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Presence of the HNK-1 Epitope on Poly(*N*-acetyllactosaminyl) Oligosaccharides and Identification of Multiple Core Proteins in the Chondroitin Sulfate Proteoglycans of Brain†

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ABSTRACT: The chondroitin sulfate proteoglycans of brain contain several core proteins bearing HNK-1 antibody epitopes. Endo-β-galactosidase treatment resulted in the almost complete disappearance of HNK-1 staining of proteoglycan immunoblots, indicating that a significant portion of the 3-sulfated sugar residues recognized by this antibody are present on poly(*N*-acetyllactosaminyl) oligosaccharides. However, after treatment with chondroitinase ABC followed by endo-β-galactosidase, several proteoglycan species showed HNK-1 reactivity, presumably due to the presence of this epitope on other oligosaccharides which are both resistant to endo-β-galactosidase and inaccessible to the antibody in the native proteoglycan. Immunostaining of the endo-β-galactosidase degradation products after separation by thin-layer chromatography demonstrated that HNK-1 reactivity was confined to a minor population of large oligosaccharides. Only a relatively small portion of the native chondroitin sulfate proteoglycans of brain enter a 6-12% SDS-polyacrylamide gel. However, after treatment of the proteoglycans with chondroitinase ABC (or chondroitinase and endo-β-galactosidase) in the presence of protease inhibitors, seven bands with molecular sizes ranging from 80 to 200 kDa appear in Coomassie Blue stained gels, and two additional bands with molecular sizes of 67 and 350-400 kDa are apparent in fluorographs of sodium [^{35}S]sulfate labeled proteoglycans. Most of these components probably represent individual proteoglycan species rather than different degrees of nonchondroitin sulfate/keratan sulfate glycosylation of a single protein core, since [^{35}S]methionine-labeled proteins of comparable molecular size were synthesized by an in vitro translation system. These findings suggest that chondroitin sulfate proteoglycans which differ in molecular size and composition may be specific to particular cell types in brain.

The chondroitin sulfate proteoglycans of brain range in molecular size from approximately 260 to 325 kDa, on the basis of their gel filtration behavior under dissociative conditions (Krusius et al., 1987). They are mostly soluble in a phosphate-buffered saline extract and account for less than 1% of the soluble brain protein (Kiang et al., 1981). Immunoelectron microscopic studies have demonstrated that the chondroitin sulfate proteoglycans are present in the extracellular space of early postnatal brain, after which period they progressively assume an intracellular (cytoplasmic) localization in neurons and astrocytes (Aquino et al., 1984a,b). Although biochemical assays have demonstrated only a limited degree of aggregation with hyaluronic acid, recent comparative studies on the localization of hyaluronic acid, hyaluronic acid binding region, link protein, and chondroitin sulfate proteoglycans in developing rat cerebellum suggest that much of the chondroitin sulfate proteoglycan of brain may occur in the form of aggregates with hyaluronic acid (and link protein) in situ (Ripellino et al., 1988, 1989).

The chondroitin sulfate proteoglycans contain an average of 56% protein, 24% glycosaminoglycans (predominantly chondroitin 4-sulfate, accompanied by smaller amounts of chondroitin 6-sulfate and keratan sulfate), and 20% N- and O-glycosidic glycoprotein oligosaccharides (Kiang et al., 1981;

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Klinger et al., 1985; Krusius et al., 1986, 1987). The asparagine-linked oligosaccharides are almost exclusively of the tri- and tetraantennary types (Klinger et al., 1985), whereas the O-glycosidic oligosaccharides consist of both conventional galactosyl(β1–3)*N*-acetylgalactosaminyl units and their mono- and disialyl derivatives, as well as a series of novel mannosyl-*O*-serine/threonine-linked oligosaccharides which can be isolated by mild alkaline–borohydride treatment of the proteoglycan glycopeptides and have the sequence GlcNAc(β1–3)Manol at their proximal ends (Finne et al., 1979; Krusius et al., 1986, 1987). These latter include GlcNAc(β1–3)Manol itself, Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Manol, NeuAc(α2–3)Gal(β1–4)GlcNAc(β1–3)Manol, and both short (3000–4500 Da) and long (10000 Da) mannose-linked keratan sulfate chains composed of disaccharide repeating units consisting of Gal(β1–4)GlcNAc–6–SO_4(β1–3).

Immunoblots of the chondroitin sulfate proteoglycans of brain and a number of nervous tissue glycoproteins are strongly stained by the HNK-1 monoclonal antibody, which has been shown to recognize a glucuronic acid 3-sulfate epitope in certain glycosphingolipids (Chou et al., 1986; Ilyas et al., 1986; Ariga et al., 1987), and considerably weaker reactivity is also seen with chondroitinase-treated cartilage or chondrosarcoma proteoglycans (Margolis et al., 1987). However, other than the observation that staining was not affected by chondroitinase treatment, no information was available as to which of the diverse oligosaccharide units present in the brain proteoglycans might contain the 3-sulfated sugar residues recognized by this antibody. This paper provides data which indicate that a large portion of these residues are components of poly(*N*-acetyllactosaminyl) oligosaccharides, and that the chondroitin sulfate proteoglycans of brain contain multiple core proteins that may be specific to particular cell types.

Experimental Procedures

Isolation of the Chondroitin Sulfate Proteoglycans. Chondroitin sulfate proteoglycans from rat brain were prepared either as described by Kiang et al. (1981) or together with the heparan sulfate proteoglycans (Klinger et al., 1985), since the products obtained by both procedures had identical properties. In the latter case, a deoxycholate extract was chromatographed on DEAE-cellulose, followed by affinity chromatography on lipoprotein lipase–Sepharose (to isolate the heparan sulfate proteoglycans). The unbound fraction from the lipoprotein lipase affinity column, eluted with 0.2 M NaCl, contained almost exclusively chondroitin sulfate proteoglycan and nucleic acid. Residual Tween 80 (from the original DEAE-cellulose chromatography step) was removed by reabsorption and elution of the proteoglycans from a small column of DEAE-cellulose, and after dialysis and lyophilization, they were dissolved in 0.2 M sodium acetate buffer, pH 5.6, for gel filtration on Sepharose CL-6B to remove nucleic acids and some smaller proteins (Kiang et al., 1981). The proteoglycans were biosynthetically labeled by intracerebral administration of carrier-free sodium [^{35}S]sulfate 18 h prior to decapitation (Klinger et al., 1985).

Enzyme Treatments. Chondroitinase ABC, endo-β-galactosidase from *Escherichia freundii*, and keratanase from *Pseudomonas* were obtained from Seikagaku Kogyo Co. (Tokyo), through ICN Immunobiologicals. The chondroitin sulfate proteoglycans of brain were treated for 1.5 h at 37 °C with chondroitinase ABC in a ratio of 1 milliunit/μg protein, which results in maximal release of [^{35}S]sulfate-labeled disaccharides (amounting to 65% of the total radioactivity) from biosynthetically labeled proteoglycans. All chondroitinase digestions were carried out in the presence of protease inhibitors, as described by Oike et al. (1980).

The brain proteoglycans were treated for 6 h at 37 °C with endo-β-galactosidase (5–6 milliunit/100 μg of protein) in 50 mM sodium acetate buffer, pH 6.0, which released 20% of the [^{35}S]sulfate radioactivity from labeled proteoglycans in the form of lower molecular size oligosaccharides. Keratanase digestions were preformed for 6 h at 37 °C in 50 mM Tris-HCl buffer, pH 7.4 with 3–4 conventional milliunits (200 Seikagaku Kogyo Co. milliunits) per 100 μg of proteoglycan protein and produced the same degree of degradation as was obtained from endo-β-galactosidase. By use of ^{14}C-labeled protein standards (Amersham), it could be demonstrated that there was no detectable protease activity in any of the glycosidases under the incubation conditions used in our studies. Enzyme degradation products were separated from residual proteoglycan by ultrafiltration on a Centricon-30 membrane (Amicon) and desalted by gel filtration on Sephadex G-15 before use in thin-layer chromatography.

Periodate Oxidation. Oligosaccharides were oxidized in 1 mL of 60 mM sodium periodate for 16 h at 4 °C in the dark. The reaction was terminated by addition of 10 μL of 50% ethylene glycol, and the samples were desalted by gel filtration on Sephadex G-50. Chondroitin sulfate proteoglycans (40–50 μg) were treated with 1 mL of 100 mM sodium periodate for 15 h at 4 °C in the dark. After addition of 10 μL of 50% ethylene glycol, the solution was allowed to stand for 1 h at 4 °C, filtered on a Centricon-30 membrane, and exchanged with water.

In Vitro Translation Studies. Total cytoplasmic RNA was extracted from adult rat brains after Polytron homogenization in 6 M guanidine isothiocyanate, followed by centrifugation for 20 h at 35000 rpm and 20 °C in a Beckman SW 41 rotor (Maniatis et al., 1982). Poly(A+) RNA was prepared by affinity chromatography on oligo(dT)–cellulose (Bethesda Research Laboratories, Gaithersburg, MD) and dissolved in water at a concentration of 1 μg/μL.

mRNA (36 μg) was translated for 1 h at 37 °C (total volume of 0.45 mL) in a nuclease-treated rabbit reticulocyte lysate (New England Nuclear, Boston, MA) in the presence of 1 mCi of [^{35}S]methionine (Pelham & Jackson, 1976). The reaction mixture was diluted with an equal volume of 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.02% NaN_3, and 5 mM methionine and precleared twice with 10 mg of preswollen protein A–Sepharose (Pharmacia) for 1 h at 4 °C. The supernatant was incubated overnight at 4 °C with 20 μL of preimmune rabbit serum and then twice for 1 h at 4 °C with 10 mg of protein A–Sepharose. The resulting supernatants were combined and immunoprecipitated by incubation overnight at 4 °C with 20 μL of a specific rabbit antiserum to the chondroitin sulfate proteoglycans (Aquino et al., 1984a), and the immune complexes were collected as described above. The protein A–Sepharose beads were washed with 10 mM Tris-HCl buffer, pH 8.6, containing 0.5 M NaCl, 0.05% NP-40, 0.1% SDS, and 5 mM methionine and boiled for 5 min in SDS-containing sample buffer prior to SDS–PAGE and fluorography.

Electrophoresis and Immunoblotting. Proteoglycan samples were heated for 5 min at 100 °C in sample buffer containing mercaptoethanol and sodium dodecyl sulfate and electrophoresed on 7% or 6–12% SDS–polyacrylamide slab gels in the discontinuous buffer system of Laemmli (1970).

Nitrocellulose immunoblots were stained with a 1:30 dilution of the HNK-1 monoclonal antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA), in conjunction with biotinylated anti-mouse IgM, avidin DH, and biotinylated

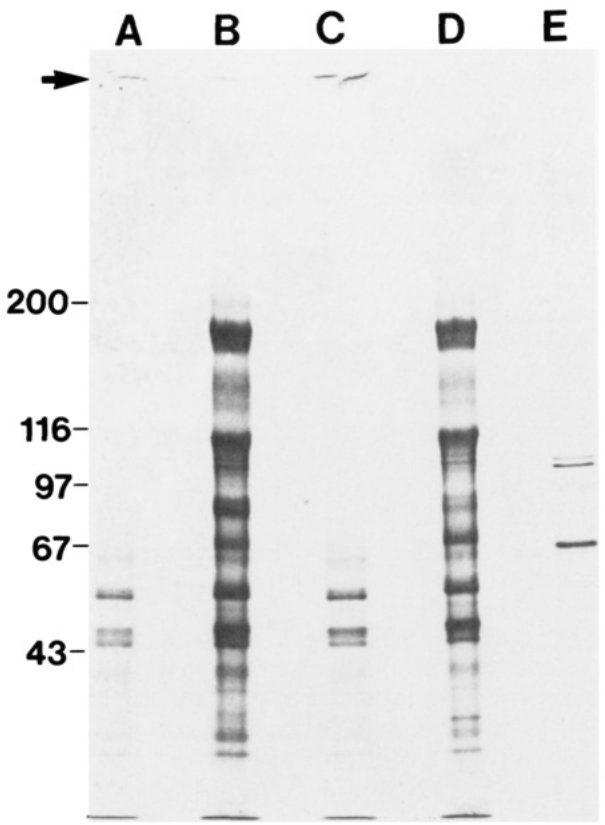

FIGURE 1: Coomassie Blue stained 6–12% SDS–polyacrylamide gradient gel: (lane A) untreated chondroitin sulfate proteoglycans (50 μg of protein); (lane B) chondroitinase-treated proteoglycans (100 μg of protein); (lane C) proteoglycans (75 μg) treated with endo-β-galactosidase; (lane D) proteoglycans (100 μg) treated with chondroitinase followed by endo-β-galactosidase; (lane E) 100 milliunits of chondroitinase ABC. Proteoglycans in lanes B and D were treated with 100 milliunits of chondroitinase ABC and those in lanes C and D with 6 and 8 milliunits of endo-β-galactosidase, respectively. Numbers at the left indicate the positions of molecular weight standards (given as $M_r \times 10^{-3}$), and the arrow indicates the interface between the stacking and separating gels.

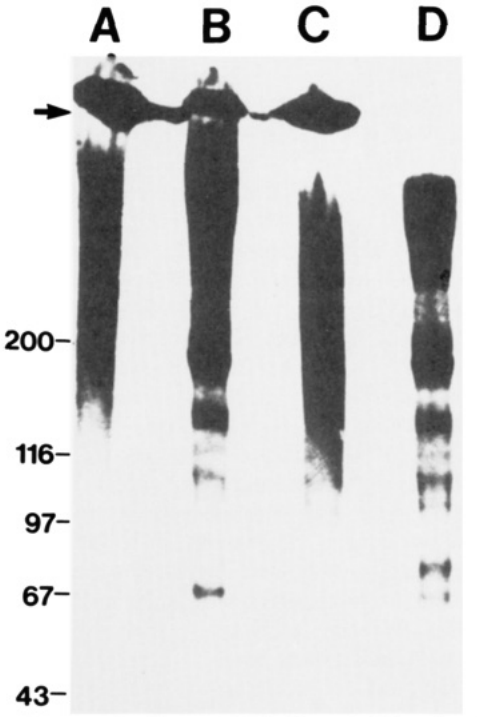

FIGURE 2: Fluorograph of lanes A–D in Figure 1. Note that different amounts of proteoglycan were applied to each lane (cf. legend to Figure 1), so as to more nearly equalize the amounts of radioactivity which remained in the samples after the various enzyme treatments.

horseradish peroxidase (all from Vector Laboratories, Burlingame, CA). Nonspecific binding sites on the nitrocellulose were blocked with 1% nonfat dry milk, and color development was with H_2O_2 and 3,3′-diaminobenzidine.

Thin-Layer Chromatography. Thin-layer chromatography was performed on silica gel 60 HPTLC plates or, for immunostaining, on amino-bonded silica gel 60 HPTLC plates (both from Merck), developed with ethanol/1-butanol/pyridine/water/acetic acid (100:10:10:30:3 v/v). Immunostaining was as described by Magnani (1987), using a 1:5 dilution of the HNK-1 antibody, followed by ^{125}I-labeled goat anti-mouse IgM (New England Nuclear, Boston, MA) at a concentration of 2 μCi/mL. For fluorography of [^{35}S]sulfate-labeled oligosaccharides, the plate was sprayed with Enhance (New England Nuclear) before exposure to Kodak XAR-5 X-ray film.

Materials. Human costal cartilage and bovine corneal keratan sulfates, which were kindly supplied by Drs. M. B. Mathews and J. A. Cifonelli (University of Chicago), were prepared by papain digestion and anion-exchange chromatography, the 3 M NaCl eluate from the ion-exchange column being used for isolation of the purified preparation (Rodén et al., 1972). They contained 20–21% galactose and 28% glucosamine by weight and had a molar ratio of sulfate to hexosamine of 1.26 and 1.48, respectively. Antibodies to the native chondroitin sulfate proteoglycans of brain (i.e., without chondroitinase treatment) were raised in rabbits, and their specificity was demonstrated by double immunodiffusion, rocket immunoelectrophoresis, crossed immunoelectrophoresis, ELISA, and immunoblotting (Aquino et al., 1984a).

Results

Effects of Treatment with Chondroitinase ABC and Endo-β-galactosidase. Coomassie Blue staining of the native proteoglycans after SDS–PAGE revealed three major bands with apparent molecular sizes of 55, 48, and 45 kDa (Figure 1, lane A). The 45-kDa component is likely to be a link protein with close structural homologies to those present in cartilage, since on immunoblots it stains with the 9/30/8-A-4 monoclonal antibody (Caterson et al., 1985) which recognizes two epitopes in the polypeptide portion of chondrosarcoma link protein. The 55-kDa band may be a further proteolytic degradation product of a less intensely stained component at 65 kDa which is recognized by the 12/21/1-C-6 monoclonal antibody (Caterson et al., 1986) to the hyaluronic acid binding region of cartilage, muscle, aorta, and other proteoglycans (Ripellino et al., 1989).[1] No significant Coomassie Blue staining was seen at the top half of the gel, where essentially all of the [^{35}S]sulfate-labeled proteoglycans appear (Figure 2, lane A). This is presumably due to interference by the chondroitin sulfate chains with dye binding to the protein cores.

After treatment of the proteoglycans with chondroitinase ABC in the presence of protease inhibitors, there is strong Coomassie Blue staining of several newly apparent bands with molecular sizes of 80–85, 115, and 165–175 kDa, as well as less intensely stained bands at 130, 140, 200, and approximately 350–400 kDa (Figure 1, lane B). The diffuse appearance of many of these bands is probably due to differing degrees of glycosylation with N- and O-linked glycoprotein

[1] It should be noted that the usual 65-kDa hyaluronic acid binding region fragment resulting from trypsin digestion of cartilage proteoglycans has a size of 55 kDa when obtained by digestion with certain clostripain preparations (Stevens & Hascall, 1986; Ripellino et al., 1988).

oligosaccharides (Finne et al., 1979; Klinger et al., 1985; Krusius et al., 1986, 1987). The doublet at 98–100 kDa represents chondroitinase ABC, and the prominent band at 67 kDa is due at least in part to bovine serum albumin added as a stabilizer for the enzyme (compare Figure 1, lane E).

After chondroitinase treatment alone (which removed 65% of the radioactivity as labeled disaccharides), most of the residual [^{35}S]sulfate radioactivity, present in keratan sulfate and in sulfated glycoprotein type oligosaccharides (Krusius et al., 1986, 1987), remains in the top half of the gel, although new bands at 67, 110, and 130–140 kDa also appear (Figure 2, lane B). These generally correspond to diffuse bands which were poorly stained by Coomassie Blue. Endo-β-galactosidase alone (which removed 20% of the [^{35}S]sulfate radioactivity) did not alter the Coomassie Blue staining of the proteoglycans (Figure 1, lane C), but it did eliminate [^{35}S]sulfate labeling from a population of proteoglycans which just barely entered the separating gel (compare lanes A and C in Figure 2). Treatment with chondroitinase ABC followed by endo-β-galactosidase (Figure 1, lane D) produced very little additional effect on Coomassie Blue staining as compared to that with chondroitinase ABC alone. However, fluorography of this material (containing only 15% of the original [^{35}S]sulfate radioactivity) showed the appearance of labeled protein bands at 67, 80, 110, 130–140, 180–200, 200–220, and approximately 350–400 kDa (Figure 2, lane D).

After isolation by ion-exchange chromatography and gel filtration, the 350–400-kDa component was found to represent approximately 30% of the total proteoglycan protein (unpublished results). However, it probably remains heavily glycosylated even after combined treatment with chondroitinase ABC and endo-β-galactosidase, accounting for the relative absence of Coomassie Blue staining in this region (Figure 1), though it reacts strongly on immunoblots with a polyclonal antibody to the proteoglycans. This assumption is consistent with our knowledge that N- and O-linked glycoprotein oligosaccharides represent 20% of the mass of the native proteoglycans (Kiang et al., 1981) and therefore more than 25% of that of the core "glycoproteins" remaining after chondroitinase treatment.

In Vitro Translation of Proteoglycan Core Proteins. In an attempt to determine what proportion of the glycoprotein bands detected after chondroitinase treatment of the proteoglycans represents different core proteins, as distinguished from different degrees of glycosylation of a single protein core, in vitro translation studies were performed. Antibodies to the chondroitin sulfate proteoglycans specifically immunoprecipitated at least eight [^{35}S]methionine-labeled proteins from a rabbit reticulocyte lysate system using mRNA from adult rat brain. The translation products ranged in size from 80 to >200 kDa, the most prominent one having a molecular size of 147 kDa (Figure 3). Although some core proteins smaller than 70 kDa may also be present, we have not attempted to identify these against a background of nonspecifically immunoprecipitated material in the low molecular size region of the gel, which becomes apparent after longer fluorographic exposures.

Our results demonstrate that most of the products obtained by chondroitinase treatment reflect the presence of distinct protein moieties, rather than merely different degrees of non-glycosaminoglycan glycosylation. Although the antibodies used in our in vitro translation studies reacted on immunoblots with the different core glycoproteins generated by chondroitinase treatment, due to the absence of N- and O-glycosidically linked oligosaccharides and the presence of an approximately

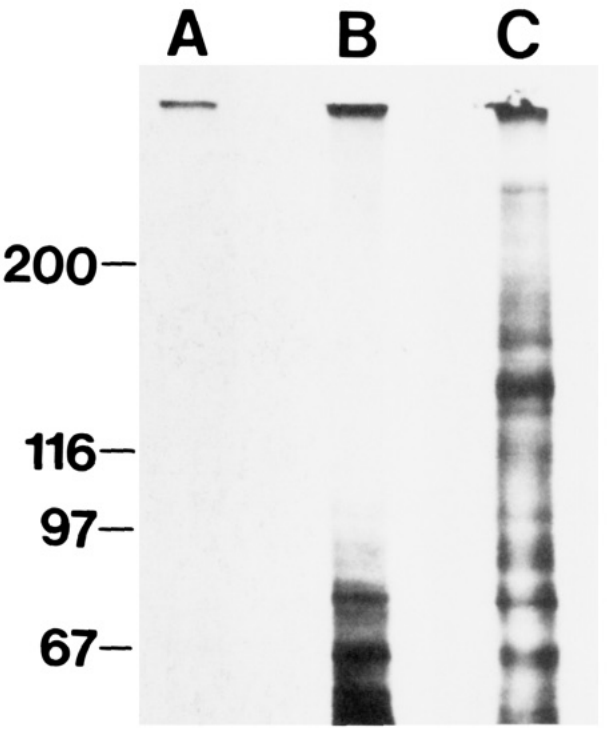

FIGURE 3: Fluorograph of in vitro translation products obtained with mRNA isolated from adult rat brain and electrophoresed on a 7% gel (for details, see Experimental Procedures): (lane A) complexes bound by protein A–Sepharose with preimmune rabbit serum; (lane B) preclearing protein A–Sepharose beads; (lane C) immunoprecipitate formed in the presence of rabbit antiserum to the chondroitin sulfate proteoglycans of brain. Numbers at the left indicate the positions of molecular weight standards (given as $M_r \times 10^{-3}$).

15–30 amino acid signal peptide, it is impossible to make reliable correlations between the in vitro translation products and those obtained by chondroitinase ABC treatment of the proteoglycans. It should also be noted in this connection that chemical deglycosylation using trifluoromethanesulfonic acid (Edge et al., 1981) was not effective in generating discrete protein species from the chondroitin sulfate proteoglycans of brain, and significant deglycosylation by peptide-*N*-glycosidase was also precluded by the high proportion of O-glycosidically linked oligosaccharides.

Localization of the HNK-1 Epitope. We have previously reported reactivity of the HNK-1 monoclonal antibody, which recognizes a glucuronic acid 3-sulfate epitope in certain glycosphingolipids (Chou et al., 1986; Ilyas et al., 1986; Ariga et al., 1987), with chondroitinase-treated chondroitin sulfate proteoglycans of brain, cartilage, and chondrosarcoma (Margolis et al., 1987). In a more detailed study on the localization of these epitopes in the brain proteoglycans, we have now found that in the absence of chondroitinase treatment very little HNK-1 reactivity is detectable after treatment with endo-β-galactosidase (Figure 4, lane B), indicating that 3-sulfated HNK-1 epitopes are present on poly(*N*-acetyllactosaminyl) oligosaccharides. (This result cannot be ascribed to decreased binding of endo-β-galactosidase-treated proteoglycans to nitrocellulose, since both untreated and endo-β-galactosidase-treated proteoglycans are stained with equal intensity by the polyclonal antiserum to the brain chondroitin sulfate proteoglycans and none of the enzyme treatments affected the binding of [^{35}S]sulfate-labeled proteoglycans to nitrocellulose, which amounted to 70–80% of the total radioactivity in every case.) Keratanase from *Pseudomonas* produces a similar decrease in HNK-1 reactivity but has very little effect on two proteoglycan species with apparent mo-

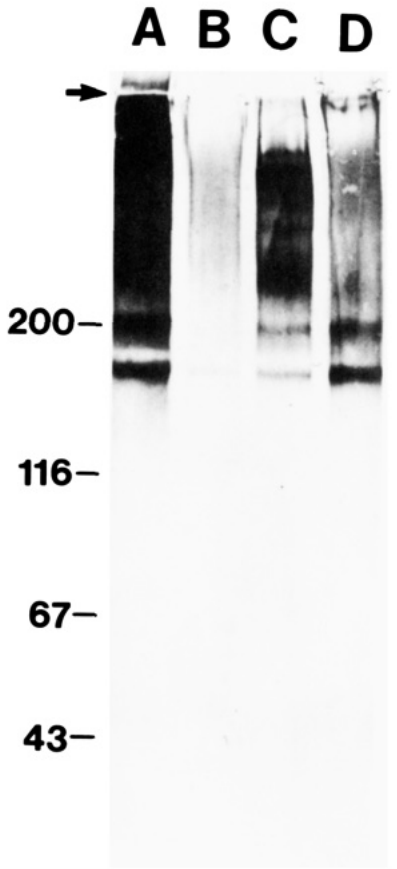

FIGURE 4: Immunoblot of 6–12% gradient gel, stained with the HNK-1 monoclonal antibody: (lane A) chondroitinase-treated chondroitin sulfate proteoglycans; (lane B) proteoglycans treated with endo-β-galactosidase; (lane C) proteoglycans treated with chondroitinase ABC followed by endo-β-galactosidase; (lane D) proteoglycans treated with keratanase. Although 25 μg of proteoglycan protein was applied to each lane to best illustrate the effects of enzyme treatments, nanogram quantities of proteoglycan were easily detectable by the antibody. HNK-1 staining of native proteoglycans (untreated with any glycosidase) is as seen in lane A, although a much greater proportion of stained material remains in the stacking gel (Margolis et al., 1987). Numbers at the left indicate the positions of molecular weight standards (given as $M_r \times 10^{-3}$), and the arrow indicates the interface between the stacking and separating gels.

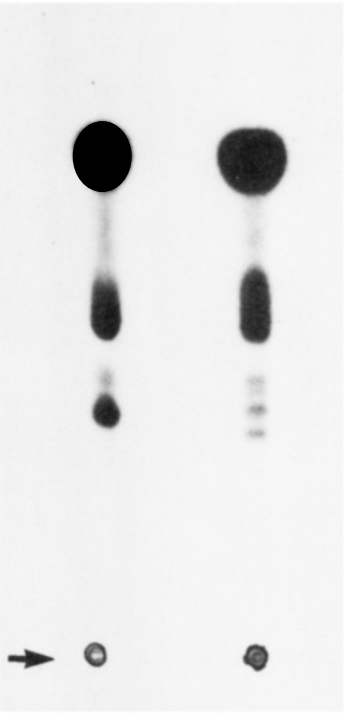

FIGURE 5: Fluorograph of di- and oligosaccharide digestion products obtained by treating [^{35}S]sulfate-labeled proteoglycans with endo-β-galactosidase (left) or keratanase (right), followed by thin-layer chromatography on high-performance silica gel 60 developed with ethanol/1-butanol/pyridine/water/acetic acid (100:10:10:30:3 v/v). The origin is indicated by the arrow.

lecular sizes of 170 and 200 kDa (Figure 4, lane D). When the brain proteoglycans are treated with chondroitinase ABC followed by endo-β-galactosidase, significant HNK-1 reactivity again appears in the 300–400-kDa area, indicating that some additional (endo-β-galactosidase resistant) epitopes are also present on the proteoglycans but are shielded from access to the antibody by the presence of chondroitin sulfate chains in proteoglycans treated with endo-β-galactosidase alone (Figure 4, lanes A–C). Since both endo-β-galactosidase and keratanase remove the same proportion (20%) of [^{35}S]sulfate radioactivity from labeled proteoglycans and an equivalent amount (approximately 50% of the residual radioactivity) from chondroitinase-treated material, it is apparent that both enzymes are similarly effective on native or chondroitinase-treated proteoglycans.

Thin-layer chromatography and fluorography of the [^{35}S]sulfate-labeled degradation products obtained after either endo-β-galactosidase or keratanase treatment demonstrate very similar patterns for the two enzymes, with the disaccharide *N*-acetylglucosaminyl-6-*O*-sulfate(β1–3)galactose presumably accounting for the major degradation product (Krusius et al., 1986), accompanied by five or more larger oligosaccharides (Figure 5). Chromatography on high-performance amino-bonded silica gel plates, followed by immunostaining with HNK-1 and ^{125}I-labeled goat anti-mouse IgM, demonstrated that immunoreactivity resided almost exclusively in a small proportion of large oligosaccharides which either remained at the origin or had a very low chromatographic mobility (Figure 6). No [^{35}S]sulfate-labeled or HNK-1-reactive material was released from the proteoglycan in the absence of enzyme.

Glycopeptides derived from the chondroitin sulfate proteoglycans were treated with alkaline borohydride, and the released O-glycosidic oligosaccharides and alkali-stable glycopeptides were fractionated by ion-exchange chromatography and gel filtration (Krusius et al., 1987). Six [^{35}S]sulfate-labeled fractions [corresponding to fractions 3, 5, 6, 7-II, 8, and 9 described by Krusius et al. (1987)] were subjected to mild periodate oxidation to generate free aldehyde groups, through which the oligosaccharides and glycopeptides were coupled to amino-bonded silica gel TLC plates for immunostaining with the HNK-1 antibody (see above) in a dot-binding assay. All of the six fractions (as well as the periodate-treated proteoglycans, but not fetuin, thyroglobulin, or α_1-acid glycoprotein) showed significant HNK-1 reactivity, demonstrating that the 3-sulfated HNK-1 epitopes are not confined to only one or a few of these sulfated oligosaccharide/glycopeptide fractions.

We previously reported that chondroitinase-treated rat chondrosarcoma and bovine nasal cartilage chondroitin sulfate proteoglycans had detectable reactivity with the HNK-1 antibody, but with an intensity which was significantly less than that of the chondroitin sulfate proteoglycans of brain (Margolis et al., 1987). Since the chondrosarcoma proteoglycans do not contain keratan sulfate and because its content in bovine nasal cartilage proteoglycans is lower than that in other types of cartilage, we examined the HNK-1 reactivity of cartilage and corneal keratan sulfates. Thin-layer chromatography and immunostaining of the endo-β-galactosidase degradation products of cartilage keratan sulfate showed some HNK-1

FIGURE 6: Autoradiograph of keratanase (left) and endo-β-galactosidase (center) digestion products obtained from the chondroitin sulfate proteoglycans of brain and of endo-β-galactosidase digestion products of cartilage keratan sulfate (right) after thin-layer chromatography on a high-performance amino-bonded silica gel 60 plate, immunostained with the HNK-1 monoclonal antibody and ^{125}I-labeled goat anti-mouse IgM. The origin is indicated by the arrow, and the direction of migration is toward the top of the figure.

reactivity (Figure 6), whereas none was seen with corneal keratan sulfate (not shown). It is probable that the lower reactivity of certain cartilage proteoglycans is not related to their keratan sulfate content, and the results of our dot-binding immunoassays (see above) also indicate that a significant portion of the HNK-1 reactivity of the brain proteoglycans is due to the presence of this epitope on other types of sulfated oligosaccharides, some of which are resistant to endo-β-galactosidase.

Discussion

Although considerable information is available concerning the structural properties (Finne et al., 1979; Kiang et al., 1981; Krusius et al., 1986, 1987), localization (Aquino et al., 1984a), and developmental changes (Margolis et al., 1975; Aquino et al., 1984b; Ripellino et al., 1988) of the chondroitin sulfate proteoglycans of brain, it was not known whether these represented a single polydisperse entity or several structurally similar macromolecules. While it was apparent that the proteoglycan molecules differ in their extent and type of glycosylation (Kiang et al., 1981), the native proteoglycans could not be fractionated into discrete species. Moreover, their moderate molecular sizes of approximately 260–325 kDa, as determined by gel filtration (Krusius et al., 1987), were not reflected in their behavior on SDS–PAGE, insofar as much of the native proteoglycan would not enter a 5% or 6% separating gel and no distinct molecular species could be detected by SDS–PAGE.

In view of the considerable heterogeneity of neuronal and glial cell types in brain, it would not be surprising that at least some of these would produce cell-specific chondroitin sulfate proteoglycans. However, since mature neurons do not divide, and therefore cannot be propagated in tissue culture, most information relevant to particular neuronal and glial proteoglycans has been obtained from the study of tumor cell lines, whose proteoglycans may differ considerably from those of normal nervous tissue.

In the present study, we have obtained evidence for the occurrence in rat brain of a number of distinct proteoglycan species which differ in their core proteins, and probably also in their degree and type of glycosylation with chondroitin sulfate, keratan sulfate, and N- and O-glycosidically linked oligosaccharides. These findings were not unexpected, but the existence of multiple species of chondroitin sulfate proteoglycans could only be demonstrated after chondroitinase treatment and by in vitro translation studies. After completion of this work, Oohira et al. (1988) have also recently reported the presence of several distinct molecular species of chondroitin sulfate proteoglycans in brain, on the basis of a comparison of tryptic peptides derived from chondroitinase-treated proteoglycans. However, in the absence of in vitro translation studies, their data do not exclude the possibility that the observed differences in peptide mapping patterns could be entirely attributable to a single core protein yielding a variety of different [^{35}S]methionine-labeled tryptic *glyco*peptides.

Recent studies have also provided evidence for developmental changes in the proteoglycan core proteins, resulting in differences in their Coomassie Blue staining pattern (unpublished results). Isolation of the core proteins and production of specific monoclonal or polyclonal antibodies should make it possible to study the structural properties and relationships of the individual proteoglycans and to obtain more detailed information concerning their specific cellular localizations and functional roles. However, in considering the biological relevance of these findings, it must also be recognized that a single cell may produce more than one type of chondroitin sulfate proteoglycan.

We have previously reported (Margolis et al., 1987) that immunoblots of the chondroitin sulfate (but not the heparan sulfate) proteoglycans of brain react with the HNK-1 monoclonal antibody, suggesting the presence in these proteoglycans of glucuronic acid 3-sulfate, which have been identified as the carbohydrate epitope recognized by this antibody in certain glycosphingolipids (Chou et al., 1986; Ilyas et al., 1986; Ariga et al., 1987). Although the HNK-1 epitope in the chondroitin sulfate proteoglycans of brain is resistant to periodate oxidation, indicating that here also the sulfate is in the 3-position, it is possible that a different 3-sulfated sugar may account for their HNK-1 reactivity. Galactose 3-sulfate can probably be excluded, since sulfatides do not react with this antibody (R. H. Quarles, personal communication). We have now demonstrated that a large proportion of the HNK-1 reactivity resides on poly(*N*-acetyllactosaminyl) oligosaccharides, which in the chondroitin sulfate proteoglycans of brains occur both as long keratan sulfate chains of approximately 10000 Da and as shorter (3000–4500 Da) sulfated oligosaccharides (Krusius et al., 1986, 1987). The HNK-1 reactivity of the brain proteoglycans is greatly reduced by endo-β-galactosidase or keratanase treatment, after which it is found in relatively large molecular size oligosaccharides which have a negligible chromatographic mobility in the TLC solvent systems which we tested. However, a significant amount of endo-β-galactosidase- (or keratanase-) resistant material remains with the chondroitinase + endo-β-galactosidase treated proteoglycans. It therefore appears that some of the 3-sulfated HNK-1 epitopes may be present on poly(*N*-acetyllactosaminyl) oligosaccharides which are resistant to endo-β-galactosidase

and keratanase due to their branching and/or sulfation pattern (Fukuda & Matsumura, 1976; Scudder et al., 1984) and on other types of sulfated N- and O-glycosidically linked oligosaccharides which we have previously identified in these proteoglycans (Kiang et al., 1981; Klinger et al., 1985; Krusius et al., 1987). Only the most acidic fraction of proteoglycan oligosaccharides, containing the 10 000-Da keratan sulfate chains which were eluted from DEAE-Sephadex with 2 M pyridine–acetate buffer, showed detectable uronic acid (approximately 6% of the total sugar in this fraction, on a molar basis), as determined by a modified carbazole reaction (Krusius et al., 1987). However, this value may be high, due to nonspecific reactivity of other sugars in the presence of a relatively small amount of actual uronic acid. Although the HNK-1 antibody also reacts weakly with cartilage proteoglycans, TLC immunostaining of the enzyme degradation products from corneal keratan sulfate showed no reactivity. It therefore appears that the 3-sulfated HNK-1 carbohydrate epitope is particularly characteristic of the chondroitin sulfate proteoglycans of brain and is not necessarily related to the presence or absence of keratan sulfate. However, it is possible that in the brain proteoglycans some of these residues may be present in the carbohydrate–protein linkage region or at branch points of keratan sulfate chains.

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