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PROTEINS AND GLYCOPROTEINS IN PLASMA MEMBRANES AND IN THE MEMBRANE LAMELLAE PRODUCED BY PURIFIED OLIGODENDROGLIA IN CULTURE

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Oligodendroglial plasma membranes are complex structures composed of a heterogeneous mixture of proteins and glycoproteins. The Coomassie stained gel patterns showed a maximum of 40 proteins with molecular weights ranging from > 200000 to 12500. Autoradiography was used to detect binding of radioiodinated lectins to glycoproteins. With concanavalin A, 5 major glycoproteins were seen; with wheat germ agglutinin, 2 major glycoproteins with approximate molecular weights of 95000 and 78000 were found; with *Ulex europaeus*, 7 major glycoproteins were observed. Additional minor bands were also seen. The impermeant probe diazodi[^{125}I]iodosulfanilic acid was used to radiolabel intact cells. It was found that 5 major proteins were radiolabeled in the plasma membranes. In all cases, the whorls of membrane lamellae produced in culture by oligodendroglia tend to have a somewhat less complicated pattern with fewer proteins and glycoproteins than the plasma membranes. However, the whorls of membrane lamellae have far more complicated protein patterns than myelin.

Introduction

The mammalian cell plasma membrane plays an important role in cell-cell interactions during development and in the disease process. During the development of the central nervous system, oligodendroglia produce myelin, a lipid rich multilamellar membrane that surrounds the axons of neurons, thereby facilitating saltatory conduction of nerve impulses. It is thought that oligodendroglia may be the target cell in many demyelinating diseases, including multiple sclerosis. In order to detect any components present in the plasma membranes only during maturation or only on cells altered in the disease state, the normal components must be identified first. Plasma membranes from any cell type are complex mixtures of proteins and glycoproteins, and we have found that those of oligodendroglia are no exception. In culture purified oligodendroglia produce whorls of membrane lamellae adjacent to the cell soma [1,2]. Both the membrane lamellae and the plasma membranes can be isolated separately using sucrose gradient centrifugation [2]. Both subfractions were analyzed by slab gel electrophoresis either after the cells were radioiodinated or by using radioiodinated lectins in a lectin overlay system. It was found that while the plasma membranes and the membrane lamellae have similar levels of incorporation of substrate into lipids [2], surprisingly the protein and glycoprotein compositions are different.

Materials and Methods

Cell isolation, culture, and subfractionation

Oligodendroglia were purified from bovine brain white matter and were maintained as sus-

pension cultures as described previously [3]. After 18–24 h in culture, the cells are likely to have replaced any membrane components affected by the isolation procedure which includes exposure of pieces of tissue to trypsin. The cells were then washed, homogenized, and subjected to subcellular fractionation [4]. After discontinuous gradients, the layers were concentrated and further purified on continuous sucrose gradients from which three bands were obtained. These were (A) a light band at 0.32 M sucrose; (B) an intermediate membrane band at 0.5–0.6 M sucrose; (C) a plasma membrane band at 1.0 M sucrose. These subfractions were concentrated in an ultracentrifuge and then further centrifuged to small pellets in an Airfuge. Characterization of these subfractions has shown the intermediate band to be the whorls of loosely compacted membrane lamellae produced by oligodendroglia in culture that are enriched in 2′,3′-cyclic nucleotide 3′-phosphodiesterase enzyme activity [2]. The plasma membrane subfraction consisted morphologically of small vesicles and upon analysis was found to be enriched in plasma membrane marker enzymes.

Myelin isolation

Myelin was prepared from bovine subcortical white matter by methods previously described [5]. After discontinuous gradient centrifugation, the crude myelin fraction was subjected to osmotic shocks and then further purified on continuous sucrose gradients. Two fractions can be obtained: light myelin at 0.5 M sucrose and heavy myelin at 0.6–0.7 M sucrose. Both myelin samples were subjected to cold ethanol extractions to precipitate proteins and partially remove lipids before preparation for electrophoresis.

Electrophoresis

The samples for electrophoresis were solubilized in 10% sodium dodecyl sulfate (SDS) by sonication. When clear solutions were obtained, aliquots were taken for protein determinations [6]. Then 50 mM Tris-HCl buffer (pH 6.8), 1% β-mercaptoethanol, 40% sucrose and 0.001% Bromophenol blue were added, and the samples were heated at 100°C for 3 min.

Linear gradient slab gels of 5–30% polyacrylamide concentration, with a 5% spacer gel, were prepared on a BioRad vertical slab gel system [7]. Solubilized samples of 30–50 μg of protein were applied in volumes of 25–50 μl per 75 μl well, prepared using a 10 well slot former in slabs of 0.75 mm thickness. A discontinuous buffer system consisting of 0.025 M Tris (base), 0.2 M glycine, pH 8.5, and 0.1% SDS was used. High and low molecular weight standards were obtained from BioRad Laboratories, Richmond, CA. The protein samples were subjected to electrophoresis for 4–6 h at increasing power (5, 12, and 15 watts) with constant cooling. The slab gels were fixed either for 2 h or overnight in methanol/acetic acid/water (45 : 45 : 10, by vol.). For staining, the slabs were placed overnight in 0.5% (w/v) Coomassie brilliant blue in water/isopropanol/acetic acid (65 : 25 : 10, by vol.). Gels were destained with several changes of fixing solution. They were placed on Gel Bond (FMC, Rockland, ME) and sealed for photography.

Lectin studies

The lectins (concanavalin A from Miles Biochemicals, Elkhart, IN; wheat germ agglutinin and *Ulex europaeus,* type I, from Sigma Chemical Co., St. Louis, MO) were radioiodinated in the presence of their respective inhibitory monosaccharides, using the radioiodination system from New England Nuclear, Boston, MA. Dialysis was used to remove unreacted iodide and the sugars. The specific activities of the iodinated lectins ranged from 70000 to 100000 cpm per μg lectin.

For binding studies, after electrophoresis to separate the proteins, the proteins were fixed and the slab gels washed several times in 0.2 M borate buffer containing 0.15 M NaCl. The slab gels were then placed on glass plates in humidified stainless steel trays. The radioiodinated lectins were diluted in the appropriate buffer and carefully pipetted over the gels until the surface of the gel was evenly covered. Usually $20 \cdot 10^6$ cpm of lectin in 6 ml of buffer were applied evenly to a 0.75 mm thick (100 mm long × 140 mm wide) originally sized slab gel. The slabs were incubated for 24 h at 25°C, were washed extensively in 0.2 M borate buffer, then in fixative solution, and then stained in Coomassie blue as described. After destaining, the slabs were treated with methanol/acetic acid/glycerol/water (50 : 75 : 100 : 775, by vol.) and then dried on a

BioRad gel slab dryer. The binding was detected by autoradiography using either Kodak X-omat R or AR film and Dupont Cronex Lighting Plus intensifying screens [8]. Exposure times varied between several days to several weeks at −70°C. X-rays were developed in the Kodak automated processor.

In all the experiments the molecular weights of oligodendroglial proteins were determined by comparison of their electrophoretic mobilities with known standards (myosin, 200000; β-galactosidase, 116500; phosphorylase B, 94000; bovine serum albumin, 68000; ovalbumin, 43000; carbonic anhydrase, 30000; soybean trypsin inhibitor, 21000; and lysozyme, 14300). Logarithmic plots of the molecular weights of the standards vs. their mobility were constructed; the estimated molecular weights of oligodendroglial proteins were determined from these plots. Nonspecific binding was assessed by incubating the gels in the presence of the inhibitory monosaccharide and lectin (fucose for *Ulex europaeus,* type I; *N*-acetylglucosamine for wheat germ agglutinin; D-glucose for concanavalin A). There was no binding in the control experiments.

Cell labeling

Cells were radioiodinated using the probe diazodi[^{125}I]iodosulfanilic acid [9]. Oligodendroglia were removed from culture and washed twice in phosphate-buffered saline with added sugars to remove fetal calf serum [3]. The cells (approx. $1.5 \cdot 10^9$) were suspended in 1 ml of phosphate-buffered saline with added sugars and the freshly prepared probe ($N_2^{125}I_2C_6H_2SO_3$) was added. The cells were mixed at frequent intervals over the next two hours, while incubated at 4°C. The cells were washed extensively and the plasma membranes and other membrane subfractions were prepared as described previously. The subfractions were solubilized and subjected to electrophoresis on slab gels as outlined above. The proteins were fixed and stained; the slabs were dried, and set up for autoradiography. Exposure time varied from 1 to 3 months at −70°C.

Results

Coomassie stained protein patterns

Both the glial plasma membranes and the glial membrane lamellae subfractions have numerous proteins, ranging in molecular weights from over 200000 to 12000. The plasma membranes have some 40 proteins that are readily visible after staining with Coomassie blue; there are 14 major bands (Table I). The plasma membranes have more proteins than the whorls of membrane lamellae (Table II). The whorls of membrane lamellae have 8 major proteins, but 23 minor bands are visible. Both membrane subfractions appear to have major proteins with molecular weights of 40000 and 32000. By no means is this a complete assessment of the total number of proteins found in each subfraction as other types of gels (5–15%, two-dimensional gels) will probably reveal additional proteins that are not clearly separated in this system. The protein patterns are completely reproducible from one membrane preparation to another.

Lectin binding studies

There are multiple bands of glycoproteins that bind to the three lectins used in this study. There are 12 glycoproteins that bind to concanavalin A in the plasma membranes, 7 that bind to wheat germ agglutinin, and 16 that bind to *Ulex europaeus* (Table I). There are 14 major glycoproteins in this group. The binding patterns of concanavalin A for the two subfractions appear quite similar when the autoradiograms are examined. However, after calculation of the molecular weights, the glycoproteins in the two subfractions have slightly differing molecular weights (Table II). The membrane lamellae subfraction has 13 glycoproteins that bind to this lectin with 5 major ones (Table II).

Two heavy bands of staining appear when separated proteins from the two subfractions are exposed to radioiodinated wheat germ agglutinin. The molecular weights of these glycoproteins are approximately 95000 and 78000. There are also other bands of proteins that react with less intensity. Such intensely stained glycoproteins are only observed with this lectin and not with the other lectins that were used.

The patterns of binding are quite distinct be-

TABLE I

ESTIMATED MOLECULAR WEIGHT DETERMINATIONS ON PLASMA MEMBRANES

Plasma membranes were purified from oligodendroglia in culture, were solubilized in detergent, and the proteins separated by gradient slab gel electrophoresis. The slab gels were either (a) stained with Coomassie blue; (b) exposed to radioiodinated lectins as described in Methods and binding assessed after autoradiography; or (c) oligodendroglía were radiolabeled using the probe, diazodi[^{15}I]iodosulfanilic acid, before subfractionation and the radiolabeled proteins identified by autoradiography. The values presented are estimated molecular weights ($\times 10^{-3}$) determined from calculations from 5–9 slab gels. Major proteins are defined as bands of proteins that are more intensely stained. Coom. blue, Coomassie blue; Con A, concanavalin A; WGA, wheat germ agglutinin; *U. eur.*, *Ulex europaeus*; ^{125}I probe, radioiodination using the probe.

Coom. blue	Con A	WGA	*U. eur.*	^{125}I probe
> 200	> 200	> 200	> 200	
200 [a]				
165 [a]	160			
155			153	
140				
	132	130		
125 [a]				
115 [a]				
110	113 [a]		107	104
99	95 [a]	95 [a]	92	
88 [a]				
83				
80			80	80
76 [a]	77 [a]	78 [a]		
73			73	72 [a]
70 [a]				
64 [a]	63		63	67 [a]
60 [a]				
55			55 [a]	57
54.5		54		
52	52 [a]		50 [a]	
48 [a]				
46				
44		43	43 [a]	44 [a]
40 [a]	41			
38			37	37 [a]
36				
32 [a]	33	33		
31			31 [a]	31
29				
27			27 [a]	26
25	25 [a]			
24			23 [a]	
22				
21 [a]			20	
19 [a]				18 [a]
17	17		17 [a]	
15				
14				
12.5				

[a] Major protein.

tween the two subfractions when *Ulex europaeus* is used. There is a major glycoprotein of 16 kDa in the membrane lamellae subfraction with 11 minor bands. In the plasma membranes there are 7 major

TABLE II

ESTIMATED MOLECULAR WEIGHT DETERMINATIONS ON MEMBRANE LAMELLAE

The whorls of membrane lamellae produced by oligodendroglia in culture were purified, solubilized in detergents, and the proteins separated by slab gel electrophoresis. The slab gels were treated as described in the legend to Table I. The values presented are estimated molecular weights ($\times 10^{-3}$).

Coom. blue	Con A	WGA	*U. eur.*	^{125}I probe
	> 200	> 200		
135		140		
120	125			
104	110		110	
95	97 [a]	99 [a]	99	98
87			87	
80 [a]	80 [a]	78 [a]	76	76
			70	
68 [a]	67			66 [a]
62	63			
56	54 [a]		56	55
52 [a]		53	51	
48	46 [a]			
44				43 [a]
40 [a]			41	
37 [a]	39	39		
32.5 [a]			34	35 [a]
27.5 [a]	27		27	29
26				
24				
22	23 [a]		21	
18	18			18
16 [a]			16 [a]	
13				
12				

[a] Major proteins.

TABLE III

ESTIMATED MOLECULAR WEIGHT DETERMINATIONS ON BOVINE MYELIN

Bovine light and heavy myelin were purified as described in the Methods section. After solubilization, the proteins were separated by gradient slab gel electrophoresis and were then exposed to radioiodinated lectins. Binding was assessed by autoradiography. The values presented are estimated molecular weights ($\times 10^{-3}$) determined from calculations obtained from 5–8 autoradiographs.

Concanavalin A	Wheat germ agglutinin	*Ulex europaeus*
Light myelin		
120		
102	108	
	76	74
		66
59		
54	54	54
44		45 [a]
41		
		39
32		34
		28
25 [a]		24 [a]
21		
18		18 [a]
14		14 [a]
Heavy myelin		
> 200	> 200	> 200
	180 [a]	
150		
118 [a]		112
108 [a]	104	
90 [a]		90
70	79	
64		66
50	54	56
		46 [a]
43		41
35		36.5
		30
25		25 [a]
20		20 [a]

[a] Major protein.

glycoproteins with 9 minor bands.

Purified bovine light and heavy myelin have a somewhat less complicated array of glycoproteins (Table III). Eleven to 12 glycoproteins bind to concanavalin A, 3–5 bind to wheat germ agglutinin, and 10–11 bind to *Ulex europaeus* in the myelin subfractions. The patterns of binding appear quite different from those seen in the oligodendroglial membrane subfractions

Radioiodination of intact cells

The probe diazodi[^{125}I]iodosulfanilic acid was used to radiolabel intact oligodendroglia. Membrane subfractions were then prepared from the radiolabeled cells and analyzed for radioactivity. Preliminary studies showed at least 2 h of exposure to the probe were needed for detectable levels of covalently bound radiolabeled proteins. Since the binding occurred at 4°C, there was little cell damage. The molecular weights of the radiolabeled proteins are presented in Tables I and II. Heavily stained bands of proteins appear at 72 000, 67 000, 44 000, 37 000 and 18 000 estimated molecular weights in the plasma membranes. There were fewer proteins (66, 43, and 35 kDa) in the membrane lamellae subfraction that were radiolabeled.

Discussion

Oligodendroglial membrane subfractions are complex structures composed of heterogeneous mixtures of proteins and glycoproteins, ranging in molecular weight from > 200 000 to 11 000. Coomassie stained patterns of either subfraction show numerous proteins with many major proteins. This is quite unlike myelin which consists of two major proteins with molecular weights of 24 000 and 18 000. There are a small number of minor bands present in myelin which vary from one species to another. In bovine myelin these minor bands are at a minimum. Whether the myelin proteins are present in the vast array of proteins in the oligodendroglial membrane subfractions is not clear at this time. There are minor bands at 24 000 and 18 000 or 19 000 molecular weights in both subfractions. However, they are minor components. It is to be noted that the whorls of membrane lamellae produced by oligodendroglia in culture are primarily uncompacted membranes. Possibly myelin proteins appear in the whorls of membrane lamellae at the time of compaction into mature myelin.

The use of radioiodinated lectins is a very sensitive method to detect nanogram amounts of glycoproteins [10–13]. Many other samples including membrane subfractions and myelin isolated from

human control and pathological brain tissue have been analyzed in our laboratory using this technique [14]. Each sample has a unique staining pattern with individual lectins, and the samples can be easily identified by examination of their autoradiographs. Control experiments whereby inhibitory monosaccharides were incubated with the corresponding lectins showed no binding. Moreover, organic solvent extraction of the samples produced no changes in the overall binding characteristics.

The reactions of the glycoproteins with their corresponding lectins occurred to varying degrees, suggesting different concentrations of glycoproteins or different oligosaccharide contents between the two membrane subfractions. With concanavalin A (specificity: primarily D-mannose, D-glucose, *N*-acetyl-D-glucosamine, mannobiosyl-*N*-glucosamine structures) 5 to 6 major and 7 minor glycoproteins were observed in the plasma membranes. In contrast, wheat germ agglutinin (specificity: primarily *N*-acetyl-D-glucosamine, *N*-acetylneuraminic acid, *N*,*N*′,*N*′-triacetylchitotriose) displayed intense binding to 2 adjacent glycoproteins in the plasma membranes, with approximate molecular weights of 95 000 and 78 000 and 99 000 and 78 000 in the membrane lamellae. It is not known at this time whether the areas of intense staining consist of multiple glycoproteins or are very high concentrations of these two components. There is no trace of these two glycoproteins in bovine myelin. There are few low molecular weight glycoproteins that bind to this lectin in either oligodendroglial subfraction.

Surprisingly *Ulex europaeus* (specificity: α-L-fucose) exhibited binding to numerous glycoproteins in both the oligodendroglial membrane subfractions and in the bovine myelin subfractions. Both the plasma membranes and the membrane lamellae have in common a major glycoprotein at 43 kDa and one at 27 kDa. There appear to be common minor glycoproteins at 56 and 41 kDa between bovine heavy myelin and the oligodendroglial membrane lamellae. The myelin associated glycoprotein [15] that can be labeled with fucose and has a molecular weight of 100 000 is not readily apparent in bovine myelin. There are minor glycoproteins in the oligodendroglial membrane lamellae with molecular weights of 110 000 and 99 000 that bind to *Ulex europaeus*. These are not present in the plasma membranes.

In general the membrane lamellae subfraction has fewer proteins and glycoproteins than the plasma membranes. The glycoproteins in the membrane lamellae differ slightly in molecular weight from those in the plasma membranes, indicating either differences in the glycoproteins themselves or in their oligosaccharide sidechains. For example, with concanavalin A major glycoproteins are found at 97 000, 80 000, 54 000 and 23 000 molecular weights in the plasma membranes, while in the membrane lamellae, they are at 95 000, 77 000, 52 000 and 25 000 molecular weights. For the moment it is assumed that these are different glycoproteins, although they are similar in molecular weights. The most obvious changes occur in the binding to *Ulex europaeus*, where the pattern of binding is quite different. There is more intense binding to low molecular weight glycoproteins in the membrane lamellae subfraction.

The impermeant probe, diazodi[^{125}I]iodosulfanic acid, has been shown by other investigators to be less disruptive when radiolabeling intact cells [8,9]. It can be produced to high specific activity and can be used at 4°C. It is thought to react primarily with tyrosine, histidine, and lysine residues in exposed proteins. Five experiments were performed using this probe with oligodendroglia, and the resulting radiolabeled proteins were similar in each case. Five major proteins with 5 minor components are radiolabeled in the plasma membranes. Interestingly, the membrane lamellae are radiolabeled less well; only 3 prominent bands are found. Possibly the configuration of the whorls of membrane lamellae reduce the amount of membrane that is exposed to the probe. With this procedure additional components were revealed that were not evident or were only very minor constituents when analyzed by the other methods.

Using these techniques, 13 major glycoproteins and 14 prominent proteins have been noted in oligodendroglial plasma membranes and 5 major proteins have been radiolabeled using a surface labeling probe. It is not known at this time whether the proteins and glycoproteins that have been identified thus far are separate constituents or whether there are common proteins between the groups. In contrast, the whorls of membrane

lamellae have a more simple pattern, consisting of 8 major proteins, 8 major glycoproteins, and 3 proteins exposed to the surface probe.

From our studies thus far it is clear that the protein and glycoprotein composition of oligodendroglial plasma membranes and of the whorls of membrane lamellae are quite complex, which is typical of plasma membranes of other cell types. Surprisingly the whorls of membrane lamellae produced by oligodendroglia in culture have a far more complicated pattern of proteins than mature compacted myelin. Our lipid studies suggest that the synthesis and assembly of mature myelin involves a multistep process that is quite ordered [2]. If the whorls of membrane lamellae are involved in this process, their production must be a very early step. Specific myelin proteins may be more involved in the compaction of the whorls of membrane lamellae into mature myelin. Whether membrane subfractions similar to the whorls of membrane lamellae produced in culture can be purified from developing brain tissue just initiating myelin formation in vivo is under investigation.

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