Relation of C-6 Glial Cells in Culture to Myelin

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C-6 glial cells were shown to contain enriched in their plasma membranes an enzyme, 2':3'-cyclic nucleotide 3'-phosphohydrolase, characteristic of myelin, and, in addition, proteolipid protein and two basic proteins that are identical in their electrophoretic mobilities with the respective proteins found in myelin. The data indicate that C-6 cells exhibit features of myelin-producing glia as well as astrocytes.

Growth of glial and neural cells in culture provides an exceptional opportunity to elucidate biochemical functions characteristic of these specific components of the central nervous system. For such purposes we have utilized C-6 glial cells derived from a rat astrocytoma and neuronal cells derived from a mouse neuroblastoma. The present study deals particularly with C-6 glial cells. We have found that the plasma membranes isolated from C-6 glial cells, which have astrocytic lineage and properties (Benda *et al.*, 1968, 1971; DeVellis & Kukes, 1973), exhibit biochemical features characteristic of myelin. Thus these membranes are enriched in the myelin marker enzyme 2':3'-cyclic nucleotide 3'-phosphohydrolase, and contain proteins of electrophoretic mobilities identical with those of proteolipid protein and basic protein of myelin.

Experimental

Culture media and methodology were described previously (Volpe & Marasa, 1975). Subcellular fractionation was carried out by the basic scheme of Gray & Whittaker (1962).

2':3'-Cyclic nucleotide 3'-phosphohydrolase (Kurihara & Tsukada, 1967), acetylcholinesterase (Ellman *et al.*, 1961) and (Na⁺+K⁺)-activated ATPase† activities (Schimmel *et al.*, 1973) were assayed. One unit of enzymic activity is defined as the amount needed to catalyse the following, in $\mu\text{mol/h}$ at 37°C: for 2':3'-cyclic nucleotide 3'-phosphohydrolase, production of 2'-AMP, for acetylcholinesterase, the hydrolysis of acetylthiocholine, and for ATPase, the production of P_i.

Lipids were extracted (Folch *et al.*, 1957) and fractionated (Rouser *et al.*, 1967), and cholesterol (Zlatkis *et al.*, 1963), phospholipids (Rouser *et al.*,

1966) and glycolipids (Hess & Lewin, 1965) were analysed. Protein was determined by the method of Lowry *et al.* (1951). Chloroform-methanol-soluble proteolipid proteins from C-6 cells were isolated by the method of Folch *et al.* (1957) and basic proteins by that of Martenson *et al.* (1971). Purification of myelin and polyacrylamide-gel electrophoresis in sodium dodecyl sulphate were carried out as described by Agrawal *et al.* (1972, 1974). Basic proteins were electrophoresed in the acetic acid system described by Martenson *et al.* (1971).

Results and discussion

Crude extracts of C-6 cells were found to contain a relatively high specific activity of 2':3'-cyclic nucleotide 3'-phosphohydrolase, i.e. 65-80 units/mg of protein, as previously reported (Zanetta *et al.*, 1972; Pfeiffer & Wechsler, 1972). This specific activity is similar to that observed in crude extracts of the Schwann cell line RN-2 (Pfeiffer & Wechsler, 1972) and for biopsied samples of oligodendroglioma (61.8) and astrocytoma (43.9) (Kurihara *et al.*, 1974). The value of the enzyme as a glial cell marker is indicated by our inability to detect this activity (limit of detectability, 2 units/mg of protein) in cultured neuroblastoma cells. Similarly, the neuronal marker, acetylcholinesterase, was undetectable in C-6 cells (limit of detectability, 0.2 unit/mg of protein), but has a specific activity of 11.7 units/mg of protein in the neuroblastoma cells.

Since 2':3'-cyclic nucleotide 3'-phosphohydrolase is particularly enriched in the myelin membrane (Kurihara & Tsukada, 1967), we next investigated the possibility that the enzyme might also be enriched in glial plasma membrane. Thus C-6 cells were subjected to the subcellular fractionation scheme of Gray & Whittaker (1962). When the crude mitochondrial fraction was subjected to fractionation on a discontinuous sucrose density gradient, we observed two distinct fractions, one equilibrating at the 1.2M- and 0.8M-sucrose interface and the other at

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† Abbreviation: ATPase, adenosine triphosphatase.

Table 1. 2':3'-Cyclic nucleotide 3'-phosphohydrolase activity in subcellular fractions of C-6 glia

Subcellular fractionation and assays were carried out as described in the text; 63% of 2':3'-cyclic nucleotide 3'-phosphohydrolase activity of the homogenate was recovered in the various fractions; 60% of ouabain-sensitive ($\text{Na}^+ + \text{K}^+$)-activated ATPase activity of the homogenate was recovered in the various fractions.

	2':3'-Cyclic nucleotide 3'-phosphohydrolase activity		($\text{Na}^+ + \text{K}^+$)-activated
	Specific activity (units/mg of protein)	(% of recovered activity)	ATPase activity (% of recovered activity)
Homogenate	68.4	—	—
Nuclear fraction	59.8	25	11
Microsomal fraction	95.6	3	1
100000g supernatant	22.3	18	1
Mitochondrial fraction	103	12	21
Fraction M ₁	376	12	23
Fraction M ₂	298	30	43

the 0.32M- and 0.8M-sucrose interface. The heavier of these two, designated fraction M₂, has been shown by Cotman *et al.* (1971) with C-6 cells to consist of plasma membrane. We found that the distinct additional band, designated fraction M₁, which sediments in virtually the same position as does myelin when whole brain is subjected to this procedure (Gray & Whittaker, 1962), contained approx. 40% as much protein as fraction M₂.

The specific activity of 2':3'-cyclic nucleotide 3'-phosphohydrolase in all of these subcellular fractions is shown in Table 1. Several points are apparent from these data. First, the two 'membrane' fractions, M₁ and M₂, contain 2':3'-cyclic nucleotide phosphohydrolase of highest specific activity: in fraction M₂ it is enriched 4-5-fold over the homogenate, and in fraction M₁ the enrichment is 5-6-fold. Secondly, approx. 40% of the recovered enzymic activity is present in these two membrane fractions, with significant additional contributions from the nuclear and soluble supernatant fractions. We also assayed the activities of ouabain-sensitive ($\text{Na}^+ + \text{K}^+$)-activated ATPase, a plasma-membrane marker, and it is particular noteworthy that the recoveries and enrichment of this enzyme share important similarities with recoveries of 2':3'-cyclic nucleotide 3'-phosphohydrolase. Thus approx. 66% of the total recovered ATPase activity was in the M₁ and M₂ membrane fractions. The specific activities in these fractions (M₁, 6.63 units/mg of protein, M₂, 4.74 units/mg of protein) were approx. 9- and 6-fold higher respectively than in the homogenate (0.76 unit/mg of protein). The major difference between ATPase and 2':3'-cyclic nucleotide 3'-phosphohydrolase recoveries was the virtual absence of ATPase from the 100000g supernatant fraction. This should be contrasted with the presence of approx. 20% of recovered 2':3'-cyclic nucleotide 3'-phosphohydrolase in this fraction. Possible explanations for the presence of 2':3'-cyclic nucleotide 3'-phosphohydrolase in the soluble fraction include solubiliza-

tion of the enzyme during the fractionation procedure or occurrence of a soluble form of the enzyme in the intact glial cell. Thus we concluded from these data that at least the major portion of 2':3'-cyclic nucleotide 3'-phosphohydrolase is associated with the plasma membrane of C-6 glial cells.

The findings of a C-6 glial membrane fraction with sedimentation properties similar to those of the myelin membrane and with the greatest enrichment of the typical myelin enzyme 2':3'-cyclic nucleotide 3'-phosphohydrolase prompted us to seek in these cells other characteristics of myelin. One such characteristic is a typical protein composition. Thus C-6 glial cells were subjected to the Folch-Lees chloroform-methanol extraction procedure for the isolation of proteolipid proteins (Folch *et al.*, 1957) and the extracted proteins separated by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. Examination of the gels shown in Plate 1(a) leads to several conclusions. First, pure myelin (gel 1) contains several major protein components, e.g. Wolfram protein, proteolipid protein, protein DM-20 and the large and the small basic proteins. Secondly, C-6 glial cells (gel 2) contain a protein that migrates to the same position as does myelin proteolipid protein. Thirdly, when pure myelin proteolipid protein is isolated by preparative gel electrophoresis in sodium dodecyl sulphate, and then added to the glial cell proteins of gel 2, the co-migration is clearly apparent, as seen in gel 3.

Pursuing the analogy with the myelin membrane further, we determined whether the encephalitogenic basic proteins characteristic of myelin are present in C-6 glial cells. The characteristic procedure for isolation of basic proteins makes use of their solubility in dilute acid (Martenson *et al.*, 1971). Thus C-6 glial cells and myelin were subjected to this characteristic isolation procedure and the extracts applied to gels run in an acetic acid system (Plate 1b). The gel containing the myelin extract shows the characteristic pattern of two major basic protein

EXPLANATION OF PLATE I

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of chloroform-methanol-extractable (a) and acid-extractable (b) proteins of C-6 glial cells

The experimental procedure followed is given in the text. The three gels in (a) were run simultaneously under identical conditions and consist of the proteins of purified myelin (1), the chloroform-methanol-extractable proteins of C-6 cells (2) and a portion of the same sample as was applied to gel (2) to which had been added pure myelin proteolipid protein (3). Identical results were obtained in two separate experiments. WP, Wolfgram protein; PLP, proteolipid protein; BP, basic protein. The two gels in (b) were run simultaneously under identical conditions and consist of the acid-extractable proteins of C-6 cells (1) and of myelin (2). Identical results were obtained in two separate experiments.

bands (indicated by arrows), the first appearing as a doublet. Strikingly, C-6 glial cells were shown to contain bands that correspond exactly to the myelin basic proteins, the first of which also appeared as a doublet. That these basic proteins of C-6 cells are not contaminating histones is suggested by several facts. First, C-6 cells extracted at pH 3.0 and pH 1.0 show the same protein pattern on acid gels; more histones would be extracted at pH 1.0 (Martenson *et al.*, 1971). Secondly, neuroblastoma cells extracted in an identical manner show no protein bands on acid gels in the region of the basic proteins (results not shown). Thirdly, the major co-migrating basic protein band of the C-6 cells had the blue-green colour shown previously to be characteristic of myelin basic protein (Martenson *et al.*, 1971).

Although the membranes of C-6 cells are analogous to myelin with respect to 2':3'-cyclic nucleotide 3'-phosphohydrolase, proteolipid protein and basic proteins, this is not the case for lipid composition. Thus the molar proportions of cholesterol/phospholipid/glycolipid for purified myelin (1.00:1.16:0.61) differed markedly from those for the fractions M₁ (1.00:1.80:0) and M₂ (1.00:1.97:0); they contained negligible quantities of glycolipid and, like other plasma membranes, their molar ratio of cholesterol/phospholipid showed a distinct predominance of phospholipid.

Thus these data indicate that C-6 glial cells contain in their plasma membranes an enzyme, 2':3'-cyclic nucleotide 3'-phosphohydrolase, characteristic of myelin, and, in addition, a proteolipid protein and two basic proteins that are identical in their electrophoretic mobilities with the respective proteins found in myelin. What is the significance of these data *in vivo*? The answer to this important question depends primarily on the nature of C-6 glial cells in culture. The morphological and biochemical data available now indicate that these cells have properties of both astrocytes and oligodendroglia. It is likely, in fact, that C-6 glial cells in culture are most nearly comparable with the less differentiated stem cell that gives rise to these glial types. Our studies of regulation of fatty acid synthesis in these cells support this contention (Volpe & Marasa, 1975). Thus the data presented appear to relate more to properties of glia of the developing nervous system and set the stage for further investigations into the

relationship of such glia to central-nervous-system development, particularly myelination.

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