

Expression of Unique Sets of GPI-linked Proteins by Different Primary Neurons In Vitro

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Abstract. We have surveyed the proteins expressed at the surface of different primary neurons as a first step in elucidating how axons regulate their ensheathment by glial cells. We characterized the surface proteins of dorsal root ganglion neurons, superior cervical ganglion neurons, and cerebellar granule cells which are myelinated, ensheathed but unmyelinated, and unensheathed, respectively. We found that the most abundant proteins are common to all three types of neurons. Reproducible differences in the composition of the integral membrane proteins (enriched by partitioning into a Triton X-114 detergent phase) were detected. These differences were most striking when the expression of glycosylphosphatidylinositol (GPI)-anchored membrane proteins by these different neurons was compared. Variations in the relative abundance and degree of glycosylation of several well known GPI-anchored proteins, including Thy-1,

F3/F11, and the 120-kD form of the neural cell adhesion molecule (N-CAM), and an abundant 60-kD GPI-linked protein were observed. In addition, we have identified several potentially novel GPI-anchored glycoproteins on each class of neurons. These include a protein that is present only on superior cervical ganglion neurons and is 90 kD; an abundant protein of 69 kD that is essentially restricted in its expression to dorsal root ganglion neurons; and proteins of 38 and 31 kD that are expressed only on granule cell neurons. Finally, the relative abundance of the three major isoforms of N-CAM was found to vary significantly between these different primary neurons. These results are the first demonstration that nerve fibers with diverse ensheathment fates differ significantly in the composition of their surface proteins and suggest an important role for GPI-anchored proteins in generating diversity of the neuronal cell surface.

THE development and continued integrity of the nervous system depends on complex and reciprocal interactions between its various cellular elements. A striking example is found in the interactions of axons with myelinating glial cells; i.e., Schwann cells in the peripheral nervous system and oligodendrocytes in the central nervous system. Contact with the axon regulates the proliferation of these glial cells (Wood and Bunge, 1975; Salzer et al., 1980; Wood and Williams, 1984) and determines their state of differentiation, most dramatically, their elaboration and maintenance of a myelin sheath (Aguayo et al., 1976; Weinberg and Spencer, 1976; Macklin et al., 1986; Kidd et al., 1990).

The mechanisms of signaling between these cells has not been elucidated. In the case of the axonal specification of myelination, which has been most clearly demonstrated by cross anastomosis experiments of myelinated and unmyelinated nerve fibers in the peripheral nervous system (Aguayo et al., 1976; Weinberg and Spencer, 1976), two major theories have been proposed. One hypothesis, based on studies showing that primarily large caliber axons become myelinated, posits a critical axonal diameter (usually considered to

be a diameter $>1 \mu\text{m}$) that triggers myelination (Friede, 1972; Voyvodic, 1989). An alternate theory is that distinct cell surface components are present on the subset of axons that specify myelination (Spencer and Weinberg, 1978). Several observations on the formation of myelin *in vivo* and on its induction *in vitro* favor this latter possibility.

During myelination, the first anatomically resolved step occurs when the glial cell extends a process of its plasma membrane around an axon (Webster et al., 1973). In the peripheral nervous system, this extending process of the Schwann cell segregates off a large caliber axon that will be myelinated into a 1:1 relationship, apart from the smaller diameter fibers that will remain ensheathed. This sorting of fibers implies that axons possess distinct surface molecules that determines their differential ensheathment by Schwann cells. In the central nervous system, the oligodendrocyte extends multiple processes (up to 40) into the neuropil that are able to select individual axons to be myelinated (Remahl and Hildebrand, 1990). As oligodendrocytes and Schwann cells are able to myelinate the same nerve fibers (Aguayo et al., 1978), both cells may recognize the same axonal surface components during myelination.

In vitro studies have also demonstrated that only a subset of nerve fibers are capable of inducing the myelination pro-

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gram of Schwann cells. Schwann cells cocultured with sensory neurons of dorsal root ganglia (DRG)¹ myelinate a substantial proportion of the nerve fibers, whereas Schwann cells cocultured with sympathetic neurons of the superior cervical ganglion do not (Roufa et al., 1986). These results suggest that cell surface components of nerve fibers that are destined to be myelinated are likely to differ from those that will remain ensheathed. Such differences however, have not been defined. A number of neural adhesion molecules have been implicated in the cell interactions of axons with Schwann cells or oligodendrocytes, but none of these are expressed in an appropriately restricted pattern to account for the nerve fiber specificity described above. For example, neural cell adhesion molecules (N-CAM), LI, N-cadherin, and integrins have all been implicated in various interactions of neurons with Schwann cells including fiber outgrowth (Bixby et al., 1988; Letourneau et al., 1990) and ensheathment (Seilheimer et al., 1989). However the widespread expression of these proteins throughout the nervous system, including their presence on both myelinated and unmyelinated fibers (Mirsky et al., 1986), suggests that while they may be required for the early events of ensheathment or myelination (Wood et al., 1990), they probably do not induce myelinogenesis themselves.

We have therefore sought to identify axonal proteins with a restricted distribution that might correlate with the different ensheathment fates of nerve fibers. To this end, we have surveyed by biochemical methods, proteins displayed at the surface of three different types of primary neurons. These included sensory neurons of the DRG, sympathetic neurons of the superior cervical ganglion (SCG), and cerebellar granule cell neurons (CGC). In the adult, these fibers are respectively myelinated, unmyelinated but ensheathed, and unensheathed by glial cells. We now report that, while the majority of cell surface proteins expressed on all three classes of neurons are identical, reproducible differences between the integral membrane proteins of these neurons were identified. These differences were most striking when the expression of glycosylphosphatidyl-inositol (GPI)-linked membrane proteins was compared. There is significant variation in both the surface abundance and extent of glycosylation of several well known GPI-anchored proteins. In addition, we have identified several apparently novel GPI-anchored glycoproteins that have a restricted expression. Finally, the alternative splicing pattern of N-CAM varies in a characteristic manner between these different primary neurons. These results are the first demonstration that nerve fibers with different ensheathment fates differ in the composition of their cell surface proteins.

Materials and Methods

Tissue Culture Methods

Sensory and sympathetic neuron cultures were established as described (Estridge and Bunge, 1978; Eldridge et al., 1989). DRGs (sensory neurons) were removed from embryonic day-15 rats and SCGs (sympathetic neurons) were removed from embryonic day-21 rats under aseptic conditions. In each

1. *Abbreviations used in this paper:* CGC, cerebellar granule cell; DRG, dorsal root ganglion; GPI, glycosylphosphatidyl-inositol; N-CAM, the neural cell adhesion molecule; PIPLC, phosphoinositol phospholipase C; SCG, superior cervical ganglion.

case, the ganglia were dissociated before plating onto an ammoniated rat tail collagen substrate. The cells were fed three times per week with MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS, 0.4% glucose, and 50 ng/ml 2.5s NGF (Bioproducts for Science, Inc., Indianapolis, IN). During the first two weeks of culture, the cells were treated with 5-fluorodeoxyuridine and uridine (both at 10^{-5} M), on alternate feedings to eliminate the mitotically active non-neuronal cells.

Granule cells were isolated from the cerebellum of postnatal day-seven rats. The cerebella from 10 rats were removed, the meninges were stripped off, and the cerebella were sliced into $<5\text{-mm}^3$ fragments. The tissue was then digested with 0.25% Trypsin for 1 h at 37°C and mechanically dissociated in the presence of DNAase type 1 ($50\ \mu\text{g/ml}$) (Sigma Chemical Co., St. Louis, MO). After trituration, large tissue fragments were permitted to settle and the supernatant was reserved. Trituration and pooling of supernatants was repeated four more times. Cells in the pooled supernatants were pelleted and rinsed with media (DME with 10% FBS, and 20 mM KCl) and then plated at a density of 2×10^6 cells/2 ml onto 35-mm dishes coated with poly-L-lysine. 60 h later the culture media was supplemented with cytosine arabinoside to a final concentration of 10^{-5} M. Cultures were studied after 7–10 d in vitro with no change of the media. The antimetabolic treatment eliminated most, but not all, of the non-neuronal cells. To characterize the small number of non-neuronal cells that remained, we eliminated neurons by feeding cultures every other day for an additional 2 wk in vitro. The remaining cells, which were flat and polygonal in shape, were replated onto coverslips for immunofluorescent staining with antibodies against glial fibrillary acidic protein and Thy-1.

Labeling of Surface Proteins

Surface proteins were labeled either with ^{125}I or biotin. External labeling of neuronal cultures with ^{125}I using lactoperoxidase was performed as described (Estridge and Bunge, 1978). To biotinylate surface proteins (Lisanti et al., 1988), cultures were first rinsed with Dulbecco's modified PBS twice at 4°C . Sulfo-NHS-Biotin (Pierce Chemical Co., Rockford, IL) was dissolved first in DMSO (200 mg/ml) then just before addition, diluted in Dulbecco's modified PBS to 0.5 mg/ml. Cells were biotinylated twice for 15 min, rinsed once with Dulbecco's modified MEM, and once with Dulbecco's modified PBS. All buffer was removed, and the labeled cells were stored at -20°C until needed. Biotinylated cultures were subsequently extracted with 1% SDS in 150 mM NaCl, 10 mM Tris, pH 7.4, at room temperature for 1 h. Solubilized proteins were precipitated in 90% acidified acetone (0.14 M HCl) at -20°C . Proteins were separated on SDS-PAGE, electroblotted, and probed with ^{125}I streptavidin.

Detection of GPI-linked Proteins

Proteins attached to the membrane via a GPI linkage were assayed as described by Lisanti et al. (1988). Briefly, biotinylated cultures were solubilized with 1% Triton X-114, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA containing the protease inhibitors antipain, pepstatin A, and leupeptin at a concentration of 0.1% (all three inhibitors were present throughout the entire procedure until the final phase separation). The Triton X-114 extract was warmed to 37°C for 3 min, and microfuged for 2 min to yield aqueous and detergent phases. The detergent phase was reserved and rinsed once with 900 μl of 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and once with 900 μl of 10 mM Tris, pH 7.4, 150 mM NaCl, 0.25 M methyl-alpha-D-mannoside, 1 mM EDTA. 400 μl of 10 mM Tris, pH 7.4, 150 mM NaCl, 0.25 M methyl-alpha-D-mannoside, 1 mM EDTA was prewarmed and then added to the detergent phase ($\sim 100\ \mu\text{l}$) and the sample was incubated at 37°C . Phosphatidylinositol specific phospholipase C (PIPLC) (a gift of Dr. M. Low, Columbia University, New York) was added to the aqueous solution at a final concentration of 6 U/ml (an amount determined empirically to release $>90\%$ of Thy-1). Samples were then vortexed continuously for 1 h at 37°C to disperse the detergent phase during the enzyme treatment. Samples were then microfuged briefly and proteins in the detergent phase (i.e., integral membrane proteins not digested by PIPLC) were acetone precipitated as described above. The aqueous phase (enriched in GPI-anchored proteins) was rinsed two more times with 100 μl of Triton X-114, incubated with 250 μl of phenyl sepharose overnight at 4°C , and the remaining proteins were precipitated with TCA; Na deoxycholate was added as a carrier. In each case, proteins were separated on SDS-PAGE and electroblotted onto nitrocellulose membranes. Blots were blocked with 1 M glucose, 10% glycerol, 0.5% Tween-20, 1% Carnation non-fat dried milk, 3% BSA in PBS for 1 h at room temperature. Blots were then probed with ^{125}I streptavidin, washed extensively with 0.5% Tween-20 in PBS, and exposed

for autoradiography. To determine the relative expression of these proteins, some autoradiographs were scanned with a densitometer (Model GS300) and scanning software (Model GS370; both from Hoeffer Scientific Instruments, San Francisco, CA). Data was analyzed with Igor (WaveMetrics, Lake Oswego, OR) on a Macintosh IICx computer. In some cases, blots of biotinylated proteins were probed with primary antibodies as described below.

Immunoblotting and Immunoprecipitation Procedures

Rabbit polyclonal antibodies specific for the following GPI-linked proteins were used: anti-rat Thy-1 (1:100, gift of N. Barclay, University of Oxford); anti-rat N-CAM (1:50, a gift of Urs Rutishauser, Case Western Reserve University); anti-rat TAG-1 (1:1,000, gift of T. Jessel, Columbia University); Acetylcholinesterase (1:100, gift of J. Grassi, Commissariat A L'Energie Atomique); anti-rat F3 (1:100, a gift of C. Goridis, Centre d'immunologie de Marseille-Luminy); Prion protein (1:1,000, a gift of Paul Bendheim, Staten Island Institute for Basic Research); and anti-human placental alkaline phosphatase (1:100, Dako Corp., Santa Barbara, CA). Samples to be immunoprecipitated were first solubilized in Triton X-114 or with 1% SDS, before diluting with 4 vol buffer A (190 mM NaCl, 50 mM Tris, pH 7.4, 6 mM EDTA, 2.5% Triton X-100). Samples were incubated with the primary antibody for 2 h at room temperature and then incubated with protein A coupled to Sepharose beads (Sigma Chemical Co., St. Louis, MO) for 2 h at room temperature. Beads were washed with buffer A five times for 5 min, washed once with 10 mM Tris, pH 7.4, and boiled in Laemmli's sample buffer for 3 min. Samples were separated on SDS-PAGE, electroblotted, and probed with ¹²⁵I streptavidin. In our hands, the antibodies to N-CAM and F3, unlike those to Thy-1, did not quantitatively immunoprecipitate all of the N-CAM and F3 present in the sample, but yielded strong signals by immunoblotting procedures (Towbin et al., 1979). Immunoreactive proteins were then detected using secondary antibodies conjugated to alkaline phosphatase (Kirkegard and Perry Laboratories, Inc., Gaithersburg, MD) and treating with 5-bromo-4-chloro-3-indoyl-phosphate and Nitroblue tetrazolium.

Image Processing

Autoradiographs and photomicrographs were scanned with a Truvel scanner at 600 dpi. Images were cropped with Adobe Photoshop. QuarkExpress was used for page layout to create images that were printed by a commercial typesetter on a linotronics printer at 2,540 dots per inch and a 150 line per inch halftone screen.

Deglycosylation of Samples

To determine the size of the core polypeptide, immunoprecipitates of specific proteins or TCA precipitates (containing deoxycholate as a carrier) of the GPI-linked proteins were enzymatically deglycosylated in 50 μ l with *N*-Glycosidase F (Boehringer Mannheim Corp., Indianapolis, IN) for 18 h at 37°C as per the manufacturer's instructions.

Results

Characterization of Primary Neurons

To identify surface proteins that might be differentially expressed on neurons with different ensheathment fates, we established cultures containing defined populations of neurons. We studied three types of neurons: DRG neurons, SCG neurons, and CGCs. Age of isolation varied with each neuron, corresponding to embryonic day 15 for DRG, embryonic day 21 for SCG, and 1 wk postnatal for CGC. To ensure maximal accessibility to labeling reagents, ganglia were enzymatically dissociated before plating in vitro. Although DRG and SCG neurons remained viable indefinitely, they were routinely characterized at 3 wk in culture. Granule cell neurons were viable for up to 2 wk in culture, and were routinely studied at 10 d when cell viability and neurite outgrowth appeared to be optimal.

All cultures were treated with antimetotics to suppress the

growth of non-neuronal cells. DRG and SCG cultures were consistently free of any non-neuronal cells and were nearly identical morphologically (Fig. 1, *a* and *b*). The CGC neurons (Fig. 1 *c*) had smaller somas that tended to reaggregate, and thinner, shorter processes than the DRG or SCG neurons. In addition, the CGC cultures were often contaminated with small numbers of non-neuronal cells that persisted despite treatment with antimetotics. The non-neuronal cells were predominately astrocytes and fibroblasts as indicated by their morphology and their staining with antibodies

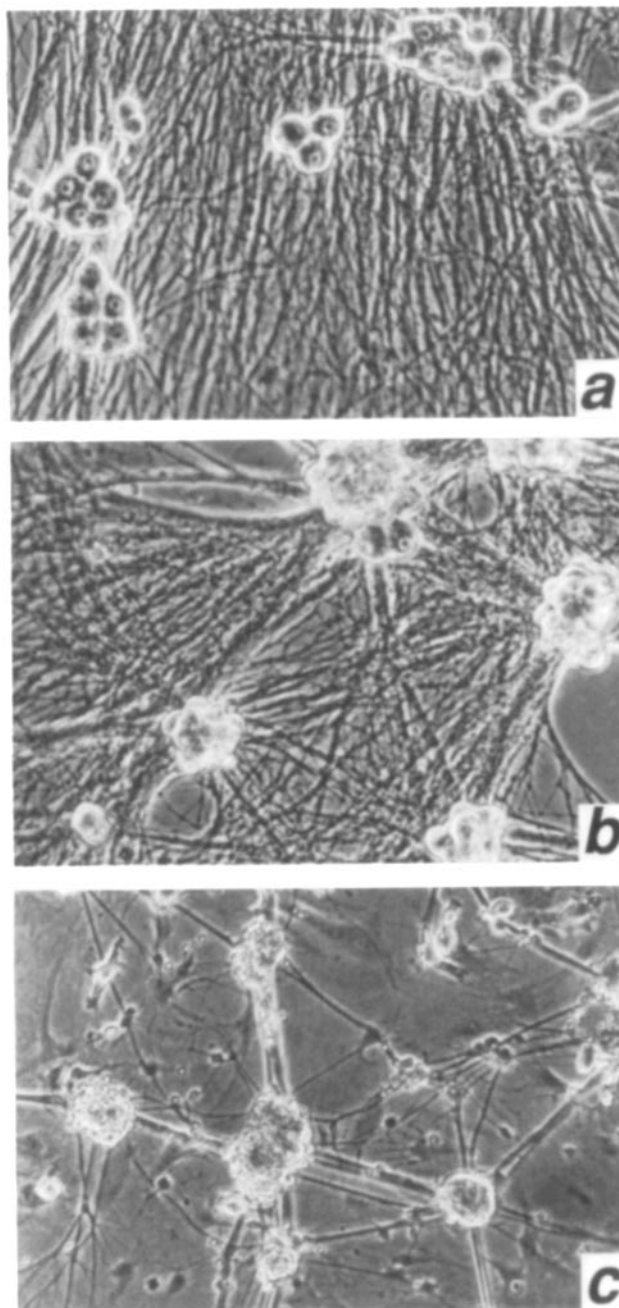


Figure 1. Primary neurons in vitro. The appearance of different primary neurons in tissue culture. (a) DRG neurons, 3 wk in vitro; (b) SCG neurons, 3 wk in vitro; and (c) CGCs, 10 d in vitro. All cultures were exposed to antimetotic agents to eliminate proliferating non-neuronal cells, although some persist in *c*.

against glial fibrillary acidic protein and Thy-1.1, respectively (data not shown).

Different Classes of Neuron Display Similar Cell Surface Proteins

We first characterized the cell surface proteins of DRG and SCG by iodinating surface accessible tyrosines using the lactoperoxidase method (Estridge and Bunge, 1978). These studies revealed a highly complex banding pattern with prominent bands with relative molecular masses of ~120, 75, 60, 40, 35, and 32 kD (Fig. 2). These findings are in general agreement with the banding pattern reported in an earlier study on the proteins of SCG neurons (Estridge and Bunge, 1978), although more complex. Surprisingly, we were unable to discern any significant differences between DRG and SCG surface proteins utilizing this procedure, which labels the accessible tyrosines of membrane proteins (Hubbard and Cohn, 1972), but can also result in the labeling of cytosolic proteins to a lesser degree (Thompson et al., 1987).

We next labeled surface proteins of sensory, sympathetic, and cerebellar granule cell neurons with the membrane impermeant reagent Sulfo-NHS-Biotin, which labels free amino groups. This labeling procedure is highly specific for surface proteins and, because it results in extensive labeling of all proteins, it may better reflect their relative abundance. After labeling, the cultures were extracted with 1% SDS, fractionated by SDS-PAGE, transferred to nitrocellulose, and surface proteins were visualized with ¹²⁵I-streptavidin to determine the protein composition of the different classes of neurons. Again, the proteins in the three cultures were remarkably similar (data not shown). However, the analysis was complicated by the presence of serum proteins in the media that had adsorbed onto the collagen and polylysine substrata as demonstrated in companion dishes without cells which were carried in parallel.

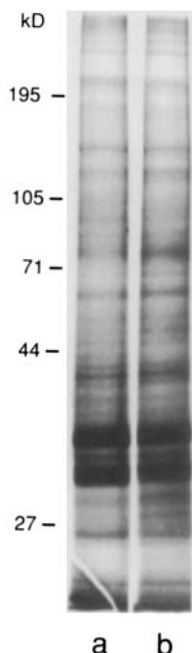


Figure 2. Labeling of neuronal surface proteins with ¹²⁵I. DRG cultures (a) and SCG cultures (b) were labeled with ¹²⁵I using lactoperoxidase. The cultures were solubilized in sample buffer, separated on SDS-PAGE, and exposed for autoradiography. Molecular weight markers are listed to the left.

To avoid the confounding effects of substrate-adsorbed proteins and to define differences in the integral membrane proteins exposed at the cell surface of these neurons, we partitioned the surface-labeled proteins with Triton X-114. By solubilizing these cultures in Triton X-114 at 4°C and then shifting to 37°C to effect a phase partition, we obtained a detergent phase highly enriched in integral membrane proteins (Bordier, 1981). This material was then fractionated by SDS-PAGE, blotted onto nitrocellulose and probed with ¹²⁵I streptavidin. The detergent phases from these different neurons consisted of complex mixtures of integral membrane proteins (Fig. 3). As in the iodination studies, the most abundant proteins on all three neurons appear to be identical. Major protein species are present with relative molecular masses corresponding to 155, 110, 58, 49, 46, 32, and 29 kD. On careful inspection however, subtle differences between the integral membrane composition of these neurons were evident. These differences include proteins of ~95 and 85 kD that are present on DRG and CGC neurons, but not on sympathetic neurons, a protein of ~72 kD that is more prominent on SCG and CGC than on DRG neurons, and a protein of ~25 kD that is present on DRG and SCG neurons but not on CGC's. Taken together however, these results indicate that there is a remarkable degree of similarity in the proteins displayed at the surface of different types of neurons.

Differential Expression of GPI-linked Proteins on Neurons

We next surveyed those integral membrane proteins that are covalently attached to the plasma membrane via a glycosylphosphatidyl-inositol linkage. This is a diverse group of integral membrane proteins that frequently mediate cell-cell interactions (Ferguson and Williams, 1988; Low and Saltiel, 1988). To identify such proteins, we took advantage of several of their features including: (a) their cell surface localization; (b) their sensitivity to cleavage by PIPLC, and (c) their conversion from a hydrophobic to a hydrophilic protein as a consequence of this cleavage. Specifically, primary cultures of neurons were biotinylated, proteins were partitioned into the Triton X-114 detergent phase, the detergent phase

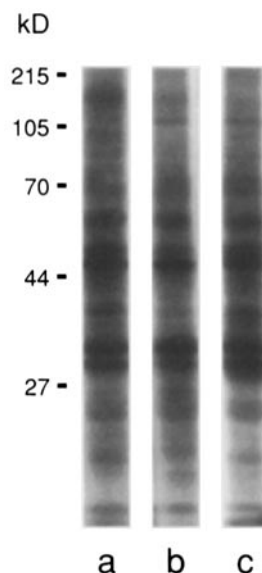


Figure 3. Characterization of the integral membrane proteins expressed by DRG, SCG, and granule cell neurons. Sensory neurons (a), sympathetic neurons (b), and cerebellar neurons (c) cultured in vitro were biotinylated with Sulfo-NHS-Biotin, and solubilized with 1% Triton X-114. A detergent phase (at 37°C), enriched in the integral membrane proteins was precipitated, separated on SDS-PAGE, electroblotted, and probed with ¹²⁵I-streptavidin. The blots were then exposed for autoradiography.

was treated with PIPLC, and those proteins partitioning into the aqueous phase were visualized with ^{125}I -streptavidin after blotting onto nitrocellulose. These studies were highly reproducible and revealed striking differences in the expression of GPI-linked proteins by these three different primary neurons. A representative set of experiments is shown in Fig. 4.

In general, the most abundant GPI-linked proteins fell into three molecular mass ranges: 120–140 kD, 60–70 kD, and 25–30 kD. In the case of the DRG, two closely migrating bands of nearly equal intensity and corresponding to 135 and 120 kD were detectable on linear gradient gels (Fig. 4 A, lane b). An additional doublet migrating at 69 and 65 kD and a single band of 25 kD were also present on DRG neurons. In the case of SCG neurons, the major protein present was 27 kD. In the granule cells, two abundant proteins were detected: a protein of 130 kD, and one of 60 kD.

A number of minor GPI-linked proteins were also observed on these neurons following longer autoradiographic exposures (Fig. 4 B). These included a 90 kD protein present on the SCG and not detected on the DRG or CGC's, and bands of 38 and 31 kD that were specific for the CGC neurons. The SCG neurons also appeared to express three bands that comigrated with the DRG bands of 135, 120, and 69 kD, but at substantially lower levels (Fig. 4 B, lane d). Analysis by densitometry of a number of such experiments found these results to be highly reproducible with respect to the abundance and relative expression of the GPI-anchored proteins. Densitometry of a representative set of autoradiographs, in which the expression of various GPI-anchored proteins is quantitated, is shown in Fig. 5.

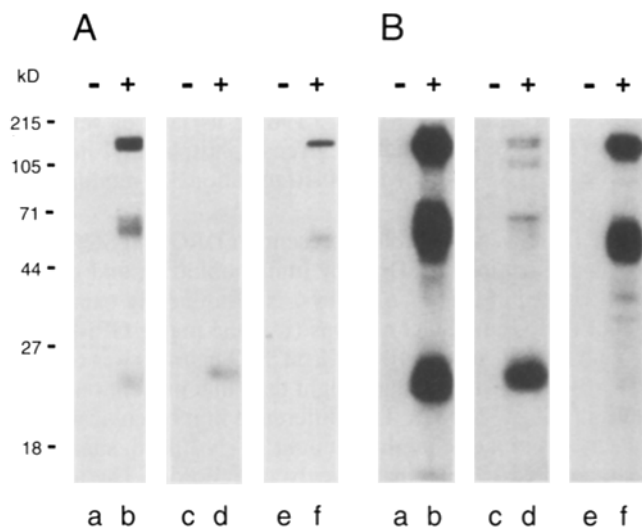


Figure 4. GPI-anchored proteins expressed by DRG, SCG, and granule cell neurons. Cultures of sensory neurons (a and b), sympathetic neurons (c and d) and cerebellar granule cells (e and f) were biotinylated and solubilized with 1% Triton X-114. In each case, the detergent phase was then treated with (+) or without (–) PIPLC for 1 h at 37°C. Proteins released from the detergent phase into the aqueous phase were precipitated, separated by SDS-PAGE, electrophoretically, and probed with ^{125}I streptavidin. The same autoradiograph was then exposed for either 6 h (A), or 28 h (B) to permit the detection of both abundant or minor proteins respectively.

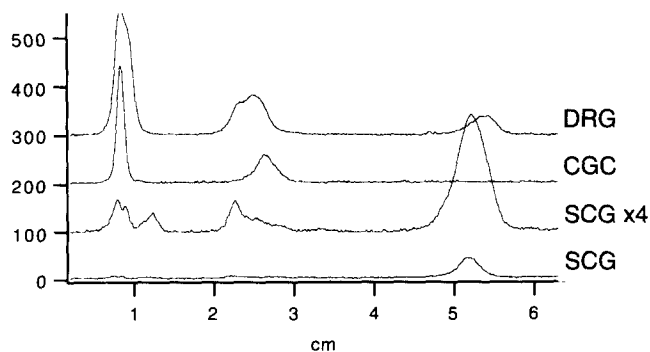


Figure 5. Quantitation of the expression of GPI-anchored proteins. The autoradiographs of GPI-anchored proteins shown in Fig. 4 were scanned with a densitometer. The absorption was plotted vs the length of the gel. Lanes were chosen to demonstrate the level of expression of GPI-anchored proteins when standardized to total membrane before extraction (curves labeled DRG, SCG, and CGC) or standardized to an arbitrary level to allow for the comparison of GPI-anchored proteins normally expressed by SCG neurons at low levels (curve labeled SCG $\times 4$).

To examine the potential localization of these proteins on processes, we explanted undissociated ganglia into culture to facilitate the subsequent removal of cell somas by mechanical dissection. This technique provides a fraction comprised entirely of neurites (Salzer et al., 1980). The profile of GPI-anchored proteins in this preparation of sensory neurites was identical to that of the total DRG preparation (data not shown) and indicates that all these proteins are expressed in abundance on nerve fibers.

Characterization of the Major Neuronal GPI-linked Proteins

To identify these various GPI-anchored proteins, we used antibodies to known GPI-linked proteins in immunoblotting or immunoprecipitation studies. Immunoprecipitation and immunoblotting identified the upper band on DRG and CGC neurons as containing F3, a GPI-linked neural adhesion molecule (Fig. 6 A). The band on SCG neurons also faintly stained with antibodies to F3. Immunoblots of DRG neurons revealed that F3 on these cells is comprised of a doublet of 135 and 125 kD of equivalent intensity. Both forms were found to be GPI-linked and released from detergent extracts by PIPLC (Fig. 6 B). However, when cultures of DRG neurons were incubated with PIPLC, only the upper band was released from the cell surface of sensory neurons, and the lower band remained associated with the cells. The lower molecular weight form of F3 on DRG neurons is therefore likely to be an intracellular, incompletely processed form of F3 recently described in CHO cells transfected with a rat F3 cDNA (Gennarini et al., 1991). Interestingly, the surface exposed F3 (upper band) on sensory neurons was consistently of higher molecular weight than on granule cells (135 vs 130 kD, respectively). Presumably, this reflects variability in the extent of the glycosylation of F3 on these different classes of neurons. We were unable to detect any TAG-1, a developmentally regulated GPI-linked glycoprotein of 135 kD (Furley et al., 1990), which would be expected to comigrate with F3. TAG-1 has recently been reported to be persistently

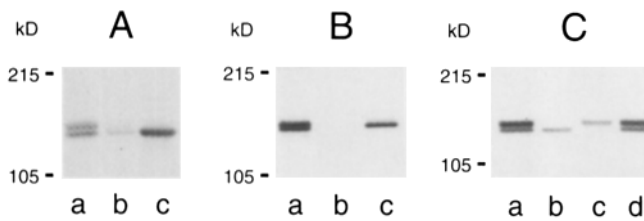


Figure 6. Expression of F3 by primary neurons. (A) Immunoblots of detergent extracts (1% SDS) of cultures of sensory neurons (a), sympathetic neurons (b), and cerebellar granule cell neurons (c). In each case, 100 μ g of the solubilized proteins were fractionated, blotted, and probed with a rabbit polyclonal antibody to F3. The blots were then visualized with a secondary antibody conjugated to alkaline phosphatase. (B) Immunoblots of GPI-anchored proteins. Proteins released from the detergent phase of Triton X-114 into the aqueous phase by PIPLC were probed with antibodies to F3. a, DRG neurons; b, SCG neurons; c, CGC neurons. (C) Differential release of the two forms of F3 from cultures of sensory neurons. a, detergent extract of DRG neurons; b, material remaining on a culture of DRG neurons after treatment with PIPLC; c, material released by PIPLC into the culture supernatant; and d, material remaining on cultures of DRG neurons after mock digestion (no PIPLC).

expressed by sensory neurons in vitro (Karagogeos et al., 1991); our failure to detect it in these studies may reflect a lower level of expression under the conditions of culture used here.

Other well characterized GPI-linked proteins in the nervous system include N-CAM (Hemperly et al., 1986) and Thy-1 (Tse et al., 1985). The prominent 120-kD band on sensory neurons was identified as corresponding to the GPI-linked form of N-CAM (Fig. 7 A). A faint band comigrating

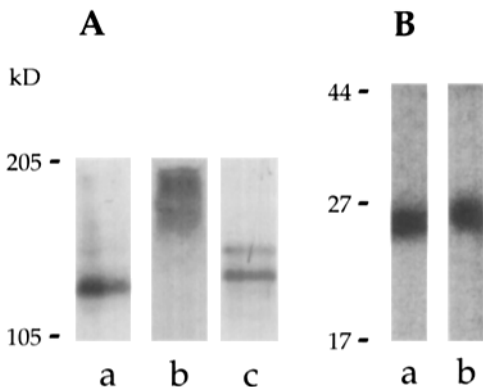


Figure 7. Expression of N-CAM isoforms and Thy-1 by primary neurons. (A) Expression of N-CAM isoforms. Cultures of sensory neurons (a), sympathetic neurons (b), and cerebellar granule cell neurons (c) were solubilized with 1% SDS. 100 μ g of the solubilized proteins were fractionated, blotted, and probed with a rabbit polyclonal antibody to N-CAM which recognizes all three isoforms. The blots were then visualized with a secondary antibody conjugated to alkaline phosphatase. (B) Immunoprecipitation of Thy-1 from neuron cultures. Biotinylated proteins of DRG neurons (a) or SCG neurons (b) were released from the detergent phase of Triton X-114 into the aqueous phase by PIPLC and quantitatively immunoprecipitated with antibodies to Thy-1. Precipitated proteins were fractionated by SDS-PAGE, blotted, and visualized with 125 I-streptavidin.

with this isoform of N-CAM was also seen on SCG, but at markedly reduced levels, and was not present on granule cells.

The finding that the GPI-linked form of N-CAM was prominently expressed by DRG but not by SCG or cerebellar granule cell neurons suggested that N-CAM might be alternatively spliced differently on these three neurons. We therefore investigated the expression of the major N-CAM isoforms on these neurons. Cultures of primary neurons were solubilized with 1% SDS, proteins were fractionated by SDS-PAGE, blotted, and probed with a polyclonal antibody to N-CAM that recognizes the three major isoforms (Fig. 7 A). While all three neurons express substantial amounts of N-CAM, the major isoforms expressed by these neurons are remarkably different. Sensory neurons primarily express the GPI-anchored (120 kD) form of N-CAM, and little, if any, of the two other transmembrane forms of N-CAM. By contrast, SCG neurons express minimal amounts of the GPI-linked form, but abundant amounts of higher molecular weight forms of N-CAM. (Based on its migration as a broad smear of 140 to 200 kD, the SCGs may express sialylated forms of both the 140- and 180-kD isoforms of N-CAM.) The pattern of N-CAM expression on cerebellar granule cells is intermediate between DRG and SCG. Cerebellar granule cells express nearly equal amounts of the 120- and the 140-kD form of N-CAM, but very little if any of the 180-kD form.

Interestingly, the 120-kD form of N-CAM on cerebellar granule cells was not released into the aqueous phase of Triton X-114 after treatment with PIPLC. To rule out the possibility that this N-CAM was cleaved by PIPLC, but remained in the detergent phase because of a hydrophobic modification, we treated intact cultures of granule cells with PIPLC. We were unable to detect release of N-CAM from the cells into the media, whereas similar treatment of sensory neurons released much of the N-CAM from the cell surface (data not shown). If, as is likely, this N-CAM isoform on granule cells is GPI-linked (He et al., 1987), its lack of sensitivity to PIPLC suggests that the glycosylphosphatidylinositol linkage of N-CAM may be posttranslationally modified in these cells.

Finally, the 25-kD protein present on DRG and SCG neurons was identified as Thy-1 by immunoblotting and immunoprecipitation (Fig. 7 B). Thy-1 is prominently expressed by both DRG and SCG neurons (it is the major GPI-linked protein on SCG neurons). Thy-1 on SCG neurons was of consistently higher molecular weight than that present on DRG neurons (27 vs 25 kD). This difference in molecular weight reflects differences in the amount of N-linked sugars as demonstrated by enzymatic deglycosylation of Thy-1 from SCG and DRG neurons with *N*-glycanase. After deglycosylation, the core polypeptide in each case was equivalent in size (data not shown). We were unable to detect any Thy-1 on CGC neurons. This is not simply because of its failure to be released into the Triton X-114 aqueous phase by PIPLC as it is also not detected by immunoblotting total detergent extracts of granule cells.

In addition to these well characterized GPI-linked proteins, we have also identified a number of potentially novel GPI-linked proteins by this procedure. Most prominently, DRG neurons expressed a large amount of a 69-kD protein that was expressed at very low levels on SCG neurons and

was not detectable on granule cells. The DRG also expressed a 65-kD protein that was absent from the SCG. To determine whether these two proteins of DRG neurons are differentially glycosylated forms of the same protein or whether the lower band was proteolytically derived from the larger protein, we performed deglycosylation experiments (Fig. 8). Enzymatic deglycosylation of the two nearly comigrating bands on DRG neurons revealed that they are indeed two distinct proteins. *N*-glycanase treatment reduced the upper band to ~65 kD and the lower band to ~39 kD. Chemical deglycosylation reduced the upper band to ~55 kD, suggesting that it contains mostly O-linked sugars, whereas the lower band, which again shifted to 39 kD, presumably contains only N-linked sugars (data not shown). Interestingly the major GPI-linked protein on CGC neurons of 60 kD also shifted to 39 kD after either chemical or enzymatic deglycosylation, suggesting that it is the same (except for the extent of glycosylation) as the 65-kD protein on sensory neurons. The larger (69 kD) protein on DRG neurons failed to react with antibodies to alkaline phosphatase and acetylcholinesterase, two GPI-anchored proteins of similar molecular mass.

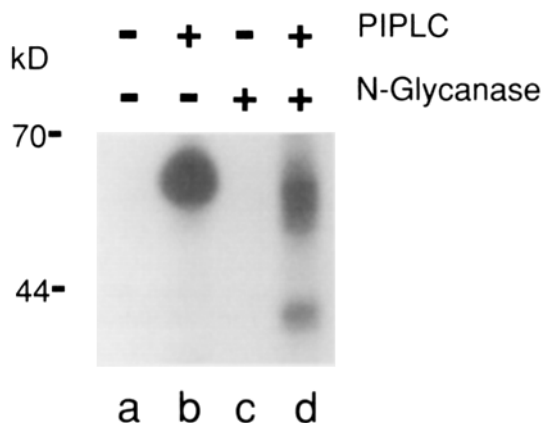


Figure 8. Deglycosylation of the 69- and 65-kD GPI-linked proteins expressed by sensory neurons. The GPI-linked protein fraction was isolated by adding PIPLC (*b* and *d*) to a Tx-114 detergent extraction of a biotinylated DRG sample. Samples were then enzymatically deglycosylated with *N*-Glycosidase (*c* and *d*) before SDS-PAGE, electroblotting, and probing with ¹²⁵I-streptavidin.

On longer autoradiographic exposures (Fig. 4 *B*), we also observed minor proteins of 90 kD present only on SCG neurons (lane *d*) and 38 and 31 kD present only on CGC neurons (lane *f*). Although the 38-kD granule cell protein is of similar molecular mass to prion protein, we were unable to detect any immunoreactivity with two different antiprion protein antibodies consistent with the lack of expression of the prion protein in the cerebellum in vivo (Bendheim et al., 1992). The 31-kD granule cell protein may correspond to a previously described GPI-anchored cerebellar protein of the same molecular mass that is developmentally regulated (Kuchler et al., 1989).

The distribution of these major GPI-anchored proteins is summarized in Table I.

Discussion

In this paper, we have characterized the surface proteins of

Table I. Differential Expression of GPI-linked Proteins

Protein	DRG	SCG	CGC
F3 (135 kD)	++++	+	++++
NCAM (120 kD)	++++	-	****
90 kD	-	++	-
69 kD	++++	tr	-
65 kD	++++	-	++++
39 kD	-	-	++
31 kD	-	-	+
Thy-1 (25 kD)	++++	++++	tr

The relative abundance of various GPI-linked proteins on primary neurons was estimated from four different experiments. Neurons used were sensory neurons of the DRG, sympathetic neurons of the SCG, and CGC. (In companion cultures containing only cerebellar glial cells, none of the proteins listed in the last column were detected indicating that they are all of neuronal origin). Abundance is indicated accordingly: -, not detected; tr, barely detectable on the longest exposures; and +, low abundance to (++++), high level expression. In one case (****) the 120-kD form of N-CAM was readily detectable by immunoblotting but was not recovered in the final aqueous phase after PIPLC treatment.

three types of primary neurons that are differentially ensheathed by glial cells: DRG neurons whose processes are heavily myelinated in vivo and in vitro; SCG neurons whose processes are ensheathed but unmyelinated in vivo and in vitro; and cerebellar granule cells whose fibers remain unmyelinated and unensheathed in vivo. This study is, to our knowledge, the first direct comparison of the composition of cell surface proteins of different neurons in vitro and the first demonstration that nerve fibers with different ensheathment fates display distinct cell surface proteins. The results reported here indicate that these neurons vary both in the combination of widely expressed adhesion molecules they display and by the presence of potentially novel GPI-anchored proteins that are highly restricted in their fiber-type expression. We consider these findings in more detail below.

GPI-anchored Proteins on Neurons

Among the major findings of this paper are that N-CAM isoforms are differentially expressed on different primary neurons. N-CAM is perhaps the most intensively characterized cell adhesion molecule in the nervous system (Edelman, 1986). N-CAM, a member of the immunoglobulin gene superfamily, is believed to promote, via a homophilic mechanism, nerve fiber outgrowth and fasciculation, neuromuscular junction formation, and neuron-glia adhesion. N-CAM exists as three major isoforms that are encoded by a single gene whose primary transcript is alternatively spliced (Cunningham et al., 1987). These isoforms share the same extracellular segment but differ in the size and sequences of their cytoplasmic segments (362 amino acids for the 180-kD form and 101 amino acids for the 140-kD form). The distinct functions of these alternatively spliced cytoplasmic segments have not been defined. The largest isoform, 180 kD, may be enriched at sites of synaptic contact where it could function to stabilize cell contacts (Persohn et al., 1989). It is of interest that this largest isoform is relatively abundant in the cultures of SCG neurons, which form synapses when dissociated in vitro (Johnson et al., 1981), but is absent in the cultures of CGC and DRG neurons which may not.

We found that the GPI-linked form of N-CAM is the major

isoform of sensory neurons. This is not an artifact of the tissue culture system as recent studies using peripheral nerve material also suggest that this is the major isoform present on sensory nerve fibers in vivo (Rosen, C., G. DeVries, and J. Salzer, unpublished observations). These findings emphasize that the GPI-linked N-CAM isoform is not primarily a glial isoform as had been suggested by several earlier studies (Keilhauer et al., 1985; Noble et al., 1985; Bhat and Silberberg, 1988). The synthesis of the GPI-anchored form of N-CAM has been described to increase postnatally in a region-specific manner (Chuong and Edelman, 1984) that parallels myelination (Bhat and Silberberg, 1988); it is the principal N-CAM isoform present on oligodendrocytes (Bhat and Silberberg, 1988). Taken together, these results suggest that the GPI-anchored form of N-CAM may have an important role in promoting the axonal-glial interactions of myelination. However, the abundant expression of the 120-kD form of N-CAM on CGC neurons indicates that it does not have an exclusive role in myelination.

Interestingly, we were only able to detect the 120-kD form of N-CAM on granule cells by immunoblotting detergent extracts of these cells; it was not released by PIPLC from living cultures or from detergent extracts. As previous studies have indicated that granule cells have a GPI-linked form of N-CAM that is at least partially sensitive to release from the cells by the PIPLC from *Staph aureus* (He et al., 1987), our results suggest that either the GPI-anchored form of N-CAM on granule cells is resistant to PIPLC isolated from *B. thuringiensis* (see Low et al., 1988 for discussion) or that it is cleaved but not released, perhaps as a result of cell specific interactions (which are not exhibited by sensory neurons). The most intriguing possibility would be that the N-CAM on granule cells is selectively modified and as a consequence is resistant to cleavage by PIPLC, in contrast to the other GPI-anchored proteins expressed by the granule cells that remain sensitive to PIPLC. While such modifications, including palmitoylation of the GPI anchor, are well described, they have been thought to affect all proteins within the same cell (Toutant et al., 1990). Confirmation of a selective modification and its possible functional significance will require further investigation.

F3/F11 is another GPI-linked member of the immunoglobulin gene superfamily (Brummendorf et al., 1989; Gennarini et al., 1989) that was detected in varying levels on these neurons. We found that F3 is abundantly expressed on both DRGs and CGCs, but is expressed at much lower levels on SCGs (Fig. 6). Like N-CAM, F3 appears to be differentially glycosylated in different neurons as the F3 detected at the cell surface of DRG neurons is larger than that expressed on granule cells. F3 was originally identified because of its ability to promote nerve fiber outgrowth (Chang et al., 1987) and was recently shown to promote cell adhesion (Gennarini et al., 1991), although it is not yet clear whether the mechanism is homophilic or heterophilic. Its role in mediating axonal-glial interactions has not yet been characterized.

We have also found an abundant GPI-anchored protein of ~60 kD on cerebellar granule cells and ~65 kD on sensory neurons of the DRG that was not detected on sympathetic neurons of the SCG. This protein, which has a core polypeptide size of 39 kD after chemical or enzymatic deglycosylation of both the DRG and SCG bands, corresponds closely in size to the Opiate Binding Protein Cell Adhesion Mole-

cule (OBCAM) (Cho et al., 1986). OBCAM was originally isolated based on its potential opiate binding properties, and was subsequently found on cDNA cloning to be a GPI-linked member of the immunoglobulin gene superfamily (Schofield et al., 1989), suggesting that it may function as a cell adhesion molecule. If this protein is confirmed as OBCAM, this would be the first demonstration that it is indeed GPI anchored (as had been predicted from its primary sequence) and its abundance and distribution would support a proposed role as a mediator of cell-cell interactions in the nervous system.

Thy-1 was found to be abundantly expressed on both DRGs and SCGs, but not to be present on CGC neurons. The lack of expression of Thy-1 on CGC neurons is consistent with previous studies demonstrating that it is absent on cerebellar granule cells in vivo (Bolin and Rouse, 1986). The expression of Thy-1 has been correlated with the cessation of axonal outgrowth in vivo (Xue et al. 1991) and with the inhibition of nerve fiber outgrowth in vitro (Mahanthappa, N. K., and P. H. Patterson, 1989. *Soc. Neurosci. Abstr.*). Its precise role in mediating cell interactions, including axonal-glial interactions, is not yet known.

In addition to these abundant GPI-linked proteins, each class of neurons expresses one or more proteins that are highly restricted in their expression. These proteins include an abundant GPI-linked protein of 69 kD on DRGs that is present at markedly reduced levels on SCGs (<1% of the level on sensory neurons), and not detectable on granule cells. This protein is currently the only protein that we have identified that distinguishes sensory neurites from sympathetic and granule cell neurites and it may therefore have a role in promoting the axonal-glial interactions of myelination. We have also detected several minor proteins that are highly restricted in their expression. These include a 90-kD protein that is specifically expressed on sympathetic neurons and proteins of 38 and 31 kD that were detected only on granule cell neurons. The 31-kD protein may correspond to a previously described GPI-anchored protein, present on cerebellar granule cells (Kuchler et al., 1989), that may be important in the migration of granule cells on astroglial processes during development (Lehmann et al., 1990). For each of the proteins, it remains possible that, like N-CAM, their apparently restricted pattern of expression may reflect the existence of alternatively spliced transmembrane isoforms that are differentially expressed or the presence of a GPI anchor that is differentially sensitive to cleavage with PIPLC. Confirmation of the restricted expression of these four proteins and identification of any alternatively spliced isoforms awaits further characterization of each of these proteins, including generation of specific antibodies.

Significance of the GPI Anchor

The striking differences in the expression of GPI-anchored proteins by these three neurons is in sharp contrast to their otherwise similar composition of integral membrane proteins (Fig. 3), and suggests that GPI-anchored proteins have an important role as generators of neuronal diversity. While the precise function of the GPI linkage is not known (Low, 1989), this type of linkage may be particularly well suited for molecules that function in neuronal interactions. GPI-linked proteins, which are anchored to the outer leaflet of the

plasma membrane, are believed to be more mobile in the plane of the membrane than are transmembrane proteins (Ishihara et al., 1987; Noda et al., 1987). As such, GPI-linked proteins are attractive candidates for mediating the dynamic remodeling of membranes that occur during nerve fiber outgrowth, synaptogenesis, and particularly in myelination by allowing for a stable but potentially fluid adhesion between the plasma membranes of apposed cells. In addition, the increased mobility of GPI-anchored proteins within the membrane may promote adhesion by facilitating the recruitment of these proteins into membrane domains engaged in adhesion (Chan et al., 1991).

The GPI linkage is a signal for targeting proteins to the apical membrane domain of polarized epithelial cells (Lisanti et al., 1989); e.g., the GPI-anchored form of N-CAM is expressed at the apical surface of transfected MDCK cells whereas the transmembrane forms are expressed basolaterally (Powell et al., 1991). The GPI anchor could potentially serve a similar function in the nervous system (Lisanti and Rodriguez-Boulan, 1990) by targeting such proteins to specific sites of cell-cell interactions. In some studies, GPI-linked proteins in the nervous system have been found to be spatially restricted in their expression. Thy-1 and TAG-1, for example, have been reported to be present on axons, while being excluded from cell bodies and dendrites (Dodd et al., 1988; Dotti et al., 1991). However, other studies suggest that the distribution of such proteins in vivo may be quite complex and significantly modified as the result of specific cell-cell interactions (Xue et al., 1991; Faivre-Sarrailh et al., 1992). In our studies, we have found that all the major GPI-anchored proteins of DRG neurons are abundantly expressed on their processes and that F3, N-CAM, and Thy-1 are also present on their cell somas (Rosen, C., and J. Salzer, unpublished observations).

Another potential role of the GPI anchor may be to provide a mechanism for regulating the abundance of these proteins at the cell surface and their release into the extracellular space by serving as a substrate for anchor specific phospholipases (Low, 1989). For example the abundance of fasciclin I associated with the plasma membrane during *Drosophila* embryogenesis varies during development, possibly by a developmentally regulated cleavage of its GPI anchor (Hortsch and Goodman, 1990). Proteins thus released from the cell surface might in turn bind to their cognate receptor on other cells thereby inhibiting cell interactions (Furley et al., 1990). Cleavage of the GPI-anchor by endogenous phospholipases would generate free diacylglycerol that could act as a second messenger. While such effects would have clear significance for signaling in cell-cell interactions, they have not yet been confirmed experimentally.

In addition to their role in cell adhesion discussed above, GPI-anchored proteins have a variety of other functions (Ferguson and Williams, 1988). Thus, a number of hydrolytic enzymes are known to be GPI linked as are a growing number of membrane receptors (Selvaraj et al., 1988; Simmons and Seed, 1988; Rothberg et al., 1990; Ploug et al., 1991) including the recently cloned ciliary neurotrophic factor receptor (Davis et al., 1991). Most of the proteins described in this study are relatively abundant, suggesting that they have a noncatalytic role. Although the functions of the apparently novel GPI-anchored proteins described in this report are not known, by analogy to the other abundant GPI-

linked proteins on these neurons (i.e., F3/F11, N-CAM, and Thy-1) and GPI-anchored proteins with a restricted distribution in the invertebrate nervous system (fasciclin I [Hortsch and Goodman, 1990], chaoptin [Krantz and Zipursky, 1990], and amalgam [Seeger et al., 1988]), we favor a role for these proteins in mediating cell interactions specific to their fiber type.

While we have identified differences in the composition GPI-anchored proteins on three types of neurons, we do not wish to imply that these are the only or even the major differences between these classes of neurons. Other integral membrane proteins present at lower abundance may vary between these fiber types (Fig. 3) but are difficult to detect against the highly complex pattern of surface proteins. In addition, differences in other membrane constituents notably glycolipids and proteoglycans, which are also likely to play critical roles in cell interactions and in some cases are known to differ between different types of neurons (Herndon and Lander, 1990; Jessell et al., 1990), would not be detected by the approaches used here. Finally, in these studies we have only examined those proteins that are constitutively expressed by neurons in vitro in the absence of normal in vivo developmental cues. Other cell adhesion molecules may be expressed on each of these neurons in vivo as a result of heterologous cell interactions, for example with ensheathing glial cells, or in response to local soluble factors such as neurotrophins.

In summary, we have found significant differences in the cell surface proteins displayed by three types of neurons, particularly in the composition of their GPI-linked proteins. While these compositional differences are likely to be relevant to the different ensheathment fates of these nerve fibers, they may also reflect other differences in the cell-cell interactions exhibited by these fibers, including their outgrowth, pathfinding and choice of synaptic targets during development. Future studies should clarify the role of these GPI-anchored proteins in specifying the mode of ensheathment of these nerve fibers and in mediating specific cell-cell interactions in the nervous system.

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