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The distribution of 215-kilodalton mannose 6-phosphate receptors within cis (heavy) and trans (light) Golgi subfractions varies in different cell types

(lysosomal enzymes/protein sorting/intracellular traffic)

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ABSTRACT **The distribution of mannose 6-phosphate (Man-6-*P*) receptors for lysosomal enzymes was investigated in Golgi subfractions prepared from three different cultured cell lines. Total microsomal fractions from clone 9 hepatocytes, normal rat kidney, or Chinese hamster ovary cells were subfractionated by flotation in sucrose density gradients, which resolves Golgi membranes into heavy (cis), intermediate, and light (trans) subfractions. The distribution of Man-6-*P* receptors within the subfractions was assessed by quantitative immunoprecipitation, and the results were compared to those obtained by immunoperoxidase localization of the receptors in Golgi cisternae of intact cells. In all cases, the results obtained by Golgi subfractionation and by immunoelectron microscopy were in agreement. In clone 9 cells, Man-6-*P* receptors were enriched in heavy (cis) Golgi subfractions, whose peak density (ρ = 1.17) was greater than those containing either galactosyltransferase activity, a trans Golgi marker, or α-mannosidase II, a middle Golgi marker. By immunoelectron microscopy, the receptors were localized to a single cis Golgi cisterna. In Chinese hamster ovary cells, Man-6-*P* receptors were concentrated in Golgi membranes of low density (1.12 g/ml) overlapping the peak of galactosyltransferase activity. By the immunoperoxidase technique, the receptors were usually localized to a single trans Golgi cisterna. In normal rat kidney cells, Man-6-*P* receptors were found to be broadly distributed across Golgi membranes (ρ = 1.12–1.17), and by immunoperoxidase localization they were found to be broadly distributed across the stacked Golgi cisternae. It is concluded that the distribution of Man-6-*P* receptors within the Golgi complex varies from one cell type to another. These differences in receptor distribution may reflect variations in lysosomal enzyme trafficking among different cell types.**

The Golgi complex serves as the cell's main intracellular sorting station for newly synthesized secretory, membrane, and lysosomal proteins, facilitating their transport to their correct resident destinations (1, 2). At present the only known Golgi sorting mechanism is that for lysosomal enzymes. Sorting of lysosomal enzymes and their removal from the secretory pathway is known to be effected by a receptor-mediated process involving the binding of lysosomal enzymes bearing the mannose 6-phosphate (Man-6-*P*) recognition marker to Man-6-*P* receptors (3, 4). Because they constitute the only known Golgi sorting mechanism, considerable attention has been paid recently to characterizing Man-6-*P* receptors. Two different Man-6-*P* receptors have been identified—a 215-kDa (cation-independent) receptor (5, 6) and a newly discovered 46-kDa (cation-dependent) receptor (7, 8) about which much less is known. We have been studying the trafficking of the 215-kDa Man-6-*P* receptors under various conditions and have obtained evidence that they cycle between Golgi cisternae (the sorting site for lysosomal enzymes) and endosomes (identified as the delivery site) via coated vesicle carriers (9, 10). These results agree with those of others who also have found these receptors to be concentrated in Golgi cisternae, prelysosomal structures, and coated vesicles (11–16).

The question of where in the Golgi complex the 215-kDa Man-6-*P* receptors are located has proved to be controversial. When we localized the receptors by immunoelectron microscopy using an immunoperoxidase procedure, we found their distribution to vary from one cell type to another: They were concentrated in cis Golgi cisternae in several cell types (9, 17) and were broadly distributed throughout the Golgi stack in others (18). Using different immunocytochemical procedures, other investigators reported that 215-kDa Man-6-*P* receptors were located throughout the stacked Golgi cisternae (12, 13) but were more concentrated in the trans cisternae (11–13). Similarly variable findings were obtained when Man-6-*P* receptors were localized in Golgi subfractions (15, 16). These studies have raised the question as to whether the conflicting results are due to differences in the techniques used or whether they represent true differences in the distribution of the receptor from one cell type to another. The question is of considerable interest because it has been assumed that the site of highest receptor concentration in the Golgi complex should correspond to the site of sorting. To investigate this problem, we have analyzed the distribution of Man-6-*P* receptors in Golgi subfractions prepared from three different cultured cell lines, and we have compared the results to those obtained by immunocytochemical localization of the 215-kDa Man-6-*P* receptor in the same cell types *in situ*.

MATERIALS AND METHODS

Materials. Rat clone 9 cells were from the American Type Culture Collection (ATCC CRL 1439). Normal rat kidney (NRK) (epithelial-like) and Chinese hamster ovary (CHO-K1) cells were kindly provided by Efraim Racker (Cornell University). [^{35}S]Methionine (1375 Ci/mmol; 1 Ci = 37 GBq) and UDP-[^{3}H]galactose (10.2 Ci/mmol) were obtained from New England Nuclear. Ovalbumin (type V), ATP, and protein A-Sepharose CL-4B were purchased from Sigma; *p*-nitrophenyl α-D-mannopyranoside was from Boehringer Mannheim.

Antibodies. Rabbit polyclonal antibodies were raised against purified rat liver Man-6-*P* receptors as described (17);

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Abbreviations: Man-6-*P*, mannose 6-phosphate; NRK, normal rat kidney; CHO, Chinese hamster kidney; PNS, postnuclear supernatant.

they recognize only the 215-kDa Man-6-*P* receptor by immunoprecipitation (17) and immunoblotting. Rhodamine-conjugated sheep anti-rabbit IgG was purchased from Cooper Biomedical (Malvern, PA), and Fab fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase were obtained from Biosys (Compiegne, France).

Cell Culture. All cells were maintained at 37°C in 95% air/5% CO_2. Clone 9 and NRK cells were grown in Eagle's minimal essential medium with Earle's salts and 10% fetal calf serum (MEM/FCS), and CHO cells were grown in Dulbecco's minimal essential medium and 10% FCS. For immunocytochemistry, cells were grown for 4–6 days to confluency and were fixed in formaldehyde as described (19). For cell fractionation experiments, cells were plated in 15 Petri dishes (150 × 25 mm) and grown for 4–6 days to subconfluency. Cells in one of the dishes were washed (three times) with methionine-free MEM and incubated for 45 min at 37°C, after which 500 μCi of [^{35}S]methionine (≈30 μCi/ml) was added and incubation was continued for 14–16 hr at 37°C. The labeled cells were then washed (three times) with MEM/FCS and incubated in MEM/FCS containing 10× (1 M) methionine for an additional 2 hr at 37°C, after which they were harvested with a rubber policeman and pooled with the unlabeled cells.

Homogenization and Fractionation. Cells were pelleted by centrifugation (2000 × *g* for 5 min) and then resuspended in cold swelling buffer (10 mM triethanolamine/1 mM EDTA/0.25 M sucrose, pH 7.4) for 5 min, after which they were centrifuged (2000 × *g* for 10 min), yielding ≈1 ml of packed cells. All manipulations were done at 4°C. The supernatant was discarded, and the cells were resuspended in 5 ml of homogenization buffer (10 mM Hepes/1 mM dithiothreitol/15 mM KCl/0.25 M sucrose, pH 7.4) and homogenized by 2–5 passes through a stainless-steel-block ball-bearing cell homogenizer (20), which resulted in ≈90% cell disruption as judged by phase-contrast microscopy. The homogenate was centrifuged (2000 × *g* for 10 min), yielding a postnuclear supernatant (PNS) that was recovered and saved and a pellet that was gently resuspended with a Pasteur pipet in 5 ml of homogenization buffer and then centrifuged (2000 × *g* for 10 min) yielding another PNS. The two PNSs were pooled and diluted to 35 ml with 10 ml of TBS (10 mM Tris/10 mM NaCl/1.5 mM $MgCl_2$, pH 7.4) and centrifuged at 150,000 × g_{avg} for 90 min in a Beckman 70 Ti rotor. The organelles in the pellet were resuspended and lysed in TBS with a Potter–Elvehjem homogenizer and recentrifuged (150,000 × *g* for 60 min). This centrifugation and membrane washing procedure was repeated twice, first with 10 mM Tris/10 mM EDTA, pH 8.2, and then with 1 mM Tris/1 mM EDTA, pH 8.0. The final microsomal membrane pellet was resuspended by homogenization in 10 ml of 50% (wt/wt) sucrose/1 mM Tris/1 mM EDTA, pH 8.0. Gradients were formed by overlaying this material with either a sucrose step gradient (21) or a continuous gradient [20–45% (wt/wt)] in Beckman SW 27.1 ultracentrifuge tubes. All sucrose solutions were in 1 mM Tris/1 mM EDTA, pH 8.0. After centrifugation (85,500 × g_{avg} for 18–20 hr) in a Beckman SW 27.1 rotor (to reach isopycnic conditions), 24 fractions of 700 μl each were collected from the top with a Buchler (Lake Buff, IN) Auto-DensiFlo collector and frozen at −70°C until assays were performed. Both gradients gave essentially the same resolution of Golgi subfractions.

Immunoprecipitation. From each gradient fraction, 200 μl was solubilized with 25 μl of 10× RIPA buffer (22) (1× = 0.1% $NaDodSO_4$/1% Triton X-100/150 mM NaCl/25 mM Tris·HCl, pH 7.2/10 mM EDTA/1% deoxycholate). Anti-Man-6-*P* receptor IgG (25 μl) was added to each fraction, and the mixture was incubated for 1.5 hr at room temperature. Preswollen protein A-Sepharose beads [10 mg (dry weight)] were added to each fraction and incubated for 1.5 hr at room temperature. The beads were collected by centrifugation in a Microfuge and washed six times with RIPA buffer, followed by one wash with 20 mM Tris (pH 7.4). The immunoprecipitates were dissociated by heating in a boiling water bath for 1 min in 2× Laemmli sample buffer (23) and resolved on 8% acrylamide gels by the Laemmli procedure (23). Gels were fixed and stained with Coomassie blue, soaked in sodium salicylate (24), dried, and fluorographed. The total amount of labeled receptor and the amount recovered in each fraction were determined by scanning densitometer (Quik-Scan R and D, Helena Laboratories, Beaumont, TX) of the fluorographs.

Other Assays. Galactosyltransferase activity was assayed by a modification (25) of the method of Brew *et al.* (26) with ovalbumin as acceptor. α-Mannosidase II was determined by the method of Tulsiani *et al.* (27). Proteins were measured by the Lowry method (28).

Immunocytochemistry. Indirect immunofluorescence and immunoperoxidase localization of the 215-kDa Man-6-*P* receptors were performed as described (9, 19).

RESULTS

Localization of the 215-kDa Man-6-*P* Receptors by Immunocytochemistry. Immunofluorescence staining gave essentially the same result on all three cell types. Man-6-*P* receptors were concentrated in the juxtanuclear Golgi region (Fig. 1). Immunoperoxidase localization of Man-6-*P* receptors at the electron-microscopic level revealed that (*i*) the

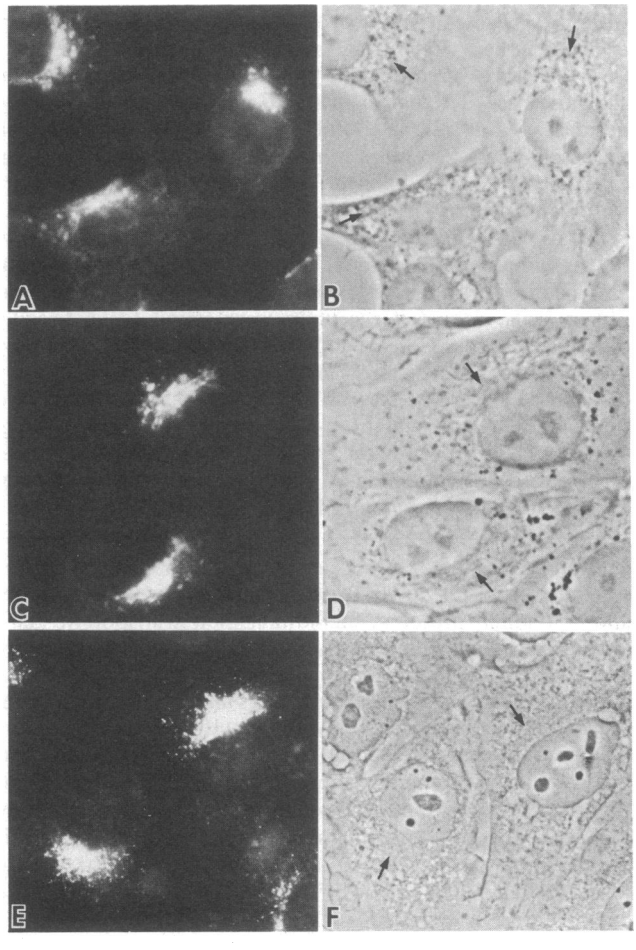

FIG. 1. Immunofluorescence localization of Man-6-*P* receptors in clone 9 hepatocytes (*A* and *B*), NRK cells (*C* and *D*), and CHO cells (*E* and *F*). In all three cell types, the receptors are concentrated in the Golgi region (*A*, *C*, and *E*) located to one side of the nucleus [see arrows in the comparison phase-contrast micrographs (*B*, *D*, and *F*)].

"Golgi" staining pattern was due to the presence of receptors not only in the stacked Golgi cisternae but also in endosomes and coated vesicles that were concentrated in the Golgi region, and (*ii*) the distribution of the receptors among Golgi elements varied from one cell type to another (Fig. 2). As previously reported (9, 10) for clone 9 cells, receptors were highly polarized in their distribution within the pile of Golgi cisternae; they were primarily confined to one or two cisternae located on one side of the stack (Fig. 2*A*), which were identified as cis elements based on their location in relation to centrioles (9).

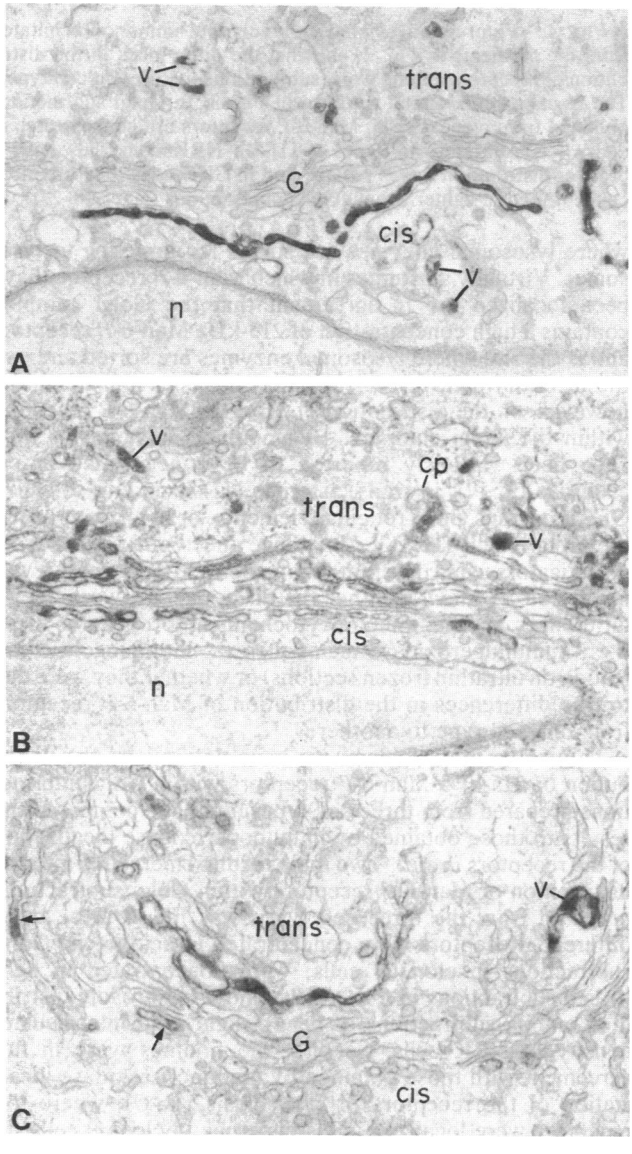

FIG. 2. Immunoperoxidase localization of Man-6-*P* receptors among Golgi elements in clone 9 hepatocytes (*A*), NRK cells (*B*), and CHO cells (*C*). Receptors are detected in Golgi cisternae (G) and in associated vesicular structures (v) in all three cell types. The distribution of receptors within the stacked cisternae varies from one cell type to another. In clone 9 cells, they are concentrated in a single cisterna on one side of the stack, which has been identified (9) as the cis side based on its relationship to centrioles. By contrast, in NRK cells (*B*) receptors are detected throughout the Golgi stack—i.e., in cis, middle, and trans cisternae. The latter includes the transmost cisterna, which corresponds morphologically to "GERL" or the trans Golgi reticulum or network because it is set apart from the stack and often shows coated regions (cp). In CHO cells (*C*) as in clone 9 cells, receptors are concentrated in a single cisterna on one side of the stack; however, in this case the reactive cisterna is located on the trans side. Smaller amounts of reaction product are also seen in middle and cis Golgi cisternae (arrows). n, Nucleus. (*A*, ×23,000; *B*, ×20,000; *C*, ×33,000.)

By contrast, in NRK cells the distribution of Man-6-*P* receptors was much more variable. Most often they were found in nearly all cisternae with no obvious concentration on one or the other side of the stack (Fig. 2*B*). However, in some cells they were concentrated in cis and trans cisternae, with the middle cisternae remaining unlabeled.

In CHO cells they were largely concentrated in trans cisternae (Fig. 2*C*) that morphologically corresponded to elements variously called "GERL" (11, 29), trans Golgi reticulum (12, 13, 30), or trans Golgi network (31). Similar findings have been found in CHO cells by Willingham *et al.* (11).

Distribution of 215-kDa Man-6-*P* Receptors in Golgi Subfractions. It has been shown that membranes derived from cis, middle, and trans Golgi elements can partially be separated by centrifugation of total microsomal membranes in sucrose density gradients; the buoyant density of the Golgi elements decreases from cis to trans (15, 21). When the location of two established Golgi enzymes—i.e., α-mannosidase II, a middle Golgi marker (15), and galactosyltransferase, a trans Golgi marker (30)—was determined in Golgi subfractions prepared from clone 9, NRK, and CHO cells, galactosyltransferase activity was always found in fractions of slightly lower density than those containing the α-mannosidase II peak. This indicates that, although the shapes and mean densities of the activity peaks varied somewhat, these enzymes are reliable Golgi subcompartment markers in these cell types.

When the distribution of Man-6-*P* receptors was determined by immunoprecipitation, there were distinct differences in the distribution of receptors among Golgi subfractions prepared from the three different cell types (Figs. 3 and 4). These differences can be seen clearly when plotted relative to the marker enzymes (Fig. 3), but they are even more pronounced in the fluorograms of immunoprecipitated receptors (Fig. 4). In clone 9 cells, the two marker enzymes and the Man-6-*P* receptors were separated from one another into distinct peaks. The receptors were found in the most-dense Golgi subfractions (mean density ≈ 1.17 g/ml, containing ≈80% of the total precipitable receptor recovered in the gradient) that were clearly denser than those of the α-mannosidase II and galactosyltransferase peaks. α-Mannosidase II activity occurred in a broad peak (ρ = 1.15 g/ml, containing ≈50% of the total activity). Galactosyltransferase activity peaked at an even lower density (1.13–1.14 g/ml). The fact that the receptors were concentrated in the most-dense Golgi subfractions (i.e., denser than those containing α-mannosidase II) is consistent with their location in cis Golgi cisternae and is in keeping with our immunocytochemical results (Fig. 2*A* and ref. 9).

In NRK cells, the distribution of the receptors was quite different. They were found in several peaks: a major one (ρ = 1.12 g/ml, containing ≈45% of the total precipitable receptor) that coincided with the peak of galactosyltransferase activity and two minor ones that coincided with α-mannosidase II, which also occurred in two peaks (1.15 and 1.17 g/ml). These results demonstrate that Man-6-*P* receptors are broadly distributed among Golgi subfractions and are in accord with the immunocytochemical data indicating they are broadly distributed across the Golgi stack (Fig. 2*B*).

In CHO cells a different distribution of Man-6-*P* receptors was seen. They occurred as a single peak at a slightly lower density (ρ = 1.12 g/ml) than the peak of galactosyltransferase activity, with significant amounts of receptor also being found in the galactosyltransferase-enriched fractions. Nearly all of the Man-6-*P* receptors were found in this single peak (≈60% of the total precipitable receptor), and few were

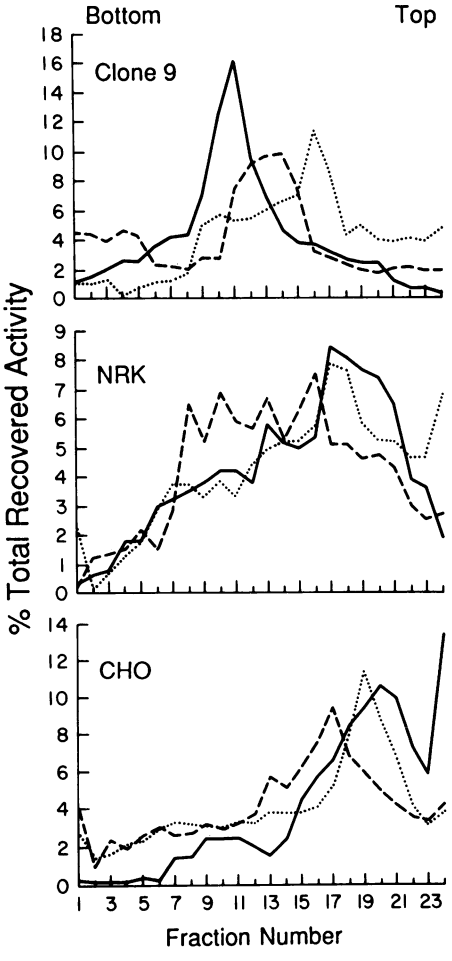

FIG. 3. Subfractionation of Golgi from clone 9, NRK, and CHO cells. Total microsomal membranes from each cell type were separated by flotation in 20–50% sucrose gradients. The distribution of Man-6-*P* receptors (—) is shown relative to α-mannosidase II (– – –), a middle Golgi marker enzyme, and galactosyltransferase (· · · ·), a trans Golgi marker. The top and bottom of the gradients are indicated. (*Top*) In Golgi subfractions from clone 9 cells, Man-6-*P* receptors are concentrated in a single peak toward the bottom of the gradient (ρ = 1.17 g/ml), corresponding to cis Golgi membranes. α-Mannosidase II and galactosyltransferase activities are found as single peaks in lighter fractions (ρ = 1.15 and 1.13–1.14 g/ml, respectively). (*Middle*) In NRK cells, significant amounts of Man-6-*P* receptors are found throughout the Golgi subfractions with a major peak coinciding with the peak of galactosyltransferase (ρ = 1.12 g/ml) and two minor peaks coinciding with two of the peaks of α-mannosidase II activity, which has a broad distribution in these cells. (*Bottom*) In the case of CHO cells, Man-6-*P* receptors are concentrated in lighter fractions corresponding to trans Golgi membranes because they occur in a single major peak (ρ = 1.12 g/ml) that overlaps but is of slightly lower density than the peak of galactosyltransferase activity. α-Mannosidase II is found as a single major peak of higher density (ρ = 1.13–1.14 g/ml).

located in the fractions containing the peak of α-mannosidase II activity (ρ = 1.13–1.14 g/ml). Thus, in CHO cells Man-6-*P* receptors are concentrated in light Golgi subfractions corresponding to trans Golgi elements and in membranes of even lower density. These results are in accord with their immunocytochemical localization to trans Golgi cisternae (Fig. 2*C* and ref. 11).

DISCUSSION

Considerable attention has been devoted to attempting to identify the most proximal location along the exocytic or so-called endoplasmic reticulum-to-plasmalemma pathway

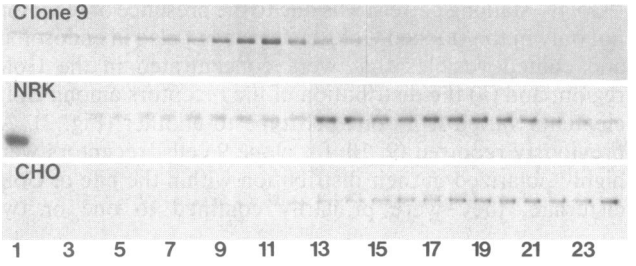

FIG. 4. Fluorogram of Man-6-*P* receptors immunoprecipitated from the gradients in Fig. 3, showing the differences in the distribution of receptors among Golgi subfractions in different cell types. The fractions correspond to those in Fig. 3. In Golgi subfractions obtained from clone 9 cells, Man-6-*P* receptors are concentrated in dense (cis) subfractions (lanes 9–11). In NRK cells they have a broader distribution (lanes 13–21), and in CHO cells they are more concentrated in lighter (trans) Golgi subfractions (lanes 18–24).

where lysosomal enzymes are sorted for targeting to lysosomes. Virtually all studies in which Man-6-*P* receptors have been localized are in agreement that the Golgi complex contains a high concentration of 215-kDa Man-6-*P* receptors and is the site where lysosomal enzymes are sorted and exit the secretory pathway (9–18). There has been no agreement, however, on the distribution of 215-kDa Man-6-*P* receptors within the Golgi complex. By immunocytochemistry they have been variously reported to be concentrated in cis cisternae (9, 17), in trans cisternae (11) including so-called GERL or trans Golgi reticular elements, or in all cisternae of the Golgi stack (12, 13, 18). Since the reports were from investigators using different techniques and cell types, it was impossible to know whether the discrepancies were due to differences in the immunocytochemical procedures used (i.e., immunoperoxidase techniques vs. immunogold localization on ultrathin frozen sections) or whether they were due to true differences in the distribution of Man-6-*P* receptors from one cell type to another.

To resolve this problem, we have determined the distribution of 215-kDa Man-6-*P* receptors within Golgi subfractions prepared from three cell types and have compared the results to those obtained by immunoperoxidase localization of the receptors *in situ*. Two main results emerged. First, the distribution of Man-6-*P* receptors within Golgi subfractions prepared from the three cell types was found to be quite different. Receptors were concentrated in heavy (cis) Golgi subfractions in clone 9 cells, were concentrated in light (trans) subfractions in CHO cells, and were broadly distributed among subfractions of heavy, light, and intermediate density in NRK cells. Second, the findings were in full agreement with those obtained by immunoperoxidase localization of the receptors *in situ* (Figs. 2 *A–C*), where the receptors were localized in cis cisternae in clone 9 cells, in trans cisternae in CHO cells, and broadly distributed across the stack in NRK cells. Thus, the differences in receptor distribution reported in the literature appear to reflect true differences related to cell type rather than limitations of certain immunocytochemical procedures. In this as well as in our earlier studies (17, 18), variations in distribution were demonstrated by using the same immunocytochemical procedure. Differences in Man-6-*P* receptor distribution in Golgi subfractions prepared from different cell types also have been reported; they were found to be concentrated in middle and light membrane subfractions in BW 5417 lymphocytes (15) but were present in both cis and trans Golgi subfractions and absent from middle Golgi subfractions prepared from rat liver (16).

How can the differences in receptor distribution, especially the cis localization, be explained? The simplest explanation is that there may be differences in Man-6-*P*-mediated lyso-

somal enzyme traffic from one cell type to another. Such differences have been suggested by the fact that most cultured cell lines and a few cell types *in situ* [e.g., osteoclasts (18)] have plasmalemmal Man-6-*P* receptors and secrete a large fraction of their lysosomal enzymes outside the cell (3). By contrast, most cell types *in situ*, including exocrine pancreatic and epididymal epithelial cells (17), lack immunodetectable plasmalemmal receptors (215 kDa) and primarily sort lysosomal enzymes intracellularly for delivery to lysosomes. The distribution of Man-6-*P* receptors might be expected to vary with the different patterns of lysosomal enzyme traffic. This is suggested by the fact that rapid redistribution of Man-6-*P* receptors is induced by treatments that affect lysosomal enzyme trafficking (9, 10, 18).

Can lysosomal enzymes exit the secretory pathway from the cis Golgi cisternae without passing through the trans cisternae? It is commonly assumed (31, 32) that all sorting and exit of biosynthetic products including lysosomal enzymes from the Golgi complex occurs on the trans side of the Golgi stack in cisternae that are variously known as GERL (29), trans Golgi reticulum (12, 13, 30), or trans Golgi network (31). The assumption that lysosomal enzymes exist exclusively from the trans side of the Golgi stack is based on the immunocytochemical finding that lysosomal enzymes and Man-6-*P* receptors can be colocalized in trans cisternae in some cell types (12, 13). This assumption is also supported by the finding that a large fraction of the lysosomal enzymes produced by most cultured cells contain complex-type oligosaccharides (33), implying that they must pass through the trans cisternae where the terminal transferases reside (30) before reaching their final destination. However, most cultured cells secrete a considerable fraction (20–60%) of their lysosomal enzymes (3); if only a small fraction of the lysosomal enzymes were sorted on the cis side for direct delivery to lysosomes, the detection of this fraction might be difficult. Moreover, in some cell types, including clone 9 hepatocytes (34) and spleen cells (35–37), intracellular lysosomal enzymes have been found to possess primarily high mannose-type oligosaccharides; in most cases examined, some fraction of the lysosomal enzyme oligosaccharides are of the high mannose-type even under circumstances (i.e., exoglycosidase deficiencies) where degradation of terminal sugars by lysosomal glycosidases is prevented (38).

An alternative explanation for the cis location of receptors in some cell types is that cis cisternae contain a storage reservoir of unoccupied receptors. In this case the assumption is that, when newly synthesized lysosomal enzymes arrive from the endoplasmic reticulum and the recognition marker is added, they bind to the receptors, and the receptor–ligand complexes move rapidly across the stack to the trans side and are not detectable in this site. This possibility cannot be ruled out but seems unlikely.

It also should be pointed out that recent findings (39, 40) suggest that membrane recycling from the plasmalemma operates to all of the stacked cisternae in the Golgi complex. If the same applies to recycling between endosomes/lysosomes and the Golgi complex, the need for precise sorting sites is obviated, and diversity in receptor distribution is possible.

In summary, we have documented here by Golgi subfractionation and immunocytochemistry that there are valid differences in the distribution of Man-6-*P* receptors from one cell type to another. It seems reasonable to assume that these differences reflect differences in the amounts and/or predominant routes of lysosomal enzyme trafficking among various cell types. Determining the basis for these differences in receptor distribution and lysosomal enzyme trafficking represents a great challenge for the future.

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Note Added in Proof. Experiments carried out on clone 9 cells to determine the location of endosomal membranes in the Golgi subfractions indicate that the major peak of Man-6-*P* receptors was well separated from the peaks of ^{125}I-labeled endosomal membranes.

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