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PLASMA MEMBRANES FROM FIBROBLASTIC CELLS IN CULTURE ISOLATION, MORPHOLOGICAL AND ENZYMATIC IDENTIFICATION

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SUMMARY

Plasma membranes were isolated from murine plasmocytoma cells in culture, by a procedure involving lysis in hypoosmotic medium leaving the nuclei intact, and separation of surface membranes from the lysate constituents on a discontinuous sucrose gradient.

The purity of the fractions was assessed by electron microscopy and by assaying enzymes for cross-contaminants. Phosphohydrolases, including the $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase (EC 3.6.1.3) and 5'-nucleotidase (EC 3.1.3.5), were concentrated in the plasma membrane-rich fractions. These fractions were essentially free from NADH: cytochrome *c* reductase, lysosomes and mitochondrial membrane enzymes.

INTRODUCTION

Plasma membranes of animal cells are essential for at least two functions. One is related to transport mechanisms, the other to cell contact. Evidence has already been given that during neoplastic transformation both functions may be profoundly altered (see Burger[1] and Pardee[2]). However, very little is known about the reciprocal effect or interrelation between the impairment of one of those functions with respect to the other.

From mouse plasmocytoma two cell lines have been obtained *in vitro*, which display many diversities in their surface properties. The fibroblast-like cell line (MF2) does not show contact inhibition of growth[3], in contrast to the epithelial-like cell line. These two cell types are therefore a suitable system in which to study some of the cell surface properties related to transport function, in respect to the loss of contact inhibition of growth. A more detailed analysis of membrane functions and structure is permitted by the isolation of a subcellular fraction representative of plasma membranes of mouse plasmocytoma. The present paper describes a method of isolation and the study of some enzymic markers of the plasma membrane-rich fraction derived from MF2 cells, in comparison with other subcellular fractions.

We have chosen to study a number of enzymes which, by analogy with tissues, could be concentrated in plasma membranes and endoplasmic reticulum.

$(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase (EC 3.6.1.3)[4], 5'-nucleotidase (EC 3.1.3.5)[5], and $(K^+ + Mg^{2+})$-stimulated *p*-nitrophenylphosphatase (EC 3.1.3.1)[5–7]

were found in plasma membranes, while UDPase (EC 3.6.1)[8], alkaline phosphatase (EC 3.1.3.1)[9], NADH: cytochrome *c* reductase (EC 1.6.99.3)[10,11] and glucose-6-phosphatase (EC 3.1.3.9)[12] were measured in endoplasmic reticulum membranes.

MATERIALS AND METHODS

Cell line

A continuous cell line, MF2, which originated from myeloma MOPC 173 was used; it grows on plastic or glass. MF2 has a fibroblast-like appearance, and forms multiple cell layers; it is able to induce tumours in Balb/c mice. Cells were cultivated under the conditions described previously[3], washed in saline and collected by scraping the surface of the flask with a piece of rubber.

Cell membrane isolation

Surface membranes were isolated by a modification of the procedure used by Emmelot and Benedetti[13] for rat hepatoma plasma membranes. All steps were carried out at 4 °C. The cells were centrifuged at $300 \times g$ for 10 min. The pellets were washed twice in physiological saline and then resuspended in the lytic medium (4 ml/g of cells), containing 1 mM $NaHCO_3$ and 2 mM $CaCl_2$, adjusted to pH 8.0 with Na_2CO_3. Cells were further dissociated in a loose Potter homogenizer with a Teflon pestle. The cell suspension was diluted with 4 vol. of the hypoosmotic medium and dispersed by rapid magnetic stirring for 20–30 min. The final volume of the lytic medium was adjusted to 15–20 ml/g of cells. The lysed cell suspension was centrifuged at $27000 \times g$ for 20 min and the enzymatic activities of the supernatant immediately determined. The pellet (P_1) was suspended in a minimum volume (V) of 8% (w/w) sucrose in 5 mM Tris–HCl buffer at pH 8.0. These conditions were chosen because the sucrose solutions used for the subsequent gradient centrifugation were hyperosmotic, and according to Steck *et al.*[14], a rather alkaline pH (pH 8.0 in 5 mM Tris buffer) and omission of divalent cations would favour the separation of plasma membrane vesicles from endoplasmic reticulum membranes.

Three volumes V of 63% sucrose solution were gradually added to the resuspended pellet which, after stirring, was transferred to siliconised 38 ml cellulose tubes.

Discontinuous gradient

Using six buckets in the SW 27 rotor, the total amount of protein in the crude preparation compatible with a good membrane yield corresponded to 230 mg.

Onto a 6-ml cushion of 52% sucrose suspension of cell material were layered successively: 12 ml of 44%, 8 ml of 40%, 10 ml of 36% and 2 ml of 32% sucrose solutions. Centrifugation was performed in a Spinco SW 27 rotor for 90 min at $130000 \times g$. The pellet (P_2) and four bands (A_1, B_1, C_1 and D_1, of densities 1.14/1.16, 1.16/1.18, 1.18/1.20 and 1.20/1.22, respectively) were collected. Each fraction was diluted with 15 vol. of 1 mM $NaHCO_3$ solution and recentrifuged in a rotor at $10000 \times g$ for 20 min. The pellets were suspended in a storage medium containing 10 mM Tris–HCl (pH 7.6), 50% glycerol, 1 mM ATP Tris, 1 mM AMP. Glycerol prevented the formation of ice crystals at −20 °C. ATP and AMP were added to stabilize the corresponding enzyme activity[15].

Enzyme activities

Enzymic assays were performed with about 100 μg of protein. The glycerol concentration in the incubation medium did not exceed 1.5%. $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase activity was measured under the conditions described by Bakkeren and Bonting[16]. 5'-Nucleotidase was measured by the method of Emmelot and Bos[17]. $(K^+ + Mg^{2+})$-stimulated *p*-nitrophenylphosphatase activity was determined as previously described[18]. Because of possible interference between this activity and residual alkaline phosphatase at pH 7.4, the latter was determined at the same time; *p*-nitrophenyl phosphate was used as substrate at a concentration of $2 \cdot 10^{-3}$ M in a 50 mM Tris buffer (pH 8.9). Conversion to *p*-nitrophenol was estimated by measuring its absorbance at 410 nm.

Uridine diphosphatase was measured according to Plaut[19], and glucose-6-phosphatase according to Swanson[20]. NADH: cytochrome *c* reductase was determined as described by Dallner[21]. Acid phosphatase (EC 3.1.3.2) activity was measured by the method of Appelmans *et al.*[22]. Enzyme activities were expressed as μmoles of product (reduced cytochrome *c*, P_i or *p*-nitrophenol) liberated per h per mg protein. Protein concentrations were determined by the method of Lowry *et al.*[23].

Phosphorus present in the reaction medium was determined by the method of Fiske and SubbaRow[24].

Electron microscopy

Pellets of the various fractions were fixed in glutaraldehyde, 3.5% in phosphate buffer (pH 7.2), post fixed in 2% OsO_4 in phosphate buffer (pH 7.1), and embedded in Epon 212. Thin sections stained with uranyl acetate and lead hydroxyde were studied in a Philips EM 300.

RESULTS AND DISCUSSION

Protein yield and protein distribution

1 g (wet weight) of cells yielded up to 4 mg of plasma membrane protein. The final yield of supernatants and pellets protein with respect to the total protein of the lysate was 80–90%.

The distribution of protein between the primary fractions and the sucrose interfaces was as follows: S, 35–45%; P_1, 45%, $A_1 + B_1$, 1.7–4.6%; $C_1 + D_1$, 4%; P_2, 27–40%.

Enzyme activities

For a given enzyme, two criteria should be considered: (a) the ratio between the specific activity of a fraction to that of the lysate, which is an indication of the purification, and (b) the ratio of the total activity of each fraction to the total activity of the lysate.

In Tables I and II, the distribution of the different enzyme activities found in each fraction are listed.

Plasma membranes enzymes

$(K^+ + Na^+)$-stimulated Mg^{2+}-ATPase and 5'-nucleotidase activities were found in Fractions A_1 and B_1 (interfaces 1.14/1.16 and 1.16/1.18). This distribution in two

TABLE I

MF$_2$ CELLS: SPECIFIC ACTIVITIES OF $(Na^+ + K^+)$-STIMULATED Mg^{2+}-ATPase, 5'-NUCLEOTIDASE AND $(K^+ + Mg^{2+})$-STIMULATED *p*-NITROPHENYLPHOSPHATASE IN FRACTIONS OBTAINED DURING THE ISOLATION PROCEDURE

The mentioned values below represent the average of three separate determinations performed on 9 preparations. Standard deviation around these mean values was about 15%. N.D., not detectable. Abbreviations: L, lysate; P, pellet; S, 27000 × *g* supernatant. (a) Preparations with one sucrose gradient. (b) Preparation with two sucrose gradients.

Enzymes		*Primary fractions*			*Sucrose interfaces*					
		L	*S*	P_1	A'_2 *1.12/1.14*	A_1 A_2 *1.14/1.16*	B_1 B_2 *1.16/1.18*	C_1 C_2 *1.18/1.20*	D_1 D_2 *1.20/1.22*	P_2 *1.22*
$(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase	(a)	0.8	0	1.5		3.2	2.2	0	0	0
	(b)	1	0	2	6.8	14	0	0	0	0
5'-Nucleotidase*	(a)	220	15	420	940	610	15	15	N.D.	0
$(K^+ + Mg^{2+})$-stimulated *p*-nitrophenylphosphatase**	(a)	0.66	2	0.3		0	0.3	1	0.1	0.1

* Specific activities expressed in μmoles P_i/h per mg protein.

** Specific activities expressed in μmoles p-nitrophenol/h per mg protein.

TABLE II

MF$_2$ CELLS: SPECIFIC ACTIVITIES OF ENDOPLASMIC RETICULUM ENZYMES IN FRACTIONS OBTAINED DURING THE ISOLATION PROCEDURE

The mentioned values below represent the average of three separate determinations performed on 9 preparations. Standard deviation around these mean values was about 15%. N.D., not detectable. Abbreviations: L, lysate; P, pellet; S, 27000 × *g* supernatant. (a) Preparation with one sucrose gradient. (b) Preparation with two sucrose gradients.

Enzyme		*Primary fractions*			*Sucrose interfaces*					
		L	*S*	P_1	A'_2 *1.12/1.14*	A_1 A_2 *1.14/1.16*	B_1 B_2 *1.16/1.18*	C_1 C_2 *1.18/1.20*	D_1 D_2 *1.20/1.22*	P_2 *1.22*
UDPase*	(a)	254	96	300		420	400	420	600	340
Alkaline phosphatase*	(a)	0.14	0.14	0		0	0	0	0	0
	(b)	0.16	0.25	0.1	0	0.5	1	0.9	0.2	0.2
NADH: cyt. *c* reductase**	(a)	0.05	0.01	0.06		0	0.04	0	0	0.06
Glucose-6-phosphatase*	(a)	2	N.D.	2.9		0	0	7	8.1	N.D.
	(b)	2.5	0	3.5	0	0	0	6.7	6.4	0
Acid phosphatase*	(a)	0.75	0.8	N.D.		0	0	0	0	0

* Specific activities expressed in μmoles P_i/h per mg protein.
** Specific activities expressed in μmoles reduced cyt. *c*/h per mg protein.

interfaces may be due to the variable amounts of contaminants trapped during vesicular formation.

In Fractions A_1 and B_1 some 30% of the initial activity of both enzymes was recovered. This result showed that a considerable amount of the $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase and 5'-nucleotidase activities had been inactivated during the purification, since the enzymes could not be demonstrated in the $27000 \times g$ and $10000 \times g$ supernatants. The loss and/or inactivation seemed to be related to the drastic change in osmolarity to which the membrane fraction was submitted during the isolation procedure.

The time spent in transferring the membranes suspension from hypo- to iso-osmotic medium affects activity. If this is longer than 10 min, 99% of $(K^+ + Na^+)$-stimulated Mg^{2+}-ATPase activity is lost; if less than 10 min this is only 70%. We cannot rule out an alternative explanation that the vesicular structure (inside out) prevents free access of substrates to these enzymes, resulting in an apparent loss of activity.

The $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase activity consistently showed 70% inhibition in the presence of 1 mM ouabain. Stimulation by Na^+ and K^+ increased the activity of Mg^{2+}-dependent ATPase by 50% at pH 7.4.

$(K^+ + Mg^{2+})$-stimulated *p*-nitrophenylphosphatase was found in liver and erythrocyte plasma membranes[5-7]. In our case, most of this activity (70% with respect to the 89% recovered) was found in the $27000 \times g$ supernatant. It was absent in the gradient Fractions A_1 and B_1. This localisation in the first supernatant may reflect an association with the lightest fragments of surface membranes or a rapid solubilisation, depending upon the lytic procedures used.

Endoplasmic reticulum enzymes

The specific activity of UDPase was highest in the D_1 fraction where 600 μmoles phosphorus were released/mg protein per h. 60% of the total activity of the lysate was found in the P_2 pellet (nuclei). The final yield was 87%. UDPase would seem to be a good marker for endoplasmic reticulum.

A major part, if not all, of the alkaline phosphatase activity was found in the S fraction (60–100%). Peaks of activity were found at interfaces 1.14/1.16 and 1.18/1.20, corresponding to 3- and 6-fold purification. The final yield was 100%.

NADH: cytochrome *c* reductase activity was highest in the P_2 fraction, probably because of the presence of reticulum associated with the nuclear envelope. The other sucrose layers were devoid of activity (less than 1%). 6% of the activity of the cell lysate was lost on treatment with hypoosmotic medium and 40% was lost on the gradient. The NADH: cytochrome *c* reductase profile through the gradient was very similar to those of UDPase and alkaline phosphatase, suggesting that this enzyme can be used as a marker for endoplasmic reticulum.

In contrast, in the isolation procedure employed here, 89% of the total cellular glucose-6-phosphatase activity was solubilized. Residual activity was mainly concentrated in the C_1 and D_1 fractions (1.18/1.20 and 1.20/1.22) with a specific activity of 6.7 μmoles P_i/h per mg, which is three times as high as the lysate. We shall discuss the significance of such results in the last part of this paper.

Acid phosphatase was found only in the $27000 \times g$ supernatant. Attempts to detect its activity in Fractions P_1 and the layers of the gradient have been unsuccessful.

Electron microscopy

Electron microscopic observations of the various fractions gathered at the different interfaces showed that most of the plasma membranes were concentrated at the interface between 1.14 and 1.16. The plasma membranes appeared as large sheets of different shapes or as vesicles of various size (Fig. 1). Most of the vesicles were empty. Some others, in particular those of larger size, contained few "filaments", with some fibrillar material attached to the inner leaflet of the membrane. The triple-layered structure of the membrane element was often visible (Fig. 1). Junctional complexes were very infrequently detected. In Fraction B_1, plasma membrane fragments were also present, but the contamination with other membranous organelles (endoplasmic reticulum fractions) was higher. Moreover, the plasma vesicles in Fractions B_1 contained more heterogeneous material either attached to the inner surface of the membrane or free than Fraction A_1.

The observed accumulation of $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase and 5'-nucleotidase in Fractions A_1 and B_1 suggests the presence of plasma membranes in these fractions. The major part of UDPase, alkaline phosphatase and NADH: cytochrome *c* reductase is found in Fractions C_1, D_1 and P_2, suggesting the presence of endoplasmic reticulum membranes in these fractions. The relative contamination of the plasma membranes in both Fractions A_1 and B_1 may be estimated by comparing the total amounts of these three enzymes in $A_1 + B_1$ with the total activities found in the original lysate. Plasma membrane preparations contain 4%, 6% and 1% of the initial activities.

However, if the estimation of the contamination by endoplasmic reticulum is based on specific activities, UDPase and alkaline phosphatase are found in the A_1 and B_1 layers which should contain plasma membranes.

It was reported[25] that UDP may be hydrolysed by liver plasma membranes and, according to Emmelot and Benedetti[13], these organelles also contain alkaline *p*-nitrophenylphosphatase activity.

Thus it is difficult at this point to decide whether the plasma membranes are contaminated with 1–6% of endoplasmic reticulum, or whether they contain UDPase and alkaline phosphatase. In the case of glucose-6-phosphatase, we have found that whereas 90% of the total activity is lost, the specific activity is three times higher in C_1 and D_1 than in the lysate. It is difficult to decide in this case whether or not this enzyme should be considered as a good marker of the endoplasmic reticulum membrane.

The significance of a given enzyme as a membrane marker for a given cell will be discussed more extensively in the second paper.

Since neither acid phosphatase nor monoamine oxidase were found in the purified plasma membrane fractions, these appear to be free of contamination by lysosomes or by outer mitochondrial membranes.

To check the density of the plasma membrane preparations on sucrose gradients and to increase the final yield and the separation of endoplasmic reticulum from plasma membranes, the Fractions A_1, B_1, C_1 and D_1 were layered onto an identical discontinuous sucrose gradient of density 1.12 to 1.22. The fractions A'_2, A_2, B_2, C_2 and D_2 (densities 1.12/1.14, 1.14/1.16, 1.16/1.18, 1.18/1.20 and 1.20/1.22, respectively) were collected as described previously. Enzyme assays indicated that floatation through a second gradient did not affect the localization nor the activities of the

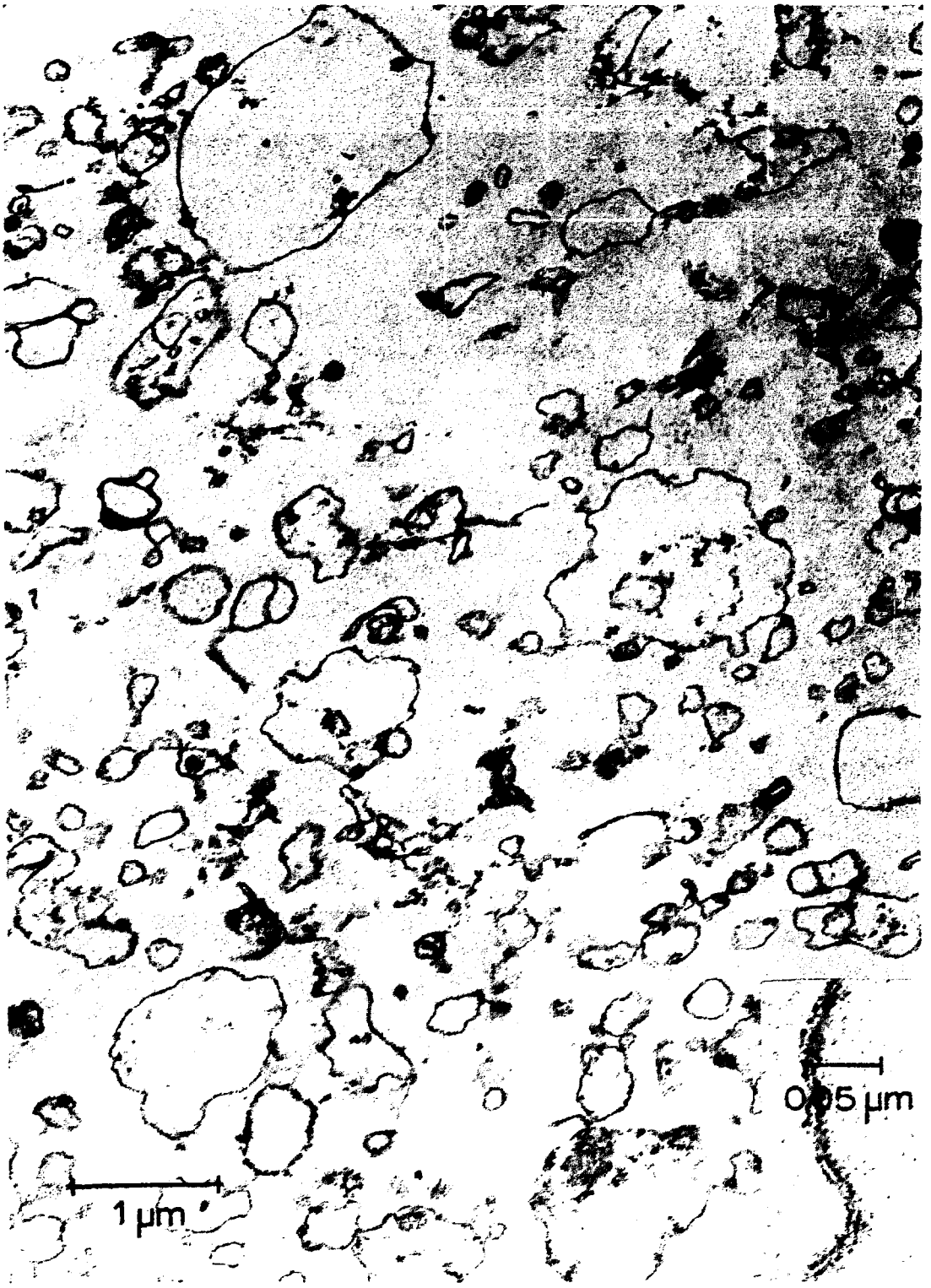

Fig. 1. Characteristic aspect of the membrane fraction concentrated at interface 1.14/1.16. The plasma membrane fragments appear as sheets or vesicles of various dimension (× 20000). In the insert is a high magnification picture (× 180000) showing the triple layered structure of the membrane elements.

endoplasmic reticulum enzymes. However, $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase and 5'-nucleotidase were only present in Fractions A'_2 and A_2, instead of A_1 and B_1. This new distribution was accompanied by a significant loss of protein (50%) without any loss of total activity or any variations in plasma membrane purity. The decrease in plasma membrane density can be attributed to an increase in the lipid–protein ratio as described by Perdue and Sneider[26].

Finally, this procedure can be carried out on such a small scale that electron microscopic analysis or measurements of the three enzymes activities are possible on plasma membranes isolated from as few as $3 \cdot 10^6$ MF_2 cells.

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