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Endosomes differ from plasma membranes in the phospholipid molecular species composition

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^{125}I-Labeled epidermal growth factor was incorporated into and highly concentrated in endosomes of Chinese hamster V79-UF cells during incubation at 37°C for 8 min after binding to its receptors on the cell surface at 4°C. From the labeled cells, endosomes were isolated by isopycnic centrifugation on a Percoll density gradient and then sucrose density gradients. The isolated endosomes were mostly free from contamination by Golgi, endoplasmic reticulum, lysosome, plasma membrane and mitochondria. Endosome membranes were found to differ from plasma membranes in the phospholipid composition. Sphingomyelin and phosphatidylserine were enriched in endosomes, compared with plasma membranes. Diacylphosphatidylcholine and diacylphosphatidylethanolamine were major phospholipids of the membranes in both organelles. The contents of molecular species of diacylphosphatidylcholine and diacylphosphatidylethanolamine with two monoenoic fatty acids were lower in endosomes than in plasma membranes. The differences in the polar head group and molecular species compositions of phospholipids between endosomes and plasma membranes did not change, regardless of whether or not the proportions of phospholipid molecular species in plasma membranes changed. The significance of the lipids in endosomes is discussed.

Introduction

The endocytic pathway plays a central role in the internalization of extracellular molecules into cells. Many different types of molecules, including low density lipoprotein, transferrin, growth factors, hormones, lysosomal enzymes, asialoglycoproteins, fluid phase components, viruses and toxins, are taken into cells through endocytosis [1–4]. Endosomes serve as intermediates in the endocytic pathway. The endocytic processes, which comprise invagination and budding from membranes, followed by pinching off of vesicles, and fusion of the vesicles with specific target membranes, may be closely regulated by the properties of the proteins and lipids which constitute the membranes. However, the functions of proteins in each of the endocytic processes are unclear, apart from the case of clathrin-coat proteins and an ATP-driven proton pump. On the other hand, the dynamic functions of membranes in the endocytic pathway might be influenced by the physical properties of the membrane themselves. There have

Abbreviations: EGF, epidermal growth factor; BSA, bovine serum albumin; MEM, Eagle's minimum essential medium; SDS, sodium dodecylsulfate; TLC, thin-layer chromatography; 14:0, myristoyl; 16:0, palmitoyl; 16:1, hexadecenoyl; 18:0, stearoyl; 18:1, octadecenoyl; 20:1, eicosenoyl; 22:1, docosenoyl. The molecular species of the phospholipids are denoted as the two acyl groups separated by a slash, such as 18:1/18:1.

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been several studies which suggested the importance of the membrane lipid composition in endocytosis. Changes in the polar head group [5,6] and acyl group [7] compositions of phospholipids and the amount of sterols [8,9] in membranes affect endocytic activities. The phospholipid molecular species composition is the most dominant factor as to the physical properties of membranes. Thus, in order to understand the mechanisms underlying the formation and the functions of endosomes, it is necessary to clarify whether or not there is any similarity in the phospholipid molecular species composition between endosomes and plasma membranes. However, the relationships between the membrane lipids of endosomes and plasma membranes have not yet been defined.

In this study, the phospholipid molecular species in endosomes and plasma membranes from V79-UF cells were analyzed using a highly sensitive method for quantitative analysis of phospholipid molecular species [10], to show that endosome membranes contain a lower content of phospholipid molecular species with two monoenoic fatty acids than plasma membranes.

Materials and Methods

Materials

^{125}I-EGF (100 μCi/μg), carrier-free Na^{125}I and CDP[*N-methyl*-^{14}C]choline (52 mCi/mmol) were obtained from Amersham Corp. UDP[1-^{3}H]galactose (11.4 Ci/mmol) was from Du Pont New England Nuclear, and [^{32}P]P_i was from the Japan Radioisotope Association (Tokyo, Japan). Percoll and density marker beads were purchased from Pharmacia Fine Chemicals, fetal calf serum was from Whittaker M.A. Bioproducts, Inc. (Walkerville, MD), and fatty acid-free BSA was from Miles Laboratories (Elkhart, IN). Lactoperoxidase and aprotinin were from Sigma, and glucose oxidase was from Oriental Yeast Co., Ltd. (Tokyo, Japan). Thin-layer plates were from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade.

Cell culture

Chinese hamster V79-UF cells, which require monounsaturated fatty acids for growth [11], were used throughout this study. The cells were maintained in MEM supplemented with 6% delipidated serum [12] (medium A) and 50 μM oleic acid complexed to BSA [13].

Internalization studies

Cells were grown in MEM containing 10% heated delipidated serum (at 56°C for 30 min) and 50 μM oleic acid in a 3.5-cm tissue culture dish, and were used for an experiment when 70% confluent. To label endosomes with ^{125}I-EGF, cultures were preincubated for 1 h at 37°C in serum-free MEM, cooled to 4°C and then incubated for 1 h at 4°C with 2.5 μCi of ^{125}I-EGF in 1 ml of Dulbecco-Vogt's medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.3) and 0.1% BSA (medium B). After washing five times with 2 ml of medium B, the cultures were incubated in fresh medium B at 37°C for various times and then rapidly cooled to 4°C. Of the cell-associated radioactivity of ^{125}I-EGF, the internalized and cell surface-bound radioactivities were determined by the method of Haigler et al. [14]. Nonspecific binding, as determined by measuring the binding in the presence of excess unlabeled EGF (3 μg/ml), accounted for approx. 20% of the total binding. Since nonspecifically bound ^{125}I-EGF was released on treatment with 0.2 M acetic acid containing 0.5 M NaCl for 6 min at 4°C for determination of the cell surface-bound radioactivity, the radioactivity that bound specifically to the receptors on the cell surface after the incubation at 37°C was estimated by subtracting the nonspecific value from the total radioactivity released on treatment with a sodium acetate solution.

Labeling procedure

To label endosomes with ^{125}I-EGF, ten 70% confluent 10-cm tissue culture dishes were treated as described under Internalization studies. After binding of ^{125}I-EGF and washing at 4°C, the cultures were incubated for 8 min at 37°C. Cell surface proteins were iodinated with carrier-free Na^{125}I by the lactoperoxidase-glucose oxidase method [15]. The iodination reaction was carried out on ice for 15 min to prevent endocytosis of ^{125}I-labeled proteins [16]. For metabolic labeling of phospholipids of cells with [^{32}P]P_i, the cells

were plated on ten 100-mm tissue culture dishes at a density of $1.2 \cdot 10^4$ cells/cm^2 in 10 ml of medium A supplemented with 50 μM oleic acid. After 48-h cultivation, the medium was replaced with 10 ml of fresh medium A containing carrier-free [^{32}P]P_i (20 μCi/ml), supplemented with or without 50 μM oleic acid. The cultures were further incubated for 24 h.

Membrane fractionation

$1.8 \cdot 10^7$ cells were seeded into a roller culture bottle (850 cm^2) containing 150 ml of medium A

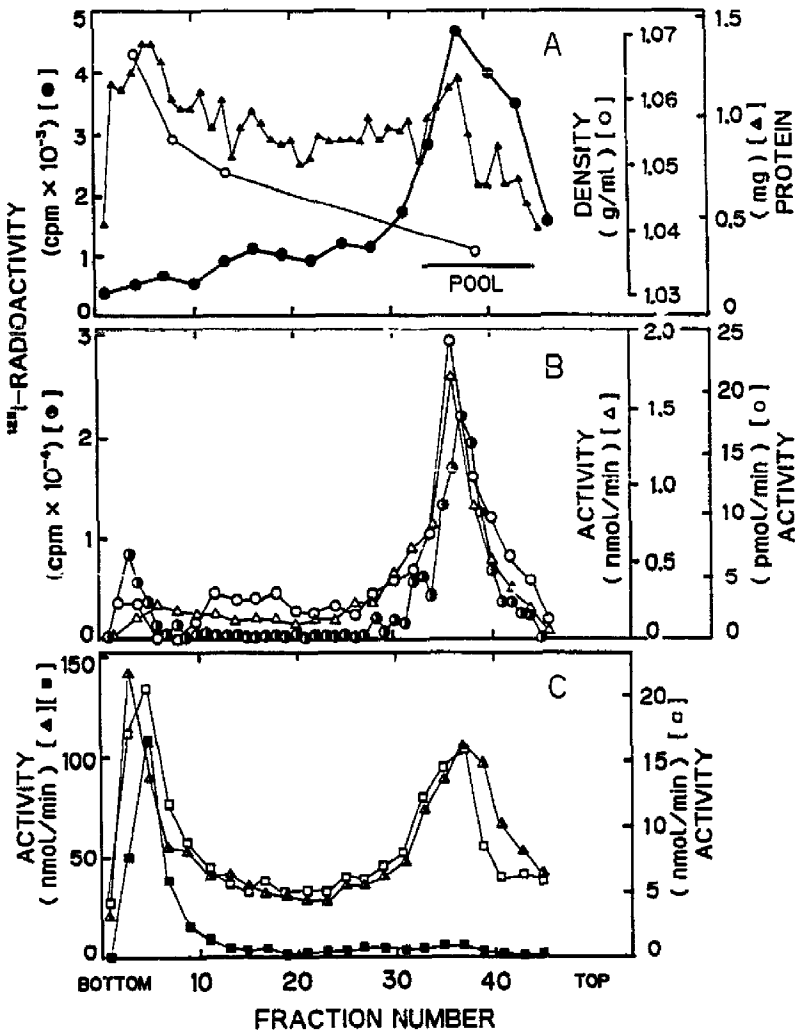

Fig. 1. Fractionation of membranes from V79-UF cells on a Percoll gradient. The postnuclear supernatant was fractionated on a Percoll gradient, and the gradient fractions were assayed for membrane marker activities as described under Materials and Methods. The Percoll density was determined using density marker beads. Protein was determined by Bradford's method. (A) ^{125}I-EGF (●), protein (▲), Percoll density (○); (B) ^{125}I-surface protein (◑), galactosyltransferase (○), cholinephosphotransferase (△); (C) succinate dehydrogenase (■), acid phosphatase (▲), β-*N*-acetylhexosaminidase (□). The ^{125}I-EGF peak fractions were pooled.

supplemented with 50 μM oleic acid. The bottle was incubated at 0.1 rev./min 37°C for 48 h. The medium was replaced with 150 ml of fresh medium A supplemented with or without 50 μM oleic acid. After 24-h incubation, the culture was incubated with 0.1 μg/ml unlabeled EGF in 30 ml of the same medium at 18°C for 1.5 h. The culture was washed with 1 mM EDTA/Ca^{2+}-Mg^{2+}-free Hank's solution (washing buffer) and then the cells were harvested by scraping with a rubber policeman at 18°C. The unlabeled cells from five bottles were combined with cells labeled with ^{125}I-EGF, $Na^{125}I$ or [^{32}P]P_i, which had been washed with the washing buffer, scraped and collected by centrifugation ($1000 \times g_{max}$, 5 min, 4°C). The combined cells were washed once with 10 mM triethanolamine (pH 6.7)/1 mM EDTA/0.25 M sucrose/0.02% sodium azide/1% aprotinin (vol/vol) (Tes) by centrifugation ($1000 \times g_{max}$, 5 min, 4°C). The cells were resuspended in Tes at $(1–1.5) \cdot 10^8$ cells/ml and then homogenized on ice in a glass tight-fitting Dounce homogenizer with 60 strokes, i.e., until > 80% disruption had occurred. The homogenate was centrifuged ($500 \times g_{av}$, 10 min, 4°C) to remove nuclei and unbroken cells, and then the pellet was reextracted. The post-nuclear supernatant (19.68 ml) was mixed with 4.32 ml of a Percoll stock solution (90% Percoll/Tes), and then the mixture was centrifuged ($33000 \times g_{av}$, 30 min, 4°C) in a RP50-2 angle rotor (Hitachi Koki Co., Ltd., Tokyo, Japan). Fractions of 0.5 ml were collected from the bottom of the tube, and fractions 33–44 (corresponding to the peak of ^{125}I-EGF, see Fig. 1) were pooled. The pooled fraction was adjusted to 38% (w/w) sucrose with 65% (w/w) sucrose/Tes. The Percoll fraction obtained from $(5–7.5) \cdot 10^8$ cells was loaded, per centrifuge tube, in a final volume of 5 ml onto 0.2 ml of a 55% (w/w) sucrose cushion. This was overlaid with a step gradient of 32% (2.5 ml), 30% (3 ml), 28% (2.5 ml) and 9% (w/w) sucrose in Tes (0.5 ml). The gradient was then centrifuged ($152000 \times g_{av}$, 17 h, 2°C) in a RPS40T swing rotor (Hitachi Koki Co., Ltd., Tokyo, Japan). This procedure is referred to as the first sucrose gradient centrifugation. The bands formed at the 28%/30% and 32%/38% sucrose interfaces were collected as the crude endosome and plasma membrane fractions, respectively. The fractions, 3 ml, from the bottom of tube were pooled as the endoplasmic reticulum fraction. The crude endosome fraction was adjusted to 40% (w/w) sucrose with 65% sucrose/Tes and then 2 ml of it was overlaid with 11 ml of a 27–35% continuous sucrose gradient/Tes. The crude plasma membrane fraction was adjusted to 43% (w/w) sucrose and then 2 ml of it was overlaid with 11 ml of a 20–40% continuous sucrose gradient/Tes. Both gradients were centrifuged ($152000 \times g_{av}$, 17 h, 2°C) in the same swing rotor, and 0.2 ml fractions were sequentially collected from the top by pumping a 55% (w/w) sucrose solution into the bottom of the centrifuge tube with a density gradient fractionator (ISCO). The absorbance at 280 nm was determined sequentially using a flow cell. The fractions in the 1.120–1.135 g/cm^3 density range of the 27–35% sucrose gradient and in the 1.146–1.156 g/cm^3 density range of the 20–40% sucrose gradient were collected as the endosome and plasma membrane fractions, respectively. This procedure is referred to as the second sucrose gradient centrifugation. This plasma membrane preparation and a different plasma membrane preparation obtained by the method of Kaplan and Simoni [17] gave the same results as to the polypeptide and phospholipid molecular species compositions. Throughout the experiment, the sucrose concentration was estimated with a refractometer at 20°C.

Marker assays

β-*N*-Acetylhexosaminidase (EC 3.2.1.52) with *p*-nitrophenyl β-*N*-acetylglucosaminide as a substrate and acid phosphatase (EC 3.1.3.1) (lysosome) were determined as described [18,19]. NADPH–cytochrome-*c* reductase (EC 1.6.2.4) (endoplasmic reticulum) was determined spectrophotometrically [20]. Since Percoll interfered with this assay, the other marker enzyme for endoplasmic reticulum, cholinephosphotransferase (EC 2.7.8.2) [21], was assayed by the method of Coleman and Bell [22]. Galactosyltransferase (EC 2.4.1.67) (Golgi) was assayed with ovalbumin as an acceptor [23]. Succinate dehydrogenase (EC 1.3.99.1) (mitochondria) was determined as described [24]. The radioactivity of ^{125}I-iodinated cell surface proteins (plasma membrane) was mea-

sured by means of trichloroacetic acid precipitation [25].

Protein analysis

Protein was determined by the method of Bradford [26] or Lowry et al. [27] with modifications [28]. SDS-polyacrylamide gel electrophoresis was carried out under reducing conditions in 10% acrylamide gel [29]. Pooled crude endosome and endoplasmic reticulum fractions from the first sucrose gradient, and endosome and plasma membrane fractions from the second sucrose gradient were diluted with Tes and then centrifuged ($200000 \times g_{av}$, 1 h, 2°C). The pellets were dissolved in the sample buffer for SDS-polyacrylamide gel electrophoresis. The gels were fixed and stained with Coomassie brilliant blue for scanning at 600 nm with a Dual-Wavelength TLC Scanner CS-910 (Shimadzu, Kyoto, Japan) or with silver for photography.

Lipid analysis

Lipids were extracted by the method of Bligh and Dyer [30]. Phospholipids were separated by TLC as described previously [11]. For determination of the phospholipid composition, phospholipids were prepared from cells labeled with [^{32}P]P_i. After separation by TLC, the subfraction of each phospholipid was visualized by autoradiography and then scraped off. The radioactivity was measured in a toluene scintillation mixture. To analyze the phospholipid molecular species, phosphatidylcholine and phosphatidylethanolamine were extracted from the silica three times with 10 ml of chloroform/methanol (2:1, by vol.). Dinitrobenzoyl derivatives of diradylglycerols were prepared from the extracted phospholipids as previously described [10]. The dinitrobenzoyl derivatives of diacylglycerol, alkenylacylglycerol and alkylacylglycerol were separated by TLC with a solvent system of hexane/diethyl ether (80:20, by vol.). The dinitrobenzoyl derivatives of diacylglycerol extracted from the silica with diethyl ether, were determined by reversed-phase high performance liquid chromatography [10].

Electron microscopy

The endosome fraction obtained on the second sucrose gradient centrifugation was diluted, so as to the become an isotonic solution, with sucrose-free Tes and then centrifuged ($100000 \times g_{av}$, 30 min, 2°C) to yield a pellet. The pellet was fixed in 1% OsO_4 in Kellenberger's buffer at 4°C overnight and then stained en bloc with aqueous uranyl acetate for 2 h at room temperature. The sample was then dehydrated at 4°C and embedded in Epon. Thin sections were cut and viewed under a H-700H transmission electron microscope (Hitachi Co., Ltd., Tokyo, Japan) after grid-staining in uranyl acetate and lead citrate.

Results

Isolation of endosomes

Endosomes of V79-UF cells were labeled by means of receptor-mediated endocytosis of ^{125}I-EGF. Under the conditions described in Materials and Methods, almost all of the ^{125}I-EGF bound to cell surface receptors was internalized into the cells (data not shown). The homogenate of the labeled cells was fractionated on a Percoll gradient. The gradient fractions were assayed for membrane markers (Fig. 1). The radioactivity of ^{125}I-EGF was distributed as a single peak in the low-density region of the gradient (Fig. 1A), where plasma membranes, Golgi membranes and endoplasmic reticulum comigrated (Fig. 1B). The activity profiles of two marker enzymes for lysosomes showed two peaks, one each in the high- and low-density regions (Fig. 1C). The enzyme activities of the dense material might be derived from intact lysosomes, whereas the activities of the light material might be derived from disrupted lysosomes, released during the homogenization and subfractionation procedures [31]. ^{125}I-EGF appeared to have not reached the lysosomes within 8-min incubation at 37°C, since it was not detected in the high-density region. The subfractionation of the cell homogenate on a Percoll gradient resulted in clear separation of mitochondria from endosomes (Fig. 1C). However, by this method, it was difficult to separate plasma membranes, endoplasmic reticulum and Golgi from endosomes (Fig. 1B). Thus, we used two different sucrose density gradient centrifugations to isolate endosomes from the pooled fraction from the Percoll gradient. On the first sucrose gradient centrifugation, plasma membranes were com-

TABLE I

PURIFICATION OF ENDOSOMES

Endosomes were purified as described under Materials and Methods. At each step, the activities of markers and protein were determined, and expressed as % of the initial total cell homogenate values. Protein in the Percoll gradient pool fraction was determined by Bradford's method. The numbers in parentheses indicate the fold-enrichment. All values have been normalized as to the specific activity (relative to protein) in the total cell homogenate. Data are the averages for three experiments. The amount of protein in the total cell homogenate was 150 mg. The activities of markers per mg of protein were: ^{125}I-EGF, 991 cpm; ^{125}I-surface protein, 2945 cpm; *β-N*-acetylhexosaminidase, 12.2 nmol/min; acid phosphatase, 66.3 nmol/min; NADPH–cytochrome-*c* reductase, 6.1 nmol/min; cholinephosphotransferase, 0.53 nmol/min; succinate dehydrogenase, 3.7 nmol/min; galactosyltransferase, 10.4 pmol/min.

Marker	Total cell homogenate	Supernatant (500 × g)	Percoll	First sucrose gradient	Second sucrose gradient
Protein	100	27.0	6.6	0.18	0.06
^{125}I-EGF	100 (1)	45.9 (1.7)	33.5 (5.1)	9.4 (52.0)	3.3 (55.5)
^{125}I-Surface protein	100 (1)	29.7 (1.1)	18.4 (2.8)	n.d. [a]	n.d. [a]
β-N-Acetylhexosaminidase	100 (1)	23.2 (0.9)	5.6 (0.9)	0.3 (1.6)	
Acid phosphatase	100 (1)	23.0 (0.9)	5.2 (0.8)	0.3 (1.8)	0.01 (0.2)
NADPH–cytochrome-*c* reductase	100 (1)	30.2 (1.1)		0.8 (4.5)	
Cholinephosphotransferase	100 (1)	30.3 (1.1)	7.6 (1.2)	0.8 (4.4)	0.04 (0.7)
Succinate dehydrogenase	100 (1)	35.6 (1.3)	2.0 (0.3)	n.d. [a]	n.d. [a]
Galactosyltransferase	100 (1)	16.9 (0.6)	7.6 (1.2)	0.3 (1.5)	0.04 (0.7)

[a] n.d., there was no detectable activity.

pletely removed from the endosome fraction, and ^{125}I-EGF was highly condensed in the interface fraction between 28% and 30% sucrose. The enrichment of ^{125}I-EGF in this fraction was 52-fold, as to the total cell homogenate (Table I). Most of the marker activities for organelles other than endosomes was distributed in the heavier-density region above 30% sucrose. Although markers for Golgi, endoplasmic reticulum and lysosome were present in the endosome fraction (28%/30% sucrose interface), their enrichment was only equivalent to 1/35th, 1/12th and 1/29th that of ^{125}I-EGF, respectively (Table I). The combined endosome pool was further processed by means of second sucrose gradient centrifugation. A single peak of ^{125}I-EGF appeared on the gradient, which coincided with that of protein (Fig. 2). The second sucrose gradient procedure resulted in a slight enrichment of ^{125}I-EGF, from 52- to 55-fold. The final yield of ^{125}I-EGF amounted to 3.3% of the total cell homogenate (Table I). The extent of the purification is probably an underestimate, since partial disruption of endosomes during purification may have led to the loss of ^{125}I-EGF. Significant decreases in the enrichment of markers

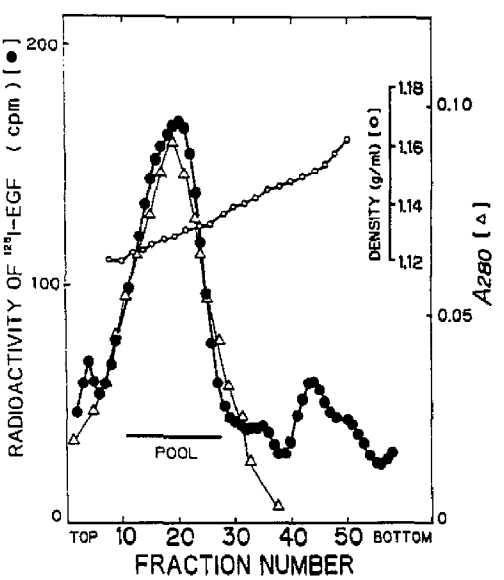

Fig. 2. Distributions of ^{125}I-EGF and protein on isopycnic centrifugation on a continuous-density sucrose gradient. The crude endosome fraction from the first sucrose gradient was fractionated by continuous-density sucrose gradient centrifugation, as described under Materials and Methods. The ^{125}I-EGF peak fractions were pooled. Similar distributions of ^{125}I-EGF and protein were observed in six separate experiments.

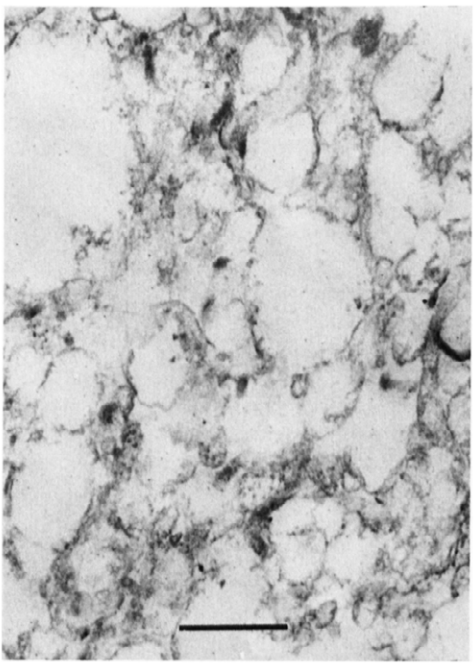

Fig. 3. Electron micrograph of isolated endosomes. Endosomes were isolated and viewed under a transmission electron microscope as described under Materials and Methods. Bar, 0.5 μm.

for Golgi (0.6), endoplasmic reticulum (0.8) and lysosome (0.2) were observed in this fraction.

An electron micrograph of the endosome preparation shows vesicles of 0.2–0.7 μm in diameter (Fig. 3).

Polypeptide composition of endosomes

The polypeptide composition of endosomes was examined by SDS-polyacrylamide gel electrophoresis on 10% gels. The composition of endosomes (Fig. 4A, lane 5) is different from those of the total cell homogenate (lane 1) and endoplasmic reticulum (lane 7). The polypeptide profile of endosomes was similar to that of plasma membranes (lane 6). However, there were differences in the amounts of some polypeptides (Fig. 4B). Polypeptides with apparent M_r of 106000, 80000 and 43000 were more enriched in endosomes than in plasma membranes.

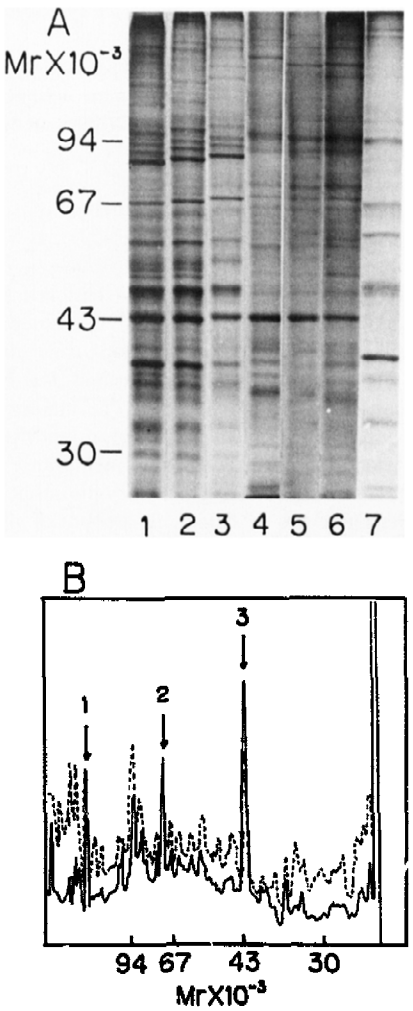

Fig. 4. SDS-polyacrylamide gel electrophoresis of membrane fractions at various stages of purification. (A) Membranes were fractionated, and SDS-polyacrylamide gel electrophoresis was carried out on 10% gels as described under Materials and Methods. The gels were stained with silver. Lane 1, total cell homogenate; lane 2,postnuclear supernatant; lane 3, Percoll centrifuged pool; lane 4, first sucrose gradient centrifuged pool (crude endosome fraction); lane 5, second sucrose gradient centrifuged pool (endosomes); lane 6, plasma membranes; lane 7, endoplasmic reticulum. (B) After SDS-polyacrylamide gel electrophoresis, the gels were stained with Coomassie brilliant blue and then scanned at 600 nm. Plasma membranes (dotted line); endosomes (solid line). The absorbance at 600 nm in arbitrary units is shown on the ordinate. Arrows 1, 2 and 3 indicate polypeptides with apparent M_r of 106000, 80000 and 43000, respectively.

Polar head group composition of phospholipids of endosomes

V79-UF cells, which require monoenoic fatty acids for growth [11], were grown in delipidated serum medium (serum lipoprotein-free medium) supplemented with oleic acid. Assaying of the polar head group composition of phospholipids was carried out by means of labeling of phospholipids with [^{32}P]P_i for 24 h (Table II), at which time the incorporation of ^{32}P into phospholipids was at plateau. Endosomes were enriched with sphingomyelin and phosphatidylserine, compared with plasma membranes and endoplasmic reticulum. This increase was compensated for by a decrease in phosphatidylcholine. The majority of phosphatidylcholine was composed of diacylphosphatidylcholine throughout the membrane preparations, the rest being alkylacylphosphatidylcholine. There was no detectable amount of alkenylacylphosphatidylcholine. Phosphatidylethanolamine was composed of diacyl molecular species and a small amount of alkylacyl and alkenylacyl molecular species in all the membrane preparations.

Molecular species composition of phospholipids of endosomes

The molecular species compositions of the major phospholipids, diacylphosphatidylcholine and diacylphosphatidylethanolamine (Table II), were determined. The major molecular species of diacylphosphatidylcholine were 18 : 1/ 18 : 1, 16 : 0/18 : 1 and 18 : 0/18 : 1 (Table III). However, the proportion of 18 : 1/18 : 1 was lower in plasma membranes than in the total cell homogenate or endoplasmic reticulum. In addition, endosomes contained a lower proportion of molecular species with two monoenoic fatty acids than plasma membranes did. This is mostly due to the difference in the proportion of 18 : 1/18 : 1 between endosomes and plasma membranes. The proportions of molecular species with two saturated fatty acids and with one saturated and one monoenoic fatty acid were higher in endosomes than in plasma membranes.

The major molecular species of diacylphosphatidylethanolamine were 18 : 1/18 : 1, 16 : 0/18 : 1 and 18 : 0/18 : 1 (Table IV). Contrary to in the case of diacylphosphatidylcholine, the proportion of 18 : 1/18 : 1 was higher in plasma membranes (53.0%) than in the total cell homogenate (39.3%) or endoplasmic reticulum (44.5%). However, the proportion of molecular species with two monoenoic fatty acids, of which 18 : 1/18 : 1 was a major component, was lower in endosomes (58.6%) than in plasma membranes (63.4%). The difference was smaller than that observed in the case of diacylphosphatidylcholine.

TABLE II

PHOSPHOLIPID COMPOSITION OF MEMBRANES FROM V79-UF CELLS GROWN IN THE PRESENCE OF OLEIC ACID

Cells were grown in MEM containing 6% delipidated serum and 50 μM oleic acid and then labeled with [^{32}P]P_i. The phospholipids were extracted from the isolated membranes, separated and then determined, as described under Materials and Methods. The data are the mean values ± S.E. ($n = 4$). The incorporated radioactivity of [^{32}P]P_i amounted to 68248 cpm/μg of P_i. Above 99% of the applied radioactivity on the thin-layer plate before development was recovered on the spots of phospholipids.

Phospholipid	mol%			
	plasma membranes	endosomes	total cell homogenate	endoplasmic reticulum
Sphingomyelin	5.7 ± 0.8	9.5 ± 1.1 [a]	4.5 ± 0.3	5.7 ± 0.5
Phosphatidylcholine	56.8 ± 0.8	50.7 ± 1.3 [b]	67.3 ± 0.5 [c]	59.1 ± 0.5
Phosphatidylserine	5.7 ± 0.6	9.5 ± 1.0 [a]	2.1 ± 0.3 [d]	5.5 ± 0.4
Phosphatidylinositol	3.7 ± 0.4	3.0 ± 0.3	3.0 ± 0.4	5.2 ± 0.4 [a]
Phosphatidylethanolamine	27.1 ± 1.9	25.7 ± 0.5	20.4 ± 1.0 [a]	21.4 ± 1.2 [a]
Cardiolipin	1.0 ± 0.3	1.6 ± 0.4	2.7 ± 0.5 [d]	3.1 ± 0.4 [b]

[a–d] The statistical significance of values compared to in the case of plasma membranes was determined by the paired *t*-test: [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$; [d] $P < 0.005$.

TABLE III

MOLECULAR SPECIES COMPOSITIONS OF DIACYLPHOSPHATIDYLCHOLINE IN MEMBRANES FROM CELLS GROWN IN THE PRESENCE OF OLEIC ACID

Cells were grown in MEM containing 6% delipidated serum and 50 μM oleic acid for 72 h. Membranes were isolated, and the molecular species of diacylphosphatidylcholine were determined as described under Materials and Methods. The data are the mean values ± S.E. ($n = 2$).

Molecular species	mol%			
	plasma membranes	endosomes	total cell homogenate	endoplasmic reticulum
18:1/16:1	5.7±0.3	9.2±3.4	5.1±0.1	5.2±0.2
16:0/16:1	5.7±0.3	5.0±0.3	5.6±0.3	5.6±0.3
16:0/14:0	2.5±0.0	3.7±0.3 [a]	1.9±0.1 [b]	2.0±0.0 [b]
18:1/18:1	30.6±0.1	21.0±1.7 [b]	37.0±0.9 [b]	34.8±0.2 [c]
16:0/18:1	32.2±0.4	34.1±2.3	30.0±0.8	30.5±0.1 [a]
16:0/16:0	4.8±0.0	8.0±0.7 [b]	3.1±0.2 [b]	4.0±0.0 [d]
20:1/18:1	4.0±0.1	2.5±0.3 [a]	4.7±0.4	4.4±0.1
18:0/18:1	12.1±0.3	14.2±0.5 [a]	10.6±0.1 [b]	10.8±0.3 [a]
18:0/16:0	1.2±0.0	1.9±0.3	0.8±0.0 [b]	1.3±0.1
22:1/18:1	1.2±0.0	0.4±0.4	1.2±0.1	1.4±0.2
Total UFA/UFA [e]	41.5±0.4	33.1±1.7 [b]	48.0±1.3 [b]	45.8±0.3 [b]
Total SFA/UFA [f]	50.0±0.4	53.3±3.0	46.2±1.0 [a]	46.9±0.1 [b]
Total SFA/SFA [g]	8.5±0.0	13.6±1.3 [a]	5.8±0.3 [b]	7.3±0.2 [b]

[a–d] The statistical significance of values compared to in the case of plasma membranes was determined by the paired t-test: [a] $P < 0.1$; [b] $P < 0.05$; [c] $P < 0.005$; [d] $P < 0.001$.

[e] UFA/UFA, molecular species with two monoenoic fatty acids.

[f] SFA/UFA, molecular species with one saturated and one monoenoic fatty acid.

[g] SFA/SFA, molecular species with two saturated fatty acids.

Effects of changes in the composition of phospholipid molecular species in plasma membranes on the lipid composition of endosomes

There was little difference in the polar head group composition of phospholipids in the total cell homogenate between cells grown in the presence and absence of oleic acid. The enrichment of sphingomyelin and phosphatidylserine with a decrease in phosphatidylcholine in endosomes was observed, compared with in plasma membranes (data not shown), similar to in the case of cells grown in the presence of oleic acid (Table II). The composition of phospholipid molecular species in plasma membranes was altered by growing V79-UF cells in the oleic-acid deficient medium. With a shift-down from normal to oleic-acid deficient medium, the proportion of the 18:1/18:1 species of diacylphosphatidylcholine in the total cell homogenate decreased from 37.0 to 28.5% (Tables III and V). However, the proportion of molecular species with two monoenoic fatty acids was lower in endosomes (26.1%) than in plasma membranes (35.6%), whereas those of molecular species with one saturated and one monoenoic fatty acid, and with two saturated fatty acids were higher in endosomes (59.2 and 14.7%) than in plasma membranes (53.0 and 11.4%).

There were small differences in the molecular species composition of diacylphosphatidylethanolamine in the total cell homogenate between cells grown in the presence and absence of oleic acid (Tables IV and VI). However, with a shift-down from normal to oleic-acid deficient medium, the proportion of the 18:1/18:1 species in plasma membranes decreased from 53.0 to 41.4%. A remarkable difference in the proportion of the 18:1/18:1 species was observed between endosomes (31.8%) and plasma membranes (41.4%), even in the case of cells grown in oleic-acid deficient medium.

TABLE IV

MOLECULAR SPECIES COMPOSITIONS OF DIACYLPHOSPHATIDYLETHANOLAMINE IN MEMBRANES FROM CELLS GROWN IN THE PRESENCE OF OLEIC ACID

Cells were grown in MEM containing 6% delipidated serum and 50 μM oleic acid for 72 h. Membranes were isolated, and the molecular species of diacylphosphatidylethanolamine were determined as described under Materials and Methods. The data are the mean values ± S.E. ($n = 2$).

Molecular species	mol%			
	plasma membranes	endosomes	total cell homogenate	endoplasmic reticulum
18:1/16:1	5.5±0.7	5.9±1.6	3.9±0.3	5.0±0.1
16:0/16:1	2.0±0.0	2.3±0.5	2.0±0.0	2.7±0.2 [a]
16:0/14:0	0.2±0.1	0.6±0.3	0.5±0.0	1.1±0.5
18:1/18:1	53.0±1.6	48.0±2.1	39.3±0.9 [b]	44.5±0.7 [b]
16:0/18:1	15.8±0.6	17.1±0.0	21.0±1.0 [b]	22.4±1.3 [b]
16:0/16:0	1.1±0.1	2.3±0.8	1.4±0.1	2.0±0.1 [b]
20:1/18:1	3.0±0.2	3.2±0.4	2.6±0.2	2.4±0.1 [a]
18:0/18:1	16.6±0.2	18.2±0.1 [b]	26.5±0.5 [c]	17.5±0.2 [a]
18:0/16:0	0.9±0.0	0.9±0.3	1.3±0.2	0.9±0.1
22:1/18:1	1.9±0.1	1.5±0.2	1.5±0.1 [a]	1.5±0.1 [b]
Total UFA/UFA [e]	63.4±1.0	58.6±1.1 [a]	47.3±0.8 [d]	53.4±1.0 [b]
Total SFA/UFA [f]	34.4±0.9	37.6±0.4 [a]	49.5±0.5 [c]	42.6±1.3 [b]
Total SFA/SFA [g]	2.2±0.2	3.8±0.7	3.2±0.3	4.0±0.3 [b]

[a–d] The statistical significance of values compared to in the case of plasma membranes was determined by the paired t-test: [a] $P < 0.1$; [b] $P < 0.05$; [c] $P < 0.005$; [d] $P < 0.01$.

[e] UFA/UFA, molecular species with two monoenoic fatty acids.

[f] SFA/UFA, molecular species with one saturated and one monoenoic fatty acid.

[g] SFA/SFA, molecular species with two saturated fatty acids.

Discussion

In the present study, we isolated endosomes from V79-UF cells by Percoll density gradient centrifugation and then two sucrose density gradient centrifugations. The isolated endosomes were mostly free from contamination by Golgi, endoplasmic reticulum and plasma membranes, which are known to have similar buoyant densities to that of endosomes (Table I). The enrichment of ^{125}I-EGF in endosomes was more than 55-fold. This degree of enrichment is comparable to in the case of purified endosomes from human KB cells [32] and Chinese hamster ovary cells [33]. Marsh et al. [33] used free flow electrophoresis to separate endosomes from other organelles based on a difference in surface charge of the particles. They reported that the treatment of crude membranes with 0.25% trypsin at 37°C for 5 min was required for production of the difference in surface charge between endosomes and other organelles. However, the trypsin treatment of endothelial cells and green monkey kidney cells caused the release of phospholipids from the cells [34]. In order to determine the phospholipid composition of endosomes, it does not appear desirable to use free flow electrophoresis, which requires trypsin treatment.

It has been assumed that endosome membranes have the same phospholipid composition as that of plasma membranes, since the membrane phospholipids of endosomes are derived from plasma membranes. However, sphingomyelin and phosphatidylserine were enriched in endosomes (Table II) compared with in plasma membranes. In hepatocytes, the enrichment of sphingomyelin in endosomes has been demonstrated by Luzio and Stanley [35], Evans and Hardison [36], and Belcher et al. [37]. However, the physiological significance of the enrichment of these phospholipids in endocytic vesicles remains obscure.

In this study, we demonstrated that the molecu-

lar species compositions of the major phospholipids in endosomes differ from those in plasma membranes. The differences between endosomes and plasma membranes remained, regardless of whether or not the proportions of phospholipid molecular species in the plasma membranes changed. The most remarkable results were obtained for diacylphosphatidylcholine (Tables III and V). The proportion of molecular species with two monoenoic fatty acids in endosomes was 73 and 80% of those in the plasma membranes of cells grown with and without oleic acid, respectively.

TABLE V

MOLECULAR SPECIES COMPOSITIONS OF DIACYLPHOSPHATIDYLCHOLINE IN MEMBRANES FROM CELLS GROWN AFTER A SHIFT-DOWN FROM NORMAL TO OLEIC-ACID DEFICIENT MEDIUM

Cells were grown in MEM containing 6% delipidated serum and 50 μM oleic acid for 48 h, and then the medium was replaced with oleic-acid deficient medium. After a further 24-h cultivation, membranes were isolated, and the molecular species of diacylphosphatidylcholine were determined as described under Materials and Methods. The data are the mean values ± S.E. ($n = 3$).

Molecular species	mol%		
	plasma membranes	endosomes	total cell homogenate
18:1/16:1	7.5±1.0	5.2±1.4	8.1±0.8
16:0/16:1	8.7±0.5	7.6±0.5	8.4±0.1
16:0/14:0	4.2±0.3	5.2±0.2 [a]	2.9±0.2 [a]
18:1/18:1	23.4±2.1	16.9±1.7 [b]	28.5±2.2
16:0/18:1	34.9±0.5	39.8±1.1 [a]	33.9±0.4
16:0/16:0	5.7±1.2	7.8±1.0	3.7±0.4
20:1/18:1	3.6±0.2	2.8±0.2 [b]	4.0±0.3
18:0/18:1	9.4±0.4	11.8±0.6 [a]	8.5±0.7
18:0/16:0	1.5±0.3	1.7±0.2	1.0±0.2
22:1/18:1	1.1±0.1	1.2±0.4	1.0±0.1
Total UFA/UFA [c]	35.6±1.3	26.1±2.3 [a]	41.6±1.7 [a]
Total SFA/UFA [d]	53.0±0.6	59.2±1.3 [a]	50.8±0.9
Total SFA/SFA [e]	11.4±1.6	14.7±1.2	7.6±0.8

[a,b] The statistical significance of values compared to in the case of plasma membranes was determined by the paired t-test: [a] $P < 0.05$; [b] $P < 0.1$.

[c] UFA/UFA, molecular species with two monoenoic fatty acids.

[d] SFA/UFA, molecular species with one saturated and one monoenoic fatty acid.

[e] SFA/SFA, molecular species with two saturated fatty acids.

TABLE VI

MOLECULAR SPECIES COMPOSITIONS OF DIACYLPHOSPHATIDYLETHANOLAMINE IN MEMBRANES FROM CELLS GROWN AFTER A SHIFT-DOWN FROM NORMAL TO OLEIC-ACID DEFICIENT MEDIUM

Cells were grown in MEM containing 6% delipidated serum and 50 μM oleic acid for 48 h, and then the medium was replaced with oleic-acid deficient medium. After a further 24-h cultivation, membranes were isolated, and the molecular species of diacylphosphatidylethanolamine were determined as described under Materials and Methods. The data are the mean values ± S.E. ($n = 3$).

Molecular species	mol%		
	plasma membranes	endosomes	total cell homogenate
18:1/16:1	10.2±0.9	7.9±1.5	7.3±0.9 [a]
16:0/16:1	4.4±0.7	4.7±1.8	3.2±0.8
18:1/18:1	41.4±2.2	31.8±3.3 [a]	35.6±1.4 [a]
16:0/18:1	20.4±0.9	21.1±0.7	23.2±1.4
16:0/16:0	2.7±0.7	4.8±0.5 [a]	2.6±0.4
20:1/18:1	2.4±0.1	2.4±0.1	2.3±0.0
18:0/18:1	14.7±1.1	20.0±5.2	22.3±3.2 [a]
18:0/16:0	2.3±1.0	4.9±1.3	2.0±0.5
22:1/18:1	1.5±0.2	2.4±0.2 [b]	1.5±0.1
Total UFA/UFA [c]	55.5±2.7	44.5±4.7	46.7±1.3 [b]
Total SFA/UFA [d]	39.5±1.3	45.8±4.2	48.7±1.9 [b]
Total SFA/SFA [e]	5.0±1.7	9.7±1.6	4.6±0.8

[a,b] The statistical significance of values compared to in the case of plasma membranes was determined by the paired t-test: [a] $P < 0.1$; [b] $P < 0.05$.

[c] UFA/UFA, molecular species with two monoenoic fatty acids.

[d] SFA/UFA, molecular species with one saturated and one monoenoic fatty acid.

[e] SFA/SFA, molecular species with two saturated fatty acids.

The differences in the polar head group and molecular species compositions of phospholipids between endosomes and plasma membranes may be explained by (1) transfer of particular phospholipids from plasma membranes to endosomes and/or (2) recycling of ones from endosomes to plasma membranes. Phospholipids of plasma membranes are known to be distributed asymmetrically across the plane of the bilayer [38–40]. If either the inner or outer leaflet is used in preference to the other for the formation of coated vesicles and/or pinocytic vesicles, particular phospholipids must be enriched in the vesicles formed. The relatively small early endocytic vesicles may

contain more lipids derived from the inner half of the plasma membrane because of their strong curvature. In addition, heterogeous lateral distribution causing the existence of lipid [41] and protein [42] domains in biological membranes has been indicated. The results of these studies imply the assembly of particular phospholipids at sites on plasma membranes, which are used for invagination on vesicle formation.

On the other hand, fibroblasts interiorize membrane equivalent to 0.5- to 2-fold their surface area during each hour of pinocytic activity [43]. Thus, the bulk of the interiorized membrane is recycled to the plasma membrane within minutes. This membrane recycling occurs during the initial fusion between early endocytic vesicles and immediately before the fusion of more mature perinuclear endosomes with lysosomes [44]. The endosome preparation used in this study may have contained the entire population of prelysosomal vacuoles, i.e., from the early peripheral endosomes to the mature perinuclear endosomes. Thus, if specific phospholipid molecular species, such as 18:1/18:1 phosphatidylcholine, were predominantly recycled from endosomes to plasma membranes, the phospholipid molecular species of isolated endosomes would be poor in the recycled phospholipid molecular species, compared with in plasma membranes.

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References

1 Helenius, A., Mellman, I., Wall, D. and Hubbard, A. (1983) Trends Biochem. Sci. 8, 245–250.
2 Pastan, I. and Willingham, M.C. (1983) Trends Biochem. Sci. 8, 250–254.
3 Bergeron, J.J.M., Cruz, J., Khan, M.N. and Posner, B.I. (1985) Annu. Rev. Physiol. 47, 383–403.
4 Steinman, R.M., Mellman, I.S., Muller, W.A. and Cohn, Z.A. (1983) J. Cell. Biol. 96, 1–27.
5 Schroeder, F. (1981) Biochim. Biophys. Acta 649, 162–174.
6 Mazière, C., Mazière, J.C., Morá, L., Auclair, M., Salmon, S. and Polonovski, J. (1986) Biochem. Biophys. Res. Commun. 137, 43–49.
7 Mahoney, E.M., Hamill, A.L., Scott, W.A. and Cohn, Z.A. (1977) Proc. Natl. Acad. Sci. USA 74, 4895–4899.
8 Heiniger, H.-J., Kandutsch, A.A. and Chen, H.W. (1976) Nature 263, 515–517.
9 Esfahani, M., Scerbo, L., Lund-Katz, S., DePace, D.M., Maniglia, R., Alexander, J.K. and Phillips, M.C. (1986) Biochim. Biophys. Acta 889, 287–300.
10 Takamura, H., Narita, H., Urade, R. and Kito, M. (1986) Lipids 21, 356–361.
11 Hayashi, Y., Urade, R. and Kito, M. (1987) Biochim. Biophys. Acta 918, 267–273.
12 Cham, B.E. and Knowles, B.R. (1976) J. Lipid Res. 17, 176–181.
13 Rintoul, D.A. and Simoni, R.D. (1977) J. Biol. Chem. 252, 7916–7918.
14 Haigler, H.T., Maxfield, F.R., Willingham, M.C. and Pastan, I. (1980) J. Biol. Chem. 255, 1239–1241.
15 Hynes, R.O. (1973) Proc. Natl. Acad. Sci. USA 70, 3170–3174.
16 Mellman, I.S., Steinman, R.M., Unkeless, J.C. and Cohn, Z.A. (1980) J. Cell Biol. 86, 712–722.
17 Kaplan, M.R. and Simoni, R.D. (1985) J. Cell Biol. 101, 446–453.
18 Li, Y.-T. and Li, S.-C. (1972) Methods Enzymol. 28, 702–713.
19 Hübscher, G. and West, G.R. (1965) Nature 205, 799–800.
20 Phillips, A.H. and Langdon, R.G. (1962) J. Biol. Chem. 237, 2652–2658.
21 Jelsema, C.L. and Morré, D.J. (1978) J. Biol. Chem. 253, 7960–7971.
22 Coleman, R. and Bell, R.M. (1977) J. Biol. Chem. 252, 3050–3056.
23 Rothman, J.E. and Fries, E. (1981) J. Cell Biol. 89, 162–168.
24 Pennington, R.J. (1961) Biochem. J. 80, 649–654.
25 Hubbard, A.L. and Cohn, Z.A., (1975) J. Cell Biol. 64, 438–460.
26 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
28 Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
29 Laemlli, U.K. (1970) Nature 227, 680–685.
30 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
31 Wilcox, C.A. and Olson, E.N. (1987) Biochemistry 26, 1029–1036.
32 Dickson, R.B., Beguinot, L., Hanover, J.A., Richert, N.D., Willingham, M.C. and Pastan, I. (1983) Proc. Natl. Acad. Sci. USA 80, 5335–5339.
33 Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I. and Helenius, A. (1987) J. Cell Biol. 104, 875–886.
34 Kirkpatrick, C.J., Melzner, I. and Goller, T. (1985) Biochim. Biophys. Acta 846, 120–126.
35 Luzio, J.P. and Stanley, K.K. (1983) Biochem. J. 216, 27–36.
36 Evans, W.H. and Hardison, W.G.M. (1985) Biochem. J. 232, 33–36.

37 Belcher, J.D., Hamilton, R.L., Brady, S.E., Hornick, C.A., Jaeckle, S., Schneider, W.J. and Havel, R.J. (1987) Proc. Natl. Acad. Sci. USA 84, 6785–6789.
38 Martin, O.C. and Pagano, R.E. (1987) J. Biol. Chem. 262, 5890–5898.
39 Van Meer, G., Simons, K., Op den Kamp, J.A.F. and Van Deenen,L.M. (1981) Biochemistry 20, 1974–1981.
40 Bishop, W.R. and Bell, R.M. (1985) Cell 42, 51–60.
41 Storch, J. and Kleinfeld, A.M. (1985) Trends Biochem. Sci. 10, 418–421.
42 Yechiel, E. and Edidin, M. (1987) J. Cell Biol. 105, 755–760.
43 Thilo, L. (1985) Biochim. Biophys. Acta 822, 243–266.
44 Haylett, T. and Thilo, L. (1986) J. Cell Biol. 103, 1249–1256.