Identification of a Membrane Glycoprotein Found Primarily in the Prelysosomal Endosome Compartment

John E. Park, Jacqueline M. Lopez, Edward B. Cluett, and William J. Brown

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

Abstract. Cells contain an intracellular compartment that serves as both the "prelysosomal" delivery site for newly synthesized lysosomal enzymes by the mannose 6-phosphate (Man6P) receptor and as a station along the endocytic pathway to lysosomes. We have obtained mAbs to a ~57-kD membrane glycoprotein, (called here plgp57), found predominantly in this prelysosomal endosome compartment. This conclusion is supported by the following results: (*a*) plgp57 was primarily found in a population of late endosomes that were located just distal to the 20°C block site in the endocytic pathway to lysosomes (~83% of the prelysosomes were positive for plgp57 but <5% of the early endosomes had detectable amounts of this marker); (*b*) plgp57 and the cation-independent (CI) Man6P receptor were located in many of the same intracellular vesicles; (*c*) plgp57 was found in the membranes of an acidic compartment; (*d*) immunoelectron microscopy showed that plgp57 was located in characteristic multilamellar- and multivesicular-type vacuoles believed to be prelysosomal endosomes; and (*e*) cell fractionation studies demonstrated that plgp57 was predominantly found in low density organelles which comigrated with late endosomes and CI Man6P receptors, and only ~10–15% of the antigen was found in high density fractions containing the majority of secondary lysosomes. These results indicate that plgp57 is a novel marker for a unique prelysosomal endosome compartment that is the site of confluence of the endocytic and biosynthetic pathways to lysosomes.

LYSOSOMES are a heterogeneous group of organelles consisting of a membrane-enclosed compartment containing acid hydrolases and substrates for those enzymes (11, 43). The study of lysosome biogenesis has the explicit challenge of understanding how and where lysosomal enzymes (LEs)[1] meet up with substrates for those enzymes to make a new lysosome. One mechanism for the biogenesis of lysosomes involves the vesicular transport of newly synthesized LEs via mannose 6-phosphate (Man6P) receptors from the *trans*-Golgi network to a recently identified prelysosomal compartment (for review see ref. 25). Biochemical and morphological studies have suggested that this delivery site is not a mature, secondary lysosome, but is instead an acidic "prelysosomal" compartment where the enzymes dissociate from Man6P receptors due to the low compartmental pH (19, 37, 44). In this acidic compartment, the enzymes remain soluble in the lumen while the receptors recycle back to the Golgi complex for multiple rounds of enzyme delivery (8, 14). In addition, it has recently been shown that this compartment also serves as a station along the endocytic pathway to lysosomes because newly internalized molecules are also delivered there before reaching secondary lysosomes (8, 18, 21, 22). Thus, it has been proposed that this unique compartment may serve as an intracellular confluence between the biosynthetic (Man6P receptor–mediated) and endocytic pathways to lysosomes (8, 21). This scheme provides a mechanism for the biogenesis of a new lysosome by allowing newly synthesized LEs to encounter recently endocytosed substrates for those enzymes.

The biochemical properties of this prelysosomal compartment are not well understood, nor is it exactly clear how this compartment is related to secondary lysosomes. To better understand this compartment and its role in lysosome biogenesis, we have sought to find an endogenous marker for this unique organelle and report here on the identification of mAbs directed against a membrane glycoprotein primarily found in prelysosomal endosomes.

Materials and Methods

Materials

Trans^{35}S-label ([^{35}S-methionine: ^{35}S-cysteine] at ~1,000 Ci/mmol) was obtained from ICN Biochemicals, Inc. (Costa Mesa, CA). Lucifer yellow (LY), protein A–Sepharose CL-4B, diaminobenzidine hydrochloride (Type II), saponin, and reagents for selecting and growing hydridomas were from Sigma Chemical Co. (St. Louis, MO). Fluorescent antibody reagents were from Organon Teknika-Cappel (Malvern, PA), Fab fragments of sheep anti-mouse IgG conjugated with HRP were from Biosys S. A. (Compiegne,

1. *Abbreviations used in this paper*: BTRS, bovine testicular cells from primary cultures; CF, cationic ferritin; CI, cation independent; IF, immunofluorescence; IP, immunoperoxidase; LE, lysosomal enzyme; LY, lucifer yellow; Man6P, mannose 6-phosphate; MDBK, Madin-Darby bovine kidney; MVB, multivesicular body.

France), and goat antirabbit conjugated with alkaline phosphatase was from Chemicon (Temecula, CA). Cationized ferritin was from Miles Scientific Co. (Naperville, IL). Nu-serum was from Collaborative Research (Bedford, MA). Rabbit polyclonal antibodies were raised against purified bovine liver cation-independent (CI) Man6P receptors and characterized as described (6).

Cells

Madin-Darby bovine kidney (MDBK) cells were grown in MEM containing 10% Nu-serum and primary cell cultures from bovine testis (BTRS cells), turbinate and lung were grown in MEM containing 10% FBS (Gibco Laboratories, Grand Island, NY), at 37°C in an atmosphere of 95% air, 5% CO_2. PAI myeloma cells were maintained in DME containing nonessential amino acids, 10% FBS, 0.004% β-mercaptoethanol, 10 mg/ml gentamycin, and supplemented with hypoxanthine and aminopterin essentially as described (24).

Immunization and Screening Hybridoma Supernatants

Membranes enriched in CI Man6P receptors from MDBK cells were prepared as described (5, 6) (~0.4 mg total protein), solubilized with 0.1% Triton X-100, mixed with Freund's complete adjuvant, and then delivered to Balb/c mice (Jackson Labs, Bar Harbor, ME) by i.p. injection. Mice were boosted with similar material but in Freund's incomplete adjuvant two times over the next 6 wk, and sera were screened by immunofluorescence (IF) on MDBK cells. 2 wk later, mice were injected with solubilized membranes in Freund's incomplete adjuvant and 3 d later spleen cells were obtained and fused with PAI myeloma cells. The cell suspension was placed into DME containing HAT selection media and plated into 96-well microtiter plates along with a feeder layer of peritoneal macrophages (24).

Supernatants from the microtiter plates were screened by IF on cultured MDBK cells. To do this, MDBK cells were grown as monolayers on the inside surfaces of the covers of 96-well microtiter plates (Costar Corp., Cambridge, MA), each of which contains 96 small rings that form a replica of the wells below. These rings served as "mini"-wells for the MDBK cells that had attached and spread inside. Cells on the plate tops were fixed in 3.7% formalin in PBS for 30 min, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. In all of these steps, solutions were simply poured on and off. Supernatants from hybridoma cultures were harvested and then 8–10 μl of each were carefully placed in each mini-well in such a way as to generate a replica of the original microtiter plate from which the supernatants were obtained. After a 1-h incubation, cells were washed with PBS, incubated with goat anti-mouse IgG coupled with rhodamine for 1 h, washed as above, and inspected by epifluorescence microscopy using a water immersion lens. Hybridomas that gave potentially interesting antibodies were cloned by limiting dilution thrice and rescreened by IF each time.

Cell Fractionation

To label late endosomes, a 75-cm^2 flask of confluent MDBK cells was washed twice with MEM, incubated with MEM containing 2 mg/ml HRP for 5 min at 37°C, rapidly rinsed with 20 ml of MEM (~30 s total), and incubated for another 10 min at 37°C to chase the HRP into late endosomes. The media was replaced with 4°C MEM and cells were harvested with a cell scraper and kept on ice. To metabolically radiolabel cells, a 75-cm^2 flask of confluent MDBK cells was labeled overnight with 50 μCi/ml Trans^{35}S-label (in methionine-free MEM). To obtain a large number of cold, carrier cells, MDBK cells were grown to confluency in 4–6, 24 × 24 cm tissue culture dishes and harvested with a rubber policeman. Cells from HRP- and ^{35}S-labeled flasks were collected by centrifugation and combined with the cold, carrier cells. Cells were washed twice with 0.25 M sucrose in 10 mM Tris, pH 7.4, homogenized in a Balch-Rothman apparatus (1) in the same solution, and a postnuclear supernatant (PNS) was obtained by low speed centrifugation. The PNS (4.5 ml) was loaded onto a linear 20–45% (wt/wt) sucrose gradient (in 10 mM Tris, pH 7.4) prepared in SW28.1 buckets (Beckman Instruments, Inc., Palo Alto, CA), and centrifuged at 25,000 RPM for 2 h at 4°C. Fractions (0.7 ml) were collected and subjected to immunoprecipitation and enzymatic analyses as described (6).

Other Procedures

IF and immunoperoxidase (IP) cytochemistry were performed on cultured bovine cells as described (7). However, for IF labeling, cells were permeabilized with 0.1% Triton X-100 in PBS for 5–10 min after fixation and washing, and saponin was omitted from the antibody incubations. Hybridoma culture supernatants were used with no dilution for IF. For double-IF labeling, goat anti-mouse IgG conjugated with fluorescein was used to detect the mAbs, and goat anti-rabbit IgG coupled conjugated with rhodamine was used to detect the polyclonal antibodies. Appropriate controls were done to ensure that no cross-reactivity occurred between the species-specific reagents and the first antibodies. To label compartments of the endosome-lysosome apparatus for light and electron microscopy, MDBK and/or BTRS cells were incubated with LY or cationic ferritin (CF), respectively, for various periods of time, and stained by indirect IF or IP as described (8).

To immunoprecipitate proteins, metabolically radiolabeled cells were extracted with RIPA buffer (9), the extracts incubated with hybridoma culture supernatant for 1 h, followed by incubation with rabbit anti-mouse Ig for 1 h, and proteins precipitated by protein A-Sepharose beads. Beads were extensively washed with 1× RIPA and immunoprecipitates analyzed by PAGE. For Western immunoblotting, proteins were transferred to nitrocellulose paper essentially as described (42) except that a different transfer buffer was used (39) and the mAb did not recognize samples that had reduced disulfide bonds, therefore, all transfers and blots were done on nonreduced samples. Nitrocellulose paper with transferred proteins was stained with Ponceau S to identify molecular weight standards, destained with dH_2O, blocked with 0.15 M NaCl, 0.05 mM Tris, pH 7.4 (TBS) containing 3% BSA, 10% goat serum, 0.05% Tween-20, probed with mABs (as culture supernatants) for 1 h, washed with TBS containing 0.05% Tween-20 (TBST), and incubated with goat anti-mouse Ig conjugated with alkaline phosphatase (1:10,000 dilution in TBST) for 1 h. The blots were washed with TBST, rinsed with TBS, and developed with 5-bromo-4-chloro-3-indolyl phosphate (0.165 mg/ml) and nitroblue tetrazolium (0.33 mg/ml) in 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5. The reaction was stopped with 5 mM EDTA.

The association of plgp57 with membranes was investigated in two ways. First, total microsomes from bovine kidney were prepared and washed by homogenization (2×) in 10 mM Tris, pH 7.4, and centrifugation at 100,000 *g* for 1 h. The membrane pellets were washed (by homogenization) in low concentrations of various detergents or alkali (0.15 M sodium carbonate, pH 11.3). Soluble and insoluble material was separated by centrifugation at 100,000 g for 1 h and the supernatants and pellets were analyzed by Western blotting using the mAbs. Second, MDBK cells were labeled overnight with ^{35}S-methionine and subjected to extraction and phase partitioning with Triton X-114 according to Bordier (3) and immunoprecipitation.

HRP activity was determined using o-dianisidine as a substrate (46), *N*-acetyl-β-D-glucosaminidase using p-nitrophenyl-*N*-acetyl-β-D-glucosamine (13), and protein by the method of Lowry et al. (27).

Results

Biochemical Characterization of the Antigen

To identify antigens specifically located in compartments of the endosome–lysosome pathway, mice were immunized with highly washed membranes enriched in Man6P receptors (a crude mixture of endosome and Golgi membranes), and hybridoma supernatants were screened by IF on cultured cells. From ~1,000 microtiter wells (representing ~10,000 hybridomas), four candidate mAbs (all IgG_1 isotypes) were identified as ones which appeared to predominantly stain endosomal compartments: three of these recognized the same protein by immunoprecipitation and stained the same organelles by immunocytochemistry. Immunoprecipitation and Western blotting with these mAbs (two shown here are called 12B and VG) specifically identified a protein that migrated as a diffuse band between ~45–66 kD (average ~57 kD) (Fig. 1). However, when cells were radiolabeled in the presence of tunicamycin, an inhibitor of *N*-asparagine–linked glycosylation, a sharper band at ~24 kD was immunoprecipitated indicating that this antigen is a glycoprotein with a core protein of ~24 kD. This antigen will be designated plgp57 for prelysosomal glycoprotein, 57 kD. plgp57 appeared to be tightly associated with membranes because

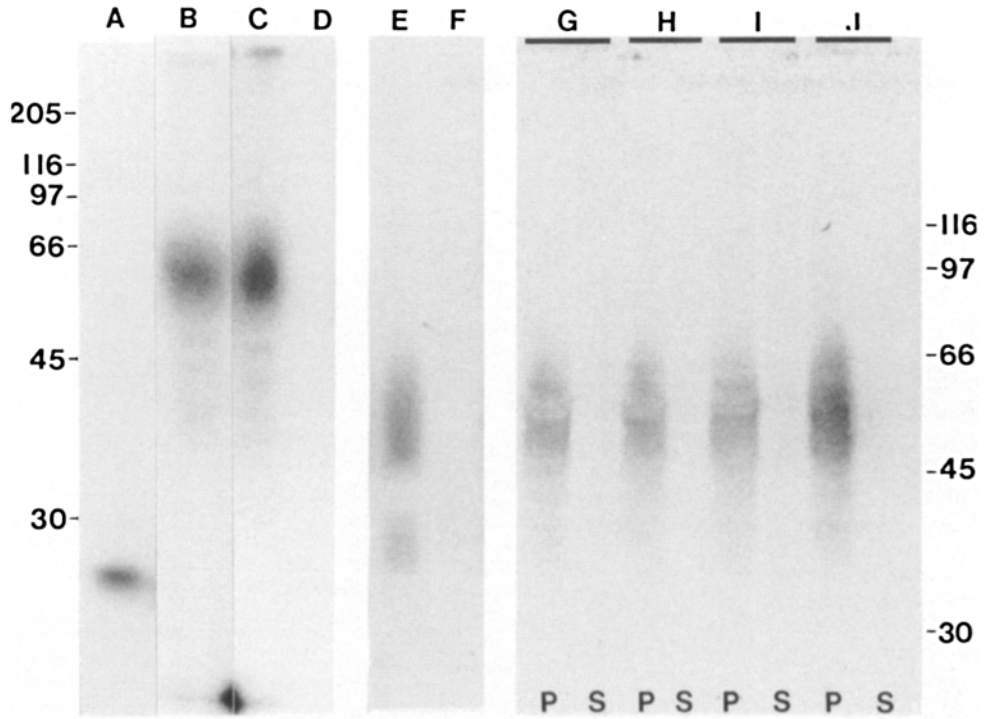


Figure 1. Biochemical characterization of plgp57 using mAbs. Lanes *A–D* were immunoprecipitates from ^{35}S-labeled MDBK cells. In lane *A*, cells were radiolabeled in the presence of the glycosylation inhibitor, tunicamycin, and a band at ~24 kD was precipitated with mAb 12B. In lanes *B–D*, cells were radiolabeled in the absence of tunicamycin and immunoprecipitated with mAb 12B (*B*), mAb VG (*C*), and a control hybridoma culture supernatant (*D*). The reactive mAbs specifically immunoprecipitated a more diffuse band between 45 and 66 kD with an average M_r~57,000. The molecular weight markers indicated on the left were from protein standards on the Coomassie blue-stained 12% acrylamide gel and relate to lanes *A–D*. Lanes *E* and *F* were from ^{35}S-methionine-labeled MDBK cells which were subsequently extracted with Triton X-114, subjected to phase partitioning to yield detergent (lane *E*) and aqueous (lane *F*) phases, followed by immunoprecipitation. plgp57 was only present in the detergent phase (lane *E*). Lanes *G–J* illustrate the sedimentation of plgp57 with washed membranes. Total microsomal membranes from bovine kidney were washed by homogenization in one of several different solutions, centrifuged to yield pellet and supernatant fractions (*P* and *S* below), and each was analyzed by immunoblotting with mAb 12B. (lane *G*) 0.05% saponin; (lane *H*) 0.05% deoxycholate; (lane *I*) 0.05% Triton X-100; (lane *J*) 150 mM Na_2CO_3, pH 11.3. The results show that plgp57 was always found in the pellet fraction. The molecular weight markers indicated on the right were from 10% acrylamide gels and relate to lanes *E–J*.

it partitioned into the detergent phase of Triton X-114 extracts, and it sedimented with membranes after extraction with low concentrations of various detergents or Na_2CO_3 (Fig. 1). plgp57 has been found in all bovine tissues examined, however, the mAbs have not been found to cross-react with other species.

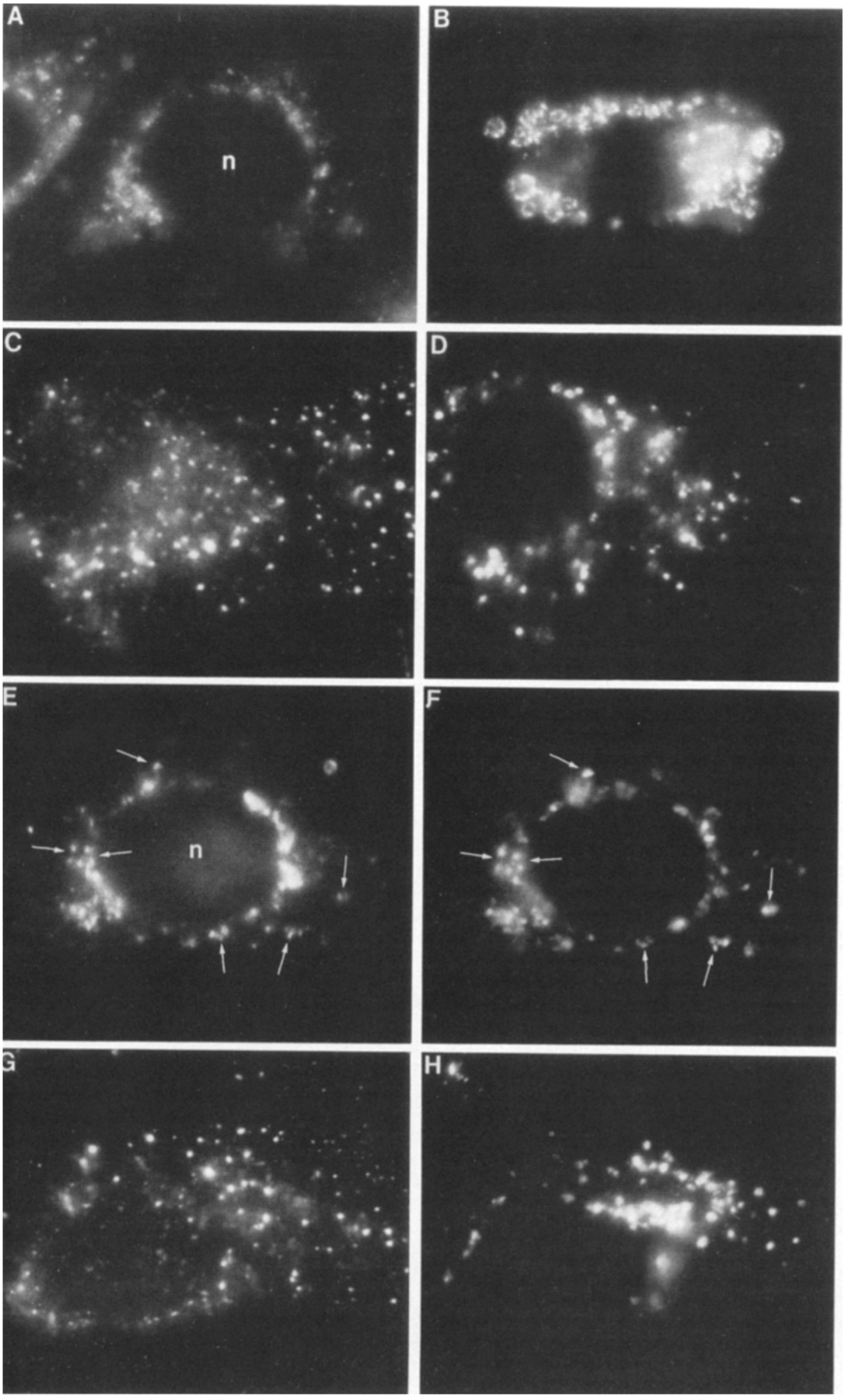
plgp57 Is Primarily Found in Acidic Late Endosomes Enriched in Man6P Receptors

IF with mAb 12B on cultured bovine kidney (MDBK) and testicular cells revealed a punctate, juxta-, or perinuclear distribution (Fig. 2), however, pretreatment of cells with 25 μM chloroquine for 3 h before fixation caused mAb 12B to stain the rims of large, dilated vacuoles indicating that plgp57 was found associated with the membranes of intracellular organelles that are normally acidic, such as endosomes and lysosomes.

To determine in what part of the vacuolar apparatus plgp57 was located, MDBK cells were pulse labeled with the fluorescent, fluid phase tracer LY followed by chases for various periods of time (all at 37°C), and then fixed and processed for IF (Fig. 2). When cells were pulsed for 5 min with LY and immediately fixed, numerous small vesicles located throughout the cytoplasm were seen to contain the tracer but not plgp57 (Fig. 2 *C* and *D*). When cells were pulsed for 5 min with LY followed by a 10–15-min chase, LY was found in fewer, larger vesicles which were located primarily in the juxta- and perinuclear Golgi region which are characteristic of late endosomes (23). Under these conditions it appeared that the majority of LY-filled late endosomes were also positive for plgp57 (Fig. 2, *E* and *F*). In cells incubated with LY continuously for 2 h followed by a 45-min chase, at which time the tracer was primarily located in mature lysosomes (40), there was much less coincidental staining with mAb 12B (Fig. 2, *G* and *H*). Identical results were obtained with BTRS cells.

It has previously been shown that 20°C inhibits the transport of molecules from endosomes to lysosomes (15), and more recent studies indicate that this site of blockade lies before endocytic tracers reach the Man6P receptor-enriched prelysosome, late endosome compartment (21). To see where the plgp57-enriched compartment was located relative to the 20°C blockage, cells were incubated with LY at 20°C for 2 h followed by IF staining with mAb 12B. In this case, there was little overlap of the two fluorescence signals (Fig. 3, *A* and *B*). However, if cells were pulse labeled at 20°C for 2 h followed by a brief chase for 13 min at 37°C, a significant number of vesicles were seen to contain LY and plgp57 (Fig. 3, *C* and *D*). Similar pulse-chase experiments with LY but using anti-CI Man6P receptor antibodies yielded identical results (not shown). These results showed that plgp57 was found primarily in late endosomes that lie just distal to the 20°C block site in the endocytic pathway to lysosomes.

To further characterize this plgp57-enriched, late endocytic compartment, and to determine if it might be involved


A
n
B
C
D
E
n
F
G
H

in Man6P receptor-mediated trafficking as would be expected for a prelysosomal endosome, MDBK cells were double labeled with mAb 12B and polyclonal antibodies against the CI Man6P receptor (Fig. 3 *E* and *F*). The staining patterns with these antibodies were remarkably similar, and it was possible to resolve many vesicles that were positive for both molecules. To investigate the relationship between the plgp57-enriched organelles and secondary lysosomes, double-IF labeling was performed using mAb 12B and polyclonal antibodies against the LE α-glucosidase (Fig. 3, *G* and *H*). The results showed that labeling with these two markers was quite different as plgp57 and α-glucosidase appeared to be located in distinct intracellular compartments. For example, α-glucosidase was occasionally found in tubular lysosomes which were not stained by mAb 12B (Fig. 3 *H*, *large arrows*). However, it was possible to identify some vesicles that contained plgp57 and that were also faintly positive for a α-glucosidase (Fig. 3, *G* and *H*, *thin arrows*). Identical results were obtained with BTRS cells.

Immunoelectron Microscopy of plgp57 and Its Presence in Unusual Multilamellar Organelles

By IP at the electron microscopic level, plgp57 was primarily located in two types of membrane-bound vacuoles that were generally somewhat spherical (0.3–0.9 μm in diameter) (Fig. 4): one contained numerous intralumenal membrane vesicles and thus resembled classically described multivesicular bodies (MVBs) (Fig. 4, *a* and *b*); the other contained multiple layers of intraluminal membrane sheets which appeared as whorls (myelin-like figures) or as parallel stacked membranes (lamellar-like) (Figs. 4 and 5). Both of these were generally located in the Golgi region. It was also not uncommon to find plgp57-stained organelles that contained both membrane lamellae and vesicles (Fig. 4). No appreciable staining was seen in the ER (rough or smooth), nuclear envelope, Golgi complex, or the plasma membrane (Fig. 4 *a*).

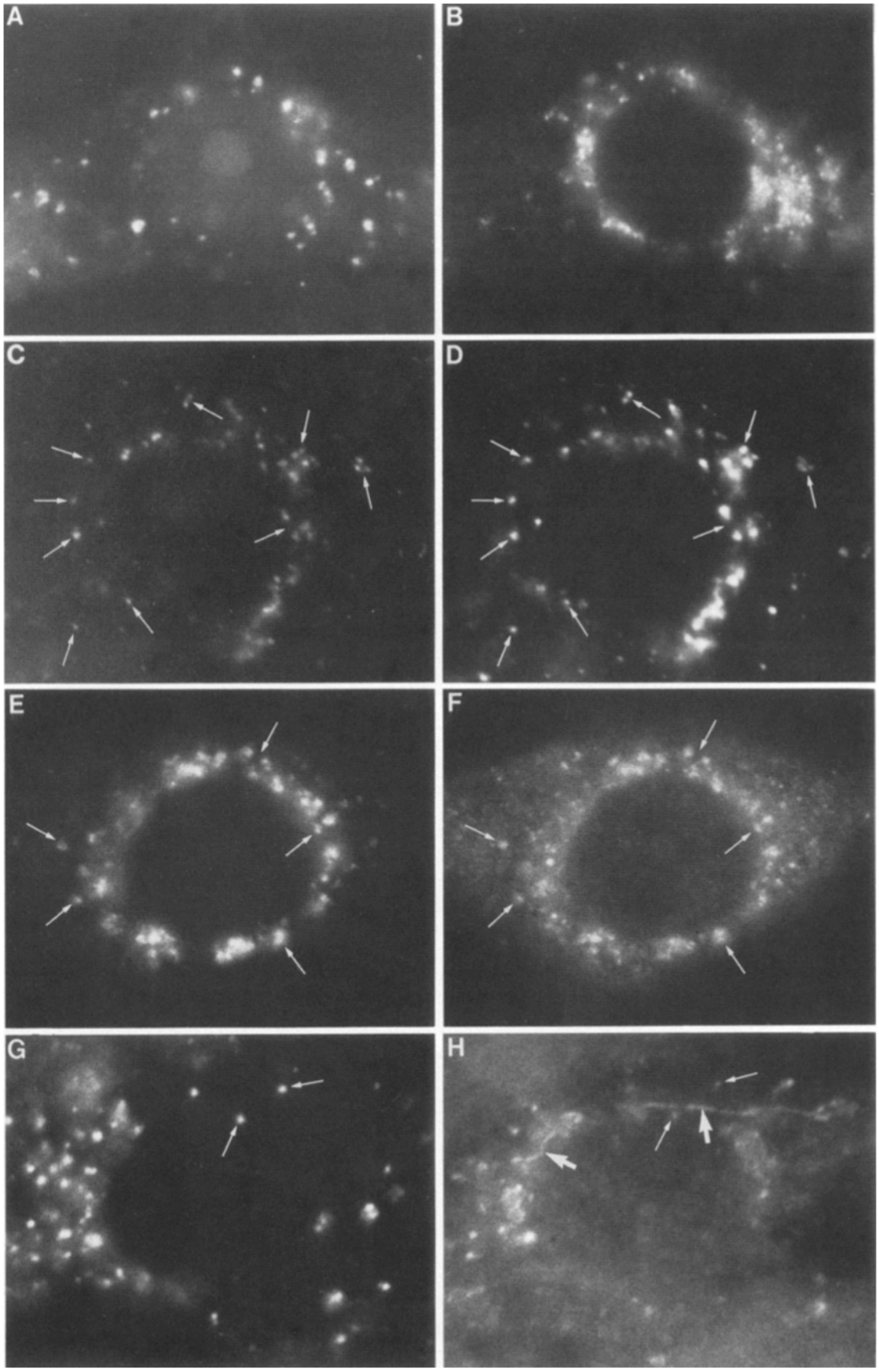
To confirm the LY-uptake experiments seen by light microscopy, plgp57 was localized by IP at the EM level along with CF that had been taken up by cells under a variety of conditions (Figs. 4 and 5). Also, to determine the proportion of vacuoles that contained both markers, electron micrographs were examined and organelles were scored as either containing just one of the markers, i.e., plgp57-positive (plgp57$^+$) or CF-positive (CF$^+$), or as containing both markers, i.e., plgp57- and CF-positive (plgp57$^+$/CF$^+$) (Fig. 6). When cells were incubated with CF at 20°C for 2 h and then fixed, many organelles were found to contain CF or plgp57, but very few were found to contain both markers (Fig. 4 *b*). Only 3–5% of these CF$^+$ early endosomes also stained for plgp57 (Fig. 6). However, when CF was allowed to reach late endosomes (10-min pulse, 10-min chase) (Fig. 5), the large majority of total plgp57$^+$ vacuoles were also plgp57$^+$/CF$^+$ (71.6%); when normalized to the total number of CF$^+$ organelles, an even higher percentage were found to be plgp57$^+$/CF$^+$ (83.5%) (Fig. 6). When cells were incubated with CF for 2 h at 37°C followed by a 1-h chase to deliver CF to secondary lysosomes, far fewer plgp57$^+$/CF$^+$ organelles were seen when compared to the 10-min label, 10-min chase protocol (Fig. 6).

plgp57 Primarily Resides in Light Density Organelles of Intracellular Origin

The subcellular distribution of plgp57 was explored by cell fractionation of MDBK cells on sucrose gradients. In these gradients, HRP-loaded late endosomes migrated as a single major peak in fractions 11–16 (Fig. 7). LE activity exhibited a bimodal distribution similar to that originally found by Rome et al. (35). The major peak contained most of the total enzyme activity and was found in fractions of high density (number 17–20). A second, minor peak of activity comigrated with HRP-labeled endosomes and contained ~10–12% of the total activity. CI Man6P receptors were found to comigrate with HRP-loaded endosomes with little or no receptor found in the dense lysosome fractions. The results of immunoprecipitations (and immunoblotting in other experiments) using mAb 12B showed that the majority of plgp57 (~70% of the total) comigrated with the peaks of HRP activity and Man6P receptors (in fractions of density ~1.14–1.16 g/ml). It should be noted that the peak of plgp57 was consistently broader than either of those for HRP or Man6P receptors, exhibiting a reproducible shoulder primarily on the dense side of the peak. In addition to that found in light density organelles, ~10–15% of the recovered plgp57 was consistently found in the dense lysosome region of the gradient.

To determine the relative amounts of plgp57 on the cell surface versus that which was intracellular, saturating concentrations of mAb VG were added to permeabilized and nonpermeabilized MDBK cells, and the bound antibody was quantitated (Fig. 8). The results showed that nonpermeabilized cells had a very small but saturable amount of binding, however, permeabilization resulted in almost a 200-fold increase in antibody binding, indicating that >99% of the total plgp57 was found intracellularly and <1% was on the cell surface.

Figure 2. Fluorescence double-labeling experiments showing that plgp57 is located in acidic late endosomes. In BTRS cells, mAb 12B stained small vesicles located primarily in the juxta- and perinuclear regions (*A*), however, when BTRS cells were first pretreated with 25 μM chloroquine for 3 h before fixation and immunostaining, mAb 12B was found on the rims of large, dilated vacuoles that were also located in the center of the cell (*B*). *C–H* show that plgp57 is located in late endosomes as revealed by double-fluorescent labeling. *C* and *D* show a cell that was incubated for 5 min with LY at 37°C, and then immediately fixed for IF. In *C*, numerous small, early endosomes located throughout the cytoplasm are seen to contain LY, however, in *D* mAb 12B appears to stain a different population of plgp57-enriched vesicles. *E* and *F* show a cell that was incubated with LY for 5 min at 37°C, chased in LY-free media for 10 min, and then fixed for IF. In this case, LY (*E*) was located in fewer vesicles primarily located in the perinuclear region, and it was easy to detect numerous organelles which also stained for plgp57 (*F*) (see arrows for several examples). Panels *G* and *H* are micrographs of cells labeled with LY for 2 h at 37°C followed by a 45-min chase in LY-free media to deliver the tracer to lysosomes. Under these labeling conditions many fewer vesicles were found to contain both labels (LY in *G* vs. plgp57 in *H*). These results indicate that plgp57 is primarily located in a compartment that lies between early endosomes and secondary lysosomes (*n* = nucleus).


A
B
C
D
E
F
G
H

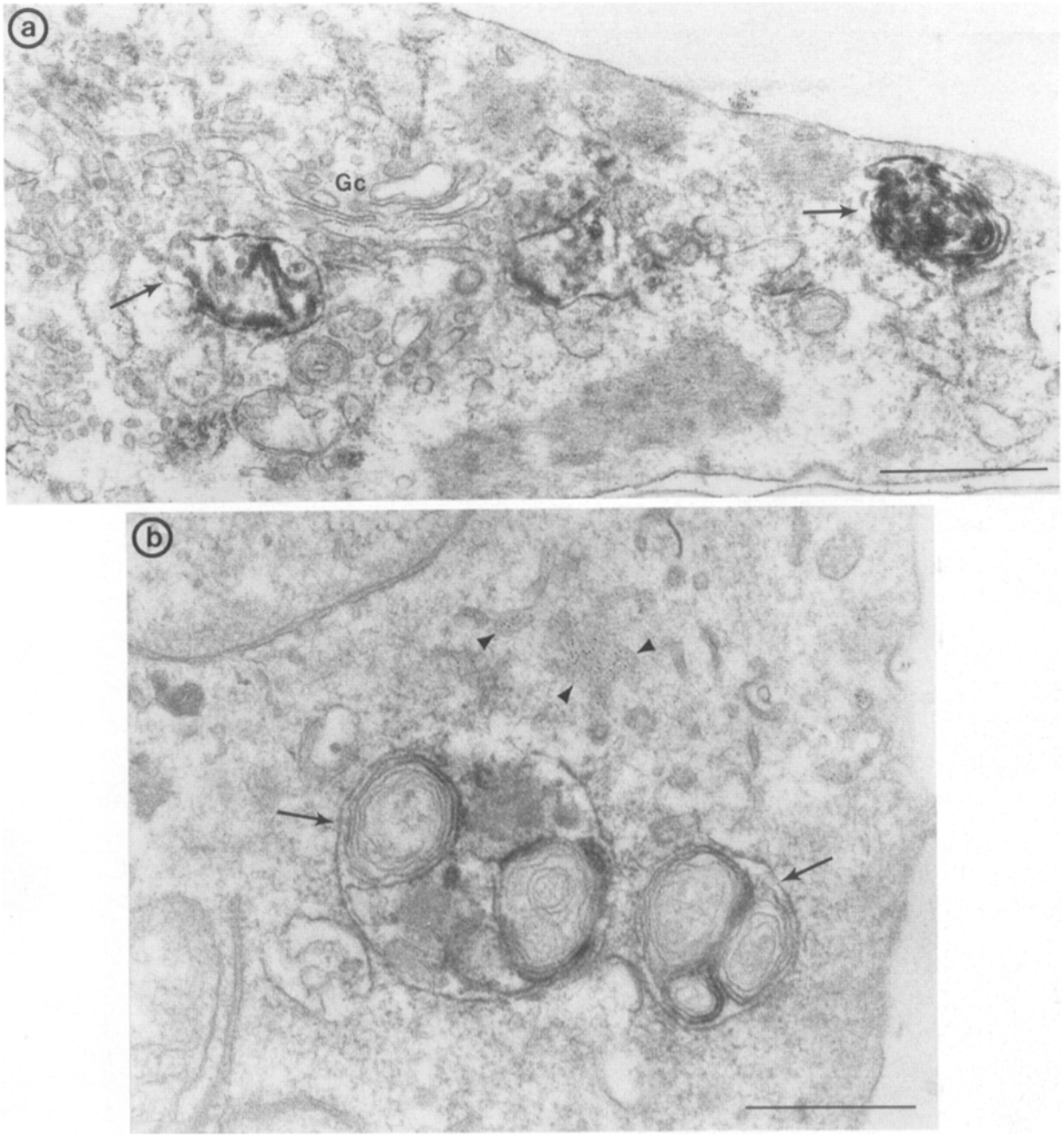


Figure 4. Immunoperoxidase localization of plgp57 with mAb 12B in MDBK (*a*) and BTRS (*b*) cells at the electron microscopic level. In both cell types, most of the organelles that contained DAB reaction product were generally located in the Golgi region and spherical in shape (0.3–1.0 μm in diameter). In most cases, these stained vacuoles also contained within their lumen numerous membrane vesicles or lamellae (*arrows*). The ER, nuclear envelope, Golgi complex (*Gc*), and plasma membrane were unstained by the antibody. (*b*) Delivery of endocytosed CF to the plgp57-enriched compartment is inhibited at 20°C. Cells were incubated with CF for 2 h at 20°C and then processed for IP staining with mAb 12B. Numerous plgp57-positive organelles were seen but they did not contain CF (*arrows*). The endosomes containing CF which were negative for plgp57 staining could be either MVBs or more tubular structures (*arrowheads*). Bars: (*a*) 0.5 μm; (*b*) 0.25 μm.

Figure 3. The plgp57-enriched compartment is distal to the 20°C block in endosome-to-lysosome pathway and also enriched in Man6P receptors. *A* and *B* show a cell that was incubated with LY 20°C for 2 h and then fixed for IF with mAb 12B. After uptake under these conditions there was little coincidental localization of LY (*A*) and mAb 12B (*B*) as each appeared to label separate populations of vesicles. However, when cells were pulse labeled at 20°C for 2 h followed by a chase in LY-free media at 37°C for just 13 min, there were numerous vesicles that contained LY (*C*) and stained with mAb 12B for plgp57 (*D*). The arrows point to several examples of vesicles that contain both markers.

When MDBK cells were double labeled with anti-plgp57 mAb (*E*) and anti-Man6P receptor polyclonal antibodies (*F*), numerous vesicles could be resolved which contained both proteins (*E* and *F, arrows*). Overall, there was a remarkable degree of coincidental staining with these two antibodies. In cells double labeled with anti-plgp57 mAb (*G*) and polyclonal antibodies against the LE α-glucosidase (*H*), only occasionally were the plgp57-enriched vesicles found to be weakly stained for α-glucosidase (*g* and *H, thin arrows*). Most of the α-glucosidase–enriched lysosomes, including several tubular lysosomes, did not appear to contain detectable amounts of plgp57 (*G* and *H, thick arrows*).

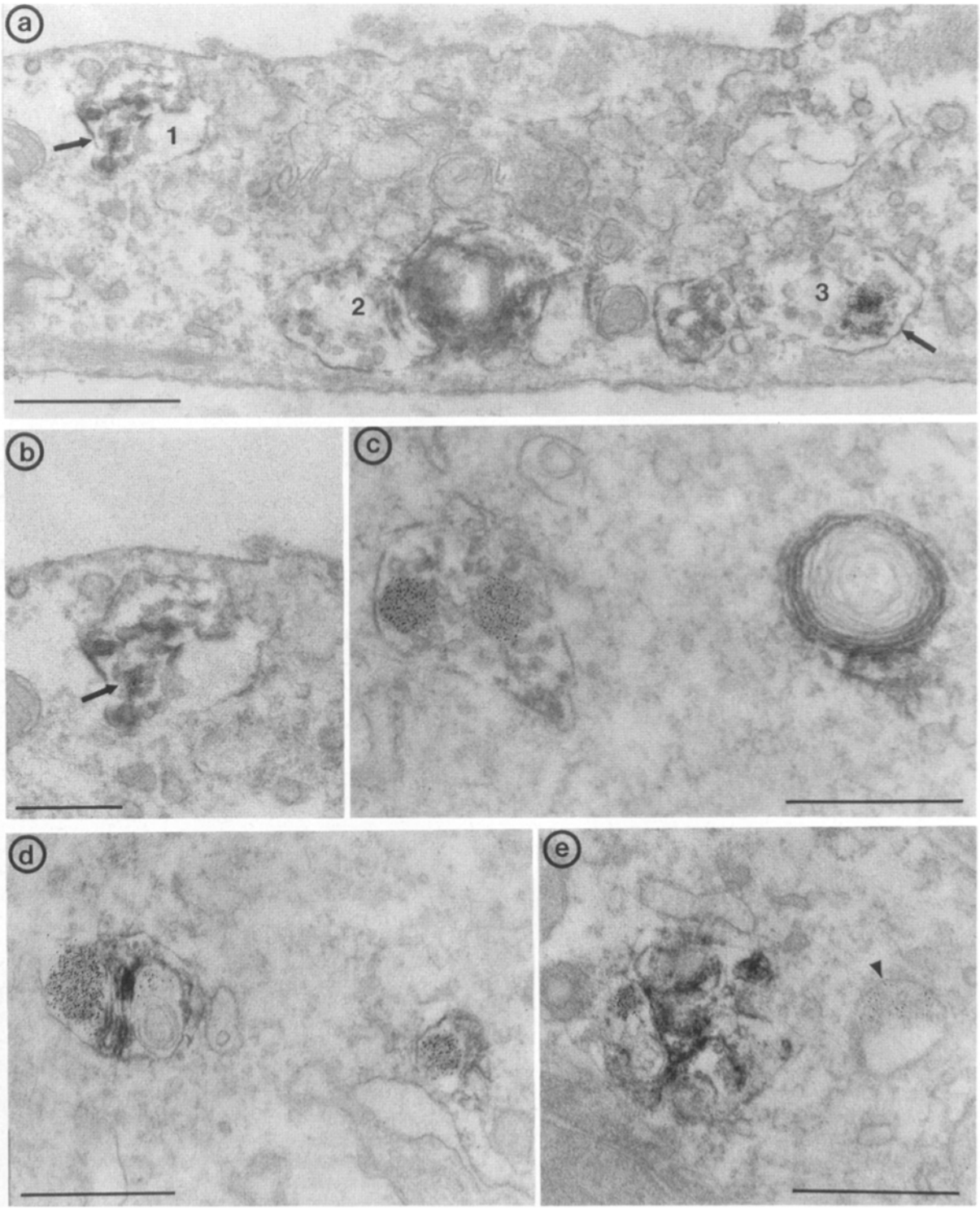


Figure 5. Delivery of endocytosed cationic ferritin (*CF*) to the plgp57-enriched, late endosomal compartment. BTRS cells were incubated with CF for 10 min followed by a 10-min chase CF-free media (both at 37°C) to label late endocytic compartments. *a* is a low power micrograph of such a treated cell showing three immunostained organelles (*1–3*). Two of these vacuoles (*1* and *3*) can be seen to also contain endocytosed CF (*arrows*). Organelle 1 is shown at higher magnification in *b* to better visualize the group of CF particles against the background of electron-dense DAB reaction product. In *c–e*, a survey of organelles containing CF and immunoreactive plgp57 is shown. These organelles can be either MLBs or MVBs (see *c* and *d*). In *e*, a CF-positive but plgp57-negative endosome can be seen (*arrowhead*). Bars: (*a*, *c–e*) 0.5 μm; (*b*) 0.25 μm.

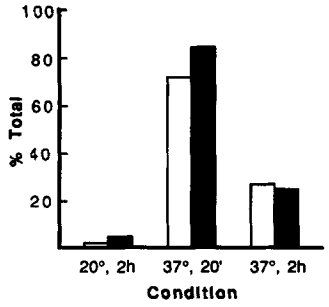


Figure 6. plgp57 is primarily found in late endosomes: quantitation from electron micrographs. To further resolve where plgp57 is located in the endosome-to-lysosome pathway, cells were incubated with CF under a variety of conditions and immunostained for plgp57. From electron micrographs, the number of organelles containing only CF (CF^+), only plgp57 ($plgp57^+$), or CF and plgp57 ($CF^+/plgp57^+$) were counted. The percentage of $CF^+/plgp57^+$ as a function of total $plgp57^+$ organelles (*open bars*) or total CF^+ organelles (*solid bars*) was plotted for each experimental condition. These conditions of CF uptake were: 20°C for 2 h to label early endosomes (*20°C, 2 h* on graph; 317 organelles counted); 10 min uptake followed by a 10-min chase both at 37°C to label late endosomes (*37°C, 20'* on graph; 315 organelles counted); uptake for 2 h followed by a 1-h chase both at 37°C to label lysosomes (*37°C, 3 h* on graph; 524 organelles counted).

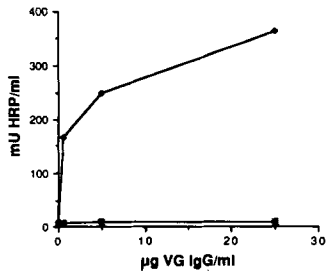


Figure 8. Very little plgp57 is found on the cell surface. To determine the relative amounts of plgp57 found intracellularly versus on the cell surface, MDBK cells were grown in 35-mm dishes, fixed with 3.7% formalin in PBS, washed in PBS, and treated (or not) with 0.05% saponin to permeabilize cells. Cells were then incubated with varying concentrations of anti-plgp57 MaB (*VG*) in culture media or culture media alone (as a negative control), washed, and incubated with a saturating concentration of goat anti-mouse IgG-HRP conjugate. Cells were washed, the bound antibodies were eluted with 100 mM citrate buffer, pH 2.7, containing 0.1% Triton X-100, and eluted HPR-conjugated antibody was quantitated by colorimetric assays using o-dianisidine as a substrate. The amount of background peroxidase activity obtained from the nonimmune control dishes was subtracted from each of the corresponding permeabilized and nonpermeabilized samples that were incubated with mAb VG to give the specific binding as shown. Permeabilized cells (◆–◆); nonpermeabilized cells (⊡–⊡).

Discussion

We have identified a membrane glycoprotein that displays a heretofore unique intracellular distribution within organelles of the endosome-lysosome apparatus. Based on the kinetics of endocytic tracer delivery to the plgp57-enriched organelles, we conclude that plgp57 was predominantly found in late endosomes. Moreover, this plgp57-enriched compartment was found to lie just distal to the 20°C block site in the endocytic pathway to lysosomes, and it was distinguished by a high concentration of CI Man6P receptors and low concentration of LEs. By cell fractionation, plgp57 was primarily found in light density organelles which comigrated with Man6P receptors and late endosomes; much lower amounts of plgp57 were found in dense secondary lysosomes. Taken together, these results show that plgp57 displays a unique intracellular distribution being primarily located in the Man6P receptor-enriched, prelysosomal compartment. To our knowledge, plgp57 is currently the most specific marker for this compartment. Moreover, these studies also provide independent evidence for the existence of a unique intracellular compartment, positioned between early endosomes and secondary lysosomes, that is intimately involved in the biogenesis of lysosomes.

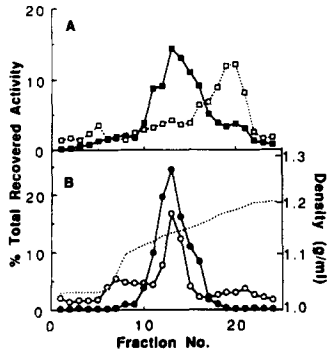


Figure 7. plgp57-enriched organelles primarily comigrate with the Man6P receptor and late endosomes, but not with dense secondary lysosomes on sucrose gradients. The majority of plgp57 (■–■) coincided with HRP-loaded, late endosomes (○–○) and CI Man6P receptors (●–●) in fractions 11-16. A second, smaller peak of plgp57 (variably 10-15% of the total recovered) was found to comigrate in denser fractions (numbers 17-20) along with the majority of lysosomal β-*N*-acetyl-glucosaminidase activity (□–□). Density (- - -). The amounts of activity or material recovered after gradient fractionation were: HRP, 90.3%; β-*N*-acetyl-glucosaminidase, 78.6%; plgp57, 86.7%; Man6P receptor, 77.2%.

The idea that newly synthesized LEs may first be delivered to a "prelysosomal" compartment after leaving the Golgi complex was initially suggested by the work of Sahagian and Neufeld (37) and Willingham et al. (44), and later others (8, 17) who discovered that mature lysosomes contained few, if any, Man6P receptors. Instead, the receptors were found in other unidentified, acidic vacuoles. From early studies, Sahagian (36) hypothesized that endosomes may serve as an intracellular delivery site for both the biosynthetic (Man6P receptor-mediated) and endocytic pathways to lysosomes. Brown et al. (8) obtained evidence for this hypothesis by showing that Man6P receptors cycled between the Golgi complex and a subpopulation of late endosomes. Recently, Griffiths et al. (21, 22) and Geuze et al. (18) further refined our understanding of this compartment by showing that it also contains small amounts of LEs (as predicted by the presence of Man6P receptors) and variable amounts of some lysosomal membrane proteins. The work of Griffiths and collaborators (21, 22), is also noteworthy because their immunocytochemical localization experiments showed that the Man6P receptor-enriched, "intermediate" compartment often had an unusual internal morphology consisting of "thin worm-like tubules" of membranes packed to high density. In addition, they also found that endocytosis at 20°C, a condition previously shown to inhibit the transport of material from endosomes to lysosomes (15), inhibited the transport of tracers to the Man6P receptor-enriched compartment (our unpublished results confirm this finding). Thus, all of the evidence is consistent with existence of a prelysosomal compartment in which endocytosed molecules and newly synthesized LEs become intermixed.

In our studies here, the plgp57-positive organelles were most frequently found to be exactly like those Man6P receptor-enriched vacuoles densely packed with membranes observed by Griffiths et al. (21, 22). The origin and significance of these luminal membranes is uncertain at present. They

have now been found to be hallmarks of prelysosomal endosomes in several cell types, and do not appear to be fixation artifacts (29, 41). In addition, plgp57 was also found within classic multivesicular bodies (MVBs), shown previously to be endosomes (16, 28, 31, 34). The distribution of plgp57 immunoreaction product within the multilamellar prelysosomes suggests that the antigen can be found throughout the luminal membranes, as is the Man6P receptor (21, 22), however, artifactual relocation of DAB reaction product due to diffusion cannot be ruled out in our studies. We have not been able to resolve this issue using immunogold methods due to poor labeling with the anti-plgp57 mAbs. Our studies also demonstrated that the plgp57-enriched compartment was located just distal to the 20°C block site in the endocytic pathway to lysosomes. Thus, the distribution of plgp57 is consistent with its being an endogenous marker of the prelysosomal, late endosome compartment.

A variety of experiments have provided evidence that endosomal membranes are not merely random samples of the plasma membrane, but instead contain a unique set of protein components (4, 12, 32). In addition, Schmid et al. (38) demonstrated that the membranes of early and late endosomes differed in protein content. Recently, membranes have been isolated which were enriched in Man6P receptors by immunoabsorption with antibodies to the cytoplasmic tails of the CI and CD Man6P receptors (30). Analysis of these membranes revealed a wide array of proteins, some located in the region of 57 kD, that appeared to be specifically coprecipitated by the antibodies. Because plgp57 is a diffuse band on gels, its presence in these membranes would probably go undetected due to the large number of other proteins in the region of 57 kD. So far, the only other well-characterized, endogenous marker for a compartment of the endosomal apparatus is a glycoprotein of 55–61 kD found in the intestinal absorptive cells of neonatal rat ileum (45). In contrast to plgp57, this 55–61-kD protein appears to be located in early endosomes and is developmentally restricted to fetal and neonatal intestinal cells, and thus it may perform a function specific to those cells. Thus, it is clear that different populations of endosomes are endowed with special compositional features, and the challenge will be to correlate these with the unique functional properties of the different types of endosomes.

The biochemical properties of plgp57 do not provide any insight into its function or other special properties of prelysosomal endosomes. plgp57 is somewhat similar to several well-characterized lysosomal membrane proteins and a protein called endolyn-78, in that all are found to some extent in prelysosomal endosomes and mature lysosomes (10, 18, 21). However, plgp57 is distinguished from these other proteins because the great majority of it is located in prelysosomal endosomes and only a small amount is in dense lysosomes (10–15% of the total). The relative proportions of several lysosomal membrane proteins and endolyn-78, when compared to plgp57, is essentially the opposite in these two compartments, as determined from cell fractionation experiments (10, 20). The reason for the small amount of plgp57 in mature lysosomes is not clear but cannot be accounted for by cross-contamination. It is possible that plgp57 primarily resides in prelysosomal endosomes, but that it also cycles to and from mature lysosomes; or, the lysosomal pool could represent that which is undergoing degradation. Preliminary studies on the half-life of plgp57 argue against the latter possibility (unpublished data). Moreover, it is worth noting that plgp57, like one of the well-characterized lysosomal membrane proteins, LEP100, is also found in small amounts on the cell surface (26). In addition, <5% of the total plgp57$^+$ organelles could be defined as early endosomes based on tracer uptake experiments at 20°C. All of these studies suggest that many membrane proteins of the endosome–lysosome system are continually moving between different compartments, however, at steady state their concentrations within specific sites may greatly vary.

How the composition of these endosomal membranes change enroute to lysosomes is not clear but must include the selective removal and addition of some proteins. For example, the transition from early to late endosome involves the removal of some internalized receptors by recycling back to the cell surface and the appearance of other proteins such as Man6P receptors and plgp57 (for discussion of functional differences between different types of endosomes see ref. 47). Then, during the transition from prelysosomal endosome to lysosome, the constituent membrane becomes depleted of plgp57 and Man6P receptors and enriched in lysosomal membrane proteins. How these transitions occur has not yet been determined. For example, is the prelysosomal endosome a biosynthetic intermediate which "matures" into a dense, secondary lysosome by filling the lumen with newly synthesized LEs and remodeling the membrane, or is it a more stable compartment whose contents are passed on to lysosomes either by vesicular traffic to, or direct fusion with, a preexisting lysosome? To date, no definitive experiment has been done to determine which of these models, the "maturation" and "vesicular" models, respectively, prevail in vivo (23, 33, 34a). The further study of plgp57 may enable us to better understand these mechanisms and the role played by prelysosomal endosomes in the formation of lysosomes.

The authors would like to thank Margot Szalay and Marian Strang for their excellent technical assistance with the EM, Lisa Heller for sharing immunoprecipitation data, and Dr. Anthony Bretscher for suggesting the immunofluorescence screening method.

This research was supported by Public Health Service Grant AM 37249 to W. J. Brown.

Received for publication 6 March 1990 and in revised form 2 October 1990.

References

1. Balch, W. E., and R. E. Rothman. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system. *Arch. Biochem. Biophys.* 240:413–425.
2. Deleted in proof.
3. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604–1607.
4. Bretscher, M. S., J. N. Thomson, and B. M. F. Pearse. 1980. Coated pits act as molecular filters. *Proc. Natl. Acad. Sci. USA.* 77:4156–4159.
5. Brown, W. J. 1990. Cation-independent mannose 6-phosphate receptors are concentrated in trans Golgi elements in normal human and I-cell disease fibroblasts. *Eur. J. Cell Biol.* 51:201–210.
6. Brown, W. J., and M. G. Farquhar. 1987. The distribution of 215-kilodalton mannose-6-phosphate receptors within cis (heavy) and trans (light) Golgi subfractions varies in different cell types. *Proc. Natl. Acad. Sci. USA.* 84:9001–9005.
7. Brown, W. J., and M. G. Farquhar. 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. *In* Methods in Cell Biology. Vol. 31, Part A. A. Tartakoff, editor. Academic Press, NY. 553–567.
8. Brown, W. J., J. Goodhouse, and M. G. Farquhar. 1986. Mannose-6-phos-

phate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. *J. Cell Biol.* 103:1235–1247.
9. Collet, M. S., and R. L. Ericson. 1978. Protein kinase activity associated with the avian sarcoma virus src gene product. *Proc. Natl. Acad. Sci. USA.* 75:2021–2024.
10. Croze, E., I. E. Ivanov, G. Kreibich, M. Adesnik, D. D. Sabatini, and M. G. Rosenfeld. 1989. Endolyn-78, a membrane glycoprotein present in morphologically diverse components of the endosomal and lysosomal compartments: implications for lysosome biogenesis. *J. Cell Biol.* 108:1579–1614.
11. de Duve, C. 1975. Exploring cells with a centrifuge. *Science (Wash. DC).* 189:186–194.
12. Dickson, R. B., L. Beguinot, J. A. Hanover, N. D. Richert, M. C. Willingham, and I. Pastan. 1983. Isolation and characterization of a highly enriched preparation of receptosomes (endosomes) from a human cell line. *Proc. Natl. Acad. Sci. USA.* 80:5335–5339.
13. Distler, J. J., and G. W. Jourdian. 1978. B-galactosidase from bovine testes. *Methods Enzymol.* 50:514–523.
14. Duncan, J. R., and S. Kornfeld. 1988. Intracellular movement of two mannose 6-phosphate receptors: return to the Golgi apparatus. *J. Cell Biol.* 106:617–628.
15. Dunn, W. A., A. L. Hubbard, and N. N. Aaronson. 1980. Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophay of ^{125}I asialofetuin by the perfused rat liver. *J. Biol. Chem.* 255:5971–5978.
16. Dunn, W. A., T. P. Connolly, and A. L. Hubbard. 1986. Receptor-mediated endocytosis of epidermal growth factor by rat hepatocytes: receptor pathway. *J. Cell Biol.* 102:24–36.
17. Geuze, H. J., J. W. Slot, G. J. A. M. Strous, A. Hasilik, and K. von Figura. 1985. Possible pathways for lysosomal enzyme delivery. *J. Cell Biol.* 101:2253–2262.
18. Geuze, H. J., W. Stoorvogel, G. J. Strous, J. W. Slot, J. Zijderhand-Bleekemolen, and I. Mellman. 1988. The sorting of mannose 6-phosphate receptors and lysosomal membrane proteins occurs in endocytic vesicles. *J. Cell Biol.* 107:2491–2502.
19. Gonzalez-Noriega, A., J. H. Grubb, V. Talkad, and W. S. Sly. 1980. Choloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J. Cell Biol.* 85:839–852.
20. Green, S. A., K. P. Zimmer, G. Griffiths, and I. Mellman. 1987. Kinetics of intracellular transport and sorting of lysosomal membrane and plasma membrane proteins. *J. Cell Biol.* 105:1227–1240.
21. Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell.* 52:329–341.
22. Griffiths, G., R. Matteoni, R. Back, and B. Hoflack. 1990. Characterization of the cation-independent mannose 6-phosphate receptor-enriched prelysosomal compartment. *J. Cell Sci.* 95:441–461.
23. Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1985. Endosomes. *TIBS (Trends Biochem. Sci.)* 8:245–250.
24. Kohler, G. 1981. The technique of hybridoma production. *Immunol. Methods.* 2:285–298.
25. Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483–525.
26. Lippincott-Schwartz, J., and D. M. Fambrough. 1986. Lysosomal membrane dynamics: structure and interorganellar movement of a major lysosomal membrane glycoprotein. *J. Cell Biol.* 102:1593–1605.
27. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:258–272.
28. Marsh, M., D. Bolzau, and A. Helenius. 1983. Penetration of Semliki forest virus from acidic prelysosomal vacuoles. *Cell.* 32:931–940.
29. McDowall, A., J. Gruenberg, K. Romisch, and G. Griffiths. 1989. The structure of organelles of the endocytic pathway in hydrated cryosections of cultured cells. *Eur. J. Cell Biol.* 49:281–294.
30. Messner, D. J., G. Griffiths, and S. Kornfeld. 1989. Isolation and characterization of membranes from bovine liver which are highly enriched in mannose 6-phosphate receptors. *J. Cell Biol.* 108:2149–2162.
31. Mueller, S. C., and A. L. Hubbard. 1986. Receptor-mediated endocytosis of asialoglycoproteins by rat hepatocytes: receptor-positive and receptor-negative endosomes. *J. Cell Biol.* 102:932–942.
32. Muller, W. A., R. A. Steinman, and Z. A. Cohn. 1983. Membrane proteins of the vacuolar system. III. Further studies on the composition and recycling of endocytic vacuole membrane in cultured macrophages. *J. Cell Biol.* 96:29–36.
33. Mullock, B. M., W. J. Branch, M. van Schaik, L. K. Gilbert, and J. P. Luzio. 1989. Reconstitution of an endosome-lysosome interaction in a cell-free system. *J. Cell Biol.* 108:2093–2100.
34. Neutra, M. R., A. Ciechanover, L. S. Owen, and H. F. Lodish. 1985. Intracellular transport of transferrin- and asialoorosomucoid-colloidal gold conjugates to lysosomes after receptor-mediated endocytosis. *J. Histochem. Cytochem.* 33:1134–1144.
34a. Roederer, M., J. R. Barry, R. B. Wilson, and R. F. Murphy. 1990. Endosomes can undergo an ATP-dependent density increase in the absence of dense lysosomes. *Eur. J. Cell Biol.* 51:229–234.
35. Rome, L. H., A. J. Garvin, M. M. Allietta, and E. F. Neufeld. 1979. Two species of lysosomal organelles in cultured human fibroblasts. *Cell.* 17:143–153.
36. Sahagian, G. G. 1984. The mannose 6-phosphate receptor; function, biosynthesis, and translocation. *Biol. Cell.* 51:207–214.
37. Sahagian, G. G., and E. F. Neufeld. 1983. Biosynthesis and turnover of the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA.* 258:7121–7128.
38. Schmid, S. L., R. Fuchs, P. Male, and I. Mellman. 1988. Two distinct subpopulations of endosomes involved in membrane recycling and transport of lysosomes. *Cell.* 52:73–83.
39. Small, G. M., T. Imanaka, and P. B. Lazarow. 1988. Immunoblotting of hydrophobic integral membrane proteins. *Anal. Biochem.* 169:405–409.
40. Swanson, J. A., B. D. Yirinec, and S. C. Silverstein. 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. *J. Cell Biol.* 100:851–859.
41. Tooze, J., M. Hollinshead, T. Ludwig, K. Howell, B. Hoflack, and H. Kern. 1990. In exocrine pancreas the basolateral endocytic pathway converges with the autophagic pathway immediately after the early endosomes. *J. Cell Biol.* 111:329–345.
42. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
43. von Figura, K., and A. Hasilik. 1986. Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* 55:167–193.
44. Willingham, M. C., I. H. Pastan, and G. G. Sahagian. 1983. Ultrastructural immunocytochemical localization of the phosphomannosyl receptor in Chinese hamster ovary (CHO) cells. *J. Histochem Cytochem.* 13:1–11.
45. Wilson, J. M., J. A. Whitney, and M. R. Neutra. 1987. Identification of an endosomal antigen specific to absorptive cells of suckling rat ileum. *J. Cell Biol.* 105:691–703.
46. Worthington. 1988. Worthington Enzyme Manual. Worthington Biochemical Corp., Freehold, N.J. 254–259.
47. Yamashiro, D. J., and F. R. Maxfield. 1987. Acidification of morphologically distinct endosomes in mutant and wild-type Chinese hamster ovary cells. *J. Cell Biol.* 105:2723–2733.