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Morphologic study of the internalization of a lysosomal enzyme by the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells

(coated pit/receptosome/Golgi/GERL/lysosome)

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ABSTRACT **The binding and internalization of a model lysosomal enzyme, β-galactosidase, was visualized by use of rabbit anti-β-galactosidase and goat anti-rabbit IgG; the second antibody was labeled with rhodamine or fluorescein (for detection by fluorescence) or with horseradish peroxidase (for electron microscopy). Chinese hamster ovary cells were incubated with β-galactosidase at 4°C, and then were washed and sequentially incubated in the cold with the two antibodies. The β-galactosidase was found primarily in coated pits. The binding of the enzyme was completely inhibited by 5 mM mannose 6-phosphate. After the reaction with enzyme and antibodies, the cells were warmed to 37°C; within 1 minute, the β-galactosidase–antibody complex had begun to move to uncoated vesicles (receptosomes). After 8 min, the β-galactosidase–antibody complex was seen in receptosomes near tubular elements in the Golgi/GERL area, within such tubular elements and at times, in vesicular elements that may correspond to coated structures of the GERL system. After 15 min, the enzyme–antibody complex was found in lysosomes near the Golgi/GERL area and a half-hour later it was in lysosomes distributed throughout the cytoplasm. Double-label experiments using β-galactosidase and gold/α_2-macroglobulin showed the presence of the two ligands in the same coated pits and receptosomes. Thus, the pathway for internalization of β-galactosidase via the mannose 6-phosphate receptor is similar to the pathway established for other ligands such as low density lipoprotein and α_2-macroglobulin.**

The surfaces of fibroblastic cells contain receptors for a wide variety of ligands. Receptors have been identified for many hormones and growth factors, low density lipoprotein, α_2-macroglobulin (α_2M), toxins, and viruses (for review, see ref. 1). Shortly after the ligand binds to its receptor, the ligand begins to be internalized. A combination of fluorescence and electron microscopic studies have shown that α_2M (2, 3), epidermal growth factor (4, 5), low density lipoprotein (6), insulin (7), *Pseudomonas* toxin (8), and some viruses (9, 10) enter the cell by the same pathway. After ligand binding, the ligand–receptor complexes rapidly diffuse within the plasma membrane until they become trapped and concentrated in coated pits (1, 2). The ligand is then transferred to uncoated vesicles termed receptosomes that carry it to the general region of the Golgi and GERL, and in many cases, further on to lysosomes (3).

Lysosomal enzymes are also internalized by cultured fibroblasts, provided the enzymes carry mannose 6-phosphate residues (11–18). The initial step involves binding to a surface receptor that specifically recognizes mannose 6-phosphate moieties (19–21). In cultured human and Chinese hamster ovary (CHO) fibroblasts, binding leads to endocytosis and delivery of the enzyme to lysosomes (22, 23); however, surface binding to the receptor on rat hepatocytes is not followed by internalization (24). The relationship of the endocytosis pathway to the transport of newly made endogenous enzymes into lysosomes is not yet clear (25–27).

To investigate the pathway by which exogenous lysosomal enzymes are transferred to lysosomes and to compare it to the pathway established for the entry of α_2M and the other ligands mentioned above, we have carried out fluorescence and electron microscopic studies in CHO cells with β-galactosidase purified from bovine testes. This enzyme was selected because its binding and uptake by the mannose 6-phosphate receptor has been studied previously (14, 17, 24), and because it can be detected by an affinity-purified antibody.

MATERIALS AND METHODS

Cells. Chinese hamster ovary (CHO) cells were gifts of A. R. Robbins (National Institutes of Health) (clone WTT2) and M. M. Gottesman (National Institutes of Health) (clone K1 and subclone 10001). Cells were grown in Eagle's minimal essential medium/5% fetal calf serum containing nonessential amino acids, penicillin, and streptomycin at 37°C in 95% air/5% CO_2.

Preparation of β-Galactosidase and Affinity Purification of Antibodies. Bovine testicular β-galactosidase and a testicular inhibitor glycoprotein fraction were purified as described (17, 24). Antibodies directed against β-galactosidase were raised in rabbits (21) and affinity purified by chromatography on a column of β-galactosidase-Sepharose 4B. The antibodies were eluted from the column with 1.0 M potassium thiocyanate and immediately dialyzed against buffer A (0.136 M NaCl/2.7 mM KCl/8 mM NaH_2PO_4/1.5 mM KH_2PO_4/0.5 mM $MgCl_2$/0.68 mM $CaCl_2$ pH 7.4) without Ca or Mg salts. To remove a minor antibody contaminant, the antibody preparation was passed through a second affinity column of inhibitor glycoprotein covalently coupled to CNBr-activated Sepharose 4B.

Goat anti-rabbit IgG conjugated to rhodamine was obtained from Cappel Labs and affinity purified on a column using rabbit IgG coupled to CNBr-activated Sepharose 4B (Pharmacia).

Labeling of Cells: Fluorescence Video Microscopy. Cells grown in 35-mm plastic culture dishes were washed in buffer A and incubated at 4°C for 30–60 min in 2 ml of buffer A/5 mM mannose 6-phosphate containing bovine serum albumin at 1 mg/ml (Pentex). This procedure was used to dissociate endogenous lysosomal enzymes from occupied surface receptors (19).

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Abbreviations: CHO, Chinese hamster ovary; α_2M, α_2-macroglobulin.

Cells were then washed with cold (4°C) buffer A and incubated sequentially in buffer A/albumin at 4°C with (*i*) a saturating amount of β-galactosidase (100 μg/ml) for 30 min, (*ii*) affinity-purified rabbit anti-β-galactosidase (100 μg/ml) for 15 min, and (*iii*) affinity-purified goat anti-rabbit IgG-rhodamine (100/μg/ml) for 15 min. After each incubation, the cells were washed five times with cold buffer A. For double-label experiments, the cells were incubated at 4°C for 15 min with rhodamine-labeled α_2M prior to incubation with β-galactosidase and fluorescein-labeled anti-rabbit IgG was substituted for the rhodamine-labeled antibody. The specificity of binding was demonstrated by including 5 mM mannose 6-phosphate during incubation with β-galactosidase or excess α_2M (1mg/ml) during incubation with rhodamine-α_2M.

After the final wash, the labeled cells were brought to 37°C with warm buffer A, incubated for 8–10 min, and then fixed with 1.5% formaldehyde in buffer A. The dishes were mounted with a 25-mm circular no. 1 coverslip using glycerol and viewed with a Zeiss epifluorescence microscope equipped with a 63X, N.A. 1.4, phase-contrast oil-immersion planapochromat objective and a silicon intensifier-target video camera as described (29). All images were recorded on video tape and direct photographs from the monitor were made with a 35–mm camera using Pan-X film (Kodak).

Labeling of Cells: Electron Microscopy. Cells were incubated at 4°C as in the fluorescence experiments described above, except that a horseradish peroxidase conjugate of anti-rabbit IgG (Cappel) was used in place of the fluorescent antibody conjugate. After labeling, the cells were either immediately fixed at 4°C with 1% glutaraldehyde in buffer A for 15 min or warmed to 37°C for 8, 15, or 45 min and then fixed with glutaraldehyde. After fixation, the cells were incubated with diaminobenzidine in 50 mM phosphate buffer pH 6.0, for 30 min at 23°C (30). Then, the cells were treated with 1.5% OsO_4 in buffer A, dehydrated with ethanol, and embedded in Epon 812 at 58°C. Thin sections were cut using a Sorvall MT2-B ultramicrotome and a diamond knife and counterstained with lead citrate. The specimens were viewed at 50 kV with a Hitachi HU-12A electron microscope using a 20–μm objective aperture.

For double-label electron microscopy, cells were incubated at 4°C with colloidal gold/α_2M (α_2M, 50 μg/ml) (31) for 90 min and then incubated sequentially at 4°C with β-galactosidase, rabbit anti-β-galactosidase, and goat anti-rabbit IgG conjugated to horseradish peroxidase. The cells were then warmed to 37°C for 1 min, fixed with 2% glutaraldehyde, and processed as described above. Specificity of binding in this double-label experiment was controlled by incubating other samples with excess unlabeled α_2M (1 mg/ml) during the gold/α_2M incubation or including 5 mM mannose 6-phosphate with the β-galactosidase incubation.

RESULTS

Fluorescence Studies. β-Galactosidase was bound to the surface of CHO cells and labeled with rabbit anti-β-galactosidase and rhodamine-labeled goat anti-rabbit IgG. When the labeled cells were incubated at 37°C for 8–10 minutes, fluorescence was found concentrated in many discrete structures (Fig. 1). Previously, using α_2M, such a pattern was shown to result from concentration of the ligand in unique intracellular vesicles termed receptosomes (3). Mannose 6-phosphate (5 mM), a competitive inhibitor of the receptor-mediated uptake of lysosomal enzymes (11–14), prevented the appearance of β-galactosidase in receptosomes (Fig. 1*C*) whereas 5 mM glucose 6-phosphate, which is not an inhibitor (11–13), had no effect (not shown). No fluorescence was detected in the absence of added bovine β-galactosidase (Fig. 1*B*), showing that, under the conditions

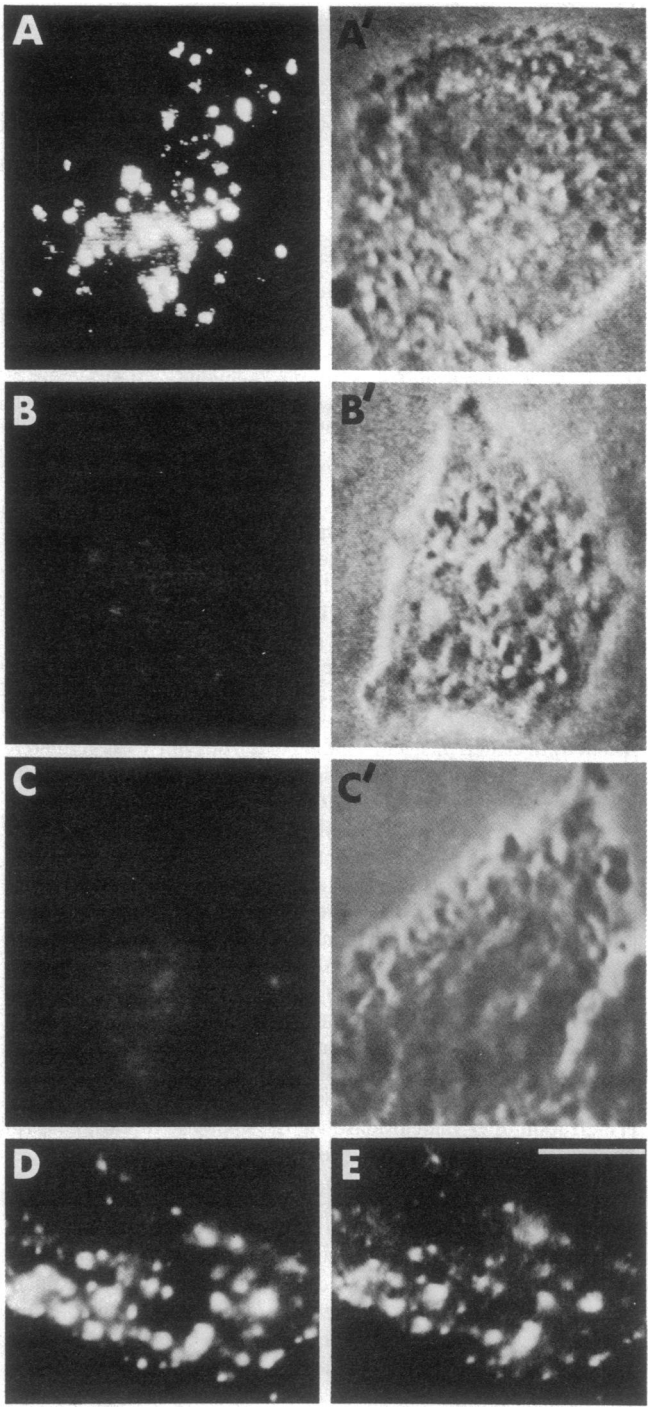

FIG. 1. Detection of internalized β-galactosidase by fluorescence video intensification microscopy. CHO cells were incubated with β-galactosidase and labeled with fluorescent antibodies at 4°C. After warming to 37°C for 8 min the cells were fixed with formaldehyde and viewed by video intensification fluorescence microscopy. (*A–C*) Fluorescence images. (*A'–C'*) Phase contrast images of the same cells. In *A*, β-galactosidase was included in the first incubation. In *B*, β-galactosidase was omitted from the first incubation. In *C*, β-galactosidase was added along with 5 mM mannose 6-phosphate. (*D* and *E*) Double-label experiment using rhodamine-labeled α_2M and fluorescein-labeled antibody to β-galactosidase; both ligands were added at 4°C prior to warming to 37°C. The distribution of α_2M on the rhodamine channel is shown in *D*, and the distribution of β-galactosidase on the fluorescein channel is shown in *E*. Most of the spots correspond in position. All of the fluorescent punctate spots seen in this figure represent internalized label present in intracellular vesicles, most likely receptosomes. (×1425; bar = 10μm.)

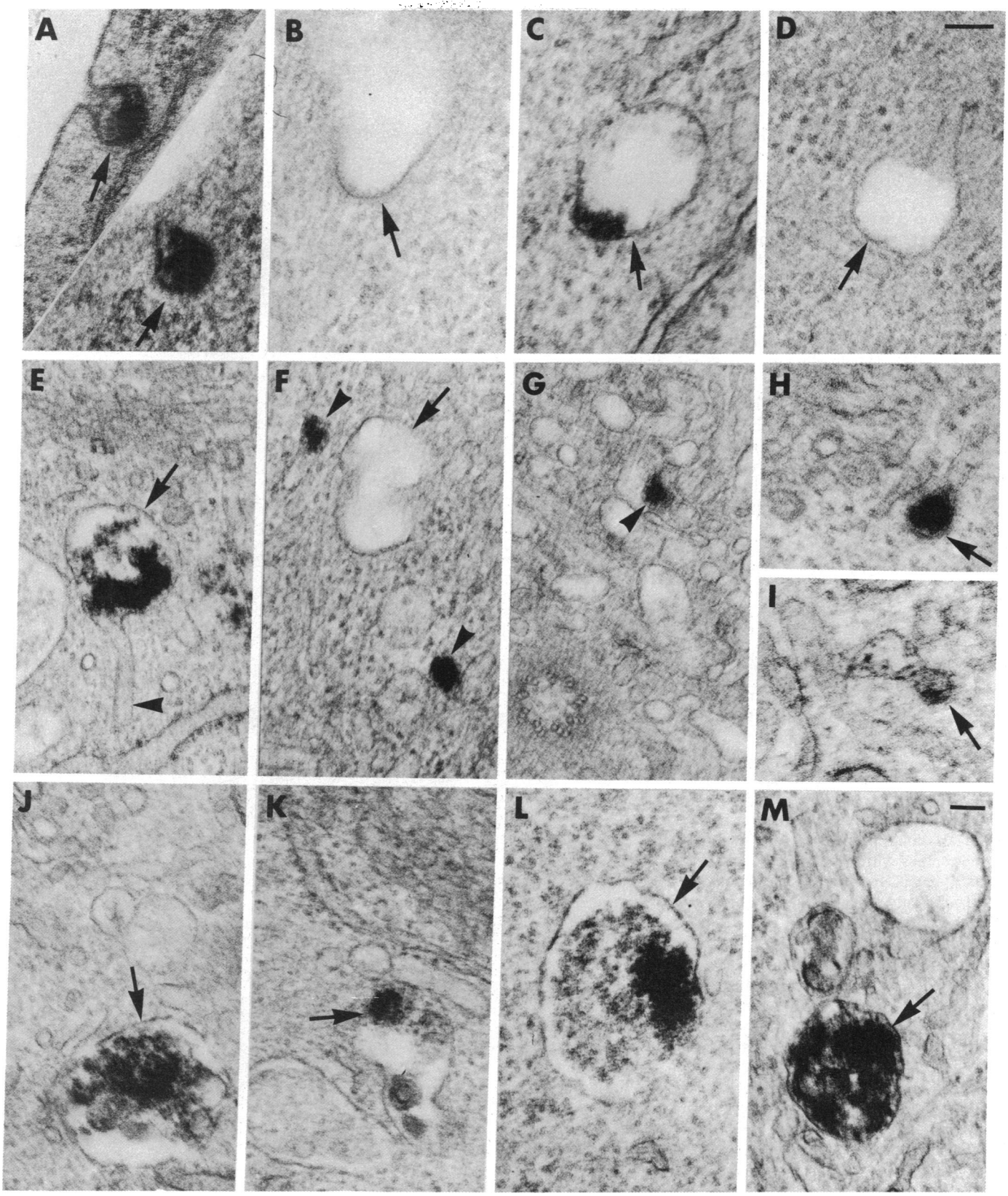

FIG. 2. Internalization of β-galactosidase observed by immunocytochemical labeling and electron microscopy. Cells were labeled at 4°C with β-galactosidase, which was visualized by peroxidase-antibody. When the cells were fixed at 4°C, the label was present in coated pits (arrows) on the cell surface (*A*). Simultaneous incubation with β-galactosidase and 5 mM mannose 6-phosphate abolished the labeling (*B*). After the cells were warmed to 37°C for 8 min, most of the label was found in receptosomes (*C*, arrow). Cells incubated in 5 mM mannose 6-phosphate together with β-galactosidase and then warmed to 37°C for 8 min contained only unlabeled receptosomes (*D*). Occasional images were found showing the close proximity of receptosome-like labeled structures near tubular elements in the cytoplasm (arrowhead), suggesting continuity of these two structures (*E*). (*F* and *G*) Label contained within tubular elements. After the cells were warmed to 37°C for 8 (*H*) or 15 (*I*) min, label was occasionally found concentrated in vesicular structures, which may correspond to the coated structures of the GERL system. At 15 min after warming, most of the label was in structures near the GERL system, which had the morphologic characteristics of lysosomes (arrows; *J, K, L*). Forty-five minutes after warming, the label was still in these lysosomal structures (*M*). (Lead citrate; *A–D, H, I*, ×90,000; *E–G, J–M*, ×63,000; bars = 0.1 μm)

used, the antibody did not react with endogenous β-galactosidase nor with other surface antigens of CHO cells.

A double-labeling experiment, in which cells were exposed to α_2M labeled with rhodamine and β-galactosidase indirectly labeled with fluorescein, showed the two ligands within the same vesicles (Fig. 1 *D* and *E*). The appearance of cell-associated β-galactosidase or α_2M was specifically inhibited by mannose 6-phosphate (5 mM) or α_2M (100 μg/ml), respectively (not shown).

Electron Microscopic Experiments. *Surface Binding.* The location of β-galactosidase at the ultrastructural level was determined from the product of the diaminobenzidine reaction generated by peroxidase-labeled antibody. This method has been used previously to follow the entry of α_2M in Swiss 3T3 cells (2). When cells were kept at 4°C to prevent cellular entry, β-galactosidase was found clustered and concentrated in coated pits (Fig. 2*A*). This binding was specific; in the presence of 5 mM mannose 6-phosphate, no cell-associated β-galactosidase could be detected (Fig. 2*B*). The presence of β-galactosidase in coated pits at 4°C should not be interpreted to mean that the receptors for β-galactosidase were preclustered in the pits prior to binding of the enzyme. For example, unoccupied α_2M receptors are diffusely distributed but accumulate in coated pits at 4°C when occupied by α_2M (2).

Eight Minutes after Entry. When labeled cells were warmed to 37°C for 8 min, the ligand was found predominantly in uncoated intracellular vesicles (Fig. 2*C*). These vesicles had the same characteristic appearance as the receptosomes first described in mouse fibroblasts (3). Their previously demonstrated morphologic characteristics include a clear center with a diameter of 2000–4000 Å, a fuzzy cytoplasmic border eccentrically placed at one edge of the vesicle, a single small intralumenal vesicular structure, and the presence of a concentrated cluster of ligand at one edge of the lumen of the receptosome. No β-galactosidase was found in receptosomes if incubation with the enzyme had been carried out in the presence of 5 mM mannose 6-phosphate (Fig. 2*D*).

Some receptosomes containing β-galactosidase were observed close to tubular structures in the Golgi/GERL region. On occasion, images were seen suggesting fusion of labeled receptosomes with tubular elements (Fig. 2*E*). Eight min after entry, β-galactosidase was also found in such tubular elements (Fig. 2 *F* and *G*). Fig. 2*G* shows a labeled tubular element in the vicinity of the centriole.

Clathrin-coated structures (32) are associated with the GERL system (33, 34). Fig. 2*H* and *I* shows β-galactosidase concentrated in vesicular structures that are thought, from their shape and size, to correspond to these coated structures.

Fifteen and 45 Minutes after Entry. By 15 min after entry, most of the β-galactosidase was found in lysosomes near the Golgi/GERL region (Fig. 2 *J–L*). In CHO cells, lysosomes appear to have an interrupted membrane after fixation and a dense irregular interior often containing myelin figures; they are readily distinguished from receptosomes (see Fig. 2*C*), which have a smooth uniterrupted membrane, coated on its interior with proteinaceous material, and an empty center (3).

At 45 min, the labeled β-galactosidase was present in lysosomes (Fig. 2*M*). These lysosomes were distributed throughout the cytoplasm, including peripheral regions of the cell.

Double-Label Experiment. The fluorescence studies described above suggested that β-galactosidase and α_2M were internalized in the same vesicles. This was demonstrated conclusively by a double-label electron microscopic experiment using gold-α_2M and antibody-labeled β-galactosidase. Both ligands could be found in the same coated pit (Fig. 3*A*) or receptosome (Fig. 3*B*). These images were found 1 min after warming to 37°C, at which time, some ligand was still present in coated pits and some had been transferred from coated pits to receptosomes. Labeling of coated pits and receptosomes by β-galactosidase or gold/α_2M was abolished by the presence of 5 mM mannose 6-phosphate or excess α_2M, respectively (Fig. 3*C–F*).

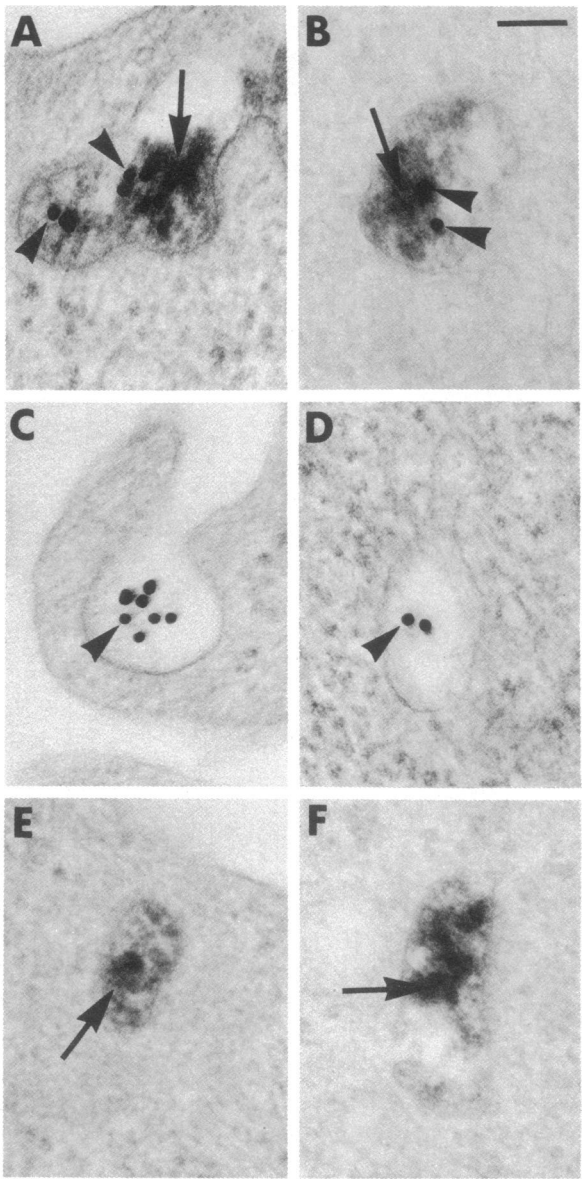

FIG. 3. Localization of β-galactosidase and α_2M in the same structures by double-label electron microscopy. Cells were labeled with gold/α_2M and β-galactosidase (indirectly labeled with horseradish peroxidase) at 4°C and then warmed to 37°C for 1 min prior to fixation. (*A*) α_2M/gold (arrowheads) and β-galactosidase–peroxidase (arrows) are present in the same coated pit. (*B*) Both ligands are present in the same receptosome. (*C* and *D*) A coated pit and a receptosome in cells that were incubated with 5 mM mannose 6-phosphate show the absence of β-galactosidase but the continued presence of α_2M/gold. (*E* and *F*) A similar competition experiment in which excess unlabeled α_2M was used instead of mannose 6-phosphate shows the absence of gold/α_2M but the presence of β-galactosidase. (Lead citrate; ×90,000; bar = 0.1 μm.)

DISCUSSION

We have examined the entry of a lysosomal enzyme by way of the mannose 6-phosphate receptor. We find that the pathway of entry is similar to that previously established by electron microscopy for α_2M, epidermal growth factor, and low density

lipoprotein (for review see ref. 1). β-Galactosidase was detected clustered in plasma membrane-associated coated pits, next in receptosomes, later in the Golgi/GERL region, and finally in lysosomes. Because many lysosomal enzymes use the mannose 6-phosphate recognition system, we assume that other lysosomal enzymes follow the same entry route.

The time course of intracellular transfer of β-galactosidase from coated pits to lysosomes in CHO cells differs slightly from that of other ligands in 3T3 cells. β-Galactosidase was detected in lysosomes 15 min after entry into CHO cells, whereas it required 30 min for α_2M to appear in the lysosomes of 3T3 cells. This appears to be due to the difference in cell type because studies with gold/α_2M in CHO cells have shown similar rapid kinetics (data not shown).

It should be noted that the internalized ligand was not β-galactosidase itself but rather β-galactosidase to which two antibodies had been bound after the initial interaction of the enzyme with the receptor. We assume, although it has not been proved, that the piggyback transport of the antibodies affects neither the rate nor the pathway of internalization of the enzyme.

We have not yet examined the location of unoccupied β-galactosidase receptors to see whether they are diffusely distributed in the plasma membrane as has been found for α_2M (2), epidermal growth factor (35), and insulin and triiodothyronine (36) or partially preclustered in coated pits as has been reported for low density lipoprotein (6). Nor do we know whether the receptor accompanies the lysosomal enzyme from cell surface to lysosome.

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