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Rapid Report

Isolation of plasma membrane exovesicles from BHK cells using merocyanine 540

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Treatment of cultured BHK cells with merocyanine 540 caused the non-lytic release of vesicular material having the phospholipid composition characteristic of plasma membrane. The protein composition of the vesicles closely resembled that of the soluble fraction of the cell, as expected for exovesicles budding from the cell surface. Vesicles prepared from cells surface-iodinated with ^{125}I contained no obvious iodinated membrane polypeptides, suggesting that no major proteins in the plasma membrane of the BHK cell are free to diffuse with lipids. The procedure described should represent a general method, applicable to a wide range of cell types, for isolating plasma membrane vesicles.

The lipid bilayer portion of the plasma membrane is of particular interest and significance because it represents not only the primary barrier between the cell and the external medium but also because it accomodates a variety of proteins whose function is to mediate interactions between the cell and its environment. Thus a knowledge of the composition and properties of this bilayer is important for the understanding of a wide range of cellular phenomena. Plasma membrane exovesicles representative of the lipid bilayer and free of skeletal proteins can be released from red cells [1–4] and some other cell types including platelets [5] and thymocytes [6] by a variety of treatments which cause expansion of the outer lipid leaflet relative to the inner leaflet and thus induce local membrane curvature according to the model of Sheetz and Singer [7]. However, some of these techniques are unsatisfactory in certain respects because they involve either long periods of treatment or gross changes in the metabolic status of the cells e.g. exposure to metabolic inhibitors. To avoid some of these problems we have recently used merocyanine 540 (MC540), a fluorescent amphipath which concentrates in the outer lipid leaflet, and which is capable of rapidly releasing cytoskeleton-free vesicles from red cells without affecting cellular metabolism. This technique has now been applied to to metabolically active BHK cells growing in culture which can be made to release plasma membrane vesicles by incubation with low concentrations of MC540.

Materials and Methods. BHK 21 cells were cultured in 10-cm diameter dishes and labelled to equilibrium with ^{32}P (5 μCi/ml) as described previously [8]. Cells were washed three times with 10 ml 150 mM NaCl, 10 mM phosphate buffer (pH 7.4) (PBS) and then were incubated at 37°C with the same medium containing 50 μM MC540 (Sigma Chemical Co. Poole, Dorset, UK). The solution of MC540 was sonicated briefly to remove any visible aggregates before addition to the cells. After various times up to 1h the medium was removed and centrifuged at 500 × *g* for 5 min to remove any cells present and then at 100000 × *g* for 1 h to sediment any vesicular material. The low and high-speed pellets were resuspended separately in 1 ml of 150 mM NaCl and lipids were extracted with 3.75 ml of methanol/chloroform (2:1, v/v). The same volume of methanol/chloroform was used to extract lipids from the remnant cells in the dishes. Carrier phospholipid from rat liver was added to the extracts of the vesicular material but not to the extract of the whole cells. Phospholipids were separated and radioactivity analysed as described previously [12].

In other experiments cells were labelled to equilibrium (2 days) with 20 μCi [^{35}S]methionine in 10-cm dishes, washed three times with PBS and then either

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reincubated in the same medium or in the same medium plus 50 μM MC540. After 30 min at 37°C vesicles were isolated as above. The pellets were dissolved in SDS sample buffer and polypeptides were separated by PAGE [9]. Samples of whole cells (treated and untreated) and of soluble fractions from these cells were also analysed. For preparation of the soluble fraction, cells were scraped from the dishes and broken by passing repeatedly through a narrow bore syringe needle. The soluble fraction was taken as the supernate obtained after centrifugation of the broken cells for 1 h at $100000 \times g$.

Iodination in the presence of lactoperoxidase was carried out on two samples of cells grown to near confluence in 3.5-cm dishes and then washed three times in PBS. The procedure of Philips and Morrison [10] was employed but using 100 μCi of carrier-free ^{125}I and three aliquots of 8 μM hydrogen peroxide added at 5-min intervals. The cells were washed twice with PBS containing 1 mM KI and then one sample was incubated in PBS alone while the other was incubated with 50 μM MC540 in the same medium. After 30 min at 37°C the medium was removed and vesicles isolated as above. Samples for PAGE were prepared

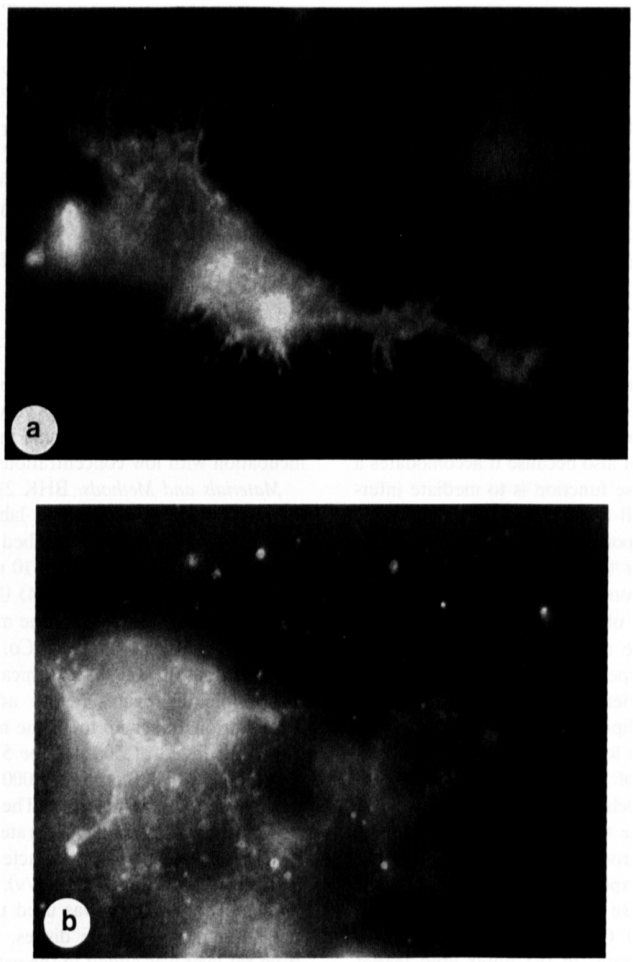

Fig. 1. Light micrographs of (a) BHK cells treated with MC540 and (b) free vesicles present in the medium surrounding the cells. Cells were grown on cover slips in 3.5 cm dishes and incubated for 30 min in serum-free Minimal Essential Medium containing 10 μM MC540. The cover slips were rinsed quickly in cold phosphate-buffered saline and then were examined in a Leitz fluorescence microscope using a magnification of $\times$1000. The fluorescence bleached rapidly but images were captured successfully using a Hamamatsu video system. For Fig. 1b it was necessary to focus put of the plane of the cells in order to see the free vesicles which underwent rapid movement across the field. Scale of micrograph: 1 cm = 2 μm.

by dissolving the remnant cells and the vesicle pellets in SDS sample buffer. Gels with iodinated samples were autoradiographed for 3–16 h and gels with ^{35}S-labelled samples were autoradiographed for 1–4 days using Hyperfilm (Amersham International plc). Autoradiographs were scanned using a Bio-Rad Model 1650 densitometer connected to a Shimadzu computing integrator.

Cells treated with MC540 were also examined in the light microscope (Leitz) using phase contrast and fluorescence imaging.

Results and Discussion. When BHK cells were treated for 1 h with 50 μM MC540 in PBS the cells gradually became rounded but there was no visual evidence for cell breakage and viability was at least 98% as judged by exclusion of Trypan blue. Under a fluorescence microscope using a green filter, the cells were seen to be microspiculated (Fig. 1a) and a multitude of tiny particles close to the limit of resolution in the light microscope were seen moving in the surrounding medium (Fig. 1b). In some cases it was possible to observe particles about 0.1–0.2 μm in diameter being released from spiculated areas of the cells. About 1% of the cells became detached from the dishes during incubation but there was no difference in this respect between treated and control cells. Detached cells were removed by a low-speed centrifugation. High speed centrifugation of the resulting supernatant solution from the treated cells gave a pellet which contained some MC540 together with small amounts of phospholipid-containing material.

Using cells labelled to equilibrium with ^{32}P the distribution of radioactivity between the various phospholipid classes should reflect mass distribution [11] and this made it possible to quantify the phospholipid in the vesicles. Thus the total amount of phospholipid and the relative masses of each phospholipid class was measured from the radioactivity in the intact cells and in the vesicles. Fig. 2 shows the time course of phospholipid release from cells treated with MC540. After an initial faster phase, release rose approximately linearly between 10 and 45 min when the vesicles accounted for approx. 0.8% of total cell phospholipid or 4% of plasma membrane phospholipid (assuming that plasma membrane lipid accounted for 20% of the total in the cells [12]). The incubation was not extended further because in treated and control cells there was an increased tendency for the cells to detach from the dishes after 1 h.

The phospholipid class distribution for intact cells and for the vesicles is shown in Table I. It is important to note that the composition of the vesicles is quite distinct from that of cells, especially because the vesicles are depleted in cardiolipin and phosphatidylinositol compared with cells and much richer in sphingomyelin and phosphatidylserine which are characteristic plasma membrane phospholipids. In these respects the composition of the vesicles closely resembled that of certain enveloped viruses which are also produced as 'exovesicles' budding from the host cell plasma membrane (Table I). This is the primary evidence that the material released by MC540 is derived from the plasma membrane. Assuming that cardiolipin (a characteristic mitochondrial phospholipid) is absent from plasma membrane the comparative compositions of cells and vesicles suggest that more than 85% of vesicle phospholipids are derived from plasma membrane.

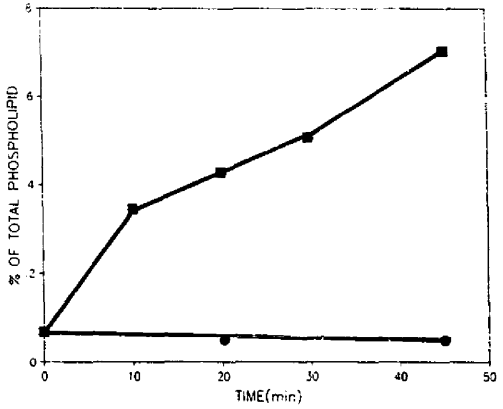

Fig. 2. Release of phospholipid from BHK cells treated with merocyanine 540. Cells labelled to equilibrium with [^{32}P]phosphate were incubated with or without merocyanine 540 for up to 1 h as described under Methods and vesicles were isolated from the low speed supernate. Results are expressed as the percentage of total cellular lipid radioactivity recovered in the vesicles. Similar results to those illustrated were obtained in three other experiments.

In terms of polypeptide content the vesicle fraction looked very much like the soluble fraction (Fig. 3) and distinctly different from the overall polypeptide pattern

TABLE I

Phospholipid composition of BHK cells vesicles released in the presence of Merocyanine 540 and of Semliki Forest virus

Cells were treated with MC540 for 45 min, vesicles were isolated and phospholipids analysed as described under Fig. 1. Results represent the means ± S.D. for four experiments for cells and vesicles. Data for virus were largely from Ref. 8 with additional data for cardiolipin. * none detected.

	Phospholipid composition (%)		
	BHK cells	vesicles	virus
Sphingomyelin	8 ± 1	23 ± 2	26 ± 2
Phosphatidylcholine	45 ± 2	25 ± 2	22 ± 1
Phosphatidylserine	8 ± 1	17 ± 1	18 ± 1
Phosphatidylinositol	8 ± 1	2 ± 1	1 ± 1
Phosphatidylethanolamine	26 ± 2	32 ± 3	28 ± 2
Cardiolipin	5 ± 1	1 ± 1	*

Fig. 3. Polyacrylamide gel patterns for cells, soluble fraction and vesicles. Cells were labelled to equilibrium with [^{35}S]methionine and polyacrylamide gel electrophoresis was carried out on samples of cytosol (A), vesicles released by MC540 prepared as described in the text (B) and cells (C). This figure was obtained by densitometric scanning of radioautographs of the gels. The molecular masses of the major bands (a, 85 kDa; b, 75 kDa; c, 69 kDa; d, 54 kDa; e, 43 kDa; f, 39 kDa) were assessed by comparison with standards which included chicken gizzard actin (43 kDa) bovine serum albumin (67 kDa), porcine pepsin (35 kDa) and human red cell membrane proteins.

of the cells. The vesicles accounted for only about 0.1% of total cell protein. These results are what would be expected for small vesicles which bud from the cell surface encapsulating a proportion of the cytosol which accounts for most of the protein of the vesicles. Very similar findings have been made with red cells and platelets [1–5]. Unlike red cells, it was not apparent that the vesicles from BHK cells contained any characteristic membrane polypeptides i.e. polypeptides which were not present in the soluble fraction.

The pattern of polypeptides of whole cells or of the cytosol fraction was very different from the spectrum of surface-iodinated polypeptides of whole cells (Fig. 4), which resembled that reported previously for BHK cells [13,14]. A high-molecular mass iodinated polypeptide (X) at 230 kDa seems likely to be fibronectin which has previously been shown to be involved in the substrate attachment of many cell types. Prominent iodinated bands (Y and Z) at 130 kDa and 110 kDa may represent the subunits of integrin, the receptor which binds fibronectin at the cell surface [15,16]. However, none of the major iodinated bands in the whole cells was evident in the vesicle fraction; only two polypeptides were visible, one of which coincided in mobility with fibronectin. The other with an apparent molecular weight of 74 kDa had the same mobility as iodinated lactoperoxidase (not shown) which was likely to be a contaminant from the iodination procedure. More extensive washing decreased the proportion of both iodinated components suggesting that they were both contaminants from the medium. It was not clear why these components should be so much more prominent in the high speed pellet from merocyanine-treated cells compared with that from untreated iodinated cells.

We have concluded previously that the membrane of vesicles released from red cells by treatment with MC540 (or other agents which cause a unilateral expansion of the outer lipid leaflet of the plasma membrane) contain a representative sample of plasma membrane lipids together with those membrane proteins which are free to diffuse in the lipid phase [17,18]. By analogy we consider that the membrane of vesicles derived from BHK cells by treatment with MC540 is likely to contain only those plasma membrane proteins which are relatively free to diffuse i.e. not bound either directly or indirectly to cytoskeleton. Since no iodinated polypeptides characteristic of

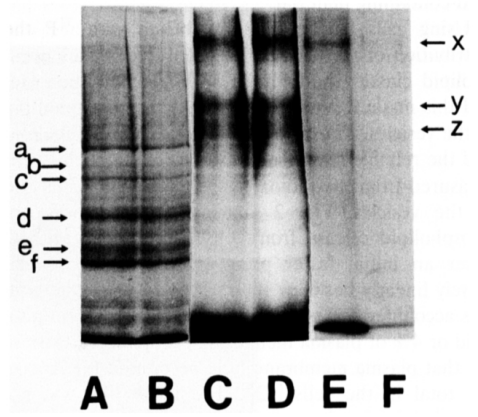

Fig. 4. PAGE patterns of cells labelled with ^{125}I and of vesicles derived from these cells. Cells were labelled with ^{125}I and vesicles prepared as described under Methods. Samples corresponding to 1% of the total cells on a 3.5-cm dish and 50% of the vesicles derived from these cells were run on 7.5% polyacrylamide gels and were radioautographed after staining with Coomassie blue. Molecular mass standards were run as for Fig. 3. (A and C). Cells treated with MC540, (B and D) untreated cells, (E) vesicles derived from cells treated with MC540, (G) high-speed pellet from supernate of untreated cells. Lanes A and B are stained with Coomassie blue; lanes C–F are radioautographs.

plasma membrane could be identified in the vesicles, it seems therefore that most of the surface protein components of the BHK cell must be associated with cytoskeleton. This would certainly be true for integrin which is known to have interactions with cytoskeleton. However, there may be minor components or proteins not labelled significantly with iodine which are not detected by these procedures. Possibly some could be identified by enzymatic methods. Although it is disappointing not to be able to identify characteristic proteins associated with the vesicle membrane this finding may be an interesting and significant indication that cell plasma membranes in general do not have many proteins which are freely mobile in the lipid phase. This suggestion contrasts with evidence derived from human erythrocytes, where substantial amounts of protein are apparently mobile in the lipid phase [18] but erythrocytes are unusual compared with other cell types in possessing such large amounts of anion exchanger protein (Band 3) which is the chief mobile protein of red cell membranes. There are suggestions that proteins linked to plasma membrane via a phosphatidylinositol glycan anchor are likely to be freely mobile [19] but even in the red cell such proteins are only minor components.

The results presented here show for the first time that using MC540 it is possible to rapidly obtain a vesicular fraction from a cultured cell which contains a representative sample of plasma membrane lipids. Previous work has demonstrated release of plasma membrane vesicles from various nucleated cell types [20] but only by prolonged treatment with aldehydes or sulphydryl reagents which are likely to inhibit normal cellular metabolism and introduce artefactual cross-links. Preliminary results using MC540 suggest that this method can be applied to other cell types and open up the general possibilities of isolating a representative sample of lipids and other components mobile in the lipid phase from a variety of nucleated cells.

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