The Endosomal Concentration of a Mannose 6-Phosphate Receptor Is Unchanged in the Absence of Ligand Synthesis

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Abstract. The cation-independent mannose-6-phosphate (Man-6-P) receptor is involved in the targeting of newly synthesized lysosomal hydrolases. To investigate the intracellular distribution of this receptor, a conjugate of lactoperoxidase coupled to asialoorosomucoid was used to catalyze its iodination within the endosomes of human hepatoma (HepG2) cells. The 215-kD, cation-independent Man-6-P receptor was iodinated by this procedure as shown by pentamannosyl-6-phosphate–Sepharose affinity chromatography and by immunoprecipitation of labeled cell extracts. The amount of this receptor detected in endosomes was found to be unchanged after inhibition of protein synthesis with cycloheximide. If the Man-6-P receptor accumulates in the Golgi apparatus in the absence of lysosomal hydrolase synthesis, it should have been correspondingly depleted from endosomes after a period of cycloheximide treatment, because these pools of receptor are in rapid equilibrium. Therefore, these data suggest that newly synthesized ligands are not required for the transport of the cation-independent Man-6-P receptor from the Golgi apparatus to endosomes.

PROTEINS destined for the plasma membrane, secretory vesicles, and lysosomes traverse a common pathway of intracellular transport until they reach the Golgi apparatus, where they are somehow sorted to their correct destinations. The targetting of proteins to lysosomes is presently understood in the greatest detail (see 20, 30, 37 for review). Upon arrival in the Golgi apparatus, newly synthesized lysosomal hydrolases are recognized by an *N*-acetylglucosaminyl phosphotransferase (16, 28) which adds *N*-acetylglucosamine phosphate to terminal α-1,2 mannose residues (7). A specific phosphodiesterase (35, 38) then removes the GlcNAc to generate the mannose-6-phosphate (Man-6-P)[1] residues that enable lysosomal enzymes to bind to Man-6-P receptors in the Golgi apparatus (20, 30, 37). It is likely that receptor-bound hydrolases are then transported to an acidic prelysosomal compartment, within which the ligand is released for transport to lysosomes, and the free receptor recycles back to the Golgi apparatus (20, 30).

Two types of Man-6-P receptors have been identified. One has been purified from a number of sources, and is a single polypeptide of 215,000 daltons (31, 34). However, the 215-kD receptor is not present in several cultured cell lines that adequately target their hydrolases to lysosomes (11). These cells enabled the discovery of a second Man-6-P receptor (17) which appears to be a glycoprotein composed of three 46-kD subunits (18). This receptor requires divalent cations, and has thus been termed Man-6-P receptor "CD" (cation dependent) to distinguish it from the 215-kD cation-independent, Man-6-P receptor "CI" (17).

1. *Abbreviations used in this paper*: ASOR, asialoorosomucoid; LPO, lactoperoxidase; Man-6-P, mannose-6-phosphate.

At steady state, the Man-6-P receptor^{CI} is located in the Golgi apparatus and in endosomes; a small fraction is present on the cell surface (3, 14, 41). Recent experiments suggest that the Man-6-P receptor^{CI} equilibrates rapidly between these compartments, because at least 90% of the 215-kD Man-6-P receptors contact extracellularly administered receptor antibodies within 90 min (29, 36). However, several reports have indicated that the receptor may accumulate in the *cis* Golgi under conditions in which synthesis of lysosomal hydrolases or the Man-6-P recognition marker is blocked (2, 4). These findings could only be explained if the Man-6-P receptor^{CI} was selectively retained in the Golgi complex and only exported after ligand binding.

To study the intracellular transport of the Man-6-P receptor^{CI}, we have employed an endosome-specific radiolabeling procedure to detect biochemically the 215-kD Man-6-P receptor when it is present in endosomes. To our surprise, we found that the steady-state concentration of the Man-6-P receptor^{CI} in endosomes of HepG2 cells was quantitatively unaltered under conditions in which lysosomal hydrolase synthesis was completely blocked. Thus, it appears that the 215-kD Man-6-P receptor cycles constitutively between intracellular compartments, independent of the presence of newly synthesized ligands.

Materials and Methods

Materials

Chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Na^{125}I and [^{35}S]methionine were from Amersham Corp. (Arlington Heights, IL). Phosphomannan from *Hansenula holstii* (NRRL

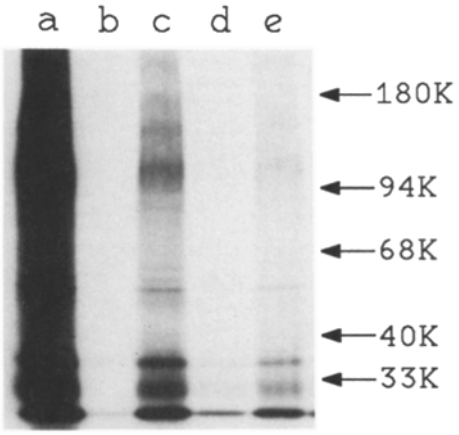

Figure 1. ASOR-LPO-mediated iodination. Reactions were carried out after incubating cells at 4°C for 90 min with (*a*) 18 μg/ml ASOR-LPO, (*b*) no ASOR-LPO, (*c*) 18 μg/ml ASOR-LPO plus 600 μg/ml ASOR, or (*d*) 18 μg/ml ASOR-LPO followed by a 5 mM EDTA wash at the end of the pretreatment period. The sample in lane *e* was incubated at 23°C instead of 4°C for 90 min with 18 μg/ml ASOR-LPO and then washed as in lane *d* to remove surface-bound conjugate. Numbers at right correspond to molecular mass in kilodaltons.

Y-2448) was the generous gift of Dr. Morey Slodki, U. S. Department of Agriculture, Northern Regional Laboratory, Peoria, IL. *Dictyostelium discoideum* cultures were provided by Dr. Catherine Berlot, Stanford University.

Cell Culture

HepG2 cells were grown and maintained as described by Schwartz et al. (32). Cells were harvested by passage through a 25-gauge needle after trypsinization to obtain uniform monolayers. 5E9 clone 11 cells (American Type Culture Collection, Rockville, MD) were grown as an ascites tumor in BALB/c mice.

Antibodies

Monoclonal transferrin receptor antibodies were purified from 5E9 clone 11 cell ascites fluid by DEAE-Affigel Blue (BioRad Laboratories, Richmond, CA) chromatography (5). Antibodies to the Man-6-P receptor^{CI} were generously provided by Yukiko Goda of this laboratory, and were prepared using Man-6-P receptor^{CI} purified to homogeneity from a detergent-solubilized, bovine liver total microsome fraction (9) by chromatography on a column of *D. discoideum* lysosomal enzymes linked to Affigel-10 (BioRad Laboratories) as described (18). The receptor was eluted with 2 mM Man-6-P in a buffer containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate instead of Triton X-100 to facilitate detergent removal by dialysis before immunization.

Preparation of Conjugate

Asialoorosomucoid (ASOR) coupled to lactoperoxidase (ASOR-LPO) was prepared exactly as described by Watts (39). The product corresponding to one molecule of LPO conjugated to one or two molecules ASOR was purified by chromatography on a 250-ml Sephacryl S-300 column run at 16 ml/h. The conjugate retained >95% of its original LPO activity, measured by the procedure of Hubbard and Cohn (19).

Endosome Labeling

The procedure of Watts (39) was employed using 10-cm dishes per reaction. Monolayers were incubated with conjugate for 2 h in a 23°C waterbath to "load" endosomes; iodinations were carried out at 4°C for 2 h. Culture dishes were placed onto ice-cooled steel blocks covered with water-soaked paper towels to maintain an iodination reaction temperature of ≤4°C. After terminating the reaction, cells were harvested directly into 1 ml, 0.15 M NaCl, 20 mM Na phosphate, 1% Triton X-100, 0.1% deoxycholate (buffer A) plus 5 mM KI, and frozen in liquid nitrogen for subsequent analyses.

Affinity Chromatography

Pentamannosyl-6-phosphate fragments of yeast phosphomannan were prepared according to Murray and Neville (27), and were derivatized with *p*-(aminophenyl)ethylamine (Aldrich Chemical Co., Milwaukee, WI) and coupled to cyanogen bromide activated Sepharose CL-4B (Sigma Chemical Co.) as described (3). Labeled extracts (0.4 ml) were chromatographed at 4°C on 0.5-ml columns in 50 mM imidazole, pH 7, 150 mM NaCl, 5 mM β-glycerophosphate, 0.05% Triton X-100 according to the procedure of Hoflack and Kornfeld (18).

Metabolic Labeling

Cell monolayers were rinsed once in PBS and then incubated for 16–18 h with [^{35}S]methionine (0.1 mCi/ml) in methionine-free medium containing 10% dialyzed fetal calf serum and supplemented with 5 μM methionine. At the end of the labeling period, the radioactive medium was replaced with complete alpha-MEM containing 10% fetal calf serum.

Other Techniques

Immunoprecipitations were carried out as described (22) except that *Staphylococcus aureus* cells were substituted for protein A-Sepharose. Protein was determined by the method of Lowry et al. (23) or Bradford (1) using BioRad reagent and bovine serum albumin as standard. Polyacrylamide gel electrophoresis was carried out by the method of Laemmli (21) using myosin (205 kD), bovine brain clathrin (180, 36, 33 kD), phosphorylase B (94 kD), bovine serum albumin (68 kD), tubulin (55 kD), ovalbumin (43.5 kD), and alcohol dehydrogenase (37 kD) as standards. Densitometry was carried out using a Quick Scan JR (Helena Laboratories, Beaumont, TX) scanning densitometer. Care was taken to ensure linearity of the response.

To determine the fraction of Man-6-P receptor that had contacted extracellularly administered antibody, cell monolayers were labeled with [^{35}S]methionine and then incubated with antisera for various times. Cells were then washed three times with PBS, harvested by addition of 1 ml of buffer A, and centrifuged at 150,000 *g* for 5 min. Samples were then split into two portions: one portion was incubated directly with fixed *S. aureus* cells plus 2 μl of preimmune serum; the other portion was incubated with an additional 2 μl of anti-Man-6-P receptor serum for 2 h at 20°C, followed by *S. aureus* cells. Samples were then handled as described for immunoprecipitations.

Results

Mellman and colleagues have shown that lactoperoxidase internalized by fluid-phase endocytosis can be used to label endocytic vesicle proteins selectively from within intact cells (24, 26). By conjugating lactoperoxidase to a specific ligand, Watts has adapted this procedure to make use of the advantages of receptor-mediated endocytosis (39). Unlike fluid-phase uptake, ligands internalized by receptor-mediated endocytosis can be concentrated within cells by virtue of their high-affinity association with specific cell surface receptors.

The receptor-mediated uptake and labeling procedure described by Watts (39) was employed to iodinate proteins within the endosome compartment of human hepatoma (HepG2) cells. Cells were incubated with a conjugate of asialoorosomucoid coupled to lactoperoxidase (ASOR-LPO). As shown previously (39), the conjugate bound to cell surface asialoglycoprotein receptors, because after binding at 4°C, ASOR-LPO could catalyze a cell surface iodination reaction in the presence of ^{125}I, glucose oxidase, and glucose (Fig. 1 *a*) that depended entirely upon the presence of the conjugate (Fig. 1 *b*), was greatly diminished by addition of excess ASOR (Fig. 1 *c*) and was eliminated after washing the cells with 5 mM EDTA, a treatment that interferes with receptor–ligand association (Fig. 1 *d*). If cells were allowed to endocy-

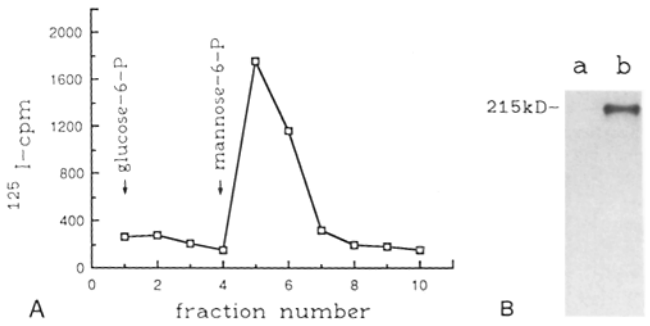

Figure 2. Pentamannosyl-6-phosphate–Sepharose chromatography of endosomal proteins. HepG2 cells were processed for endosome labeling as in Fig. 1 *e*. 1-ml fractions were collected; fractions were analyzed on a 6.5% acrylamide gel and a portion of the resulting autoradiograph is shown. Lane *a*, fraction 2; lane *b*, fraction 5.

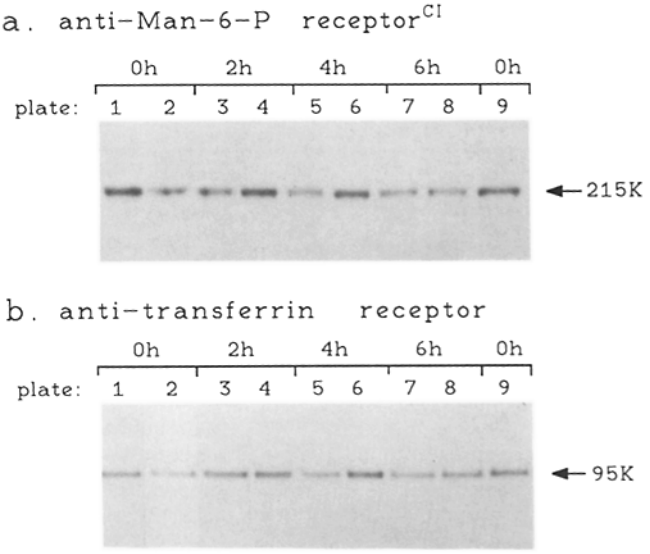

Figure 3. Effect of cycloheximide on endosomal Man-6-P and transferrin receptors. Individual plates of HepG2 cells were pretreated for the indicated times with 100 μg/ml cycloheximide and then processed for endosomal protein iodination as in Fig. 1 *e*. (*a*) Immunoprecipitation of Man-6-P receptor^{CI}; (*b*) immunoprecipitation of transferrin receptor. The plate samples used for immunoprecipitation in *a* are identical with those used in *b*. Plate 9 was incubated with ASOR-LPO in the absence of cycloheximide; all other ASOR-LPO incubations were carried out in the continued presence of cycloheximide.

tose ASOR-LPO at 23°C and were then washed with EDTA to remove cell-surface conjugate, endosome-specific iodination could be achieved after incubating intact cells with ^{125}I, glucose oxidase, and glucose (Fig. 1 *e*). Under these conditions, most of the ASOR-LPO is not transported beyond endosomes to lysosomes (8), and the ^{125}I and extracellularly generated H_2O_2 must permeate endosomal and plasma membranes for intraorganellar iodination (26). Given these permeability barriers, it is not unexpected that the level of endosomal iodination (Fig. 1 *e*) is much lower than that obtained at the cell surface (Fig. 1 *a*).

To verify further that the observed labeling was occurring intracellularly, an endosome-labeling reaction was carried out as before (as in Fig. 1 *e*), and at the end of the labeling period, cells were washed again with EDTA to remove any residual ASOR-LPO that might have remained on the cell surface. ASOR-LPO is self-iodinated during the labeling procedure, and can be used to monitor the cellular topography of the reaction (24). Gel electrophoresis and autoradiographic analysis did not detect any ASOR-LPO in the EDTA wash, even after long exposure (data not shown), confirming that the labeling reaction occurred within an intracellular compartment.

Watts has shown that the transferrin receptor is among the major polypeptides labeled within endosomes of HepG2 cells using this procedure (39). To determine whether the Man-6-P receptor^{CI} could also be detected in endosomes, HepG2 cells were processed for endosome labeling (as in Fig. 1 *e*), and the resulting extract was subjected to affinity chromatography on a pentamannosyl-6-phosphate-Sepharose column. The Man-6-P receptor^{CI} should bind selectively to this resin in the absence of divalent cations. As can be seen in Fig. 2 *A*, a peak of radioactive material was eluted from this column after addition of Man-6-P but not glucose-6-P, characteristic of this receptor. Analysis of the fractions by SDS-PAGE and autoradiography confirmed that the eluted material corresponded to the 215-kD Man-6-P receptor (Fig. 2 *B*). Therefore, the endosome-radiolabeling protocol led to iodination of the Man-6-P receptor^{CI} in HepG2 cell endosomes.

If the 215-kD Man-6-P receptor accumulates in the Golgi apparatus in the absence of lysosomal hydrolase synthesis, it should be correspondingly depleted from endosomes after a period of cycloheximide treatment. To test this, cells were treated with cycloheximide (100 μg/ml) for up to 6 h to permit complete transit of newly synthesized lysosomal hydrolases to lysosomes. A variety of hydrolases require only 1–3 h for complete lysosome delivery (10, 12, 33). The conditions employed blocked at least 98% of protein synthesis as measured by [^{35}S]methionine incorporation, and did not affect Man-6-P receptor turnover (data not shown).

Cells treated with cycloheximide for various times were incubated with ASOR-LPO for 2 h at 23°, washed with EDTA to remove surface-bound conjugate, and then processed as before for endosomal radiolabeling. Individual samples were then analyzed for both the Man-6-P receptor^{CI} (Fig. 3 *a*) and the transferrin receptor (Fig. 3 *b*). The level of the transferrin receptor in endosomes should not be affected by cycloheximide treatment in that this protein has been shown to cycle through the endocytic pathway at the same rate in the presence or absence of bound transferrin (40), and cycloheximide has no effect on the recycling of this receptor in the same cell line under virtually identical conditions (6). Therefore, the transferrin receptor serves as an internal standard for endosomal labeling.

The ratio of labeled Man-6-P receptor to transferrin receptor was found to be constant (compare Fig. 3 *a* with *b*). The observed differences between plates were due entirely to differences in cell number, as shown by protein determination; the radioactive incorporation per milligram protein was comparable for all samples (data not shown). Quantitation of the data presented in Fig. 3 showed that the relative endosomal concentrations of the Man-6-P receptor^{CI} and the transferrin receptor were unaffected by cycloheximide treatment (Fig. 4).

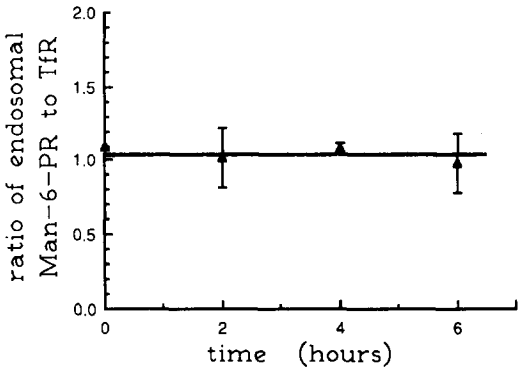

Figure 4. Quantitation of the effect of cycloheximide on endosomal Man-6-P and transferrin receptors. Data presented in Fig. 3, *a* and *b* were quantitated by densitometric scanning. The relative amounts of the two labeled receptors in a single sample after various times of treatment are shown in arbitrary units; *triangles* represent the average of duplicate samples.

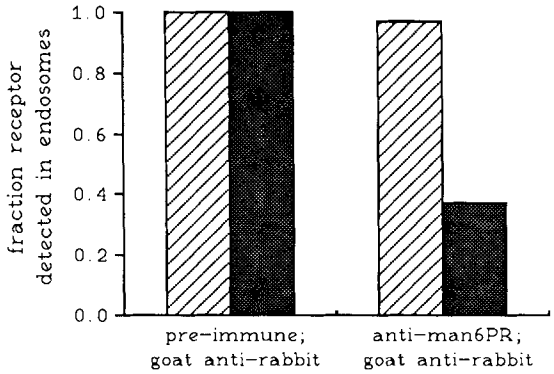

Figure 6. Effect of anti-Man-6-P receptor antibody treatment on the level of endosomal Man-6-P receptors. HepG2 cell monolayers were treated with either preimmune serum followed by goat anti-rabbit IgG (*left*) or anti-Man-6-P receptor serum followed by goat anti-rabbit IgG (*right*) as described in Fig. 5. Endosomal proteins were then radiolabeled with ^{125}I (as in Fig. 3) and analyzed by immunoprecipitation, gel electrophoresis, autoradiography, and densitometry. ^{125}I-transferrin receptor is shown in *hatched bars*; ^{125}I-Man-6-P receptor is shown in *solid bars*. The fraction of receptor detected after receptor antibody treatment (*right*) is presented relative to the control sample (*left*).

To determine whether the endosome labeling procedure would have been able to detect a decrease in the level of Man-6-P receptors, we sought alternative conditions under which the level of endosomal Man-6-P receptors would be decreased. Mellman and colleagues have demonstrated that the Fc receptor can be directed to lysosomes when cross-linked by bound antibodies (25). von Figura and co-workers have obtained similar results for the Man-6-P receptor, and have shown that fibroblasts can be virtually depleted of the Man-6-P receptor after antibody treatment (13, 36). As shown in Fig. 5, antibodies can also be used to deplete HepG2 cells of a large fraction of their total complement of Man-6-P receptors. When compared with cells treated with preimmune serum and goat anti-rabbit IgG (Fig. 5, *left*), HepG2 cells treated with anti-Man-6-P receptor serum contained 33% less receptor after a 5-h incubation; only one-third of the total receptor remained in these cells if a cross-linking second antibody was also employed. Treatment of cells with anti-Man-6-P receptor serum had no effect on the level of total cellular transferrin receptors (Fig. 5). The observed rate of antibody-induced, total receptor loss in HepG2 cells was very similar to that measured for fibroblasts (13).

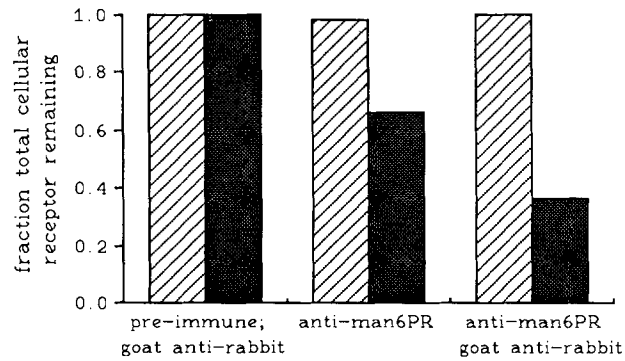

Figure 5. Antibody-mediated depletion of total cellular Man-6-P receptors. [^{35}S]methionine-labeled HepG2 cell monolayers were treated with either 10% preimmune serum followed by 50 μg/ml goat anti-rabbit IgG (*left*), 10% anti-Man-6-P receptor serum followed by preimmune serum (*center*), or 10% anti-Man-6-P receptor serum followed by 50 μg/ml goat anti-rabbit IgG (*right*). Primary antibody incubations were for 5 h; plates were then washed three times with PBS, and incubated for 3 h with the second antibody. Monolayers were then washed, harvested, and analyzed for total cellular [^{35}S]transferrin receptors (*hatched bars*) or total cellular [^{35}S]Man-6-P receptors (*solid bars*) by immunoprecipitation, gel electrophoresis, autoradiography, and densitometry.

To investigate the sensitivity of the endosome-labeling procedure, antibody treatment was used to deplete cells of a large fraction of their total cellular Man-6-P receptors, and the level of Man-6-P receptors remaining in endosomes was then determined. Under conditions that deplete cells of two-thirds of their Man-6-P receptors (Fig. 5), the level of Man-6-P receptors detected by the endosome-radiolabeling procedure also decreased by two-thirds, whereas the level of transferrin receptors was unchanged (Fig. 6). This experiment demonstrates that the endosome-specific radiolabeling procedure can detect a change in endosomal receptor content. Furthermore, the correspondence between the loss of total cellular Man-6-P receptors (66%) and the loss of endosomal Man-6-P receptors (67%) indicates that the pool of Man-6-P receptors labeled in this experiment was in equilibrium with the total cellular Man-6-P receptor pool.

The experiments described above demonstrate that the level of Man-6-P receptors present in endosomes does not change in the absence of new ligand synthesis. These findings are consistent with the notion that the Man-6-P receptor cycles continuously between cellular compartments, whether or not it is occupied. This conclusion would only be valid if the receptor remained in equilibrium with other cellular compartments in the absence of ligand synthesis. The ability of intracellular Man-6-P receptors to contact extracellular antibodies was used as a measure of the cycling of this receptor between cellular compartments. In this type of experiment, antibodies are used to mark the appearance of the receptor at the cell surface (or in endosomes), rather than to stimulate receptor degradation.

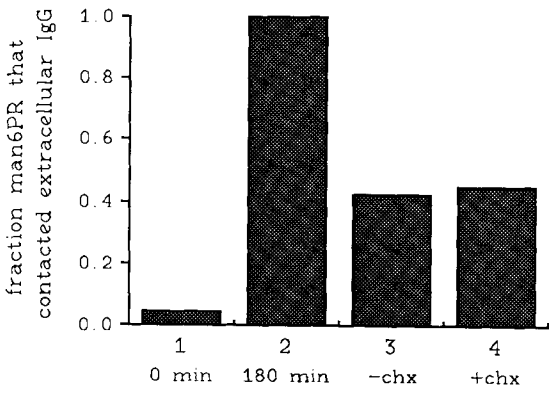

Figure 7. Effect of cycloheximide on the cycling of Man-6-P receptors to the cell surface and endosomes. Lanes *1* and *2*, [^{35}S]methionine-labeled Chinese hamster ovary cells were treated for either 0 or 180 min in the presence of 10% anti-Man-6-P receptor serum. Cells were then washed, harvested, and analyzed for the fraction of total cellular Man-6-P receptors that had contacted antibody. Lanes *3* and *4*, [^{35}S]methionine-labeled HepG2 cells were incubated for 3 h in the presence or absence of 100 μg/ml cycloheximide, washed three times in PBS, and then incubated for an additional 180 min with 10% anti-Man-6-P receptor serum in the continued presence or absence of cycloheximide. The fraction of the total Man-6-P receptor that had contacted receptor antibody was then determined.

As shown previously (29), virtually all of the Man-6-P receptors in Chinese hamster ovary (CHO) cells contact extracellular antibody within 180 min (Fig. 7). The process is somewhat slower in HepG2 cells: only 42% of the Man-6-P receptor contacted extracellular antibody in an equivalent time period. Most importantly, the same amount of receptor (44%) contacted extracellular antibodies in cells treated with cycloheximide. Gartung et al. (13) have obtained similar results using human fibroblasts. Therefore, the equilibrium of the Man-6-P receptor between cellular compartments appears to be unchanged in the absence of protein synthesis.

Discussion

Immunocytochemical experiments indicated that the Man-6-P receptor^{CI} accumulates in the Golgi apparatus under conditions in which synthesis of lysosomal hydrolases or the Man-6-P recognition marker is blocked (2, 4, but see also 15). This would require that the departure of the Man-6-P receptor from the Golgi apparatus be coupled to ligand binding. In an attempt to confirm these observations using biochemical methods, we have used an endosome-specific labeling procedure to detect and quantify the Man-6-P receptor^{CI} as well as the transferrin receptor in HepG2 cell endosomes. If the Man-6-P receptor accumulates in the Golgi complex, it should be concomitantly depleted from endosomes, because the receptor is in equilibrium between these compartments. Under conditions in which 98% of protein synthesis was blocked, and after incubation periods that should have been far beyond what was necessary to transfer most newly synthesized lysosomal hydrolases to lysosomes (10, 12, 33), we were unable to detect any alteration in the steady-state levels of either the Man-6-P or the transferrin receptor within endosomes. Analogous results were obtained using tunicamycin to block *N*-linked oligosaccharide addition and thus attachment of the Man-6-P recognition marker (data not shown).

Our inability to detect a change in the level of these receptors was not due to a lack of sensitivity of our detection method, because a decrease in endosomal Man-6-P receptors was observed in cells depleted of these receptors by cross-linking antibody treatment. Furthermore, the receptor-depletion experiments demonstrated that the pool of receptors labeled by ASOR-LPO was in equilibrium with total cellular Man-6-P receptors, in that the decrease in endosomal Man-6-P receptors paralleled the loss of total Man-6-P receptors within these cells. We cannot explain the discrepancy between these biochemical data and the immunocytochemical experiments of Brown and Farquhar (2, 4).

Endosomal levels of both labeled and unlabeled transferrin receptors were unchanged in the absence of protein synthesis (this work and Ciechanover et al. [6]). Therefore, the concentration of ASOR-LPO in endosomes must also have remained roughly constant, in that iodination levels are proportional to ASOR-LPO concentration. Because, at least by this criterion, cycloheximide treatment did not significantly alter endosome volume, we can conclude that not only the level, but also the actual concentration of the Man-6-P receptor remained unchanged.

The Man-6-P receptor transports lysosomal hydrolases from the Golgi complex to a prelysosomal compartment, and then returns to the Golgi complex for another round of transport. Yet a small amount of this intracellular receptor can also be detected at the cell surface and in peripheral endosomes, and such endocytic Man-6-P receptors equilibrate rapidly with those that are Golgi complex-derived. We do not yet know whether the prelysosomal compartment used by the Man-6-P receptor to unload lysosomal enzymes is in fact an endosome compartment shared with the endocytic pathway, or whether it represents a distinct, low pH compartment that communicates with late endosomes. The experiments described here involved radiolabeling of receptors within cellular compartments that had access to endocytosed ASOR-LPO after 2 h at 23°C. In that this temperature is somewhat higher than that originally reported for blockage of transport beyond endosomes (8), it is possible that the labeled receptors we studied were present not only in peripheral and late endosomes, but also in more distal compartments that might include a potentially distinct, prelysosome compartment. Further experiments will be needed to determine the precise localization of the radiolabeled Man-6-P receptors.

The steady-state measurements reported here indicate that the cellular distribution of the Man-6-P receptor is not affected by the presence or absence of transiting lysosomal enzymes. Furthermore, Man-6-P receptors in the Golgi apparatus, endosomes, and at the cell surface are in rapid equilibrium (29, 36), even in the absence of protein synthesis (this work and Gartung et al. [13]). Taken together, these findings strongly suggest that the Man-6-P receptor cycles continuously between intracellular compartments, with or without bound ligand. A major challenge for the future will be to identify the signals used to target Man-6-P receptors in the Golgi apparatus to a prelysosomal compartment, and then return them to the Golgi complex for another round of lysosomal hydrolase transport.

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Note Added in Proof: Concurrent with the submittal of this article for publication, other authors reported in this journal that unoccupied endosomal Man-6-P receptors recycle rapidly to the Golgi complex (Brown, W. J., J. Goodhouse, and M. G. Farquhar, 1986, *J. Cell Biol.* 103:1235–1247).

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