and 18). At E6, the expression of keratan sulfate became weak, concomitantly with the disappearance of the groove (Fig. 12*C*). These observations suggest that keratan sulfate may play a role in the formation of the fovea isthmi. At the cellular level, keratan sulfate expression was

observed on the cell bodies and the processes. I tentatively defined these processes as radial glial fibers which are considered to be the scaffold used to direct the migration of neurons (Rakic, 1971, 1972; Hatten and Mason, 1990; Misson et al., 1991). In principle, the formation of grooves and swellings in the neural tube is largely the result of disproportionate cell proliferation along the tube. This idea is true to the case of hindbrain development. In hindbrain, an axial series of swellings, termed rhombomeres, manifest in early developmental stage. It was reported that the density of mitotic figures is greater toward the centers of rhombomeres than in the boundary regions (Guthrie et al., 1991). I examined the correlation between the keratan sulfate immunoreactivity and the mitotic state of cells in the mes-metencephalic boundary. Keratan sulfate was expressed on both mitotic-active and post-mitotic cells, suggesting that keratan sulfate does not function to suppress proliferation nor to maintain the mitotic-active state. However, more detailed study concerning proliferation and cell-lineage analysis in the mes-metencephalic region will be required.

Several genes encoding transcription factors such as *Otx-1/-2*, *En-1/-2*, *Pax-5/pax(zf[b])* and secreted proteins *Wnt-1* and *Fgf-8* are known to be expressed in the mes-metencephalic border region (Bally-Cuif and Wassef, 1995). Among these, there seems to be a spatiotemporal correlation between the expression of *Wnt-1*, *Fgf-8*, and keratan sulfate. In the chick embryo, *Wnt-1* expression in the mes-metencephalic region is confined to a ring of cells localized immediately rostral to the isthmus from E3 to E7 (Bally-Cuif and Wassef, 1994). These cells encircle the neural tube except for the ventral midline, which is quite similar to the keratan sulfate expression pattern. The characteristic expression of keratan sulfate at the mes-metencephalic boundary was also observed in the mouse embryos from E10.5 to E13.0 (Fig. 11*E*), although I could not determine the precise time of the onset of expression here because I did not examine stages earlier than E10.5. *Wnt-1* expression in mouse in this region was observed from E9.5 to E12.5 (Bally-Cuif et al., 1992).

Furthermore, from E9.0 to E12.5, *Fgf-8* expressing cells were localized immediately caudal to the ring of *Wnt-1* expressing cells (Crossley and Martin, 1995).

Disruption of the *Wnt-1* gene revealed that this molecule plays a crucial role in the early development of the mes-metencephalic region (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Moreover, at stages when its expression domain is restricted to a narrow ring, this protein is thought to function as a boundary to stabilize and maintain two differentiated domains, *i.e.* the mesencephalon and the metencephalon (Bally-Cuif and Wassef, 1994; Bally-Cuif et al., 1995). Recently, Crossley et al. (1996) reported surprising findings concerning the function of *Fgf-8* in brain organization; FGF8-coated beads implanted in the caudal forebrain of chick embryo induced development of a complete midbrain by establishing an ectopic isthmus-like organizing center in the forebrain, indicating that endogenous FGF8 protein expressed at the isthmus organizes mes-metencephalic development.

Keratan sulfate expression was also observed at the zona limitans intrathalamica which represents the border between the ventral and dorsal thalamus. It has been reported that *sonic hedgehog (Shh)*, a secreted signaling molecule implicated in floor plate and motor neuron induction (Echelard et al., 1993; Roelink et al., 1994), is expressed at the zona limitans in mouse embryos around E10.5 (Shimamura et al., 1995) and in chick embryos at stage 18-19 (Ericson et al., 1995). Rubenstein et al. (1994) suggested that the neuromere boundaries such as the zona limitans and the isthmus may be neuroepithelial organizers which have inductive properties and may play a role in patterning along the rostrocaudal axis of the neural tube. Considering this idea, it is tempting to speculate that keratan sulfate-containing 6B4 PG and/or PTPζ may play a role in the signal transduction required for axial patterning in the neural tube in combination with other signaling molecules such as *Shh* in the zona limitans, or *Wnt-1* and *Fgf-8* in the isthmus. Alternatively, keratan sulfate on

these molecules at the boundary may act as a barrier that restricts the diffusion of morphogenetic signals during patterning of the neural tube. Such a diffusion barrier has been suggested to be present at the zona limitans intrathalamica (Crossley et al., 1996). In this context, it should be noted that 6B4 PG specifically binds to peptide factors such as pleiotrophin (Maeda et al., in press).

Keratan sulfate and neurite outgrowth

There is controversy concerning the physiological role of keratan sulfate. Some researchers reported that keratan sulfate functions as an inhibitory factor for neurite outgrowth and cell adhesion (Snow et al., 1990a; Cole and McCabe, 1991; Geisert and Bidanset, 1993). However, we have previously shown that keratan sulfate on 6B4 PG does not affect the neurite outgrowth-promoting activity of this molecule on rat cortical neurons (Maeda and Noda, 1996). Dou and Levine (1995) demonstrated that keratan sulfate inhibited neurite outgrowth from both rat cerebellar and dorsal root ganglia neurons on laminin-coated substrates, but the inhibitory effect was not evident on L1-coated substrates. They therefore suggested that keratan sulfate does not exert strong negative influences over axonal outgrowth in the developing central nervous system where L1 glycoprotein is abundant. The results of the present study support this idea. As shown in Figure 13*C* , many neurofilament-positive fibers passed through the keratan sulfate-rich mes-metencephalic boundary region, where these neurofilament-positive fibers were also intensely stained with anti-Ng-CAM/L1 antibody (Fig. 13*F*). This finding suggests that keratan sulfate in the mes-metencephalic boundary is not a barrier for nerve fiber growth in this Ng-CAM/L1-rich region.

Modification of 6B4 PG and PTPζ with Le^x carbohydrate

6B4 PG and PTPζ were the only Le^x carbohydrate-containing proteins detected in the developing chick brain. The same results were obtained from

the neonatal rat brain (data not shown). These findings were quite surprising considering that many Le^X-containing proteins were detected in other systems. Immunoblot analysis of mouse teratocarcinoma cells, for instance, demonstrated that polydisperse high-molecular-weight glycoproteins (apparent molecular masses > 100 kDa) and a number of discrete protein bands reacted with anti-Le^X Ab (Childs et al., 1983). In this study, these molecules could not be detected, suggesting that 6B4 PG and PTPζ play unique functions through Le^X carbohydrate in the developing brain. I showed that the Le^X carbohydrate structure on 6B4 PG is susceptible to digestion by endo-β-galactosidase. Since endo-β-galactosidase does not have exoglycosidase activity, this suggests that Le^X is carried on the carbohydrate chains containing internal N-acetyllactosamine sequences (Fukuda et al., 1978). Keratanase II digestion of 6B4 PG did not remove the Le^X carbohydrate, indicating that this structure is not carried on keratan sulfate as a chain-capping structure.

Le^X has been demonstrated to play roles in compaction of the mouse embryo at the morula stage and adhesion of embryonal carcinoma cells (Fenderson et al., 1984; Eggens et al., 1989). This carbohydrate may be recognized by a lectin (Grabel et al., 1983), and further, a homotypic Le^X-Le^X interaction was reported in the presence of Ca^{2+} and Mg^{2+} (Eggens et al., 1989). In addition to the protein-bound form, Le^X carbohydrates have been shown to be present in the form of glycolipids in both the human and rat brain (Yamamoto et al., 1985; Schwarting et al., 1989). Therefore, various Le^X-mediated interactions among PTPζ, 6B4 PG, and Le^X-containing glycolipids might lead to the modification of the PTPase activity of PTPζ. Alternatively, Le^X-specific lectins, although not yet identified, may be involved in cell signaling as ligands for PTPζ. It is also possible that PTPζ molecules form aggregates by Le^X-mediated side by side interaction, which may trigger cell signaling. All these processes would be modulated by Le^X-containing

glycolipids on the cell surface. Further studies are required to reveal the functional importance of Le^X carbohydrate on 6B4 PG and PTPζ.

Concluding remarks

I demonstrated that 6B4 PG and PTPζ are the major, if not the only, keratan sulfate- and Le^X carbohydrate-containing molecules in the developing chick brain, and modification of these proteoglycans with keratan sulfate and Le^X carbohydrate was developmentally regulated. Keratan sulfate addition on these proteoglycans was region-specific, especially at the boundary areas such as the roof plate of the tectum, the zona limitans intrathalamica in the diencephalon and the mes-metencephalic boundary. These results suggest that locally expressed carbohydrates modify the signal transduction pathway at restricted regions related with morphogenetic boundaries. Future studies of the involvement of carbohydrates in signal transduction, in for example ligand binding, may reveal new aspects of the mechanism of neural development.

ACKNOWLEDGEMENTS

I would like to express sincere gratitude to Prof. Masaharu Noda for his support and encouragement throughout my graduate research. I am also grateful to Dr. Nobuaki Maeda for his constant advice and guidance during the past several years. Drs. Masahito Yamagata, Eiji Watanabe and Shinji Hirano gave me useful advices and discussions. I would also like to thank my friends and colleagues including Dr. Masakazu Takahashi, Jyunichi Yuasa, Takafumi Shintani and Taeko Nishiwaki for their advices and enlightening discussions.

Of foremost importance, I owe a special debt of gratitude to my family for their unfailing support and encouragement throughout my graduate research.

REFERENCES

Akeson, R. and Warren, S. L. (1986). PC12 adhesion and neurite formation on selected substrates are inhibited by some glycosaminoglycans and a fibronectin-derived tetrapeptide. *Exp. Cell Res.* **162**, 347-362.

Bally-Cuif, L., Alvarado-Mallart, R. M., Darnell, D. K. and Wassef, M. (1992). Relationship between *Wnt-1* and *En-2* expression domains during early development of normal and ectopic met-mesencephalon. *Development* **115**, 999-1009.

Bally-Cuif, L. and Wassef, M. (1994). Ectopic induction and reorganization of *Wnt-1* expression in quail/chick chimeras. *Development* **120**, 3379-3394.

Bally-Cuif, L. and Wassef, M. (1995). Determination events in the nervous system of the vertebrate embryo. *Curr. Opin. Genet. Dev.* **5**, 450-458.

Bally-Cuif, L., Cholley, B. and Wassef, M. (1995). Involvement of *Wnt-1* in the formation of the mes/metencephalic boundary. *Mech. Dev.* **53**, 23-34.

Barnea, G., Grumet, M., Milev, P., Silvennoinen, O., Levy, J. B., Sap, J. and Schlessinger, J. (1994). Receptor tyrosine phosphatase β is expressed in the form of proteoglycan and binds to the extracellular matrix protein tenascin. *J. Biol. Chem.* **269**, 14349-14352.

Burg, M. A. and Cole, G. J. (1994). Claustrin, an antiadhesive neural keratan sulfate proteoglycan, is structurally related to MAP1B. *J. Neurobiol.* **25**, 1-22.

Carbonetto, S., Gruver, M. M. and Turner, D. C. (1983). Nerve fiber growth in culture on fibronectin, collagen, and glycosaminoglycan substrates. *J. Neurosci.* **3**, 2324-2335.

Caterson, B., Christner, J. E. and Baker, J. R. (1983). Identification of a monoclonal antibody that specifically recognizes corneal and skeletal keratan sulfate. *J. Biol. Chem.* **258**, 8848-8854.

Childs, R. A., Pennington, J., Uemura, K., Scudder, P., Goodfellow, P. N., Evans, M. J. and Feizi, T. (1983). High-molecular-weight glycoproteins are the major

carriers of the carbohydrate differentiation antigens I, i and SSEA-1 of mouse teratocarcinoma cells. *Biochem. J.* **215**, 491-503.

Cole, G. J. and McCabe, C. F. (1991). Identification of a developmentally regulated keratan sulfate proteoglycan that inhibits cell adhesion and neurite outgrowth. *Neuron* **7**, 1007-1018.

Crossley, P. H. and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-68.

Dou, C.-L. and Levine, J. M. (1995). Differential effects of glycosaminoglycans on neurite growth on laminin and L1 substrates. *J. Neurosci.* **15**, 8053-8066.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Eggens, I., Fenderson, B., Toyokuni, T., Dean, B., Stroud, M. and Hakomori, S. (1989). Specific interaction between Le^x and Le^x determinants. *J. Biol. Chem.* **264**, 9476-9484.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell* **81**, 747-756.

Fenderson, B. A., Zehavi, U. and Hakomori, S. (1984). A multivalent lacto-*N*-fucopentaose III-lysyllysine conjugate decompacts preimplantation mouse embryos, while the free oligosaccharide is ineffective. *J. Exp. Med.* **160**, 1591-1596.

Fukuda, M. N., Watanabe, K. and Hakomori, S. I. (1978). Release of oligosaccharides from various glycosphingolipids by endo-β-galactosidase. *J. Biol. Chem.* **253**, 6814-6819.

Geisert, E. E. and Bidanset, D. J. (1993). A central nervous system keratan sulfate proteoglycan: localization to boundaries in the neonatal rat brain. *Dev. Brain Res.* **75**, 163-173.

Grabel, L. B., Singer, M. S., Martin, G. R. and Rosen, S. D. (1983). Teratocarcinoma stem cell adhesion: the role of divalent cations and a cell surface lectin. *J. Cell Biol.* **96**, 1532-1537.

Grumet, M., Milev, P., Sakurai, T., Karthikeyan, L., Bourdon, M., Margolis, R. K. and Margolis, R. U. (1994). Interactions with tenascin and differential effects on cell adhesion of neurocan and phosphacan, two major chondroitin sulfate proteoglycans of nervous tissue. *J. Biol. Chem.* **269**, 12142-12146.

Guthrie, S., Butcher, M. and Lumsden, A. (1991). Patterns of cell division and interkinetic nuclear migration in the chick embryo hindbrain. *J. Neurobiol.* **22**, 742-754.

Hamburger, V. and Hamilton, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hatta, K., Takagi, S., Fujisawa, H. and Takeichi, M. (1987). Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. *Dev. Biol.* **120**, 215-227.

Hatten, M. E. and Mason, C. A. (1990). Mechanisms of glial-guided neuronal migration *in vitro* and *in vivo*. *Experientia* **46**, 907-916.

Heinegard, D. (1972). Extraction, fractionation and characterization of proteoglycans from bovine tracheal cartilage. *Biochim. Biophys. Acta* **285**, 181-192.

Hu, H., Tomasiewicz, H., Magnuson, T. and Rutishauser, U. (1996). The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. *Neuron* **16**, 735-743.

Kinnunen, T., Raulo, E., Nolo, R., Maccarana, M., Lindahl, U. and Rauvala, H. (1996). Neurite outgrowth in brain neurons induced by heparin-binding growth-

associated molecule (HB-GAM) depends on the specific interaction of HB- GAM with heparan sulfate at the cell surface. *J. Biol. Chem.* **271**, 2243-2248.

Krueger, N. X. and Saito, H. (1992). A human transmembrane protein-tyrosine phosphatase, PTP ζ, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7417-7421.

Krueger, R. C., Hennig, A. K. and Schwartz, N. B. (1992). Two immunologically and developmentally distinct chondroitin sulfate proteolglycans in embryonic chick brain. *J. Biol. Chem.* **267**, 12149-12161.

Kuhlenbeck, H. (1973). The central nervous system of vertebrates,. Vol 3, Part II (ed. F. Karger), Berlin.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lagenaur, C., Schachner, M., Solter, D. and Knowles, B. (1982). Monoclonal antibody against ssea-1 is specific for a subpopulation of astrocytes in mouse cerebellum. *Neurosci. Lett.* **31**, 181-184.

Lagenaur, C. and Lemmon, V. (1987). An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7753-7757.

Lemmon, V., Staros, E. B., Perry, H. E. and Gottlieb, D. I. (1982). A monoclonal antibody which binds to the surface of chick brain cells and myotubes: cell selectivity and properties of the antigens. *Dev. Brain Res.* **3**, 349-360.

Maeda, N., Matsui, F. and Oohira, A. (1992). A chondroitin sulfate proteoglycan that is developmentally regulated in the cerebellar mossy fiber system. *Dev. Biol.* **151**, 564-574.

Maeda, N., Hamanaka, H., Shintani, T., Nishiwaki, T. and Noda, M. (1994). Multiple receptor-like protein tyrosine phosphatases in the form of chondroitin sulfate proteoglycan. *FEBS Lett.* **354**, 67-70.

Maeda, N., Hamanaka, H., Oohira, A. and Noda, M. (1995). Purification, characterization and developmental expression of a brain-specific chondroitin sulfate proteoglycan, 6B4 proteoglycan/phosphacan. *Neuroscience* **67**, 23-35.

Maeda, N. and Noda, M. (1996). 6B4 proteoglycan/phosphacan is a repulsive substratum but promotes morphological differentiation of cortical neurons. *Development* **122**, 647-658.

Maeda, N., Nishiwaki, T., Shintani, T., Hamanaka, H. and Noda, M. 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein tyrosine phosphatase ζ/RPTPβ, binds pleiotrophin/HB-GAM. *J. Biol. Chem.*, in press.

Marcus, R. C., Blazeski, R., Godement, P. and Mason, C. A. (1995). Retinal axon divergence in the optic chiasm: uncrossed axons diverge from crossed axons within a midline glial specialization. *J. Neurosci.* **15**, 3716-3729.

Matsumoto, K., Wanaka, A., Takatsuji, K., Muramatsu, H., Muramatsu, T. and Tohyama, M. (1994). A novel family of heparin-binding growth factors, pleiotrophin and midkine, is expressed in the developing rat cerebral cortex. *Dev. Brain Res.* **79**, 229-241.

Maurel, P., Rauch, U., Flad, M., Margolis, R. K. and Margolis, R. U. (1994). Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 2512-2516.

McMahon, A. P. and Bradley, A. (1990). The *Wnt-1* (*int-1*) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.

Mehmet, H., Scudder, P., Tang, P. W., Hounsell, E. F., Caterson, B. and Feizi, T. (1986). The antigenic determinants recognized by three monoclonal antibodies to keratan sulphate involve sulphated hepta- or larger oligosaccharides of the poly(*N*-acetyllactosamine) series. *Eur. J. Biochem.* **157**, 385-391.

Meyer-Puttlitz, B., Milev, P., Junker, E., Zimmer, I., Margolis, R. U. and Margolis, R. K. (1995). Chondroitin sulfate and chondroitin/keratan sulfate proteoglycans of nervous tissue: developmental changes of neurocan and phosphacan. *J. Neurochem.* **65**, 2327-2337.

Milev, P., Friedlander, D. R., Sakurai, T., Karthikeyan, L., Flad, M., Margolis, R. K., Grumet, M. and Margolis, R. U. (1994). Interactions of the chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. *J. Cell Biol.* **127**, 1703-1715.

Misson, J.-P., Edwards, M. A., Yamamoto, M. and Caviness, V. S. (1988). Identification of radial glial cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker. *Dev. Brain Res.* **44**, 95-108.

Ono, K., Tomasiewicz, H., Magnuson, T. and Rutishauser, U. (1994). N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. *Neuron* **13**, 595-609.

Oudega, M., Marani, E. and Thomeer, R. T. W. M. (1992). Transient expression of stage-specific embryonic antigen-1 (CD15) in the developing dorsal rat spinal cord. *Histochem. J.* **24**, 869-877.

Peles, E., Nativ, M., Campbell, P. L., Sakurai, T., Martinez, R., Lev, S., Clary, D. O., Schilling, J., Barnea, G., Plowman, G. D., Grumet, M. and Schlessinger, J. (1995). The carbonic anhydrase domain of receptor tyrosine phosphatase β is a functional ligand for the axonal cell recognition molecule contactin. *Cell* **82**, 251-260.

Rakic, P. (1971). Guidance of neurons migrating to the fetal monkey neocortex. *Brain Res.* **33**, 471-476.

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.* **145**, 61-84.

Rauch, U., Gao, P., Janetzko, A., Flaccus, A., Hilgenberg, L., Tekotte, H., Margolis, R. K. and Margolis, R. U. (1991). Isolation and characterization of developmentally regulated chondroitin sulfate and chondroitin/keratan sulfate proteoglycans of brain identified with monoclonal antibodies. *J. Biol. Chem.* **266**, 14785-14801.

Raulo, E., Julkunen, I., Merenmies, J., Pihlaskari, R. and Rauvala, H. (1992). Secretion and biological activities of heparin-binding growth-associated molecule. Neurite outgrowth-promoting and mitogenic actions of the recombinant and tissue-derived protein. *J. Biol. Chem.* **267**, 11408-11416.

Rauvala, H. (1989). An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. *EMBO J.* **8**, 2933-2941.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and Dodd, J. (1994). Floor plate and motor neuron induction by *vhh-1*, a vertebrate homolog of *hedgehog* expressed by the notochord. *Cell* **76**, 761-775.

Rubenstein, J. L. R., Martinez, S., Shimamura, K. and Puelles, L. (1994). The embryonic vertebrate forebrain: the prosomeric model. *Science* **266**, 578-580.

Rutishauser, U. and Landmesser, L. (1991). Polysialic acid on the surface of axons regulates patterns of normal and activity-dependent innervation. *Trends Neurosci.* **14**, 528-532.

Schachner, M. and Martini, R. (1995). Glycans and the modulation of neural-recognition molecule function. *Trends Neurosci.* **18**, 183-191.

Schwarting, G. A., Williams, M. A., Evans, J. E. and McCluer, R. H. (1989). Characterization of SSEA-1 glycolipids from the brain of a patient with fucosidosis. *Glycoconj. J.* **6**, 293-302.

Shimamura, K. and Takeichi, M. (1992). Local and transient expression of E-cadherin involved in mouse embryonic brain morphogenesis. *Development* **116**, 1011-1019.

Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. R. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.

Sieber-Blum, M. (1989). SSEA-1 is a specific marker for the spinal sensory neuron lineage in the quail embryo and in neural crest cell cultures. *Dev. Biol.* **134**, 362-375.

Snow, D. M., Lemmon, V., Carrino, D. A., Caplan, A. I. and Silver, J. (1990a). Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*. *Exp. Neurol.* **109**, 111-130.

Snow, D. M., Steindler, D. A. and Silver, J. (1990b). Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. *Dev. Biol.* **138**, 359-376.

Thomas, K. R. and Capecchi, M. R. (1990). Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**, 847-850.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350-4354.

Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryo (*Gallus domesticus*). *Ergeb. Anat. Entwicklungsgesch.* **41**, 1-88.

Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **3**, 97-130.

Yamamoto, M., Boyer, A. M. and Schwarting, G. A. (1985). Fucose- containing glycolipids are stage- and region-specific antigens in developing embryonic brain of rodents. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3045-3049.

TABLES AND FIGURES

Table 1.

Structure of glycosaminoglycans

Glycosaminoglycan	Repeating disaccharide unit (Major sulfation site)
Hyaluronic acid	4 GlcUA β 1 → 3 GlcNAc β 1 →
Chondroitin sulfate	4 GlcUA β 1 → 3 GalNAc (4S) (6S) β 1 →
Dermatan sulfate	4 IdUA (2S) α 1 → 3 GalNAc (4S) (6S) β 1 →
Heparan sulfate and heparin	4 GlcUA β 1 → 4 GlcNAc (NS) (6S) α 1 →
	4 IdUA (2S) α 1 → 4 GlcNAc (NS) (6S) α 1 →
Keratan sulfate	3 Gal (6S) β 1 → 4 GlcNAc (6S) β 1 →

Abbreviations used: GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; Gal, D-galactose; GlcNAc, D-*N*-acetylglucosamine; GalNAc, D-*N*-acetylgalactosamine.

Figure 1. Schematic drawing of 6B4 PG and PTPζ. 6B4 PG consists of an N-terminal carbonic anhydrase (CAH)-like domain, a fibronectin type III domain and the large cysteine-free, serine glycine-rich region. PTPζ is a splicing variant of 6B4 PG, which has a transmembrane region and an intracellular region containing two repeated PTPase domains. It has been shown that only the membrane-proximal PTPase domain is catalytically active. 6B4 PG is known to be modified with several kinds of carbohydrates including chondroitin sulfate, keratan sulfate, HNK-1 epitope, and N- and O-linked oligosaccharides. PTPζ is known to be modified with chondroitin sulfate and HNK-1 epitope. In this study, I demonstrated that PTPζ is also modified with keratan sulfate, and that both molecules bear Le^X carbohydrate.

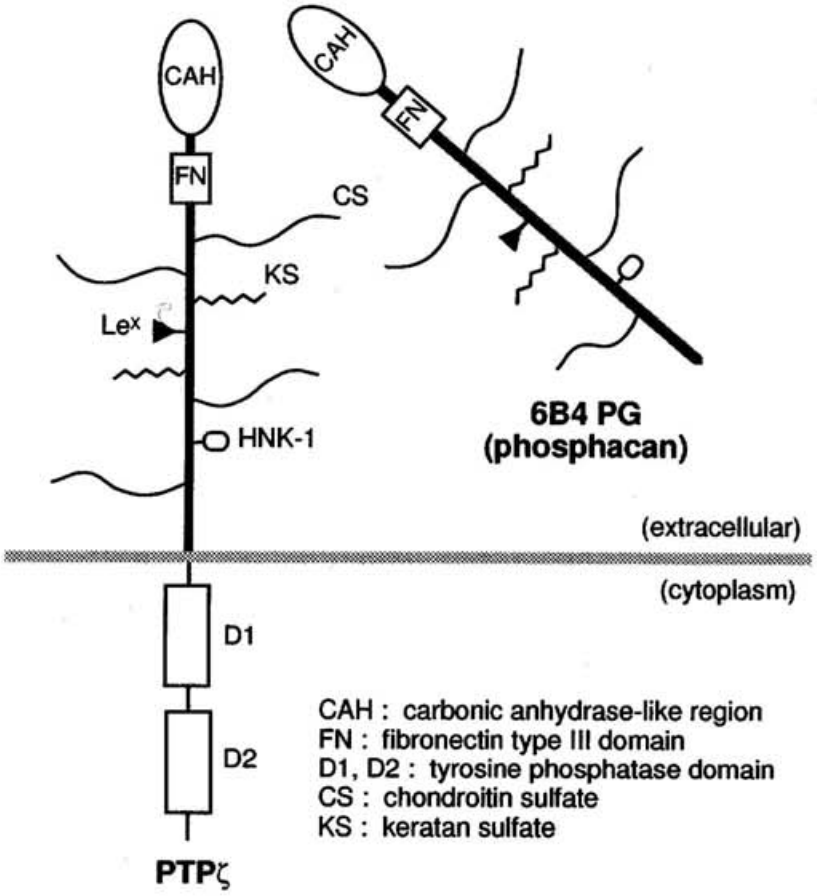

CAH
FN
CS
KS
Lex
HNK-1
CAH
FN
6B4 PG
(phosphacan)
(extracellular)
(cytoplasm)
D1
D2
PTPζ
CAH : carbonic anhydrase-like region
FN : fibronectin type III domain
D1, D2 : tyrosine phosphatase domain
CS : chondroitin sulfate
KS : keratan sulfate

Figure 2. Purification of chick 6B4 PG, and production of the antibody against purified 6B4 PG. *A*, Chick 6B4 PG was purified and treated with chondroitinase ABC as described in Materials and Methods. The sample (2 μg of protein) was applied to 5% SDS-PAGE, and the proteins were stained with Coomassie Brilliant Blue. The preparation showed a single band of 300 kDa (*arrowhead*). The 100-kDa band was chondroitinase ABC. *B*, Immunoblotting of purified 6B4 PG using MAb 6B4 (*lane 1*) and polyclonal antibody against purified chick 6B4 PG (*lane 2*). Positions of molecular weight markers (in kDa) are shown at left.

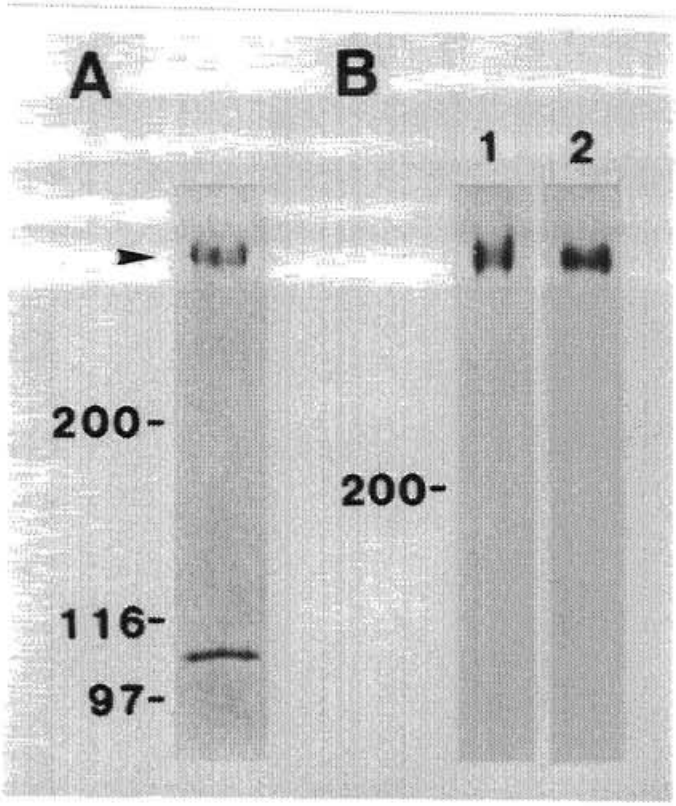

A
B
1
2
200-
116-
97-
200-

Figure 3. Comparison of N-terminal amino acid sequence of 6B4 PG. The N-terminal sequence of the purified chick 6B4 PG corresponds well to that of rat 6B4 PG. This indicates that MAb 6B4 recognizes the chick homolog of 6B4 PG. Amino acids conserved in chick and rat 6B4 PG are shown in boxes.

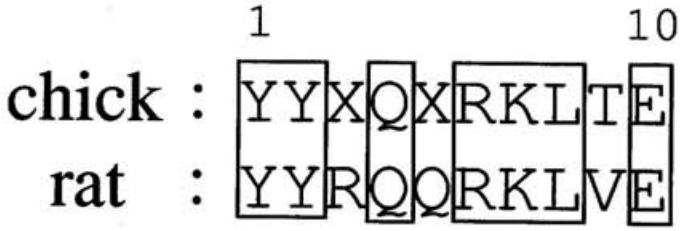

1
10
chick : YYXQXRKLTE
rat : YYRQQRKLVE

Figure 4. Production of PTPζ–specific antibody, anti-PZ1. Immunoblotting of E14 brain homogenates (50 μg protein) using anti-PZ1, before (*lane 1*) or after (*lanes 2* and *3*) chondroitinase ABC digestion. Only after chondroitinase ABC digestion, a single band of 300 kDa was observed (*arrowhead*). The immunoreactivity was abolished by preincubation of anti-PZ1 with 100 μg/ml antigenic peptide (*lane 3*). The bands at 116 kDa and 70 kDa were nonspecific because they appeared even without incubation with anti-PZ1. Positions of molecular weight markers (in kDa) are shown at left.

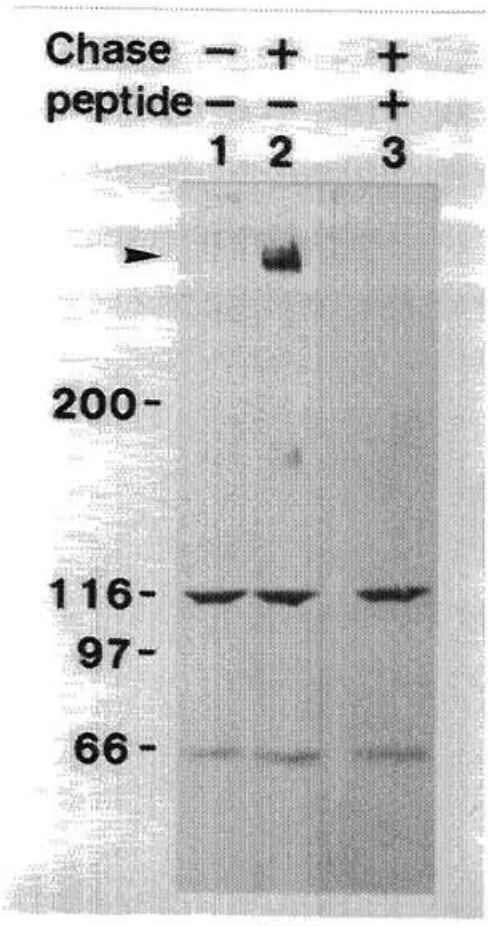

Chase − + +
peptide − − +
1 2 3
200-
116-
97-
66-

Figure 5. 6B4 PG and/or PTPζ are the major keratan sulfate-containing molecules in the developing chick brain. *A*, E14 brain homogenates (10 μg) were analyzed by immunoblotting using MAb 5D4. Intact (*lane 1*), heparitinase- (*lane 2*), chondroitinase ABC- (*lane 3*) and heparitinase + chondroitinase ABC- (*lane 4*) digested samples were analyzed. A single band of 300 kDa (*arrowhead*) appeared after digestion with chondroitinase ABC, but not with heparitinase. *B*, The immunoprecipitate (*lane 1*) and the supernatant (*lane 2*) from E14 brain extracts (10 μg) with anti-6B4 PG were analyzed by immunoblotting using MAb 5D4. Almost all of the MAb 5D4-epitope was precipitated with anti-6B4 PG. *C*, Chondroitinase ABC-digested total protein extracts (10 μg protein) from E14 whole brain were subjected to immunoprecipitation with MAb 5D4. The proteins recovered in the precipitate (*lane 1*) and the supernatant (*lane 2*) were analyzed by immunoblotting using MAbs 5D4 (a) and 6B4 (b). The whole amount (*lane 2, a*) and an aliquot corresponding to one fifth (*lane 2, b*) of the supernatant were loaded. Only a portion of 6B4 PG and/or PTPζ was modified with keratan sulfate. In *A*, *B*, positions of molecular weight markers (in kDa) are shown at left.

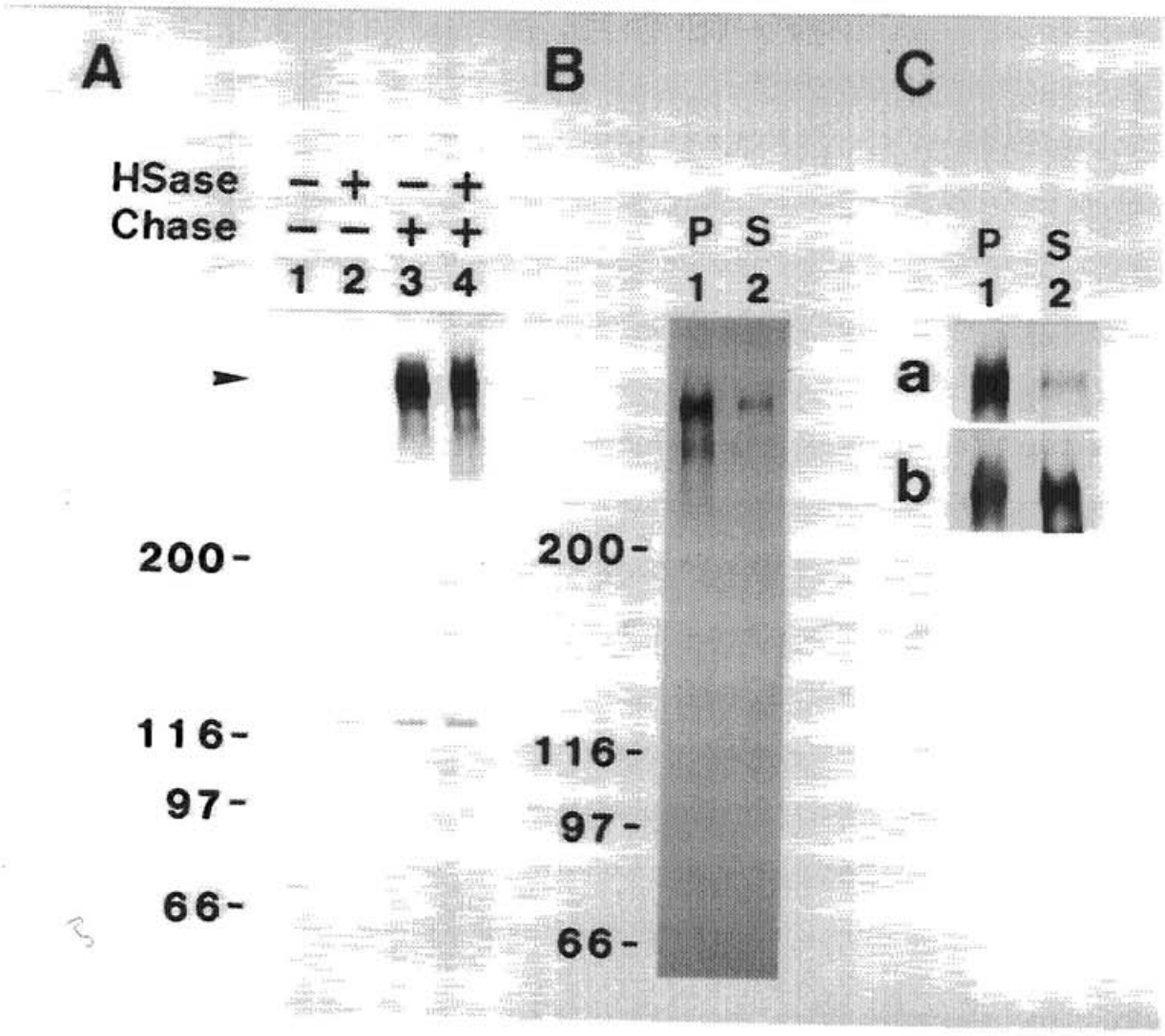

A
B
C
HSase – + – +
Chase – – + +
1 2 3 4
200-
116-
97-
66-
P S
1 2
200-
116-
97-
66-
P S
1 2
a
b

Figure 6. Both of 6B4 PG and PTPζ are modified with keratan sulfate. Chondroitinase ABC-digested total protein extracts (50 μg protein) from E14 chick brain were subjected to immunoprecipitation with anti-PZ1. The proteins recovered in the precipitate (*lane 1*) and the supernatant (*lane 2*) were analyzed with immunoblotting using MAb 6B4 (*A*) and MAb 5D4 (*B*). PTPζ recovered in the precipitate as well as 6B4 PG in the supernatant showed MAb 5D4-reactivity .

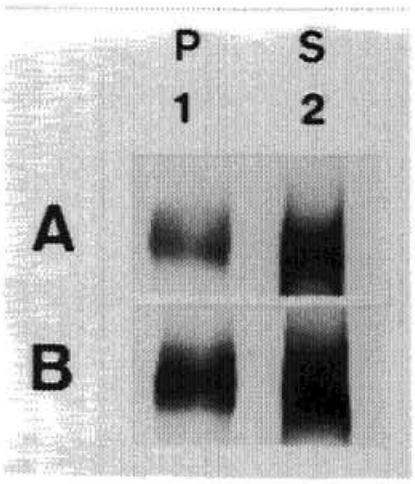

P
S
1
2
A
B

Figure 7. 6B4 PG is modified with Le^x carbohydrate. Purified 6B4 PG was digested with chondroitinase ABC (*lane 1*) or with chondroitinase ABC + endo-β-galactosidase (*lane 2*), and then analyzed by immunoblotting using anti-Le^x Ab. Le^x carbohydrate was degraded by endo-β-galactosidase.

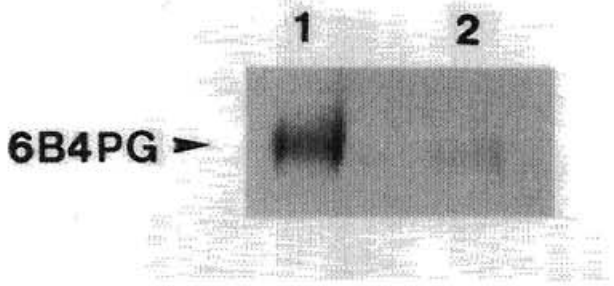

1
2
6B4PG

Figure 8. 6B4 PG and PTPζ are the major Le^x-containing proteins in the developing chick brain. *A*, E14 brain homogenate (30 μg protein) was untreated (*lane 1*) or treated with heparitinase (*lane 2*), chondroitinase ABC (*lane 3*) and heparitinase + chondroitinase ABC (*lane 4*), and then analyzed by immunoblotting using anti-Le^x Ab. A chondroitin sulfate proteoglycan with a 300-kDa core protein reacted with anti-Le^x Ab (*arrowhead*). The bands at 116 and 70 kDa were nonspecific signal because they did not depend on the primary antibody. *B*, E14 brain extracts were subjected to immunoprecipitation with anti-6B4 PG. The proteins recovered in the precipitate (*lane 1*) and the supernatant (*lane 2*) were analyzed by immunoblotting using anti-Le^x Ab. The results indicated that 6B4 PG and/or PTPζ are the major Le^x-containing proteins in the chick brain. *C*, The proteins immunoprecipitated with anti-Le^x Ab from chondroitinase ABC-digested E14 brain extracts were analyzed by immunoblotting using anti-Le^x Ab (*lane 1*), MAb 6B4 (*lane 2*) and anti-PZ1 (*lane 3*). This indicates that PTPζ contains Le^x carbohydrate. *D*, Chondroitinase ABC-digested total protein extracts from E14 chick brain were subjected to immunoprecipitation with anti-PZ1. The proteins recovered in the precipitate (*lane 1*) and the supernatant (*lane 2*) fractions were analyzed by immunoblotting using MAb 6B4 (*a*) and anti-Le^x Ab(*b*). Both PTPζ in the precipitate and 6B4 PG in the supernatant reacted with anti-Le^x Ab. In *A*, *B*, positions of molecular weight markers (in kDa) are shown at left.

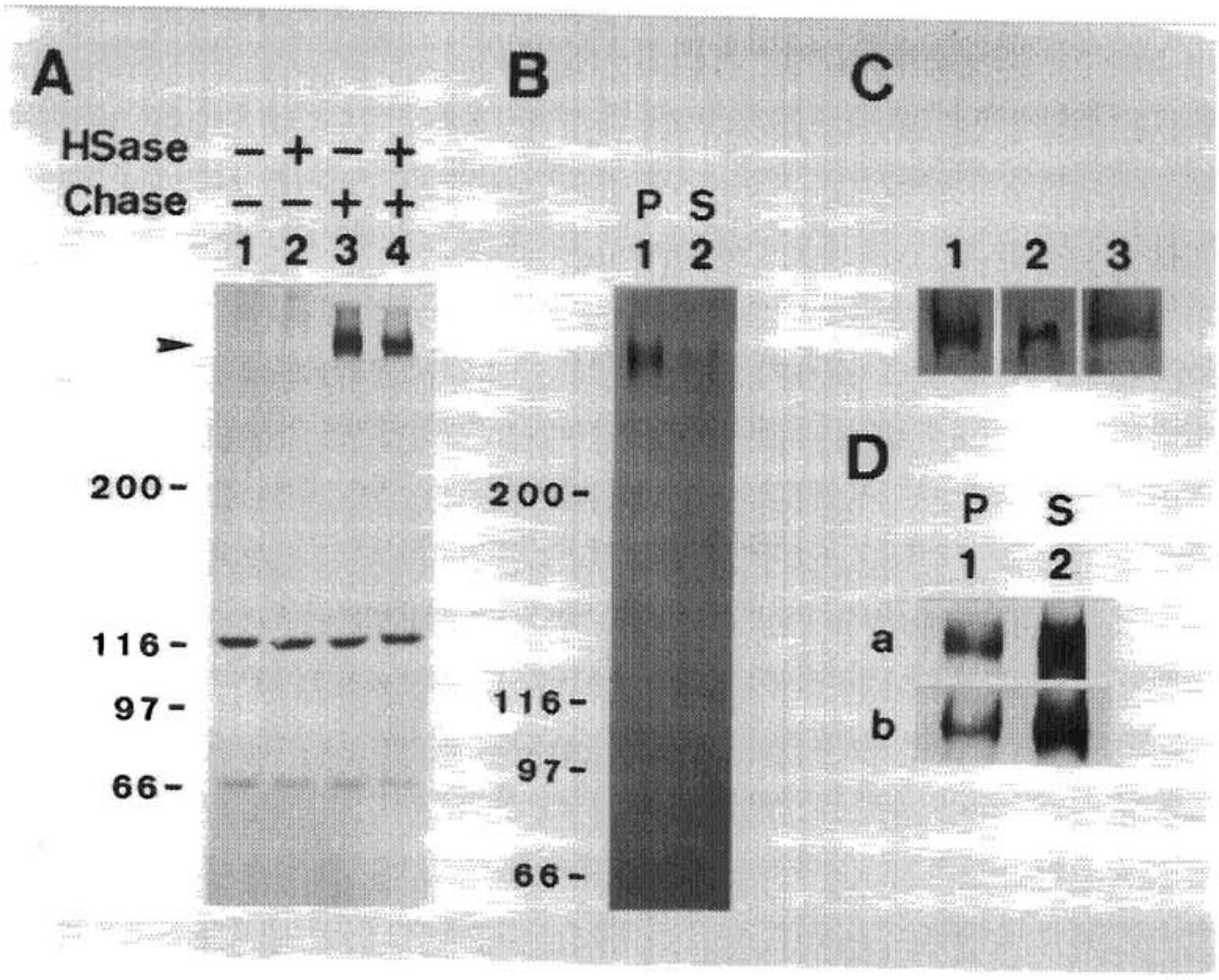

A
HSase – + – +
Chase – – + +
1 2 3 4
200-
116-
97-
66-
B
P S
1 2
200-
116-
97-
66-
C
1 2 3
D
P S
1 2
a
b

Figure 9. Developmental changes in the modification pattern of 6B4 PG and PTPζ with carbohydrates. The immunoprecipitates with anti-6B4 PG from total brain extracts at various embryonic stages (E5-E18) were digested with chondroitinase ABC and then analyzed by immunoblotting using antibodies to carbohydrates. *A*, MAb 6B4; *B*, MAb 5D4; *C*, anti-Le^X Ab; *D*, HNK-1 MAb.

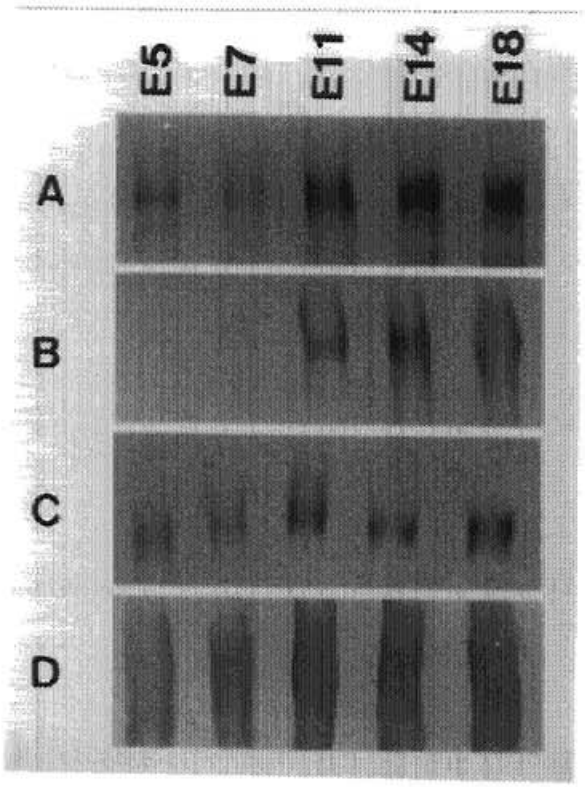

E5
E7
E11
E14
E18
A
B
C
D

Figure 10. Immunohistochemical localization of keratan sulfate and 6B4 PG epitopes in the developing chick tectum. Frontal sections of the developing tectum at various stages were stained immunohistochemically with MAbs 5D4 (*A-C*) and 6B4 (*D-F*). Stages analyzed were: *A* and *D*, E10; *B* and *E*, E11; *C* and *F*, E13. At all stages, only a part of the 6B4 PG-positive regions were keratan sulfate-positive. In *A* and *D* (at E10), the left side in panel is the lateral side of the tectum, and dorsal is on the right. In *B* and *E* (at E11) and in *C* and *F* (at E13), ventral is on the left and dorsal is on the right. This directional change was derived from rotation of the tectum during development. Scale bar: 1 mm.

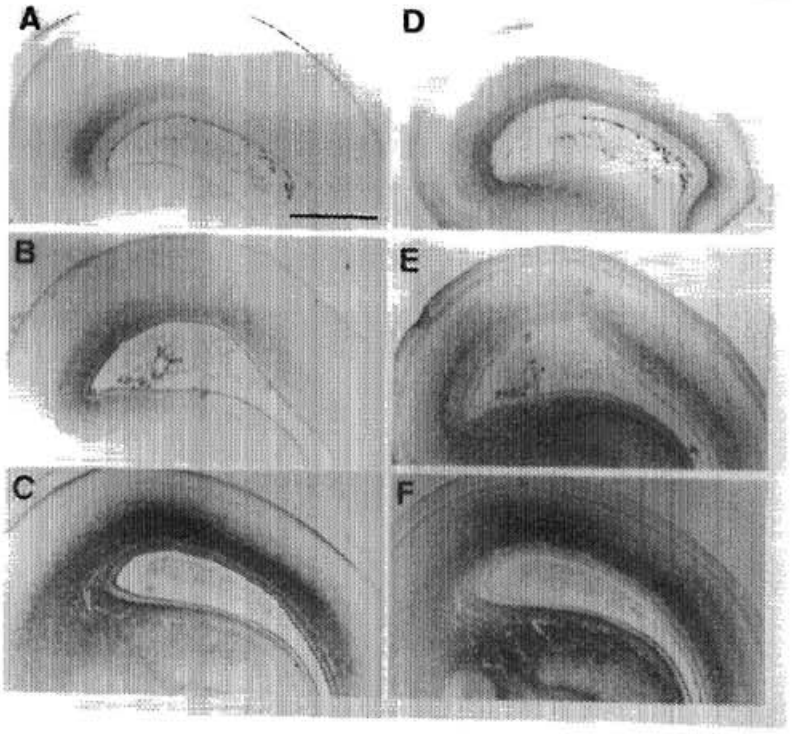

A
D
B
E
C
F

Figure 11. Whole-mount immunostaining of E5 chick brain with MAb 5D4. *A*, Overview of the preparation. The arrowheads indicate the zona limitans intrathalamica. The arrow indicates the position of the isthmic region. *B*, The ventricular surface in mes-metencephalic region. *C*, Dorsal view of the tectum and the pretectal area. Staining was observed in the roof plate of the rostral part of the tectum (*arrowheads*), but not at the caudal part. *D*, Magnified view of the mes-metencephalic boundary region seen from the luminal side. *E*, Whole-mount immunostaining of E13.0 mouse embryo with MAb 5D4. The mes-metencephalic boundary region viewed from the luminal side. In *D* and *E*, the arrows indicate the position of the groove of the fovea isthmi which develops at the basal plate of the neural tube. Positive immunoreactivity was observed along this groove. di, diencephalon; is, isthmus; mes, mesencephalon; met, metencephalon; pt, pretectal area; rh, rhombencephalon; rp, roof plate; tect, tectum; tel, telencephalon.

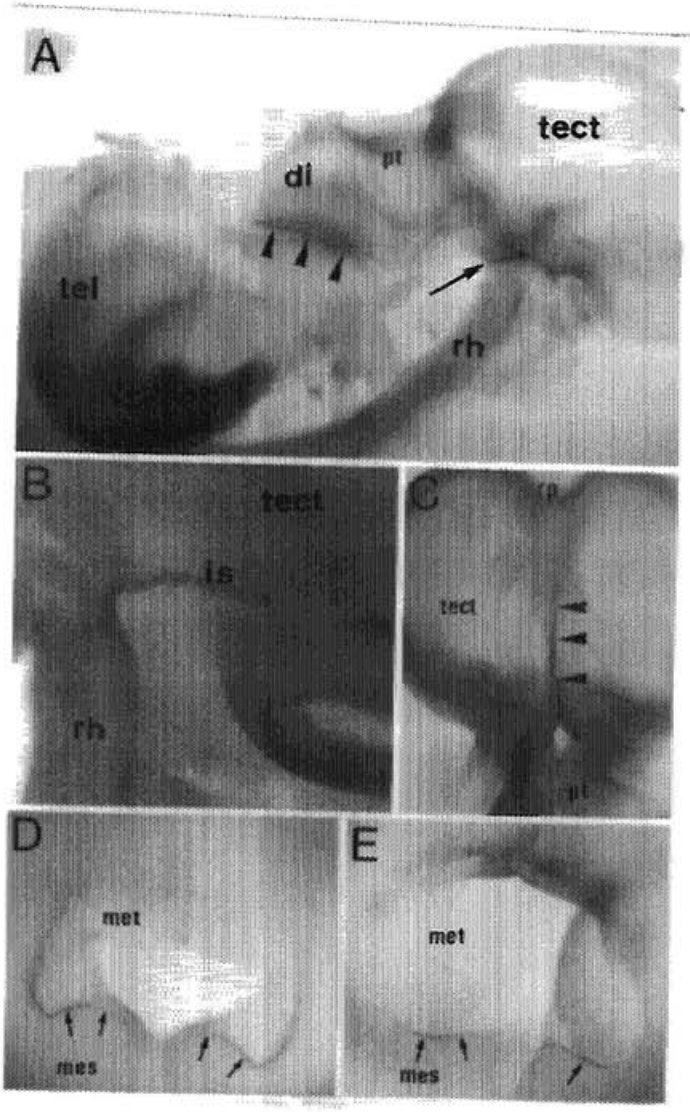

A
tect
dl
pt
tel
rh
B
tect
is
rh
C
tect
D
met
mes
E
met
mes

Figure 12. Developmentally regulated expression of keratan sulfate at the mes-metencephalic boundary. Parasagittal sections of the mes-metencephalic region at various stages, E3(*A*), E4(*B*), E6(*C*) and E7(*D*), were stained immunohistochemically with MAb 5D4. Arrowhead in *A* indicates the position where keratan sulfate expression began. Marks in *B* indicate the following: Large arrowhead, fovea isthmi; small arrowhead, the flexure formed between the caudal tectum and the rostral cerebellar anlage; arrow, the stained fiber structure which runs along the neural tube rostrocaudally. Arrowhead in *C* indicates the apical surface which is stained with MAb 5D4. mes, mesencephalon; met, metencephalon. Scale bars: *A*, *B*, 50 μm; *C*, 100 μm; *D*, 200 μm.

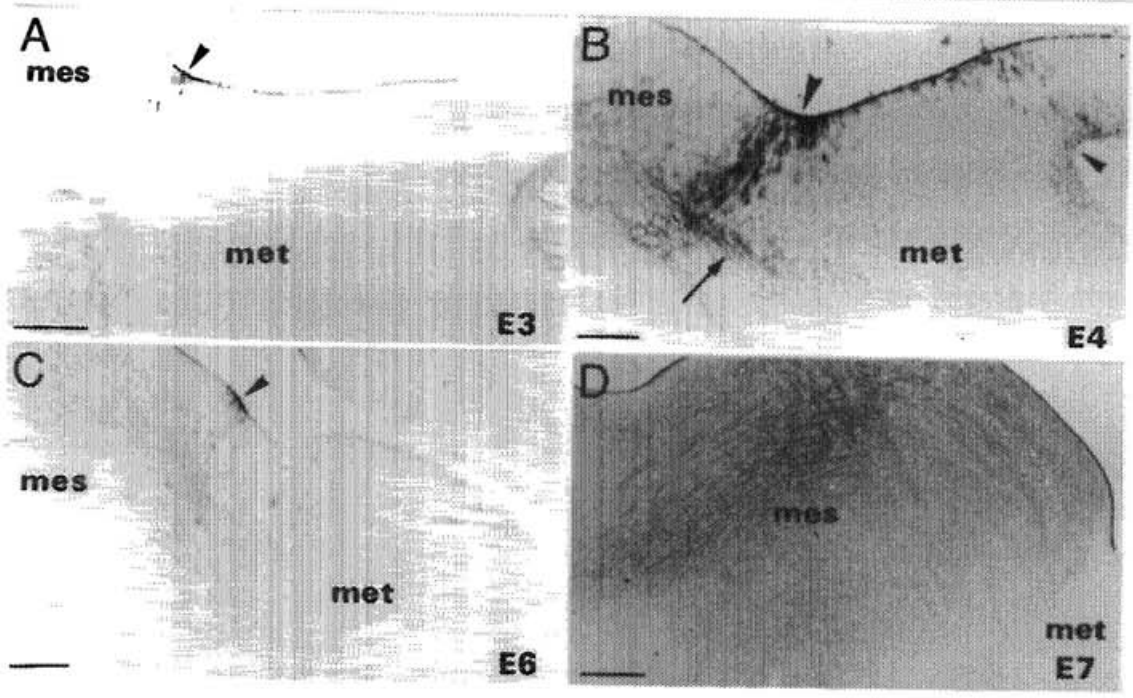

A
mes
met
E3
B
mes
met
E4
C
mes
met
E6
D
mes
met
E7

Figure 13. Characterization of keratan sulfate expressing cells. Serial sections (*A-C* and *D-F*) of E5 chick brain were stained with MAb 5D4 (*A,D*), anti-vimentin (*B*), anti-neurofilament (*C*), anti-N-CAM (*E*), and anti-Ng-CAM/L1 (*F*). Arrowheads in *A* and *B* indicate the keratan sulfate and vimentin-positive processes, respectively, which extended from the ventricular zone to the pial surface. mz, mantle zone; vz, ventricular zone; v, ventricle. Scale bars: *A-C* (shown in *A*), 25 μm; *D-F*(shown in *D*), 50 μm.

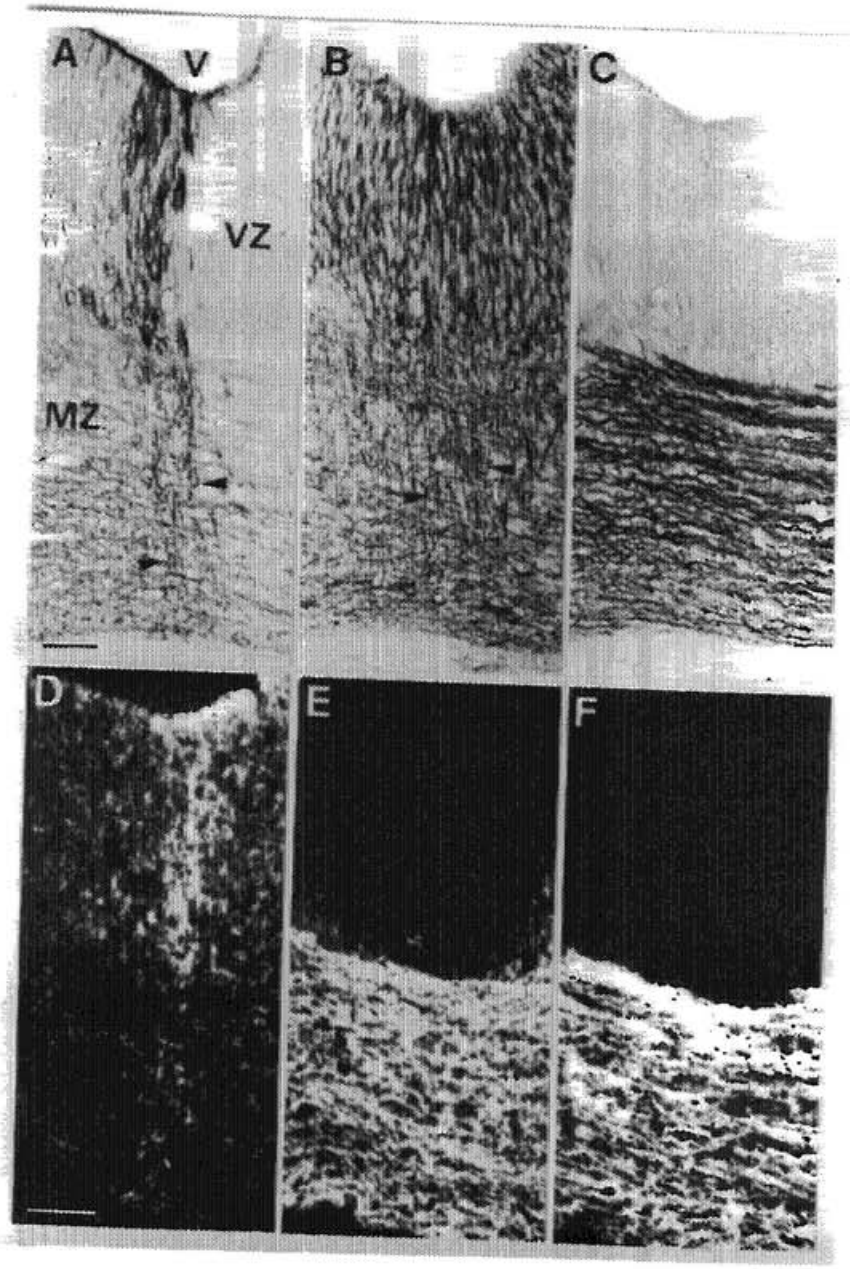

A
V
VZ
MZ
B
C
D
E
F

Figure 14. Keratan sulfate is expressed on both mitotic-active and post-mitotic cells in the mes-metencephalic boundary. A section of E5 chick embryo was stained with MAb 5D4 and hematoxylin. Arrowhead indicates a cell in M-phase. Arrows indicate the post-mitotic migrating neurons. Scale bar: 10 μm.

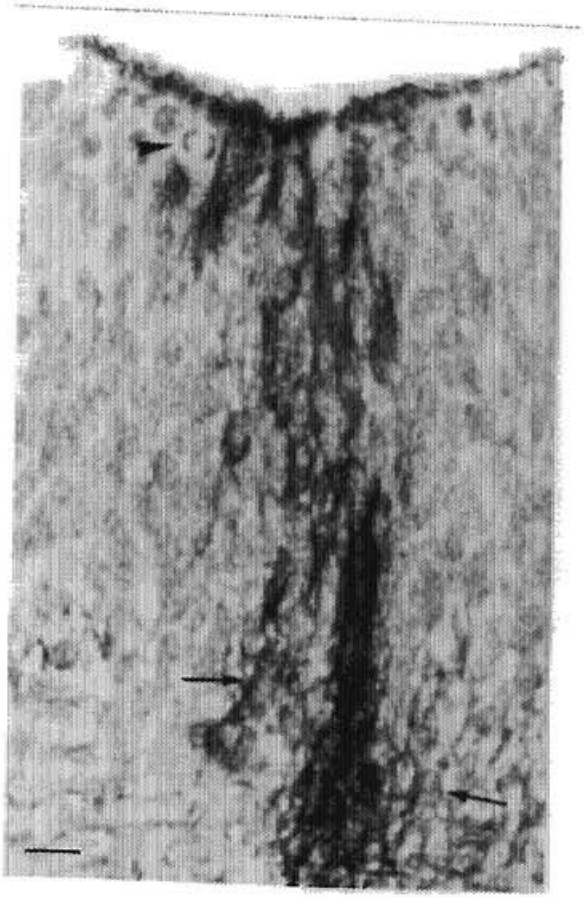


Figure 15. Specificity of MAb 5D4 that recognizes keratan sulfate in immunohistochemistry. Parasaggital sections of E5 embryos were incubated in the presence (*B*) or absence (*A*) of endo-β-galactosidase, and then processed for immunohistochemical staining with MAb 5D4. Immunoreactivity at the mes-metencephalic boundary (*arrowhead* in *A*) and the cartilaginous tissue (*arrow* in *A*) was abolished in *B*. This indicates that MAb 5D4 specifically recognizes keratan sulfate. mes, mesencephalon; met, metencephalon; v, ventricle. Scale bar: 250 μm.

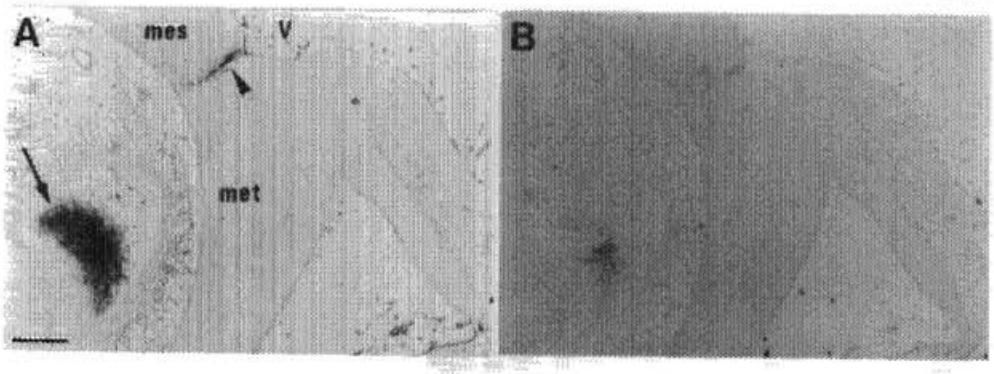

A
mes
V
met
B

Figure 16. Immunohistochemical analysis of E5 chick embryos. Parasagittal sections of E5 chick embryos were stained with MAbs 5D4 (*A*) and 6B4 (*B*). di, diencephalon; is, isthmic region; rh, rhombencephalon; tect, tectum; tel, telencephalon; tg, trigeminal ganglion. Scale bar: 500 μm.

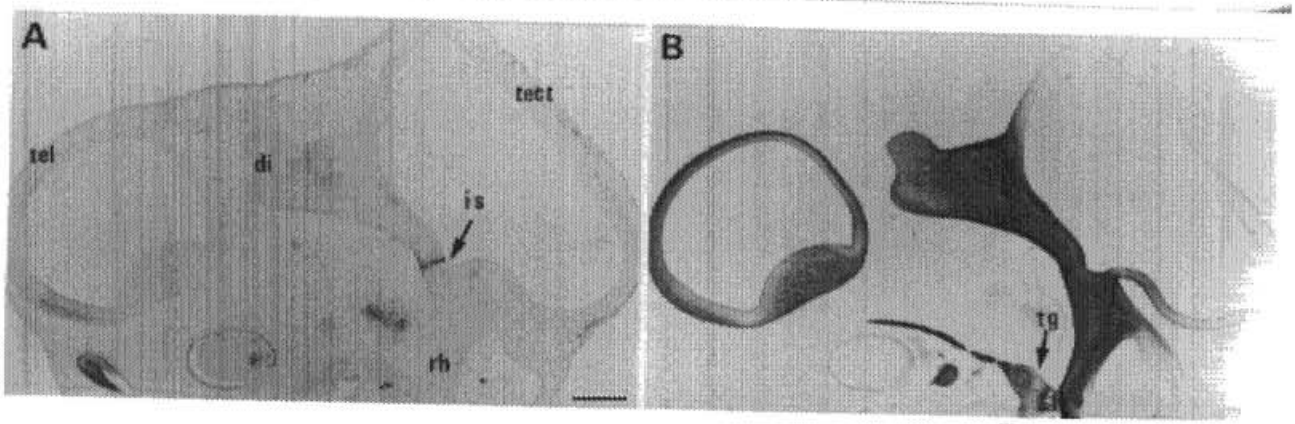

A
tel
di
tect
is
rh
B
tg

Figure 17. The region-specific expression of keratan sulfate was attributed to the modification of 6B4 PG and PTPζ. *A-F*, Tissue homogenates were prepared from the following regions of E5 chick brain: telencephalon (*lane 1*), mes-metencephalic boundary portion (*lane 2*), the rostral (*lane 3*) and caudal (*lane 4*) portions of the roof plate in the tectum, and rhombencephalon (*lane 5*). Total protein extracts (*A, C, E*) or immunoprecipitates with MAb 5D4 (*B, D, F*) were treated with chondroitinase ABC and analyzed by immunoblotting with MAb 5D4 (*A, B*), MAb 6B4 (*C, D*) and anti-PZ1 (*E, F*). These experiments indicated that locally expressed keratan sulfate is attached to PTPζ. *G*, Chondroitinase ABC-digested total protein extracts from E5 mes-metencephalic region were used for immunoprecipitation with anti-PZ1. The proteins recovered in the precipitate (*lane 1*) and the supernatant (*lane 2*) fractions were analyzed by immunoblotting with MAb 6B4 (*a*) and MAb 5D4 (*b*). This indicated that both PTPζ (in the precipitate) and 6B4 PG (in the supernatant) were substituted with keratan sulfate. *H*, The homogenates from E5 mes-metencephalic boundary, untreated (*lane 1*), treated with chondroitinase ABC (*lane 2*), chondroitinase ABC + keratanase II (*lane 3*), and endo-β-galactosidase (*lane 4*), were analyzed by immunoblotting with MAb 5D4. Both keratanase II and endo-β-galactosidase treatments weakened the signal, indicating that MAb 5D4 specifically recognized keratan sulfate on immunoblots.

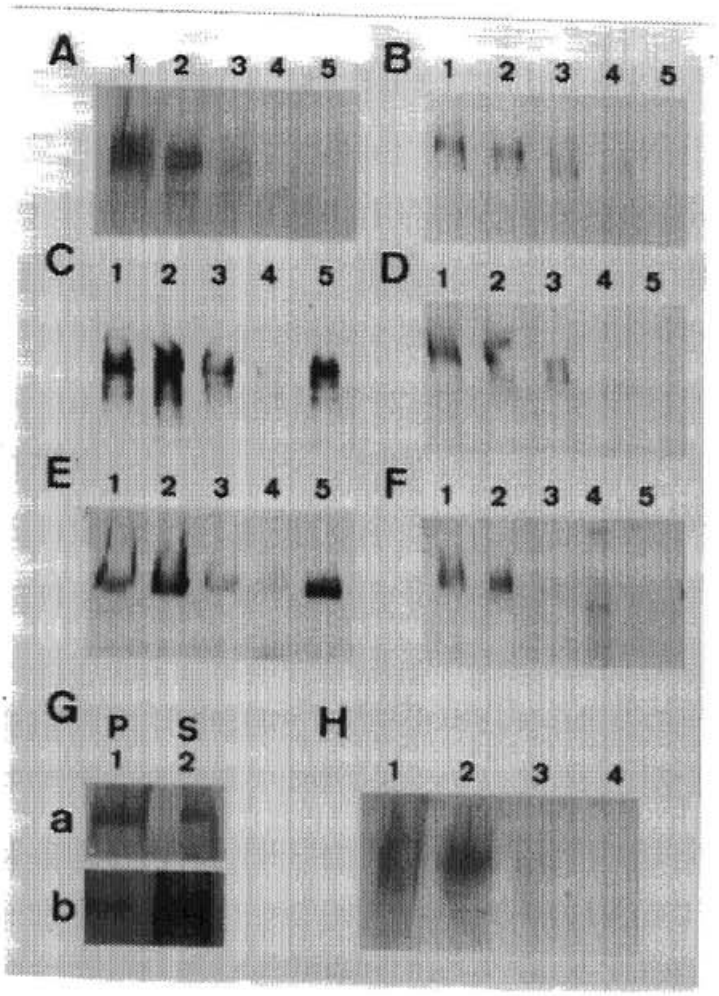
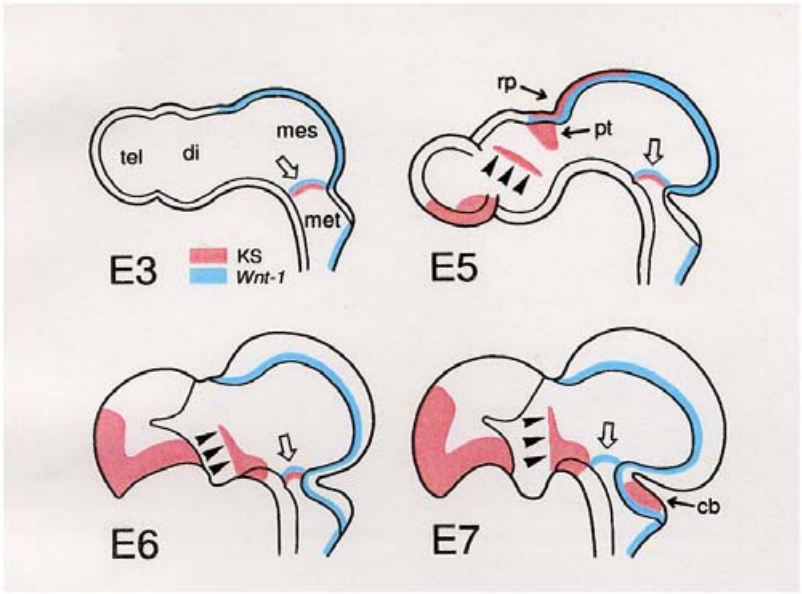

A
1 2 3 4 5
B
1 2 3 4 5
C
1 2 3 4 5
D
1 2 3 4 5
E
1 2 3 4 5
F
1 2 3 4 5
G
P S
1 2
a
b
H
1 2 3 4

Figure 18. Schematic representation showing the developmental profile of localization of keratan sulfate and *Wnt-1*. Keratan sulfate (KS) was expressed in several restricted regions such as a part of the telencephalon, the zona limitans (*arrowheads*), the mes-metencephalic boundary (*open arrows*), and the roof plate of the tectum. In the mes-metencephalic boundary region (*open arrows*), keratan sulfate and *Wnt-1* were expressed in nearly the same regions from E3 to E6. Keratan sulfate and *Wnt-1* seemed to be co-expressed in parts of the roof plate of the tectum at E5. I did not examine whether keratan sulfate is expressed in the roof plate at E6 and E7. At E7, keratan sulfate expression was also seen in the cerebellum. The keratan sulfate expression patterns at E3, E6 and E7 were reconstructed from the staining of tissue sections. *Wnt-1* expression patterns were drawn based on the data of Bally-Cuif and Wassef (1994). cb, cerebellum; di, diencephalon; mes, mesencephalon; met, metencephalon; pt, pretectal area; rp, roof plate; tel, telencephalon.


tel
di
mes
met
E3
KS
Wnt-1
rp
pt
E5
E6
cb
E7