Isolation of a Neural Chondroitin Sulfate Proteoglycan with Neurite Outgrowth Promoting Properties

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Abstract. Proteoglycans are expressed in various tissues on cell surfaces and in the extracellular matrix and display substantial heterogeneity of both protein and carbohydrate constituents. The functions of individual proteoglycans of the nervous system are not well characterized, partly because specific reagents which would permit their isolation are missing. We report here that the monoclonal antibody 473HD, which binds to the surface of early differentiation stages of murine astrocytes and oligodendrocytes, reacts with the chondroitin sulfate/dermatan sulfate hybrid epitope DSD-1 expressed on a central nervous system chondroitin sulfate proteoglycan designated DSD-1-PG. When purified from detergent-free postnatal days 7 to 14 mouse brain extracts, DSD-1-PG displays an apparent molecular mass between 800–1,000 kD with a prominent core glycoprotein of 350–400 kD. Polyclonal anti-DSD-1-PG antibodies and monoclonal antibody 473HD react with the same molecular species as shown by immunocytochemistry and sequential immunoprecipitation performed on postnatal mouse cerebellar cultures, suggesting that the DSD-1 epitope is restricted to one proteoglycan. DSD-1-PG promotes neurite outgrowth of embryonic day 14 mesencephalic and embryonic day 18 hippocampal neurons from rat, a process which can be blocked by monoclonal antibody 473HD and by enzymatic removal of the DSD-1-epitope. These results show that the hybrid glycosaminoglycan structure DSD-1 supports the morphological differentiation of central nervous system neurons.

THE formation of specific intercellular connections between defined neuronal assemblies is a crucial step during the development of the central nervous system (CNS)[1]. Current concepts suggest that advancing growth cones are at least partially guided by selective interactions with their local environment (Bixby and Harris, 1991). Several adhesion molecules have been identified which play a role in this context, for example members of the Ig-superfamily which support neurite fasciculation and promote advancement of growth cones along axonal pathways (Jessell, 1988; Hortsch and Goodman, 1991; Rathjen, 1991; Schachner, 1991; Walsh and Doherty, 1991), or cadherins which regulate neuronal process extension on astrocyte and muscle cell surfaces (Bixby et al., 1987; Tomaselli et al., 1988; Takeichi, 1991). These latter processes also involve integrins, a family of extracellular matrix (ECM) receptors, implying that the ECM contributes to growth cone guidance. Consistent with this assumption several ECM glycoproteins such as laminin, thrombospondin, vitronectin, and tenascin have been shown to promote neurite outgrowth from a variety of peripheral nervous system (PNS) and CNS neurons (Sanes, 1989; Hortsch and Goodman, 1991; Reichardt and Tomaselli, 1991; Hynes and Lander, 1992). In addition proteoglycans (PGs) of the ECM which consist of a glycoprotein core with covalently linked glycosaminoglycan (GAG) chains (Hassel et al., 1986; Ruoslahti, 1988), may also play a role in neuronal fiber formation. For example, heparan sulfate proteoglycans (HSPGs) support neurite growth, in several cases as components of laminin-containing glycoprotein complexes (Lander et al., 1985*a*,*b*; Chiu et al., 1986; Hantaz-Ambroise et al., 1987; Matthiessen et al., 1989).

Tissue fractionation studies performed with rat brain revealed that most HSPGs are tightly associated with cell membranes, whereas chondroitin sulfate proteoglycans

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1. *Abbreviations used in this paper*: CNS, central nervous system; CS, chondroitin sulfate; DS, dermatan sulfate; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; GFAP, glial fibrillary acidic protein; HS, heparan sulfate; KS, keratan sulfate; PG, proteoglycan; PORN, poly-DL-ornithine; pDSD-1-PG, polyclonal antibodies to DSD-1-PG; PNS, peripheral nervous system; pTN, polyclonal antibodies to tenascin; PVDF, polyvinylidene difluoride; PVP, polyvinylpyrolidone.

(CSPGs), which constitute the major population of PGs in the CNS, are recovered in detergent-free salt extracts (Kiang et al., 1981; Klinger et al., 1985; Margolis and Margolis, 1989; Herndon and Lander, 1990). Not very much is known about the contribution of CSPGs to neurite growth or to the development of neural tissues. A major CSPG of rat brain has been characterized in more detail and found to be expressed first by Bergmann glia and subsequently also by granule cells in the developing rat cerebellum (Aquino et al., 1984*a*,*b*). Recent observations show that chondroitin sulfate/keratan sulfate proteoglycans (CS/KSPGs) are transiently expressed in discrete areas, for example in the roof plate of the developing spinal cord or alongside forming peripheral axonal pathways, where they may act as barriers to axon advance (Snow et al., 1990*a*,*b*; Cole and McCabe, 1991; Oakley and Tosney, 1991; Brittis et al., 1992). CSPGs are upregulated in CNS lesions and it has been proposed that their inhibitory properties might contribute to impairment of axonal regeneration in astrocytic scar areas (McKeon et al., 1991; Bovolenta et al., 1993; Pindzola et al., 1993). The structural characteristics of these PGs, however, have only partially been elucidated. It is known that soluble CSPG preparations from postnatal rat brain contain at least eight core glycoproteins, which are differentially expressed during rat CNS development (Herndon and Lander, 1990). According to peptide mapping studies, some of these represent distinct molecular species (Oohira et al., 1988). Interestingly, several of the core glycoproteins carry the L2/HNK-1 epitope, a carbohydrate structure also expressed by neural recognition molecules (Kruse et al., 1984, 1985; Chou et al., 1986; Gowda et al., 1989; Rauch et al., 1991). These reports indicate a substantial heterogeneity of CSPGs in the CNS.

The detailed structural and functional analysis of the growing number of brain CSPGs has hitherto been hampered by the lack of tools which would permit their characterization (Margolis and Margolis, 1989; Rauch et al., 1991). Several mAbs have been described that react specifically with individual PGs, such as 1D1, 3H1, and 3F8. These mAbs have been used to purify distinct PGs from neural tissues and one of these has been characterized with recombinant techniques and named neurocan (formerly 1D1; Rauch et al., 1991, 1992). The cellular sources of these PGs have, however, not yet been worked out in detail. In this regard, more is known about CAT 301 and 6B4 which identify neuronal CSPGs (Hockfield and McKay, 1983; Maeda et al., 1992) and NG2 which recognizes a glial CSPG of neural tissues (Stallcup and Beasley, 1987; Zaremba et al., 1989). Neuronal expression of CAT 301 early during spinal cord development depends on the activation of the NMDA receptor, suggesting a role of PGs in neuronal plasticity (Kalb and Hockfield, 1990). In support of a physiological role of PGs in the developing nervous system, it has recently been reported that a CSPG isolated from rat optic tectum supports survival of retinal neurons (Schulz et al., 1990). Furthermore, the astroglial CSPG astrochondrin, which has originally been defined by the mAb L5 directed to an N-linked carbohydrate component, is involved in cerebellar granule cell migration and mediates extension of astrocyte processes on collagen and laminin, but not on fibronectin substrates (Streit et al., 1990, 1993). Finally, it has recently been reported that the CSPGs neurocan and 3F8 of rat neural tissues inhibit homophilic interactions of Ng-CAM and N-CAM and the binding of neurons to these cell adhesion molecules (Grumet et al., 1993). In the light of these findings, we have studied in more detail DSD-1-PG recognized by mAb 473HD on the surface of immature CNS glia (formerly mAb 473) (Faissner, A. 1988. *Soc. Neurosci. Abstr.* 14:920). We show here that DSD-1-PG purified from postnatal mouse brain promotes neurite outgrowth by embryonic day 14 (E14) mesencephalic and E18 hippocampal neurons through the dermatan sulfate (DS)-containing hybrid epitope DSD-1 (for dermatan sulfate dependent). Part of this work has been presented in abstract form (Faissner, A. 1988. *Soc. Neurosci. Abstr.* 14:920 and Faissner, A., A. Lochter, A. Streit, A. Clement, C. Mandl, and M. Schachner. 1993. *Soc. Neurosci. Abstr.* 19:435).

Materials and Methods

Animals

For the preparations of single cells from embryonic or postnatal brains, CD1 rats or NMRI mice were used. The day a vaginal plug was found was designated embryonic day 0 (E0). Female LouXSD rats and New Zealand rabbits were used for immunization. All animals were kept at the local facility (Versuchstieranlage des Zentralbereichs Theoretikum, Heidelberg, Germany).

ECM Molecules

Human serum fibronectin was purchased from Boehringer Mannheim GmbH (Mannheim, Germany) or purified according to standard protocols (Ruoslahti et al., 1982) from outdated human plasma provided by the local blood and serum bank. Laminin isolated from Engelbreth-Holm-Swarm mouse sarcoma cells was a kind gift of Dr. K. Kühn (Max-Planck-Institute for Biochemistry, Munich, Germany) or acquired from GIBCO BRL (Eggenstein, Germany). Tenascin was purified as described (Faissner and Kruse, 1990). Chondroitin sulfates A (CS A), B (CS B, dermatan sulfate), and C (CS C), keratan sulfate (KS), heparan sulfate (HS), heparin and dextran sulfate were purchased from Sigma Chemical Co. (Deisenhofen, Germany) or ICN Biomedicals GmbH (Meckenheim, Germany).

Enzymes

Chondroitinase ACI from Flavobacterium heparinum was reconstituted in 20 mM Tris-HCl, 100 μg/ml BSA, 10 μg/ml ovomucoid, pH 7.4, and digestions were carried out in 50 mM Tris-HCl, 50 mM Na-acetate, 100 μg/ml BSA, and 10 μg/ml ovomucoid, pH 7.4. Chondroitinase ACII from Arthrobacter aurescens was taken up in 20 mM Na-acetate, 100 μg/ml BSA, 10 μg/ml ovomucoid, pH 6.0, and digestions were performed in 100 mM Na-acetate, 100 μg/ml BSA, and 10 μg/ml ovomucoid, pH 6.0. Chondroitinase B (dermatanase) from Flavobacterium heparinum was reconstituted in 50 mM Tris-HCl, 500 μg/ml BSA, pH 8.0, and digestions were performed in the same buffer. Chondroitinase ABC from Proteus vulgaris was dissolved in 20 mM Tris-HCl, 100 μg/ml BSA, and 10 μg/ml ovomucoid, pH 8.0, and digestion was carried out in 50 mM Tris-HCl, 50 mM Na-acetate, 100 μg/ml BSA, 10 μg/ml ovomucoid, and 1 mM PMSF, pH 8.0. Keratanase from Pseudomonas species was reconstituted in digestion buffer consisting of 100 mM Tris-HCl, 100 mM Na-acetate, 1 mM EDTA, 100 μg/ml BSA, pH 8.0, with the protease inhibitors 1 mM PMSF, 100 mM ε-aminocaproic acid, 5 mM benzamidine-HCl, 0.1 mM pepstatin. Heparinase from Flavobacterium heparinum and heparitinase were taken up in 50 mM Tris-HCl, 50 mM Na-acetate, 5 mM $CaCl_2$, 100 μg/ml BSA, pH 7.4, supplied with the additives 1 mM PMSF, 0.1 mM pepstatin, 1.0 mM leupeptin. The same buffer was used for digestion experiments. Enzymes were acquired from Sigma Chem. Co. or ICN Biomedicals GmbH and aliquots stored at −70°C.

Analytical Procedures

Protein concentrations were measured according to Lowry (Lowry et al., 1951) and Bradford (1976), with the Bio Rad protein assay (Bio Rad Laboratories, Munich, Germany) or with the Micro BCA reagent (Pierce Chem.

Co., Rockford, IL). Uronic acid concentrations were determined with a colorimetric assay (Blumenkrantz and Asboe-Hansen, 1973). Chondroitin-sulfate A, C, or heparin were used as standard reference with identical results.

SDS-PAGE and Western Blots

SDS-PAGE was performed on 4–10% gradient slab gels (Laemmli, 1970). The gels were stained with reducing silver ions (Merril et al., 1982) or Coomassie Blue (Serva, Heidelberg, Germany) according to standard protocols. Western blots were carried out as described and developed with Peroxidase-derivatized secondary antibodies or ^{125}I-labeled Protein A (Faissner et al., 1985, 1988). In some cases polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore Corp., Bedford, MA) were used instead of nitrocellulose and prepared according to the manufacturers instructions. Molecular mass standards were myosin, 205 kD; β-galactosidase, 116 kD; phosphorylase b, 97.4 kD; bovine serum albumin, 66 kD; egg white albumin, 45 kD; carbonic anhydrase, 29 kD (kit for molecular masses 30–200 kD; Sigma Chem. Co.); α2-macroglobulin, 180 kD; β-galactosidase, 116 kD; fructose-6-phosphate kinase, 84 kD; pyruvate kinase, 58 kD; fumarase, 48.5 kD; lactic dehydrogenase, 36.5 kD; triosephosphate isomerase, 26.6 kD (prestained kit for molecular masses 27–180 kD; Sigma Chem. Co.) or ^{14}C-methylated proteins myosin, 205 kD; phosphorylase b, 97.4 kD; BSA, 69 kD; ovalbumin, 46 kD; carbonic anhydrase, 30 kD; lysozyme, 14.3 kD (Amersham Buchler GmbH & Co. KG, Braunschweig, Germany; molecular mass markers for electrophoresis, 10–50 μCi/mg protein).

mAbs

mAb CS-56 reactive with chondroitin sulfates A and C (Avnur and Geiger, 1984) was purchased from Sigma Chem. Co., a mAb specific for KS was obtained from ICN Biomedicals GmbH and a mAb to GFAP was acquired from Boehringer Mannheim. mAbs O1 and O4 to oligodendrocyte surface antigens, to the L2/HNK-1 epitope (clones 334, 336) and to the cell adhesion molecule L1 (clone 324) have been described (Sommer and Schachner, 1981; Kruse et al., 1984; Rathjen and Schachner, 1984). Rat IgM mAb clone H1 which was used as control in immunoprecipitation experiments stains the surface of GFAP-positive astrocytes in culture. For production of monoclonal antibodies female LouXSD rats were immunized intraperitoneally and subcutaneously with 50 μg "rest-L2" glycoprotein fraction from adult mouse brains (Kruse et al., 1985) emulsified in complete and two times at two-week intervals with the same amount of immunogen dissolved in incomplete Freund's adjuvant. One week later 50 μg of the antigen in PBS, pH 7.4, was injected into the tail vein and fusions were carried out 4 d thereafter using the mouse myeloma line X-Ag8-653 as described (Lagenaur et al., 1980; Faissner and Kruse, 1990). Supernatants reactive with the antigen were detected with an enzyme-linked immunosorbent assay (ELISA, see below) and further characterized by immunocytology on mouse postnatal cerebellar cultures. Antibody subclasses were determined as described (Faissner and Kruse, 1990) and the rat IgM mAb 473HD (Faissner, A. 1988. *Soc. Neurosci. Abstr.* 14:920) was chosen for further study. For large scale production of antibody the hybridoma clone was grown in RPMI containing 1% vol/vol Nutridoma (Boehringer Mannheim) and the supernatant concentrated by $(NH_4)_2SO_4$ precipitation. SDS-PAGE revealed a purity of more than 95% of the antibody.

Polyclonal Antibodies

Polyclonal antibodies to vimentin and glial fibrillary acidic protein (GFAP) were purchased from Sigma Chem. Co. and Dako Diagnostika GmbH (Hamburg, Germany), respectively, polyclonal antibodies to N-CAM (Trotter et al., 1989) were a kind gift of Dr. J. Trotter (Department of Neurobiology, Heidelberg, Germany), and polyclonal antibodies to L1, laminin, fibronectin, and tenascin (pTN) have been described elsewhere (Rathjen and Schachner, 1984; Faissner et al., 1985; Pesheva et al., 1989; Faissner and Kruse, 1990). For production of polyclonal antibodies to the DSD-1-proteoglycan (pDSD-1-PG), 50–100 μg of uronic acid equivalents of the purified material (see below) emulsified in Freund's adjuvants were injected into rabbits at multiple sites subcutaneously on the back and the animal was boosted at three-week intervals with the same amount of antigen in incomplete Freund's adjuvants. One week after the third boost a first bleed was collected (KAF13[1]). Subsequently, the rabbit was boosted repeatedly after recovery phases of six weeks and serum was collected one week after each boost. IgG-fractions were prepared by affinity chromatography on protein A–Sepharose following standard protocols (Faissner and Kruse, 1990). After dialysis against PBS, 0.05% wt/vol NaN_3 the IgG fractions were concentrated to 4.0–5.0 mg/ml by pressure dialysis using PM30 membranes (Amicon, Witten, Germany) and stored at −70°C. IgGs from the fourth bleed (KAF13[4]) were used throughout for all the experiments detailed in this manuscript. Peroxidase- and alkaline phosphatase-derivatized secondary antibodies to rabbit, mouse and rat Igs were obtained from Promega Corp. (Madison, WI), Cappel Labs. (Cochranville, PA), or Dianova (Hamburg, Germany).

Purification of DSD-1-PG

For preparation of DSD-1-PG detergent-free extracts (Hoffman and Edelman, 1987; Faissner and Kruse, 1990) of postnatal day 1 (P1) to P15 mouse brains were sequentially circulated with a flow rate of 20 ml/h at 4°C over gelatin Sepharose, Sepharose 4B coupled with 5 mg/ml rat IgG, or 1 mg/ml mAb 473HD (10-ml bed volume each). Columns were washed with 30-bed volumes PBS, 10-bed volumes PBS containing 0.5 M NaCl followed by 10-bed volumes PBS and finally eluted with 2-bed volumes 0.1 M diethylamine, 0.1 M NaCl, 1 mM EDTA, and 1 mM EGTA, pH 11.5. The column eluates were neutralized by addition of 0.1 n HCl, dialyzed against PBS, concentrated in Amicon chambers, and stored at −70°C. Protein concentration (Micro BCA; Pierce Biochemicals) was about 20 μg/ml; 3 ml of eluate were obtained per preparation. For ion exchange chromatography on MONO Q columns (Pharmacia LKB, Freiburg, Germany) 2 ml of mAb 473HD eluate were dialyzed against 8 M Urea, 50 mM Na-acetate, pH 6.0 (loading buffer), concentrated to 0.5 ml in Centricon tubes (Amicon), loaded on the column, and subjected to a linear salt gradient from 0 to 1.5 M NaCl in loading buffer. 40 1-ml fractions were collected. Gel filtration was carried out on Superose 6 (Pharmacia LKB) with 0.5 ml of mAb 473HD-positive material in 4.0 M guanidinium-hydrochloride, 50 mM Na-acetate, pH 6.0, and 45 fractions were obtained. Fractions containing mAb 473HD immunoreactivity were identified by ELISA (see below). For preparative purposes eluates from several preparations were loaded on Q Sepharose with a super-loop device and eluted in two steps of 0.5 and 1.0 M NaCl (Streit et al., 1990). Immunoreactive fractions (all at 1.0 M NaCl) were pooled, dialyzed against H_2O, lyophilized, and taken up in PBS. In such preparations, concentration of uronic acid and protein were about 40 μg/ml and 5–7 μg/ml (or not measurable), respectively. About 500 μg uronic acid equivalents were isolated from 5,000 mouse brains and the resulting material was defined as DSD-1-PG. In some cases, supernatants of subcultured human embryonic fibroblasts (Faissner and Kruse, 1990) instead of postnatal mouse brain extracts were used as starting material for purification of DSD-1-PG, following the same protocol.

ELISAs

Clone supernatants resulting from the fusion experiments were tested by an immunospot test on nitrocellulose filters using the immunogen "rest-L2," 50 μg/ml, 3 μl per spot (Hawkes et al., 1982). For analysis of DSD-1-PG by ELISA with mAb 473HD and other mono- or polyclonal antibodies, 100-μl aliquots were taken from fractions of ion exchange or size exclusion chromatography columns, cell culture supernatants, tissue extracts or DSD-1-PG preparations (the latter at 2 μg/ml uronic acid equivalents) and spotted onto nitrocellulose sheets using the Minifold dot blot device (Schleicher & Schuell, Dassel, Germany). For investigation of mAb 473HD binding to immobilized GAGs, carbohydrates were directly applied to the filter at 1, 10, or 100 μg/ml in PBS, with 1–2 μl per spot. In some cases, GAGs were digested with GAG-lyases prior to application to nitrocellulose. Subsequently, the nitrocellulose sheets were washed twice with 50 mM Tris–HCl, 150 mM NaCl, pH 7.4 (TBS), treated with blocking buffer consisting of delipidated milk powder, 4% wt/vol in TBS supplemented with 0.1% wt/vol Tween 20, washed once with TBS, 0.5% wt/vol Tween 20 (TBS-Tween), and incubated overnight with mAb 473HD (1–5 μg/ml) in blocking buffer. Thereafter, filters were rinsed three times with TBS-Tween, incubated for 2 h with peroxidase-derivatized anti-rat Ig in blocking buffer, washed three times with TBS-Tween, and developed with ABTS or diaminobenzidine color substratum. Alternatively, mAb 473HD column eluates or DSD-1-PG preparations were adsorbed overnight to polyvinyl-pyrolidone (PVP; Falcon Plastics, Cockeysville, PA) plates at 0.5 μg/ml uronic acid equivalents in 0.1 M $NaHCO_3$, pH 8.0, 100 μl per well. The wells were blocked for at least 1 h with 0.1 M $NaHCO_3$, 1% wt/vol BSA, pH 8.0, 200 μl per well and washed twice with 0.1 M $NaHCO_3$, pH 8.0. Subsequently, PVP plates were incubated with antibodies diluted in PBS, 0.5% wt/vol BSA, 100 μl per well, for at least two hours or overnight, washed four times with PBS, 0.05% wt/vol Tween 20 (PBS-Tween), incubated for two hours with peroxidase-derivatized secondary antibodies in PBS, 0.5% wt/vol BSA,

washed four times with PBS-Tween, and developed with ABTS (Faissner and Kruse, 1990). The colored reaction product was quantified with an ELISA reader at 405 nm (Titertek multiscan; Flow Labs., Inc.). For digestion of DSD-1-PG with specific GAG-lyases before antibody incubation, nitrocellulose filters, or PVP plates containing the PG were washed twice with the corresponding digestion buffer (see Enzymes) after the blocking step. Thereafter, filters or microtiter wells were incubated with chondroitinases ACI, ACII, ABC, or keratanase at 10–100 mU/ml or heparinase and heparitinase at 1 U/ml for 3 h at 37°C, washed twice with adequate buffers, and processed for ELISA with mAbs 473HD or 336 (directed to the L2/HNK-1-epitope) as described above. For competition assays, 1 μg/ml of purified mAb 473HD was preincubated with defined GAGs (50 μg/ml if not indicated otherwise) for 1 h at 37°C and used for ELISA on DSD-1-PG in the presence of the competitors.

Iodination of DSD-1-PG

For iodination using the Bolton-Hunter or the iodogen procedure DSD-1-PG (20–30 μg of uronic acid equivalents) was dialyzed against 8.0 M urea, 0.1 M NaH_2PO_4/Na_2HPO_4, pH 8.1, and concentrated to 300 μl in Centricon 30 tubes (Amicon). The Bolton-Hunter reagent was dried under a stream of nitrogen, the PG was incubated with the ester for 30 min at room temperature and the reaction was stopped by the addition of 40 μl 1.0 M glycine. Unbound radioactivity was removed by filtration on Sephadex G10 (coarse) in 8.0 M Urea, 0.1 M NaCl, 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4 (Faissner et al., 1990). Peak fractions were monitored by γ-counting, pooled, and stored at 4°C. Under these conditions, 10–25 μCi were incorporated into the PG and the specific activity was 5–10 μCi per μg protein. For iodination according to the iodogen procedure (Salacinski et al., 1981), the concentrated PG was incubated with 1 mCi $Na^{125}I$ and one iodogen bead (Pierce Chem. Co.) in a test tube coated with iodogen (1,3,4,6-tetrachloro-3,6,-DI-phenylglucouril; Sigma Chem. Co.; 40 μg/ml in methylene chloride [dichloromethane, Sigma Chem. Co.], 0.5 ml per test tube dried under an N_2 stream) for 15 min and subsequently treated as described for the Bolton-Hunter procedure. Incorporation was 25–50 μCi per μg of protein. For digestion with GAG-lyases, the iodinated PG was precipitated twice with 70% vol/vol ethanol in water, dried, and incubated with chondroitinases ACI (100 mU), ACII (100 mU), ABC (100 mU), keratanase (100 mU), heparinase (1.0 U), or heparitinase (1.0 U) for 3 h at 37°C in adequate incubation buffers as detailed in Enzymes. The reaction was interrupted by addition of twofold concentrated sample buffer for PAGE. The digested material was resolved on 4–10% gradient slab gels and the gels were processed for autoradiography as described (Faissner et al., 1988).

Tissue Fractionation and Digestion

To analyze the solubilization properties of DSD-1-PG, P6-P14 mouse brains were homogenized by 10 strokes with a dounce homogenizer at a 1:5 (wt/vol) ratio in the following buffers: A, 10 mM Tris-HCl, 4 M guanidinium-HCl, and 5 mM EDTA, pH 7.4; B, 140 mM NaCl, 4 mM KCl, 15 mM $NaHCO_3$, 10 mM glucose, 0.2 mM NaH_2PO_4-H_2O, and 0.2 mM KH_2PO_4, pH 7.4; C, 100 mM diethylamine, 100 mM NaCl, and 2 mM EDTA, pH 11.5; D, 100 mM diethylamine, 60 mM *N*-octylglucopyranosid, and 2 mM EDTA, pH 11.5; E, 20 mM Tris-HCl, 5 mM EDTA, and 60 mM *N*-octylglucopyranosid, pH 8; F, 10 mM Tris-HCl, 2 M Urea, 2 mM EDTA, and 2 mM EGTA, pH 8.5; and G, 50 mM Tris-HCl, 50 mM Na-acetate, and 60 mM *N*-octylglucopyranosid, pH 8. Buffers A–G were replenished with 10 μg/ml uvomucoid, 1 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml SBTI, 5 μg/ml leupeptin, 0.1 μg/ml pepstatin, 1 μg/ml antipain, and 1 μg/ml α2-macroglobulin. Homogenates were stirred at 4°C for 60 min and subsequently cleared by centrifugation at 10,000 *g* for 10 min. Supernatants were carefully removed and extracts in buffers A–F were dialyzed twice against a 100-fold volume of 50 mM Tris-HCl, 50 mM Na-acetate, and 1 mM PMSF, pH 8, cleared again at 10,000 *g*, 4°C, for 10 min, and aliquots removed for digestion with GAG-lyases. To this end, cleared extracts in buffers A–G were replenished with 100 μg/ml BSA (RIA-grade), 10 μg/ml uvomucoid, and 1 mM PMSF, pH 8, and incubated with chondroitinase ABC (15 mU per 50 μl of extract) or without addition of enzyme (control) for 2 h at 37°C. Digestion was terminated by addition of 4× concentrated SDS-PAGE sample buffer and probes were resolved on 4–10% SDS-PAGE gradient slab gels, subjected to Western blotting and the filters finally developed with mAb 473HD or pDSD-1-PG antibodies and ^{125}I-derivatized secondary reagents (see above). For documentation of DSD-1-PG in rat CNS, E18 hippocampi were obtained by microdissection and transferred to ice-cold HBSS, freed from meninges and immediately frozen in an Eppendorf tube placed into liquid nitrogen. 12 pooled hippocampi were homogenized in 2 ml of buffer G and resulting tissue extracts (containing 200 μg/ml protein according to the Bio Rad protein assay) were subsequently processed for digestion with chondroitinase ABC and Western blotting essentially as described for postnatal mouse brain extracts (see above). In some cases, 50-μl aliquots of chondroitinase ABC treated and control extracts were used for dot blot and subsequent ELISA as detailed earlier (see ELISAs).

Preparation of Cell Culture Substrates

For preparation of coated substrates, multichamber culture slides (Falcon Plastics) were incubated for 1–2 h at 37°C with 1.5 μg/ml poly-DL-ornithine (PORN; Sigma Chem. Co.) in 0.1 M borate buffer, pH 8.2 (Collins, 1978). Preliminary control experiments had shown that coating with low concentrations of PORN as compared to uncoated plastic does not notably impair embryonic hippocampal neuron differentiation. The wells were washed twice with distilled water, air dried, incubated overnight at 37°C with PBS or PBS containing 5 μg/ml uronic acid equivalents of DSD-1-PG (both at 100 μl/well) sterilized by passage through filters with low protein-binding capacity (Millex GV4; Millipore Corp.), and finally washed twice with PBS. For some experiments DSD-1-PG was heat-treated by boiling for 10 min before coating. When cell culture was carried out in the presence of antibodies or for digestion with GAG-lyases, the culture substrates were blocked for 2 h with heat-inactivated BSA (15 min at 80°C, 5 mg/ml in PBS) after the coating step. Digestion was subsequently carried out with chondroitinases ACII or ABC with 100 μl of enzyme per culture well (2 U/ml in incubation buffer) for 3 h at 37°C. Thereafter, culture wells were washed with ddH_2O before cell plating. The adsorption of DSD-1-PG to the PORN-conditioned culture substrates after overnight coating, after incubation with antibodies or after enzyme treatments, both before cell plating and after the 24-h culture period, was monitored by ELISA. To this end, mAbs 473HD and 336 or pDSD-1-PG were applied in a final volume of 250 μl per microchamber well. In addition, the efficiency of chondroitinase ACII and ABC treatment in removing the GAG chains of DSD-1-PG was assessed with mAb CS-56. Development of the ELISA was performed as described above and the soluble color product was measured with an ELISA reader after transfer to microtiter plates.

Cell Culture

Hippocampal cell cultures were established from embryonic day 18 (E18)-E19 rat brains (Banker and Cowan, 1977) with some modifications (Lochter et al., 1991). Hippocampi were obtained by microdissection, placed in HBSS, freed from meninges, and incubated in HBSS containing 0.25% wt/vol trypsin (GIBCO BRL) for 10 min at 37°C. After three washes in HBSS, hippocampi were dissociated by trituration in HBSS containing 1 mM Mg_2SO_4 and 0.025% wt/vol DNase I (Boehringer Mannheim) using fire-polished Pasteur pipettes. The resulting cell suspension was centrifuged at 80 *g* for 10 min at room temperature and the pellet resuspended in chemically defined medium (but without putrescine) (Rousselet et al., 1988) consisting of DME with Ham's F12 solution (DME/F12 1:1; GIBCO BRL), 2.4 mg/ml $NaHCO_3$, 33 mM D-glucose, 2 mM L-glutamine, 25 μg/ml insulin, 100 μg/ml transferrin, 2×10^{-8} M progesterone, 3×10^{-8} M selenium, 0.1% (wt/vol) ovalbumin, 5 mM Hepes, 5 IU/ml penicillin, and 5 μg/ml streptomycin. Monolayer cultures from single cell suspensions from mesencephalon were prepared from E14-E15 rats and plated in defined medium (Chamak and Prochiantz, 1989). 90% of the mesencephalic and 98% of the hippocampal cells were viable as judged by trypan blue exclusion. All cells were plated at a density of 10,000 cells/cm^2 in 250 μl medium per well and maintained in this medium for 24 h. The plating efficiency of hippocampal neurons 1.5 and 24 h after plating was ~70% on all substrates. Viability of cells as determined by trypan blue exclusion after 24 h of culture was 60% on PORN and 80% on PORN with substratum-bound DSD-1-PG. Cultures of both hippocampal and mesencephalic cells were estimated to be 98% pure neurons by morphological (Banker and Cowan, 1977, for hippocampal cultures) or immunological (for mesencephalic cultures, Chamak and Prochiantz, 1989) criteria. To test the influence of mAbs on the effect of substratum-bound DSD-1-PG, mAb 473HD was dialyzed against DME/F12 and sterilized by filtration (Millex GV4; Millipore Corp.). The wells were incubated for 2 h at 37°C with 20 μg/ml of mAb 473HD in PBS and washed twice with PBS prior to cell plating. An equal concentration of antibodies was added to the culture medium 1 h thereafter. The mAb 473HD did not affect the survival rate of hippocampal neurons. Primary mouse cerebellar and E14 mouse or subcultured human embryonic fibroblast cultures were established as described and cultivated on poly-l-

lysine-conditioned coverslips for immunocytological studies (Schnitzer and Schachner, 1981; Kruse et al., 1985; Faissner and Kruse, 1990).

Histochemical and Immunocytological Staining of Cultured Cells

For histochemical staining, cultures were fixed in PBS containing 2.5% (vol/vol) glutaraldehyde for 1 h at room temperature. After two washes with PBS, cells were stained for 15 min with 0.5% (wt/vol) toluidine blue (Sigma Chem. Co.) in 2.5% (wt/vol) Na_2CO_3, washed twice with distilled water and air dried. For immunochemical staining with peroxidase-derivatized secondary antibodies, cultures were fixed with 4% (wt/vol) paraformaldehyde in PBS for 15 min at room temperature, washed twice with PBS, and blocked for 10 min with 10% (vol/vol) horse serum in PBS. Cultures were then incubated for 30 min at 37°C with polyclonal N-CAM antibodies (1:200) in PBS containing 10% (vol/vol) horse serum, washed twice with PBS, and incubated for 30 min at 37°C with peroxidase-coupled secondary antibodies to rabbit IgG (1:1,000) in PBS containing 10% (vol/vol) horse serum. Subsequently, the cells were washed twice with PBS, incubated with 0.5 mg/ml diaminobenzidine, 0.015% (vol/vol) H_2O_2 in 10 mM Tris-HCl, pH 7.4, at room temperature, washed twice with distilled water, and air dried. For double immunofluorescence labeling, cultures were fixed with 4% (wt/vol) paraformaldehyde and subsequently stained with mAb 473HD or mAb 324 (anti-L1) followed by anti-rat-FITC, and counterstained with polyclonal antibodies to L1, DSD-1-PG, vimentin, or GFAP which were visualized with anti-rabbit-Texas Red, according to published protocols (Schnitzer and Schachner, 1981). Cells were permeabilized with ethanol (10 min at 4°C) prior to application of antibodies directed against the cytoskeleton. When mAbs O1 or O4 were compared with mAb 473HD in double immunofluorescence-staining experiments, subclass specific anti-mouse IgM and anti-rat IgM secondary antibodies derivatized with FITC or Texas red were used. These were diluted in the presence of serum from the species corresponding to the mAb of the counter-stain in order to minimize cross-reactions. Thus, anti-mouse IgM-Texas red diluted in the presence of 10% (vol/vol) rat serum did not recognize mAb 473HD on cell surfaces expressing the DSD-1 epitope. In some cases, fixation was performed after application of the first or the second antibody or at the end of the staining procedure. Coverslips were embedded with Moviol 4-88 (Hoechst, Frankfurt, Germany) dissolved in PBS, and replenished with the protectant Citifluor (Amersham Buchler). Cultures were viewed with a Zeiss epifluorescence microscope (Axiophot) and photographed with an integrated camera system.

Morphometry and Statistical Analysis

For quantitative morphometry only singly growing cells were measured and only neurites exceeding one cell diameter in length were taken into account. Neurite outgrowth was determined as the fraction of process-bearing cells from at least 100 neurons per well chosen at random and given in percent. Neurons extending processes were further analyzed by evaluating the total length of neurites per cell using an Ai Tectron image analysis system (VIDS III software). Samples of 50 randomly selected neurons were investigated per well. Since the single values obtained were not normally distributed, neurite lengths of individual neurons grown under different conditions within an experiment were compared with the Mann-Whitney U-Test, a non-parametric statistical procedure. For comparison between independent experiments, total neurite lengths of 50 neurons for defined experimental conditions were summed and the resulting sums were evaluated with the Mann-Whitney U-Test. All statistical tests were taken from the textbook by Clauss and Ebner (1977). Graphical representations of results were performed using Statview II TM (Abacus Concepts Inc., Berkeley, CA), Cricket Graph TM (Cricket Software, Malvern, PA), and Canvas TM (Deneba Software Inc., Miami, FL) at the Zentrum für Molekulare Biologie Heidelberg, University of Heidelberg.

Biosynthetic Labeling of Cell Cultures and Immunoprecipitation

For biosynthetic labeling of sulfate groups, postnatal cerebellar cultures were incubated overnight with 50 μCi/ml $^{35}SO_4$ in low sulfate basal medium Eagle, 10% (vol/vol) horse serum. Thereafter, cultures were washed twice with CMF-HBSS and then detergent solubilized in 1 ml ice-cold solubilization buffer (0.15 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 10 mM methionine, 0.5% NP-40, and 0.05% NaN_3, pH 7.4) containing aprotinin, soybean trypsin inhibitor, turkey eggwhite trypsin inhibitor (all at 10 μg/ml), PMSF, and iodoacetamide (both at 5 mM), and pepstatin (at 2 μM). After 10 min on ice, cells were gently scraped off the Petri dish, transferred to test tubes, and kept on ice for another 30 min. Detergent lysates were cleared by centrifugation at 800 *g* for 10 min and at 100,000 *g* for 1 h at 4°C and then either stored at −70°C or immediately used for immunoprecipitation. After the end of the labeling period culture supernatants were processed separately, e.g., treated with protease inhibitors and centrifuged as described above. Immunoprecipitation was carried out as described (Faissner et al., 1985). In brief, 1-ml aliquots of biosynthetically labeled detergent-solubilized cell extracts or culture supernatants were mixed with 200 μg pDSD-1-PG antibodies, 20 μg mAb 473HD, or 20 μg mAb clone H1 (negative control) and incubated for at least 1 h on a shaker. This and all subsequent steps were carried out at 4°C. The aliquots were then incubated for another hour with 120 μl of preswollen Sepharose-protein A conjugate (Sigma Chem. Co.) in case of polyclonal rabbit antibodies. For precipitation of mAb 473HD, monoclonal anti-rat-kappa-light-chain antibody coupled to cyanogen bromide activated Sepharose 4B at 3.0 mg per 1.0 ml of swollen gel was used (MARK-1) (Bazin, 1982), and 100 μl of MARK-1-Sepharose prepared as 10% vol/vol suspension in solubilization buffer were added to a single aliquot. Sepharose beads were washed several times and finally resuspended in 65 μl SDS-sample buffer, boiled for 4 min at 100°C and centrifuged (8,000 *g*, 1 min). The supernatants were counted in a β-scintillation counter and in some cases supernatants of precipitates collected in parallel experiments were run on 4–10% gradient SDS-polyacrylamide slab gels. For sequential immunoprecipitation, this procedure was repeated several times with the same aliquot (Faissner et al., 1988). After eight-cycles of precipitation with pDSD-1 antibodies aliquots were cleared with Sepharose-protein A conjugate in order to remove residual polyclonal antibodies and finally subjected to one cycle of precipitation with mAb 473HD. In the reverse experiment, mAb 473HD was used for eight cycles of immunoprecipitation and ensuing clearance was carried out with MARK-1-Sepharose prior to the final precipitation with pDSD-1-PG. Both sequences of immunoprecipitation were performed in parallel, starting with split aliquots from the same labeled culture. For fluorography, gels were fixed in 10% acetate, 30% methanol in water and subsequently treated with "Amplify" as described by the producer (Amersham Buchler). Kodak XAR-5 films were exposed at −70°C and developed according to the supplier's instructions. For determination of incorporated activity, two 50-μl aliquots from both detergent extracts or culture supernatants were incubated with acetone (80% vol/vol, −20°C overnight) or TCA (20% wt/vol, 60 min on ice). Precipitates were collected by centrifugation at 12,000 *g* for 15 min, washed once with ethanol/diethyl ether (1:1 vol/vol) and finally recovered at 12,000 *g* for 10 min. The resulting pellets were dried, dissolved in heated SDS-PAGE sample buffer, boiled for 5 min, and finally quantified in a β-scintillation counter.

Results

mAb 473HD Identifies DSD-1-PG in the Mouse CNS

The rat IgM mAb 473HD reactive with the molecule designated DSD-1-PG was obtained by generating mAbs against glycoproteins carrying the L2/HNK-1-epitope, a carbohydrate structure common to several recognition molecules and PGs of the rodent central nervous system. mAb 473HD immunoprecipitates chondroitinase ABC-sensitive component(s) from $^{35}SO_4$-labeled G26/20 glioma cell culture supernatants which migrate as a polydisperse smear at 1,000 kD, suggesting that DSD-1-PG is soluble in the absence of detergents (Faissner, A. 1988. *Soc. Neurosci. Abstr.* 14:920). For biochemical characterization, the molecule was therefore enriched from detergent-free, physiological saline extracts of early postnatal day 8 (P8) to P15 mouse brains by immunoaffinity chromatography on a mAb 473HD column. The resulting column eluates were further purified by ion exchange chromatography where DSD-1-PG eluted at 1.0 M NaCl (Fig. 1 *A*). In gel filtration experiments, DSD-1-PG obtained by this two-step procedure migrated as peak between 800 and 1,000 kD in the presence of 4.0 M guanidinium-HCl (dissociative conditions) (Fig. 1 *B*). Analogous results were

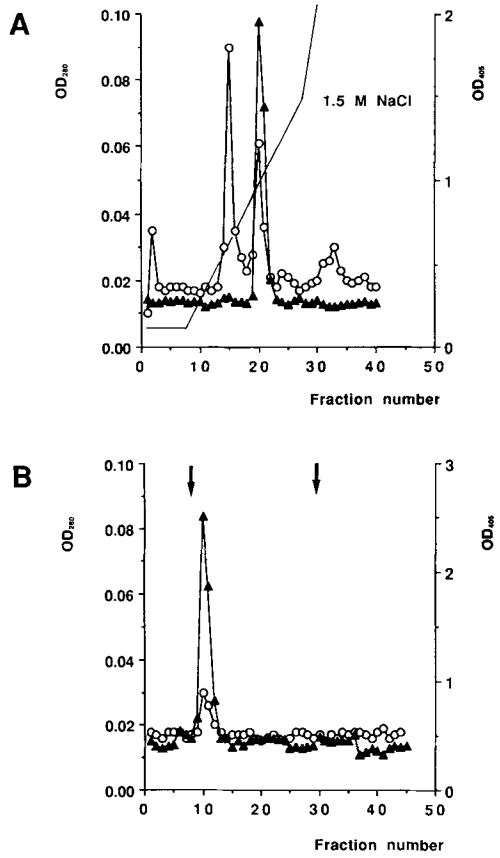

Figure 1. Analysis of DSD-1-PG by fast performance liquid chromatography. DSD-1-PG–containing glycoprotein fractions were enriched by immunoaffinity chromatography on a mAb 473HD column, as described in Materials and Methods. Column eluates were further processed by ion exchange chromatography (*A*) and subsequent size exclusion chromatography (*B*). (*A*) mAb 473HD immunoreactive fractions (OD_{405}, *black triangles*) eluted at 1.0 M NaCl (linear slope) from MONO Q Sepharose, in contrast to the majority of proteins (OD_{280}, *open circles*). (*B*) Immunoreactive material obtained in *A* was recovered as one peak between 800 and 1,000 kD in 4.0 M guanidinium-HCl on Superose 6. Arrows indicate V_o and V_t.

obtained when the sequence of chromatography steps was reversed (not shown). Our purification protocol yielded ~200 ng uronic acid equivalents of DSD-1-PG per 1 gram brain tissue (wet weight). Because of this low yield, purified DSD-1-PG had to be radioactively labeled with ^{125}I for visualization and enzymatic digestion studies. The radioiodinated molecule appeared as a broad smear commencing at 1,000 kD by SDS-PAGE and was sensitive to chondroitinase ACII and ABC, but not to other GAG-lyases (Fig. 2). Removal of GAGs both by chondroitinase ACII and ABC resulted in a prominent component of molecular mass 350–400 kD and, in a minority of cases (not shown), in faint, diffuse bands in the range of 240 and 180 kD with less than 10% of the total radioactivity. These might represent degradation products of the large component or additional species reactive with mAb 473HD which are expressed at lower levels or not recovered in substantial amounts by the isolation procedure. Thus, variations in the migration properties of DSD-1-PG which were also observed within the same batch may reflect the technical difficulties to resolve highly

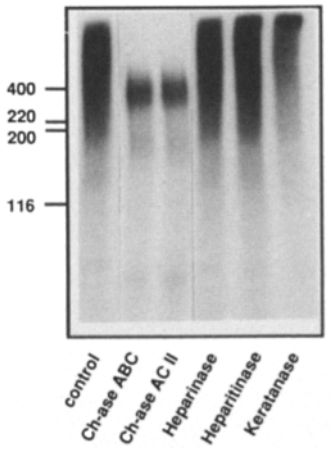

Figure 2. Analysis of DSD-1-PG by digestion with glycosaminoglycan lyases. ^{125}I-labeled DSD-1-PG (10^6 cpm per lane) was precipitated with ethanol and incubated with chondroitinase reaction buffer devoid of enzyme or digested with chondroitinase ACII, chondroitinase ABC, heparinase, heparitinase, or keratanase as indicated in the figure, boiled in sample buffer, and resolved by SDS-PAGE on 4–10% gradient slab gels. An autoradiography of the gel is shown. Molecular mass markers indicated at the left margin were laminin A chain (400 kD), fibronectin (220 kD), laminin B chain (200 kD), α2-macroglobulin (180 kD), and β-galactosidase (116 kD).

charged polydisperse molecules by SDS-PAGE. In contrast to chondroitinase ABC, heparinase, heparitinase, and keratanase did not significantly affect the migration behavior of DSD-1-PG by SDS-PAGE, consistent with the notion that it constitutes a CSPG and did not react with an mAb to keratan sulfate (KS) in ELISAs (Fig. 2, and not shown). The yield of DSD-1-PG from postnatal mouse brain comprised 200 ng uronic acid equivalents and 20–40 ng of protein per g wet weight. Soluble P0 rat brain extract encompass at least nine distinct PGs with an overall concentration of 15 μg protein per g of tissue (wet weight) (Herndon and Lander, 1990) which corresponds to an average content of 170 ng PG protein. From this point of view, and considering its restricted distribution, the estimated concentration of DSD-1-PG in postnatal mouse brain is not surprisingly low.

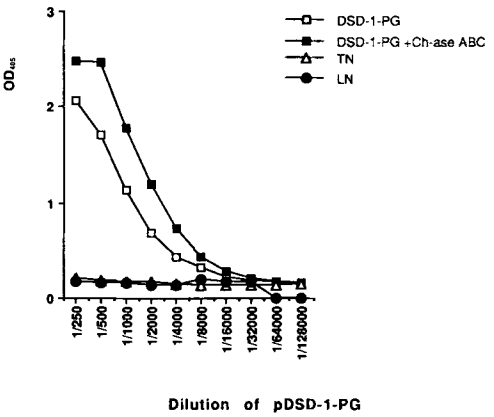

Figure 3. Specificity of polyclonal anti–DSD-1-PG antibodies. Laminin (*LN*, *open triangles*) and tenascin (*TN*, *filled circles*, both at 1.0 μg/ml) and DSD-1-PG (0.5 μg/ml uronic acid equivalents) were coated to wells of a PVP plate. The PG was not further treated (*open squares*) or digested with chondroitinase ABC (*filled squares*) after adsorption to the substrate. The ELISA was developed with a dilution row of pDSD-1-PG and a soluble color substrate (*ABTS*, OD at 405 nm). Note that the antibodies do not cross-react with laminin or tenascin and show higher affinity to DSD-1-PG after removal of the GAG complement. Starting concentration (1:250) corresponded to 20 μg/ml of IgG.

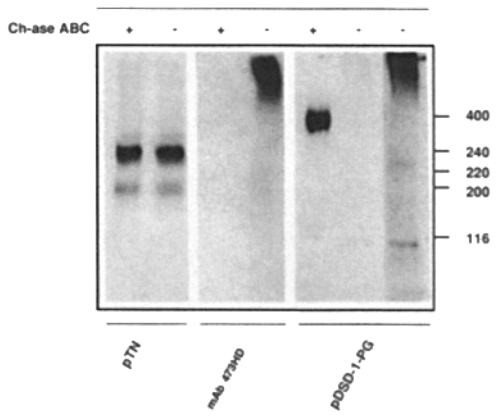

Figure 4. Analysis of DSD-1-PG by Western blotting. Extracts of postnatal day 8–15 mouse brains (prepared in 50 mM Tris–HCl, 50 mM Na-acetate, 60 mM N-octylglucopyranoside, pH 8, including protease inhibitors) were incubated with chondroitinase ABC or without addition of enzyme (control) as indicated in the figure. Samples were resolved by SDS-PAGE in 4–10% gradient slab gels, transferred by Western blotting and filters were developed with mAb 473HD, pTN, and pDSD-1-PG antibodies and ^{125}I-derivatized secondary reagents. Note that the DSD-1 epitope is present on polydisperse material migrating from 600–1000 kD and is removed by enzyme treatment. Digestion of GAGs yields one core glycoprotein of 350–400 kD. Tenascin is not affected by enzyme treatment, as expected. An autoradiograph is shown, exposures had to be prolonged for visualization of the pDSD-1-PG signal on the undigested extracts, presumably because of reduced affinity for the intact PG (last lane on the right). Under these conditions, faint bands at 230 and 110 kD emerge which are negligible in comparison to the intensity of the signal obtained on the chondroitinase ABC treated material. Molecular mass markers indicated at the right margin were laminin A chain (400 kD), fibronectin (220 kD), laminin B chain (200 kD), α2-macroglobulin (180 kD), and β-galactosidase (116 kD).

To establish that DSD-1-PG represents a defined entity, pDSD-1-PG against the purified molecule were raised in rabbits. Expectedly, the polyclonal antibodies reacted with DSD-1-PG adsorbed to PVP plates but did not recognize the ECM glycoproteins tenascin, laminin, or fibronectin in ELISA (Fig. 3 and not shown). The polyclonal antibodies showed a higher affinity towards the core glycoprotein of DSD-1-PG than for the intact molecule, as evidenced by increased reactivity after treatment of the PG with chondroitinase ABC (Fig. 3). Enzyme treatment and the release of the GAG-chains did not detach the core protein from the PVP wells (Fig. 3). The specificity of the polyclonal antibodies to DSD-1-PG was confirmed in Western blot experiments. Extracts from P8 to P15 mouse brains were digested with chondroitinase ABC prior to SDS-PAGE. The polyclonal antibodies reacted with a polydisperse smear beginning in the range of 1,000 kD apparent molecular mass in untreated samples and visualized a major band of 350–400 kD in CNS homogenates digested with chondroitinase ABC, in accordance with the characteristics of purified DSD-1-PG (Figs. 2 and 4). To exclude that further components reactive with the polyclonal antibodies are expressed in compartments of the CNS not accessible to physiological saline extraction, P6-P14 mouse brains were homogenized under various conditions including urea-, guanidinium hydrochloride-, or detergent-containing buffers and high pH. The homogenates were subsequently processed for chondroitinase digestion and SDS-PAGE and analyzed by Western blotting, with essentially the same results as described above (Table I). It has to be kept in mind, however, that additional DSD-1-PG components may be hidden in the insoluble pellets after extraction. These are not accessible to our analytical strategy because stronger solubilization conditions such as SDS-containing buffers inactivate the chondroitinase ABC enzyme used to reveal the core glycoprotein(s). In parallel experiments, mAb 473HD reacted with a polydisperse smear of 1,000 kD comigrating with the material recognized by the polyclonal antibodies in undigested CNS homogenates. The epitope was, however, removed by chondroitinase ABC treatment, indicating that mAb 473HD recognizes the GAG complement of DSD-1-PG (Fig. 4, and see below). Analogous results were obtained when E18 rat hippocampi were used instead of postnatal mouse brains for the preparation of CNS tissue extracts, subsequent digestion with chondroitinase ABC and Western blotting with mAb 473HD or pDSD-1-PG, documenting that the molecule is expressed in this region of the nervous system during the period of neuritogenesis (not shown).

To examine whether this epitope is only expressed on a subpopulation of DSD-1-PG, mAb 473HD and the polyclonal pDSD-1-PG antibodies were compared in immunofluorescence double-labeling studies performed on mouse cerebellar cultures. As already reported, mAb 473HD reacted with glial, but not with neuronal surfaces in cultures prepared from embryonic, peri-, and postnatal tissue maintained in culture for various time periods. Thus, ~50% of 473HD-positive cells contained vimentin, in contrast to 1–10% which overlapped with GFAP, markers of immature and mature astrocytes in this culture system. Furthermore, 50% of mAb 473HD-positive cells expressed O4 and 10% expressed O1, markers of immature and mature oligodendrocytes, respectively (Fig. 5 and not shown). In contrast, no overlap with L1, a marker of postmitotic neurons, was observed (Fig. 6). Less than 1% of mAb 473HD-positive cells expressed fibronectin, indicating that DSD-1-PG is absent from surfaces of fibroblast-like cells which may represent meningeal cells (not shown). Consistent with this result, mAb 473HD did not stain the surface of E14 mouse fibroblasts and no mAb 473HD-positive material could be enriched from the supernatant of subcultured human embryonic fibroblasts by immunoaffinity chromatography on a mAb 473HD affinity column. Finally, in embryonal cerebellar cultures some mAb 473HD-positive cells did not express any of the aforementioned markers (Fig. 5 and not shown). These observations suggest that DSD-1-PG is surface-expressed by the astrocyte and oligodendrocyte lineages during earlier differentiation stages. The polyclonal DSD-1-PG antibodies recognized the same cells as the mAb 473HD (Fig. 6). In some cases, mAb 473HD showed a more pronounced reactivity with the culture substrate than the polyclonal antibodies, presumably because the latter display a lower affinity towards the intact as compared to the GAG-free molecule (Fig. 3). To ascertain that lower expression levels of DSD-1-PG on seemingly unstained cells are not overseen because of this reduced affinity, the cultures were digested with chondroitinase ABC prior to the immunofluorescence studies. This treatment removed the mAb 473HD-binding site from the cell surface, as expected, but did not

Table I. Differential Extraction of DSD-1-PG from Postnatal Mouse Brains

Extraction buffer	Western blot with mAb 473HD Control kD	mAb 473HD Ch-ase ABC	pDSD-1-PG Control kD	pDSD-1-PG Ch-ase ABC kD
A, 10 mM Tris-HCl, 4 M guanidinium-HCl, 5 mM EDTA, pH 7.4	600–1,000	none	600–1,000	350–400
B, 140 mM NaCl, 4 mM KCl, 15 mM $NaHCO_3$, 10 mM glucose, 0.2 mM NaH_2PO_4-H_2O, and 0.2 mM KH_2PO_4, pH 7.4	600–1,000	none	600–1,000	350–400
C, 100 mM diethylamine, 100 mM NaCl, and 2 mM EDTA, pH 11.5	600–1,000	none	600–1,000	350–400
D, 100 mM diethylamine, 60 mM *N*-octylglucopyranosid, and 2 mM EDTA, pH 11.5	600–1,000	none	600–1,000	350–400
E, 20 mM Tris-HCl, 5 mM EDTA, and 60 mM *N*-octylglucopyranosid, pH 8	600–1,000	none	600–1,000	350–400
F, 10 mM Tris-HCl, 2 M Urea, 2 mM EDTA, and 2 mM EGTA, pH 8.5	600–1,000	none	600–1,000	350–400
G, 50 mM Tris-HCl, 50 mM Na-acetate, and 60 mM *N*-octylglucoside, pH 8	600–1,000	none	600–1,000	350–400

Postnatal mouse brains were homogenized in buffers A–G and extracts incubated with chondroitinase ABC or under identical conditions without addition of enzyme (*control*) for 2 h at 37°C. After SDS-PAGE probes were subjected to Western blotting and filters developed with mAb 473HD or pDSD-1-PG and ^{125}I-derivatized secondary reagents. As expected, the DSD-1 epitope was sensitive to chondroitinase ABC while pDSD-1-PG reacted with a core glycoprotein of 350–400 kD. Note that all extraction protocols yielded identical results.

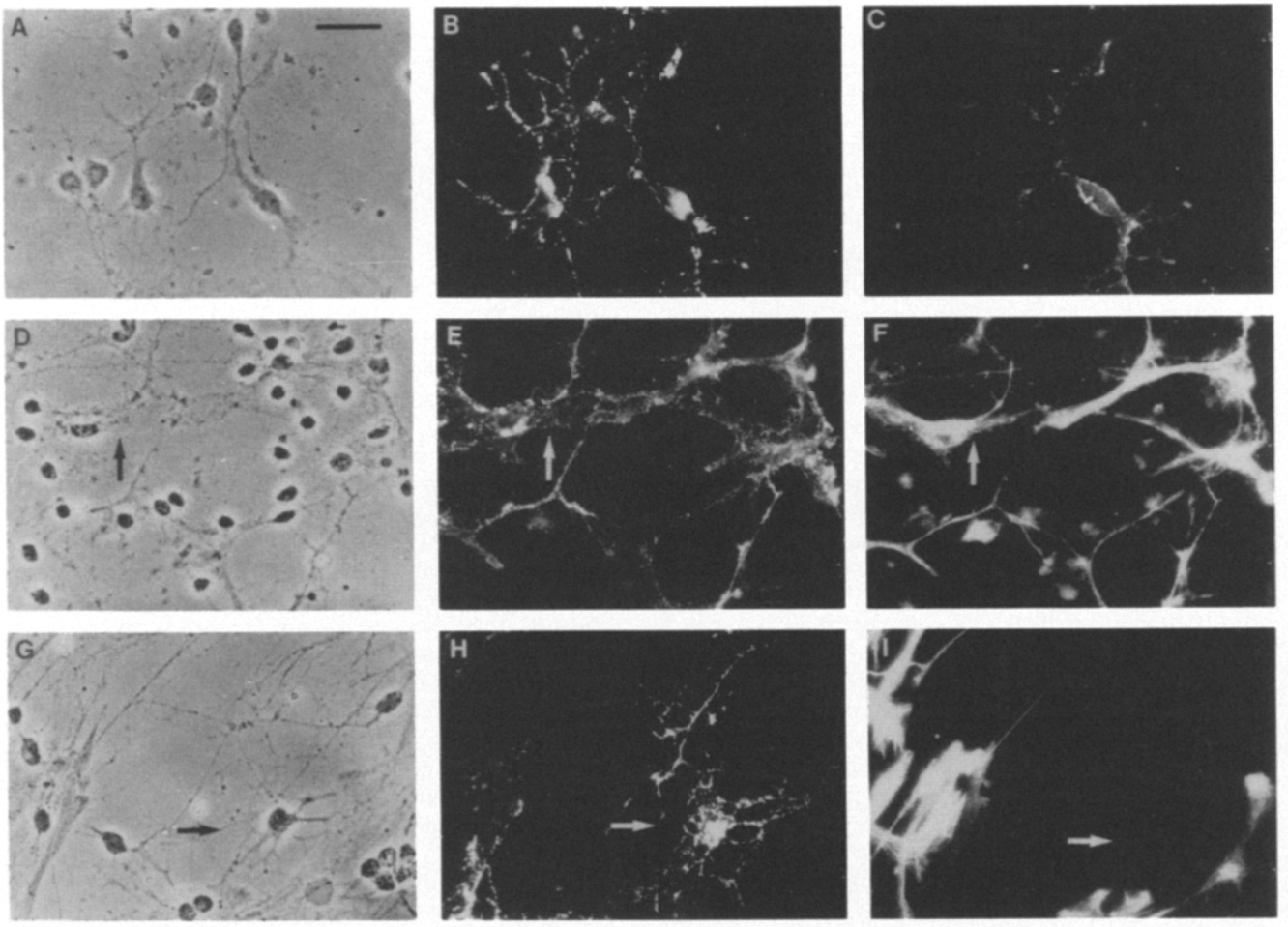

Figure 5. Expression of DSD-1-PG on glial cell surfaces. Cerebellar cells from E17 (*A–C*) and P5 (*D–I*) mice were maintained for 3 (*A–F*) or 5 d in vitro, respectively. Cultures were stained with 473HD followed by anti-rat FITC (*B*, *E*, and *H*), fixed and counterstained with O4 and anti-mouse TRITC (*C*) or additionally permeabilized and labeled with rabbit polyclonal antibodies to vimentin (*F*) or GFAP (*I*) and anti-rabbit TRITC. Note that part of the DSD-1-PG expressing cells carry O4, a marker for immature oligodendrocytes and that overlap of the DSD-1-epitope is more pronounced with vimentin than with GFAP, markers of immature and mature astrocytes, respectively. Bar, 30 μm.

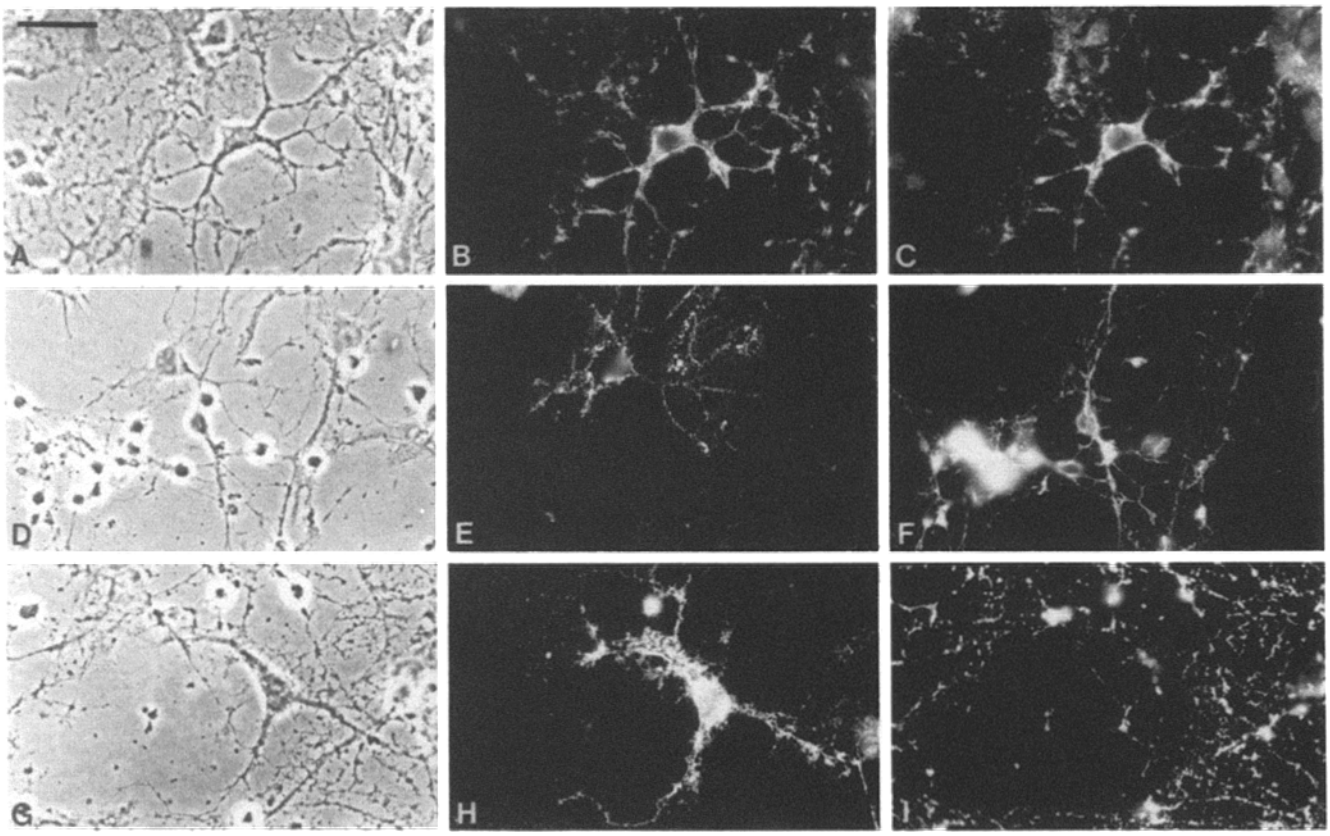

Figure 6. Absence of DSD-1-PG from cerebellar neuron surfaces. Postnatal cerebellar cells were cultivated for 3 d and surface stained with mAb 473HD (*B* and *E*) or mAb 324 to the adhesion molecule L1 (*I*) and anti-rat FITC, and subsequently counterstained with pDSD-1-PG (KAF13[4]) (*C* and *H*) or polyclonal L1-antibodies (*F*) and anti-rabbit Texas red. Note that mAb 473HD and pDSD-1-PG show overlapping staining patterns and do not react with neuronal surfaces. Bar, 20 μm.

modify the marker profile of cells immunostained with the polyclonal antibodies (not shown). The combined observations indicate that mAb 473HD and pDSD-1-PG react with the same or closely related molecular species. This conclusion was supported by sequential immunoprecipitations carried out on the supernatants of biosynthetically labeled postnatal mouse cerebellar cultures. Both mAb 473HD and pDSD-1-PG antibodies precipitated material comigrating as polydisperse smear of 1,000 kD in SDS-PAGE (Fig. 7, *inset*). In contrast, the rat IgM mAb clone H1 which stains the surface of GFAP-positive astrocytes in culture did not yield a detectable immunoprecipitate, underlining the specificity of the procedure (not shown). Eight cycles of immunoprecipitation with mAb 473HD removed virtually all material reactive with pDSD-1-PG while, in a parallel experiment, eight cycles of immunoprecipitation with the polyclonal antibodies removed nearly to completion the antigen bound by mAb 473HD. Based on these measurements, we estimate that mAb 473HD and pDSD-1-PG antigens are more than 95% identical or, in other words, that the mAb 473HD GAG-epitope is expressed on the majority, if not all, DSD-1-PG molecules and not restricted to a subpopulation of DSD-1-PG. The total immunoprecipitated radioactivity for both the mono- and the polyclonal antibody corresponded to less than 3% of the incorporated activity, which was independently determined by TCA or acetone precipitation. Thus, DSD-1-PG represents a minor fraction of the labeled components in the culture supernatant, excluding unspecific depletion of the molecule by repeated immunoprecipitation cycles.

mAb 473HD Reacts with the Chondroitin Sulfate/Dermatan Sulfate Hybrid GAG Structure DSD-1

To characterize the site recognized by mAb 473HD, antibody binding was determined by ELISA after digestion of DSD-1-PG with GAG-lyases. In these experiments, the reactivity of mAb 473HD proved resistant to treatment of DSD-1-PG with heparinase, heparitinase, and keratanase, which did not significantly modify the migration behavior of DSD-1-PG in SDS-PAGE (Figs. 2 and 8 *A*). Chondroitinase ACII, which degrades CS A and CS C, but not CS B, slightly reduced, whereas chondroitinase ABC, which cleaves CS A, CS B, and CS C abolished the binding of mAb 473HD to the molecule (Fig. 8 *A*). This hints towards a participation of DS (CS B) in the formation of the epitope. Notably, the selective removal of GAGs did not detach the residual PG core from the ELISA plate, as demonstrated by the specific binding of pDSD-1-PG (Fig. 3) or of mAb 336 against the L2/HNK-1 carbohydrate epitope after treatment of DSD-1-PG with chondroitinase ABC (Fig. 8 *A*). The latter observation indicates that the PG core is a glycoprotein and shares the N-linked L2/HNK-1 carbohydrate with several cell adhesion molecules and neural proteoglycans. Partial reduction

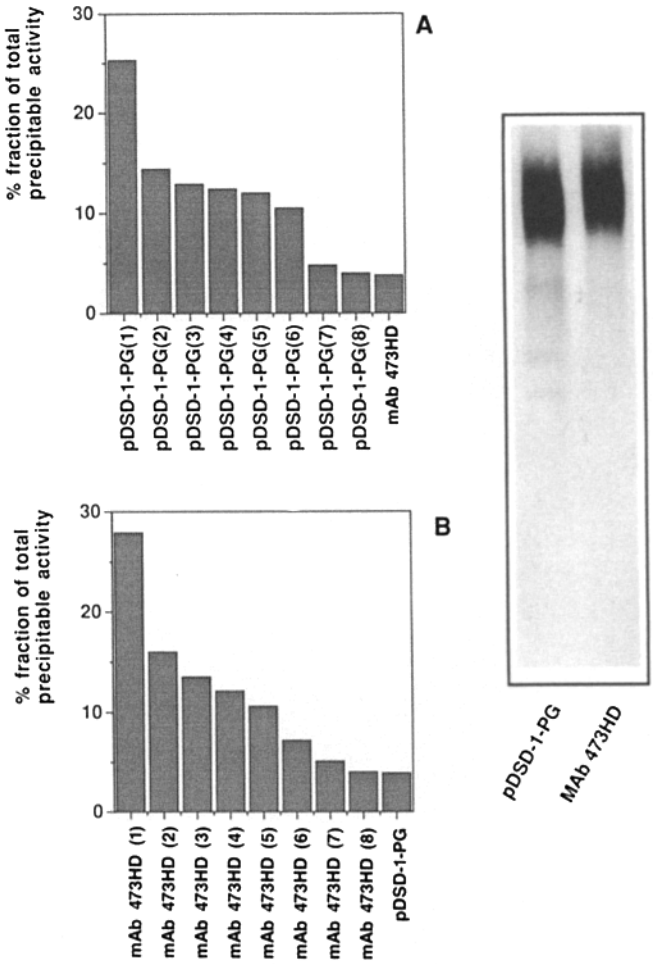

Figure 7. Comparison of mAb 473HD and pDSD-1-PG by sequential immunoprecipitation. P3 mouse cerebellar cultures were biosynthetically labeled with $^{35}SO_4$ and supernatants were used for several cycles of immunoprecipitation with pDSD-1-PG followed by mAb 473HD (*A*) or for the reverse order (*B*). Note that precipitates obtained with mAb 473HD and pDSD-1-PG comigrate in SDS-PAGE on 4–10% gradient slab gels (fluorography, *inset*) and that each antibody virtually removed the precipitable activity for the other. Bars detail the fractions of the total precipitated activity collected in the steps of one sequence of immunoprecipitations.

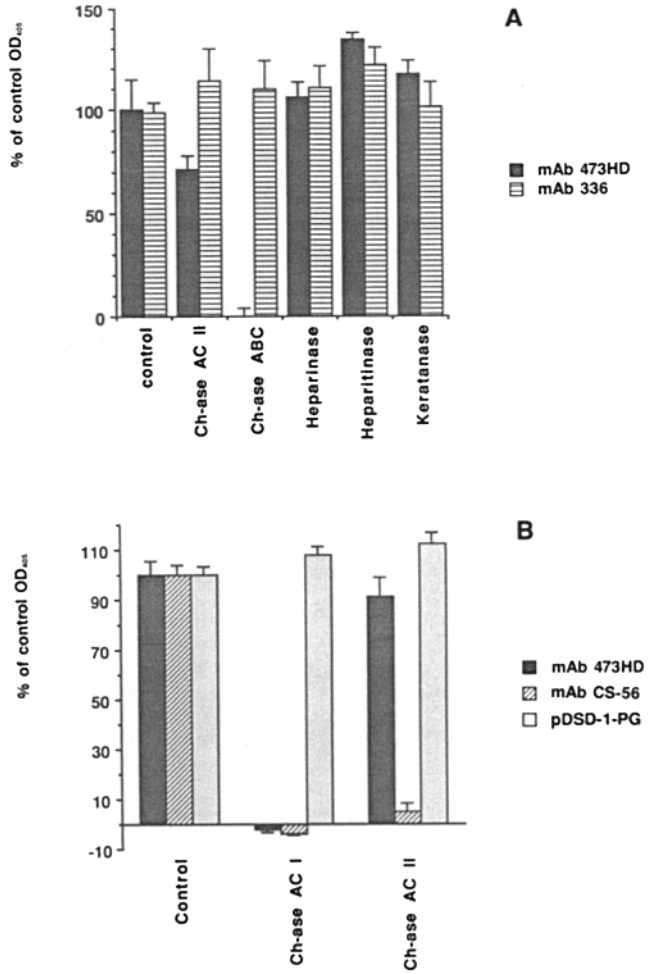

Figure 8. Sensitivity of the DSD-1 epitope to glycosaminoglycan lyases. (*A*) Purified DSD-1-PG (2 μg/ml uronic acid equivalents, 100 μl per spot) was adsorbed to nitrocellulose filters using a dot blot apparatus and digested with glycosaminoglycan lyases as indicated under each column. ELISAs were performed with mAb 473HD (10 μg/ml) and mAb 336 (specific for the L2/HNK1 carbohydrate epitope [Kruse et al., 1984], 10 μg/ml) as indicated in the figure. Note that the DSD-1 epitope is specifically removed by chondroitinase ABC, in contrast to the L2/HNK-1 epitope. (*B*) Purified DSD-1-PG (0.5 μg/ml uronic acid equivalents, 100 μl per well) was adsorbed to PVP plates. ELISA was performed with mono- and polyclonal antibodies on untreated DSD-1-PG (control conditions) or DSD-1-PG digested (test conditions) with chondroitinases ACI (0.1 unit/ml) and ACII (0.1 unit/ml) as detailed in the figure. Note that the mAb 473HD epitope is specifically removed by chondroitinase ACI but not by chondroitinase ACII, in contrast to the CS-56 epitope which is degraded by both enzymes. In all experiments (*A* and *B*) OD_{405} values obtained for each antibody on untreated DSD-1-PG (control) were set 100%. OD_{405} was determined for antibody binding after various enzyme treatments (test conditions) and expressed as %-fraction of control using the equation % of control = $OD_{405test}/OD_{405control}$. At least four measurements were performed per treatment, bars indicate standard deviations. The results of representative experiments are shown.

of mAb 473HD binding to DSD-1-PG by chondroitinase ACII may reflect that CS A or CS C sequence motives contribute, in addition to CS B, to the structure of the epitope. To evaluate this contention, the effects of chondroitinase ACI and of chondroitinase ACII, which degrade CS A and CS C by random and by stepwise attack, respectively, on mAb 473HD binding to DSD-1-PG were compared (Hiyama and Okada, 1976). Both chondroitinase ACI and ACII removed the reactivity for mAb CS-56, which is specific for CS A and CS C (Avnur and Geiger, 1984), confirming efficient removal of these GAGs from DSD-1-PG (Fig. 8 *B*). As expected, the binding of pDSD-1-PG was not affected by treatment with GAG-lyases (Fig. 8 *B*). These enzymes, however, exhibited differential effects towards the mAb 473HD-binding site in that chondroitinase ACI eliminated whereas chondroitinase ACII only slightly altered reactivity of mAb 473HD with DSD-1-PG (Fig. 8 *B*). Neither enzyme degrades intact DS polymers (Fransson and Havsmark, 1970; Schwarz et al., 1990; Gu et al., 1993). Furthermore, it is known that chondroitinase ACII is competitively inhibited by iduronic acid containing dimers, the building blocks of DS, and in particular does not cleave iduronidic linkages (Heinegard and Sommarin, 1987; Seikagaku Company, 1991).

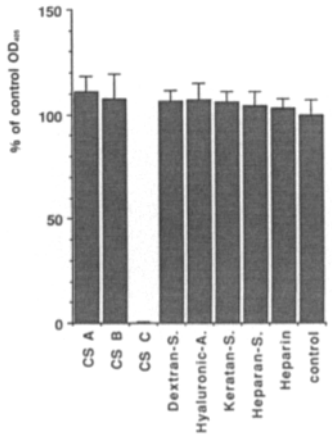

Figure 9. Competition of mAb 473HD binding by soluble GAGs. Purified DSD-1-PG (0.5 μg/ml uronic acid equivalents, 100 μl per well) was adsorbed to PVP-plates. mAb 473HD (10 μg/ml) was preincubated with GAGs (50 μg/ml) as indicated under the hatched columns and subsequently used for ELISA in the presence of the carbohydrate chains. Note that CS C competitively inhibits the binding of mAb 473HD to the DSD-1 epitope, in contrast to the other components. The % of control OD_{405} was calculated as described in legend to Fig. 7.

Thus, this result is consistent with the view that mAb 473HD binds to a CS/DS-hybrid GAG structure on DSD-1-PG. Therefore, the structure identified by mAb 473HD on the glial CNS proteoglycan was named DSD-1-epitope (for dermatan-sulfate-dependent no. 1). The conclusion that DSD-1 constitutes a GAG hybrid is supported by the inability of dermatanase, a GAG-lyase which attacks GAGs composed of DS polymers, to eliminate the DSD-1-epitope (not shown). Consistent with this result, preincubation of mAb 473HD with DS polymers did not prevent the binding of the antibody to DSD-1-PG (Fig. 9). Similarly, CS A, heparin, HS, KS or dextran sulfate did not interfere with the binding of mAb 473HD to DSD-1-PG when added as soluble competitors to the ELISA (Fig. 9). In contrast, CS C successfully suppressed the interaction of mAb 473HD with DSD-1-PG. These combined results suggest that the DSD-1-epitope is presumably created by insertion of iduronic acid into a CS C framework, probably by epimerization. This assertion is supported by experiments performed with GAGs adsorbed to nitrocellulose carriers. Indeed, mAb 473HD readily bound to immobilized CS C but did not react with CS A, CS B, KS, and dextran sulfate as determined by ELISA (not shown). mAb 473HD also reacted with CS C spotted onto nitrocellulose after digestion with chondroitinase ACII, but not with CS C degraded by chondroitinase ABC, indicating that the DSD-1-epitope might be contained in CS C preparations (not shown). It is noteworthy that the residual DSD-1-epitope accounts only for a minor portion of the GAG-moieties, because both chondroitinase ACI and ACII digestion of DSD-1-PG result in a major component of comparable molecular weight (Fig. 2), although chondroitinase ACII leaves the GAG-epitope DSD-1 intact.

DSD-1-PG Promotes Neurite Outgrowth

To gain insight into the function of DSD-1-PG, its influence on the morphological differentiation of neurons was investigated. When embryonic day 18 (E18) hippocampal neurons were grown on PORN-covered plastic conditioned with purified DSD-1-PG, the fraction of process-bearing cells increased with augmenting coating concentrations of the PG. A maximal effect on neurite outgrowth was obtained at 2.5 μg/ml uronic acid equivalents and, therefore, a saturating

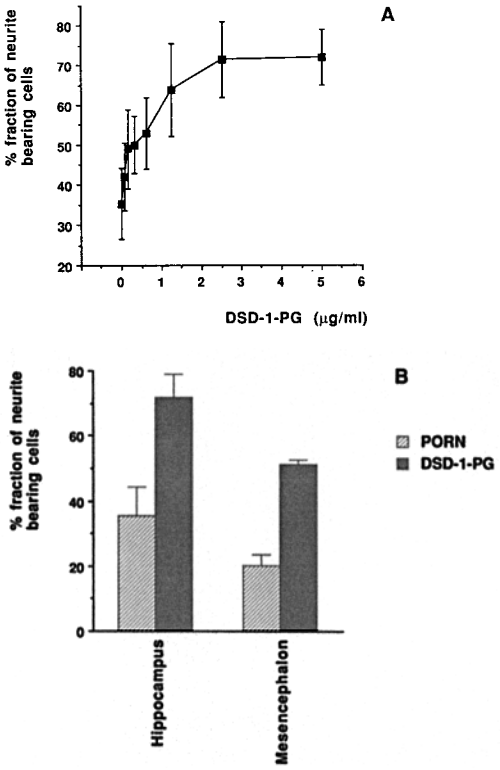

Figure 10. Concentration-dependent promotion of neurite outgrowth by DSD-1-PG. (*A*) E18 hippocampal neurons were seeded at low density in tissue culture plastic chamber slides on PORN substrates coated with DSD-1-PG at concentrations as indicated on the abscissa, fixed and stained with toluidine blue after 24 h of culture. The proportion of process bearing cells was determined as fraction of at least 100 cells per well selected at random and given in %. At least 400 neurons from at least two independent experiments were analyzed for each concentration, the curve is based on the quantitation of >5,100 neurons. (*B*) E14 mesencephalic or E18 hippocampal neurons were grown for 24 h in tissue culture plastic chamber slides on PORN or PORN coated with 5 μg/ml uronic acid equivalents DSD-1-PG, as indicated in the figure. After the culture period the neurons were fixed, stained with toluidine blue, and the fraction of process-bearing cells was determined. At least 100 neurons (n) were counted per well and at least one well was evaluated per independent experiment (N). Columns show fractions of process-bearing cells for E14 mesencephalic (control: $n > 1{,}300$, $N = 5$, DSD-1-PG: $n > 1{,}500$, $N = 9$) and E18 hippocampal (control: $n > 4{,}400$, $N = 20$, DSD-1-PG: $n > 2{,}400$, $N = 12$) neurons and bars indicate the standard deviations. For both mesencephalic and hippocampal neurons the increase of the fraction of process-bearing cells on PORN/DSD-1-PG substrates was highly significant ($p < 0.001$ according to the unpaired Student's t test).

concentration of 5 μg/ml uronic acid equivalents of DSD-1-PG was used for further experiments (Fig. 10 *A*). Under these conditions, the fraction of process bearing E14 rat mesencephalic and E18 rat hippocampal neurons was enhanced by 160 and 100%, respectively, after 24 h as compared to the PORN control without PG (Fig. 10 *B*). In addition, the process bearing neurons exhibited a more elaborate morphology and longer processes on DSD-1-PG–containing substrates than on the PORN control (Fig. 11). This impression was confirmed by the systematic morphometric analysis of process bearing neurons which demonstrated that DSD-1-

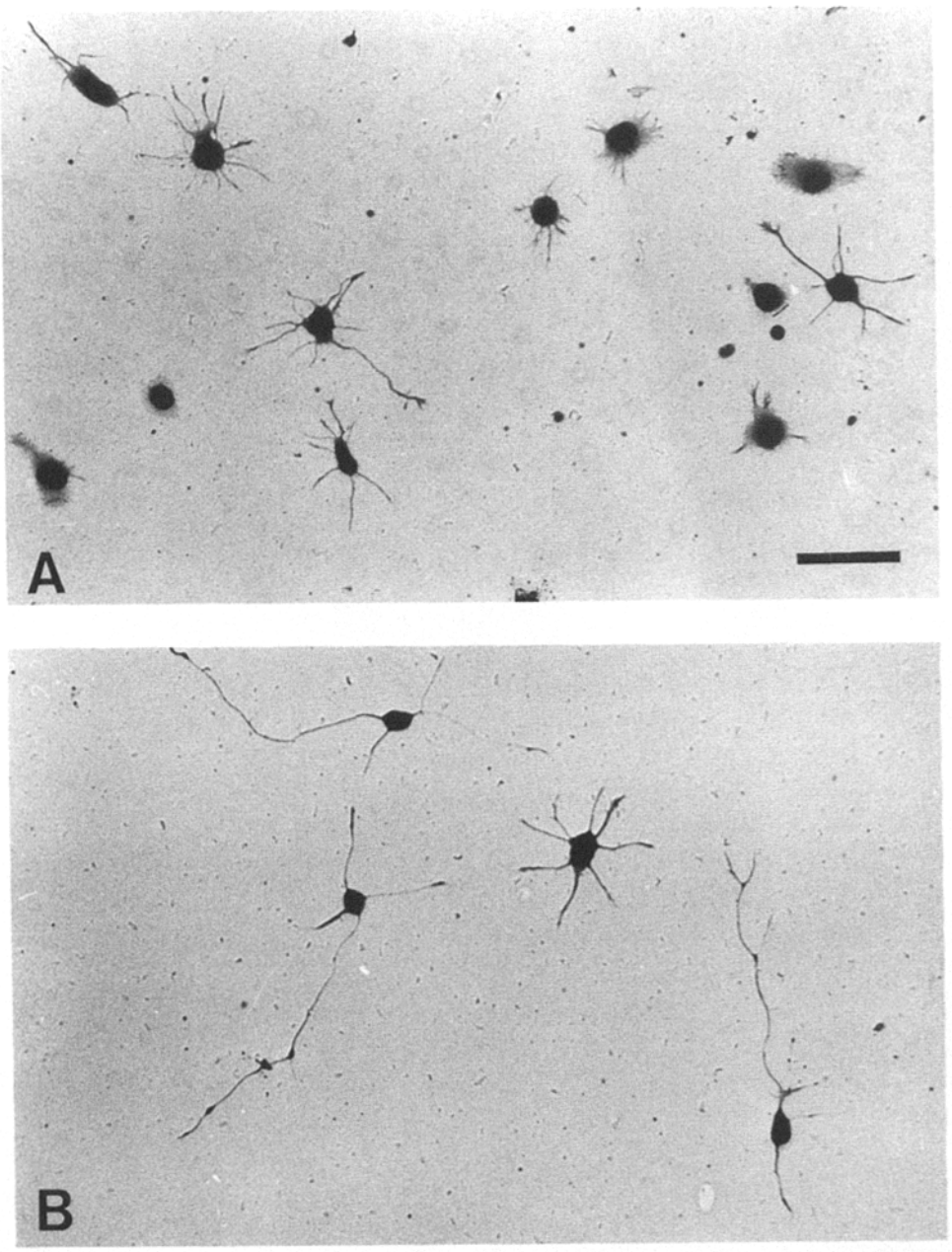

Figure 11. Differentiation of E18 hippocampal neurons on DSD-1-PG–containing polyornithine substrates. E18 hippocampal neurons were grown in tissue culture plastic chamber slides on PORN (*A*) and PORN coated with 5 μg/ml DSD-1-PG (*B*). Cells were fixed after 24 h and stained with toluidine blue. Note the increased process lengths on the PORN/DSD-1-PG substratum (*B*). Bar, 50 μm.

PG increased neurite lengths (by 65%) (Fig. 12, Table II). The stimulatory effect of DSD-1-PG on neurite elongation was strongly reduced by mAb 473HD, suggesting that the antibody reacts with a functionally important structure. To confirm that iduronic acid-containing GAGs are involved in neurite outgrowth, the DSD-1-PG/PORN culture substrate was digested with GAG-lyases prior to the addition of neurons. Under these conditions, chondroitinase ABC which cleaves, but not chondroitinase ACII which spares the DSD-1 epitope, abolished promotion of neurite elongation by DSD-1-PG (Fig. 12, Table II). The efficiency of chondroitinase ACII treatment was monitored with mAb CS-56, which confirmed removal of the majority of GAGs from the culture substrate (not shown). The neurite outgrowth promoting effect of DSD-1-PG was, like the DSD-1 epitope, resistant to heat treatment of the molecule (not shown). To exclude that the GAG-lyase or antibody treatments removed the PG, culture substrates were examined by ELISA with pDSD-1-PG antibodies after coating, after enzyme digestion prior to cell plating and at the end of the 24-h culture period. In all of these cases DSD-1-PG and/or its core glycoprotein were clearly demonstrable and no mitigation of the ELISA signal could be visualized (not shown). Thus, with respect to enzyme digestions and antibody incubations the culture substrates behaved like the carriers of the ELISAs (Figs. 3, 8, and 9). We conclude from these results that the CS/DS hybrid GAG chain DSD-1 is crucial for the neurite outgrowth and elongation promoting properties of DSD-1-PG. In view of inhibitory properties of CSPGs observed in various systems, DSD-1-PG was also examined in a repulsion assay on patterned substrates (Faissner and Kruse, 1990). When coated at 5 μg/ml uronic acid, the concentration used for the neurite outgrowth studies, no repulsive properties of DSD-1-PG were observed (not shown).

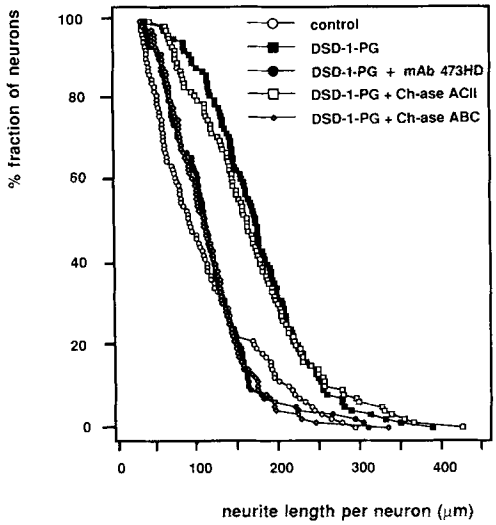

Figure 12. Length distribution of hippocampal neurites on DSD-1-PG-containing substrates. Neurites of E18 hippocampal neurons cultured as described in the legend to Fig. 10 were morphometrically analyzed and neurite lengths plotted in a frequency distribution histogram. The graph gives the relative fraction of hippocampal neurons (ordinate) with neurites longer than a given length in μm (abscissa) on different substrates as indicated in the figure. Total neurite lengths of 100 process-bearing neurons chosen at random from two wells (one experiment) were plotted for each culture condition. According to the Mann-Whitney U-test neurite length distributions on PORN, on PORN/DSD-1-PG treated with chondroitinase ABC or with mAb 473HD did not significantly differ from each other while neurite length distributions on PORN/DSD-1-PG or PORN/DSD-1-PG treated with chondroitinase ACII were statistically equivalent and clearly distinct from the first group ($p < 0.001$). Ch-ase ACII: chondroitinase ACII, Ch-ase ABC: chondroitinase ABC.

Discussion

We describe in our report that mAb 473HD identifies DSD-1-PG on the surface of young astrocytes and oligodendrocytes and can be used to isolate the CSPG from postnatal mouse brain. As salient feature, DSD-1-PG contains the hitherto undescribed CS/DS hybrid GAG structure DSD-1 which is specifically recognized by mAb 473HD. Purified DSD-1-PG promotes neurite outgrowth from embryonic CNS neurons, an effect which involves the DSD-1-epitope.

mAb 473HD Reacts with the CS/DS Hybrid Epitope DSD-1 on DSD-1-PG

The specificity of mAb 473HD has been established by Western blotting, by immunoaffinity isolation of DSD-1-PG from detergent-free physiological saline mouse brain extracts, by immunoprecipitation and by ELISA. Differential digestion of DSD-1-PG with GAG-lyases revealed that the epitope recognized by mAb 473HD is sensitive to chondroitinases ABC and ACI and resistant to chondroitinase ACII. The latter enzyme is competitively inhibited by DS with a Ki of 6.1×10^{-5} M (as moles of repeating disaccharide units) (Hiyama and Okada, 1977). Selective resistance to chondroitinase ACII and sensitivity to chondroitinase ABC can be used as operational criterium for the identification of DS-motives (Heinegard and Sommarin, 1987; Schwarz et al., 1990; Seikagaku Company, 1991; Gu et al., 1993). Therefore, we propose DSD-1 as a name for the epitope (for dermatan-sulfate-dependent no. 1). Alternatively, other biochemical modifications such as galactosaminoglycan-containing GAG sequences would have to be assumed (Hascall et al., 1972; Rauch et al., 1986). At present, it is not known whether these chemical modifications are selectively distinguished by chondroitinases ACI and ACII (Hiyama and Okada, 1976; Heinegard and Sommarin, 1987) and KS motives could not be identified in DSD-1-PG. For these reasons, the hypothesis that the mAb 473HD-binding site involves DS motives seems the most plausible at present, although a more precise assessment will require thorough and detailed structural investigations. Interestingly, chondroitinase ACII degrades GAGs in a stepwise manner and might be blocked by DS segments in the vicinity of the core glycoprotein. These might still be accessible to chondroitinase ACI which proceeds by multiple random attack (Hiyama and Okada, 1976). Digestion of DSD-1-PG with chondroitinases ABC or ACII results in major glycoprotein cores with similar molecular mass. This could indicate that the re-

Table II. Quantitative Analysis of Neurite Lengths on DSD-1-PG/PORN Substrates

Substrate	Neurite length of 50 cells μm	SD μm	SE μm	% stimulation as compared to control	% inhibition as compared to DSD-1-PG	Experiments N	Cells n
PBS (control)	4774	1095	234	–	–	12	1100
DSD-1-PG	7749	1729	397	64.7 ± 18.0*	–	12	950
DSD-1-PG + mAb 473HD	5658	1403	389	15.3 ± 10.1‡	76.6 ± 13.6*	7	650
DSD-1-PG + Ch-ase ACII	7132	1071	378	61.5 ± 13.5*	−5.3 ± 22.5 (ns)	5	400
DSD-1-PG + Ch-ase ABC	4872	1051	350	8.8 ± 14.2 (NS)	85.4 ± 24.6*	5	450

Hippocampal neurons were cultured under different conditions and morphometrically analyzed. Since neurite lengths were not normally distributed, values for 50 process-bearing neurons per individual well were collected at random and summed. At lease 400 neurons from at least five independent experiments were sampled. The average values and corresponding standard deviations of the single sums and the % increase or decrease of summed neurite lengths within independent experiments were calculated. The significance of the difference between the mean values of summed neurite lengths was estimated with the Mann-Whitney U-Test and is indicated next to the % stimulation of neurite lengths of the % inhibition of DSD-1-PG effect. Percentages were calculated as % stimulation $S = (L_{test} - L_{control})/L_{control}$ and % inhibition of DSD-1-PG effect $I = (S_{test} - S_{DSD\text{-}1\text{-}PG})/S_{DSD\text{-}1\text{-}PG}$. Ch-ase AC and ABC, chondroitinase AC or ABC, respectively; SE, standard error of the mean; N, number of independent experiments; n, number of single cells; and L, total neurite length of 50 neurons.
* $0.0001 < p < 0.001$.
‡ $0.01 < p < 0.05$.

sidual DSD-1-epitope is located close to the core of DSD-1-PG. The failure of dermatanase which requires a motive composed of several iduronic acid constituents to remove, the inability of purified DS to compete for the mAb 473HD-binding site, and the fact that chondroitinases ACI and ACII do not cleave pure DS imply that DSD-1 is a hybrid structure (Fransson and Havsmark, 1970; Michelacci and Dietrich, 1975). To our knowledge, 473HD is the first mAb with this reactivity profile. This might explain why the DSD-1-epitope shows a restricted expression while CSPGs have been found on the surface of most cell types, including neurons (Zaremba et al., 1989; Lander, 1993, for review). Systematic comparison of pDSD-1-PG and mAb 473HD by double immunofluorescence and sequential immunoprecipitation experiments performed on postnatal mouse cerebellar cultures demonstrated that the DSD-1-epitope is present on the major fraction and not only a subset of DSD-1-PG. DSD-1-PG migrates as a single peak in ion exchange and size exclusion chromatography and contains a prominent glycoprotein of molecular mass 350–400 kD upon removal of its GAG constituents. Polyclonal antibodies generated against DSD-1-PG react with polydisperse material of 1,000-kD molecular mass both in Western blotting and in immunoprecipitation experiments and detect a prominent band of 350–400 kD in CNS extracts digested with chondroitinase ABC, consistent with the properties of purified DSD-1-PG. No substantial additional bands were detected by various extraction techniques, yet the existence of further components not accessible to our procedures cannot be excluded. Therefore, the precise determination of DSD-1-PG glycoprotein core(s) and their potential variants will require molecular cloning of the PG.

DSD-1-PG Promotes Neurite Outgrowth

To obtain insight into the potential function of DSD-1-PG, we have cultured E14 mesencephalic and E18 hippocampal neurons at low density on DSD-1-PG/PORN substrates. Under these conditions, DSD-1-PG promoted neurite outgrowth in that the fraction of process-bearing neurons and total neurite lengths were augmented by 100 and 65%, respectively, in comparison to neurons growing on PORN controls. The neurite outgrowth promoting effect was resistant to heat treatment and nearly reduced to control levels by addition of mAb 473HD to the culture medium, suggesting that the DSD-1 hybrid GAG structure is involved. This conclusion was confirmed by differential digestion of the culture substrate where chondroitinase ACII spared while chondroitinase ABC removed both the DSD-1 epitope and the neurite outgrowth promoting property of DSD-1-PG. It is unlikely that the effect is caused by neutralization of the polycationic poly-DL-ornithine culture substrate because elimination of most of the negatively charged GAG chains by chondroitinase ACII does not mitigate neurite outgrowth promotion. In view of its expression, DSD-1-PG might thus contribute to the influence of immature astrocyte surfaces on the shaping of neuronal assemblies and fiber extensions (Rakic, 1988). Support of neurite growth by PGs has already been documented. For example, HS, DS, and CS GAGs potentiate neurite outgrowth by PC12 cells upon exposure to aFGF, bFGF, and NGF (Damon et al., 1988), HS, DS, and CS GAGs induce neurite outgrowth and in part stimulate the development of polarity in E14 mesencephalic neurons (Lafont et al., 1992) and the combined core proteins of three soluble postnatal rat brain CSPGs promote neurite outgrowth from E16 rat embryonic neurons (Iijima et al., 1991). The neurite growth promoting properties described in these publications and our study contrast, however, with inhibitory influences of CS GAGs or CSPGs on neurite growth reported by other groups. For example, embryonic chick DRG neurites do not grow out in a three-dimensional HEMA-gel culture system containing heparin, CS, and hyaluronic acid (Carbonetto et al., 1983), avoid DS, CS, and hyaluronic acid adsorbed to collagen type I (Verna et al., 1989) and spots of a CS/KSPG isolated from bovine cartilage on a plain laminin or N-CAM substrate (Snow et al., 1990*b*). Similarly, the developmentally regulated KSPG claustrin from embryonic chicken brain inhibits the growth of E12 optic lobe neurites from laminin-containing into laminin/claustrin-coated areas, an effect probably involving the KS chains (Snow et al., 1990*b*; Cole and McCabe, 1991). In embryonic chick epidermis, a CSPG prevents embryonic chick DRG neurite ingrowth into epidermal explants in an in vitro coculture system and antibodies to CS neutralize this effect (Verna, 1985; Fichard et al., 1991). Another report has attributed inhibition of neurite outgrowth by CSPGs to the core glycoproteins rather than to the GAG moieties (Oohira et al., 1991). The repulsive effects of GAGs have mainly been observed in choice situations where neurites were confronted with favorable substrates, e.g., collagens, laminin, and N-CAM on the one side and areas of the culture dish containing GAGs and/or PGs in addition on the other side. There are precedents that molecules displaying repulsive or inhibitory activities in choice situations may permit, or even enhance neurite growth or cell migration as homogeneous substrates, for example tenascin (Crossin et al., 1990; Faissner and Kruse, 1990; Wehrle and Chiquet, 1990; Lochter et al., 1991; Taylor et al., 1993; reviewed in Faissner, 1993; Faissner and Schachner, 1994) or laminin (Calof and Lander, 1991). Whether this is also true of DSD-1-PG will have to be studied. Our immunofluorescence double-labeling studies with different glial cell markers have established that DSD-1-PG is detectable on the surfaces of immature astrocytes and oligodendrocytes and down-regulated with maturation in both lineages. Consistent with this conclusion, preliminary studies have localized DSD-1-PG to glial boundaries, e.g., in the developing somatosensory cortex of the mouse (Steindler et al., 1990).

Relationship of DSD-1-PG to Other PGs

Judged by biochemical criteria, DSD-1-PG could be included in the soluble CSPG fractions of rat brain or in the PG core glycoproteins expressing the L2/HNK-1 carbohydrate epitope (Kiang et al., 1981; Klinger et al., 1985; Oohira et al., 1988; Gowda et al., 1989; Herndon and Lander, 1990; Rauch et al., 1991). It is unlikely that the neurite outgrowth promoting CSPGs recently described in rat CNS are related to DSD-1-PG because these express mostly CS A (80%) and CS C (20%), but not DS and do not contain a comparable core protein (Oohira et al., 1988; Iijima et al., 1991). Neurocan, which also expresses the L2/HNK-1 epitope, has a core glycoprotein of 150 kD, distinct from DSD-1-PG (Rauch et al., 1991, 1992; Grumet et al., 1993). DSD-1-PG differs also from the embryonic chick brain cytotactin-binding PG or from CAT-301 because these CSPGs are expressed by neurons, unlike DSD-1-PG which

is expressed on glial surfaces (Hoffman and Edelman, 1987; Hoffman et al., 1988; Zaremba et al., 1989). DSD-1-PG seems also distinct from the Tl antigen, a CSPG with a major core glycoprotein of 300 kD, because Tl requires denaturing conditions for purification and is barely contained in salt extracts from rodent brain, in contrast to DSD-1-PG (Iwata and Carlson, 1993). In terms of molecular weight of the core glycoprotein and of cellular specificity, DSD-1-PG is reminiscent of the CSPG NG2 (Stallcup and Beasley, 1987; Stallcup et al., 1990; Nishiyama et al., 1991). Yet polyclonal anti-NG2 antibodies do not cross-react with DSD-1-PG both before and after treatment with GAG-lyases (Streit, A., W. Stallcup, A. Faissner, M. Schachner, unpublished observations). Finally, DSD-1-PG is also distinguishable from the CSPG astrochondrin (Streit et al., 1990, 1993) which is expressed by mature, GFAP-positive astrocytes and not by oligodendrocytes, while DSD-1-PG shows a nearly reverse distribution pattern. In support of this notion, astrochondrin does not promote neurite outgrowth (Streit et al., 1993) and is hence also functionally distinct from DSD-1-PG. It is interesting that mAb 473HD detects a CS C-epitope that is sensitive to both chondroitinases ABC and ACI in human peripheral nerve proteoglycan fractions which are immunologically cross-reactive with versican and decorin antibodies (Braunewell, K.-H., R. Martini, R. LeBaron, H. Kresse, A. Faissner, B. Schmitz and M. Schachner, manuscript in preparation). However, versican so far has not been detected during embryonic CNS development and has been reported to be produced by fibroblasts, while DSD-1-PG is present in E13 mouse CNS and barely detectable in primary fibroblast cultures (Faissner, A., A. Clement, unpublished observations; Zimmermann and Ruoslahti, 1989; Bignami et al., 1993). In contrast, versican has recently been documented in peri- and postnatal rat brain (Bignami et al., 1993). The available data relating to GAG composition, molecular weight of the core glycoprotein(s), distribution, and function are compatible with the possibility that DSD-1-PG might be related to versican and represent a novel member of the growing aggrecan family (reviewed by Lander, 1993). The more precise evaluation of possible similarities to and differences from other CSPGs will require information about the primary structure of DSD-1-PG.

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