Two Membrane Protein Fractions from Rat Central Myelin with Inhibitory Properties for Neurite Growth and Fibroblast Spreading

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Abstract. Lack of neurite growth in optic nerve explants in vitro has been suggested to be due to nonpermissive substrate properties of higher vertebrate central nervous system (CNS) white matter. We have searched for surface components in CNS white matter, which would prevent neurite growth. CNS, but not peripheral nervous system (PNS) myelin fractions from rat and chick were highly nonpermissive substrates in vitro. We have used an in vitro spreading assay with 3T3 cells to quantify substrate qualities of membrane fractions and of isolated membrane proteins reconstituted in artificial lipid vesicles. CNS myelin nonpermissiveness was abolished by treatment with proteases and was not associated with myelin lipid. Nonpermissive proteins were found to be membrane bound and yielded highly nonpermissive substrates upon reconstitution into liposomes. Size fractionation of myelin protein by SDS-PAGE revealed two highly nonpermissive minor protein fractions of M_r 35 and 250-kD. Removal of 35- and of 250-kD protein fractions yielded a CNS myelin protein fraction with permissive substrate properties. Supplementation of permissive membrane protein fractions (PNS, liver) with low amounts of 35- or of 250-kD CNS myelin protein was sufficient to generate highly nonpermissive substrates. Inhibitory 35- and 250-kD proteins were found to be enriched in CNS white matter and were found in optic nerve cell cultures which contained highly nonpermissive, differentiated oligodendrocytes.

The data presented demonstrate the existence of membrane proteins with potent nonpermissive substrate properties. Distribution and properties suggest that these proteins might play a crucial inhibitory role during development and regeneration in CNS white matter.

THE differentiated central nervous system (CNS)[1] of higher vertebrates is capable of only very limited regenerative neurite growth after lesions. On the other hand, extensive regenerative growth is found in the CNS of lower vertebrates and in the peripheral nervous system of all vertebrates including man. Transplantation experiments performed by Aguayo and his colleagues have demonstrated that lack of regeneration is not an intrinsic property of CNS neurons, as these readily extend processes into implanted peripheral nervous tissue (Benfey and Aguayo, 1982; Richardson et al., 1984; So and Aguayo, 1985). Conversely, PNS neurons fail to extend processes into central nervous tissue, thus indicating the existence of fundamental differences between the two tissues (Aguayo et al., 1978; Weinberg and Spencer, 1979). While one major difference consists in a differential distribution of the neurite outgrowth promoting extracellular matrix component laminin (Liesi, 1985; Carbonetto et al., 1987), recent evidence points to additional factors. Thus, Schwab and Thoenen found drastic differences in neurite growth supporting properties of sciatic and of optic nerve explants in vitro in spite of the presence of laminin immunoreactivity in both explants (Schwab and Thoenen, 1985; Schwab, M. E., unpublished observation). As these experiments were carried out in the presence of optimal amounts of neurotrophic factors and as the differences persisted upon freezing of tested substrates, it was suggested that absence of growth into optic nerve explants may be due to the presence of unfavorable surface components. To test this hypothesis, we have examined substrate properties of adult central and peripheral nervous tissue in supporting neurite outgrowth in vitro. In a companion paper (Schwab and Caroni, 1988) we show that myelin-forming oligodendrocytes and CNS myelin are nonpermissive substrates for neurite extension and fibroblast spreading in vitro. Here we show that CNS myelin of higher vertebrates contains minor membrane-bound proteins of M_r 35 and of 250 kD, which seem to account for its non-permissive substrate properties. Such properties are not found in CNS myelin from trout or frog. Furthermore, as inclusion of small amounts of these proteins is sufficient to convert a neutral substrate into a nonpermissive one, we conclude that they are potent inhibitors of neurite outgrowth and of fibroblast spreading. This is to our knowledge the first description of minor membrane proteins conferring general nonadhesive properties to the membranes which incorporate them. We discuss the potential importance of such adhesion

1. *Abbreviations used in this paper*: medium A, 30 mM Hepes (pH 7.4); CNS, central nervous system; PLYS, polylysine; PNS, peripheral nervous system.

inhibitors in the context of the morphogenetic role played by differential adhesiveness during nervous system development.

Materials and Methods

Cell Culture

Mouse NIH 3T3 cells were cultured and assayed for spreading behavior in DME containing 10% FCS. In control experiments, use of defined serum-free medium did not alter responses of 3T3 cells to tested substrates. Mouse neuroblastoma cells (line NB-2A) were cultured in DME with 10% FCS in the presence of either 1 mM dibutyryl-cAMP or of glia-derived neurite promoting factor (Günther et al., 1985). Superior cervical and dorsal root ganglia from newborn rats were dissected and dissociated into single cells as described (Mains and Patterson, 1973; Schwab and Thoenen, 1985). Neurons were cultured in an enriched L15 medium with 5% rat serum (Mains and Patterson, 1973) and with 100 ng/ml of 2.5 S nerve growth factor. Overgrowth by contaminating dividing cells was prevented by inclusion of cytosine arabinoside (10^{-5} M) in the culture medium.

Sources of Tested Substrates

Myelin fractions were all prepared by the same procedure, involving tissue homogenization in isotonic sucrose buffer with a polytron (model PCU-2; Kinematica, Luzern, Switzerland) homogenizer (setting 4, two times 30 s, on ice) and flotation of a low speed supernatant onto a discontinuous sucrose gradient (myelin collected at 0.25 M sucrose top layer) (Colman et al., 1982). All isolation media contained trasylol (aprotinin; Sigma Chemical Co., St. Louis, MO; 100 U/ml), 5 mM iodoacetamide, and 5 mM EDTA to reduce protease digestion (this reagent mixture is designated below as protease inhibitors). Finally, myelin fractions were washed hypotonically in 30 mM Hepes (pH 7.4) (medium A) plus protease inhibitors and frozen in aliquots at −80°C. CNS myelin fractions were prepared from spinal cords carefully stripped of ventral and dorsal roots, or from rat optic nerves. The following sources were used: rat, spinal cord, 3 mo old Lewis rats, male; chick, spinal cord, P21; trout, spinal cord, 2 y; frog, spinal cord, 6 mo. PNS myelin fractions were prepared from rat sciatic nerves (3 mo, male). Rat liver cell membranes were prepared by standard procedures, involving mild isotonic homogenization and collection of membranes at the density of 0.25 M sucrose (discontinuous sucrose gradient). Rat CNS tissue enriched in gray matter was obtained from superficial neocortex layers, whereas white matter–enriched tissue consisted of the corpus callosum.

Substrate-assaying Procedure

Substrates to be tested, in 40–70 mOsmol solutions, were dried onto polylysine (PLYS)-coated tissue culture dishes. Unbound membranes and solutes were removed by three washes with Ca^{2+} Mg^{2+}-free Hank's solution. Coated dishes were then immediately used in substrate-testing assays. For most experiments, substrates were dried onto the wells of dishes (35-mm dishes with four internal wells; Greiner, Nürtingen, Federal Republic of Germany). 3T3 cells were detached from ~30% confluent cultures by brief trypsin (0.2%) treatment in 37°C PBS plus EDTA. Trypsinisation was stopped by 10-fold excess of serum-containing DME; cells were collected and resuspended in DME-10% FCS at appropriate concentrations. 30,000 cells/cm^2 were added to precoated culture dishes and experiments were scored after 1 h in culture. In some cases, due to occasional slower spreading behavior of 3T3 cell populations, scoring had to be delayed up to 2 h in culture. After periods of more than ~5 h, inhibitory properties of myelin fractions in the absence of serum were less pronounced than observed in the presence of 10% FCS, possibly due to the presence of substrate digesting proteases. If substantial spreading on PLYS-coated dishes (e.g., see Table I) was not obtained within 2 h in culture, tests were discarded and repeated with a fresh batch of cells. Quantitative evaluation of spreading was performed with a surface integration program on at least 30 cells per experimental point. Only spread cells were considered and a zero-spreading value (strongly refractory and round cells) was subtracted. Each experiment was repeated at least five times. Experiments were found to be subject to only small quantitative variations, and values from representative experiments are given. Spreading degrees are given as means plus minus standard error of the mean. When recoveries of inhibitory activity were estimated, serial dilutions of liposomes (in medium A) were assayed for nonpermissiveness. In some cases, in order to detect differences among strong inhibitory substrates, 3T3-spreading times were extended to up to 5 h. Recovery values are based on internal calibration with a CNS myelin liposome standard, and are to be considered as first approximations. When neurite extension was evaluated, neuroblastoma cells or superior cervical ganglia neurons were seeded at ~25,000 cells/cm^2 and experiments were scored after 24 h in culture.

Substrate Processing

Protease sensitivity of inhibitory fraction was determined by digesting washed, protease inhibitor–depleted membranes with trypsin. Membrane fractions (concentrations of maximally 1 mg of protein per ml) were exposed for 10 min at room temperature to 0.1% trypsin. Digestion was interrupted by the addition of 0.2% trypsin inhibitor (Sigma Chemical Co.) and membranes were either washed in medium A or separated from protease by sephadex G50 chromatography (liposomes). Under these conditions, trypsin was retarded by the column, whereas liposomes were recovered in the excluded volume. Digested, washed membranes were finally adsorbed to culture dishes and their substrate properties were analyzed as described in the previous section. In some experiments with myelin fractions, pronase (Sigma Chemical Co.) or elastase (Sigma Chemical Co.) were used. In those cases, protease was removed by three washes of the myelin in 30 mM Hepes, pH 7.4.

Extraction of peripheral membrane proteins from CNS myelin was performed by resuspending membranes in either 4 M guanidinium Cl (Merck & Co., Inc., Rahway, NJ)/30 mM Hepes or in 500 mM unbuffered Trizma base (Sigma Chemical Co.). Protease inhibitors were routinely included in the extraction buffers. After incubation for 30 min at room temperature, myelin was sedimented, washed in medium A, and assayed for its substrate properties.

Ethanol/ether (2:3 vol ratios) extraction of myelin was performed by a standard procedure (see, for example, Everly et al., 1973). Solvent-insoluble fraction was reconstituted into lipid vesicles (see next section); mainly lipid-containing soluble fraction was dried and reconstituted by the cholate method (see next section).

Liposomes

Liposomes were prepared in medium A by the cholate method (Brunner et al., 1978). Protein was solubilized in 2% SDS (in medium A plus protease inhibitors); insoluble protein was sedimented and discarded. Solubilized protein was precipitated with a 30-fold excess of acetone. To obtain reproducible yields, acetone precipitation was allowed to proceed for 15 h at 4°C. Protein extracts from tissues were prepared by homogenization of minced tissue with a glass-teflon potter in 2% SDS-containing, protease inhibitors-supplemented, medium A. Solubilized protein was then precipitated with ice-cold acetone as described above. Extracts from cultured cells were prepared by, first, detaching the cells with a rubber policeman in the presence of PBS plus EDTA plus protease inhibitors, and by then homogenizing suspended cells with a glass-teflon potter. Upon low-speed pelleting of nuclear material, 2% SDS was added to supernatants and solubilized protein was precipitated with ice-cold acetone. In all cases, acetone-precipitated protein was sedimented (10,000 *g*, 15 min) and resuspended at 1 mg/ml in medium A with 2.5% cholate. Phospholipids (phosphatidylcholine/phosphatidylserine, 10:1) dissolved in medium A with 2.5% cholate were then added (~5–10:1 ratio of added phospholipid to protein) and liposomes were formed on a Sephadex G-50 column. When gel-extracted protein was reconstituted, precipitated protein was resuspended at ~50 μg/ml and phospholipid to protein ratios were up to 100:1.

A number of control experiments were performed. Thus, acetone-

Table I. Spreading Extent of 3T3 Fibroblasts on Permissive and on Nonpermissive Substrates

Substrate	Spreading extent
	μm^2
PLYS	1,789 ± 249
Rat CNS myelin	249 ± 68
Rat PNS myelin	1,812 ± 166
Liver membranes	1,849 ± 211

3T3 cells were seeded onto substrate-adsorbed wells of dishes (Greiner), photographs were taken after 1 h in culture, and spreading extent was determined as described in the Materials and Methods section. Amounts of protein to be adsorbed were 20 μg/cm^2 for each substrate. Values given as mean ± SEM.

precipitated myelin or gel-extracted protein did not prevent 3T3 spreading when resuspended in medium A or in medium A with 2.5% cholate and adsorbed directly onto PLYS-coated dishes. Also, running of cholate-solubilized protein on the Sephadex G-50 column, in the absence of phospholipids did not yield nonpermissive fractions. Experiments with ^{125}I-labeled CNS myelin protein showed that ~30% of applied label was recovered in the liposome fraction from the G-50 column. In some experiments, lipid vesicles were formed in the presence of trace amounts of [^{3}H]cholesterol and were then dried onto the wells of tissue culture dishes (Greiner). Total culture dish associated membrane amounts ([^{3}H]cholesterol) were found to vary independently of tested protein, indicating that differences in liposome binding to culture dishes cannot be responsible for the observed differences in substrate properties (Table III).

Gel-extracted Protein Fractions as Substrate

Protein was run on 3–15% gradient gels under reducing conditions. For this purpose, samples were preincubated for 30 min at room temperature in sample buffer containing 2% SDS and β-mercaptoethanol. Thin lanes were cut and stained with Coomassie Brilliant Blue or with the silver method. Protein bands to be analyzed were carefully aligned with the unstained gel parts to be extracted. Gel regions from the unstained gel part were cut, and minced gel was extracted for 1 h with 0.5% SDS. In most experiments, 50 μg/ml of insulin (Sigma Chemical Co.) were included in order to reduce losses due to adsorption of low concentration protein. Insulin was selected for its purity and for its small size, resulting in efficient separation from the liposome fraction. In control experiments, no substrate differences could be detected when 50 μg/ml of insulin were added to various reconstitution mixtures, including protein-free liposomes. Gel-extracted protein was precipitated with 10-fold excess of ice-cold acetone (15 h) and sedimented protein was resuspended in cholate buffer. Protein was stored frozen in cholate buffer and reconstitution mixtures were prepared from these protein stocks. Reconstitution and test of substrate properties were performed as described above.

Other Methods

Protein was determined by the filter binding method (Schaffner and Weissmann, 1973) with BSA (Sigma Chemical Co.) as a standard.

Results

Rat CNS Myelin Is a Nonpermissive Substrate for Neurite Outgrowth

A comparison of the substrate properties of rat CNS (spinal cord) and PNS (sciatic nerve) myelin, of trout CNS (spinal cord) myelin, and of PLYS-coated tissue culture plastic in supporting nerve growth factor–induced neurite outgrowth from rat superior cervical ganglion neurons is shown in Fig. 1. Neurons were seeded onto a PLYS-coated dish onto which islets of substrates to be tested had been previously adsorbed. Neurons readily extended processes on rat PNS myelin and on frog CNS myelin, whereas rat CNS myelin-coated surfaces were strictly avoided. Most cells did not attach to CNS myelin-coated substrate. Those that did attach occasionally extended short, bundled processes with minimal contact with the substrate. Analogous results were obtained when myelin was prepared from rat optic nerve or from rat brain. Neuron type apparently did not influence substrate response as similar results were obtained with dorsal root ganglion neurons. Likewise, rat CNS myelin nonpermissiveness was observed for dibutyryl-cAMP-induced or glia-derived neurotrophic factor-induced outgrowth from neuroblastoma cells. Thus, nonpermissiveness of rat CNS myelin is apparently general with regard to neuron type and induction of neurite outgrowth.

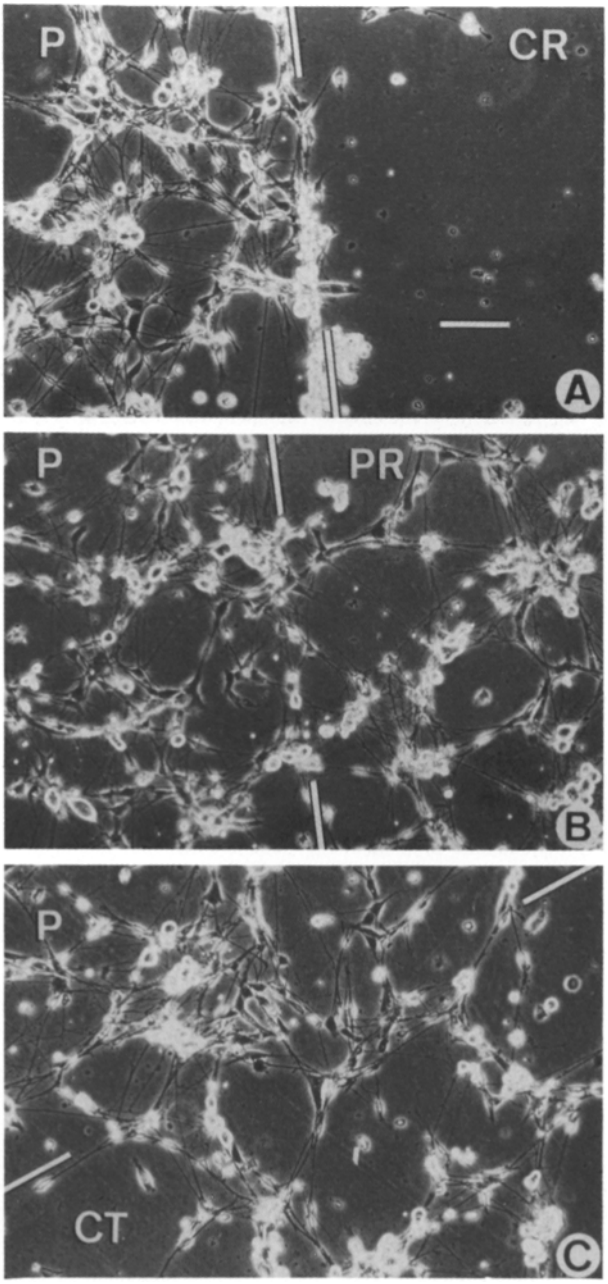

Figure 1. Rat CNS myelin is a nonpermissive substrate for cell attachment and neurite outgrowth. Tested substrates were dried onto one-half of PLYS-coated tissue culture dishes. Substrate boundary line is indicated by side bars. SCG neurons were plated in the presence of NGF and of cytosine arabinoside; cultures were examined after 24 h. (*A*) PLYS (*P*), rat CNS myelin (*CR*) boundary. (*B*) PLYS (*P*), rat PNS myelin (*PR*) boundary. (*C*) PLYS (*P*), trout CNS myelin (*CT*) boundary. Bar, 50 μm.

Impaired Spreading of Fibroblasts: Quantitative Assay for Rat CNS Myelin Nonpermissiveness

Unfavorable substrate properties of rat CNS myelin did not affect only neurons but could also be observed to affect the spreading and migration of 3T3 fibroblasts. Fibroblasts spread slowly and only to a very limited extent when seeded onto CNS myelin (Fig. 2). Again, spreading was normal on rat PNS myelin (Fig. 2), and on trout CNS myelin. Spreading

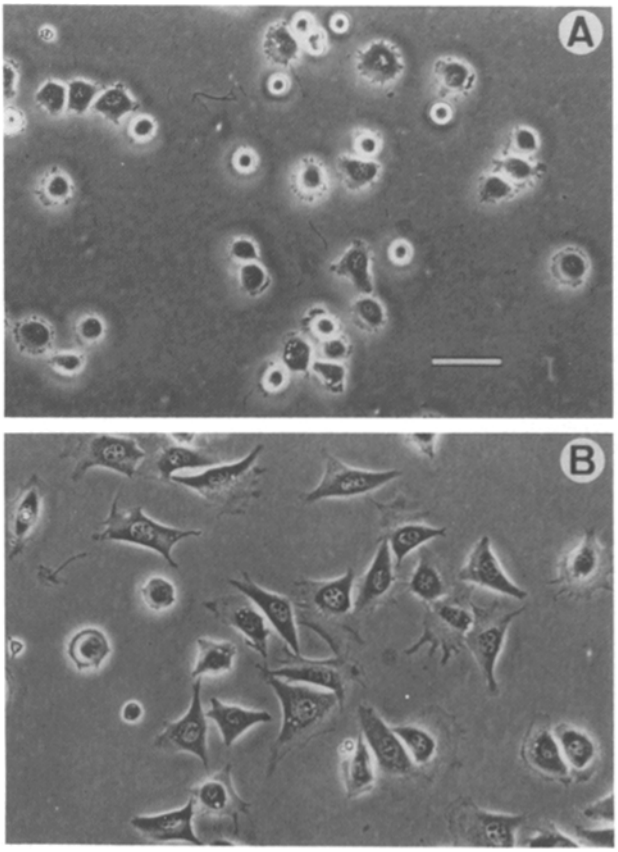

Figure 2. Rat CNS myelin is a nonpermissive substrate for 3T3 fibroblasts. 3T3 cells (1 h in culture) on (*A*) rat CNS myelin, and (*B*) rat PNS myelin. Bar, 50 μm.

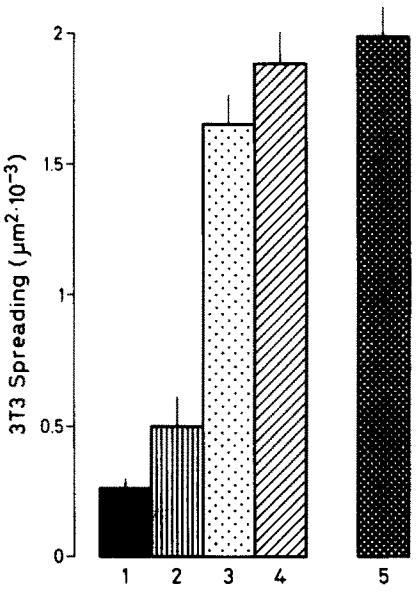

Figure 3. Substrate properties of CNS myelin fractions from rat, chick, trout, and frog. Spinal cord myelin fractions from different species were adsorbed to PLYS-coated wells of dishes (Greiner). 3T3 cells were added and experiments were scored after 1 h. Spreading values are given as mean ± SEM. Substrates: myelin fractions from: (*1*) rat CNS; (*2*) chick CNS; (*3*) trout CNS; (*4*) frog CNS; (*5*) rat PNS.

of 3T3 fibroblasts on different substrates was quantitated. As shown in Table I, 3T3 fibroblast spreading was markedly reduced on rat CNS myelin but not on peripheral myelin nor on a liver membrane fraction. Differences in the extent of spreading were most reproducibly observed after short periods (1 h) of substrate contact. Differences tended to diminish or even to vanish after prolonged exposure to the substrate (10 h). Possible interpretations of this diminution include protease digestion of substrate, matrix production by the fibroblasts, and possibly also progressive detachment of dish-adsorbed substrate. Also, it was important to seed cells at comparable densities (30,000 cells/cm²). When these precautions were observed, spreading data were found to be highly reproducible. Differences in substrate properties as determined for fibroblasts were found to vary accordingly when neurons were tested (see also Figs. 1 and 5).

Nonpermissive Substrate Effect Is Found in CNS Myelin of Higher Vertebrates (Chick, Rat), but Not of Lower Vertebrates (Trout, Frog)

Lack of regenerative fiber growth is found in the CNS of higher vertebrates but not in those of fishes and to a limited extent in those of amphibia (Bohn et al., 1982; Stensaas, 1983; Hopkins et al., 1985; Liuzzi and Lasek, 1986). We, therefore, prepared spinal cord myelin fractions from trout, frog, and chick in order to determine potential differences in substrate properties. Trout (Figs. 1 and 3) and frog (Fig. 3) CNS myelin fractions have substrate properties similar to those of rat peripheral myelin, whereas CNS myelin from the chick (spinal cord, postnatal 21) is a nonpermissive substrate, although slightly less so than its rat counterpart.

Membrane-bound Protein Fraction of Rat CNS Myelin Is Responsible for Its Nonpermissive Substrate Properties

Rat CNS myelin was processed by standard procedures in order to determine the nature of the component(s) responsible for its non-permissive substrate properties. Fractions were tested for reduction of 3T3 fibroblast spreading. Data are shown in Table II. Brief treatment of the myelin with trypsin

Table II. Nonpermissiveness of CNS Myelin Is due to Membrane-bound Protein

Substrate	3T3 spreading
	μm²
Tissue culture plastic	1,646 ± 309
CNS myelin	
Untreated	211 ± 30
Trypsin-treated	1,344 ± 181
Liposomes	
Ethanol/ether-soluble myelin fraction	1,253 ± 159
Ethanol/ether-insoluble myelin fraction	226 ± 45
Artificial lipid vesicles, no additions	1,328 ± 136

Spreading extent of 3T3 cells was estimated after 1 h in culture. Protein amounts to be adsorbed to wells of dishes (Greiner) were 20 μg of CNS myelin protein per cm². In the solvent extraction experiments, 100 μg of CNS myelin protein were extracted and one-fifth of resulting liposome-containing volume was dried onto wells. These myelin quantities represent about 10 times saturation levels with respect to observed nonpermissiveness.

abolished nonpermissiveness. Similar results were obtained with elastase or with pronase treatment (not shown).

Extraction of the myelin under conditions that solubilize peripheral membrane proteins (4 M guanidinium chloride or pH 10.5) failed to dissociate nonpermissiveness from low speed myelin membrane pellets. Lipid extraction with ethanol/ether yielded a permissive lipid fraction and a nonpermissive protein fraction (Table II). The latter required detergent to be solubilized and had to be incorporated into lipid vesicles in order to permit detection of nonpermissive substrate property. In control experiments, phosphatidylcholine/phosphatidylserine liposomes were a slightly less favorable substrate than tissue culture plastic (Table II). When CNS myelin protein-containing liposomes were subjected to trypsin treatment, their nonpermissive substrate properties were abolished (Table III). Thus, a membrane-bound protein fraction from rat CNS myelin is a nonpermissive substrate for 3T3 fibroblast spreading. That fraction can apparently be reconstituted in active form into artificial lipid vesicles. In control experiments, protein from membrane fractions with permissive substrate properties (rat PNS myelin, liver membranes, see Table I) yielded, upon reconstitution, liposomes that were permissive for 3T3 spreading (Table III).

Identification of 35- and 250-kD Minor Proteins from Myelin as Nonpermissive Substrates for Fibroblast Spreading and Neurite Outgrowth

As myelin nonpermissiveness partially survived denaturing procedures, attempts were made to identify responsible components following separation by SDS-PAGE. In preliminary experiments, it was found that solubilization of myelin proteins in SDS-PAGE sample buffer followed by reconstitution of acetone-precipitated protein yielded a fraction possessing ~30% of starting nonpermissiveness. Apparent activity recoveries were estimated by assaying serial dilutions of reconstituted protein with the 3T3 fibroblast spreading assay. As a comparison, solubilization in 2% NP-40, 0.5% Na-deoxycholate yielded apparent activity recoveries of ~80% (data not shown). When CNS myelin protein was run on SDS-PAGE and the entire gel was then extracted with 0.5% SDS, recoveries of nonpermissive substrate activity were ~20%. Activity could be recovered in approximately equal amounts (~10% of applied activity) from gel regions corresponding to the migration distance of 35- and of 250-kD proteins, respectively (Fig. 4). The inhibitory proteins were highly effective, as <10 ng of 250-kD protein per cm^2 of culture dish were required to obtain half-maximal inhibition. Neither the 250- nor the 35-kD region contained major myelin protein bands (each region contained <3% of total silver stained myelin protein). These gel regions apparently contained more than one protein species. Reconstitution of pooled gel regions depleted of 35- and of 250-kD proteins yielded permissive liposomes (Fig. 5). Thus, 35- and 250-kD proteins account for most of the nonpermissive substrate activity of gel-extracted CNS myelin protein. Similarly to unfractionated myelin, 35- and 250-kD proteins were nonpermissive substrates for fibroblast spreading and for neurite extension (Fig. 5). In control experiments, sciatic nerve protein or a liver homogenate did not generate 250- nor 35-kD nonpermissive protein fractions (Fig. 6). It seems, therefore, reasonable to conclude that the protein fractions identified above are responsible for the marked nonpermissive substrate properties of rat CNS myelin in vitro.

We next asked, whether addition of these proteins to fractions with neutral substrate properties is sufficient to generate a non-permissive substrate. As shown in Fig. 6, liver protein and sciatic nerve protein could yield non-permissive substrates for 3T3 cells when supplemented with 250- or with 35-kD proteins from rat CNS myelin. In these experiments, 250- and 35-kD proteins were added to amounts of liver (or sciatic nerve) protein equivalent to the ones of total CNS myelin protein from which they were prepared. We conclude that 35- and 250-kD proteins from rat CNS myelin act as inhibitors of neurite outgrowth and of fibroblast spreading, as their addition converts a neutral substrate into a nonpermissive one.

Nonpermissive Substrate Property Is Enriched in CNS White Matter and in Cultured Oligodendrocytes

Considering the documented poor regenerative fiber growth found in mature CNS white matter (Nornes et al., 1983; Björklund and Stenevi, 1984), it was of particular interest to determine whether 35- and 250-kD neurite outgrowth-inhibiting proteins from CNS myelin are enriched in CNS white matter and in myelin forming cells. We have prepared pro-

Table III. Nonpermissive Substrate Property of CNS Myelin Is Preserved upon Reconstitution into Artificial Lipid Vesicles

Reconstituted protein fraction	3T3 spreading	Dish-adsorbed lipids
	μm^2	*cpm [³H]cholesterol*
No protein	1,638 ± 91	521 ± 65
CNS myelin	136 ± 30	650 ± 58
CNS myelin; resulting liposomes trypsinized	1,397 ± 152	630 ± 32
PNS myelin	1,570 ± 136	620 ± 41
liver membranes	1,445 ± 121	750 ± 47

Tested protein fractions (100 μg) were reconstituted and one-fifth of resulting liposome-containing volume (60 μl) was adsorbed to wells. The adsorbed volume contained ~20,000 cpm [³H]cholesterol. Dish-adsorbed counts were determined upon SDS solubilization of adsorbed liposomes. For these experiments, liposomes were removed prior to fibroblast addition. Trypsinisation of CNS myelin liposomes and separation of inhibitor-blocked trypsin from vesicles was performed as described in the Materials and Methods section.

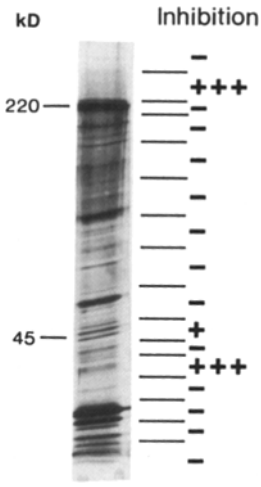

Figure 4. SDS-PAGE fractionation of rat CNS myelin protein. Nonpermissiveness comigrates with proteins of 250 and of 35 kD. 3–15% polyacrylamide-reducing gradient gel of rat CNS myelin protein. Protein from indicated gel regions was extracted and nonpermissiveness of corresponding liposomes was estimated. Activity-containing regions of ~35 and 250 kD are defined in the following in the same way as is done here. Molecular masses were estimated from commercial standards (Sigma Chemical Co.).

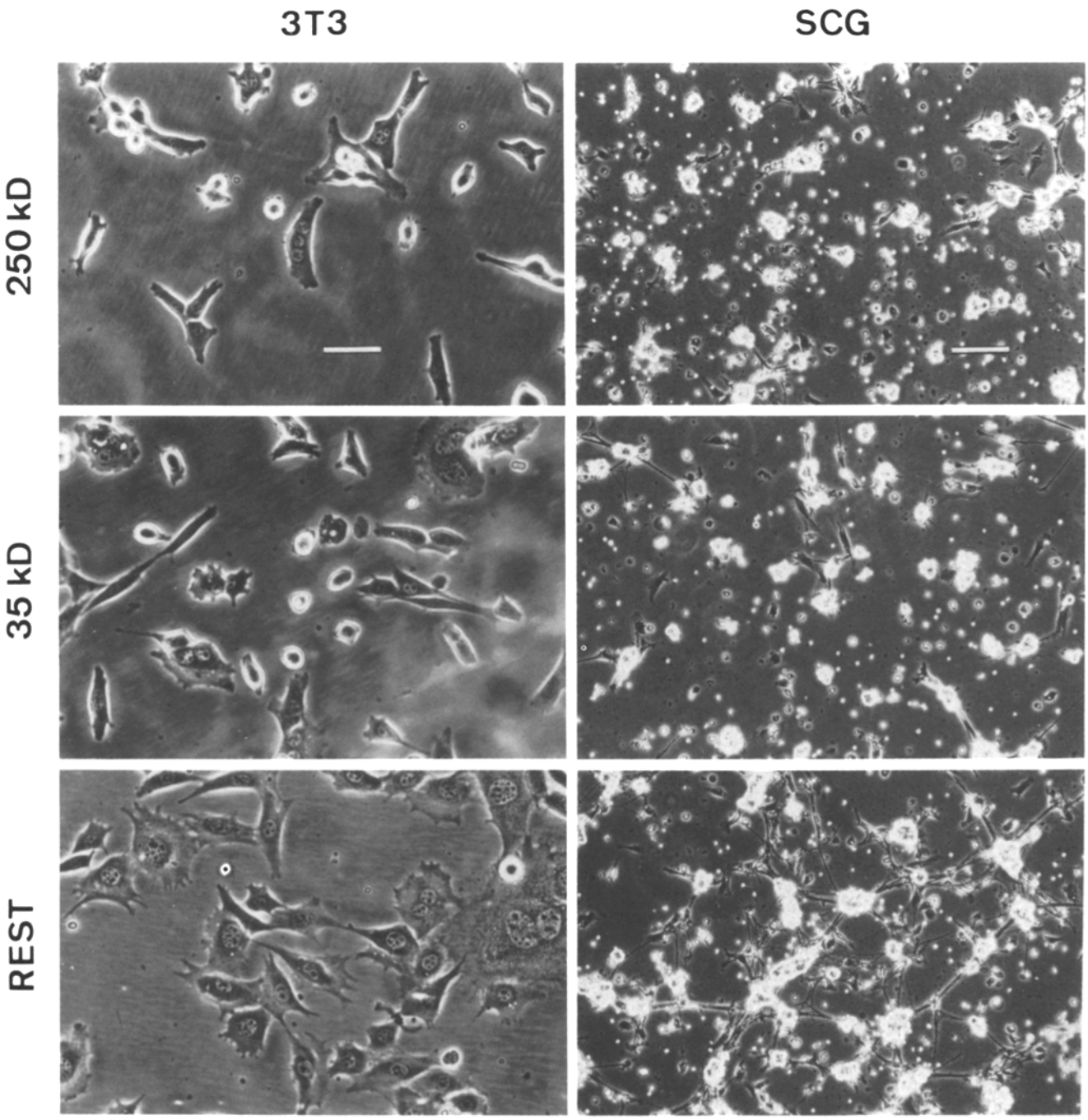

Figure 5. 35- and 250-kD protein fractions from rat CNS myelin are nonpermissive substrates for 3T3 spreading and neurite outgrowth. Liposomes formed in the presence of gel-extracted protein fractions as indicated were tested for their substrate properties. Substrate designated as rest: protein from pooled gel regions excluding the 35 and of 250-kD fractions. Incubation times were 1 (3T3 cells) and 24 h (SCG neurons in the presence of NGF), respectively. Bars: (3T3) 100 μm; (SCG) 50 μm.

tein-containing lipid vesicles from homogenates of different CNS regions and have determined their substrate properties. Rat CNS white matter material yielded highly nonpermissive liposomes containing inhibitory 250- and 35-kD protein fractions (Table IV). Gray matter–derived liposomes contained markedly less nonpermissive activity. Significantly, high quantities of inhibitory activity were extracted from optic nerve–derived cell cultures. Such cultures contain highly nonpermissive, myelin marker–positive oligodendrocytes (Schwab and Caroni, 1988). Analogous protein fractions from a Schwann cell–containing culture yielded no inhibitory proteins. Thus, nonpermissive substrate activity in the nervous system, as detected by our assay, codistributes with CNS white matter and with myelin-forming oligodendrocytes.

Discussion

We have shown in a previous report that myelin marker-positive oligodendrocytes and rat CNS myelin are non-

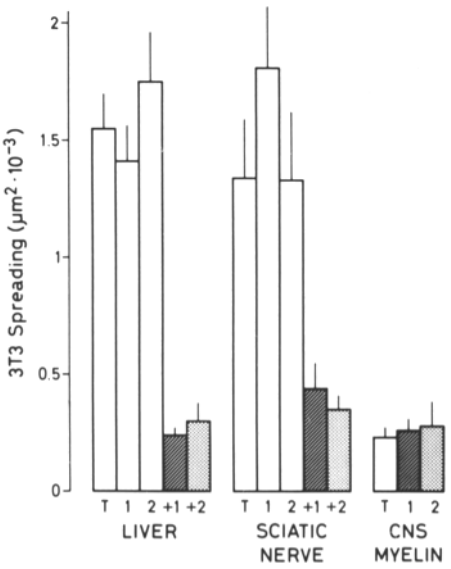

Figure 6. 35- and 250-kD protein fractions from rat CNS myelin convert permissive substrates into nonpermissive ones. Total (*T*) liposomes were formed with 100 μg of protein from each of the three sources (rat) indicated in the figure. One (250 kD) and two (35 kD) liposomes were formed with gel-extracted protein regions from 3–15% gels loaded with 500 μg protein from the same three sources. Columns labeled *+1* and *+2* indicate that 250- (*1*) or 35-kD (*2*) protein from 500 μg of rat CNS myelin were combined with 100 μg of total liver or PNS myelin protein before reconstitution.

permissive substrates for neurite outgrowth and for fibroblast spreading (Schwab and Caroni, 1988). In this study we have determined what makes rat CNS myelin a poor substrate. We first showed that brief treatment of the myelin with protease abolished nonpermissiveness, demonstrating the involvement of protein. These proteins require detergent to be separated from the myelin membranes. Solubilized myelin protein reconstituted with a phosphatidylcholine/phosphatidylserine mixture yielded liposomes with highly nonpermissive substrate properties. Liposomes with such unfavorable substrate properties were obtained from rat CNS myelin protein but not from the protein constituents of membrane fractions possessing permissive substrate properties (PNS myelin, liver). We, therefore, assume that nonpermissiveness is due to the same protein(s) in myelin and in myelin-derived liposomes. When myelin proteins were fractionated by SDS-PAGE, protein fractions with relative molecular masses of ~35 and 250 kD were found to yield highly nonpermissive liposomes upon reconstitution. These proteins are likely to be responsible for the nonpermissive substrate properties of CNS myelin in vitro. Thus, fractionated myelin proteins depleted of 35- and of 250-kD components were a permissive substrate. Furthermore, nonpermissive 35- and 250-kD protein fractions could not be found in rat PNS myelin nor in a liver-derived membrane fraction. Therefore, presence of nonpermissive 35- and 250-kD proteins and nonpermissive membrane fractions are correlated. Both protein fractions can function independently and their relationship is presently unknown.

We have determined whether the presence of the 35- and 250-kD proteins from CNS myelin can be sufficient to generate a nonpermissive substrate by combining them with other-

Table IV. Distribution of Inhibitory 250- and 35-kD Protein Fractions: Enrichment in CNS White Matter and in Oligodendrocyte-containing Cultures

	3T3 spreading on liposomes from		
Protein source	Total protein	250-kD fraction	35-kD fraction
	μm^2	μm^2	μm^2
CNS white matter	211 ± 60	158 ± 45	242 ± 51
CNS gray matter	845 ± 106	362 ± 65	460 ± 55
Optic nerve culture	240 ± 67	272 ± 52	332 ± 58
Sciatic nerve culture	1,623 ± 173	1,850 ± 250	1,261 ± 141
Trout CNS myelin	1,050 ± 110	1,150 ± 135	1,585 ± 185

Protein (source) amounts were 100 μg (total protein liposomes) and 500 μg (gel-applied protein). Sample preparation as described in the Materials and Methods section. Tested protein, if not indicated otherwise, was obtained from rat tissues.

wise permissive substrate fractions. Thus, not only is nonpermissiveness of depleted rat CNS myelin restored (not shown), but supplemented liver or sciatic nerve protein–derived liposomes become nonpermissive (Fig. 6). We therefore conclude that 35- and 250-kD proteins of rat CNS myelin are likely to be responsible for its nonpermissive substrate properties and that these proteins can be considered inhibitors of fibroblast spreading and of neurite outgrowth. Definitive proof that the proteins are indeed the cause of CNS white matter nonpermissiveness was recently obtained with specific blocking antibodies (Caroni and Schwab, 1988). Such antibodies neutralized the nonpermissiveness of gel-purified inhibitors-containing liposomes, of CNS myelin membranes, and of living cultured oligodendrocytes.

The mechanism by which myelin-derived inhibitors prevent cell spreading and neurite outgrowth is presently unknown. Some of our observations suggest that the inhibitory effect is related to poor adhesiveness. Thus, very slow attachment of cells to nonpermissive myelin and inhibitor-containing liposomes was observed. Also, brief adsorption of the myelin substrate with low concentrations (10 μg/ml) of fibronectin dramatically improved substrate properties of CNS myelin for 3T3 fibroblasts (data not shown). On the other hand, adhesive molecules like the cell adhesion molecule N-CAM are present in substantial amounts on CNS myelin membranes and on the surface of inhibitory oligodendrocytes (Caroni, P., and M. E. Schwab, unpublished observations). It would, therefore, probably be incorrect to consider CNS myelin and myelin-making oligodendrocytes as generally nonadhesive surfaces. The possible involvement of specific nonpermissiveness-mediating mechanisms is currently under investigation in this laboratory. Irrespective of the mechanism involved, however, our findings raise the provocative question of how minor membrane-bound proteins can convert a neutral substrate into a highly nonpermissive one. In neural development, where differential adhesiveness of surfaces is expected to play a crucial morphogenetic role, such proteins are of obvious interest.

Are the nonpermissive substrate properties described in the present study involved in the lack of regenerative fiber growth found in the CNS of higher vertebrates? As men-

tioned in the introductory section other factors, and in particular laminin (Liesi, 1985; Hopkins et al., 1985; Carbonetto et al., 1987) and Ng-CAM (Daniloff et al., 1986) expression, have been implied. While neutral substrate properties of CNS myelin fractions from frog and trout might be due to the presence of low amounts of laminin, no nonpermissive 35-kD protein fraction could be extracted from these sources (Table IV). The apparent absence of inhibitory substrates in CNS myelin fractions from lower vertebrates (trout and frog) suggests that absence of laminin (for example) and expression of inhibitory substrates on myelin-forming oligodendrocytes might have evolved in parallel leading to limited regenerative fiber growth in the differentiated CNS. As fiber tracts are crucial structural elements of the CNS, selective pressure to prevent invasion of already formed fiber tracts during development might have led to the expression of highly nonpermissive surface molecules on glial cells within fiber tracts, i.e., on oligodendrocytes. Our findings predict that CNS white matter would be a particularly unfavorable environment for growing neurites. Consistent with our in vitro data, neurite growth from implanted embryonic CNS tissues in adult rat CNS has been found in some cases to reach up to 14 mm within some gray matter areas but has not been found to exceed 1 mm within white matter (Nornes et al., 1983; Björklund and Stenevi, 1984; Commissiong, 1984). Limited regeneration after lesion has been seen in the retina where myelin is absent (McConnell and Berry, 1982), and in aminergic unmyelinated fiber tracts after chemical (Björklund and Stenevi, 1979), although not after mechanical, lesions (Bregman, 1987). In addition, recent experiments in this laboratory (Savio, T., and M. E. Schwab, manuscript in preparation) demonstrate a marked difference in substrate properties for neurite outgrowth when white and gray areas of the rat CNS are compared.

We thank Mrs. J. Erni and Mrs. D. Steinberg for their skillfull technical assistance and Mrs. S. Kaufmann for typing the manuscript. We are grateful to Dr. Y.-A. Barde for critically reading the manuscript.

This work was supported by the Swiss National Foundation for Scientific Research (grant No. 3.043-0.84) and the Bonizzi-Theler Foundation (Zurich).

Received for publication 5 October 1987, and in revised form 30 November 1987.

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