Plasma membrane biogenesis in eukaryotic cells: Translocation of newly synthesized lipid

(*Acanthamoeba castellanii*/sterols/phospholipids)

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Communicated by Eugene P. Kennedy, December 5, 1983

ABSTRACT We examined the transfer of sterols and phospholipids from their site of synthesis to the plasma membrane of *Acanthamoeba castellanii*. Cells were labeled with [3 H]acetate, and plasma membrane fractions were isolated under conditions that minimize the nonspecific exchange of lipids between subcellular membrane fractions. Sterols and phospholipids were purified from both whole-cell homogenates and isolated plasma membrane. In whole cells, 3 H-labeled lipids were formed, with no apparent time lag, in a linear manner up to 1 hr. Labeled sterol and phospholipids appeared in the plasma membrane, after a 30-min lag, at approximately the same rate. However, the ratio of newly synthesized sterol to phospholipid was significantly enriched in the plasma membrane relative to the whole cell, even at the earlier time points. Pulse-chase experiments indicated that sterols and phospholipids are turned over in the plasma membrane with similar, rather short half-lives. The results of these studies suggest that, although sterols and phospholipids are transported to the cell surface with similar kinetics, some sorting of the lipids must occur at an early stage in membrane biogenesis. The data are consistent with a model of lipid translocation by vesicular transport.

The problem of cellular sorting and targeting of molecular components destined for the plasma membrane has received much attention in recent years. Due largely to the introduction of a number of novel techniques, a great deal of information has emerged concerning the mechanism involved in cell processing and transporting the integral membrane proteins (1-3).

Dealing with the problem of intracellular lipid transport has been a more difficult task, primarily due to technical obstacles. Nevertheless, the problem of sorting and targeting of lipids is clearly one that requires consideration. The primary site of synthesis of both sterols and phospholipids is the endoplasmic reticulum (4); yet the sterol-to-phospholipid ratio is significantly higher in the plasma membrane than in other intracellular membranes (5, 6).

We have begun to examine the problem of lipid translocation to the surface membrane of *Acanthamoeba castellanii*. In the study reported here, we investigated the kinetics of transport of both sterol and phospholipid molecules to the plasma membrane. Our results suggest that vesicular transport may be involved in the movement of lipids to the cell surface in this organism.

METHODS

Cell Culture and Isolation of Plasma Membrane-Enriched Fractions. *A. castellanii* were cultured as described by Korn (7). Plasma membrane fractions were isolated from cell ho-

mogenates by a smaller-scale modification of the procedure described by Korn (7).

Labeling of Whole Cells and Plasma Membrane Fractions with [3 H]Acetate. Cells were collected by centrifugation, washed twice, and suspended at a density of 7.5×10^7 cells per ml in phosphate-buffered saline (pH 7.0). The incubations with [3 H]acetate were carried out at 23°C with occasional shaking. At the indicated times, 1 ml of the labeled cell suspension was added to 10 ml of ice-cold 10 mM Tris-HCl, pH 7.4, and the cells were collected at $500 \times g$ for 2 min. The cell pellets were homogenized, an aliquot was removed for analysis of whole cells, and plasma membrane fractions were isolated as described above.

The radioactive compound used was [3 H]acetic acid, sodium salt (2 Ci/mmol, ICN; 1 Ci = 37 GBq).

Lipid Analyses. Amoeba lipids were extracted by the method of Bligh and Dyer (8) and fractionated into neutral lipids and phospholipids on a small silicic acid column. Neutral lipids were further separated by TLC on plastic-backed Silica gel G strips (Brinkmann) developed in chloroform/methanol, 100:2 (vol/vol). The strips containing radioactive samples were cut into 0.5-cm sections and assayed directly in 5 ml of Ultrafluor (National Diagnostics, Somerville, NJ).

Because ergosterol and related sterols are known to be susceptible to light-induced degradation (9), care was taken to minimize the exposure of these compounds to light after the lipid extraction procedure. A purified ergosterol standard carried through the same analytical procedure gave only one spot after TLC.

Sterols were quantitated by GLC on a column of 3% OV-17 on 80-100 Gas Chrom Q (Applied Sciences, Deerfield, IL) with 5 α -cholestane as the internal standard.

The phosphate content of phospholipid fractions was determined by the method of Chen *et al.* (10) after ashing as described by Ames and Dubin (11).

Other Methods. Protein was determined by the method of Lowry *et al.* (12) with bovine serum albumin as a standard, and 5'-nucleotidase activity was measured as described by Mills (13). Alkaline phosphodiesterase was determined with p-nitrophenylthymidine 5'-monophosphate as a substrate (14).

RESULTS

Lipid Composition of *Acanthamoeba* Plasma Membrane. Plasma membrane fractions isolated from *A. castellanii* in the present study had a lipid composition (see Table 1) that did not vary significantly from that found by Ulsamer *et al.* (15). The plasma membrane marker enzymes, 5'-nucleotidase and alkaline phosphodiesterase, were enriched (4.9- and 6.5-fold, respectively) in this fraction relative to whole-cell homogenates. The molar ratio of sterol to phospholipid in our preparation (Table 1) was 0.988 and is typical of that found in other eukaryotic cell membranes. Because the purpose of the present study was to examine the biosynthesis of

Table 1. Lipid composition of whole cells and plasma membrane isolated from *A. castellanii*

	Whole cells	Plasma membrane	Enrichment
Sterol,			
$\mu\text{g}/\text{mg}$ of protein	21.4	173.2	8.1
$\text{cpm} \times 10^{-3}/\text{mg}$ of protein	2.2	24.9	11.3
Phospholipid,			
nmol/mg of protein	142.4	472.4	3.3
$\text{cpm} \times 10^{-3}/\text{mg}$ of protein	8.9	31.4	3.5
Sterol/phospholipid ratio,			
mol/mol	0.380	0.988	2.6
cpm/cpm	0.249	0.792	3.2

Cells (7.5×10^7) were labeled with 3 mCi of [^3H]acetate in phosphate-buffered saline at 23°C for 2 hr.

sterol and phospholipid components of the plasma membrane, we considered this ratio to be the most important indicator of membrane purity.

The labeling pattern of sterols and total phospholipids is also presented in Table 1. After 2 hr of incubation with [^3H]acetate, the enrichment of labeled lipids in the plasma membrane relative to the homogenate was comparable to that of total lipids, suggesting that at this point the specific radioactivity of lipids in the plasma membrane had reached a steady-state value. For ease in comparing sterols to phospholipids in the subsequent labeling experiments, most of the data are presented as cpm per mg of protein. Thus, the apparent enrichment of plasma membrane fractions over whole cells reflects the actual enrichment in lipid content relative to protein (Table 1).

When the sterol fractions were analyzed by GLC, they were found to contain predominantly ergosterol (40%) and a component (60%) with a slightly longer retention time, which Ulsamer *et al.* have identified as 7-dehydroporiferasterol (16). Also in agreement with Korn *et al.* (17), we found that

these sterols were converted to derivatives upon incubation of homogenized cells or subcellular fractions, even at 4°C. The TLC system utilized for the present studies separated these derivatives ($R_f = 0.52$) from the original sterols ($R_f = 0.44$). For quantitation of labeled sterols, these two regions were combined. Thus, the conversion of the sterols during cell fractionation does not alter the interpretation of our results in any way.

Incorporation of [^3H]Acetate into Whole-Cell and Plasma Membrane Lipids. The synthesis of sterols from [^3H]acetate in whole cells took place with no apparent lag under the conditions utilized for these experiments (Fig. 1). This synthesis occurred at a linear rate for at least 60 min before reaching a steady state. The appearance of labeled sterol in the plasma membrane was biphasic. There was an initial lag (or slower rate of appearance) for at least 30 min, followed by a much more rapid rate of 407 $\text{cpm}(\text{mg of protein})^{-1}\text{min}^{-1}$ for the next 90 min. Although the initial slower rate may represent the transport of a separate intracellular pool of sterols, it more likely reflects a minor contamination of the plasma membrane fraction with some other intracellular membrane. This interpretation is supported by the data presented in Fig. 1 *Inset*. The percentage of total labeled sterol present in the plasma membrane fraction remained constant at 1.8% for the first 30 min of continuous labeling. This would not be expected if any movement of sterol to the surface occurred during this time but might be explained by the presence of some other membrane fraction (more enriched in sterol/protein than the whole cell) present as a minor contaminant. After 2 hr of incubation, 5.4% of the total labeled sterol was recovered in the plasma membrane fraction. This value is comparable to that of the total sterol recovered in this fraction as measured by GLC and, thus, reflects the recovery of plasma membrane from the sucrose gradients.

The synthesis of phospholipids (Fig. 2) occurred at a more rapid rate and reached a higher level when normalized to protein than did the sterols (Fig. 1). However, the appearance of radioactive phospholipids in the plasma membrane was quite similar to that of the sterols. There was again an initial lag of 30 min, followed by a more rapid rate of incorpo-

FIG. 1. Incorporation of [^3H]acetate in sterols in whole cells (●) and plasma membranes (○). *Acanthamoeba* (6×10^8 cells) were incubated at 23°C in 8 ml of phosphate-buffered saline containing 3 mCi of [^3H]acetate. At the indicated times, 1 ml of cell suspension was removed and washed, and plasma membrane fractions were isolated. Aliquots were removed for protein determination, and then lipids were extracted from plasma membranes and whole-cell homogenates. Sterols were purified as described. (*Inset*) Percentage of total labeled sterol present in the plasma membrane (PM) fraction. The data shown are typical of those obtained in four separate experiments.

FIG. 2. Incorporation of [^3H]acetate into phospholipid in whole cells (●) and plasma membrane (○). Experimental details were identical to those described in Fig. 1. Phospholipids and neutral lipids were separated as described. (*Inset*) Percentage of total labeled phospholipid present in the plasma membrane (PM) fraction. The data shown are typical of those obtained in four separate experiments.

ration of 493 cpm·(mg of protein)⁻¹·min⁻¹. In contrast to the sterols, only 1.7% of the radioactive phospholipids was recovered in the plasma membrane fraction. (Fig. 2 *Inset*). This again is comparable to the recovery of total lipid phosphorus from the sucrose gradient in this fraction.

The ratio of labeled sterol to phospholipid in whole cells and plasma membrane fractions over the course of a 2-hr incubation is shown in Fig. 3. For the initial 30 min, the increase in the sterol/phospholipid ratio in the plasma membrane paralleled that of the whole cells. Thus, this initial increase could be explained by the presence of contaminating membranes as discussed above. However, after 30 min of incubation, the ratio in the whole cell remained constant at 0.25, while in the plasma membrane the ratio continued to rise to 0.79 by 2 hr.

The incorporation of [³H]mannose into trichloroacetic acid-precipitable material in the plasma membrane also displayed a 30-min lag time (data not shown). However, the rate of appearance [102 cpm·(mg of protein)⁻¹·min⁻¹] was significantly slower than that of the lipids.

Exchange of Sterols Between Populations of *Acanthamoeba* Membranes. Because of the lengthy procedure involved in the isolation of plasma membrane fractions from *Acanthamoeba* (see *Methods*), it was of some concern to rule out the possibility of transfer of labeled sterols from one population of membrane vesicles to another after homogenization. In order to do this, latex phagosomes were prepared from unlabeled cells as described by Korn (7), mixed with homogenates of cells labeled with [³H]acetate, and allowed to stand on ice for 2 hr before reisolating. The reisolated latex phagosomes contained 0.897% of the total protein in the mixture but only 0.046% of the labeled sterol. Thus, the transfer or exchange of labeled sterols between membrane populations during plasma membrane isolation is apparently not significant.

Turnover of Sterols in Whole Cells and Plasma Membranes. The turnover of labeled sterols was measured after a 30-min pulse with [³H]acetate (Fig. 4). The synthesis of sterols by whole cells continued for 5 min and then remained relatively constant, falling only slightly over the course of the experiment with a half-time of 375 min (Fig. 4). Sterol continued to appear in the plasma membrane at a rate of 450 cpm·(mg of protein)⁻¹·min⁻¹ for an additional 15 min after the initiation of the chase, before turning over rather rapidly with a half-time of 75 min. The rate and time course of arrival and turnover of phospholipid at the plasma membrane during the chase was similar to the sterols (data not shown). Because both molecules arrived at the surface after a 30-min lag time (Figs. 1

FIG. 3. Ratio of newly synthesized sterol to phospholipid in whole cells (●) and isolated plasma membrane (○). Experimental details were identical to those described in Fig. 1. The data shown are typical of those obtained in four separate experiments.

FIG. 4. Turnover of [³H]acetate labeled sterols in *Acanthamoeba* cells (●) and plasma membrane (○). Cells (7.5×10^7) were pulsed at 23°C with 10 mCi of [³H]acetate in 6 ml of phosphate-buffered saline for 30 min. Cells were washed and resuspended in 6 ml of culture medium supplemented with 20 mM sodium acetate. At the indicated times, 1-ml aliquots were removed, mixed with 1.5×10^8 unlabeled carrier cells, and washed once with 10 mM Tris-HCl (pH 7.5). Plasma membrane fractions were isolated, and lipids were extracted and purified as described. The data shown are typical of those obtained in four separate experiments.

and 2), it appears that their residence time in the plasma membrane is about 15 min.

DISCUSSION

In this study, we examined the movement of newly synthesized lipids from their site of synthesis to the plasma membrane. The protozoan *A. castellanii* proved to be a useful system for these studies. Because the amoeba obtains its nutrients by phagocytosis and pinocytosis, it is actively involved in membrane shuttling, allowing relatively easy metabolic labeling of membrane components. After 2 hr of labeling with carrier-free [³H]acetate, the enrichment of radioactively labeled lipids in the plasma membrane relative to the whole cell adequately reflects the enrichment of total lipids (Table 1). Thus, a time course of 2 hr appears to be sufficient for measuring the assembly of the lipid components of the plasma membrane in this organism.

The kinetics of transport of labeled sterol and phospholipids to the cell surface appear to be quite similar. Both appeared in the plasma membrane after 30 min of continuous labeling with [³H]acetate. Because there was no apparent lag in the synthesis of either of these molecules, this can be taken as a reasonable estimate of the transit time for the movement of these lipids from their site of synthesis to the plasma membrane. This time course is considerably different from that observed by DeGrella and Simoni in Chinese hamster ovary cells (18). In their system, cholesterol moved to the plasma membrane quite rapidly with no apparent lag. These experiments were carried out at 37°C, whereas in the present study the *Acanthamoeba* were labeled at 23°C. Although the difference in temperature might account for the slower transport of sterol in our system, the results also could be explained by the obvious differences between the cell types, particularly in the types of sterol synthesized.

An interesting and unexpected finding from the present study was that despite similar transit times and rates of transport to the surface, the radioactively labeled sterol-to-phospholipid ratio was enriched in the plasma membrane relative to whole cells, even at the earlier time points. A similar pattern was also observed among the individual classes of phospholipids, where the ratio of radioactively labeled phos-

phatidylethanolamine to phosphatidylcholine became enriched in the plasma membrane at the earlier time points (unpublished data). These results suggest that the sorting of lipids must take place at an early time point in plasma membrane assembly, quite possibly before the lipids reach the cell surface.

Sterols and phospholipids also were turned over at similar rates. The rather short half-life of these molecules in the plasma membrane is consistent with the findings of Bowers and Olszewski (19), who estimated that *A. castellanii* turns over its surface membrane at a rate of 2–10 times per hr. The fact that the half-lives for sterols and phospholipids were so similar suggests that endocytic vesicles may contain representative portions of membrane lipids.

Although the studies presented here do not directly address the question of transport mechanisms, some predictions can be made from our observations. The two mechanisms that must be considered are vesicular transport (4) and carrier or exchange protein-mediated transport (20). The latter has been postulated as a method of shuttling phospholipids between various subcellular membranes (21). However, our results are more consistent with a model of membrane assembly by vesicular transport. The appearance of newly synthesized sterols and phospholipids in the plasma membrane at the same time and with similar kinetics would be most easily explained by the fusion of a preformed vesicle with the membrane.

The [³H]mannose-labeled macromolecules arrived at the cell surface at a much slower rate than the sterols or phospholipids (data not shown). Therefore, plasma membrane assembly in this organism cannot easily be explained by the transport of a single type of vesicle to the membrane. A similar conclusion was reached by DeGrella and Simoni, who found that cholesterol and glycoproteins were moved to the surface of Chinese hamster ovary cells at very different rates (18). Therefore, one might expect to find an intracellular pool of vesicles bound for the plasma membrane and highly enriched in lipid.

Lipid-rich vesicles have been found, in fact, in *Acanthamoeba palestinensis* (22) and *Dictyostelium discoideum* (23). The latter were derived from Golgi membranes as judged by marker enzymes and were shown to be involved in the transfer of phospholipids to the cell membrane. However, the presence or absence of sterol in these vesicles was not determined. Based upon the data presented here, one might predict that the *Acanthamoeba* would contain intracellular lipid-rich vesicles comprised of both sterol and phospholipids in the same or similar proportions as found in the surface membrane.

We thank Dr. Neal Band for kindly supplying stock cultures of *A. castellanii*. We also express our gratitude to Dr. Richard Hoover for his advice on growing the cells and Ms. Iristine Roddy for excellent technical assistance. This work was supported by National Science Foundation Grant PCM-8110593, National Institutes of Health Grant GM 22175, and National Institutes of Health Fellowship HL 06455.

1. Sabatini, D. D., Kreibich, G., Morimoto, T. & Adesnik, M. (1982) *J. Cell Biol.* **92**, 1–22.
2. Strous, G. J. A. M. & Lodish, H. F. (1980) *Cell* **22**, 709–717.
3. Rothman, J. E. (1981) *Science* **213**, 1212–1219.
4. Morré, D. J., Kartenbeck, J. & Franke, W. (1979) *Biochim. Biophys. Acta* **559**, 71–152.
5. Colbeau, A., Nachbaur, J. & Bignais, P. M. (1971) *Biochim. Biophys. Acta* **249**, 462–492.
6. Green, C. (1977) in *Biochemistry of Lipids II*, ed. Goodwin, T. W. (University Park Press, Baltimore), Vol. 14, pp. 101–152.
7. Korn, E. D. (1974) *Methods Enzymol.* **31**, 686–699.
8. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917.
9. Fieser, L. F. & Fieser, M. (1959) *Steroids* (Reinhold, New York).
10. Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758.
11. Ames, B. N. & Dubin, D. T. (1960) *J. Biol. Chem.* **235**, 769–775.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
13. Mills, J. T. (1980) Dissertation (Albert Einstein College of Medicine, New York).
14. Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. & Berthet, J. (1974) *J. Cell Biol.* **61**, 188–200.
15. Ulsamer, A. B., Wright, P. L., Wetzol, M. G. & Korn, E. D. (1971) *J. Cell Biol.* **51**, 193–215.
16. Ulsamer, A. B., Smith, F. P. & Korn, E. D. (1969) *J. Cell Biol.* **43**, 105–114.
17. Korn, E. D., Ulsamer, A. B., Weighing, R. R., Wetzol, M. G. & Wright, P. L. (1969) *Biochim. Biophys. Acta* **187**, 555–563.
18. DeGrella, R. F. & Simoni, R. D. (1982) *J. Biol. Chem.* **257**, 14256–14262.
19. Bowers, B. & Olszewski, T. E. (1972) *J. Cell Biol.* **53**, 691–694.
20. Wirtz, K. W. A. (1974) *Biochim. Biophys. Acta* **344**, 95–117.
21. Sleight, R. G. & Pagano, R. E. (1983) *J. Biol. Chem.* **258**, 9050–9058.
22. Chlapowski, F. J. & Band, R. N. (1971) *J. Cell Biol.* **50**, 634–651.
23. DeSilva, N. S. & Siu, C.-H. (1981) *J. Biol. Chem.* **256**, 5845–5850.