

Antibodies against a Lysosomal Membrane Antigen Recognize A Prelysosomal Compartment Involved in the Endocytic Pathway in Cultured Prolactin Cells

C. TOUGARD, D. LOUVARD, R. PICART, and A. TIXIER-VIDAL

*Groupe de Neuroendocrinologie Cellulaire, Collège de France, 75231 Paris Cedex 05, France; and
Unité de Biologie des Membranes, Département de Biologie Moléculaire, Institut Pasteur,
75724 Paris Cedex 15, France*

ABSTRACT Antibodies against a lysosomal membrane antigen (A-Ly-M) have recently been obtained and characterized (Reggio, H., D. Bainton, E. Harms, E. Coudrier, and D. Louvard, 1984, *J. Cell Biol.*, 99:1511–1526). They recognize a 100,000-mol-wt antigen immunologically related to a purified $[H^+,K^+]ATPase$ from pig gastric mucosa. In the present study, we have localized this antigen during adsorptive endocytosis in rat prolactin cells in culture using cationized ferritin (CF) as a tracer. CF was rapidly internalized (after 5 min) in coated pits and vesicles that were labeled by antibodies against clathrin. The tracer was then delivered (after 15 min) to vacuoles and multivesicular bodies. These structures were labeled with A-Ly-M. These organelles were devoid of acid phosphatase activity. At later stages (after 30 min) CF was observed within larger structures that were strongly stained by A-Ly-M and displayed a strong acid phosphatase activity. These findings clearly indicate that A-Ly-M react with prelysosomal and lysosomal compartments involved in the endocytic pathway in cultured prolactin cells. The membrane of these structures therefore contains antigenic determinant(s) related to the 100,000-mol-wt polypeptide. Our results suggest that the prelysosomal structure stained by A-Ly-M may represent in GH₃ cells the acidic prelysosomal compartment recently described in the early steps of endocytosis in other cell types (Tycko, B., and F. R. Maxfield, 1982, *Cell*, 28:643–651).

Acidification of endocytic vesicles involved as an early step in adsorptive or receptor-mediated endocytosis has recently been demonstrated using pH-sensitive fluorescent probes (4, 12, 19, 26). This rapid acidification occurs before the fusion of endocytic vesicles with lysosomes and in the absence of lysosomal hydrolases.

To explain the molecular mechanisms responsible for the acidification of both the prelysosomal and lysosomal compartments, the existence of an ATP-dependent proton pump has been postulated (for review, see reference 16). Such an activity has recently been demonstrated pharmacologically using isolated brain clathrin-coated vesicles (3), partially isolated endosomes, and lysosomes taken from fibroblasts and macrophages (4), or intact permeabilized fibroblasts (29).

A new approach for studying membrane components that are possibly involved in the acidification of the endocytic pathway is provided by the recently characterized antibodies against a lysosomal membrane fraction that recognize a

100,000-mol-wt antigen immunologically related to a purified $[H^+,K^+]ATPase$ from pig gastric mucosa (17, 18). Immunoelectron microscope localization of this antigen in normal rat kidney cells, rat macrophages, and hepatocytes revealed its presence not only in the membrane of secondary lysosomes but also in the membranes of several intracellular organelles known to be involved in receptor-mediated endocytosis (4, 12, 19, 26). Moreover, some patches on the plasma membrane and some Golgi cisternae were also labeled (18).

This prompted us to localize this antigen during adsorptive endocytosis in rat prolactin (PRL)¹ cells in culture using cationized ferritin (CF) as a tracer. These localizations were compared to those of clathrin performed in parallel experiments using previously described antibodies (11). We found

¹ *Abbreviations used in this paper:* A-Ly-M, antibodies against a lysosomal membrane antigen; A-clathrin, antibodies against clathrin; CF, cationized ferritin; PRL prolactin.

that CF was rapidly internalized via clathrin-coated pits and vesicles. It was then delivered to vacuoles and multivesicular bodies that were labeled with antibodies against the lysosomal membrane antigen (A-Ly-M) but had no acid phosphatase activity. At later stages, CF was observed within larger structures labeled by A-Ly-M and displaying a strong acid phosphatase activity. The presents findings indicate clearly that A-Ly-M recognize a membrane component present both in prelysosomal and lysosomal compartments involved in the endocytic pathway in cultured PRL cells.

MATERIALS AND METHODS

Antibodies

A-Ly-M of rat liver tissue were prepared as described in detail elsewhere (16, 18). The purified A-Ly-M recognized an antigen with an apparent molecular weight of 100,000. Antibodies against clathrin (A-clathrin), prepared as previously described (11), detected both the heavy and light chains of clathrin.

Cell Cultures

GH₃ CELLS: GH₃B₆ cells, a subclone of GH₃ cells (7), were grown in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum.

PRIMARY CULTURES: Cells were obtained by enzymatic dispersion of normal male Wistar rat anterior pituitaries and cultured as previously described (23, 25).

TREATMENT OF GH₃ CELLS: GH₃ cells were incubated in 1 ml of Ham's F10 medium containing CF (Miles Laboratories, Elkhart, IN) (0.05 mg/ml) for 20 min at 4°C. Cells were then rapidly washed at 4°C and transferred to 37°C in 1 ml of F10 for increasing time intervals from 5 min to 2 h. Cells were then rapidly rinsed with F10 medium and fixed in situ for immunocytochemistry (see below) or for acid phosphatase cytochemistry as previously described (21).

Immunocytochemical procedure

Cells were fixed in situ in 0.4% glutaraldehyde in 0.01 M phosphate-buffered saline with 2 mM MgCl₂ and 0.08 M NaCl, pH 7.4, according to Ohtsuki et al. (14). The immunocytochemical staining was performed directly in the culture dishes as previously described (23–25) using permeabilization with saponin and the indirect immunoperoxidase technique. Cells were incubated with rabbit-specific antibodies diluted 1:50 for 90 min at room temperature, and with peroxidase-labeled sheep antibodies against rabbit immunoglobulins (Institut Pasteur Production, Paris) for 90 min at room temperature. No membrane counterstaining was performed either before or after sectioning.

To detect possible antigenic sites at the cell surface, we also used other immunocytochemical procedures. Some GH₃ cells were fixed with 2% paraformaldehyde containing 0.05% glutaraldehyde and immunocytochemically stained with A-Ly-M as above described, except that saponin was omitted at any step. Moreover, some living GH₃ cells were incubated at 4°C with A-Ly-M for 30 min then with the peroxidase-labeled second antibodies for 30 min. They were then postincubated or not at 37°C before to be fixed in 1% glutaraldehyde without permeabilization. Some of these cells were loaded at 4°C with CF before or after incubation with antibodies at 4°C.

RESULTS

Localization of the Lysosomal Membrane Antigen

In GH₃ cells, a positive reaction of variable intensity was observed on the membrane of numerous structures. The most intensely stained and the most numerous looked like secondary lysosomes (Fig. 1, *a–e*). Beneath the plasma membrane a few small vesicles were also strongly labeled (Fig. 1 *d*). In the peripheral cytoplasm, a moderate reaction was observed on the membrane of large irregularly shaped, electron-lucent vacuoles. Some of these vacuoles contained a few vesicles and resembled multivesicular bodies. In such structures, the intensity of the membrane staining greatly varied (Figs. 1 *a–e*). In

the Golgi zone the membranes of two to three saccules were clearly labeled (Figs. 1 *g* and 2 *a*). In the core of the Golgi zone only a slight reaction deposit was observed on the membranes of some small vesicles. A weak reaction was also found on the membrane of multivesicular bodies located in the vicinity of the Golgi zone (Fig. 1 *g*). The GERL cisternae, as previously identified (22), appeared unlabeled (Fig. 2 *a*). In some cells, the membrane of a few rough endoplasmic reticulum cisternae was slightly labeled. In PRL cells in primary cultures, A-Ly-M labeled the same structures as in GH₃ cells. However, the membrane of secretory granules was completely free of reaction product (Fig. 2 *b*).

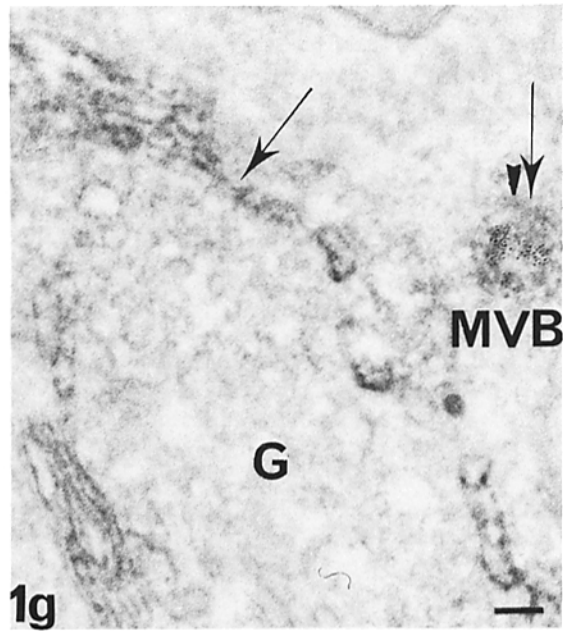
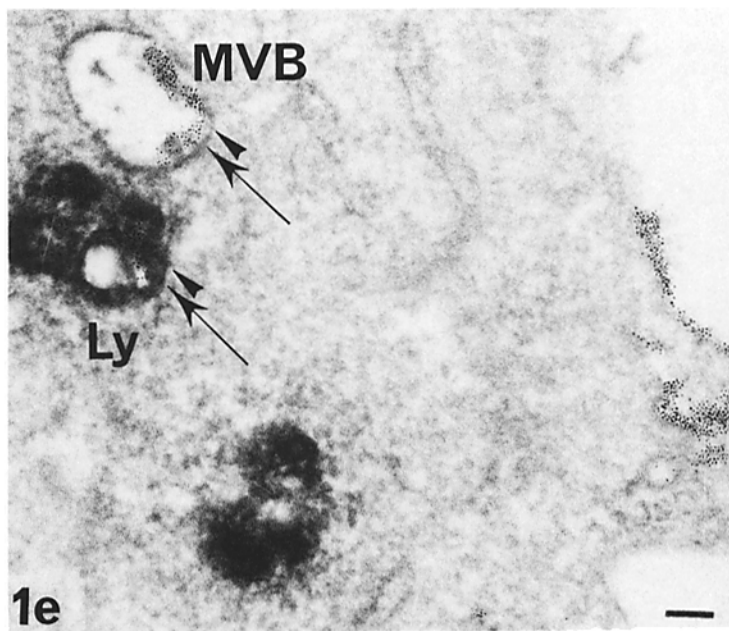
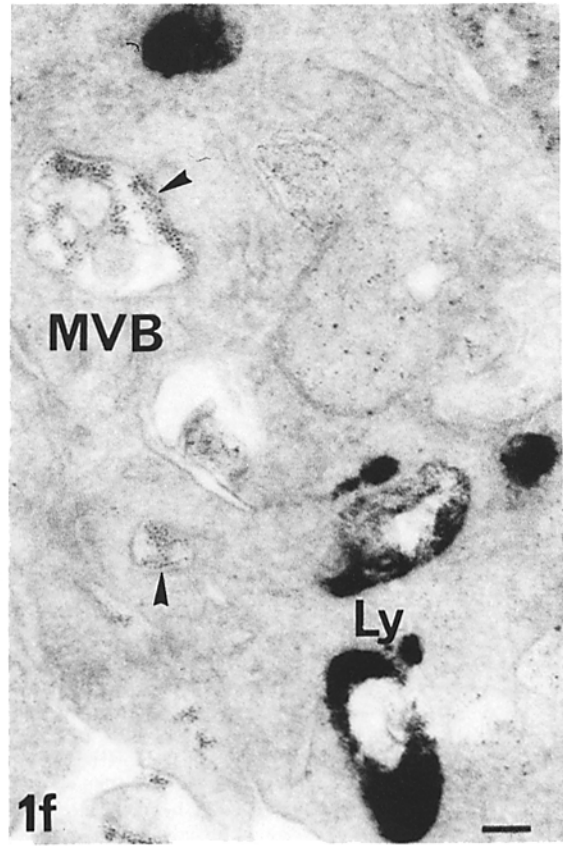
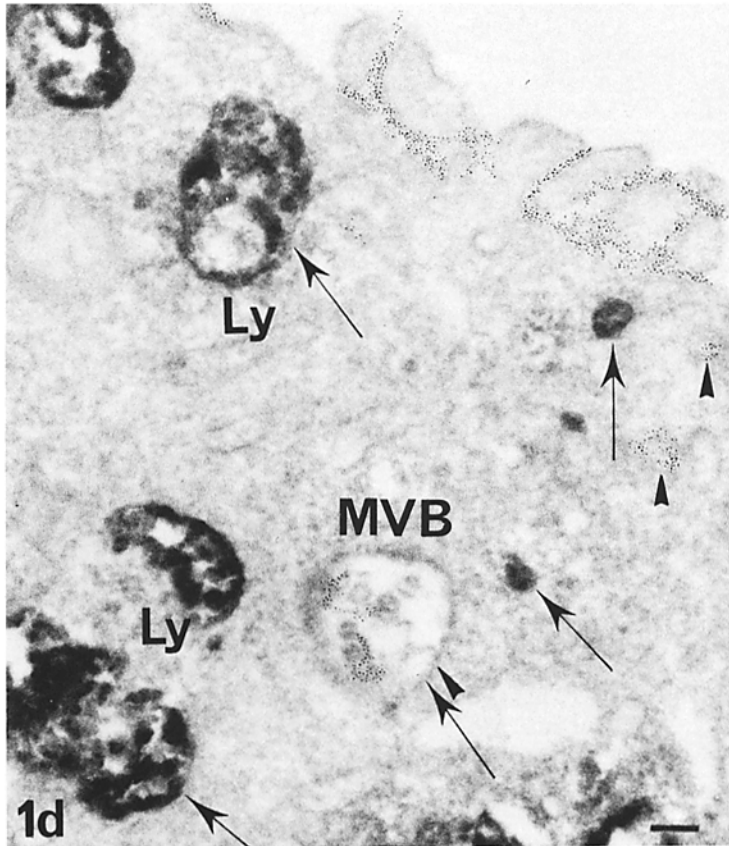
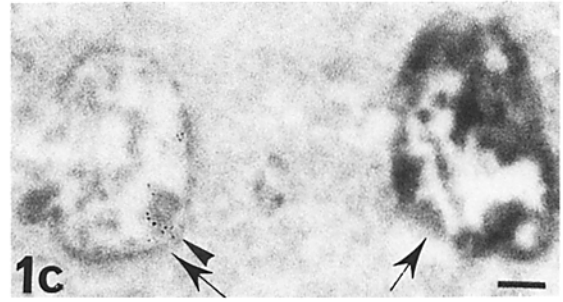
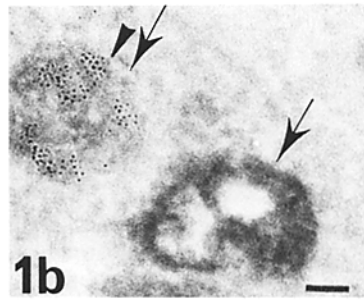
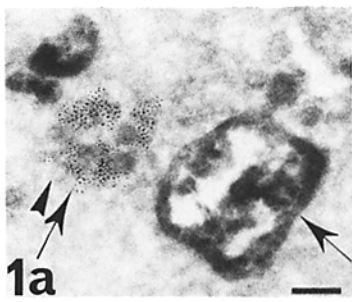
The staining of the plasma membrane varied greatly with experimental conditions (Fig. 3). This was explored on GH₃ cells only. In permeabilized GH₃ cells, the plasma membrane was not significantly labeled. In contrast, in fixed nonpermeabilized cells a conspicuous reaction product was observed on the plasma membrane (Fig. 3 *a*), suggesting that saponin can extract some antigens from the plasma membrane. A similar observation has been reported for the same antigen in another cell type (18). Such an effect of permeabilizing agents, as saponin, on the labeling of other plasma membrane antigens has been recently reported (20). When living cells were exposed to the antibodies at 4°C before fixation the overall staining of the cell surface was reduced, except on small patches and invaginations where the staining was intense (Fig. 3 *b*). Preloading with CF did not prevent the binding of antibodies at the cell surface (Fig. 3 *d*) and, vice versa, the binding of antibodies did not prevent the loading with CF. Following transfer at 37°C of cells coated at 4°C with antibodies, labeling was found inside the cell. After 15 min, the membrane of a few large irregularly shaped structures was clearly labeled (Fig. 3, *e* and *f*), suggesting that plasma membrane antigen was internalized. A very few immunoreactive vesicles could be seen inside the cell after only 5 min at 37°C. These vesicles were usually seen beneath the plasma membrane. Whether such vesicles represent true vesicles or small invaginations was not explored in this study.

Time Course of Endocytosis of CF in GH₃ Cells: Immunocytochemical Staining of Membrane Compartments

Following binding at 4°C, CF particles were exclusively located at the cell surface, where they were concentrated in clusters mainly at the base of microvilli. After transfer of the cells to 37°C, a progressive internalization of membrane associated CF particles was observed. The membrane compartments involved in such adsorptive endocytosis were immunocytochemically identified using either A-Ly-M or A-clathrin on permeabilized cells.

At 5 and 15 min, CF was seen on small pits at the cell surface and inside small vesicles near the plasma membrane. The membrane of such structures was not significantly labeled with A-Ly-M. In contrast, after staining with A-clathrin, a massive reaction deposit was associated with their cytoplasmic face (Fig. 4).

From 15 min onwards, CF was found within large vacuoles, some of which displayed typical features of multivesicular bodies; here, the CF particles were associated with the outer surface of the inner vesicles (Fig. 1). The membranes of such structures were slightly labeled by A-Ly-M (Fig. 1, *a–d*) but not by A-clathrin, except in a few cases in which a straight



area at one pole that can be related to the dense plaque (Fig. 4, *b* and *d*) was stained on the cytoplasmic face. At 15 min, acid phosphatase reaction product was never found in vacuoles and multivesicular bodies containing CF (Fig. 1*f*).

Between 30 min and 2 h at least, CF was seen in large structures that were strongly stained by A-Ly-M but never by A-clathrin (Fig. 1*e*). These structures also contained acid phosphatase activity and thus could be identified as secondary lysosomes (Fig. 1*f*). CF was also observed within small vesicles located in the Golgi zone, the membranes of which were unstained either by A-Ly-M or by A-clathrin (not shown). No CF particles were detected within the Golgi saccules at any time during the experiment. At the end of the incubation, some CF particles were still found at the cell surface.

DISCUSSION

The subcellular distribution of the immunostaining obtained in rat PRL cells in culture using A-Ly-M are similar to those recently described in normal rat kidney cells, rat macrophages, and hepatocytes (17, 18). In all these cell types, the labeling was not restricted to the membrane of secondary lysosomes. It was also found on the plasma membrane, on the membranes of small vesicles and large vacuoles, which most likely represent a prelysosomal compartment, as well as on some Golgi cisternae and vesicles nearby Golgi cisternae, which might be part of the synthetic pathway of the antigen (18). These localizations were consistent with the recent evidence obtained using several cell types for acidification of a prelysosomal compartment involved in the endocytic pathway (see introduction). They suggest that the antibodies, which are specific for a 100,000-mol-wt antigen immunologically related to a gastric purified $[H^+,K^+]ATPase$, may also recognize a component of the proton pump(s) in the cells (18).

The functional significance of the subcellular localization of the lysosomal membrane antigen in rat PRL cells in culture can be further discussed in the light of the present findings on CF internalization in GH₃ cells.

In GH₃ cells, as could be expected, the internalization of CF follows the classic sequential steps of adsorptive endocytosis: coated as well as uncoated pits and vesicles, irregularly shaped vacuoles, secondary lysosomes and small, uncoated vesicles in the Golgi zone. Unlike the endocytosis of CF in normal PRL cells (2), no CF particles could be found in Golgi saccules in GH₃ cells. This may be related to the low storage ability of those cells (8). Such a variability in the presence of CF into Golgi saccules has also been reported among myeloma cell lines (15).

The irregularly shaped vacuoles involved in the early steps of CF endocytosis in GH₃ cells are morphologically similar to

receptosomes (28) or endosomes (5, 27). In addition, the implication of multivesicular bodies in the prelysosomal step seems particularly striking in PRL cells. A similar observation was reported for the receptor-mediated endocytosis of epidermal growth factor (13) and transferrin (10) in human carcinoma (A 431) cells and transferrin in rat reticulocytes (9).

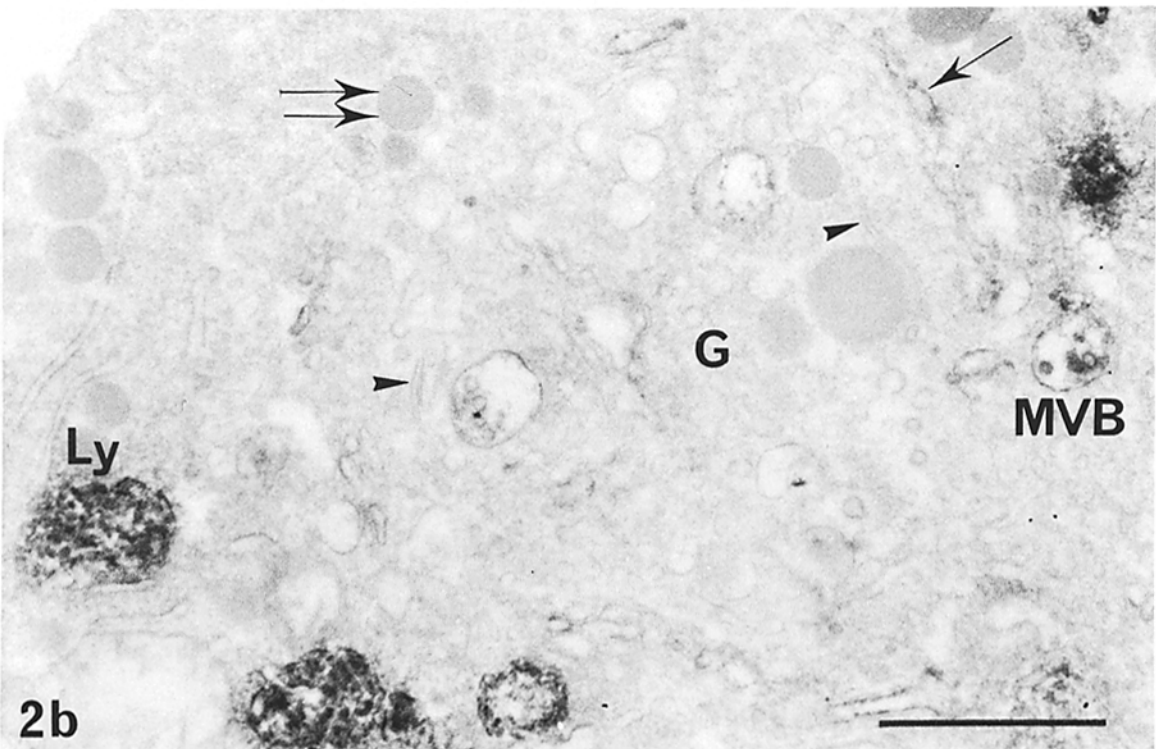
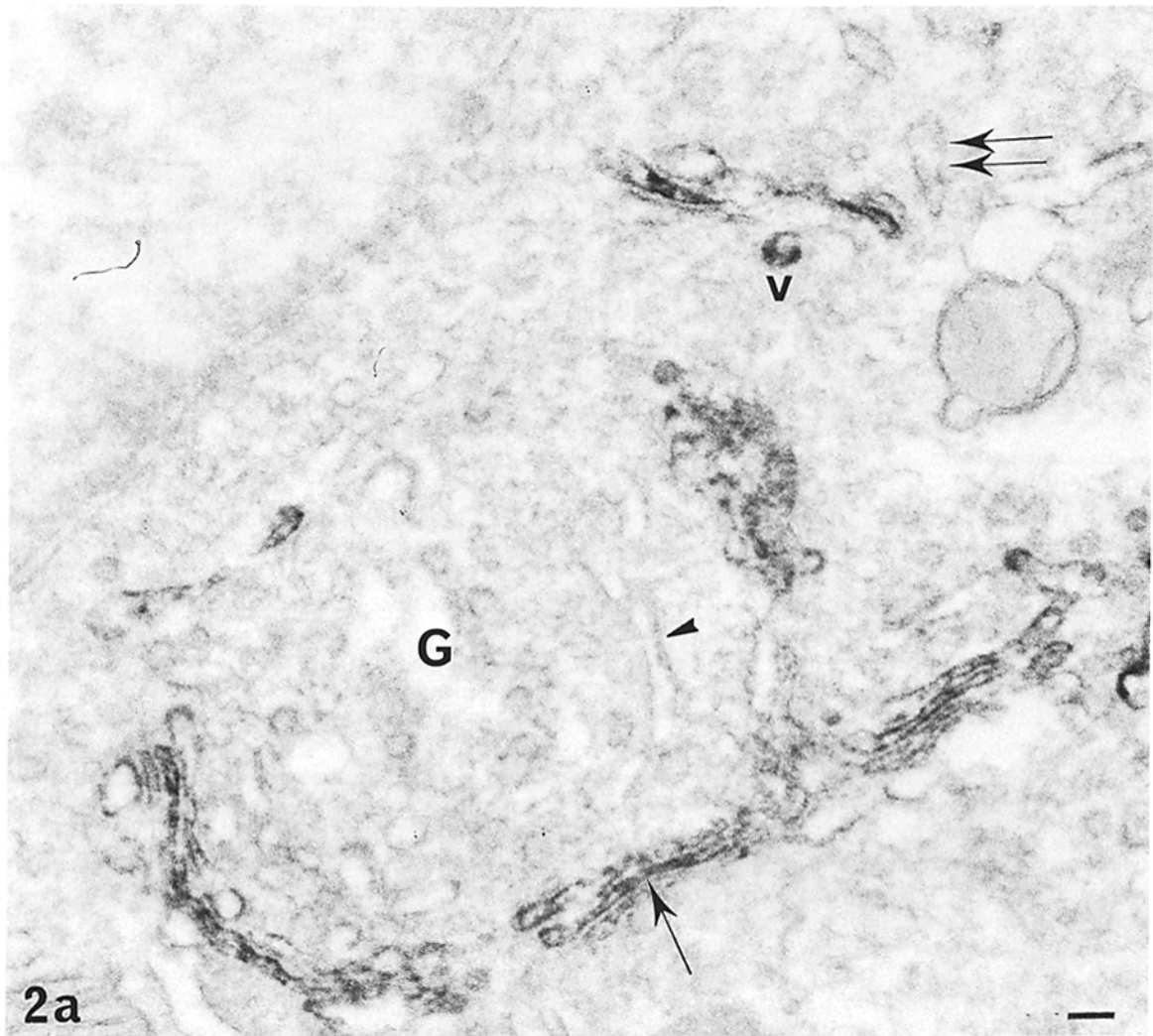
The parallel localization of a lysosomal membrane antigen, clathrin, and acid phosphatase activity allowed us to characterize functionally the membrane compartments during the course of CF endocytosis in GH₃ cells. Our results show that endosomes and multivesicular bodies possess on their membrane antigenic determinant(s) related to a 100,000-mol-wt polypeptide (17, 18), but do not contain acid phosphatase activity. This antigen was found closely associated with acidic compartments that have recently been described in the early steps of endocytosis in other cell types (4, 12, 19, 26). In GH₃ cells, the acidification of prelysosomal structures identified in our study may be driven by an ATP-dependent proton pump as recently demonstrated in macrophages and fibroblasts (4, 29).

It is intriguing to notice that the membranes of the clathrin-coated pits and vesicles that are involved in the first step of the endocytosis of CF in GH₃ cells did not seem to be significantly labeled by A-Ly-M. Even after binding of A-Ly-M at 4°C on living cells, followed by a postincubation at 37°C (a procedure leading to the internalization of this antigen), no clear-cut evidence for immunoreactive presumptive coated pits or coated vesicles could be obtained. This is in apparent contradiction with the fact that A-Ly-M have been found to recognize a 100,000-mol-wt polypeptide in a preparation of brain clathrin-coated vesicles (18). Although some coated vesicles in rat liver were immunostained using A-Ly-M, "not all coated vesicles appear to have demonstrable antigen" (18). In addition in fibroblasts in culture (normal rat kidney cells), no positive coated vesicles have been identified (18). Our observations may reveal further structural differences between coated vesicles. One may indeed obtain negative results in case of low level of immunoreactive sites in coated structures. Alternatively a partial extraction of antigen by saponin permeabilization, as it is the case for the plasma membrane (see Results) may account for our results.

It is also worth mentioning that the A-Ly-M do not recognize the membrane of secretory granules, although the presence of an electrochemical proton gradient in anterior pituitary secretory granules has been reported (1).

The presence of the lysosomal membrane antigen on some Golgi saccules is consistent with the recent evidence for an electrogenic pump able to promote in vitro acidification of vesicles derived from Golgi membranes of rat liver cells (6).

FIGURE 1 GH₃ cells incubated for increasing time intervals after binding of CF at 4°C. (*a-d*) GH₃ cells immunocytochemically stained by A-Ly-M after 15 min of endocytosis of CF. In *d*, CF particles are seen bound to the cell membrane, within small vesicles near the plasma membrane and within multivesicular bodies (MVB). The small vesicles that contain CF are not labeled by A-Ly-M, whereas other small vesicles that do not contain CF are labeled by A-Ly-M. In *a-d*, the MVB that contain CF are lightly labeled by A-Ly-M. Large structures that look like secondary lysosomes (*Ly*) do not contain CF at this stage of incubation period, but are strongly labeled by A-Ly-M. Bar, 0.1 μm . $\times 60,000$. (*e-g*) GH₃ cells are fixed after 1 h of endocytosis of CF. In *e*, the cell is immunocytochemically stained by A-Ly-M. At this later stage CF is observed within lysosomes that are labeled by A-Ly-M. Bar, 0.1 μm . $\times 60,000$. In *f*, acid phosphatase activity is detected in this cell. Acid phosphatase reaction product is observed in lysosomes (*Ly*) whereas it is never found in vacuoles and multivesicular bodies (MVB) which however contain CF. Bar, 0.1 μm . $\times 60,000$. In *g*, the cell is immunocytochemically stained by A-Ly-M. No CF is detected within Golgi saccules but some multivesicular bodies (MVB), labeled by A-Ly-M, contain CF particles in the Golgi zone (*G*). Bar, 0.1 μm . $\times 60,000$. In *a-g*, arrowheads show membrane compartments containing CF. In *a-e* and *g*, arrows show membrane compartments labeled by A-Ly-M, and arrowheads and arrows show membrane compartments containing CF and labeled by A-Ly-M.



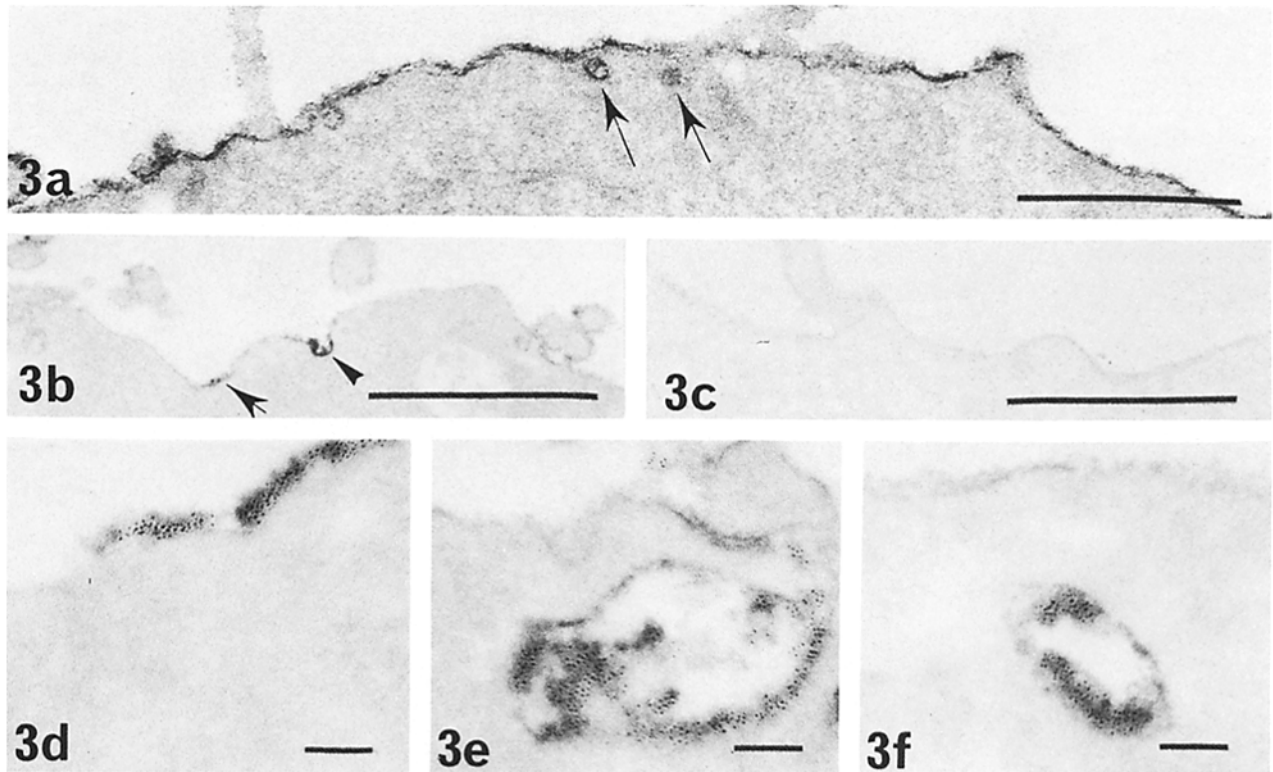


FIGURE 3 Immunocytochemical staining of the plasma membrane of GH₃ cells by A-Ly-M. (a) A GH₃ cell fixed with 2% formaldehyde-0.05% glutaraldehyde and immunocytochemically stained by A-Ly-M without permeabilization. A conspicuous reaction product is observed on the plasma membrane as well as on a few small vesicles (arrows) beneath the plasma membrane. (b) A GH₃ cell exposed to A-Ly-M at 4°C before fixation with 1% glutaraldehyde without permeabilization. In these experimental conditions, the overall staining of the cell surface is greatly reduced (compare with a), except on small patches (arrows) and invaginations (arrowheads) where the staining is intense. (c) A GH₃ cell exposed to normal rabbit serum at 4°C before fixation. In these control conditions, no reaction product is observed at the cell surface. (a-c) Bar, 1 μm . $\times 30,000$. (d) A GH₃ cell loaded with CF at 4°C then exposed to A-Ly-M at 4°C before fixation with 1% glutaraldehyde without permeabilization. Note a superimposition of the two labelings. Thus, the preloading with CF did not prevent the immunocytochemical staining of plasma membrane (compare with b). (e and f) GH₃ cells loaded with CF at 4°C, exposed to A-Ly-M at 4°C, and postincubated 15 min at 37°C before fixation. The immunocytochemical staining is found inside the cells on the membrane of large and irregularly shaped vacuoles which are also loaded with CF (compare with Fig. 1, a-d). (d-f) Bar, 0.1 μm . $\times 90,000$.

To our surprise, the membrane of the GERL cisternae, which is particularly apparent in both PRL cell types, was never labeled with the A-Ly-M, whereas it always contained abundant acid phosphatase-positive material (22). There is a large overlap between the present results and previous staining with Golgi antibodies of the same cell types (25), since both antibodies label the membranes of medial saccules, lysosomes, and endosome-like vacuoles, but not the secretory granule membrane. In contrast, the staining obtained with these two antibodies differed at the level of small vesicles and of the

plasma membranes in GH₃ cells, particularly following thyroliberin treatment (25). Work is in progress to determine the effects of physiologically relevant ligands on the distribution of these membrane-associated antigens.

In conclusion, our findings reveal a subtle subcompartmentalization of membranes involved in adsorptive endocytosis in PRL cells in culture. Since the localization of A-Ly-M has been found to correlate with an acid compartment in others cell types, our observations support the theory of an acidified prelysosomal compartment in GH₃ cells.

FIGURE 2 Immunocytochemical staining of the Golgi zone (G) by A-Ly-M. (a) In a GH₃ cell, the membrane of two or three saccules located on one face of the stacks (arrows) is intensely labeled by A-Ly-M. The presumptive GERL cisterna (arrowheads) is unlabeled. Some vesicles (v) are also labeled. Bar, 0.1 μm . $\times 60,000$. (b) In a normal PRL cell in primary culture the membrane of some Golgi saccules (arrows) is slightly labeled whereas the GERL cisternae (arrowheads) are unlabeled. The membranes of multivesicular bodies (MVB) are lightly labeled whereas those of lysosomes (Ly) are strongly labeled. Note that the membrane of secretory granules (double arrows) is completely free of reaction product. Bar, 1 μm . $\times 30,000$.

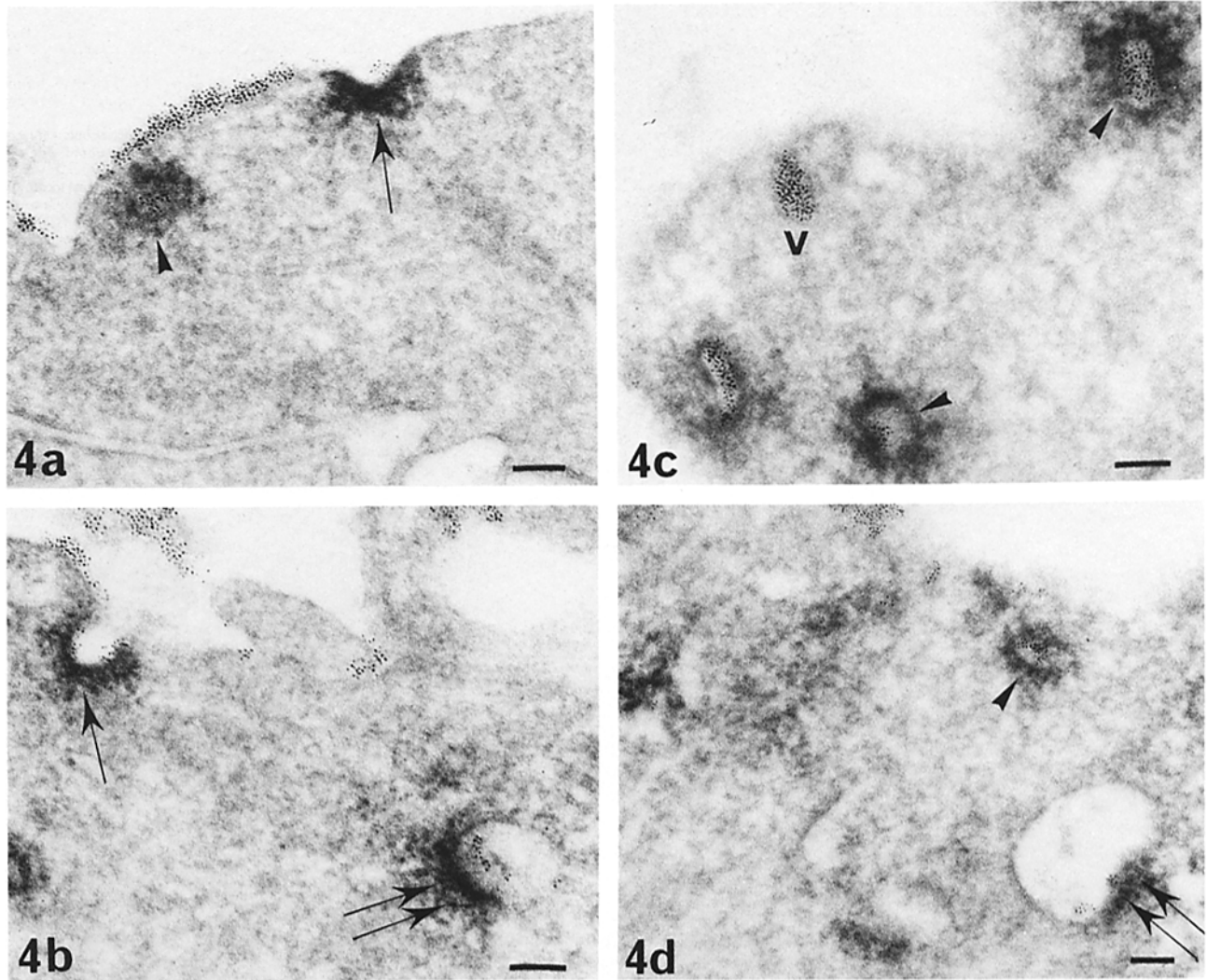


FIGURE 4 GH₃ cells incubated for increasing time intervals after binding of CF at 4°C and immunocytochemically stained by A-clathrin. After 15 min (a and b) and after 30 min (c and d) of endocytosis of CF, CF particles are seen on small pits (arrows) at the cell surface and inside small vesicles (arrowheads) near the plasma membrane. The membrane of such structures as well as fuzzy material associated with their cytoplasmic face are strongly labeled by A-clathrin. These structures correspond, therefore, to coated pits and coated vesicles. Note a vesicle or an invagination (v) in c that contains CF and is uncoated. All these small structures, which contain CF, were not labeled by A-Ly-M (compare with Fig. d). In b and d, a straight area (double arrows) at one pole of large vacuoles, which contain CF, is labeled by A-clathrin. (a-c) Bar, 0.1 μm . $\times 75,000$. (d) Bar, 0.1 μm . $\times 60,000$.

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