

# Mannose-6-Phosphate Receptors for Lysosomal Enzymes Cycle between the Golgi Complex and Endosomes

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**Abstract.** We have examined the distribution of mannose-6-phosphate (Man6P) receptors (215 kD) for lysosomal enzymes in cultured Clone 9 hepatocytes at various times after the addition or removal of lysosomotropic weak bases (chloroquine or  $\text{NH}_4\text{Cl}$ ). Our previous studies demonstrated that after treatment with these agents, Man6P receptors are depleted from their sorting site in the Golgi complex and accumulate in dilated vacuoles that could represent either endosomes or lysosomes (Brown, W. J., E. Constantinescu, and M. G. Farquhar, 1984, *J. Cell Biol.*, 99:320–326). We have now investigated the nature of these vacuoles by labeling  $\text{NH}_4\text{Cl}$ -treated cells simultaneously with anti-Man6P receptor IgG and lysosomal or endosomal markers. The structures in which the immunolabeled receptors are found were identified as endosomes based on the presence of endocytic tracers (lucifer yellow and cationized ferritin). The lysosomal membrane marker, lgpl20, was associated with a separate population of swollen vacuoles that did not contain detectable Man6P receptors. When cells were allowed to recover from weak base treatment, the receptors

reappeared in the Golgi cisternae of most cells ( $\sim 90\%$ ) within  $\sim 20$  min, indicating that as the intracellular pH drops and lysosomal enzymes dissociate, the entire population of receptors rapidly recycles to Golgi cisternae. When  $\text{NH}_4\text{Cl}$ -treated cells were allowed to endocytose Man6P, a competitive inhibitor of lysosomal enzyme binding, the receptors also recycled to the Golgi cisternae, suggesting that lysosomal enzymes can dissociate from the receptors under these conditions (high pH + presence of competitive inhibitor). From these results it can be concluded that (a) the intracellular itinerary of the 215-kD Man6P receptor involves its cycling via coated vesicles between the Golgi complex and endosomes, (b) ligand dissociation is both necessary and sufficient to trigger the recycling of Man6P receptors to the Golgi complex, and (c) endosomes rather than secondary lysosomes represent the junction where endocytosed material and primary lysosomes carrying receptor-bound lysosomal enzymes meet. The implications of these findings for the biogenesis of secondary lysosomes are discussed.

**M**ANNOSE-6-PHOSPHATE (Man6P)<sup>1</sup> receptors (215 kD) are known to function in the targeting and transport of newly synthesized lysosomal enzymes (for reviews see references 11 and 43). To identify the transport pathway followed by the 215-kD Man6P receptor, we previously localized these receptors in a variety of cell types by immunocytochemistry (4, 5, 7) and showed that they are concentrated in the Golgi complex, in small vesicles (often with clathrin-like coats), and in larger vacuoles. These organelles were assumed to represent the sorting site, the carrier, and the delivery site, respectively, for lysosomal enzymes. We also reported that treatment of cells for 3 h with lysosomotropic weak bases (chloroquine or  $\text{NH}_4\text{Cl}$ ) causes depletion of Man6P receptors from the sorting site in the Golgi complex and their accumulation at the delivery site in

large, dilated vacuoles (7). The identity of the vacuoles was not determined, but they were assumed to represent lysosomes, endosomes, or a mixture of the two. The ability to trap the receptors at the delivery site and the fact that the effects of treatment with lysosomotropic weak bases are reversible enabled us to investigate the following questions: (a) Is the delivery site for 215-kD Man6P receptors an endosome, a lysosome, or both? (b) Do these receptors recycle back to the sorting site in the Golgi complex after removal of lysosomotropic agents? and, (c) If recycling does occur, what triggers this event? We present here evidence on all three of these questions.

## Materials and Methods

### Materials

Rat Clone 9 hepatocytes were obtained from Dr. David Sabatini, New York University. Chloroquine diphosphate,  $\text{NH}_4\text{Cl}$ , cycloheximide, lucifer yel-

1. *Abbreviations used in this paper:* Glu6P, glucose-6-phosphate; IF, immunofluorescence; IP, immunoperoxidase; Man1P, mannose-1-phosphate; Man6P, mannose-6-phosphate; MEM/FCS, Eagle's minimum essential medium/fetal calf serum.

low, Man6P, and diaminobenzidine were purchased from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium (Earle's salts) and fetal calf serum were from Gibco (Grand Island, NY). Fluorescein- or rhodamine-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG were from Cooper Biomedical, Inc. (Malvern, PA) and Fab fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase were from Biosys (Compiègne, France). Cationized ferritin ( $pI \geq 8.5$ ) was from Miles Scientific Div., Miles Laboratories Inc. (Naperville, IL).

### Antibodies

Rabbit polyclonal anti-Man6P receptor IgG was characterized previously (4). It was shown to recognize by immunoprecipitation (4) and by immunoblotting (unpublished data) only the 215-kD Man6P receptor. Monoclonal anti-Igpl20 was kindly provided by Dr. Ira Mellman (Yale University). It was shown to recognize a 120-kD glycoprotein by immunoprecipitation (33) and to stain membranes of lysosomes but not endosomes in baby hamster kidney (33) and rat kidney proximal tubule (39) cells.

### Cell Culture

Clone 9 cells were grown in minimal essential medium (Earle's salts) with 10% fetal calf serum (MEM/FCS) in an atmosphere of 95% air, 5% CO<sub>2</sub>. Cells were plated onto glass coverslips for immunofluorescence (IF) or 35-mm plastic petri dishes for immunoperoxidase (IP) staining and were cultured for 3–4 d.

### Incubation with Lysosomotropic Weak Bases

Clone 9 cells were incubated with chloroquine (25  $\mu$ M) or NH<sub>4</sub>Cl (10 or 50 mM) in MEM/FCS for 5, 10, 15, 30, 60, 120, or 180 min at 37°C. These agents, which cause a rise in the internal pH and vacuolization of endosomes and lysosomes (20, 34, 37, 38), lead to the accumulation of Man6P receptors in large, dilated vacuoles and their depletion from the Golgi complex (7). For electron microscopy, cells were treated with 10 mM NH<sub>4</sub>Cl, whereas for light microscopy, they were treated with 50 mM NH<sub>4</sub>Cl to generate larger vacuoles that were easier to visualize. The cells were then either fixed for immunocytochemistry or subjected to further experimental manipulations.

### Recovery Experiments

Cells that had been treated with chloroquine or NH<sub>4</sub>Cl for at least 180 min as described above were quickly washed five times (total time 2–3 min) with MEM/FCS at 37°C and incubated in weak base-free media for 5 min to 6 h at 37°C. During this washout or recovery period, lysosomes and endosomes rapidly re-acidify (20, 34, 37). Cells were then fixed and processed for IF or IP as necessary.

In some experiments, cycloheximide (2  $\mu$ g/ml final concentration) was added to cells that had been incubated with MEM/FCS plus NH<sub>4</sub>Cl for 3 h, and the incubation was continued for another 30 min. They were then washed free of weak bases, incubated with MEM/FCS plus cycloheximide for up to 2 h, and fixed for IF or IP.

### Labeling of Endosomes for Light and Electron Microscopy

Endosomes were labeled by allowing them to take up the fluorescent marker lucifer yellow or the electron-dense marker cationized ferritin. Clone 9 cells that had been treated with weak bases as described above were incubated with either lucifer yellow (10–15 mg/ml in MEM/FCS) or cationized ferritin (50  $\mu$ g/ml), for 15 or 30 min at 37°C in the continuous presence of either chloroquine or NH<sub>4</sub>Cl. Cells were then fixed and processed for IF or IP, respectively. Lucifer yellow has been shown to be an excellent, fluorescent fluid-phase marker which is taken up sequentially into endosomes (at 5–15 min) and lysosomes (at 20–30 min) (46). It did not bind appreciably to cell membranes and was not toxic at the concentrations used.

### Incubation of NH<sub>4</sub>Cl-treated Cells with Man6P

Cells treated with MEM/FCS containing NH<sub>4</sub>Cl for  $\geq 3$  h were incubated in the same media containing either 50 mM Man6P, mannose-1-phosphate (Man1P), or glucose-6-phosphate (Glu6P) for up to 3 h at 37°C. Man6P is known to competitively inhibit lysosomal enzyme binding to Man6P receptors, whereas Man1P and Glu6P do not (43). Cells were then fixed and processed for IF or IP.

### Immunofluorescence

Clone 9 cells were fixed on coverslips in 3.7% formalin in PBS for 45 min at room temperature and permeabilized with 0.05% saponin in PBS. Cells were then incubated for 1 h either with anti-receptor IgG (50  $\mu$ g/ml) followed by rhodamine-labeled goat anti-rabbit IgG (diluted 1:50) for 1 h, or anti-Igpl20 (ascites fluid, diluted 1:100) followed by fluorescein-labeled goat anti-mouse IgG (1:50) for 1 h. Cells were doubly labeled by incubating them sequentially with anti-receptor IgG, anti-Igpl20, rhodamine-labeled anti-rabbit IgG, and fluorescein-labeled anti-mouse IgG.

In the case of cells that had been incubated with lucifer yellow before fixation, rhodamine-labeled anti-mouse IgG was used to detect anti-Igpl20. Coverslips were mounted in *p*-phenylenediamine to reduce fading (31) and photographed in a Zeiss Photomicroscope III equipped with epifluorescence illumination and appropriate barrier filters to prevent overlap of the two fluorescent signals.

### Immunoperoxidase

Cells cultured in petri dishes were fixed in McLean and Nakane's fixative and permeabilized with 0.005% saponin as previously described (7). They were then incubated for 1 h or overnight in either anti-receptor IgG (50  $\mu$ g/ml) or anti-Igpl20 IgG (as above) followed by Fab fragments of either sheep anti-rabbit or anti-mouse IgG (1:50 dilution) for 1 h, after which they were then fixed in glutaraldehyde, incubated in diaminobenzidine medium, and processed as previously described (5). Ultrathin sections were stained with lead citrate and examined at 60 kV in either a Philips 301 or 410 electron microscope.

### Quantitation of Man6P Receptor Distribution

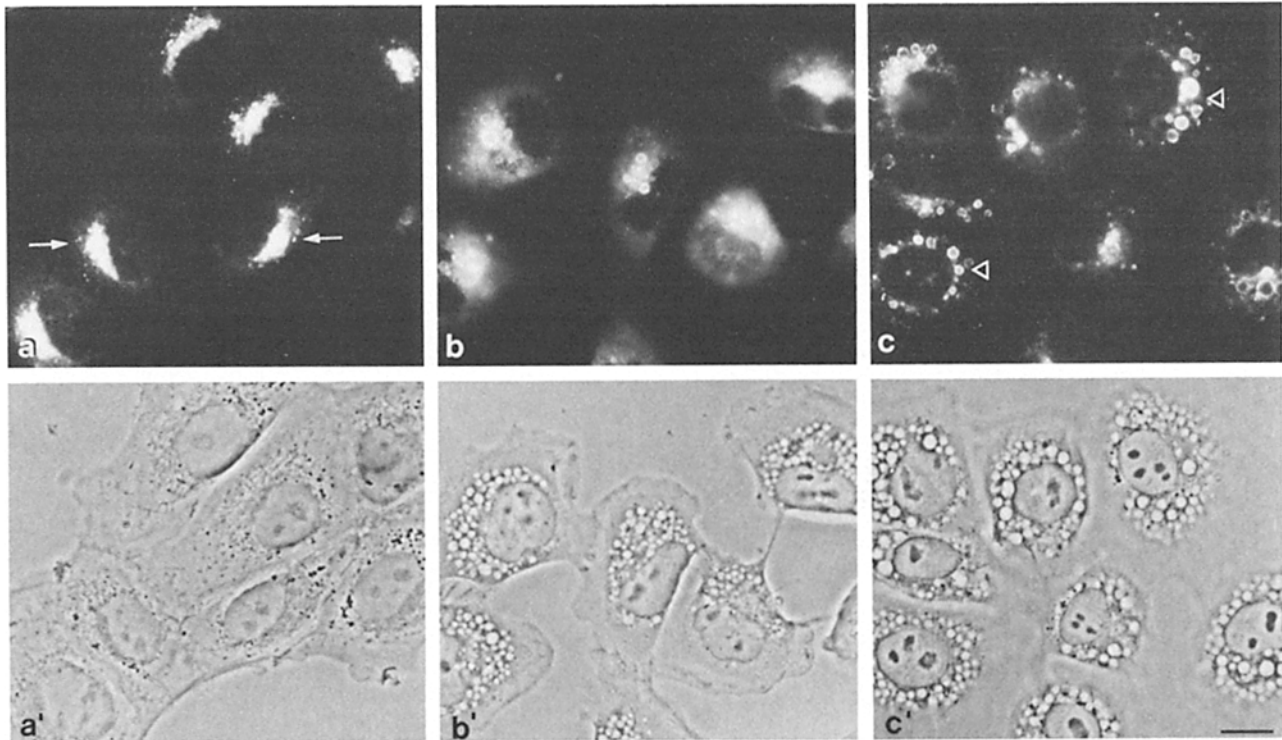
The number of cells in which receptors were concentrated in the juxtanuclear, Golgi region versus those in which receptors were concentrated in large, dilated vacuoles were counted at various times after NH<sub>4</sub>Cl treatment or its removal. Cells were scored as having either a "Golgi"- or "vacuole"-type staining pattern in IF preparations stained for Man6P receptors. At least 200 cells were scored at each time point in four to seven separate experiments.

### Results

#### Man6P Receptors Are Trapped in Endosomes by Treatment with Lysosomotropic Weak Bases

As was previously shown (4), in control (untreated) Clone 9 hepatocytes, by IF 215-kD Man6P receptors are typically concentrated in the Golgi region (Fig. 1 *a*), where they can be localized by IP at the electron microscopic level in one or two Golgi cisternae, in associated vesicles, and in larger vacuoles (Fig. 2, *a* and *b*). After treatment with lysosomotropic weak bases, they are depleted from Golgi cisternae and accumulate in large, dilated vacuoles (Figs. 1, *b* and *c* and 2 *c*). To determine the identity of the dilated vacuoles where the receptors accumulate, we performed double-labeling by IF and compared the distribution of the receptor to that of a lysosomal marker (Igpl20) and an endosomal marker (lucifer yellow) in chloroquine- or NH<sub>4</sub>Cl-treated cells.

In treated cells, which had been incubated for 15 min with lucifer yellow (to allow its uptake into endosomes) and then fixed and stained with anti-Igpl20 by indirect IF, there was staining of large vacuoles with both markers, but there was little or no overlap between lucifer yellow-labeled endosomes and the anti-Igpl20 stained lysosomes (Fig. 3, *a* and *b*). However, in cells incubated for 30 min in lucifer yellow, there was some overlap between the two signals (not shown). This demonstrates that in Clone 9 cells, as in macrophages (46), lucifer yellow is a valid endosomal marker at 15 min after its uptake, and that both endosomes and lysosomes become vacuolated after treatment with lysosomotropic weak



**Figure 1.** Immunofluorescence localization of Man6P receptors in Clone 9 hepatocytes after the addition of  $\text{NH}_4\text{Cl}$ . Pairs of micrographs are shown of the same field by fluorescence (*a-c*) or phase-contrast (*a'-c'*) microscopy. Control, untreated cells (*a* and *a'*). By phase contrast an increase in the size and number of phase-lucent vacuoles is evident, and by immunofluorescence a change in the distribution of Man6P receptors is seen at 2 h (*b* and *b'*) or 3 h (*c* and *c'*) after addition of  $\text{NH}_4\text{Cl}$ . There is a change from a juxtannuclear "Golgi" staining pattern in controls (*arrows* in *a*) to a "vacuolar" (endosome) staining pattern (*arrowheads* in *c*). Note that only some of the dilated vacuoles contain detectable receptors. Bar, 10  $\mu\text{m}$ .

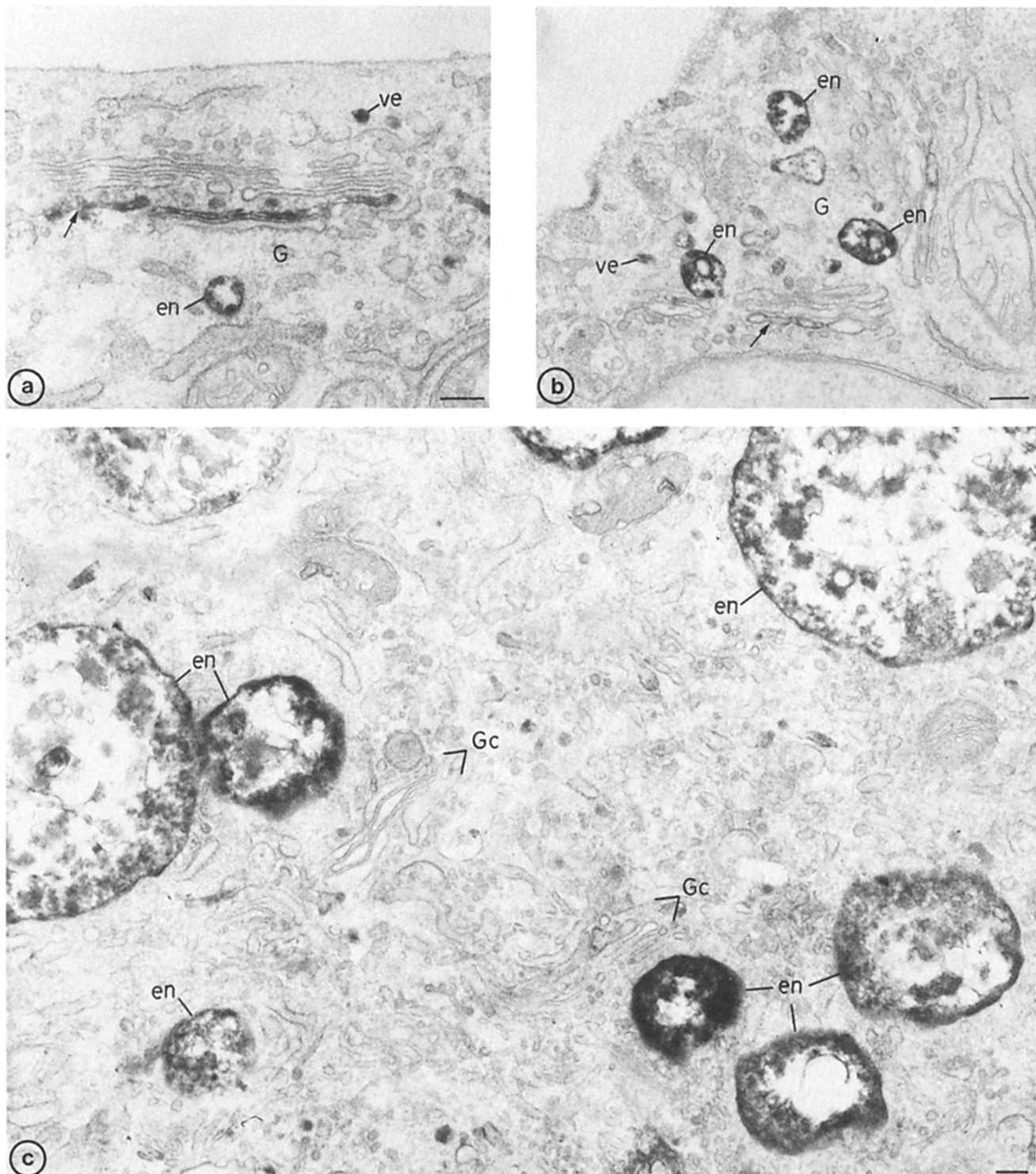
bases. In cells doubly labeled by indirect IF for Man6P receptors and Igpl20, the staining patterns clearly did not coincide: some of the numerous large vacuoles contained receptors, and others contained the lysosomal marker (Fig. 3, *d* and *e*). When  $\text{NH}_4\text{Cl}$ -treated cells were allowed to internalize lucifer yellow for 15 min and then stained for Man6P receptors, the patterns of staining for the endocytic marker and the receptors clearly overlapped (Fig. 3, *g* and *h*). The results show that staining for Man6P receptors and the endosomal marker, lucifer yellow, are nearly identical, whereas staining for the receptor and the lysosomal marker, Igpl20, are quite different. We conclude that the majority of immunodetectable Man6P receptors become trapped in endosomes, rather than in lysosomes after incubation with lysosomotropic weak bases, and accordingly, endosomes, not lysosomes, represent the delivery site for newly synthesized lysosomal enzymes transported via the 215-kD receptor in  $\text{NH}_4\text{Cl}$ -treated Clone 9 cells.

The presence of Man6P receptors and an endocytic marker in the same vacuole was verified by electron microscopy in  $\text{NH}_4\text{Cl}$ -treated cells allowed to take up cationized ferritin for 15 min. Cationized ferritin binds to the cell surface and is taken up by endocytosis in coated or smooth vesicles and is delivered to endosomes (Fig. 4, *a-c*). When such cells were stained for Man6P receptors by IP, the cationized ferritin and diaminobenzidine reaction product were regularly detected in the same dilated vacuoles (Fig. 4, *a-c*). The morphology of the dilated endosomes containing both trapped receptors and cationized ferritin was variable, but typically

they appeared electron lucent with internal vesicles and thus could be classified as multivesicular endosomes (Fig. 4, *a-d*).

#### ***Man6P Receptors Recycle from Endosomes to the Golgi Complex after $\text{NH}_4\text{Cl}$ Removal***

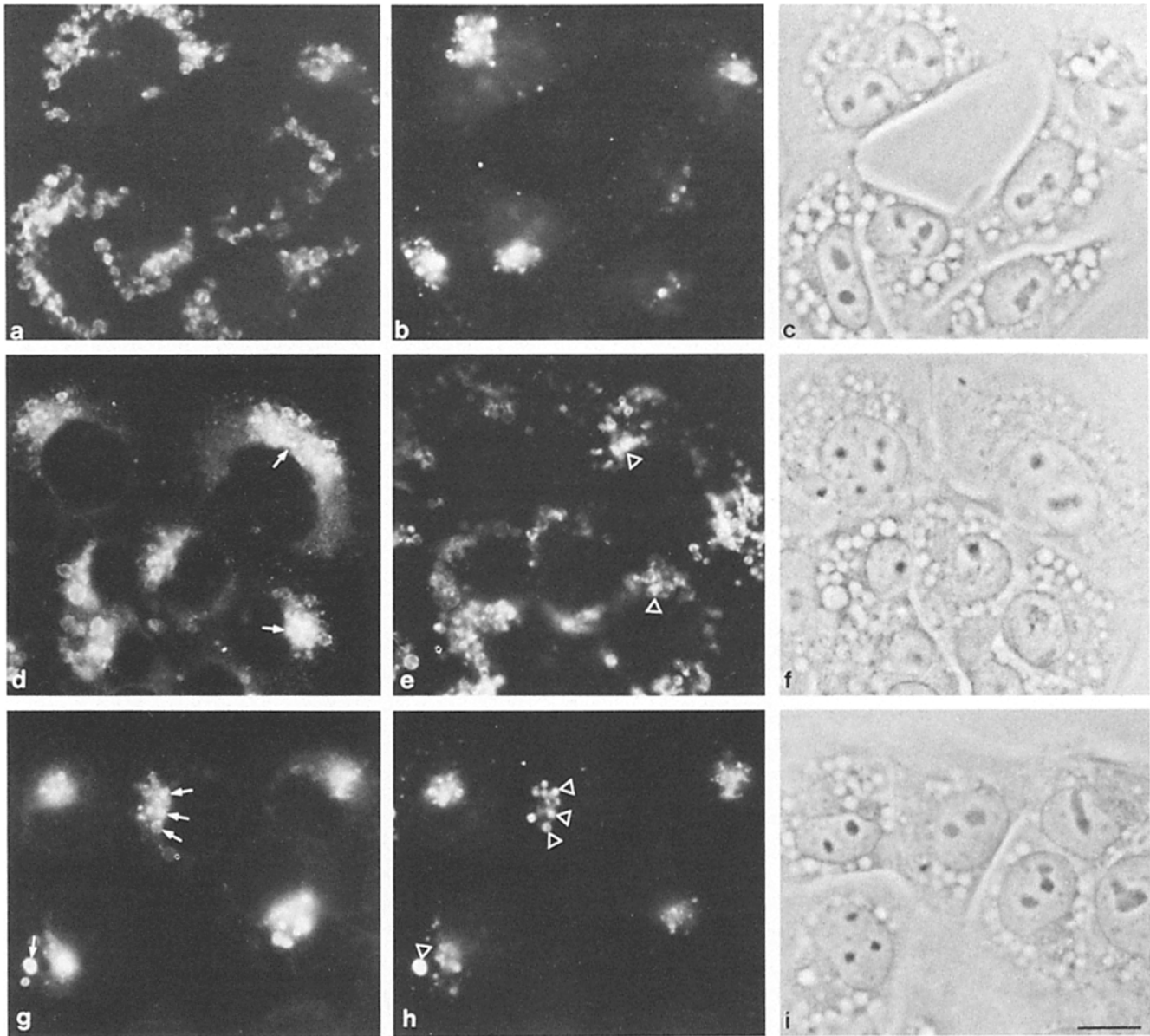
To determine the fate of Man6P receptors trapped in endosomes upon recovery from  $\text{NH}_4\text{Cl}$  treatment, their distribution was determined in cells transferred to fresh ( $\text{NH}_4\text{Cl}$ -free) media. By phase-contrast microscopy (Fig. 5, *a'-c'*), it was apparent that there was a progressive diminution in the size of the large dilated vacuoles (endosomes plus lysosomes) over a period of 1–2 h. The decrease in size was first evident by  $\sim 30$  min (Fig. 5 *c'*), but for the cell to completely return to normal,  $\sim 2$  h was required (not shown). However, by IF, receptors could already be detected in the juxtannuclear (Golgi) region of some cells as early as 5 min after washout (Fig. 5 *a*), and after 15–30 min (Fig. 5, *b* and *c*), the pattern of staining in most cells was indistinguishable from that of untreated cells (see Fig. 1 *a*). In virtually every cell, receptors were clustered in the Golgi region and were not detectable in the remaining dilated, cytoplasmic vacuoles. The pattern of staining remained unchanged in cells allowed to recover for up to 6 h. The results were the same whether or not cycloheximide was included in the washout media. The findings indicate that although endosomes and lysosomes remain dilated up to 2 h after  $\text{NH}_4\text{Cl}$  removal, the receptors rapidly recycle to the Golgi region, and this recycling occurs in the absence of protein synthesis.



**Figure 2.** Immunoperoxidase localization of Man6P receptors in control (a and b) and chloroquine-treated (c) Clone 9 hepatocytes. In control cells, the receptors are found in a single stacked Golgi cisterna (arrows), in small vesicles (ve), and in endocytic vacuoles (en) concentrated in the Golgi region. After chloroquine treatment, the receptors are found exclusively in the large, dilated vacuoles and are depleted from Golgi cisternae (Gc). Often smaller vesicles are seen in the lumen of some of the dilated multivesicular bodies (Fig. 3 c). Bars, 0.2  $\mu$ m.

The nature of the compartments in the Golgi region to which the recycling Man6P receptors are delivered was checked by electron microscopy. By 5 min after removal of  $\text{NH}_4\text{Cl}$ , immunoreactive receptors could already be detected in their normal locations in the stacked Golgi cisternae

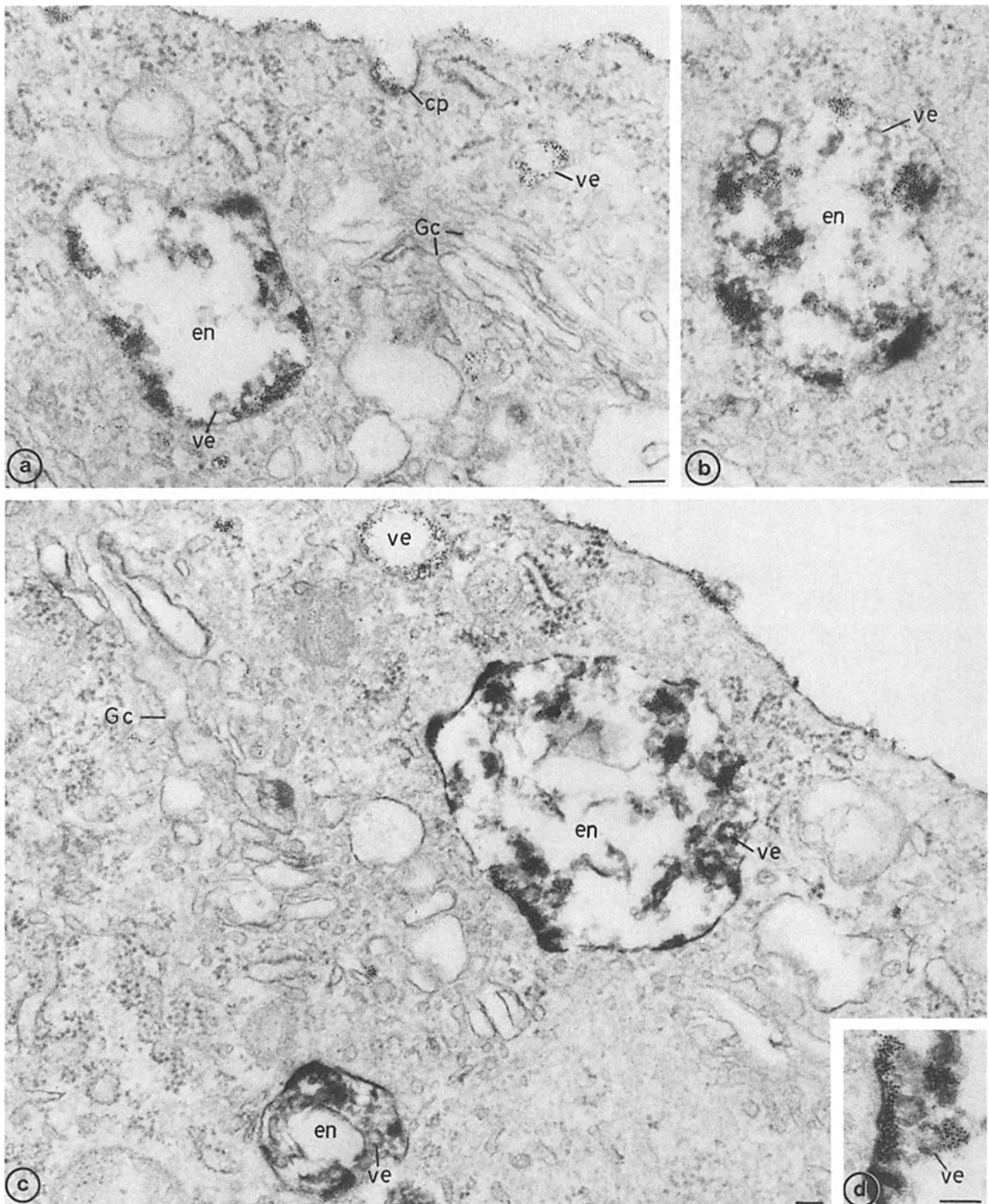
and associated vesicles of many cells (Fig. 6 a). As in controls, reaction product was largely restricted to several cisternae on one side, identified as *cis* Golgi elements in these cells by analysis of Golgi subfractions (6) and immunoelectron microscopy (7). Labeling was more extensive, however, than



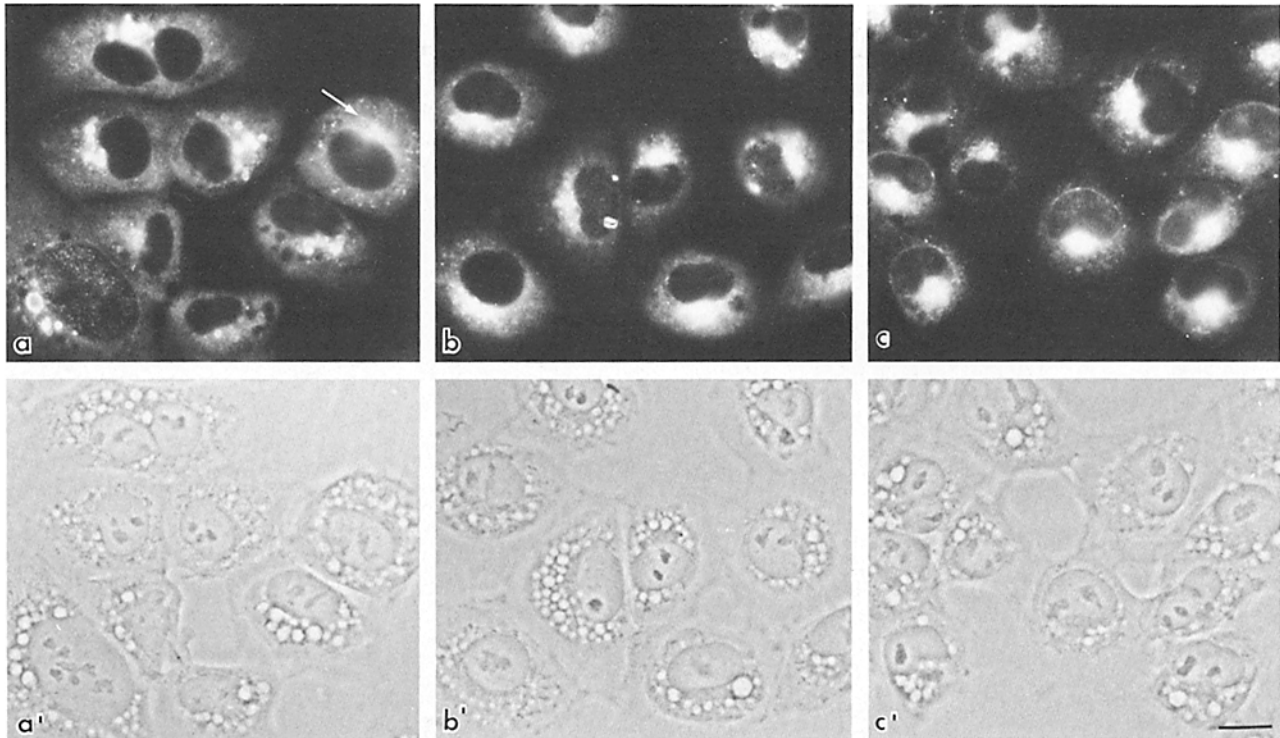
**Figure 3.** Fluorescence double-labeling of vacuolated,  $\text{NH}_4\text{Cl}$ -treated (3 h) Clone 9 cells with rabbit polyclonal anti-Man6P receptor antibodies and either with mouse monoclonal anti-Igp120, a lysosomal membrane marker, or lucifer yellow, an endosomal marker, as described in the Materials and Methods. In each case, the right panel shows the phase contrast and the two left panels the fluorescence micrographs of the same group of cells. (a-c) Cells that had been allowed to take up the fluorescent dye lucifer yellow for 15 min after which they were fixed and labeled by indirect immunofluorescence with anti-Igp120 and rhodamine-labeled anti-mouse IgG. There is very little or no overlap between the distribution of the lysosomal marker, Igp120 (a), and the endosomal marker, lucifer yellow (b). Lysosomes are typically located more peripherally, whereas endosomes are located in the juxtannuclear Golgi region. (d-f) Cells fixed and doubly immunolabeled with anti-Man6P receptor IgG detected with rhodamine-conjugated anti-rabbit IgG (d), and anti-Igp120 IgG detected with fluorescein-conjugated anti-mouse IgG (e). The labeling patterns of the two markers are very different: receptors are found in a population of swollen vacuoles (arrows) that differs from that of lysosomes labeled by Igp120 (arrowheads). (g-i) Cells allowed to endocytose lucifer yellow for 15 min after which they were fixed and stained by indirect immunofluorescence with anti-Man6P receptor IgG detected with rhodamine-labeled anti-rabbit IgG. The overall labeling patterns are nearly identical; both Man6P receptors (g) and endosomes (h) are concentrated in the juxtannuclear region. Many of the cytoplasmic vacuoles contain both receptors (arrows) and lucifer yellow (arrowheads), demonstrating that Man6P receptors are trapped in the endosomes of  $\text{NH}_4\text{Cl}$ -treated cells. Bar, 10  $\mu\text{m}$ .

in controls because typically more than one (rather than one) cisterna was labeled. The distribution of receptors within Golgi elements remained the same throughout the course of the recovery period (Figs. 6, b and c). During the same period, endosomal staining decreased until by  $\sim 1$  h after washout, little or no diaminobenzidine reaction product was

seen in the few remaining larger vacuoles or in the more numerous remaining smaller vacuoles (Fig. 6, b and c). Note that at early time points (5 min), receptors were frequently detected in coated invaginations, apparently captured in the process of budding from endosomes, and in small vesicles, some with clathrin-like coats, located throughout the cyto-



**Figure 4.** Co-localization of Man6P receptors and the endocytic tracer, cationized ferritin, in the dilated endosomes of  $\text{NH}_4\text{Cl}$ -treated Clone 9 cells. Portions of three cells (*a-c*) treated with  $\text{NH}_4\text{Cl}$ , incubated with cationized ferritin for 15 min, and then fixed and processed to visualize Man6P receptors by indirect immunoperoxidase. In all three fields both ferritin molecules and DAB reaction product are found in large, dilated endosomes (*en*). A portion of one of the vacuoles in *c* is enlarged (*d*). Typically, these doubly labeled endosomes are of the multivesicular type because they contain internal vesicles (*ve*). Ferritin is also seen in a coated pit (*cp*) at the cell surface and in endocytic vesicles (*ve*) which do not contain immunodetectable receptors. Note that immunoreactive receptors are depleted from Golgi cisternae (*Gc*). Bars, 0.1  $\mu\text{m}$ .



**Figure 5.** Redistribution of Man6P receptors at various intervals after removing  $\text{NH}_4\text{Cl}$ . Cells were treated for 3 h with  $\text{NH}_4\text{Cl}$ , after which they were incubated in  $\text{NH}_4\text{Cl}$ -free medium and then fixed and labeled with anti-receptor IgG detected with rhodamine-conjugated anti-rabbit IgG. By 5 min after removing  $\text{NH}_4\text{Cl}$  (*a* and *a'*), many cells have a "Golgi" staining pattern (*arrow*), indicating receptors have already recycled back to the Golgi region. By 15 min, a greater number of cells showed a Golgi staining pattern (*b* and *b'*) and by 30 min (*c* and *c'*) the pattern of staining was indistinguishable from untreated cells (Fig. 1 *a*). However, note that the cells remained vacuolated up to 30 min (*a'*-*c'*). Bar, 10  $\mu\text{m}$ .

plasm (*inset*, Fig. 6 *a*). These observations demonstrate that upon removal of  $\text{NH}_4\text{Cl}$ , the receptors return to the stacked Golgi cisternae and suggest that their return is via coated vesicles.

#### **Depletion of Man6P Receptors from the Golgi Complex after $\text{NH}_4\text{Cl}$ Treatment Is Slow**

To determine the time required for their depletion from the Golgi complex and delivery to endosomes, the distribution of Man6P receptors was followed with time after  $\text{NH}_4\text{Cl}$  addition by determining the percentage of cells with primarily a "Golgi" signal versus those with primarily a "vacuole" (endosome) signal (Fig. 7). The results demonstrate that there is a lag phase of  $\sim 30$  min, after which there is a gradual increase in the number of "vacuole"-stained cells. The conversion of the cell population from a "Golgi"- to an endosome-type staining pattern was slow ( $t_{1/2} = 75$  min):  $>90\%$  of the cells still showed a "Golgi" staining pattern 1 h after addition of  $\text{NH}_4\text{Cl}$ . After 2 h,  $\sim 35\%$  of the cells still showed this pattern, but by 3 h few, if any, did. By this time receptors were mainly associated with dilated endosomes (see also Fig. 1 *c*). The pattern remained the same in cells treated with weak bases for up to 15 h (data not shown).

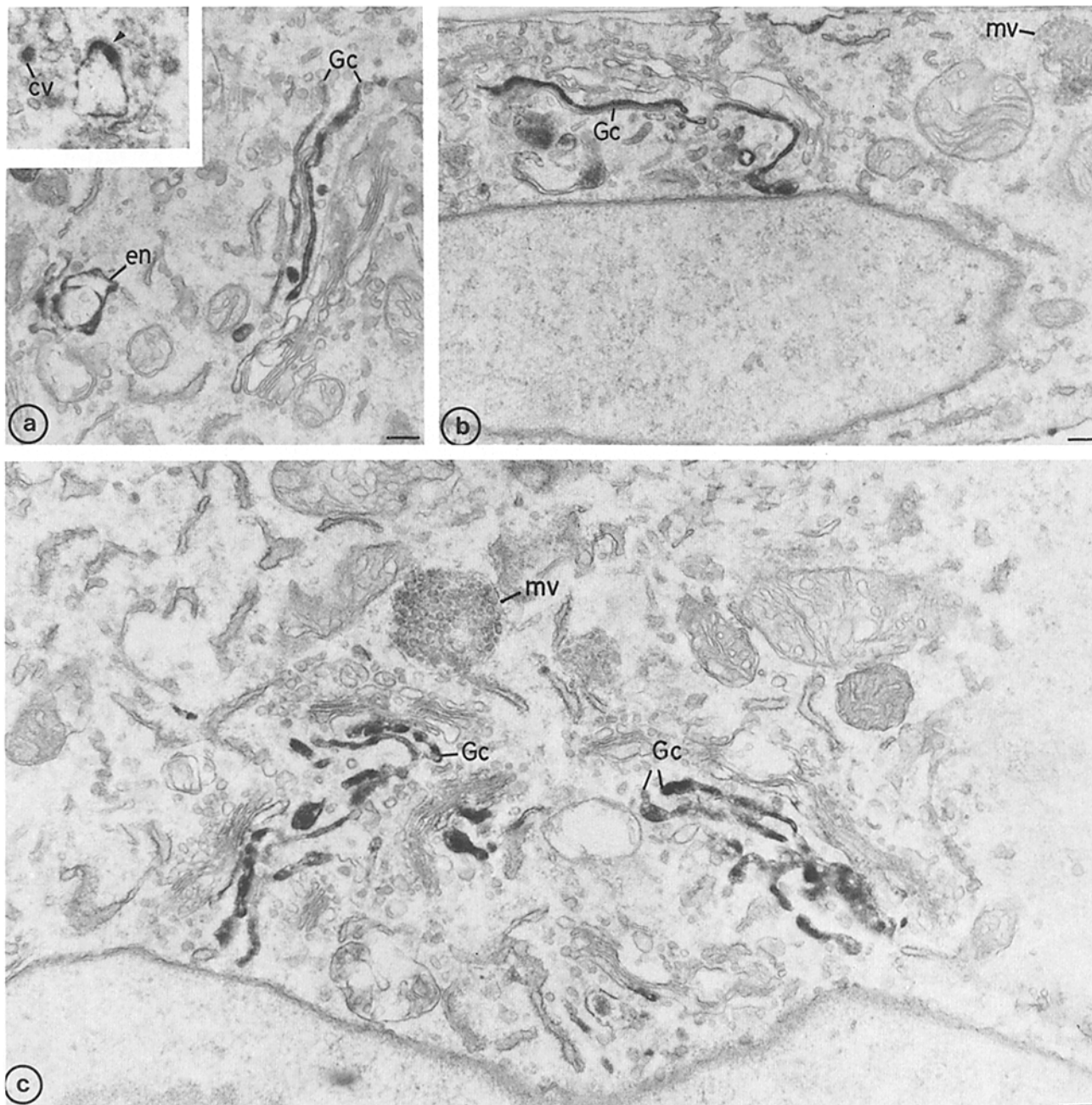
#### **Recycling of Man6P Receptors from Endosomes to Golgi Cisternae after $\text{NH}_4\text{Cl}$ Washout Is Rapid**

The time required for Man6P receptors to be depleted from endosomes and returned to the Golgi region after  $\text{NH}_4\text{Cl}$

removal was similarly determined by scoring receptor-labeled cells by IF as "Golgi"- or "vacuole"-stained (Fig. 7). Within 5–10 min after washout,  $\sim 50\%$  of the cells already showed a "Golgi" pattern of receptor staining (see Fig. 5), and by 30 min  $\sim 90\%$  of the cells showed predominantly a "Golgi" pattern with a concomitant decrease in those with a "vacuole"-staining pattern (Fig. 7). Thus, the conversion of "vacuole"- to "Golgi"-staining occurred rapidly ( $t_{1/2} = 10$  min). Although qualitatively similar results were obtained after recovery from chloroquine treatment, data obtained with this drug were more variable, and the cells recovered somewhat more slowly. However, after removal of both of these agents receptors rapidly returned to the Golgi region in the majority of cells.

#### **Ligand Dissociation Triggers the Recycling of Man6P Receptors to the Golgi Complex**

The recycling of receptors that occurs after recovery from treatment with lysosomotropic weak bases might be attributable to a direct effect of low pH on the receptor, or alternatively, to the dissociation of lysosomal enzymes from their receptors that occurs with the re-establishment of an acidic, intra-endosomal environment. To determine the effect of receptor occupancy on recycling, Man6P, a competitive inhibitor of receptor-enzyme binding (18), was added directly to the culture media of  $\text{NH}_4\text{Cl}$ -treated cells with the expectation that Man6P (like lucifer yellow and cationized ferritin) would be internalized by endocytosis and delivered to endo-



**Figure 6.** Immunoperoxidase localization of Man6P receptors in Clone 9 cells allowed to recover from  $\text{NH}_4\text{Cl}$  treatment. By 5 min after removing  $\text{NH}_4\text{Cl}$  from the medium (a), receptors can be detected in two of the Golgi cisternae (Gc), indicating that they have already recycled back to the Golgi complex. Receptors can also be detected in vesicles with clathrin-like coats (cv) and vesicular protrusions apparently budding from vacuolated endosomes (arrowhead), as shown in the inset. After 1 h of recovery (b and c), receptors are found almost exclusively in the stacked Golgi cisternae. In c, two to three cisternae contain immunodetectable receptors. Little if any reaction product was seen in multivesicular bodies (mv) present here. In these experiments, cycloheximide was added to the culture medium after removing  $\text{NH}_4\text{Cl}$  to prevent synthesis of new receptors. Bars, 0.2  $\mu\text{m}$ .

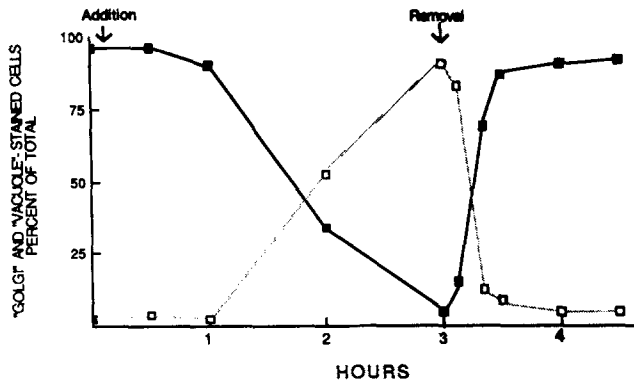
somes where it would cause dissociation of lysosomal enzymes from their receptors. After 1 h incubation with 50 mM Man6P, the cells remain vacuolated (Fig. 8 b'), but Man6P receptors could be detected by IF in the Golgi region of many cells (Fig. 8 b). The staining pattern resembled that seen when cells were merely washed free of weak base (see Fig. 5, b and c).

When  $\text{NH}_4\text{Cl}$ -treated cells were similarly incubated in the presence of 50 mM Man1P or 50 mM Glu6P (Figs. 8,

c and c'), sugars that do not inhibit the binding of lysosomal enzymes to Man6P receptors, no changes in the distribution of the receptor were detected.

Electron microscopy confirmed that in  $\text{NH}_4\text{Cl}$ -treated cells incubated for 1 h in the presence of Man6P, the receptors reappeared in Golgi cisternae (Fig. 9 a). During this period, images of endosomes with budding coated pits were often seen (Fig. 9 b) similar to those observed in cells washed free of weak bases (inset, Fig. 6 a). Also, endosomes with



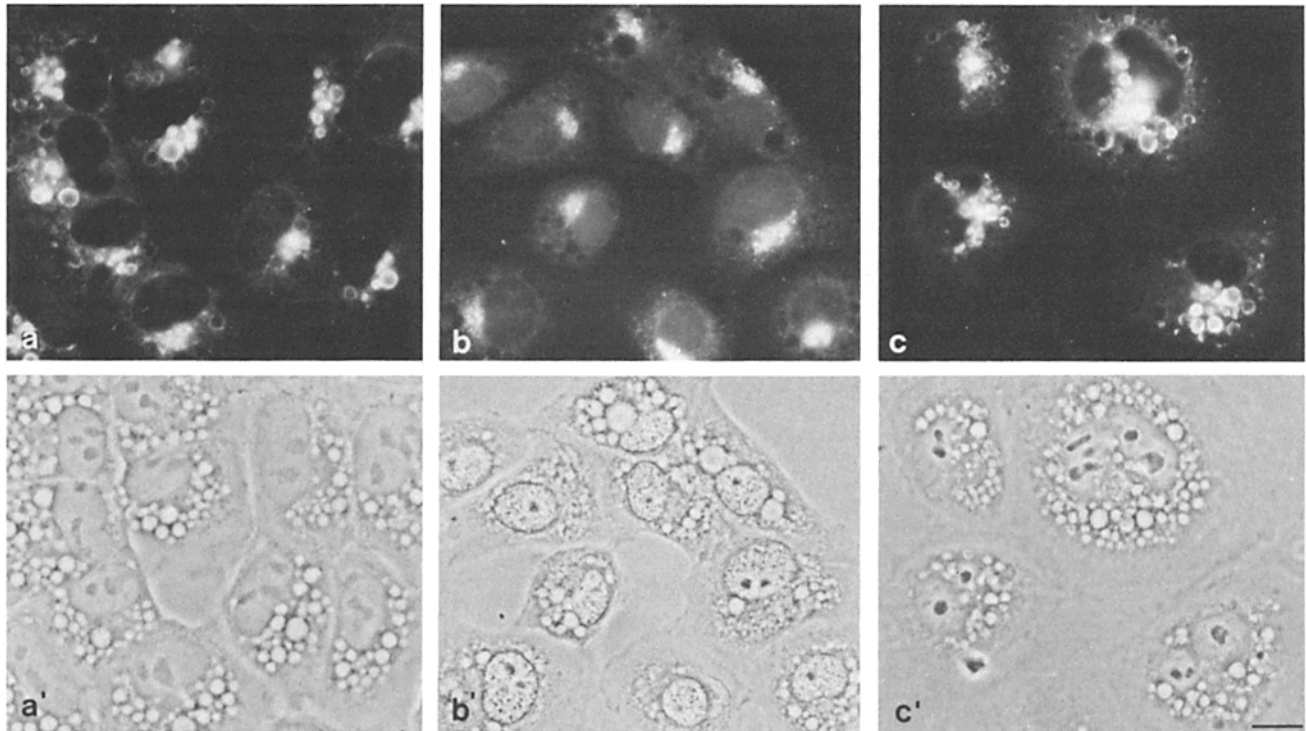


**Figure 7.** Graph expressing the percent of “Golgi”-versus “vacuole”-stained Cone 9 cells at various times after  $\text{NH}_4\text{Cl}$  addition and removal (arrows). At the time points indicated, cells were fixed, stained by immunofluorescence for Man6P receptors, and scored as being either “Golgi”- (■) or “vacuole”- (□) stained (see Fig. 1 for examples). In controls, >95% of the cells are “Golgi”-stained. When  $\text{NH}_4\text{Cl}$  was added, after a lag of  $\sim 30$  min, there was a gradual increase in the number of “vacuole”-stained cells over 3 h at which time virtually no “Golgi”-stained cells were seen ( $t_{1/2} = 75$  min). After  $\text{NH}_4\text{Cl}$  removal there was a very rapid increase in the number of “Golgi”-stained cells and a concomitant loss of “vacuole”-stained cells, indicating that Man6P receptors rapidly recycle ( $t_{1/2} = 10$  min) back to the Golgi complex.

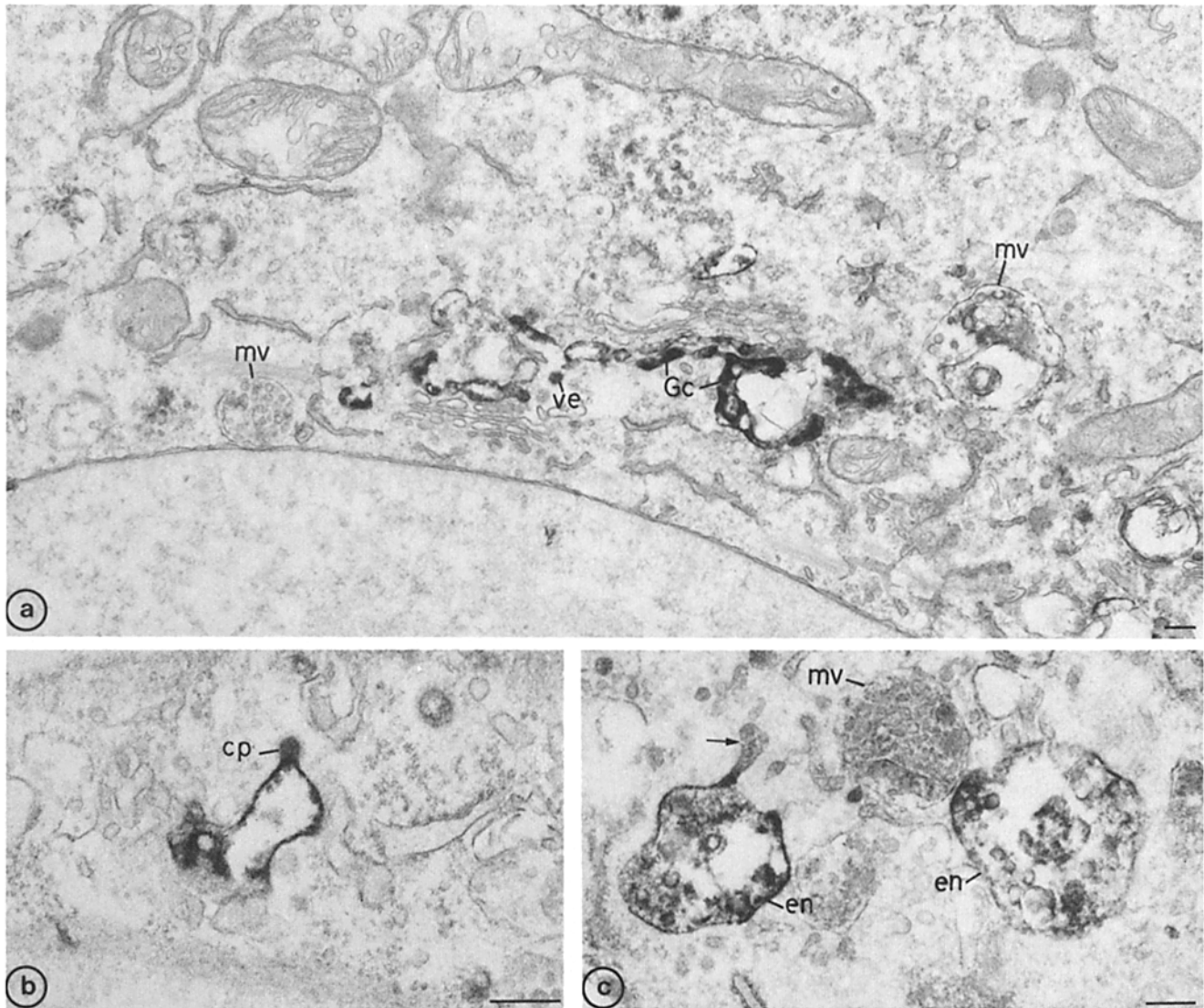
protruding, tail-like extensions containing reaction product were commonly seen (Fig. 9 c). Thus, the findings after Man6P addition were the same as in cells placed in weak base-free medium except that the endosomes remained di-

lated throughout the experiment (due to the continued presence of  $\text{NH}_4\text{Cl}$ ) and reversal occurred more slowly: return to a Golgi staining pattern required 1–3 h for completion.

When the percent of  $\text{NH}_4\text{Cl}$ -treated cells with “Golgi” staining was determined by IF 2 h after addition of sugars to the media,  $\sim 75\%$  of the cells showed this pattern with Man6P, whereas few cells (10–20%) showed “Golgi” staining after incubation with Man1P or Glu6P (Fig. 10). These findings indicate that the reversal effect was specific for Man6P, and that the receptors remained trapped in the dilated endosomes in cells incubated with Man1P or Glu6P. The simplest explanation of these results is that Man6P is delivered to endosomes by endocytosis and causes dissociation of lysosomal enzymes from their receptors thereby allowing the receptors to recycle, via coated vesicles, back to the Golgi cisternae. The delivery of Man6P to endosomes probably occurs by fluid phase- or adsorptive (nonspecific) endocytosis rather than via 215-kD Man6P receptors because our IF results suggest that in Clone 9 cells (data not shown) as in human fibroblasts (27) the cell surface is depleted of receptors after chloroquine treatment. Also, recycling to the Golgi was concentration dependent as would be expected if uptake were by fluid phase pinocytosis: it was detected with 50 mM Man6P but not 5 mM Man6P (data not shown). Preliminary results obtained with acridine orange (a fluorescent dye that labels acidic compartments) indicate that the Man6P effect is not due to the re-acidification of endosomes (or lysosomes) because no significant accumulation of the dye was detected in weak base-treated cells incubated with Man6P (data not shown). We conclude that ligand dissociation—not a pH effect—triggers the recycling of Man6P receptors.



**Figure 8.** Effect of incubation in Man6P, a competitive inhibitor of lysosomal enzyme binding, on Man6P receptor recycling in Clone 9 cells. (a and a') Cells treated with  $\text{NH}_4\text{Cl}$  for 3 h, showing the typical “vacuole”-type (endosomal), Man6P receptor, labeling pattern. (b and b') Similar  $\text{NH}_4\text{Cl}$ -treated cells incubated with Man6P for 1 h. Although they remain heavily vacuolated (b'), most cells have a “Golgi”-type, receptor staining pattern (b) indicating that Man6P receptors have recycled back to the Golgi complex. (c and c')  $\text{NH}_4\text{Cl}$ -treated cells incubated for 1 h in Glu6P, a sugar that does not inhibit the binding of lysosomal enzymes to the receptor. The staining pattern is the same as in a, indicating that there is no change in the distribution of Man6P receptors. Bar, 10  $\mu\text{m}$ .



**Figure 9.** Immunoperoxidase localization of Man6P receptors in  $\text{NH}_4\text{Cl}$ -treated Clone 9 cells incubated for 1 h with Man6P. Receptors are found mainly in Golgi cisternae (Gc), one of which is dilated, and associated vesicles (ve). Many of the multivesicular bodies (mv) no longer contain detectable receptors. Endosomes with reactive coated pits (cp) or tail-like extensions (arrow in c) were often seen, suggesting that Man6P receptors are collected and concentrated in coated pits and/or tubular extensions for recycling back to the Golgi complex. Bars, 0.2  $\mu\text{m}$ .

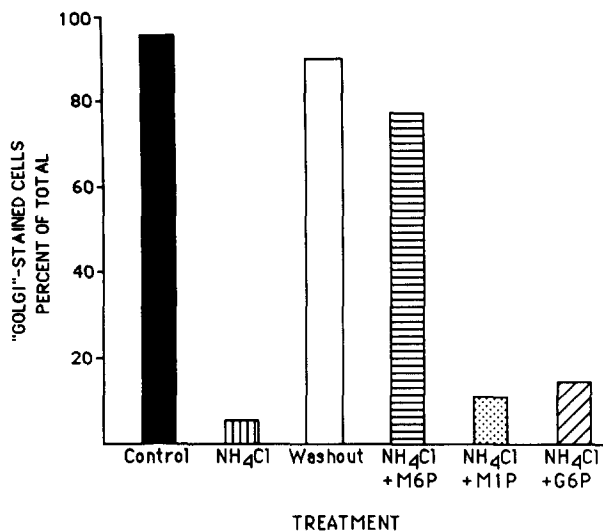
## Discussion

In this paper we have studied the intracellular traffic of 215-kD Man6P receptors for lysosomal enzymes taking advantage of our previous finding that treatment of cells with lysosomotropic weak bases results in the accumulation of receptors in dilated vacuoles, which form as a result of this treatment, and their depletion from Golgi cisternae (7). The main findings that have emerged are as follows: (a) the delivery site where Man6P receptors with their bound lysosomal enzymes are trapped by weak base treatment has been identified as an endosome, not a lysosome; (b) after weak base removal Man6P receptors were demonstrated to recycle from endosomes to the Golgi complex and to do so very rapidly; (c) evidence was obtained that coated vesicles serve as carriers for transport of recycling, unoccupied Man6P receptors back to the Golgi cisternae; and, (d) that Man6P receptor recycling is triggered by ligand dissociation. These

findings provide new information on the intracellular itinerary of the 215-kD Man6P receptor and their cargo of newly synthesized lysosomal enzymes and have a bearing on the mode of formation of secondary lysosomes.

Recently, many of the events that occur during the biosynthesis of lysosomal enzymes (15, 26, 28) and their targeting to lysosomes via Man6P receptors (11, 43) have been elucidated, and two different populations of Man6P receptors (215 and 46 kD) have been described (30, 42). Little is known as yet about the distribution of the 46-kD receptor, but the 215-kD Man6P receptor (4, 7, 21) and lysosomal enzymes bearing the Man6P recognition marker (24, 47) have been shown to be concentrated in the Golgi complex where the sorting and packaging of newly synthesized lysosomal enzymes occurs (reviewed in references 11, 16, and 43).

Our finding that the bulk of the immunoreactive 215-kD Man6P receptors are trapped in endosomes after weak base



**Figure 10.** Effect of incubation in Man6P and other sugars on distribution of Man6P receptors. Clone 9 cells were treated with NH<sub>4</sub>Cl for 3 h followed by either recovery in normal media for 1 h (*Washout*), or incubated in the continuous presence of NH<sub>4</sub>Cl with 50 mM Man6P (*NH<sub>4</sub>Cl + M6P*), Man1P (*NH<sub>4</sub>Cl + M1P*), or Glu6P (*NH<sub>4</sub>Cl + G6P*). They were then fixed, stained by IF for Man6P receptors, and the number of "Golgi"-stained cells was determined. A significant increase in the number of "Golgi"-stained cells is seen after removal or "washout" of NH<sub>4</sub>Cl (~90% of control) or after addition of Man6P (~75% of control) but not after addition of Man1P or Glu6P. In the presence of NH<sub>4</sub>Cl, recycling of Man6P receptors to the Golgi cisternae occurs only when Man6P is added.

treatment suggests that at least some of the newly synthesized acid hydrolases are delivered to endosomes and implies that secondary lysosomes can arise from endosomes.

According to the original lysosome concept (13), a primary lysosome contains only newly synthesized lysosomal enzymes derived from the Golgi complex, whereas a secondary lysosome contains both acid hydrolases and appropriate substrates (i.e., ingested materials undergoing degradation). Thus, by these definitions the coated vesicle carrier is a primary lysosome, and an endosome (which regularly contains substrates taken up by endocytosis) becomes converted into a secondary lysosome upon fusion with coated vesicles bearing receptor-bound lysosomal enzymes. There is general agreement at present (16) that in most cell types, coated vesicles serve to transport newly synthesized acid hydrolases from some part of the Golgi complex to their delivery site of action. It is usually assumed that the delivery site for lysosomal enzymes is a secondary lysosome, although there is little or no direct evidence on this point. Our results suggest that this is not the case because in NH<sub>4</sub>Cl-treated Clone 9 hepatocytes, the target organelle with which coated vesicles serving as primary lysosomes fuse is an endosome. Based on our results, we envisage the occurrence of the following sequence of events: A coated vesicle carrying newly synthesized lysosomal enzyme precursors bound to Man6P receptors fuses with an endosome, the lysosomal enzymes dissociate from their receptors due to the low pH (~5–5.5) environment encountered there (20, 34), leaving the immature precursor enzymes free in the lumen (where they are proteolytically converted to their mature forms [15,

28]), and allowing the receptors to recycle back to the Golgi complex. Our observations suggest that to convert an endosome into a secondary lysosome an additional step is required: the endosomal membrane must be replaced with a lysosomal membrane, since the two appear to be different (14, 33, 39). NH<sub>4</sub>Cl treatment appears to interfere with this conversion, which could be accomplished either by fusion with pre-existing secondary lysosomes (followed by removal of the endosomal membrane components via the pinching off of small vesicles), or by replacement of the endosome-type membrane with lysosome-type membrane, e.g., by fusion of multiple small vesicles composed of lysosomal membrane components with the endosome.

Various points of view have been put forth concerning how endosomes are related to lysosomes. Our data, indicating that primary lysosomes fuse with endosomes, favor the so-called "maturation model" alternative put forth by Helenius et al. (29), which states that endosomes are not permanent structures but are intermediates on their way to becoming lysosomes.

A number of findings are consistent with the above conclusions: (a) Electron microscopic studies in which acid phosphatase was used as a lysosomal marker have demonstrated that multivesicular bodies, a type of endosome, are initially acid phosphatase-negative, but after incorporation of tracers by endocytosis gradually become acid phosphatase-positive (19, 36, 44). (b) Recent studies by Geuze et al. (22) have confirmed that Man6P receptors accumulate in the swollen vacuoles of NH<sub>4</sub>Cl-treated cells and have demonstrated by double immunogold labeling that lysosomal enzymes are associated with the trapped receptors. (c) By cell fractionation lysosomes from human fibroblasts can be separated into two populations—a high density fraction that typically contains most of the lysosomal enzyme activity and is composed of structures that resemble electron-dense lysosomes, and a low density fraction that contains a mixture of organelles including acid phosphatase-positive multivesicular bodies (40). (d) When fibroblasts were incubated with endocytic tracers, the internalized molecules were found initially in the low density fraction and later appeared in the high density fraction, both with lysosomal enzyme activity (35). (e) Newly synthesized, but proteolytically cleaved, lysosomal enzymes first appeared in low density fractions and were later chased into high density fractions which contained most of the lysosomal enzyme activity in several cell types (3, 23, 50). (f) In Chinese hamster ovary cells, Man6P receptors were found predominantly in mixed Golgi/endosome-enriched fractions, whereas lysosome fractions had almost no detectable receptor activity (41). All of these data can be explained by assuming that Man6P receptors with their bound, newly synthesized lysosomal enzymes are delivered to endosomes, which are thereby transformed into secondary lysosomes. More specifically, endosomes, or some subpopulation of endosomes, represent the junction point between the biosynthetic pathway for newly synthesized lysosomal enzymes (215-kD Man6P receptor-mediated) and the endocytic pathways to lysosomes.

Data on the turnover of Man6P receptors indicate their  $t_{1/2}$  ≈ 20–24 h, suggesting that the receptors are reused for more than one round of lysosomal enzyme transport (10, 25, 41). The data presented here provide evidence that Man6P receptors do in fact cycle intracellularly, from the Golgi complex

to endosomes and back again. Based on our analysis it is clear that it takes the receptor population as a whole longer to travel from the Golgi complex to endosomes ( $t_{1/2} = 75$  min for the bulk of the population) than to recycle from endosomes back to the Golgi complex ( $t_{1/2} = 10$  min). The reason for the differences in the apparent transit time to and from endosomes is not apparent, however, it should be kept in mind that the measurement of the time spent in the Golgi-to-endosome leg actually represents the cumulative time it takes for the pool of Golgi receptors (as well as those returning from endosomes) to become loaded with incoming, newly synthesized lysosomal enzymes, as well as the time required for their transport to endosomes. By contrast, on the return leg, unloading and transit are synchronized; removal of lysosomotropic weak bases causes a rapid drop in intracellular pH which triggers dissociation of lysosomal enzymes simultaneously from the entire population of trapped receptors. Assuming that the maximum time it takes for a round trip time is  $\sim 85$  min, based on a receptor half-life of  $\sim 24$  h in Clone 9 cells (unpublished data), a single Man6P receptor could make at least 18–20 round trips. Recycling of cell surface receptors, such as those for low density lipoproteins (1), transferrin (12, 32), and asialoglycoproteins (2, 45, 48, 49), is well documented; however, the Man6P receptor is the only known example of a receptor that performs its main function while cycling largely or exclusively (in cell types that lack surface receptors [4]) in transport between intracellular compartments.

It should be pointed out that Clone 9 cells, like human fibroblasts (11, 43), have cell surface Man6P receptors that can mediate the uptake of the appropriate ligands, and these surface receptors exchange with those in the intracellular pool. However, in Clone 9 hepatocytes (unpublished data) as well as many other cell types (11, 17, 43), surface receptors normally account for only  $\sim 10\%$  of the total receptors, with the remaining 90% being intracellular. Moreover, we have observed that in Clone 9 cells, as in human fibroblasts (27), Man6P receptors become depleted from the cell surface and are trapped intracellularly after chloroquine or  $\text{NH}_4\text{Cl}$  treatment. Thus, it follows that the contribution of cell surface receptors to the intracellular recycling seen here by IF must be minimal, i.e.,  $\leq 10\%$  of the total signal.

A key finding was that when Man6P, a competitive inhibitor of lysosomal enzyme binding to Man6P receptors, was added to the culture medium of living,  $\text{NH}_4\text{Cl}$ -treated cells, it could induce the recycling of receptors back to the Golgi complex. This is most readily explained by the fluid phase pinocytotic uptake of the sugar into endosomes where it causes dissociation of lysosomal enzymes from their receptors. Apparently, Man6P can displace lysosomal enzymes while at the same time allowing the receptors to behave as though they are unoccupied (i.e., to recycle). This is in keeping with previous work indicating that monovalent ligands (i.e., Man6P) can competitively dissociate pre-bound, multivalent ligands (i.e., lysosomal enzymes with Man6P residues), but unlike the latter, they bind with low affinity to Man6P receptors and do not induce the pinocytosis of surface receptors (18). From these results, along with those obtained after  $\text{NH}_4\text{Cl}$  removal, we conclude that ligand dissociation is all that is required for triggering the recycling of unoccupied Man6P receptors back to the Golgi complex. Previously, we have obtained evidence that ligand loading is important in

triggering receptor movement from Golgi cisternae to endosomes (5, 7). These observations imply that (a) structural information must reside within Man6P receptors which serves to target them to endosomes when occupied and to the Golgi complex when unoccupied and (b) ligand binding must induce appropriate changes in the receptor which initiate these events.

During recovery from  $\text{NH}_4\text{Cl}$  treatment, coated vesicles bearing Man6P receptors were often observed budding from endosomes, suggesting that they serve as the carriers for transporting unoccupied receptors to the Golgi complex. Rome and co-workers (8, 9) have found both occupied and unoccupied 215-kD Man6P receptors in coated vesicle-enriched fractions from rat brain and liver which probably correspond, respectively, to those traveling from the Golgi complex to endosomes and those traveling from endosomes back to the Golgi complex, as well as some derived from the cell surface.

In summary, the data obtained provide evidence that the 215-kD Man6P receptor, which functions in the sorting out and transport of newly synthesized lysosomal enzymes, cycles between the Golgi complex and endosomes, and that endosomes represent the junction point where the biosynthetic pathway for lysosomal enzymes (Man6P receptor-mediated) and endocytic pathway to lysosomes converge.

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