Pinocytosis in Mouse L-Fibroblasts: Ultrastructural Evidence for a Direct Membrane Shuttle Between the Plasma Membrane and the Lysosomal Compartment

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ABSTRACT Mouse L-fibroblasts internalized large amounts of cationized ferritin (CF) by pinocytosis. Initially (60–90 s after addition of CF to cell monolayers at 37°C), CF was found in vesicles measuring 100–400 nm (sectioned diameter) and as small clusters adhering to the inner aspect of the limiting membrane of a few large (> 600 nm) vacuoles. After 5–30 min, CF labeling of large vacuoles was pronounced and continuous. Moreover, 70–80% of all labeled structures were tiny (< 100 nm) vesicles. However, the absolute frequency of tiny vesicles increased more than twofold from 5 min to 30 min. When the cells were incubated with CF for 30 min, then washed and further incubated for 3 h without CF, almost all CF was present in dense bodies (100–500 nm). When L-cells were first incubated with horseradish peroxidase (HRP), then washed and incubated with CF, double-labeled vacuoles were observed. Tiny vesicles also contained HRP-CF, and small HRP-CF patches were localized on the cell surface. Distinct labeling of stacked Golgi cisterns was not observed in any experiment. These observations suggest that the numerous tiny vesicles are not endocytic but rather pinch off from the large vacuoles and move toward the cell surface to fuse with the plasma membrane. Thus, ultrastructural evidence is provided in favor of a direct membrane shuttle between the plasma membrane and the lysosomal compartment.

During pinocytosis many cells internalize remarkably large amounts of surface membrane, in addition to adsorbed molecules and molecules in the fluid phase. In a stereologic electron microscope study with horseradish peroxidase (HRP) as fluid-phase marker of pinocytic vesicles (pinosomes) and secondary lysosomes, Steinman et al. (18) demonstrated that macrophages internalize the equivalent of their entire surface area every half hour, and mouse L-fibroblasts every second hour, although the surface area of the cells and of their lysosomal compartment remained constant within a 3-h period. The amount of internalized membrane (pinocytic rate) may be even higher (see references 1, 3, 12, 22). Because most plasma membrane constituents are known to have long half-lives (10–100 h), de novo membrane synthesis can hardly account for this high rate of removal of surface membrane (see references 13, 15, 19). Steinman et al. (18) therefore suggested that internalized membrane was recycled intact back to the cell surface rather than degraded within the lysosomal compartment. They suggested that the numerous tiny vesicles present in the cells were a possible vehicle for this recycling, as such vesicles have a large membrane surface area in relation to volume, but the application of the fluid-phase marker HRP did not permit the exploration of this hypothesis. Since then, mainly biochemical and kinetic evidence has accumulated in favor of a membrane shuttle between the plasma membrane and lysosomes (1, 10, 12–14, 19, 22).

In electron microscope studies, the application of cationized ferritin (CF; pI = 8.5), a polyvalent ligand binding to cell surface anionic sites (2, 8, 16, 20), has made it possible to visualize, at least to some extent, pathways followed by internalized membrane in various cellular systems (4–7, 9, 11, 23–26).

In this paper we report observations on endocytosis and membrane recycling in mouse L-fibroblasts using CF, and a double-labeling technique with HRP and CF (24). Our results indicate that newly formed pinosomes measuring 100–400 nm deliver their load and membrane to large vacuoles. When the inner membrane surface of these vacuoles has become distinctly

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CF-labeled in this manner, a very large population of tiny (< 100 nm) vesicles in the cytoplasm also show CF. Moreover, we provide evidence that these vesicles are most likely pinched off from the large vacuoles and carry membrane directly back to the cell surface. These observations are therefore taken to indicate a direct membrane shuttle between the plasma membrane and the lysosomal compartment.[1]

MATERIALS AND METHODS

Cells

L 929 mouse fibroblasts (L-cells) were obtained from the Fibiger Laboratory (Copenhagen, Denmark). They were grown in monolayers in plastic plates (Nunc, Denmark), 50 × 15 mm, at 37°C in Dulbecco's modified Eagle's medium containing 20 mM tricine-chloride and 24 mM $NaHCO_3$ and supplemented with 10% fetal calf serum (Grand Island Biological Co. [Gibco], Scotland) as previously described (26). The cells were routinely transferred once a week. For experiments, the cells were trypsinized (0.1% trypsin in 0.02% EDTA), counted and inoculated with 10^5 cells per plate, and grown for 5 d.

Tracer Experiments

In all experiments, CF (Miles Yeda; Miles Laboratories, Inc., Elkhart, IN) was used at a final concentration of 0.1 mg/ml PBS, native ferritin (NF; pI = 4.6) (Miles Yeda) at 5 mg/ml, and HRP (Sigma type II, Sigma Chemical Co., St. Louis, MO) at 1 mg/ml. The fixative contained 1% formaldehyde, 1.25% glutaraldehyde, and 0.1 M sodium cacodylate buffer, pH 7.2, and was added directly to rinsed cell monolayers. Fixation was carried out at room temperature for 60 min. All experiments included at least two plates.

Short Experiments with CF

These experiments were performed in a special room at 37°C. Cell cultures in plates were rinsed with PBS, and fresh PBS with CF was added. After exactly 10, 15, 25, 55, or 85 s, the CF-medium was poured off, the cultures were rinsed with PBS, and fixative was added. The time points for the addition of fixative thus correspond to approximately 15, 20, 30, 60, and 90 s, respectively, which are regarded as the times of CF incubation. Thereafter, fixation was carried out at room temperature. This approach was also used in preliminary experiments with incubation for 5 min.

Longer Experiments

PBS with CF was added to rinsed cells at 4°C. The cells were thereafter incubated for 5 min at 4°C and processed as follows: (*a*) the cells were rinsed three times with cold PBS and fixed; (*b*) the cells were incubated in the presence of CF for 5, 15, 30, or 60 min at 37°C, then rinsed and fixed; (*c*) the cells were incubated in the presence of CF for 30 min at 37°C, then rinsed and further incubated for 3 h at 37°C in PBS without CF, before being washed and fixed.

Experiments with NF

NF was added to cultures rinsed in PBS at 4°C. After 5 min, the cells were allowed to incubate in the presence of NF for 5, 30, or 60 min at 37°C before being washed and fixed.

Experiments with HRP and CF (Double-Labeling)

Rinsed cells were incubated with HRP for 5 or 30 min at 37°C, rinsed again, and fixed. In other experiments, cells were incubated with HRP for 30 min at 37°C, rinsed carefully three times in PBS, and then incubated with CF for 10 or 30 min at 37°C before the final rinse and fixation.

Further Processing for Electron Microscopy

After fixation the cells from CF and NF experiments were scraped off the plates and centrifuged in cacodylate buffer for 25 min at 1,600 *g*.

After the cultures were fixed in HRP and double-labeling experiments, the cells in the plates were rinsed twice in 0.05 M Tris-HCl buffer, pH 7.6. The plates were then incubated for 60 min at 37°C in a medium containing 5 ml of 0.05 M Tris-HCl buffer (pH adjusted to 7.6 in the final medium), 2.5 mg of diaminobenzidine (Sigma Chemical Co.), and 0.05 ml of 1% H_2O_2 per plate. The cultures were then rinsed with cold cacodylate buffer, and the cells were scraped from the plates and pelleted as described above.

All pellets were treated with 2% OsO_4 in cacodylate buffer, pH 7.2, for 1 h at 4°C, rinsed briefly in buffer followed by distilled H_2O, and then block-stained for 1 h at room temperature in 1% uranyl acetate in H_2O. The specimens were dehydrated in ethanol and embedded in Epon. ~30–40-nm sections were examined without further contrasting in a JEOL 100-CX electron microscope operated at 60 kV and calibrated with a carbon grating.

Quantification

All quantitative analysis of the material is based on sectioned diameter (as opposed to spherical diameter) of vesicles and vacuoles (profiles) containing CF particles. Micrographs for quantification were in principle obtained as reported by Steinman et al. (18), except that we used a higher magnification and thus analyzed a smaller sectioned cell area per micrograph. From each experiment used for quantification (90 s, 5, 15, 30 min, and 30 min followed by 3 h without CF), 40–100 micrographs (primary magnification × 24,000; total magnification

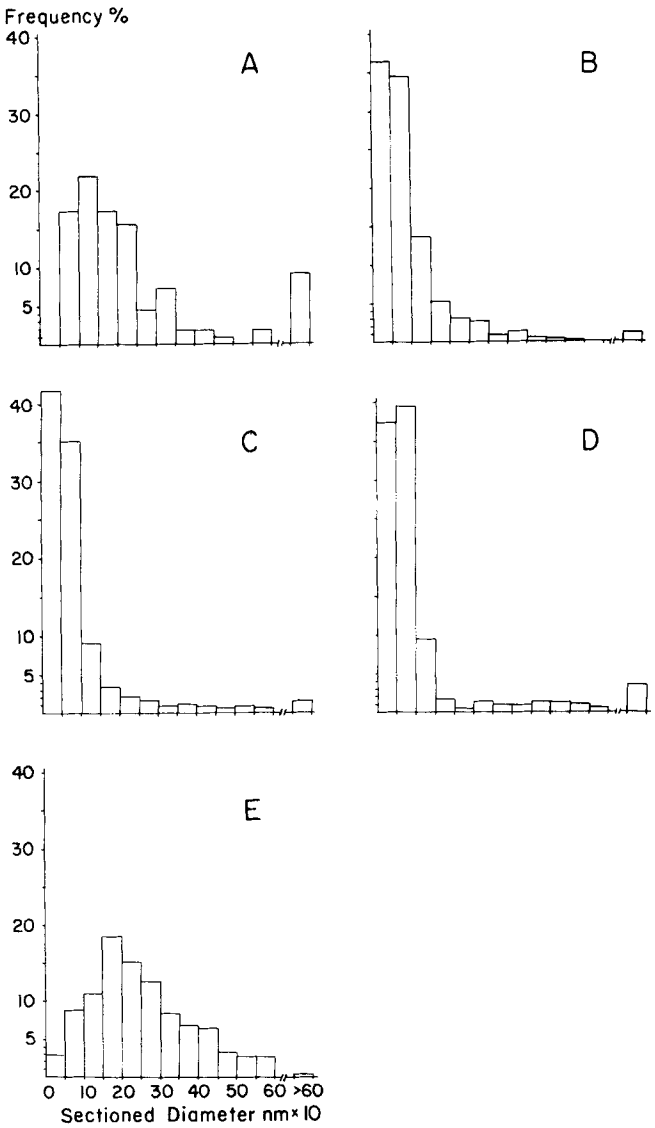

FIGURE 1 Frequency distribution of CF-labeled profile diameters from experiments with L-cells incubated in the presence of CF at 37°C for various times. *A*, $n = 109$; *B*, $n = 789$; *C*, $n = 579$; *D*, $n = 1360$; *E*, $n = 263$; where n = number of diameters measured. *A*, 90 s; *B*, 5 min; *C*, 15 min; *D*, 30 min; *E*, 30 + 180 min.

[1] We use the term lysosomal compartment collectively for vacuoles of various size and density (including dense bodies) involved in the sequestration of internalized material, although some of these presumably represent prelysosomes rather than secondary lysosomes.

67,000) were obtained (representing sections through the cytoplasm of 20–40 different cells). Diameters (in one predetermined direction) of all labeled structures not connected to the cell surface were measured (using a × 10 magnifying glass with a 0.1 mm scale for the smallest ones), and the data are presented as a relative frequency distribution of profile diameters (Fig. 1 *A–E*). Using a Hewlett-Packard digitizer, we measured the total sectioned cytoplasmic area (excluding nuclei) used for the diameter measurements in the 5- and 30-min experiments, and subsequently calculated the absolute frequency of sectioned vesicular profiles of < 100 nm in diameter per μm^2 sectioned cell (Table I).

TABLE I

*Area Density (Absolute Frequency) of CF-labeled Vesicular Profiles < 100 nm in Diameter**

	Incubation time in CF-medium at 37°C	
	5 min	30 min
Total area of sectioned cytoplasm (μm^2)	517	386
Total number of vesicular profiles <100 nm in diameter	559	1048
Number of vesicular profiles <100 nm per μm^2 sectioned cytoplasm	1.08	2.72

* The total diameter distribution of all labeled vesicles and vacuoles from the two experiments referred to here is shown in Fig. 1 B and D.

RESULTS

Pinocytosis of CF

In all experiments performed at 37°C, CF binding to the cell surface was uneven and patchy.

In the experiments in which cells were fixed 15–30 s after CF had been added at 37°C, no labeled vesicles or vacuoles were observed. After 60 s, however, a few CF-labeled profiles measuring 100–400 nm were present in the cells. Therefore, in our experimental system, the 60-s experiment appears to represent the initial phase of pinocytosis of CF.

After 90 s of incubation with CF at 37°C, labeled vesicles and vacuoles showed a wider range of diameters (Figs. 1 *A* and 2), although they were still infrequent. CF in large vacuoles appeared as small clusters adhering to the membrane (Fig. 2 *B*).

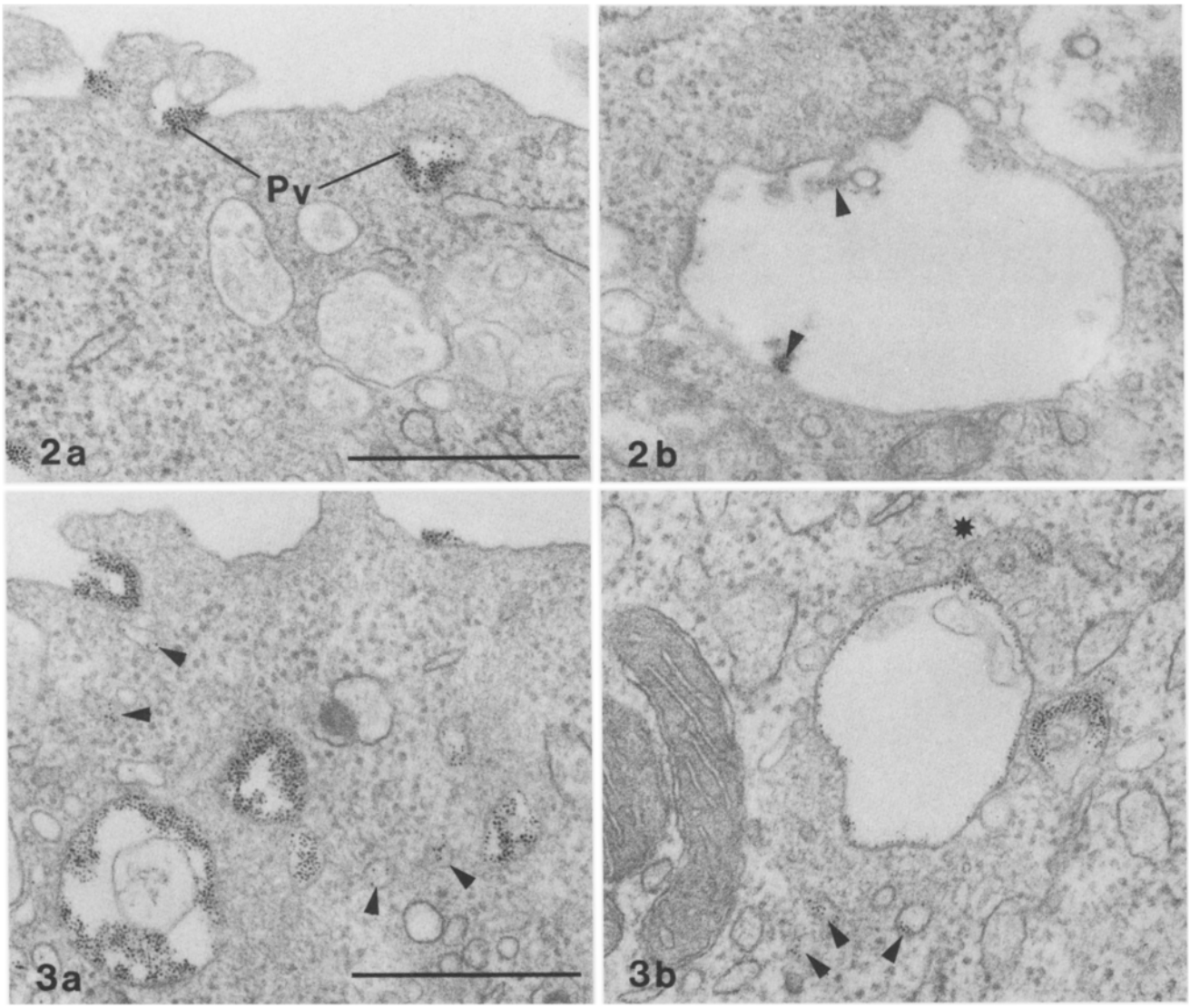

FIGURE 2 L-cells after exposure to CF for 90 s at 37°C before fixation. Presumptive pinocytic vesicles at formation are indicated (*Pv*) in *a*. In *b*, small CF clusters in a large vacuole are indicated (arrowheads). Bar, 0.5 μm. × 67,200.

FIGURE 3 L-cells after exposure to CF for 5 min at 37°C before fixation. Larger vacuoles show rather continuous CF labeling of their inner surface. In *b*, a tubular profile with CF is connected to the vacuole (asterisk). Numerous tiny vesicles with CF are also present (arrowheads). Bar, 0.5 μm. × 67,200.

Many of the 100–400-nm vesicular profiles may actually be surface-connected at another plane of sectioning and therefore may not be pinocytic. However, the CF-labeled large vacuoles clearly showed that pinocytosis had occurred at this time.

After 5 min at 37°C (irrespective of whether CF was added at 4°C or 37°C), numerous vesicles and vacuoles with CF labeling were present within the cells (Fig. 3). Their diameter distribution is shown in Fig. 1 *B*. The most remarkable difference between the 5-min and the 90-s experiments was that a large population of tiny vesicles (< 100 nm) showed labeling

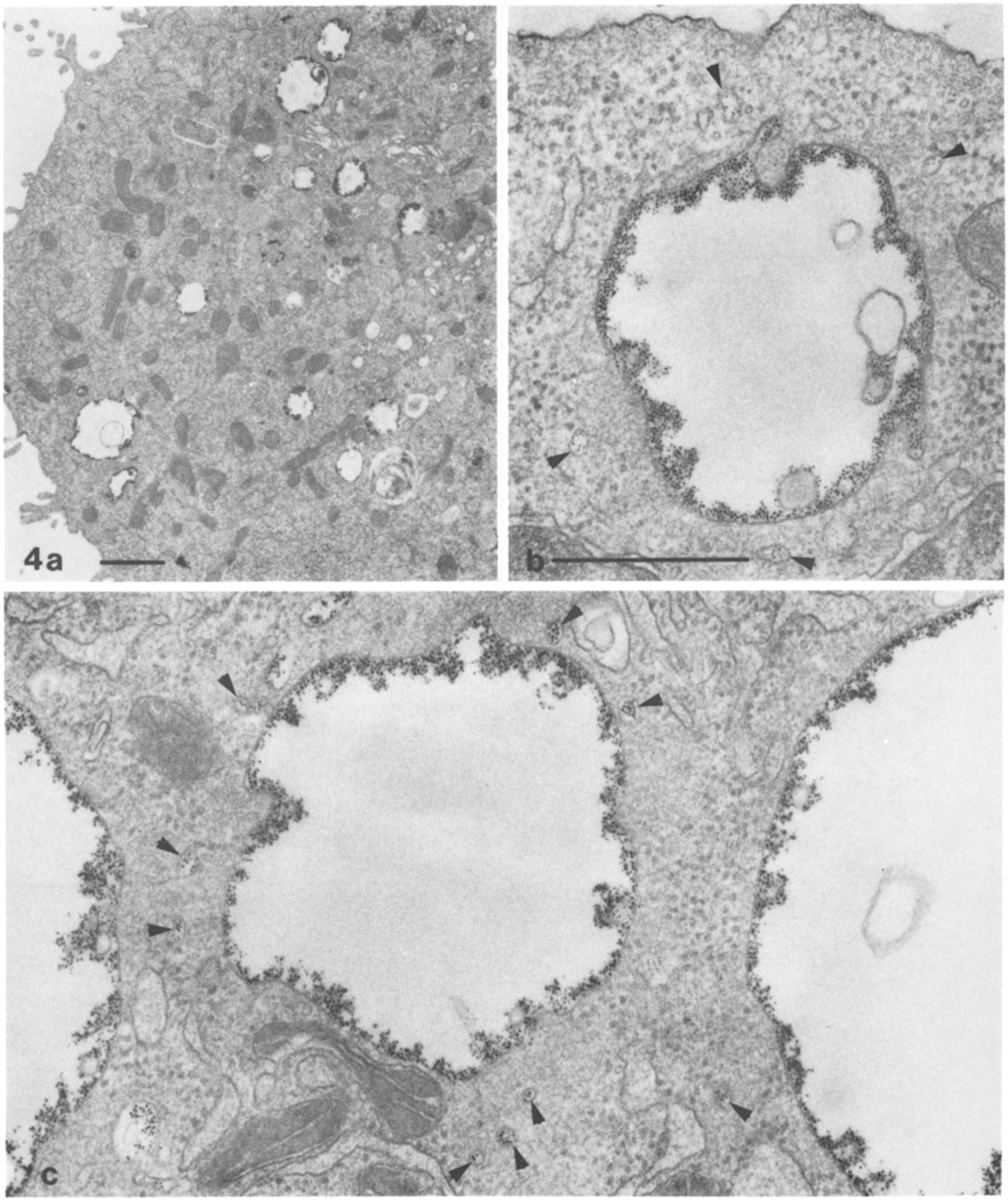

FIGURE 4 Labeling of L-cells after 30 min of exposure to CF before fixation. (*a*) Survey micrograph showing vacuoles of various sizes with peripheral CF-labeling (compare with Fig. 5 *A*). Bar, 1 μm. × 11,000. (*b* and *c*) Higher magnifications of large vacuoles with pronounced and continuous CF-labeling of the membrane. Arrowheads indicate numerous tiny vesicles with CF. Bar, 0.5 μm. × 67,200.

after 5 min (Figs. 1 *B* and 3). Moreover, larger vacuoles now often showed more or less continuous CF labeling of their inner surface (Fig. 3 *B*). After 15 min of incubation the situation with respect to relative diameter distribution of vesicles and vacuoles (Fig. 1 *C*) was the same as after 5 min, but labeled vacuoles were more frequent and showed more distinct and continuous CF labeling.

When the cells were incubated for 30 min at 37°C in the presence of CF, the distribution of sectioned diameters of labeled vesicles and vacuoles (Fig. 1 *D*) still corresponded to that after 5 min. Most large vacuoles (> 600 nm) now showed heavy and continuous CF labeling of their inner surface (Fig. 4), although nonmembrane-bound CF clusters also occurred. In addition, some smaller vacuoles with a somewhat condensed content, and dense bodies contained CF. In these bodies CF typically had detached from the membrane. Small vesicular (or tubular) profiles with CF were sometimes connected to the limiting membrane of the larger vacuoles (Fig. 4), as was also the case for vacuoles in the 15- and 5-min experiments.

In the 5-, 15-, and 30-min experiments, between 70 and 80% of all labeled profiles had a diameter of < 100 nm (Fig. 1 *B–D*). However, the absolute frequency (area density) of these tiny vesicles had increased more than twofold from 5 min to 30 min (Table I). The tiny vesicles with CF were present throughout the cytoplasm but were most frequent around large vacuoles (Fig. 4).

When the cells were incubated for 60 min, fewer very large vacuoles with peripheral CF labeling, and more smaller vacuoles (300–800 nm) with a dense matrix and a heavy load of CF, were observed.

When the cells were incubated at 37°C for 30 min in the presence of CF, then washed and further incubated for 3 h in PBS without CF, dense bodies, more or less filled with CF and typically measuring 100–500 nm, predominated (Figs. 1 *E* and 5). Small vesicles (< 100 nm) and large vacuoles (> 600 nm) with CF were rare. Larger vacuoles without peripheral CF labeling sometimes showed small CF clusters freely in a somewhat condensed matrix.

None of these experiments showed distinct CF labeling of stacked Golgi cisterns. Of ~100 Golgi complexes examined, apparent labeling of a single cistern was noticed in two cases (both after 60 min of incubation).

Pinocytosis of NF

NF appeared to be internalized by the same type of vehicle as was CF (i.e., in 100–400-nm vesicles, which in general were the smallest structures containing NF). However, NF never bound distinctly to the cell surface, and the amount of NF internalized was far below that of CF, with respect to both the number of labeled profiles and the number of ferritin particles within such profiles. Moreover, NF was typically located freely in large vacuoles (rather than being membrane-bound), and only a few tiny vesicles with NF were observed.

Double-labeling with HRP and CF

L-cells incubated in PBS with HRP for 5 or 30 min at 37°C showed tracer-labeled vesicles and vacuoles of various sizes, but reaction product could never be detected on the cell surface (see also references 17, 18). Only a few tiny vesicles showed reaction product.

When the cells were first allowed to ingest HRP for 30 min, then washed carefully, and thereafter exposed to CF for 10 or 30 min before fixation, HRP was seen mostly in vacuoles measuring 200–500 nm and more or less filled with reaction product, whereas CF was seen in vesicles and vacuoles of

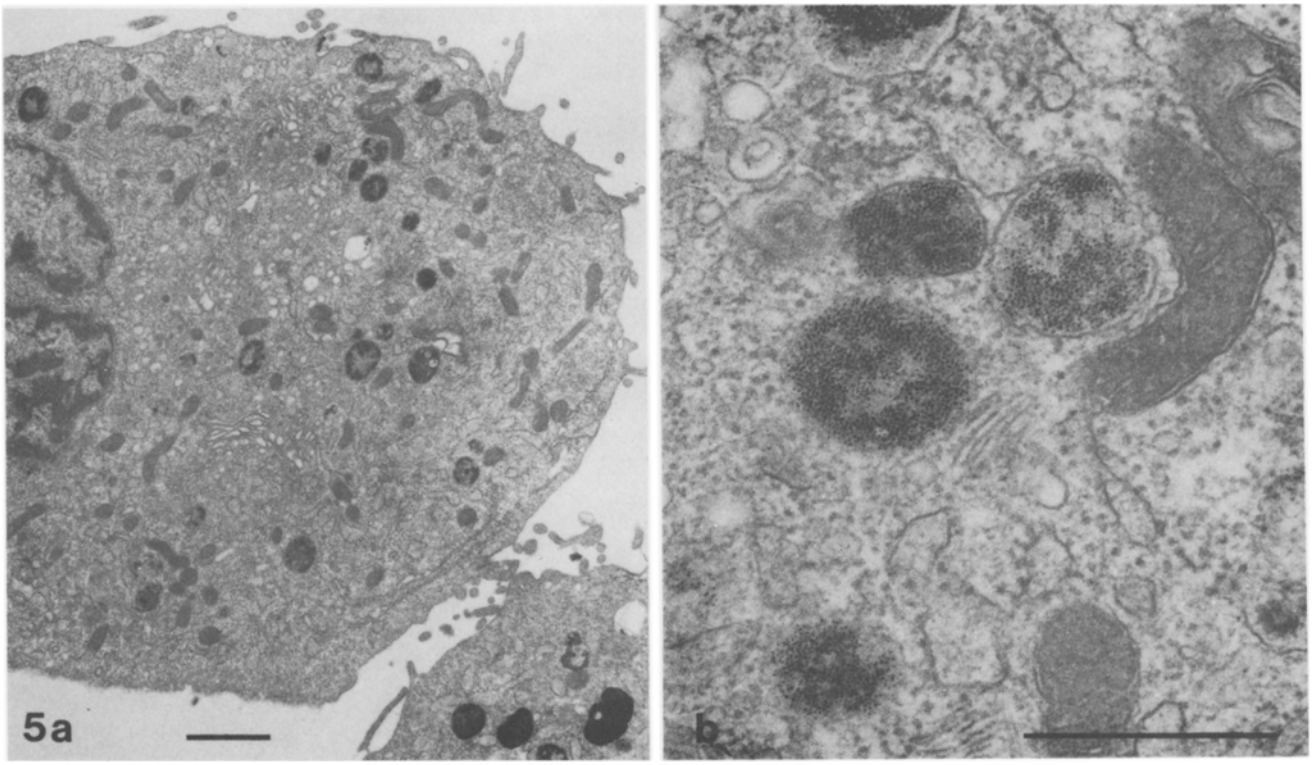

FIGURE 5 L-cells incubated with CF for 30 min at 37°C, then washed and further incubated for 3 h at 37°C without CF. (*a*) Survey micrograph showing numerous dense bodies heavily loaded with CF (compare with Fig. 4 *a*). Bar, 1 μm. × 11,000. (*b*) CF-labeled dense bodies at a higher magnification. Bar, 0.5 μm. × 67,200.

considerably variable sizes (comparable to the diameter distribution shown in Fig. 1 *C* and *D*). However, after both 10 and 30 min of CF incubation, 20–30% of the labeled vacuoles showed both HRP and CF (of 133 profiles larger than 300 nm, 36 were labeled only by HRP, 63 only by CF, and 34 by both tracers [25.6%] in experiments with 10 min of CF incubation). In these double-labeled vacuoles, HRP and CF were often found as a complex existing freely in the vacuolar lumen (Fig. 6 *B*). In some cases, however, CF was localized to the inner aspect of the vacuolar membrane, whereas some HRP was associated with the rim of CF, and some could be existing freely in the vacuole (Fig. 6 *C*). Small vesicular profiles connected with such vacuoles sometimes showed HRP-CF labeling. Also, tiny vesicles occurring freely in the cytoplasm contained HRP-CF complex (Fig. 6 *D* and *E*). For some of these small vesicles, the double labeling could be established unequivocally, whereas in other cases the density of the presumptive HRP reaction product (the "background" for the CF particles) was rather low, although not so electron-lucent as in vesicles labeled exclusively with CF (Fig. 6 *D* and *H*). On occasion, distinct HRP-CF complexes were found on the cell surface in small patches, sometimes within small plasmalemmal pits (Fig. 6 *F* and *G*). As for the cytoplasmic vesicles, the density of the HRP reaction product varied, which often made positive identification of HRP-CF complexes on the cell surface difficult.

No double-labeling experiments revealed HRP, CF, or HRP-CF complexes in stacked Golgi cisterns.

DISCUSSION

The main findings of this study are that: (*a*) following pinocytosis of CF by L-cells and subsequent labeling of large vacuoles, CF is also present in a very large population of tiny vesicles; (*b*) these vesicles are apparently pinched off from the large vacuoles rather than being endocytic, and move toward the cell surface to fuse with the plasma membrane; and (*c*) stacked Golgi cisterns remain unlabeled. We take these observations as morphological support for the existence of a direct membrane shuttle between the plasma membrane and the lysosomal compartment in L-cells (18), a recycling pathway that has recently been suggested in several biochemical studies on other cell types (1, 10, 12–14, 22).

A very important question in this study has been whether the numerous tiny vesicles (< 100 nm, sectioned diameter) are endocytic or are pinching off from the membrane of the large vacuoles. In general, presumptive pinocytic vesicles were found to be larger (100–400 nm) than these tiny vesicles, as evaluated in short experiments (60–90 s of incubation). Steinman et al. (18) showed that after 1 and 5 min of exposure of L-cells to HRP, most pinocytic vesicles (~80%) measured 100–400 nm (sectioned diameter), with the 0–100 nm class only representing

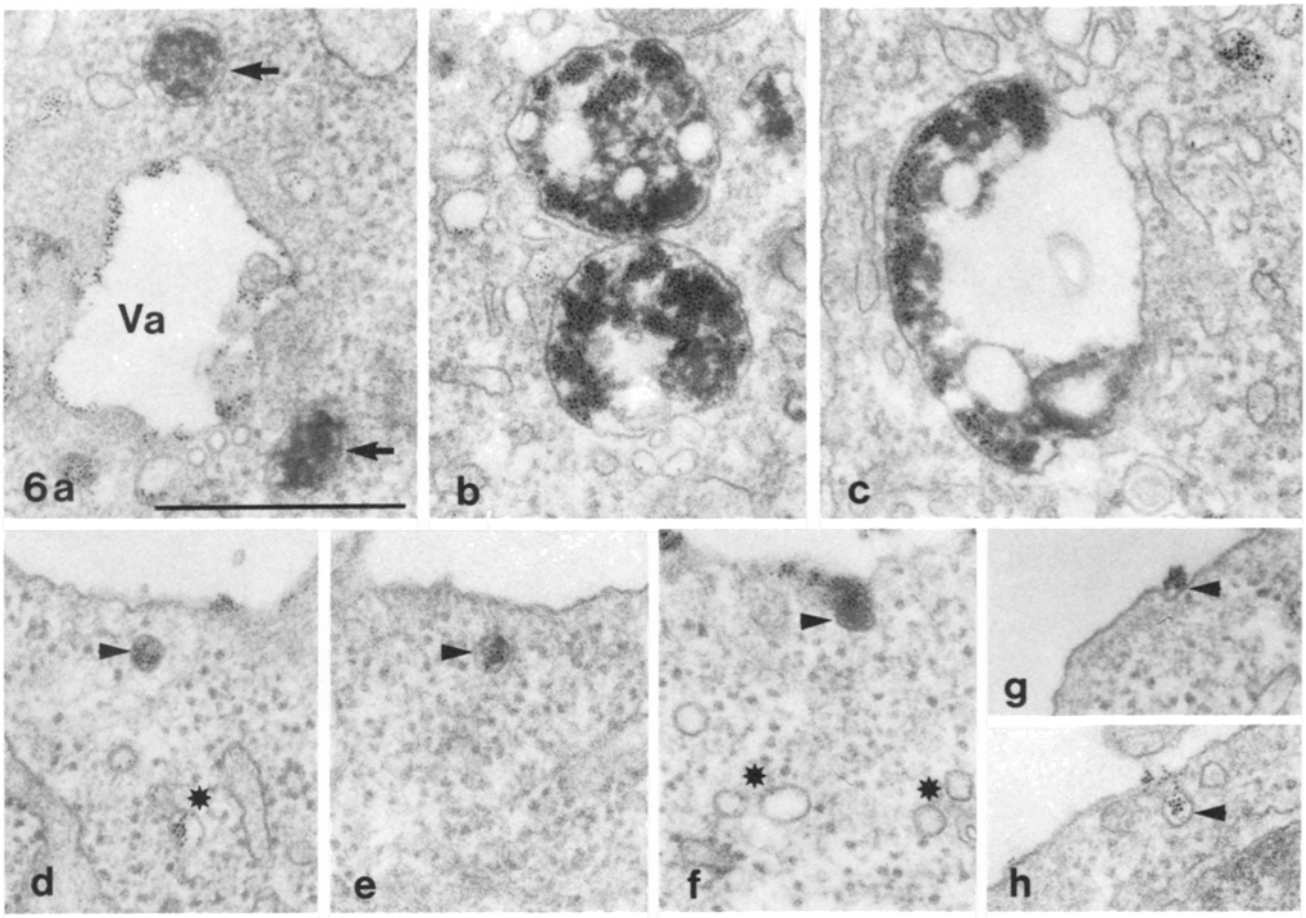

FIGURE 6 L-cells incubated with HRP for 30 min at 37°C, then washed and incubated for another 30 min with CF at 37°C before the final washing and fixation. Frame *a* shows a CF-labeled vacuole (*Va*) and two small dense bodies with HRP (arrows). Frame *b* shows two vacuoles (dense bodies) containing HRP-CF complexes. In *c* the vacuole shows peripheral labeling by CF, partly associated with HRP. Frames *d* and *e* show tiny vesicles with HRP-CF (arrowheads). Note the vesicle with CF only (asterisk) in *d*. In *f* and *g*, HRP-CF patches are present on the cell surface, associated with plasmalemmal pits (arrowheads). The asterisks in *f* indicate small unlabeled vesicles. Frame *h* shows a plasmalemmal pit containing CF only. Bar, 0.5 μm. × 67,200.

15%. Our present data (Fig. 1*A*) closely resemble those of Steinman et al. (Fig. 13 in reference 18). Converted to actual (spherical) dimensions, the average diameter of L-cell pinocytic vesicles is 200 nm, according to Steinman et al. (18). Moreover, Thilo and Vogel (22) have stressed that the role of the smallest (100–200 nm) pinosomes has been highly exaggerated and that the volume-weighted average size of pinosomes in L-cells is ~400 nm. Thus, although it appears impossible to determine whether a certain "small" vesicle (i.e., ~100 nm) containing CF is endocytic or not, our present results as well as available information in the literature strongly indicate that pinocytic vesicles in L-cells in general are significantly larger than the numerous tiny vesicles. Moreover, our results clearly revealed that the highest number (relative, Fig. 1, as well as absolute, Table I) of CF-labeled tiny vesicles correlated with heavy labeling of large vacuoles rather than with initial pinocytic events. This suggests that the tiny vesicles labeled with CF are formed from the large vacuoles and not from the plasma membrane. They probably bud off from the membrane of the vacuoles continuously, but the probability for their labeling increases significantly with heavy and continuous labeling of the vacuolar membrane, and therefore a marked labeling of the population of tiny vesicles occurs at relatively late time points.

The double-labeling experiments provide additional support for the view that the tiny vesicles are pinching off from the vacuolar membrane rather than fusing with it. The rationale of these experiments (see also reference 24) was that in cells first allowed to ingest the fluid-phase marker HRP (17, 18), then washed and thereafter incubated with CF, vacuoles with a HRP-CF complex should be found. Small vesicles budding off from such double-labeled vacuoles (but not endocytic vesicles fusing with them, because the cell surface showed no HRP labeling and the cells were washed carefully before CF administration) should therefore also contain HRP-CF complexes. This appeared to be a correct assumption, thus providing further support for the notion that tiny vesicles are pinched off from the vacuoles.

Another pertinent question was whether the tiny vesicles derived from large vacuoles fuse with the plasma membrane (exocytosis). Because CF was always present in small clusters on the cell surface (irrespective of careful washing procedures or long incubation times), a certain CF cluster present on the cell surface or in a small plasmalemmal pit may be taken to be exocytosed as well as not yet endocytosed, as also stressed by Ottosen et al. (11). However, in the double-labeling experiments, HRP-CF complexes were observed on occasion on the cell surface, sometimes associated with small pits. This supports, though not unequivocally, the view that the tiny vesicles coming from the large vacuoles fuse with the plasma membrane. Similar observations have recently been reported for macrophages (24). The relatively low frequency of HRP-CF patches on the cell surface, as well as of HRP-CF-labeled tiny vesicles, is probably explained by the low frequency of double-labeled vacuoles with HRP-CF adhering to the membrane, and possibly also by problems of detecting enzymatically very small amounts of HRP. Moreover, HRP-CF patches may detach from the cell surface following exocytosis and then be washed away.

Removal of membrane from large vacuoles would cause shrinkage of the vacuole. This was clearly documented by Steinman et al. (18) as well as by our study with CF, where the tracer eventually was found in relatively small dense bodies. Also, in double-labeling experiments, HRP-labeled vacuoles or dense bodies (presumptive secondary lysosomes, cf. reference 18) were generally smaller than the more recently formed CF-labeled vacuoles.

Our conclusion is also supported by the finding that stacked Golgi cisterns, in principle, remained unlabeled in all experiments. CF labeling of stacked Golgi cisterns has been observed in several cell types (6, 7, 9, 11, 23, 24), whereas very little or no CF labeling was found in other studies (21, 25, 26). However, because CF is not an ideal membrane marker (it often detaches from membranes after internalization; cf. references 6, 11, 25), lack of CF labeling of stacked Golgi cisterns does not necessarily indicate that some internalized membrane has not reached the Golgi complex via lysosomes (25). Compared with published information, our present experiments suggest that very little, if any, membrane passes through Golgi cisterns on its way from the lysosomal compartment back to the cell surface in L-cells.

In conclusion, our results provide new ultrastructural support for the suggestion that a population of tiny vesicles is responsible for recycling of intact membrane from the lysosomal compartment directly back to the cell surface during constitutive pinocytosis (15, 18). Whereas plasma membrane apparently recycles rapidly between the cell surface and vacuoles, lysosomal membrane may recycle more slowly between vacuoles and dense bodies. This is a simple and economical way to keep the cell surface area as well as the surface area of the lysosomal compartment constant during pinocytosis (18) and fits well with the recent kinetic studies of Besterman et al. (1), which show that pinocytosis in macrophages and fibroblasts involves two compartments operating in series, one with a rapid turnover ($t_{1/2}$ = 5–8 min), and one with a considerably slower turnover ($t_{1/2}$ = 3–10 h).

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