

# Redistribution of Mannose-6-Phosphate Receptors Induced by Tunicamycin and Chloroquine

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**ABSTRACT** The distribution of Man-6-P receptors was determined by immunoperoxidase cytochemistry in Clone 9 hepatocytes cultured in the presence or absence of tunicamycin and chloroquine, agents that perturb lysosomal enzyme sorting and lead to their secretion. In control (untreated) cells, receptors were localized in *cis* Golgi cisternae, coated vesicles, and in endosomes or lysosomes. After tunicamycin treatment, receptors were found in coated vesicles lined up along the *cis* cisternae but were not detected in endosomes or lysosomes. After chloroquine treatment, receptors were localized in large vacuolated endosomes or lysosomes but were not usually detected in Golgi cisternae or in coated vesicles. These results demonstrate a redistribution of receptors along the normal Man-6-P-dependent sorting pathway after these treatments. In ligand-deficient (tunicamycin-treated) cells, immunoreactive receptors accumulate at the presumptive sorting site in the *cis* Golgi and are depleted from endosomes and lysosomes. When the intralysosomal pH is increased (by chloroquine treatment) preventing ligand-receptor dissociation, receptors accumulate at the presumptive delivery site (lysosomes and endosomes) and are depleted from the *cis* Golgi region. The findings also suggest that (a) ligand binding triggers movement of the receptor to endosomes or lysosomes, and (b) ligand dissociation triggers their movement back to the *cis* Golgi region.

Recently we localized the mannose-6-phosphate receptor for lysosomal enzymes in several secretory and absorptive cell types by immunocytochemistry in an attempt to define the Man-6-P-dependent transport route and to identify the site at which sorting of lysosomal enzymes and secretory products takes place (1). We localized the receptor in *cis* Golgi cisternae, in coated vesicles, and in endosomes and lysosomes of several cell types. We have assumed that this distribution delineates the Man-6-P-dependent pathway by which lysosomal enzymes are delivered to lysosomes, and that the organelles mentioned represent stations along this pathway which correspond to the sorting site, the carrier, and the delivery site, respectively. On the basis of these results we proposed that normally lysosomal enzymes bearing the recognition marker are removed from the secretory pathway in the *cis* Golgi for delivery to lysosomes, bypassing the *trans* Golgi cisternae.

Our previous findings applied to the steady-state distribution of Man-6-P receptors in several epithelia (hepatocytes, exocrine pancreas, and epididymis) in which the polarity of the Golgi stacks (*cis* vs. *trans*) is clear. In this paper, we have extended our inquiry to cultured cells and have determined the distribution of the receptor both at steady state (untreated cells) and under conditions in which the traffic of lysosomal

enzymes is perturbed by tunicamycin (2, 3) or chloroquine (3-6) treatment. In cells incubated with these agents, sorting of lysosomal enzymes does not take place owing either to the production of defective ligand (2, 3) or to a deficiency of receptors (6), respectively. As a result, in both instances the newly synthesized lysosomal enzymes are secreted rather than being delivered to lysosomes (2-6).

## MATERIALS AND METHODS

**Materials:** F(ab) fragments of goat anti-rabbit IgG conjugated to rhodamine were purchased from Cappel Laboratories, Inc., (Cochranville, PA) and F(ab) fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase were obtained from the Institute Pasteur Productions (Marnes La Coquette, France). Diaminobenzidine hydrochloride (Type II), tunicamycin, cyclohexamide, chloroquine, and saponin were purchased from Sigma Chemical Co., (St. Louis, MO). Clone 9 and Fu5C8 cells (rat hepatocyte cell lines) were obtained from Dr. David Sabatini, New York University (3), and normal rat kidney (NRK) cells were obtained from Dr. Ira Pastan, National Institutes of Health.

**Cell Culture:** Cells were cultured at 37°C in Eagle's minimal essential medium, containing 10% fetal calf serum, 1% penicillin, and 1% streptomycin in an atmosphere of 95% O<sub>2</sub>, 5% CO<sub>2</sub>. They were grown on coverslips for immunofluorescence and in 35-mm plastic dishes to near confluency for immunoelectron microscopy. Agents that induce lysosomal enzyme secretion were added to the incubation media for 3 h before fixation at the same

concentrations used in previous studies (3, 6)—i.e., tunicamycin, 1–2  $\mu\text{g}/\text{ml}$ ; cycloheximide, 2  $\mu\text{g}/\text{ml}$ ; chloroquine, 25  $\mu\text{M}$ .

**Preparation of Antibodies:** Procedures for the preparation and affinity purification of anti-Man-6-P receptor IgG, designated R2, were given previously (1). Briefly, the Man-6-P receptor, a 215-kdalton protein (7, 8) was affinity purified from a detergent extract of rat liver microsomal membranes on a pentamannosyl-6-phosphate column. Antibodies were raised against this protein which specifically immunoprecipitated only the ~215-kdalton receptor protein from radiolabeled rat tissues.

**Immunofluorescence:** Cultured cells were fixed on coverslips in 3.7% formalin in PBS, pH 7.4, for 45 min at room temperature, both with and without acetone permeabilization. They were incubated for 1 h with (a) R2 anti-Man-6-P receptor IgG (40  $\mu\text{g}/\text{ml}$ ), (b) affinity-purified R2 IgG (10  $\mu\text{g}/\text{ml}$ ), or (c) R2 preimmune serum (diluted 1:100) followed by incubation with rhodamine-conjugated goat anti-rabbit F(ab) (1:50) and examined in a Zeiss photomicroscope II, equipped with epifluorescence illumination and an appropriate filter for rhodamine.

**Immunoperoxidase:** Clone 9 cells were processed as previously described (9). Briefly, they were fixed in McLean and Nakane's (10) fixative (2% formaldehyde, 0.75 M lysine, 10 mM NaIO<sub>4</sub> in phosphate buffer, 35 mM, pH 6.2) for 2–4 h, permeabilized with 0.005% saponin (11) and incubated for 1 h each in R2 antireceptor IgG or preimmune serum (as detailed above for immunofluorescence) and horseradish peroxidase-conjugated sheep anti-rabbit Fab (1:50). Cells were then fixed for 1 h in 1.5% glutaraldehyde in 100 mM sodium cacodylate, pH 7.4, containing 5% sucrose, incubated in diaminobenzidine hydrochloride (DAB) medium (0.2% DAB with 0.01% H<sub>2</sub>O<sub>2</sub>) for 3–5 min, and postfixed with ferrocyanide-reduced OsO<sub>4</sub> for 45 min at 4°C. They were then dehydrated and detached from the plastic dishes by being rinsed rapidly in 100% propylene oxide (12), collected in a Pasteur pipette, transferred to Eppendorf tubes, and centrifuged; the resultant pellets were embedded in Epon 812. Thin sections were stained with lead citrate and examined in a Philips 301 electron microscope.

## RESULTS

### Localization of Man-6-P Receptors by Immunofluorescence

When nonpermeabilized, control (untreated) Clone 9 cells were incubated with antireceptor antibodies, a uniform, bright punctate staining was observed (Fig. 1A), indicating the presence of cell surface Man-6-P receptors. When cells were permeabilized with acetone before the antibody incubation, a punctate intracellular staining was seen which was concentrated in the juxtannuclear or Golgi region (Fig. 1B).

In cells treated with tunicamycin (Fig. 1C) or cycloheximide (Fig. 1D), no morphological changes were detected by phase-contrast microscopy. By immunofluorescence the receptors were seen to be concentrated at the cell surface and in the juxtannuclear, Golgi region as in controls. However, the staining pattern in the Golgi region was altered in that instead of a punctate pattern, the receptors were distributed in a reticular network of various shapes and sizes. A similar redistribution of receptors was seen in NRK cells, human skin fibroblasts, and Fu5C8 hepatocytes after tunicamycin treatment.

In cells treated with chloroquine, large cytoplasmic vacuoles were visible by phase-contrast microscopy (Fig. 1E). These vacuoles are assumed to be lysosomes because chloroquine treatment is known to cause lysosomal vacuolation (13) and increased intralysosomal pH (14, 15). By immunofluorescence (Fig. 1F), the distribution of receptors was seen to be dramatically altered in that instead of being confined to the Golgi region, it was found throughout the cytoplasm, where it corresponded to that of the vacuolated lysosomes, many (but not all) of which were brightly fluorescent at their periphery.

No staining was seen in cells incubated with R2 preimmune serum.

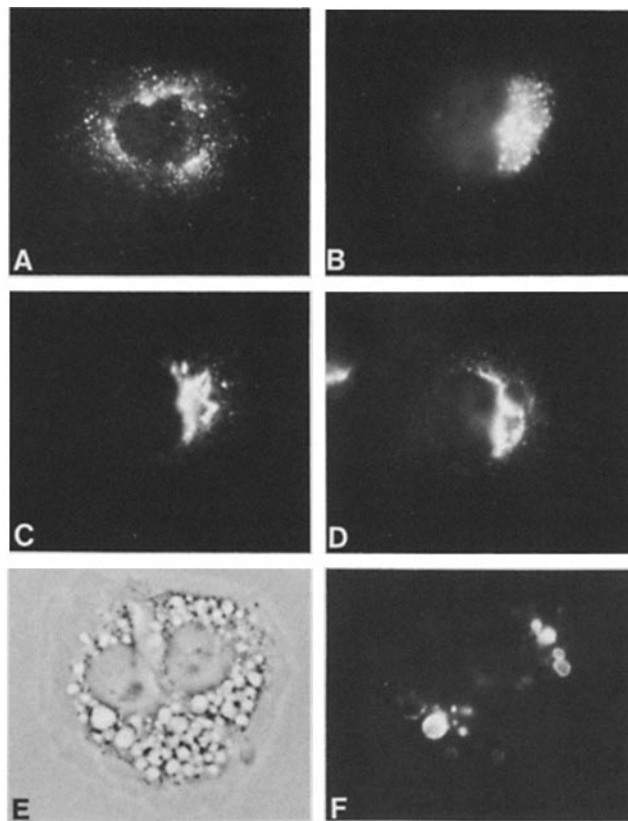


FIGURE 1 Immunofluorescence localization of Man-6-P receptors in Clone 9 hepatocytes. (A) Nonpermeabilized control. A punctate staining pattern is seen at the cell surface. Receptors are also present on the remaining cell surface which is out of the plane of focus. (B–F) Acetone-permeabilized cells. (B) Control, untreated cell. Bright punctate staining is limited to the juxtannuclear or Golgi region. (C and D) Cells treated for 3 h with tunicamycin and cycloheximide, respectively. Receptors are distributed in a reticular instead of a punctate pattern in the Golgi region. (E and F) Cell incubated with chloroquine for 3 h. By phase-contrast microscopy (E) large vacuolated lysosomes characteristic of chloroquine-treated cells (13) fill the cytoplasm. By immunofluorescence (F) it is evident that receptor staining is found at the periphery of some of the large lysosomes.  $\times 300$ .

### Localization of Man-6-P Receptors by Immunoelectron Microscopy

In untreated Clone 9 cells, receptors were detected in coated pits and vesicles located at or near the cell surface (Fig. 2) by immunoperoxidase. The concentration of receptors in coated vesicles exposed at the cell surface undoubtedly accounts for the punctate, surface staining seen by immunofluorescence and resembles the situation in hepatocytes in situ where receptors were localized in coated pits along the sinusoidal plasmalemmal domain (1). Intracellularly, immunoreactive receptors were localized in the following: (a) *cis* Golgi cisternae, (b) some of the coated vesicles found near, or budding from, the reactive Golgi cisternae (Figs. 3 and 4), and (c) in large reactive vacuoles (0.4–1.0  $\mu\text{m}$ ), assumed to be lysosomes or endosomes,<sup>1</sup> which were typically located in the Golgi

<sup>1</sup> The large immunoreactive vacuoles could represent either endosomes or lysosomes. Because there is no way to distinguish between these two types of organelles based on morphology alone, we consider them collectively here as a group.

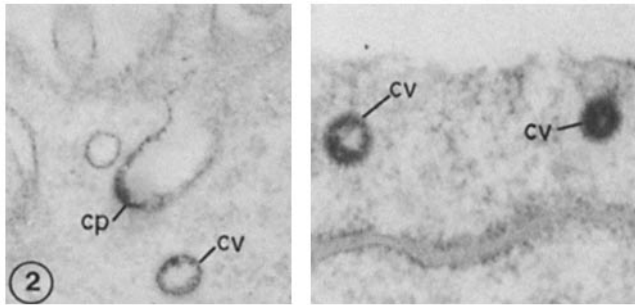


FIGURE 2 Immunoperoxidase localization of Man-6-P receptors in coated pits (cp) and coated vesicles (cv) present along the plasma membrane of control (untreated) Clone 9 hepatocytes. Note that little or no staining is present along the remaining (noncoated) regions of the cell surface.  $\times 43,000$ .

region. The rough endoplasmic reticulum, nuclear envelope, and other cell compartments did not stain for the receptor.

Within the Golgi complex, receptors were typically confined to one or two of the flattened cisternae located on one side of the Golgi stacks, with no apparent gradation of reaction product between highly reactive and nonreactive cisternae. In these cells, as in the case of other cultured cells studied to date (e.g., normal human fibroblasts and I cell fibroblasts [9]), it was difficult to determine whether the immunoreactive cisternae were located on the *cis* or *trans* side of the Golgi stack owing to the absence of appropriate landmarks such as forming secretory granules. However, in some cases centrioles were present in the plane of section, and based on their topography, the reactive cisternae could be identified as *cis* cisternae (see Figs. 8 and 9).

In cells treated with tunicamycin or cycloheximide two major differences in receptor distribution were found (Figs. 5–9): (a) The number of Golgi-associated coated vesicles that contained reaction product was greatly increased, and in some cases massive accumulations of reactive coated vesicles were seen near or in continuity with the labeled (*cis*) cisterna (Fig. 7); and (b) staining was rarely seen in lysosomes or endosomes (Figs. 5–9). The lack of lysosomal staining was not due to the absence of these organelles because vacuoles (0.4–1.0  $\mu\text{m}$ ), which morphologically resemble endosomes or lysosomes, were present (Figs. 5 and 9). Apparently the change in the Golgi staining pattern from punctate to reticular detected by immunofluorescence was created by the lining up of immunoreactive coated vesicles along Golgi cisternae together with the loss of punctate endosomal or lysosomal staining. It is of interest that the receptor distribution was the same in tunicamycin- and cycloheximide-treated cells: in both cases labeling of the Golgi stack was restricted to one or two cisternae, there was no visible gradation of staining between reactive and nonreactive cisternae, and when sections through the

Golgi complex included the centrioles (Figs. 8 and 9), the reactive cisternae could be identified as *cis* cisternae.

Chloroquine treatment also induced striking changes in the distribution of Man-6-P receptors: (a) immunoreactive receptors were found almost exclusively in huge (1–3  $\mu\text{m}$ ) vacuolated endosomes or lysosomes, and (b) they were not usually detected in Golgi cisternae or in coated vesicles (Figs. 10 and 11). Only rarely was a small amount of reaction product detected in a single Golgi cisterna or in a coated vesicle. Most often those structures were unstained.

## DISCUSSION

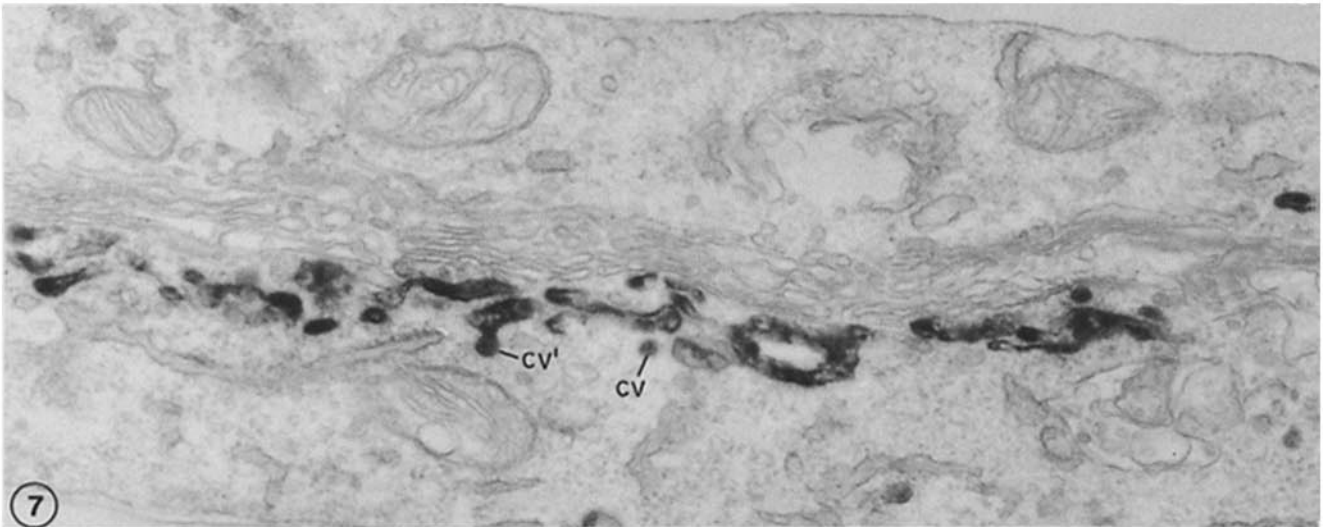
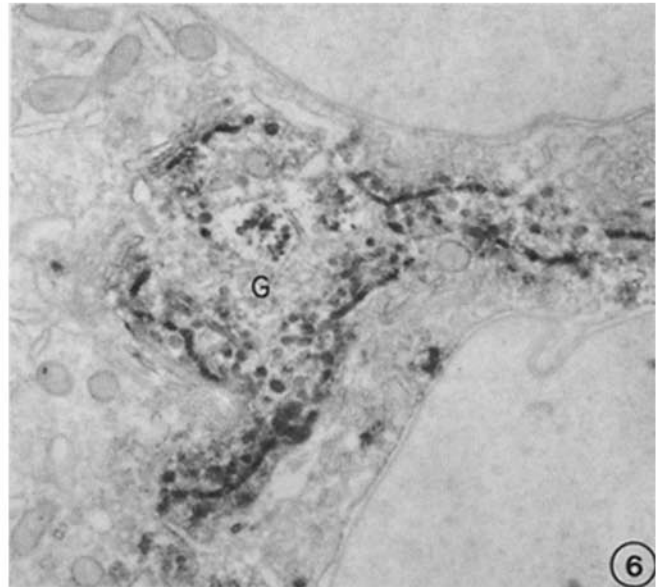
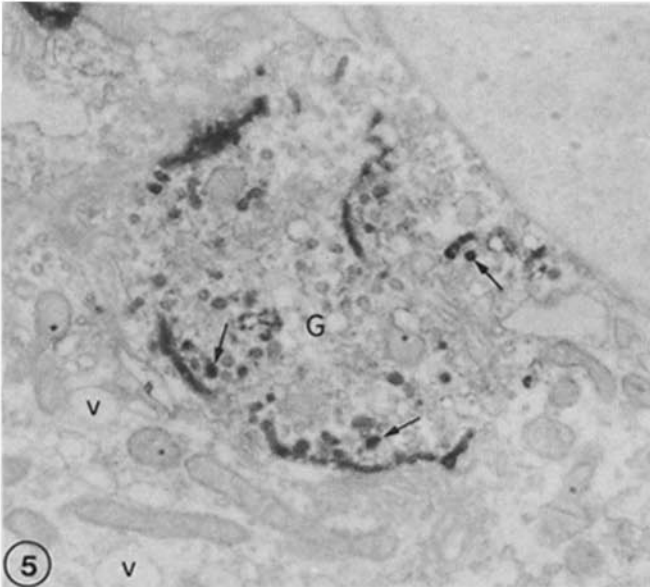
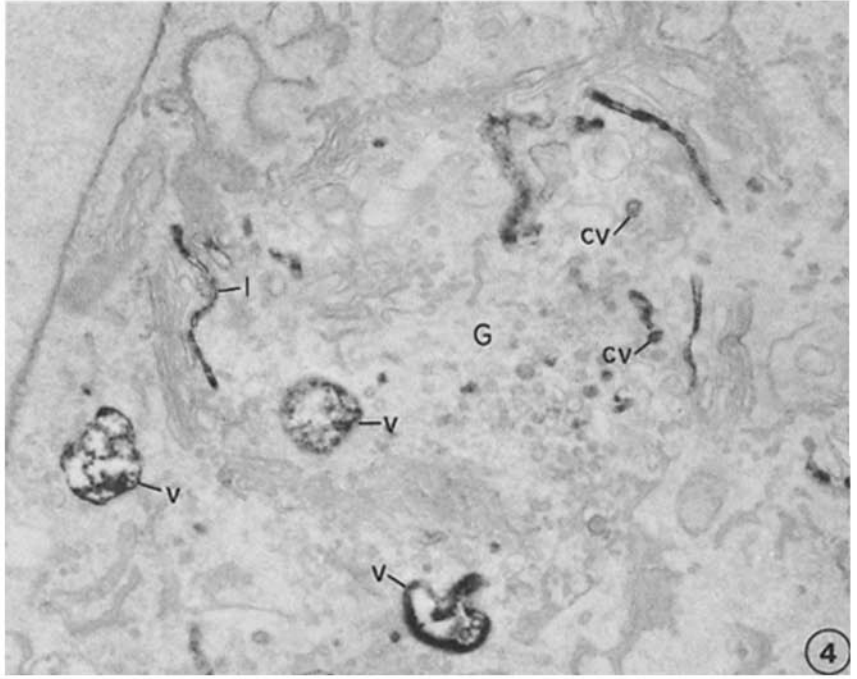
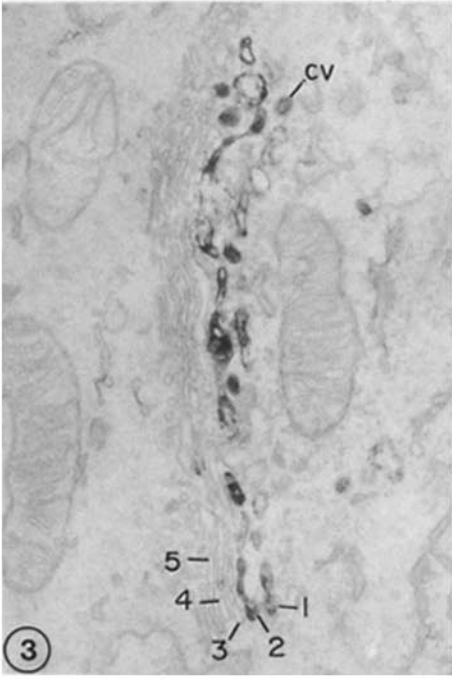
There is now abundant evidence that the interaction between phosphomannosyl residues on newly synthesized lysosomal enzymes and Man-6-P receptors results in the selective targeting of lysosomal enzymes to lysosomes (6, 16). Although some cell types lack Man-6-P receptors (17), there is no doubt that Man-6-P receptors are widely distributed and functional in most cell types tested to date (16–18). We have previously shown by immunocytochemistry (1) that at steady state the highest intracellular concentrations of Man-6-P receptors—the key element in the sorting process—are in *cis* Golgi cisternae, in some coated vesicles, and in endosomes and/or lysosomes in several cell types (hepatocytes, exocrine pancreatic, and epididymal epithelia) studied *in situ*.

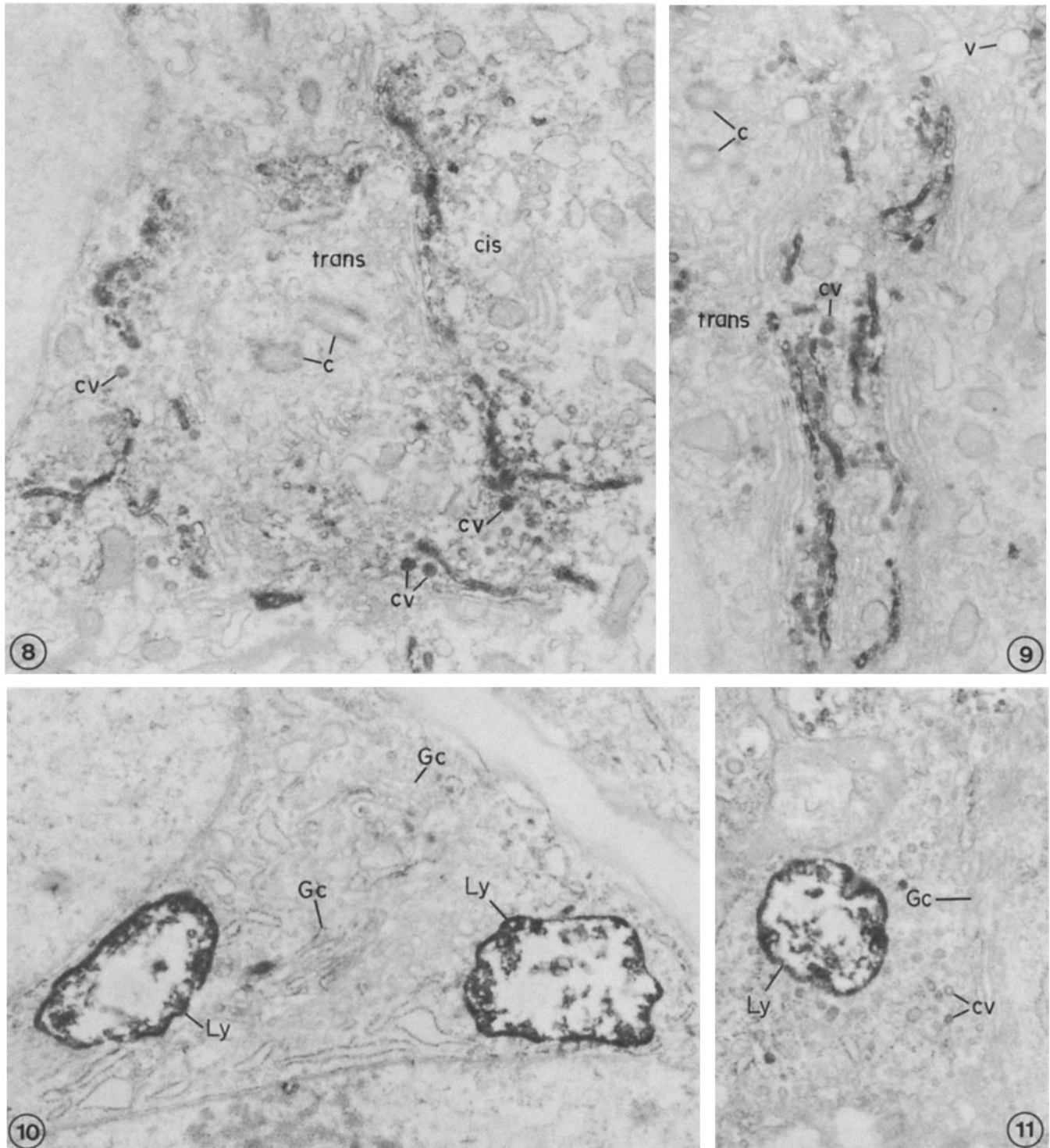
In this report we have determined the distribution of the receptor in cultured cells under conditions, established by previous investigators (2, 3, 6), in which the traffic of lysosomal enzymes is perturbed by tunicamycin and chloroquine treatments, and the enzymes are not sorted for delivery to lysosomes but are secreted into the medium. We found that both treatments lead to a redistribution of receptors: in tunicamycin- (or cycloheximide-) treated cells, receptors are found in coated vesicles lined up at the presumptive sorting site in the *cis* Golgi and are not detected in lysosomes and endosomes, whereas in chloroquine-treated cells receptors are found at the presumptive delivery site (endosomes or lysosomes) and are not detected in Golgi cisternae.

The redistribution of receptors that occurs after tunicamycin treatment can be explained by the fact that in the presence of this N-glycosylation inhibitor the recognition marker is not added to lysosomal enzymes; hence the enzymes fail to bind to the receptor for sorting and delivery to lysosomes and are secreted into the medium (2, 3) along with other secretory products. The receptor-bearing coated vesicles which normally serve as the carrier to ferry the acid hydrolases from the *cis* Golgi to lysosomes return to the sorting site in the *cis* Golgi where they gradually accumulate over time, awaiting the arrival of an appropriate ligand.

The redistribution of receptors that occurs after chloroquine treatment can be explained by the fact that in the presence of this lysosomotropic agent, receptors become trapped in lyso-

FIGURES 3–7 Immunoperoxidase localization of Man-6-P receptors in the Golgi regions (G) of Clone 9 hepatocytes. In controls (Figs. 3 and 4), immunoreactive receptors are highly concentrated in one or two Golgi cisternae (1 and 2) located on one side of the stack, in coated vesicles (cv) located near the reactive cisternae, and in 0.4–1.0- $\mu\text{m}$  vacuoles (v) that presumably correspond to endosomes or lysosomes. In tunicamycin-treated (3 h) cells (Figs. 5 and 6), there is a dramatic accumulation of coated vesicles (arrows) in the Golgi region (G), and no immunoreactive endosomes or lysosomes (v) are seen. As in controls, immunoreactive receptors are concentrated in one or two Golgi cisternae located on one side of the stacks. Fig. 7 is a higher magnification of another tunicamycin-treated cell, demonstrating receptors in coated vesicles close to (cv) or in continuity with (cv') a reactive Golgi cisterna. Note that reaction product is found in only one or two of the Golgi cisternae, and there is no gradation of reaction product across the stack. (Figs. 3 and 4)  $\times 24,000$ ; (Fig. 5)  $\times 18,000$ ; (Fig. 6)  $\times 16,500$ ; (Fig. 7)  $\times 40,000$ .





FIGURES 8-11 Figs. 8 and 9 demonstrate the effects of cycloheximide-treatment (3 h) on Clone 9 hepatocytes. As in tunicamycin-treated cells, immunoreactive coated vesicles (cv) accumulate in the Golgi region and in one or two Golgi cisternae but are not detected in endosomes or lysosomes (v). Fortuitously, in both figures the sections pass through the centrioles (c) which can be used as topographical markers for the *trans* side of the Golgi stack: immunoreactive Golgi cisternae are located on the opposite or *cis* side of the stacks. Note that the degree and direction of curvature of the Golgi stacks is quite variable. Figs. 10 and 11 demonstrate the effects of chloroquine treatment (3 h) on the distribution of Man-6-P receptors. Receptors are detected in huge (1-3  $\mu$ m) lysosomes or endosomes (Ly), and the Golgi cisternae (Gc) are virtually depleted of immunoreactive receptors. Also, relatively few of the Golgi-associated coated vesicles (cv) contain immunoreactive receptors. (Figs. 8 and 9)  $\times 23,000$ ; (Fig. 10)  $\times 20,000$ ; (Fig. 11)  $\times 26,000$ .



somes because acid hydrolases cannot dissociate from Man-6-P receptors when the pH rises above 6.0 (6). Chloroquine is known to increase intralysosomal pH (and presumably also intraendosomal pH) to > 6.0 (13, 14). Under these conditions the carrier coated vesicles, containing lysosomal enzymes bound to the receptor (19), continue to fuse with lysosomes, causing an accumulation of receptors in these organelles.

Thus, the receptors are not found in any new or different intracellular sites (e.g., *trans* Golgi or GERL [Golgi-associated endoplasmic reticulum lysosome] cisternae) under the conditions studied. Instead, it appears that there is a redistribution of the receptors along the normal delivery route with these treatments causing a depletion of receptors from some stations on the presumptive route and an accumulation at others.

The finding of increased numbers of coated vesicles bearing Man-6-P receptors in the Golgi region in tunicamycin- (and cyclohexamide-) treated cells is the same as observed previously (9) in I cell fibroblasts from patients with Mucopolidosis II which are also ligand deficient. I cells are the functional equivalent of tunicamycin-treated cells in that they do not make the recognition marker (owing to a deficiency in *N*-acetyl glucosamine phosphotransferase) (20, 21), and they secrete rather than store newly synthesized lysosomal enzymes (22). We previously noted (9) that the accumulation of coated vesicles in ligand-deficient cells implies that there is a distinctive subpopulation of coated vesicles involved as primary lysosomes in the transport of lysosomal enzymes from the *cis* Golgi to lysosomes and that ligand loading must trigger their relocation to lysosomes. The present results with chloroquine suggest that dissociation of the ligand triggers return of the receptors (presumably via coated vesicles) to the *cis* Golgi.

Based on the presence of immunoreactive receptors in *cis* Golgi cisternae and their absence from the remaining Golgi cisternae, we previously proposed (1) that the *cis* Golgi represents the site where the secretory and lysosomal pathways diverge and that normally the bulk of the intracellular lysosomal enzymes bearing the recognition marker are sorted for delivery to lysosomes in the *cis* Golgi, bypassing the *trans* Golgi cisternae. We further proposed that if sorting does not occur because of the lack of functional receptors or ligand, acid hydrolases continue along the secretory pathway, reach the *trans* Golgi cisternae where the appropriate enzymes are believed to reside (23–25), and are trimmed, and at least some of their oligosaccharide chains undergo further processing to the complex type (addition of terminal galactose and sialic acid). The results obtained on Clone 9 hepatocytes are in keeping with this proposal since Rosenfeld et al. (3) have demonstrated that in these cells, all of the intracellular, precursor acid hydrolases contain high-mannose (endoglycosidase [Endo] H-sensitive) type oligosaccharides, whereas those that are normally secreted (30–40% of the total synthesized) contain complex-type (Endo H-resistant and Endo D-sensitive) oligosaccharides. Thus, both the biochemical findings on the glycosylation state of the secreted enzymes in Clone 9 cells, as well as the present observations on the distribution of Man-6-P receptors after treatments that perturb lysosomal enzyme traffic, are in accord with the assumption that sorting of the bulk of the acid hydrolases normally occurs in the *cis* Golgi. Alternative explanations, e.g., that the bulk of the Man-6-P-dependent intracellular sorting occurs in *trans* Golgi (26–28) or GERL (29, 30) elements, are more difficult to reconcile with the information at hand.

Are there some general principles in Golgi sorting to be

learned from our findings? With great foresight, Sly and co-workers (6, 16, 31) predicted that the intracellular sorting of acid hydrolases for delivery to lysosomes occurs by a receptor-mediated “segregation” event comparable to receptor-mediated endocytosis at the cell surface, which removes them from the biosynthetic pathway. They also predicted (6) that the secretion of lysosomal enzymes by chloroquine-treated cells occurs as the result of the accumulation of Man-6-P receptors in lysosomes and their depletion from the endoplasmic reticulum or Golgi. Our findings document that this is indeed the case. Furthermore, our results pinpoint the location of the receptor-mediated event to *cis* Golgi cisternae and identify the carrier as a coated vesicle subpopulation whose membranes must be compositionally distinct from endocytic vesicles at the cell surface. It might be predicted that sorting of other cell products destined for other cell surfaces (e.g., basal instead of luminal) and membrane constituents destined for specific cell compartments might occur by a similar receptor-mediated process in a distinctive subpopulation of coated vesicles which removes them from the appropriate region of the Golgi complex by means of as-yet-unidentified specific receptors. An interesting case in point is the Man-6-P receptor—itsself an integral membrane glycoprotein with *N*-asparagine-linked, complex-type oligosaccharides (32, 33)—which must be specifically targeted to *cis* Golgi cisternae.

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