A Phosphatidylinositol-linked Peanut Agglutinin-binding Glycoprotein in Central Nervous System Myelin and on Oligodendrocytes

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Abstract. Here we report the isolation and initial biochemical characterization of a 120-kD peanut agglutinin-binding glycoprotein from the adult human central nervous system (CNS), which is anchored to membranes through a phosphatidylinositol linkage. Myelin incubated with phosphatidylinositol-specific phospholipase C released the protein as a soluble polypeptide of 105 kD, which was isolated with peanut agglutinin-agarose affinity chromatography. The protein was found to be highly glycosylated. The protein appears to be confined to the CNS, where its developmental expression is region specific and parallels myelination. It is in greater quantity in white matter than in gray matter and it is in isolated human CNS myelin. Furthermore, ovine oligodendrocytes in culture contain the protein on their surfaces and release it into the supernatant as a soluble 105-kD form. We call this protein the oligodendrocyte-myelin protein.

In the central nervous system (CNS)[1] it is likely that myelination calls for complicated interactions between oligodendrocytes and axons (Bunge et al., 1978). The chemical nature of these interactions is still largely unknown. There are, however, hypotheses about limited aspects of these interactions that may or may not be supported by circumstantial evidence. It has for example been postulated that the myelin-associated glycoprotein may mediate interactions between axons and myelin-forming cells (Quarles, 1984), and that a protein called gp 150/225 may do the same (Gulcher et al., 1986).

One approach to understanding the molecular basis of those oligodendrocyte-axon interactions that lead to myelination is to examine CNS molecules that appear at the time of myelination. Many of these molecules are likely to be only structural components of myelin but some may also (Quarles, 1984) or only (Gulcher et al., 1986) participate in the process of myelination. While the protein composition of myelin has been extensively studied and is relatively simple, with a few proteins accounting for most of the total protein, the existence of additional quantitatively minor proteins is well recognized (Lees and Brostoff, 1984). The functions of these minor proteins are not known, but they are reasonable candidates for molecules that mediate interactions between oligodendrocytes and axons.

It is generally accepted that the carbohydrate components of glycoproteins grant their polypeptides unique properties, some of which may be of functional significance (Sharon and Lis, 1982). Carbohydrate moieties may also serve as markers for proteins having certain functions. For example, the HNK-1/L2 carbohydrate is on the myelin-associated glycoprotein (McGarry et al., 1983), the neural cell adhesion molecule (N-CAM) (Kruse et al., 1984), the L_1 glycoprotein (Kruse et al., 1984), the J_1 glycoprotein (Kruse et al., 1985), cytotactin (Grumet et al., 1985), the neuron-glia cell adhesion moelcule (Tucker et al., 1984; Thiery et al., 1985), and gp 150/225 (Gulcher et al., 1986), all of which are likely to play important roles in cell adhesion.

In our previous work, we studied developmental alterations in the sizes of glycoproteins in the human CNS that bind certain lectins, hoping to establish that morphological changes are accompanied by specific changes at the molecular level (Mikol et al., 1988). One of the developmental changes we observed was the appearance of a 120-kD peanut agglutinin (PNA)-binding glycoprotein at the time of myelination. This protein proved to be present in CNS myelin. Here we describe the isolation and characterization of this protein.

Materials and Methods

Tissue Samples

Human frontal lobes, cerebella, and thoracic spinal cords were obtained at autopsies performed for diagnostic purposes, <24 h after death on individuals of varying ages, all without gross or histologic evidence of neurologic disease. CNS myelin was isolated by the method of Norton and Poduslo (1973), both from human autopsy material and from fresh ovine brains obtained from a local vendor. Human peripheral nerves, livers, kidneys, and spleens were also obtained at autopsies.

1. *Abbreviations used in this paper*: CNS, central nervous system; N-CAM, neural cell adhesion molecule; OM protein, oligodendrocyte-myelin protein; PC, phosphatidylcholine; PI, phosphatidylinositol; PLC, phospholipase C; PNA, peanut agglutinin.

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Oligodendrocyte-Myelin (OM) Protein Isolation

All steps in the isolation procedure were performed at 4°C, with the exception of the phospholipase C (PLC) incubation. Hemispheric white matter was dissected away from gray matter, homogenized 1:20 (wt/vol) in buffer (20 mM triethanolamine, 0.15 M NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.5), and centrifuged at 100,000 *g* for 30 min. The pellet and the supernatant were used in separate isolation pathways. The 100,000 *g* pellet was washed twice, and then preincubated with shaking at 37°C for 1.5 h, whereafter it was spun down and washed twice. The pellet was again homogenized 1:20 (wt/vol), incubated with 0.5 U/ml *Bacillus cereus* PLC (type III; Sigma Chemical Co., St. Louis, MO) for 3.5 h, and centrifuged at 100,000 *g* for 30 min. The PLC supernatant contained the released OM protein. At this point the isolation procedures of the OM protein from the PLC supernatant and the original white matter supernatant were identical. The OM protein was precipitated with ammonium sulfate at a concentration between 33 and 67% of saturation. The pellet was dissolved and dialyzed to equilibrium against buffer. A concentrated 1,000× stock of 0.1 M $CaCl_2/MnCl_2/MgCl_2$ was added to the dialysate which was then incubated for 1 h with PNA-agarose beads (Vector Laboratories, Inc., Burlingame, CA). The beads were packed in a disposable gravity column, washed with high salt buffer (20 mM triethanolamine, 2 M NaCl, 1 mM EDTA, 0.5 mM PMSF, pH 7.5) and finally eluted with the same buffer plus 0.5 M D-galactose. D-Galactose was removed from the eluates by dialysis and the dialysate was subjected to a second PNA affinity extraction. The final eluates contained highly purified OM protein.

Amino Acid Sequencing

The NH_2-terminal sequence of the OM protein was determined by automated Edman degradation using a gas-phase sequenator (model 470A; Applied Biosystems, Inc., Foster City, CA) with an online amino acid phenylthiohydantoin analyzer. Quantitation was done with a Nelson Analytical (Cupertino, CA) system.

SDS Electrophoresis, Immunoblots, and Lectin Binding

Protein samples were quantified with a Protein Assay (Bio-Rad Laboratories, Richmond, CA). Molecular mass standards were also from Bio-Rad Laboratories. Polypeptides were subjected to electrophoresis with the method of Fairbanks et al. (1971) using 5.6% gels, or as described by Laemmli (1970) using 7.5 or 10% gels after solubilization in 5.7 M urea, 1% (wt/vol) SDS, 1% (vol/vol) 2-mercaptoethanol, and heating at 100°C for 3 min. Gels were either directly stained with silver nitrate (Merril et al., 1984) or the polypeptides were electrically transferred from the gels onto nitrocellulose sheets using a Transblot TM cell (Bio-Rad Laboratories) at 60 V for 3 h in 20 mM Tris-HCl buffer (pH 7.5) with 20% (vol/vol) methanol (Towbin et al., 1979). The nitrocellulose was quenched overnight with 5% (wt/vol) BSA in 0.15 M NaCl-0.05 M Tris-HCl buffer at pH 7.6 (TBS). The nitrocellulose was then washed for 15 min before a 1-h incubation with either rat polyclonal antibodies against the OM protein, polyclonal rabbit antibodies against N-CAM, the HNK-1 mouse monoclonal antibody, or PNA (50 µg/ml; Vector Laboratories, Inc.). In all cases except for PNA staining, 0.15 M NaCl-0.05 M Tris-HCl (pH 7.6) containing 0.05% (vol/vol) P-40 (Particle Data, Inc., Elmhurst, IL) and 5% (vol/vol) normal goat serum was used for all washes and dilutions, whereas 0.5% (wt/vol) BSA was substituted for goat serum in washes and dilutions for PNA staining. Sheets incubated with antibodies were washed three times for 15 min each, incubated for 1 h with peroxidase-labeled antibodies against rat, rabbit, or mouse immunoglobulins (Dako Corp., Santa Barbara, CA), and then washed three times again. Alternatively, the PNA-exposed strips were washed three times and incubated with goat anti-PNA antibodies (10 µg/ml; Vector Laboratories, Inc.), and then washed three times and incubated for 1 h with peroxidase-labeled rabbit anti-goat immunoglobulins (Dako Corp.), followed by three washes. Conjugates were used at 1:100. The sheets were developed in 0.05% (wt/vol) diaminobenzidine tetrahydrochloride and 0.01% (vol/vol) H_2O_2 in 0.15 M NaCl-0.05 M Tris-HCl (pH 7.6). Various concentrations of α-methyl-D-mannoside (Sigma Chemical Co.), D-glucose (Vector Laboratories, Inc.), or D-galactose (Vector Laboratories, Inc.) were tested for their ability to block PNA binding. The monosaccharides were present with PNA during the primary incubation period and during the first two of three subsequent washes.

Antibodies

The OM protein (15 µg) was emulsified in complete Freund's adjuvant and injected into the footpads of female Lewis rats for each of three injections over 2 mo. Antibodies specific for the polypeptide backbone of the OM protein were affinity purified from deglycosylated protein on nitrocellulose using a previously described method (Olmsted, 1981). After absorption with isolated OM protein, the affinity-purified antibodies reacted with nothing on immunoblots of brain homogenates and gave no staining in immunohistochemistry. A hybridoma producing anti-HNK-1 antibody was obtained from the American Type Tissue Collection (Rockville, MD) and polyclonal antibodies against N-CAM were generously provided by Dr. Urs Rutishauser of Case Western Reserve University, Cleveland, OH.

Enzymatic Treatments

Phospholipases. Preparative release of the OM protein using *B. cereus* PLC (type III; Sigma Chemical Co.) was described above in the isolation method. To inhibit the phosphatidylcholine (PC)-specific PLC in this preparation, 5 mM *o*-phenanthroline (Sigma Chemical Co.) was used (Little, 1981), while 5 mM Zn^{++} was included to inhibit the phosphatidylinositol (PI)-specific PLC (Ikezawa and Taguchi, 1981). A pure PI-specific PLC from *Bacillus thuringiensis* (generously provided by Dr. Martin G. Low, Columbia University, New York) was used at 50 mU/ml in 50 mM Tris-maleate (pH 7.4). Highly purified PC-specific PLC from *B. cereus* was also used at 10 U/ml (type I; Boehringer Mannheim Diagnostics, Inc., Houston, TX).

Glycanases. Enzymatic cleavage of both complex and high-mannose N-linked glycans by Endoglycosidase F (endo-β-*N*-acetylglucosaminidase F; New England Nuclear, Boston, MA) or only of high-mannose N-linked chains by Endoglycosidase H (endo-β-*N*-acetylglucosaminidase H; New England Nuclear) was done as described (Elder and Alexander, 1982; Tarentino and Maley, 1974) at 37°C for 12 h using 5 µg of OM protein and 0.5 U enzyme in 100 µl total vol. Neuraminidase (type X; Sigma Chemical Co.) digestion (1 U/ml) was performed in 50 mM sodium acetate (pH 5.1) at 37°C for 4 h. For complete deglycosylation, the OM protein was first treated with Endoglycosidase F as above in 100 mM sodium phosphate (pH 6.0), followed by the addition of an equal volume of 2.5% (vol/vol) NP-40

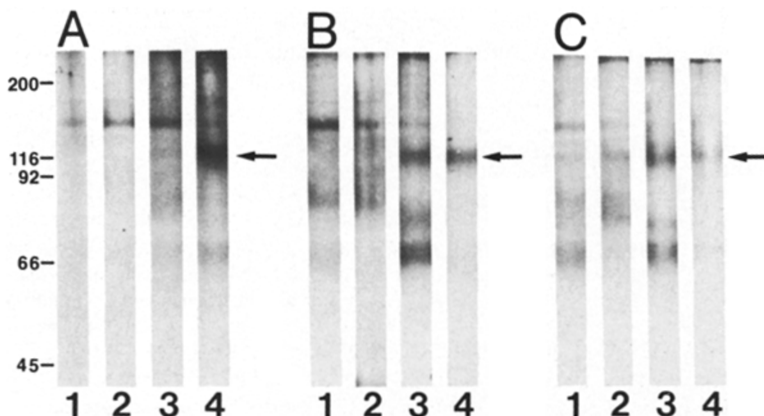

Figure 1. PNA affinity staining of developmental CNS samples. Tissue homogenates from (*A*) hemisphere, (*B*) cerebellum, and (*C*) spinal cord at various ages were separated on a Fairbanks 5.6% gel: (lanes *1*) 29-wk fetus; (lanes *2*) 37-wk fetus; (lanes *3*) 7 mo after birth; and (lanes *4*) adult. Numbers indicate the molecular mass standards and the arrows point to the OM protein: 200 kD, myosin; 116 kD, β-galactosidase; 92 kD, phosphorylase b; 66 kD, BSA; 45 kD, ovalbumin. Compare the varying temporal appearance of the OM protein in each tissue. The OM protein migrates somewhat more slowly than the 116-kD marker.

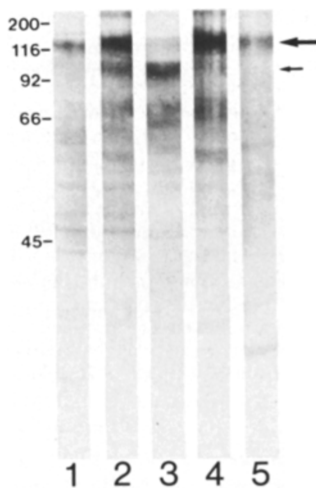

Figure 2. PNA binding to fractionated CNS tissues. Equal amounts of total protein from (lane *1*) crude gray matter, (lane *2*) crude white matter, (lane *3*) the 100,000 *g* supernatant from white matter, (lane *4*) the 100,000 *g* pellet from white matter, and (lane *5*) isolated myelin were separated on a 10% Laemmli gel, transferred to nitrocellulose, and stained with PNA. Numbers indicate molecular mass standards. The presence of two bands at 120 kD (*large arrow*) and 105 kD (*small arrow*) which partition into the pellet (120-kD band) or supernatant (105-kD band) is apparent. The OM protein is present in greater amount in white matter than in gray matter and there is more in the white matter pellet than in myelin.

in 50 mM Tris-maleate (pH 5.6) and incubation with neuraminidase for 4 h. The final step was cleavage of 0-linked glycans using 50 mU/ml of *O*-glycanase (endo-α-*N*-acetylgalactosaminidase; Genzyme Corp., Boston, MA) for 12 h.

Oligodendrocyte Isolation and Culture

Oligodendrocytes were isolated from ovine white matter and cultured as previously described (Szuchet et al., 1980). The cultures used for the studies described here contained only (>98%) cells that stained with antibodies against galactocerebroside.

Immunochemistry and Immunohistochemistry

Sections (6 μm) of formalin-fixed paraffin-embedded blocks from human autopsy material and ovine brains were stained with the rat anti-OM protein polyclonal antiserum (1:100), preimmune rat serum (1:40), or affinity-purified polyclonal antibodies against the OM protein. Sternberger's peroxidase-antiperoxidase method (Sternberger, 1979*b*) was used. Rat peroxidase-antiperoxidase complexes were obtained from Sternberger-Meyer Immunocytochemicals Inc. (Jarretsville, MD). The cultured ovine oligodendrocytes were stained with the same antibodies as well as with antibodies against galactocerebroside (generously provided by Dr. Joyce Benjamins, Wayne State University) using indirect immunofluorescence (Sternberger, 1979*a*).

Results

Identification

While studying the lectin-binding patterns of polypeptides from human CNS tissue homogenates that had been subjected to electrophoresis (Mikol et al., 1988), we observed an intensely staining 120-kD PNA-binding polypeptide, which we now call the OM protein (Fig. 1). The OM protein was clearly present in the adult spinal cord, cerebellum, and hemisphere, but showed regional variation in its developmental expression. It was detected in fetal spinal cord at 29 wk of gestation and in cerebellum at 37 wk of gestation, but only a small amount was detected in the cerebral hemisphere 7 mo after birth. This expression pattern is similar to that seen for the myelin-associated glycoprotein (Marton and Stefansson, 1984) and correlates temporally with myelination. There was no OM protein detected on immunoblots containing polypeptides from homogenates of peripheral nerve, liver, kidney, or spleen (data not shown).

There is more of the OM protein in white matter than gray matter and it is present in myelin, though in lesser amount than in a white matter membrane preparation (Fig. 2), indicating that the OM protein is not concentrated in compact myelin. A portion of the OM protein (105-kD form) clearly partitions in the 100,000 *g* supernatant of a white matter homogenate, whereas the bulk remains in the pellet (120-kD form) and can be solubilized in 1% NP-40 (not shown). The

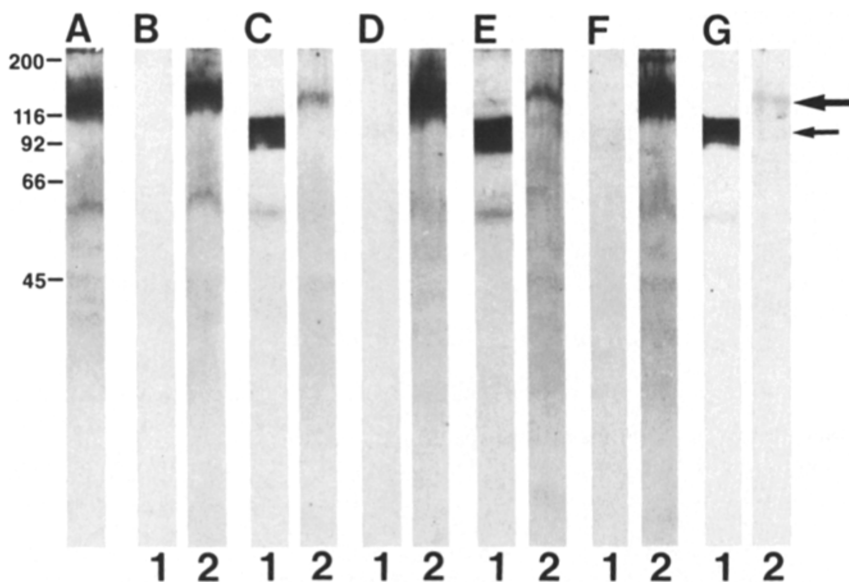

Figure 3. Phospholipase C cleavage of the OM protein. White matter pellet was homogenized to 50 mg/ml (wet wt). Equal aliquots of (*A*) this homogenate and of the (lanes *1*) 100,000 *g* supernatant and (lanes *2*) 100,000 *g* pellet of each incubation (*B–G*) were solubilized for SDS-PAGE on a 10% Laemmli gel, and subsequent PNA staining. (*B*) Incubation without enzyme; (*C*) incubation with 100 mU/ml Sigma *B. cereus* PLC alone (type III); (*D*) incubation in the presence of 5 mM Zn^{++}; or (*E*) 5 mM *o*-phenanthroline; (*F*) incubation with 10 U/ml Boehringer Mannheim *B. cereus* PLC (type I); (*G*) incubation with 50 mU/ml *B. thuringiensis*. Incubation without enzyme or with highly purified *B. cereus* PLC (Boehringer Mannheim) (PC-specific) does not release the OM protein, whereas a cruder preparation of *B. cereus* PLC (Sigma Chemical Co.) (PC-specific plus PI-specific) or purified *B. thuringiensis* PLC (PI-specific) cleaves most of the membrane-bound OM protein. Zn^{++}, an inhibitor of the PI-specific PLC of *B. cereus*, blocks release of the OM protein while *o*-phenanthroline, an inhibitor of the PC-specific PLC of *B. cereus*, does not. The molecular mass difference between the two forms of the OM protein is readily apparent on this gel system (120-kD form, *large arrow*; 105-kD form, *small arrow*). Numbers indicate molecular mass standards.

Figure 4. Purification of the OM protein. (*A*) Silver and (*B*) PNA staining of the OM protein isolation steps from white matter pellet, separated on a 7.5% Laemmli gel. The 100,000 *g* supernatant of white matter pellet incubated (lanes *1*) without enzyme shows no OM protein, while the 100,000 *g* supernatant of the sample incubated (lanes *2*) with 100 mU/ml *B. cereus* PLC contains the released Om protein, which is then concentrated by (lanes *3*) ammonium sulfate precipitation, and further purified in the 0.5 M D-galactose eluates of the (lanes *4*) first and (lanes *5*) second PNA–agarose extractions. Silver stain of the final product shows that the OM protein (*arrows*) can be purified to near homogeneity from the pellet (*A*, lane *5*) of a white matter homogenate. Numbers indicate molecular mass standards for the silver-stained gel.

membrane-bound form may also be released from the membrane (as a soluble 105-kD form) by incubation with PI-specific PLC (Fig. 3). A relatively pure PI-specific PLC from *B. thuringiensis* releases the OM protein very effectively as does a crude PLC preparation from *B. cereus* (Sigma Chemical Co.) which contains both a PI-specific PLC and a PC-specific PLC (Sundler et al., 1978*a*, *b*). Zn^{++}, an inhibitor of the PI-specific PLC of *B. cereus* (Ikezawa and Taguchi, 1981), blocks release of the OM protein, while *o*-phenanthroline, an inhibitor of the PC-specific PLC (Little, 1981) does not. Furthermore, a highly purified *B. cereus* PC-specific PLC (Boehringer-Mannheim Diagnostics, Inc.) is without effect.

Purification

Because the OM protein partitions in the supernatant as well as the pellet of a white matter homogenate, both fractions were used for purification. When the pellet was used as starting material, the OM protein was isolated to near homogeneity by two sequential PNA affinity extractions of a PLC supernatant which had first been concentrated by ammonium sulfate precipitation (Fig. 4). In contrast, isolation of OM protein from a white matter supernatant using the same approach yielded, in addition to the OM protein, a 65-kD polypeptide. In both cases, PNA affinity chromatography was a key step in the isolation procedure. The presence of 2 M NaCl in the wash buffer inhibits the nonspecific binding of most proteins to PNA-agarose but does not affect binding of the OM protein. PNA has a marked affinity for the OM protein, which is eluted specifically by concentrations of D-galactose as low as 50 mM (not shown). D-Galactose, but not D-glucose or α-methyl-D-mannoside, blocked binding of PNA to the OM protein on nitrocellulose. The purified protein did not bind polyclonal antibodies against N-CAM, nor did the affinity-purified anti–OM protein antibodies bind to polypeptides with the relative mobilities of the larger two N-CAMs in brain homogenates. It is also of importance that the OM protein does not contain the HNK-1 epitope (not shown).

Amino Acid Sequencing

Only one major sequence was obtained and there was good correlation between the amount of protein sequenced and the yield of amino acids. The NH_2-terminal amino acid sequence of human OM protein isolated to pure form as in Fig. 4 (*lanes 5*) is shown in Fig. 5. Search of the National Biomedical Research Foundation computer data base did not reveal a striking sequence similarity (Reeck et al., 1987) to any hitherto described protein.

Biochemical Characterization

The OM protein isolated as the soluble 105-kD polypeptide was subjected to several enzymatic treatments in order to assess its carbohydrate composition (Fig. 6). Neuraminidase cleavage of sialic acids resulted in a 95-kD polypeptide, and treatment with Endoglycosidase H or Endoglycosidase F yielded polypeptides of 90 or 80 kD, respectively. In all three cases, PNA binding was unaffected, which suggested the presence of O-linked carbohydrate. It is of interest here that the saccharide structure for which PNA is known to have the highest affinity (gal [β1-3]galNAc) usually resides on O-linked glycans (Pereira et al., 1976). When Endoglycosidase F cleavage of N-linked glycans was followed by incubation with neuraminidase and finally with *O*-glycanase, a 75-kD polypeptide was obtained that did not bind PNA.

Immunohistochemical Staining

Affinity-purified polyclonal rat antibodies to human OM protein stained the myelin sheaths on tissue sections from formalin-fixed paraffin-embedded human and ovine CNS (Fig. 7, *A* and *B*). There was no staining of peripheral ner-

5 10 15 20 25
Ile-Cys-Pro-Leu-Gln-(His)-Ile-(His)-Thr-Glu-Arg-His-Arg-His-Val-Asp-Cys-Ser-Gly-Arg-Asp-Leu-Ser-Thr-(Leu)-Pro-Pro-Gly

Figure 5. NH_2-terminal sequence of the OM protein. The first 28 amino acid residues of the OM protein were determined by automated Edman degradation using a gas-phase sequenator and analysis of phenylthiohydantoin derivatives. Uncertain residues are indicated in parentheses.

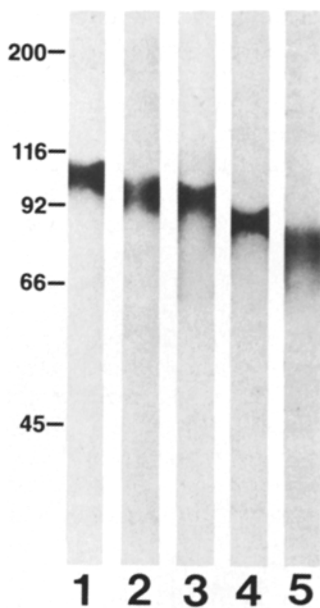

Figure 6. Characterization of OM protein carbohydrates. Enzymatic deglycosylation of the OM protein using (lane *2*) neuraminidase, (lane *3*) Endoglycosidase H, or (lane *4*) Endoglycosidase F. Lane *5* represents deglycosylation with Endoglycosidase F followed by neuraminidase and then *O*-glycanase. Lane *1* represents the OM protein incubated without enzyme. The samples were separated on a 7.5% Laemmli gel, transferred to nitrocellulose, and stained with anti–OM protein antibodies. The polypeptide backbone of the OM protein is ~75 kD. Numbers indicate molecular mass standards.

vous system myelin and no staining of other structures in the CNS.

Ovine Oligodendrocyte Cultures

Immunoblot staining of polypeptides that had been subjected to electrophoresis from sheep white matter homogenate or sheep myelin with either PNA or anti–OM protein antibodies showed the presence of the OM protein, primarily in the membrane-bound form. When ovine oligodendrocytes kept attached in culture were solubilized and similarly stained, both the membrane-bound and soluble forms were seen, the latter of which was recovered from the supernatant using PNA affinity chromatography (Fig. 7 *D*). Furthermore, the cultures of attached oligodendrocytes showed time-dependent OM protein expression. With increasing time after attachment, more of the membrane-bound OM protein was found in washed oligodendrocyte pellets. Immunofluorescence staining of unfixed oligodendrocytes demonstrated that OM protein is on the surface of the cells and increases in density after attachment (Fig. 7 *C*). The cultures used for these experiments contained only (>98%) cells that carried galactocerebroside on their surface. Double-labeling experiments showed that the same cells stained for both galactocereboside and the OM protein (data not shown).

Discussion

We have described here the isolation of a 120-kD polypeptide from human white matter. The isolation procedure takes advantage of its high affinity for PNA and a PI linkage to membranes. We call this polypeptide the OM protein, a glycoprotein present on oligodendrocytes and in CNS myelin. However, it is currently unclear whether the OM protein is confined to oligodendrocytes and CNS myelin. We found the OM protein in CNS myelin isolated by the method of Norton and Poduslo (1973) but not as much of it as in a white matter pellet (Fig. 1 *B*). Since myelin isolated according to the method of Norton and Poduslo (1973) contains mostly compact myelin, these results could mean that the OM protein is mainly found in loose myelin. It is interesting that we did not see staining of cell bodies of oligodendrocytes in tissue sections from adult human or ovine CNS. However, the OM protein was detected immunohistochemically on the surface of ovine oligodendrocytes in culture. Furthermore, there was a considerable amount of soluble 105-kD OM protein in the supernatants from these cells. There was also a substantial increase in the amount of membrane-bound OM protein with increasing time in culture, for at least the first 75 d.

The PNA binding to the OM protein was blocked by 0.01 M D-galactose which indicates that it was binding to carbohydrate on the protein. Moreover, the binding was not blocked by 0.1 M D-glucose or 0.1 M α-methyl-D-mannoside which suggests that PNA was binding to carbohydrate on the OM protein with the expected specificity. In general, binding of lectins can be blocked by monosaccharides that are somewhat characteristic for a given lectin, although the monosaccharide structure does not necessarily bear a relationship to the oligosaccharides for which they have the highest affinity (Debray et al., 1981). PNA has been shown to have highest affinity for the structure gal (β1-3)galNAc, which is most often found on O-linked glycans (Pereira et al., 1976). We used a series of glycosidases to characterize the carbohydrate components of the OM protein and their contributions to the approximate size of the OM protein (Fig. 6). Sialic acids account for 10 kD, high-mannose N-linked glycans 15 kD, complex N-linked glycans 10 kD, and O-linked glycans ~5 kD. Moreover, these results confirm that the OM protein contains O-linked glycans. PNA and the anti–OM protein antibodies also bound to 120-kD polypeptides in bovine, ovine, and canine brains, weakly in mouse and rat brains, and not at all in chicken brains. It is at present unclear to what extent this reflects species differences in amounts of the OM protein, or species differences in primary structure or posttranslational modification.

The amino-terminal sequence of the OM protein shown in Fig. 5 was compared with the National Biomedical Research Foundation protein sequence data base, with no obvious homologies identified. Thus the OM protein is clearly distinct from the 120-kD form of N-CAM, which resembles the OM protein not only in its size but also in its PI membrane attachment (He et al., 1986; Sadoul et al., 1986; Hemperly et al., 1986). The NH_2 terminus of N-CAM has been determined in several species (Rougon and Marshak, 1986), and is quite different from that seen here for the OM protein. It is also of importance here that polyclonal anti–N-CAM antibodies do not bind to the OM protein.

It is now recognized that a growing number of proteins share a PI-membrane linkage (Cross, 1987; Low, 1987), including the 120-kD N-CAM (He et al., 1986; Sadoul et al., 1986; Hemperly et al., 1986), the Thy-1 glycoprotein (Low and Kincade, 1985; Tse et al., 1985; Fatemi and Tartakoff 1986), and the variant surface glycoproteins of trypanosomes (Ferguson et al., 1985*a*, *b*). Where the functions of PI-linked proteins are known, they appear diverse, but in general the properties that such an anchor offers include lateral membrane mobility and rapid release by the action of endogenous PLC (Cross, 1987; Low, 1987). In this context, our finding of a PI-linked protein in CNS myelin is certainly of interest. The absence of OM protein from myelin of the peripheral nervous system suggests a function unique to CNS

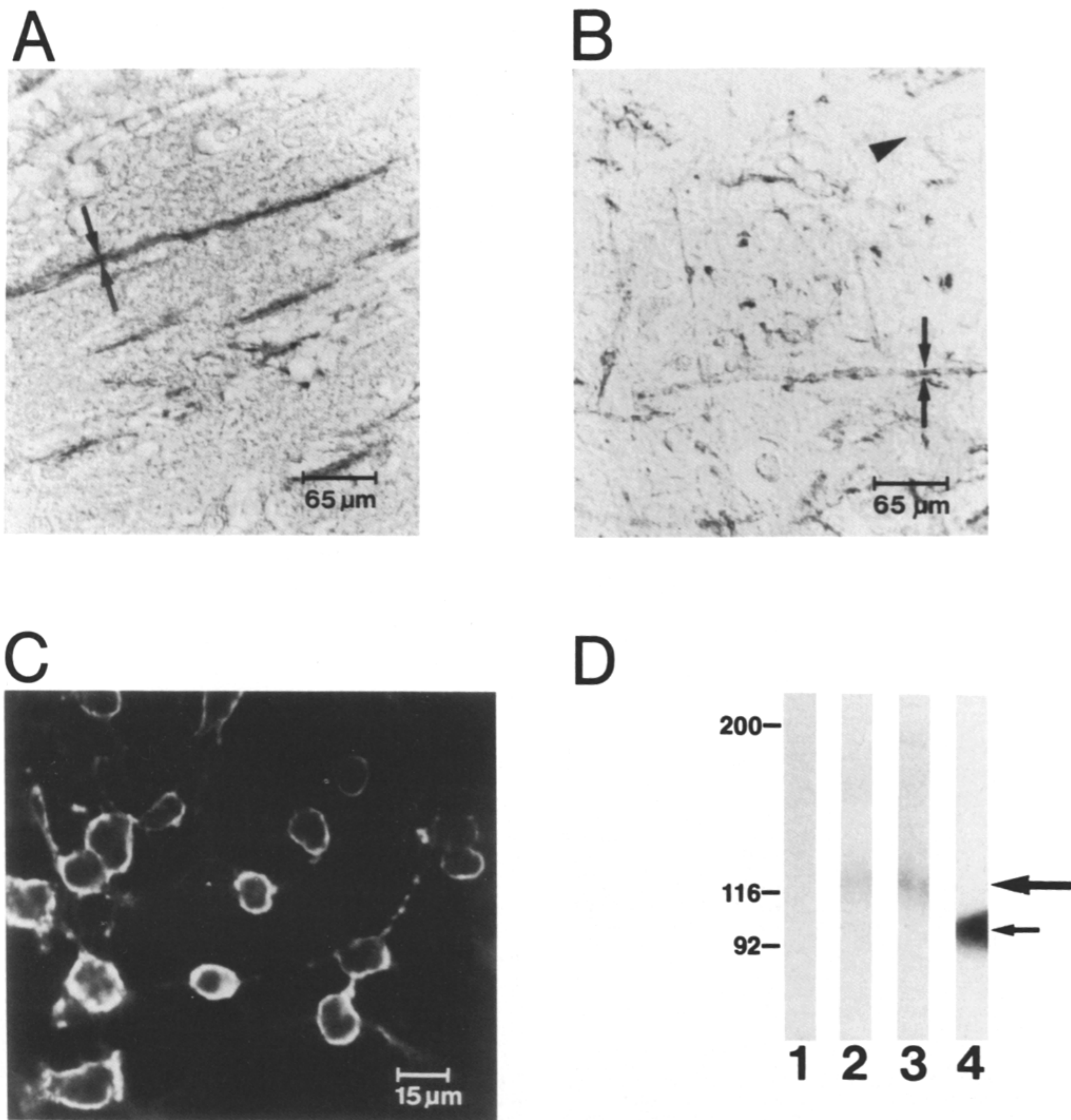

Figure 7. Localization of the OM protein using affinity-purified polyclonal antibodies. (*A* and *B*) Peroxidase-antiperoxidase staining of formalin-fixed paraffin-embedded sections of spinal cord with affinity-purified polyclonal rat antibodies against the OM protein. Myelin sheaths are intensely stained (*arrows*, *A* and *B*) while a neuron (*arrowhead*, *B*) is unstained. (*C*) Culture of ovine oligodendrocytes (2 wk) stained with anti-OM protein antibodies using immunofluorescence. (*D*) Attached cells after varying times in culture (5, 25, and 75 d; lanes *1–3*) were washed, and the same total amount of protein was added per lane and separated on a 7.5% Laemmli gel. After separation the proteins were transferred to nitrocellulose, which was then stained with antibodies to the OM protein. The supernatant from the cells after 25 d was harvested, centrifuged at 100,000 *g* for 30 min, and the OM protein extracted by PNA affinity chromatography (lane *4*). It is clear that the amount of OM protein attached to the oligodendrocytes increases with increasing time in culture and it can be harvested from the supernatants. Numbers indicate molecular mass standards and the arrows point to the locations of the OM protein.

myelin, although there may be a peripheral nervous system homologue to the OM protein.

We found a considerable amount of 105-kD OM protein in the brains used in this study. Keeping in mind that the 105-kD form may correspond to the portion of OM protein released by PLC, the possibility exists that there may be a phospholipase in the OM unit whose function it is to release the OM protein. An alternative explanation would be that rupture of the PI linkage in this case was due to postmortem autolysis. The former explanation is perhaps supported by

the observation that the oligodendrocytes in culture released a considerable amount of 105-kD OM protein into the supernatant, indicating that they may contain a phospholipase. Thus the expression of OM protein on oligodendrocytes may be regulated not only at the transcriptional/translational level but also at the level of membrane attachment and release.

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References

Bunge, R. P., M. B. Bunge, and M. Cochran. 1978. Some factors influencing the proliferation and differentiation of myelin-forming cells. *Neurology.* 28(Suppl):59-67.

Cross, G. A. M. 1987. Eukaryotic protein modification and membrane attachment via phosphatidylinositol. *Cell.* 48:179-181.

Debray, H., D. Decout, G. Strecker, G. Spik, and J. Montreuil. 1981. Specificity of twelve lectins toward oligosaccharides and glycopeptides related to N-glycosylproteins. *Eur. J. Biochem.* 117:41-55.

Elder, J. H., and S. Alexander. 1982. Endo-β-*N*-acetylglucosaminidase F: endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. *Proc. Natl. Acad. Sci. USA.* 79:4540-4544.

Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606-2617.

Fatemi, S. H., and A. M. Tartakoff. 1986. Hydrophilic anchor-deficient Thy-1 is secreted by a class E mutant T lymphoma. *Cell.* 46:653-657.

Ferguson, M. A. J., K. Haldar, and G. A. M. Cross. 1985*a*. *Trypanosoma brucei* variant surface glycoprotein has a sn-1,2-dimyristyl glycerol membrane anchor at its COOH terminus. *J. Biol. Chem.* 260:4963-4968.

Ferguson, M. A. J., M. G. Low, and G. A. M. Cross. 1985*b*. Glycosyl-sn-1,2-dimyristyl phosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 260:14547-14555.

Grumet, M., S. Hoffman, K. L. Crossin, and G. M. Edelman. 1985. Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. *Proc. Natl. Acad. Sci. USA.* 82:8075-8079.

Gulcher, J. R., L. S. Marton, and K. Stefansson. 1986. Two large glycosylated polypeptides found in myelinating oligodendrocytes but not in myelin. *Proc. Natl. Acad. Sci. USA.* 83:2118-2122.

He, H.-T., J. Barbet, J.-C. Chaix, and C. Goridis. 1986. Phosphatidylinositol is involved in the membrane attachment of NCAM-120, the smallest component of the neural cell adhesion molecule. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2489-2494.

Hemperly, J. J., G. M. Edelman, and B. A. Cunningham. 1986. cDNA clones of the neural cell adhesion molecule (N-CAM) lacking a membrane-spanning region consistent with evidence for membrane attachment via a phosphatidylinositol intermediate. *Proc. Natl. Acad. Sci. USA.* 83:9822-9826.

Ikezawa, H., and R. Taguchi. 1981. Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* and *Bacillus thuringiensis*. *Methods Enzymol.* 71:731-741.

Kruse, J., G. Keilhauer, A. Faissner, R. Timpl, and M. Schachner. 1985. The J1 glycoprotein: a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature (Lond.).* 316:146-148.

Kruse, J., R. Mailhammer, H. Wernecke, A. Faissner, I. Sommer, C. Goridis, and M. Schachner. 1984. Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature (Lond.).* 311:153-155.

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

Lees, M. B., and S. W. Brostoff. 1984. Proteins of myelin. *In* Myelin. 2nd ed. P. Morrel, editor. Plenum Publishing Corp., New York. 197-224.

Little, C. 1981. Phospholipase C from *Bacillus cereus*. *Methods. Enzymol.* 71:725-730.

Low, M. G. 1987. Biochemistry of the glycosyl-phosphatidylinositol membrane protein anchors. *Biochem. J.* 244:1-13.

Low, M. G., and P. W. Kincade. 1985. Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. *Nature (Lond.).* 318:62-64.

Marton, L. S., and K. Stefansson. 1984. Developmental alterations in molecular weights of proteins in the human central nervous system that react with antibodies against myelin-associated glycoprotein. *J. Cell Biol.* 99:1642-1646.

McGarry, R. C., S. L. Helfand, R. H. Quarles, and J. C. Roder. 1983. Recognition of myelin-associated glycoprotein by the monoclonal antibody HNK-1. *Nature (Lond.).* 306:376-378.

Merril, C. R., D. Goldman, and M. L. Van Keuren. 1984. Gel protein stains: silver stain. *Methods Enzymol.* 104:441-447.

Mikol, D. D., L. Wrabetz, L. S. Marton, and K. Stefansson. 1988. Developmental changes in the molecular weights of polypeptides in the human central nervous system that carry the HNK-1 epitope and bind phaseolus vulgaris lectins. *J. Neurochem.* In press.

Norton, W. T., and S. E. Poduslo. 1973. Myelination in rat brain: method of myelin isolation. *J. Neurochem.* 21:749-757.

Olmsted, J. B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955-11957.

Pereira, M. E. A., E. A. Kabat, R. Lotan, and N. Sharon. 1976. Immunochemical studies on the specificity of the peanut (*Arachis hypogaea*) agglutinin. *Carbohydr. Res.* 51:107-118.

Quarles, R. H. 1984. Myelin-associated glycoprotein in development and disease. *Dev. Neurosci.* 6:285-303.

Reeck, G. R., C. de Haën, D. C. Teller, R. F. Doolittle, W. M. Fitch, R. E. Dickerson, P. Chambon, A. D. McLachlan, E. Margoliash, T. H. Jukes, and E. Zuckerlandl. 1987. "Homology" in proteins and nucleic acids: a terminology muddle and a way out of it. *Cell.* 50:667.

Rougon, G., and D. R. Marshak. 1986. Structural and immunological characterization of the amino-terminal domain of mammalian neural cell adhesion-molecules. *J. Biol. Chem.* 261:3396-3401.

Sadoul, K., A. Meyer, M. G. Low, and M. Schachner. 1986. Release of the 120 kDa component of the mouse neural cell adhesion molecule N-CAM from cell surfaces by phosphatidylinositol-specific phospholipase C. *Neurosci. Lett.* 72:341-346.

Sharon, N., and H. Lis. 1982. Glycoproteins. *In* The Proteins. Vol. V. 3rd ed. H. Neurath and R. L. Hill, editors. Academic Press Inc., New York. 1-144.

Sternberger, L. A. 1979*a*. Immunocytochemistry. John Wiley & Sons, New York. pp. 24-58.

Sternberger, L. A. 1979*b*. Immunocytochemistry. John Wiley & Sons, New York. 104-169.

Sundler, R., A. W. Alberts, and P. R. Vagelos. 1978*a*. Enzymatic properties of phosphatidylinositol inositolphosphohydrolase from *Bacillus cereus*. *J. Biol. Chem.* 253:4175-4179.

Sundler, R., A. W. Alberts, and P. R. Vagelos. 1978*b*. Phospholipases as probes for membrane sidedness. *J. Biol. Chem.* 253:5299-5304.

Szuchet, S., K. Stefansson, R. L. Wollmann, G. Dawson, and B. G. W. Arnason. 1980. Maintenance of isolated oligodendrocytes in long-term culture. *Brain Res.* 200:151-164.

Tarentino, A. L., and F. Maley. 1974. Purification and properties of an endo-β-*N*-acetylglucosaminidase from *Streptomyces griseus*. *J. Biol. Chem.* 249: 811-817.

Thiery, J.-P., A. Delouvée, M. Grumet, and G. M. Edelman. 1985. Initial appearance and regional distribution of the neuron-glia cell adhesion molecule in the chick embryo. *J. Cell Biol.* 100:442-456.

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

Tse, A. G. D., A. N. Barclay, A. Watts, and A. F. Williams. 1985. A glycophospholipid tail at the carboxyl terminus of the Thy-1 glycoprotein of neurons and thymocytes. *Science (Wash. DC).* 230:1003-1008.

Tucker, G. C., H. Aoyama, M. Lipinski, T. Tursz, and J. P. Thiery. 1984. Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. *Cell Differ.* 14:223-230.