Cell- and Ligand-specific Dephosphorylation of Acid Hydrolases: Evidence That the Mannose 6-Phosphatase Is Controlled by Compartmentalization

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Abstract. Mouse L cells that possess the cation-independent mannose 6-phosphate (Man 6-P)/insulin-like growth factor (IGF) II receptor change the extent to which they dephosphorylate endocytosed acid hydrolases in response to serum (Einstein, R., and C. A. Gabel. 1989. *J. Cell Biol.* 109:1037–1046). To investigate the mechanism by which dephosphorylation competence is regulated, the dephosphorylation of individual acid hydrolases was studied in Man 6-P/IGF II receptor-positive and -deficient cell lines. ^{125}I-labeled Man 6-P–containing acid hydrolases were proteolytically processed but remained phosphorylated when endocytosed by receptor-positive L cells maintained in the absence of serum; after the addition of serum, however, the cell-associated hydrolases were dephosphorylated. Individual hydrolases were dephosphorylated at distinct rates and to different extents. In contrast, the same hydrolases were dephosphorylated equally and completely after entry into Man 6-P/IGF II receptor-positive Chinese hamster ovary (CHO) cells. The dephosphorylation competence of Man 6-P/IGF II receptor-deficient mouse J774 cells was more limited. β-Glucuronidase produced by these cells underwent a limited dephosphorylation in transit to lysosomes such that diphosphorylated oligosaccharides were converted to monophosphorylated species. The overall quantity of phosphorylated oligosaccharides associated with the enzyme, however, did not decrease within the lysosomal compartment. Likewise, β-glucuronidase was not dephosphorylated when introduced into J774 cells via Fc receptor–mediated endocytosis. The CHO and J774 cell lysosomes, therefore, display opposite extremes with respect to their capacity to dephosphorylate acid hydrolases; within CHO cell lysosomes acid hydrolases are rapidly and efficiently dephosphorylated, but within J774 cell lysosomes the same acid hydrolases remain phosphorylated. This difference in processing indicates that lysosomes themselves exist in a dephosphorylation-competent and -incompetent state. Man 6-P–bearing acid hydrolases endocytosed by the L^+ cells in the absence of serum were not distributed uniformly throughout the lysosomal compartment. The change in the dephosphorylation competence of L cells in response to serum suggests, therefore, that these cells contain multiple populations of lysosomes that differ with respect to their content of a mannose 6-phosphatase, and that serum factors affect the distribution of hydrolases between the different compartments.

THE transport of newly synthesized acid hydrolases to the lysosomal compartment requires the posttranslational attachment of a specific recognition marker, mannose 6-phosphate (Man 6-P),[1] to the enzymes' asparagine-linked high mannose oligosaccharides (Sly and Fischer, 1982; Kornfeld, 1987; von Figura and Hasilik, 1986). The Man 6-P–containing oligosaccharides mediate the subsequent interaction of the acid hydrolases with a Golgi-associated Man 6-P receptor and their diversion from the secretory to the lysosomal transport pathway (Gonzalez-Noriega et al., 1980; Brown and Farquhar, 1984; Brown et al., 1986; Willingham et al., 1981). Two separate Man 6-P receptors have been identified and purified; a 275-kD cation-independent (CI) (Sahagian et al., 1981; Steiner and Rome, 1982) and a 46-kD cation-dependent (CD) form (Hoflack and Kornfeld, 1985*a*). The CI receptor also is the receptor for insulin-like growth factor II (Tong et al., 1988; Morgan et al., 1987). Many cell lines possess both the CD and CI Man 6-P receptor while others contain only the CD form (Hoflack and Kornfeld, 1985*a*,*b*); each receptor directs newly synthesized enzymes to lysosomes but only the CI form mediates en-

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1. *Abbreviations used in this paper*: CD, cation dependent; CI, cation independent; FBS, fetal bovine serum; LY, Lucifer yellow; Man 6-P, mannose 6-phosphate; Man 6-Pase, Man 6-phosphatase; TR, Texas red.

docytosis of extracellular ligands (Stein et al., 1987; Kyle et al., 1988; Lobel et al., 1989). The existence of two separate receptors raises the possibility that they operate independently to form distinct populations of lysosomes. Lysosomal heterogeneity has been observed both morphologically and biochemically. Rat exocrine acinar lysosomes, for example, stain unequally with acid phosphatase substrates (Oliver and Yuasa, 1987) and rat adrenocortical lysosomes contain variable quantities of arylsulfatase and acid phosphatase when analyzed by x-ray microanalysis (Bacsy, 1982). In addition, separate lysosomal populations have been suggested on the basis that individual lysosomes differ with respect to entry and degradation of endocytic tracers (Storrie et al., 1986). Thus, all lysosomes are not created equal, and alternative targeting mechanisms may exist to account for this heterogeneity.

Within endosomal and lysosomal compartments, precursor forms of the acid hydrolases are proteolytically converted to their mature counterparts (Brown and Swank, 1983; Gieselman et al., 1983; Hasilik and von Figura, 1984) and the oligosaccharides may be dephosphorylated (Gabel et al., 1982; Gabel and Foster, 1986*a*,*b*). Cells deficient in the CI receptor are ineffecient in the removal of the Man 6-P recognition marker from newly synthesized acid hydrolases and accumulate phosphorylated enzymes intracellularly. In contrast, CI receptor-positive cells generally dephosphorylate newly synthesized acid hydrolases and, as a result, contain low steady-state levels of the phosphorylated species (Gabel et al., 1983). Differential dephosphorylation of acid hydrolases by the CI receptor-positive and deficient cell lines led to the suggestion that the CD receptor may operate in the formation of a class of lysosomes deficient in an acid Man 6-phosphatase (Man 6-Pase) whereas the CI receptor may transport hydrolases to Man 6-Pase–positive lysosomes (Gabel et al., 1983; Kornfeld, 1987). We previously identified a line of mouse L cells that contains the CI receptor but does not dephosphorylate endogenous acid hydrolases or molecules internalized via receptor-mediated endocytosis when the cells are maintained at high density (Gabel and Foster, 1986*a*). As such, the L cells constitute an exception to the generalization noted above. These same L cells, however, become dephosphorylation competent when they are maintained at low cell density in the presence of serum (Einstein and Gabel, 1989). To investigate the mechanism by which the L cells regulate dephosphorylation of their acid hydrolases, we compared the postendocytic processing of individual polypeptides introduced into CI receptor-positive and -deficient cells. The results are consistent with a model in which dephosphorylation of acid hydrolases is controlled, in part, by compartmentalization of the processing phosphatase and individual hydrolases to distinct endosomal and lysosomal locations.

Materials and Methods

Cells

CI Man 6-P receptor-positive mouse L cells (L^+ cells) were grown in αMEM containing 10% newborn calf serum, penicillin (100 u/ml), and streptomycin (100 meq/ml). Mouse J774 cells and L cells deficient in the CI Man 6-P receptor (L^- cells) were kindly provided by Drs. Samuel Silverstein (Columbia University, New York) and Stuart Kornfeld (Washington University, St. Louis, MO), respectively; both lines were maintained in MEM, 10% newborn calf serum. CHO cells were maintained in MEM containing 10% fetal bovine serum (FBS).

Receptor-mediated Endocytosis of ^{125}I-Ligands

^{125}I-Man 6-P–bearing proteins were prepared as described previously (Einstein and Gabel, 1989). The polypeptides were diluted with MEM and filter sterilized before addition to cells. L^+ cells were incubated with the labeled proteins at 37°C in the presence and absence of 5 mM Man 6-P on either 35- or 60-mm culture dishes; the cells were at or near confluence when exposed to the ligands unless stated otherwise. After the incubation the postuptake supernatant was removed, and the cells were washed with PBS (10 mM phosphate, pH 7.3, 150 mM NaCl) containing 2 mg/ml BSA followed by PBS alone. To identify cell-associated ligands, the cells were harvested with a rubber policeman, collected by centrifugation, and the cell pellet was dissolved directly in sample buffer (Laemmli, 1970) by bath sonication (three 15-s bursts) and boiled for 3 min. The resulting samples were analyzed by PAGE and radiolabeled polypeptides were detected by radioautography; lightning-plus intensifying screens (Dupont Instruments, Wilmington, DE) were used to enhance sensitivity. The extent of dephosphorylation of the cell-associated acid hydrolases was determined by chromatography of cell extracts on a CI Man 6-P receptor affinity column as detailed previously (Einstein and Gabel, 1989). To determine the extent of dephosphorylation of individual proteins, the nonbound and bound fractions from the affinity column were pooled separately, 20 μg of hemoglobin was added, and the samples were lyophilized. The resulting residue was suspended in 0.5 ml of ice cold 10% TCA and insoluble proteins were collected by centrifugation (8,000 *g* for 20 min). After washing twice with cold 95% ethanol the pellet was disaggregated in sample buffer and analyzed by SDS-PAGE and radioautography (Laemmli, 1970). In some cases the autoradiograms were scanned by densitometry and areas were determined by integration. Alternatively, the radioactive regions of the dried gel were excised and the radioactivity was quantitated in a γ counter. The two methods yielded comparable values as to the percent of each individual polypeptide that bound to the column.

Percoll Density Gradient Fractionation

L^+ cells (10^8 cells on a 15-cm plate) were incubated with the ^{125}I-Man 6-P–bearing ligands for 10 min, and then chased in ligand-free medium for the indicated times. The monolayers were washed as detailed above after which the cells were harvested with a rubber policeman. Cells were collected by centrifugation, washed with 0.25 M sucrose, and suspended in 7 ml lysis buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.0), and disrupted by 30 strokes of a Dounce homogenizer; the extract was centrifuged at 800 *g* for 5 min. The pellet was suspended in 4 ml lysis buffer, rehomogenized, and centrifuged. The postnuclear supernatants were combined and added to a solution containing 10.8 ml Percoll (Sigma Chemical Co., St. Louis, MO), 1.2 ml of 2.5 M sucrose, 0.4 ml of 1 M Hepes, pH 7.0; 0.25 M sucrose was added to adjust the total volume to 40 ml. The mixture was layered over 1.5 ml of 2.5 M sucrose and centrifuged in a rotor (model VTi50; Beckman Instruments, Inc., Palo Alto, CA) for 45 min at 22,000 rpm. The resulting gradient was divided into 2-ml fractions by pumping from the bottom of the tube.

Galactosyltransferase was assayed as described previously (Goldberg and Kornfeld, 1983). β-Hexosaminidase was assayed using *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide as substrate (Hall et al., 1978). The heavy (fractions 3–7) and light (fractions 18–22) peaks were pooled separately, adjusted to 0.4% in Triton X-100, and the Percoll was removed by centrifugation in a rotor (Model Ti70.1; Beckman Instruments, Inc.) for 4 h at 45,000 rpm. Proteins in the supernatants were precipitated by the addition of 100% TCA to a final concentration of 10%. The precipitates were collected by centrifugation, washed twice with cold 95% ethanol, and dissolved in sample buffer before analysis by SDS-PAGE and radioautography (Laemmli, 1970).

To localize the CI Man 6-P receptor within the Percoll gradient, a confluent 15-cm dish of L^+ cells was incubated for 3 h in 10 ml of glucose-free αMEM containing 10% FBS and 2 mCi of [2-^{3}H]mannose. To obtain sufficient quantities of the radiolabeled Man 6-P receptor, the L cells used for this experiment were maintained at a high cell density. We have shown previously that L^+ cells grown at high density exist in a dephosphorylation-incompetent state that is comparable to that observed at low density in the absence of serum (Einstein and Gabel, 1989). The labeled cells were chased for 30 min in complete αMEM, 10% FBS, and then harvested and fractionated as detailed above. Immunoprecipitation of the CI Man 6-P receptor from the Percoll gradient fractions was performed as described previously

(Gabel and Kornfeld, 1984). Briefly, the individual fractions were adjusted to 1% in Triton X-100, 50 mM in NaCl, and 0.1 mM PMSF by the addition of concentrated stock solutions. The colloidal silica subsequently was removed by centrifugation (100,000 *g* for 2 h) and the supernatants were collected and incubated overnight with rabbit anti-bovine liver CI Man 6-P receptor antiserum. Antigen-antibody complexes were recovered with Pansorbin and disaggregated in sample buffer. The immunoprecipitates were fractionated on a 7.5% polyacrylamide gel and the radiolabeled receptor was located by fluorography after soaking the gel in Amplify (Amersham Corp., Arlington Heights, IL).

Metabolic Labeling of Endogenous β-Glucuronidase

Mouse J774 cells (10-cm dishes; 60% confluence) were pulse-labeled with 0.5 mCi of [2-^{3}H]mannose in 3 ml of glucose-free MEM, 10% dialyzed FBS for 30 min at 37°C. After the pulse, the media was replaced with 7 ml of MEM, 1% newborn calf serum to initiate the chase. To harvest, the dish was placed on ice, the chase medium was removed (and clarified by centrifugation), and the attached cells were rinsed twice with cold PBS containing 2 mg/ml BSA. The cells were scraped from the dish into PBS, 2 mg/ml BSA, they were collected by centrifugation, and the pellet was frozen in a dry ice/ethanol bath and stored at −70°C. The frozen cell pellets subsequently were suspended in 1 ml of extraction buffer (25 mM Hepes, pH 7.0, 0.1 M NaCl, 1% Triton X-100, 0.2% deoxycholate, 0.2 mM PMSF, 5 mM PO_4, 5 mM Man 6-P) and the extracts were incubated for 30 min on ice before clarification for 5 min at 12,000 *g*. The supernatant was recovered and the pellet was suspended in 0.5 ml of extraction buffer by sonication and recentrifuged; corresponding supernatants were combined. β-Glucuronidase was immunoprecipitated from the detergent extracts and from the chase media as described previously (Gabel and Foster, 1986*b*). The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The regions of the dried gel that contained ^{3}H-labeled β-glucuronidase subunits were excised and the radioactivity was solubilized by pronase digestion (Gabel and Foster, 1986*b*). The resulting glycopeptides were desalted by Sephadex G-25 chromatography, and then fractionated on Con A-Sepharose. The columns (2 ml) were eluted sequentially with 10 ml of 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.02% NaN_3 (TBS; peak I), 10 ml of TBS containing 10 mM α-methylglucoside (peak II), and 12 ml of TBS containing 0.1 M α-methylmannoside at 56°C (peak III). High mannose-type glycopeptides (peak III) were desalted by Sephadex G-25 chromatography and digested with endoglycosidase H. The released oligosaccharides were applied to a QAE-Sephadex column (0.5 × 15 cm) equilibrated in 2 mM ammonium acetate, pH 5.3, and phosphorylated species that bound to the resin were eluted with a linear 200-ml gradient of ammonium acetate, from 2 to 350 mM in acetate, as described previously (Varki and Kornfeld, 1983; Gabel and Foster, 1986*b*).

Internalization of ^{125}I-β-Glucuronidase via the J774 Cell Fc Receptor

^{125}I-Man 6-P-bearing ligands were diluted with PBS to a final volume of 1 ml and filter sterilized. Rabbit anti-β-glucuronidase serum (IgG fraction, 48 mg/ml) was diluted with PBS to a final concentration of 1.17 mg/ml and filter sterilized. 0.1 ml of the ^{125}I-labeled ligand mixture was incubated with increasing amounts of antibody for 2 h at 4°C in a final volume of 0.2 ml. J774 cells, previously plated on 3.5-cm dishes, were washed with serum-free medium after which the ligand-antibody mixture was added (in a total volume of 0.3 ml of serum-free medium). After a 4-h incubation at 37°C, the post-uptake supernatant was removed and the cells were rinsed twice with ice cold PBS. The cells were scraped into 0.25 M sucrose with a rubber policeman and collected by centrifugation. The cell pellet was disaggregated in sample buffer (Laemmli, 1970), boiled for 3 min, and cell-associated ligands were analyzed by SDS-PAGE and radioautography.

Dephosphorylation of cell-associated β-glucuronidase was determined as follows. ^{125}I-ligands were incubated with anti-β-glucuronidase antibody for 2 h at 4°C (final IgG concentration of 0.39 mg/ml). The antigen-antibody mixture was added to J774 cells (6-cm dishes; 70% confluence) and incubated for 2 h at 37°C in serum-free medium. The medium was discarded and the cells were rinsed with 3 ml of MEM containing 10% serum. The cells were chased in 3 ml of MEM, 10% newborn calf serum for the indicated times, after which they were harvested as above. The cell pellets were frozen in a dry ice/ethanol bath and stored at −70°C. The samples were fractionated on the CI Man 6-P receptor affinity column as described previously (Einstein and Gabel, 1989).

Fluorescence Localization of Lysosomal Markers

Man 6-P-bearing acid hydrolases were isolated by receptor affinity chromatography as described earlier (Einstein and Gabel, 1989). 0.9 ml of ligand solution (containing 60 mU of β-glucuronidase activity in 50 mM phosphate, pH 7.0, 150 mM NaCl, 2 mM EDTA, 0.1 mM PMSF, 5 mM β-glycerophosphate, and 5 mM Man 6-P) was adjusted to 6.8 mM in biotin-*N*-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL) by the addition of 0.025 ml of a 250-mM stock solution in DMSO. After 2 h at room temperature, an additional 0.025 ml of the stock reagent was added and the reaction was continued for 2 h. The reaction mixture subsequently was dialyzed against two 1-liter changes of 20 mM Hepes, pH 7.0, 150 mM NaCl, 5 mM β-glycerophosphate at 4°C. The dialyzed ligands were adjusted to 1 mg/ml in ovalbumin and clarified by centrifugation; 50–60% of the starting β-glucuronidase activity was recovered with the biotinylated polypeptides.

L^+ cells were plated on glass coverslips at a density of 1×10^4 cells/cm^2 and cultured overnight in αMEM containing 10% FBS. The cells were incubated with 0.6 mg/ml Lucifer yellow (LY) in a modified αMEM containing 5 mM sodium bicarbonate, 20 mM Hepes, pH 7.0, 35 ng/ml transferrin, 5 ng/ml sodium selinite, 2 mg/ml BSA (supplemented serum-free medium), and 10% FBS. After 90 min at 37°C, the media was removed and the cells were washed twice with media containing 10% FBS. Fresh serum-containing media was added and the cells were incubated at 37°C for 30 min to chase the LY into lysosomes. At this point, cells to be conditioned in serum-free medium were washed twice with supplemented serum-free medium, and then incubated for 16 h in the same medium.

Biotinylated acid hydrolases (0.18 ml containing 37 mU/ml β-glucuronidase) were diluted with 0.06 ml of Texas red (TR)-streptavidin (Bethesda Research Laboratories, Gaithersburg, MD) and incubated for 45 min at 4°C. The mixture was diluted with 3.5 ml of supplemented serum-free medium and filter sterilized. 0.5 ml of this solution was diluted twofold with supplemented serum-free medium (± 10% FBS) and incubated with a coverslip of the LY-loaded cells for 90 min at 37°C; when present, Man 6-P was included at 5 mM. The cells were washed and chased with the appropriate serum-containing or serum-depleted media. Cells were photographed without fixation using a fluorescence microscope (Nikon Inc., Garden City, NY) equipped with an FITC (for LY) and TR optical cube; 5–15-s exposures were required for both fluorochromes.

Enzyme Digestions

N-glycanase digestion was performed by diluting 0.005 ml of SDS-disaggregated sample with 0.055 ml of 0.1 M sodium phosphate, pH 7.5, 0.11% SDS, 0.55% 2-mercaptoethanol, 5.5 mM EDTA, 1% Triton X-100, and adding 0.003 ml *N*-glycanase (250 u/ml from Genzyme Corp., Boston, MA). The digests were incubated overnight at 37°C after which 15 μg of an equal mixture of ovalbumin/hemoglobin was added and the proteins were precipitated with 0.007 ml of 100% TCA. Precipitates were collected by centrifugation, washed with cold acetone, dissolved in 0.05 ml of sample buffer (Laemmli, 1970), and analyzed by SDS-PAGE and radioautography.

Results

L^+ Cells Internalize and Modify ^{125}I-Man 6-P-bearing Polypeptides

Proteins that contain the Man 6-P recognition marker were isolated from the growth medium of J774 cells and were labeled with ^{125}I (Einstein and Gabel, 1989). The macrophage-like cell secretions contain many acid hydrolase activities including β-glucuronidase, β-galactosidase, β-*N*-acetylglucosaminidase, and cathepsin L (Gabel and Foster, 1986*b*; Jessup and Dean, 1980; Portnoy et al., 1986). Of the four major iodinated species, however, we were able to ascribe only one (72,000 M_r) to a known acid hydrolase, β-glucuronidase. Although their identity is unknown, the prolonged stability of the ^{125}I-polypeptides after endocytosis is consistent with their being lysosomal constituents (Einstein and Gabel, 1989), as nonlysosomal proteins are degraded rapidly within the environment of the lysosome (Murray and Neville, 1980). Accumulation of the ^{125}I-Man 6-P-bearing polypeptides by

Table I. Cell-dependent Accumulation of ^{125}I-Man 6-P–bearing Ligands

Cell type	Input cell-associated	
	−Man 6-P	+Man 6-P
	%	%
L^+	32.0	0.27
CHO	16.5	0.83
J774	0.15	0.14
L^-	0.19	0.18

The indicated cells were incubated with 10^6 cpm of the ^{125}I-Man 6-P–bearing ligands for 2 h in the presence and absence of 5 mM Man 6-P in a total volume of 1 ml, except for J774 cells, which were incubated for 3 h. The percent of the input radioactivity recovered with the cells is indicated.

individual cell lines was not universal and correlated with expression of the CI receptor. CI receptor-positive L cells (L^+ cells), for example, accumulated 32% of the input radioactivity after 2 h of incubation with the labeled ligands, and 5 mM Man 6-P reduced the accumulation to 0.27% (Table I). Likewise, CHO cells showed a large Man 6-P inhibitable accumulation of the radiolabeled polypeptides. In contrast, L^- cells and J774 macrophages which do not express the CI receptor (Gabel et al., 1983) did not accumulate radioactivity in a Man 6-P–inhibitable process (Table I). Endocytosis of the ^{125}I-Man 6-P–bearing ligands, therefore, required the presence of the CI Man 6-P receptor.

Accumulation of the ^{125}I-labeled polypeptides by the L^+ cells was linear for an initial 60 min of incubation and subsequently declined (Fig. 1 *A*). The plateau observed after 4 h suggested that high affinity ligands were depleted from the medium. Each of the major polypeptide species in the input ligand preparation was internalized by the cells (Fig. 1 *B*, compare lanes *1* and *2*), and Man 6-P blocked the accumulation of all species (Fig. 1 *B*, lane *9*). Internalization of the 38-kD species (p38), however, did not parallel its abundance within the input ligand preparation. At early times (Fig. 1 *B*, lanes *2* and *3*), for example, the cells contained similar quantities of the 70-kD polypeptide (β-glucuronidase) and p38, even though p38 was the most abundant of the input polypeptides (Fig. 1 *B*, lane *1*). Although both ^{125}I-labeled polypeptides bound to the receptor affinity column, the L^+ cells apparently internalized β-glucuronidase more efficiently than p38. At later times the cell-associated fractions contained novel protein species that were not present in the input ligand preparation (Fig. 1 *B*, compare lanes *1* and *8*). We suspected that the new species resulted from the proteolytic maturation of precursor acid hydrolases after entry into the cells (Brown and Swank, 1983; Gieselman et al., 1983; Hasilik and von Figura, 1984; Gabel and Foster, 1987). p38, for example, was converted to a 35-kD species and β-glucuronidase to a 68-kD form (Fig. 1 *B*). The 68-kD mature β-glucuronidase species was detected after only 5 min of incubation of the cells with the ligands (Fig. 1 *B*, lane *2*). In contrast, the maturation of p38 occurred more slowly. After 15 min of incubation p38 was not processed to its 35-kD form (Fig. 1 *B*, lane *3*), but the conversion was apparent at the 30-min point (Fig. 1 *B*, lane *4*). The 62-kD input species also disappeared after cell association (Fig. 1 *B*, lanes *6–8*) suggesting that it was converted to a lower molecular mass form, while the 52-kD protein remained unaltered. The electrophoretic mobility of the cell-associated and input forms of β-glucuronidase and p38 increased after *N*-glycanase digestion (Fig. 2). The difference in the apparent molecular weights between the input and cell-associated forms of the individual polypeptides, however, persisted after deglycosylation, suggesting that the postendocytic change in mobility

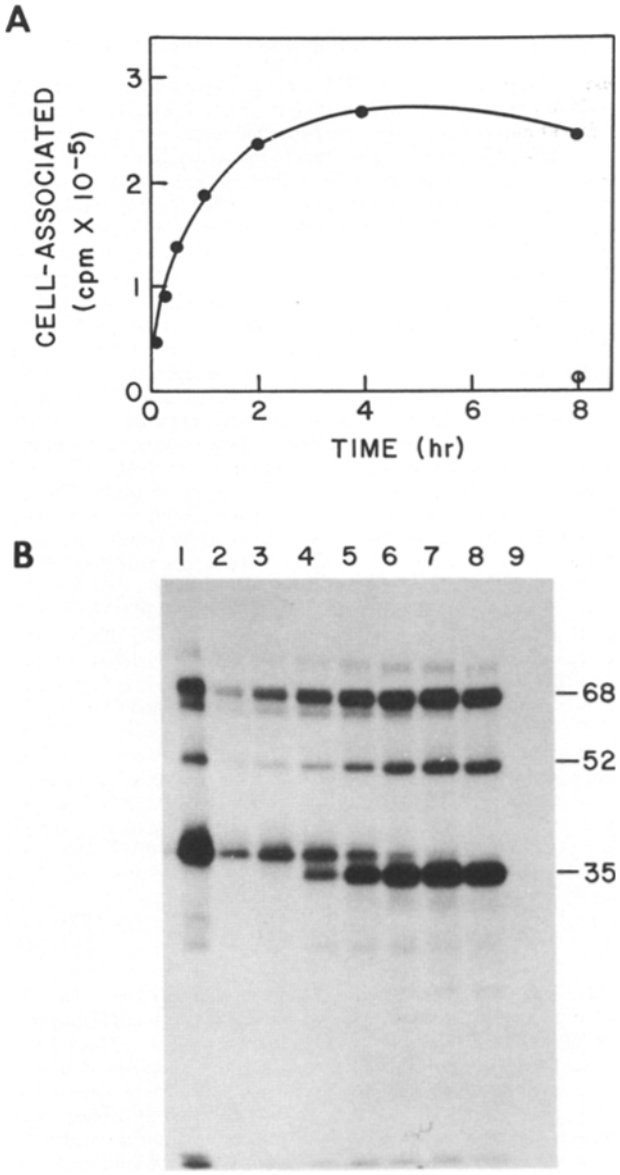


Figure 1. Analysis of cell-associated proteins after a continuous uptake of ^{125}I-Man 6-P–bearing ligands. (*A*) L^+ cells were grown to confluence on 60-mm dishes and incubated with 10^6 cpm of the ^{125}I-labeled ligands in the presence (○) and absence (●) of Man 6-P. At the indicated times, the cells were collected and disaggregated; total cell-associated radioactivity was determined for each time point. (*B*) Samples of the extracts were analyzed by SDS-PAGE. The autoradiogram shows the input ^{125}I-ligands (lane *1*), and the radiolabeled proteins recovered from the cell extracts after 5 (lane *2*), 15 (lane *3*), 30 (lane *4*), 60 (lane *5*), 120 (lane *6*), 240 (lane *7*), and 480 min of incubation in the absence (lane *8*) and presence (lane *9*) of 5 mM Man 6-P. An equal volume of each extract was loaded onto the gel. Migration positions of molecular mass standards are indicated (in kD) on the right.

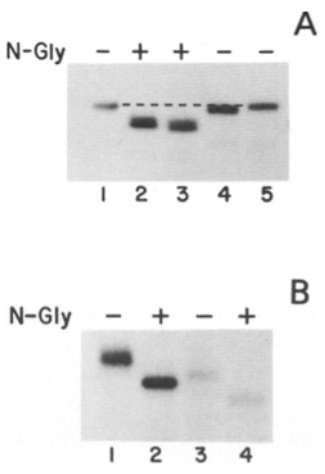


Figure 2. β-Glucuronidase and p38 are proteolytically processed after cellular association. (*A*) After a 1-h uptake and 1-h chase, β-glucuronidase was immunoprecipitated from the cell extract (lanes *3* and *4*) and from the input ligand preparation (lanes *1*, *2*, and *5*). The disaggregated immunoprecipitates were incubated in the presence (lanes *2* and *3*) and absence (lanes *1*, *4*, and *5*) of *N*-glycanase (*N-Gly*) and the digests were analyzed by SDS-PAGE; the autoradiogram is shown. (*B*) ^{125}I-labeled hydrolases recovered from the L^+ cells after a 10-min pulse and 50-min chase (lanes *3* and *4*) or from the input ligand preparation (lanes *1* and *2*) were incubated in the presence (lanes *2* and *4*) and absence (lanes *1* and *3*) of *N*-glycanase. The samples were analyzed by SDS-PAGE and the region of the autoradiogram containing p38 is shown.

corresponded to the normal proteolytic maturation of the precursor forms of the acid hydrolases and not to processing of asparagine-linked oligosaccharides (Fig. 2).

Serum-dependent Dephosphorylation

The fate of the Man 6-P recognition marker attached to ligands endocytosed by L^+ cells is affected by extracellular serum factors (Einstein and Gabel, 1989). Thus, in the presence of serum the ^{125}I-labeled polypeptides were dephosphorylated (dephosphorylation competent; dish B, Table II). In contrast, the same polypeptides were not dephosphorylated when the cells were maintained in the absence of serum (dephosphorylation incompetent; dish A, Table II). Likewise, cells cultured at high density in the presence of serum become dephosphorylation incompetent, presumably by depletion of factors from the medium that are necessary to maintain the dephosphorylation capability (Einstein and Gabel, 1989). To determine whether hydrolases endocytosed in the absence of serum would remain phosphorylated after exposure of the recipient cells to serum, low density L^+ cells were incubated in series with ^{125}I- and ^{131}I-labeled polypeptides. L^+ cells in the dephosphorylation-incompetent state were exposed to ^{125}I-labeled ligands for 1 h, after which the cells were chased in serum-free medium. After a 2-h incubation to allow the ligands to accumulate within lysosomes, serum-containing medium was added to induce dephosphorylation competence. ^{131}I-Labeled hydrolases then were incubated with the cells and chased into lysosomes, and the extent of dephosphorylation of the radiolabeled polypeptides ultimately was determined by receptor affinity chromatography. The ^{125}I- and ^{131}I-labeled species were dephosphorylated to similar extents as 12 and 14%, respectively, of the cell-associated molecules bound to the receptor affinity column (dish C, Table II). Reversal in the order of addition of the radiolabeled ligands did not alter the extent of dephosphoryla-

Table II. Serum-dependent Dephosphorylation of Cell-associated Hydrolases

Dish	First ligand introduced	Switched to serum	Second ligand introduced	Bound ^{125}I	Bound ^{131}I
				%	%
A	^{125}I	No	None	71	–
B	None	Yes	^{125}I	12	–
C	^{125}I	Yes	^{131}I	12	14
D	^{131}I	Yes	^{125}I	11	12
E	^{125}I	Yes	None	17	–

Low density L cells (10^4/cm^2) were preconditioned overnight in serum-free medium. The cells subsequently were incubated with the first radiolabeled ligand preparation for 1 h. The acid hydrolases were labeled either with ^{125}I or ^{131}I. After a 2-h chase to allow the internalized polypeptides to accumulate within lysosomes, the cells were switched to serum-containing medium. 2 h later, the monolayers were incubated with the second radiolabeled ligand preparation for 1 h and chased for an additional 6 h. At the end of the chase, the cells were harvested, disrupted by sonication, and the soluble components were fractionated on the CI Man 6-P receptor affinity column. The percentage of each isotope that bound and eluted with Man 6-P is indicated.

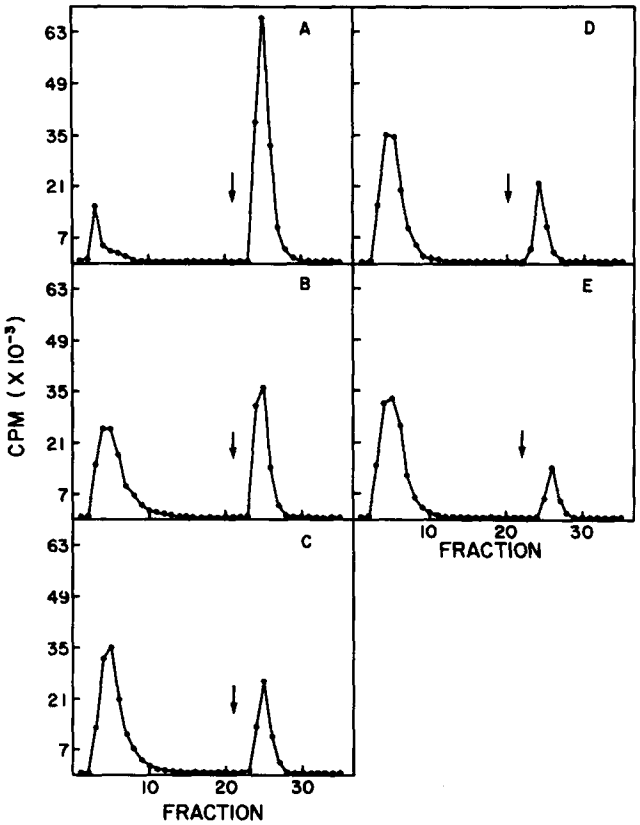


Figure 3. Man 6-P receptor affinity chromatography of cell-associated ^{125}I-labeled polypeptides. ^{125}I-Man 6-P-bearing hydrolases were incubated with L^+ cells for 1 h, and then chased in ligand-free media. At the indicated times, the cells were harvested and soluble extracts were applied to the Man 6-P receptor affinity column. The profiles show the distribution of the cell-associated ^{125}I radioactivity after 0 (*A*), 4 (*B*), 8 (*C*), 14 (*D*), and 24 (*E*) h of chase. The arrows indicate where 5 mM Man 6-P was applied. To rule out the possibility that the endogenous ligands completed with the ^{125}I-labeled polypeptides for the receptor binding sites, a small portion of the 24-h nonbound fraction was rechromatographed. 95% of the radioactivity again failed to bind to the receptor column (not shown), indicating that this fraction was devoid of high affinity ^{125}I-ligands.

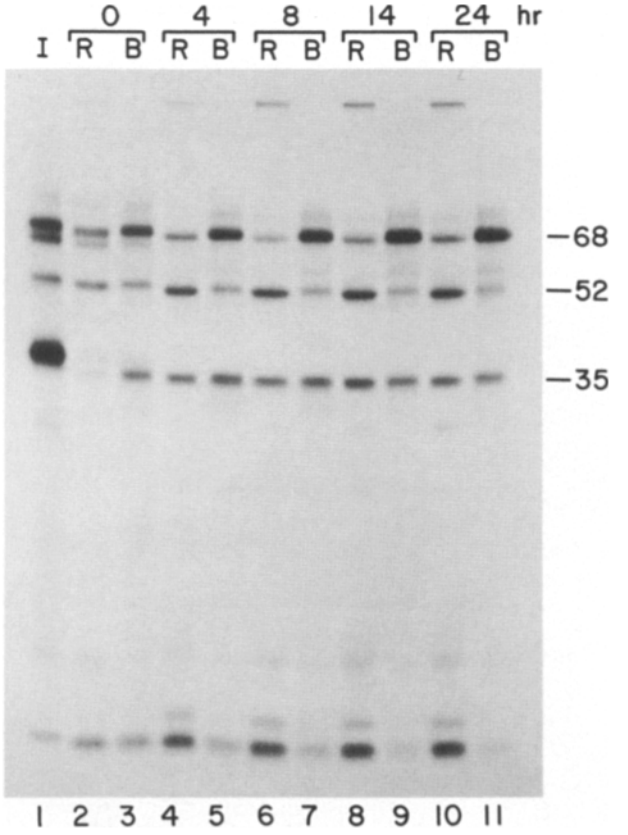


Figure 4. Comparison of the nonbound and bound fractions of the Man 6-P receptor affinity column. Proteins in the nonbound and bound fractions from Fig. 3 were analyzed on a 12.5% polyacrylamide–SDS gel. The autoradiogram of the gel shows (lane *1*) the input ligands; (lanes *2*, *4*, *6*, *8*, and *10*) the nonbound fractions at *0*-, *4*-, *8*-, *14*-, and 24-h chase, respectively; (lanes *3*, *5*, *7*, *9*, and *11*) the bound fractions at *0*-, *4*-, *8*-, *14*-, and 24-h chase, respectively.

tion (dish D, Table II). Moreover, addition of the second ligand was not required to observe dephosphorylation of the first (dish E, Table II). Thus, acid hydrolases endocytosed by dephosphorylation-incompetent cells were dephosphorylated when the cells attained dephosphorylation competence.

Individual Polypeptides Are Dephosphorylated at Separate Rates and to Different Extents

To analyze the dephosphorylation kinetics of individual glycoproteins, L^+ cells were pulse-loaded with the ^{125}I-labeled polypeptides and cell extracts were chromatographed on the Man 6-P receptor affinity column after various times of chase. The L^+ cells in these experiments were maintained at an intermediate density that resulted in a partial dephosphorylation. After a 60-min loading, 82% of the cell-associated radioactivity bound to the receptor affinity column and eluted with 5 mM Man 6-P (Fig. 3 *A*). Binding to the column, however, decreased progressively during the chase (Fig. 3, *B–D*), such that only 17% of the ^{125}I-labeled species eluted with Man 6-P after 24 h (Fig. 3 *E*). Overall, dephosphorylation of the ^{125}I-labeled polypeptides occurred with a half-time of 10.7 h. The nonbound and bound fractions of the

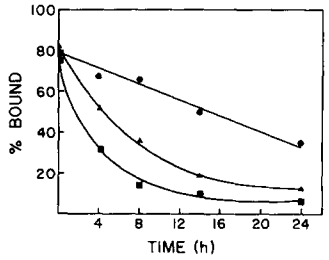


Figure 5. Quantitation of the binding of individual hydrolases to the Man 6-P receptor affinity column. The autoradiogram in Fig. 4 was scanned by densitometry and the total amount of the 68-kD protein (β-glucuronidase, ●), the 35-kD protein (p38, ▲), and the 52-kD protein (■) recovered in the nonbound and bound fractions at each time point was calculated. The percentage of each protein recovered in the bound fraction at the indicated time of chase is shown.

affinity column were analyzed by SDS-PAGE and radioautography to determine whether β-glucuronidase and p38 were dephosphorylated equally. After the 1-h pulse, the majority of the two polypeptides were proteolytically processed and bound to the receptor affinity column (Fig. 4, lanes *2* and *3*). During the subsequent chase both proteins were dephosphorylated and, as a result, were recovered in the nonbound fraction of the affinity column. p38, however, partitioned into the nonbound fraction more rapidly than β-glucuronidase; as a result, β-glucuronidase became the predominant polypeptide species recovered in the bound fractions (Fig. 4, lanes *3*, *5*, *7*, *9*, and *11*). Quantitation of individual proteins indicated that β-glucuronidase slowly appeared in the nonbound fraction at a rate of 2%/h, while p38 and a 52-kD species appeared with apparent first-order kinetics and half-times of 8.2 and 6.7 h, respectively (Fig. 5). In four separate experiments the same rank order in the rates of dephosphorylation of β-glucuronidase and p38 were observed such that β-glucuronidase was always dephosphorylated more slowly and less extensively than p38. The absolute extent of dephosphorylation, however, varied as would be expected from the density dependence (Eistein and Gabel, 1989). Many other ^{125}I-labeled proteins paralleled the behavior of p38. A group of smaller proteins (11, 12, and 16 kD), for example, were dephosphorylated much faster and to a greater extent than

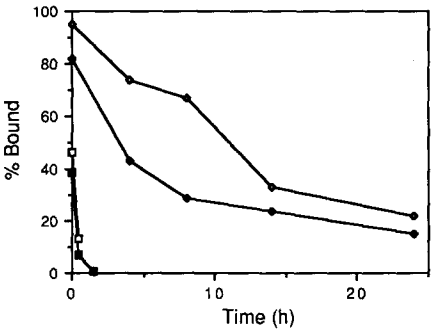


Figure 6. The rate and extent of ligand dephosphorylation is cell dependent. L^+-cells (♦,◇) or CHO cells (□,■) were incubated for 1 h or 30 min, respectively, with the ^{125}I-labeled ligands, and then chased in ligand-free media. Extracts prepared from cells harvested at the indicated times were fractionated on the Man 6-P receptor affinity column. The bound and nonbound peaks were assessed for their content of β-glucuronidase (◇,□) and the percent that bound to the affinity column is indicated. The percentage of the total cell-associated ^{125}I that bound to the affinity column also is indicated (♦,■).

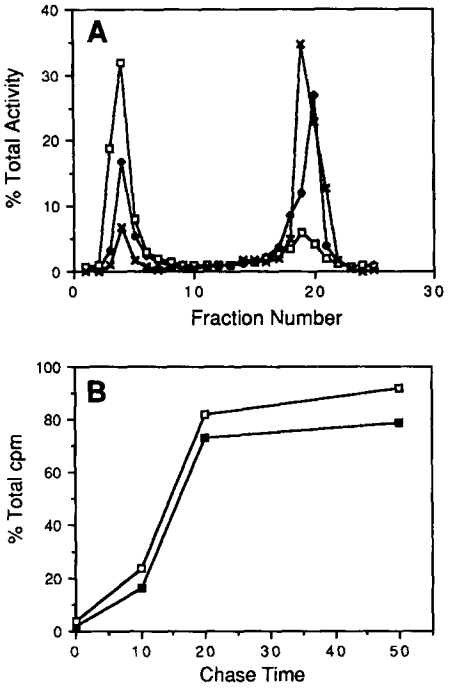


Figure 7. Endocytosed ligands accumulate within lysosomes. (*A*) Cells were incubated for 15 min with ^{125}I-ligands after which a cell extract was prepared and fractionated on a Percoll density gradient. The distribution of galactosyltransferase (*X*), β-hexosaminidase (□), and ^{125}I (♦) is indicated. Fraction 1 corresponds to the bottom of the gradient. (*B*) L$^+$ cells were incubated with the ^{125}I-ligands for 10 min, and then chased in fresh, unlabeled medium for the indicated times. The percentage of β-glucuronidase (□) and p38 (■) that was recovered in the heavy peak (fractions 3–7) as a function of chase time is shown.

β-glucuronidase (Fig. 4). Relative to all of the cell-associated radiolabeled species, a higher percentage of β-glucuronidase bound to the affinity column at all times of chase (Fig. 6).

Internalized Polypeptides Are Transported to Lysosomes

Ligands transported via the surface CI Man 6-P receptor are expected to reach lysosomes (Willingham et al., 1981). The differential rate and extent of dephosphorylation of the internalized ^{125}I-labeled hydrolases could arise, therefore, from an inability of the cells to transport β-glucuronidase to lysosomes. To determine the kinetics at which the ^{125}I-labeled polypeptides were delivered to lysosomes, extracts of the L$^+$ cells were fractionated by Percoll density gradient centrifugation (Einstein and Gabel, 1989). After a 15-min incubation with the ^{125}I-labeled polypeptides, 60% of the cell-associated radioactivity sedimented with light vesicles that were distinct from the denser lysosomal vesicles which contained endogenous β-*N*-acetylglucosaminidase (Fig. 7 *A*). Vesicles that contained the radiolabeled polypeptides sedimented to the same region of the gradient as vesicles that contained the Golgi marker activity galactosyltransferase (Fig. 7 *A*). This distribution is expected since 15 min is an insufficient amount of time for the majority of the endocytosed molecules to reach lysosomes, and endosomes are lighter than lysosomes on Percoll gradients (Schmid et al., 1988). During a subsequent chase, however, the cell-associated radioactivity accumulated within the denser lysosomal vesicles. β-Glucuronidase and p38 were transferred to the lysosomal organelles with comparable efficiency and kinetics. Within 20 min of chase, >70% of each polypeptide species sedimented with the dense lysosomal fractions (Fig. 7 *B*). 10–20% of each of the radiolabeled species remained associated with light vesicles even after 50 min of chase (Fig. 7 *B*). These molecules are assumed to be associated with a light population of lysosomes; 18% of the endogenous β-*N*-acetylglucosaminidase also sedimented in this region of the gradient (Fig. 7 *A*). Thus, both β-glucuronidase and p38 accumulated in dense lysosomes with similar kinetics after endocytosis by the L$^+$ cells.

Dissociation of phosphorylated acid hydrolases from the CI Man 6-P receptor occurs more slowly than dissociation of insulin from its receptor after endocytosis (Borden et al., 1990). The slow dissociation kinetics suggest that the Man 6-P receptor and its ligand remain associated within early endosomes and only dissociate within late, prelysosomal endosomes. Moreover, BHK cell CI Man 6-P receptor is located within late endosomal structures that are distinct morphologically from early endosomes (Griffiths et al., 1988). The CI Man 6-P receptor never is found associated with lysosomes (Brown et al. 1986). To address the possibility that the ^{125}I-labeled hydrolases internalized by the L cells in the absence of serum remained within late endosomes rather than accumulating within lysosomes, the distribution of the CI Man 6-P receptor on the Percoll gradient was assessed. [^{3}H]Mannose-labeled L cells were subjected to the same subcellular fractionation protocol described above and the receptor subsequently was recovered by immunoprecipitation. The ^{3}H-labeled receptor was found exclusively at the top of the gradient corresponding to the fractions that contained light lysosomes and galactosyltransferase (Fig. 8); no labeled receptor was recovered in fractions that contained the dense population of lysosomes. Based on this distribution, we conclude that the dense vesicles that contain the in-

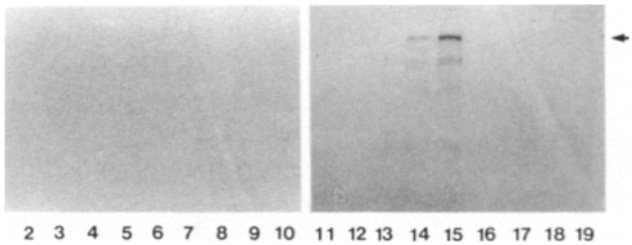


Figure 8. Distribution of the CI Man 6-P receptor on the Percoll gradient. Mouse L$^+$ cells metabolically labeled with [^{3}H]mannose were disrupted by homogenization and the resulting extract was fractionated by Percoll density gradient centrifugation. The CI Man 6-P receptor was immunoprecipitated from individual fractions recovered from the gradient, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. The fluorograms show the distribution of the ^{3}H-labeled receptor; the numbers indicate fraction number (bottom of the gradient = fraction 1) and the arrow denotes the position of purified bovine liver CI Man 6-P receptor detected by Coomassie blue staining of the SDS gel. During the immunoprecipitation fraction 16 was lost and, as a result, no sample was loaded into lane *16*.

Table III. Characterization of J774 Cell β-Glucuronidase Oligosaccharides

Source	Chase time	Total cpm	Con A-Sepharose Peak I	Peak II	Peak III	Peak III anionic	2:1 ratio
	h		%	%	%	%	
Cell	1	22,100	6	10	84	32	1.2
	3	7,100	9	16	75	33	0.65
Medium	1	18,700	9	18	73	39	3.1
	3	37,000	7	18	75	41	3.4

J774 cells were labeled for 30 min with [^{3}H]-mannose, and then chased for 1 or 3 *h* in the absence of the labeled monosaccharide. β-Glucuronidase was immunoprecipitated from detergent extracts of the cells (*Cell*) or from the chase medium (*Medium*) and the immunoprecipitates were fractionated by SDS-PAGE. Regions of the dried gel containing β-glucuronidase were excised and the radioactivity was solubilized by pronase digestion. The resulting glycopeptides were fractionated on Con A-Sepharose, and the high mannose-type units (peak III) were digested with endoglycosidase H and fractionated by QAE-Sephadex chromatography. The percentage of peak III radioactivity that bound and eluted as anionic phosphorylated oligosaccharides is indicated. The 2:1 ratio indicates the amount of radioactivity recovered as diphosphorylated oligosaccharides divided by the sum of the monophosphorylated species (Lazzarino and Gabel, 1988).

ternalized ^{125}I-hydrolases are lysosomal rather than endosomal structures.

CHO Cells Dephosphorylate ^{125}I-Man 6-P-bearing Hydrolases

The slower dephosphorylation of β-glucuronidase relative to the other polypeptides did not reflect a slower rate of transport to lysosomes. If, on the other hand, the slow dephosphorylation of β-glucuronidase reflects that this hydrolase is a poor substrate for the Man 6-Pase, then one would expect to observe the differential processing after introduction of the hydrolases into any cell (assuming that the same Man 6-Pase is present). The loss of the Man 6-P recognition marker from β-glucuronidase, therefore, was analyzed after internalization of the ^{125}I-labeled polypeptides by CHO cells. These cells efficiently dephosphorylate endogenous and endocytosed acid hydrolases, and they do not change their dephosphorylation competence in response to serum conditions (Einstein and Gabel, 1989). ^{125}I-Man 6-P-bearing polypeptides were internalized by CHO cells in a Man 6-P-inhibitable process (Table I), and the internalized ligands underwent the same proteolytic maturations as observed in the L$^+$ cells (not shown). The extent of dephosphorylation, however, was more complete in CHO cells. After a 30-min pulse, 39% of the CHO cell-associated ^{125}I-labeled polypeptides bound to the receptor affinity column, and the percentage declined after 30 and 90 min of chase to 7 and <1%, respectively (Fig. 6). Fractions from the affinity column were analyzed by SDS-PAGE and radioautography to quantitate β-glucuronidase individually. The distribution of β-glucuronidase within the nonbound and bound fractions paralleled the distribution of total cell-associated radioactivity (Fig. 6). Thus, after a 30-min pulse and a 30-min pulse/30-min chase, 48 and 14%, respectively, of β-glucuronidase bound to the affinity column. All of the radiolabeled polypeptides, therefore, were effective substrates for the CHO cell lysosomal Man 6-Pase.

J774 Cells Partially Dephosphorylate Endogenous β-Glucuronidase

By analogy to other cells that lack the CI Man 6-P receptor, J774 cells are not expected to dephosphorylate their newly synthesized acid hydrolases (Gabel et al., 1983). To verify that their endogenous hydrolases remained phosphorylated, J774 cells were labeled with [2-^{3}H]mannose and the fate of the phosphorylated oligosaccharides attached to β-glucuronidase was determined. After a 30-min pulse and a 60-min chase, 49% of the ^{3}H-labeled β-glucuronidase was recovered in the media, and 84% of the molecules were extracellular after 3 h of chase (Table III). The pronounced secretion of the ^{3}H-labeled molecules indicated that the J774 cells were inefficient in the delivery of newly synthesized acid hydrolases to lysosomes as are other CI receptor-deficient cell lines (Goldberg and Kornfeld, 1981; Gabel et al., 1983; Kyle et al., 1988; Lobel et al., 1989). Glycopeptides generated from the immunoprecipitated polypeptides were fractionated on Con A-Sepharose. 73-75% of the [^{3}H]mannose-derived radioactivity associated with the secreted molecules eluted as high mannose-type glycopeptides (peak III; Table III), and only 25% of the radioactivity was associated with complex-type units (peaks I and II; Table III). After the 1-h chase, 84% of the radioactivity recovered from the intracellular polypeptides eluted from Con A-Sepharose as high mannose-type units. The higher percentage of high mannose structures relative to the secreted polypeptides (84 vs. 75%) suggested that some of the intracellular molecules remained at a pre-Golgi location after the 1-h chase; high mannose oligosaccharides are converted to complex-type units within the Golgi apparatus (Kornfeld and Kornfeld, 1985). After 3 h of chase, however, the intracellular and secreted β-glucuronidase molecules contained the same percentage of high mannose-type units, indicating that all of the pulse-labeled molecules had passed through the Golgi apparatus.

To examine the state of phosphorylation, the high mannose-type glycopeptides were digested with endoglycosidase H and the released oligosaccharides were fractionated on QAE-Sephadex; phosphorylated high mannose-type species bind to the anion exchange resin and elute in characteristic locations (Goldberg and Kornfeld, 1983; Varki and Kornfeld, 1983). Secreted β-glucuronidase contained three major phosphorylated oligosaccharide species corresponding to units with one phosphomonoester group, units with two phosphomonoester groups, and sialylated/phosphomonoester hybrid-type units (Fig. 9, *C* and *D*). The ratio of diphosphorylated to monophosphorylated oligosaccharides (the 2:1 ratio) associated with the secreted molecules was 3.1 and 3.4 after 1 and 3 h of chase, respectively (Table III). Oligosaccharides associated with intracellular β-glucuronidase molecules after the 1-h chase contained species with one and two phosphodiester groups in addition to the phosphomonoester species (Fig. 9 *A*). The presence of the phosphodiester species provided further evidence that some of the newly synthesized molecules remained within the ER and early Golgi apparatus after the 1-h chase; the conversion of a phosphodiester to a monoester occurs within the Golgi apparatus (Goldberg and Kornfeld, 1983; Waheed et al., 1981). After the 3-h chase, however, intracellular β-glucuronidase contained predominantly the phosphomonoester species (Fig. 9 *B*), suggesting that all of the newly synthesized molecules had migrated

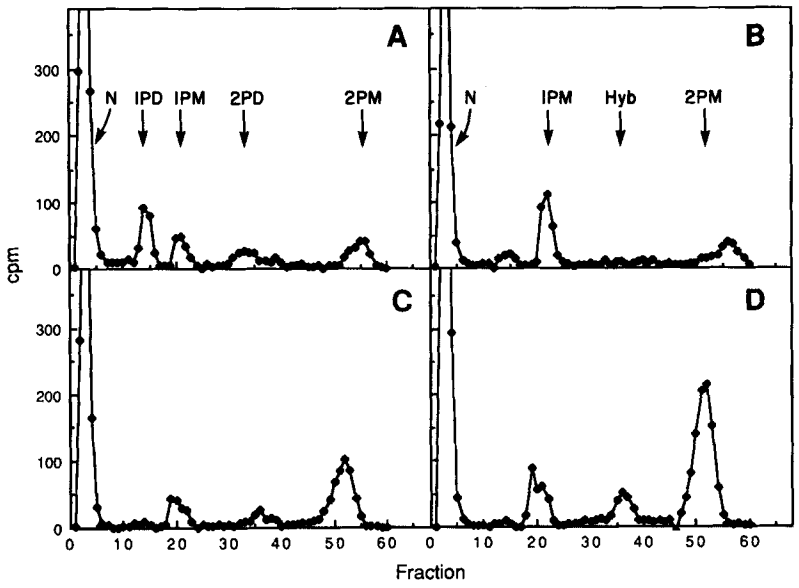


Figure 9. QAE-Sephadex chromatography of β-glucuronidase high mannose-type oligosaccharides. J774 cells were pulsed-labeled with [^{3}H]mannose for 30 min, and then chased for 1 (*A* and *C*) or 3 (*B* and *D*) h. After the chase β-glucuronidase was immunoprecipitated from the cells (*A* and *B*) or from the chase medium (*C* and *D*) and the high mannose-type oligosaccharides associated with the enzyme were fractionated by QAE-Sephadex chromatography. The elution positions of neutral oligosaccharides (*N*) and units containing 1 phosphodiester (*IPD*), 1 phosphomonoester (*IPM*), two phosphodiesters (*2PD*), one phosphomonoester/one sialic acid residue (*Hyb*), and 2 phosphomonoesters (*2PM*) are indicated.

through the Golgi apparatus and had reached lysosomes. Relative to the secreted molecules, intracellular β-glucuronidase contained a similar percentage of anionic oligosaccharides (32 vs. 40%; Table III), signifying that the intracellular and secreted forms of the acid hydrolase contained the same number of phosphorylated units. Qualitatively, however, the phosphorylated oligosaccharides differed. The 2:1 ratio of cell-associated β-glucuronidase oligosaccharides was only 0.65 after the 3-h chase and was much lower than the 3.4 ratio associated with the secreted molecules (Table III).

β-Glucuronidase Internalized via the J774 Cell Fc Receptor Retains the Man 6-P Recognition Marker

To demonstrate that endocytosed acid hydrolases could, like the endogenous molecules, remain phosphorylated within lysosomes, ^{125}I-labeled polypeptides were introduced into J774 cells. Since these cells lack the CI Man 6-P receptor and do not internalize the ^{125}I-labeled polypeptides in a Man 6-P-dependent process (Table I), an alternative receptor was used to mediate transport. Antigen–antibody com-

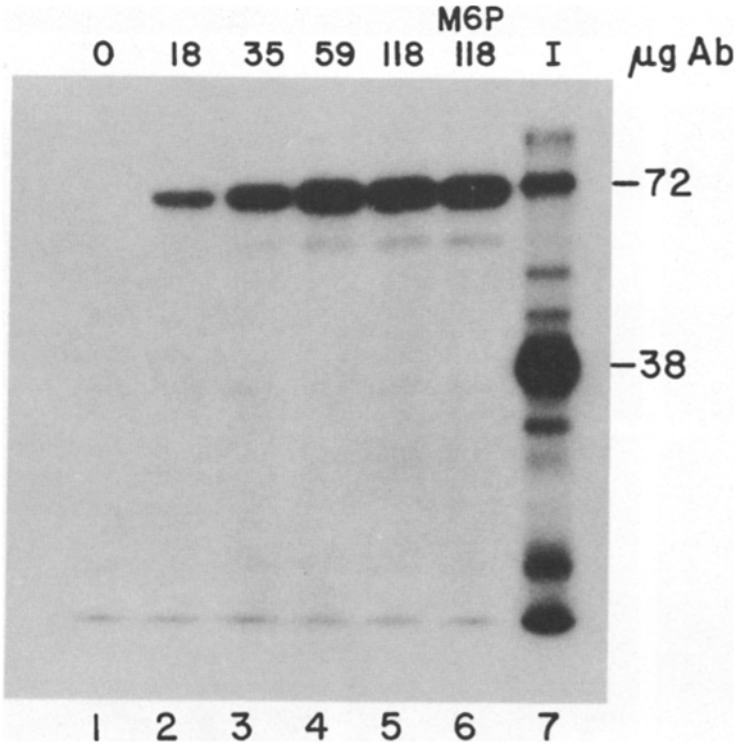
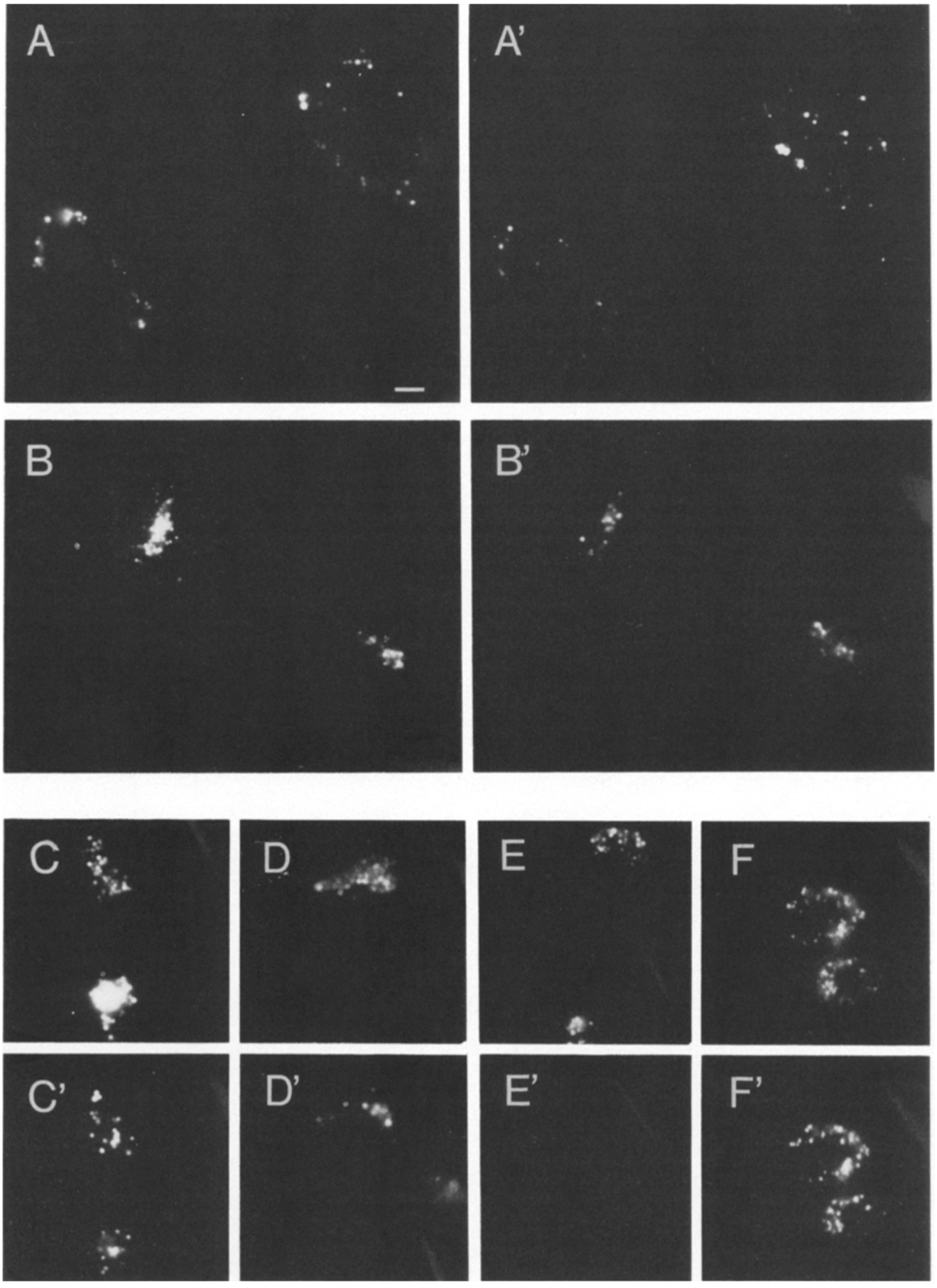


Figure 10. Internalization of ^{125}I-β-glucuronidase via the J774 cell Fc receptor. ^{125}I-Man 6-P polypeptides were mixed with the indicated amounts of anti-β-glucuronidase antibody (*Ab*) for 2 h at 4°C. Mouse J774 cells (on 35-mm dishes) were incubated with the mixture in serum-free medium for 4 h. One plate contained 5 mM Man 6-P (lane *6*; M6P). After the incubation cell extracts were analyzed by PAGE. An autoradiogram of the dried gel is shown. Input labeled polypeptides (*I*) are shown in lane *7*, and the numbers on the right represent the apparent molecular masses (in kD) of the indicated protein species.


A
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plexes are internalized and delivered to lysosomes in cells that express the Fc receptor (Mellman and Plutner, 1984). We took advantage of this receptor to selectively introduce β-glucuronidase into the Fc receptor-containing J774 cells (Mellman et al., 1984). The ^{125}I-Man 6-P polypeptides were preincubated with increasing quantities of rabbit anti-β-glucuronidase antisera after which the mixtures were added to the J774 cells. In the absence of the antisera, no β-glucuronidase became cell associated (Fig. 10, lane *1*). Preincubation with the antiserum, however, led to a dose-dependent increase in the level of cell-associated β-glucuronidase (Fig. 10, lanes *2–5*). Other polypeptides present in the input ligand preparation were not internalized by the cells in the presence of the antiserum, indicating that the cellular accumulation resulted from the formation of a specific antigen–antibody complex. Man 6-P did not inhibit internalization of ^{125}I-β-glucuronidase via the Fc receptor (Fig. 10, lane *6*).

The phosphorylation state of the cell-associated β-glucuronidase molecules was determined by Man 6-P receptor affinity chromatography. After a 2-h incubation of the cells with the antigen–antibody complexes, 93% of the cell-associated radioactivity bound to the receptor affinity column. A subsequent chase in the absence of the extracellular antigen–antibody complexes did not alter either the total quantity of cell-associated radioactivity or the percentage of the molecules that bound to the receptor affinity column. After 6 h of chase, 93% of the cell-associated ^{125}I-β-glucuronidase bound to the affinity column. Like the endogenous newly synthesized acid hydrolases, therefore, the Fc receptor–internalized β-glucuronidase molecules were stable within J774 cell lysosomes and were not dephosphorylated. The dephosphorylation-incompetent state was observed even though the J774 cells were maintained in the presence of serum during the chase.

Evidence of Lysosomal Inequality in L⁺ cells

To examine the possibility that L⁺ cells contained diversity within their lysosomal compartment, the intracellular distribution of acid hydrolases internalized via the CI Man 6-P receptor was compared to the distribution of a lysosomal content marker, LY. This fluorescent molecule enters cells via fluid phase endocytosis and is delivered to lysosomes where it persists (Swanson, 1989). L⁺ cells were loaded with LY in the presence of serum, and then conditioned overnight in the presence or absence of serum components. Biotinylated Man 6-P–bearing acid hydrolases were incubated with the LY-loaded cells for 90 min, after which the cells were chased in ligand-free medium for 30 min. Intracellular sites of biotinylated ligand accumulation were visualized with TR-streptavidin; the cells were incubated simultaneously with the fluorescent conjugate and biotinylated ligands and, as a result, were viewed directly without fixation or permeabilization. When LY-loaded cells were preconditioned in serum-free medium, the biotinylated ligands were localized within a subset of LY-positive lysosomes (Fig. 11, *A–C*). The LY-positive lysosomes were scattered throughout the cytosol (Fig. 10 *A*) or clustered near the nucleus of the serum-deprived cells (Fig. 11, *B* and *C*). Cells in both formations consistently displayed fewer TR-positive lysosomes, and these corresponded to a subset of the LY-positive lysosomes (Fig. 11, *A′–C′*). Increasing the time of chase before the cells were photographed to 210 min did not increase the level of correspondence between the LY- and TR-positive lysosomes (Fig. 11, *D* and *D′*). Moreover, when the LY-loaded cells were incubated with the biotinylated ligands in the presence of Man 6-P, no TR-streptavidin was internalized (Fig. 11, *E* and *E′*); the internalization of TR-streptavidin, therefore, was dependent on the Man 6-P receptor-mediated accumulation of the biotinylated ligands. When the LY-loaded cells were preconditioned in serum-containing medium, on the other hand, the pattern of the two lysosomal markers was similar both in number and distribution (Fig. 11, *F* and *F′*). These data indicate, therefore, that the L⁺ cells maintained two soluble lysosomal markers in a state of nonequilibrium in the absence of serum, but in the presence of serum factors the two markers achieved a similar distribution.

Discussion

After production within the rough ER, newly synthesized acid hydrolases experience a number of posttranslational modifications that affect both their protein and carbohydrate components. These modifications occur in an ordered series of reactions that reflect, in part, localization of the processing enzymes to specific compartments within the vacuolar system (Kornfeld and Kornfeld, 1985; Goldberg and Kornfeld, 1983; Waheed et al., 1981). The initial phosphorylation of high mannose–type oligosaccharides, for example, occurs within a pre-Golgi compartment and results in the attachment of a single phosphodiester group to a mannose residue positioned to the α,3-branch of the core β-linked mannose

Figure 11. Demonstration of lysosomal heterogeneity within mouse L cells. L⁺ cells were plated at 10^4 cells/cm^2 and cultured overnight. The cells subsequently were incubated with 0.6 mg/ml of LY for 90 min, and then chased from 30 min in fresh medium devoid of LY; up to this point, the cells were maintained in the presence of serum. The monolayers then were washed three times and incubated for 16 h in serum-free medium to induce dephosphorylation incompetence. The serum-deprived monolayers were incubated simultaneously with biotinylated Man 6-P–bearing acid hydrolases and TR-labeled strepavidin for 90 min (in the presence or absence of 5 mM Man 6-P) after which the cells were incubated in ligand- and serum-free media. After the appropriate chase period, representative cells were photographed sequentially under FITC (for LY fluorescence; *A–F*) and TR (*A′–F′*) optics. Cells in *A–C* were chased for 30 min in the absence of biotinylated ligand, and the cell in *D* was chased for 210 min. In every case there are fewer LY-positive vesicles than there are TR-positive vesicles. Cells in *E* were incubated simultaneously with the biotinylated acid hydrolases, TR-streptavidin, and 5 mM Man 6-P. These cells stain positively for LY (*E*) but not for TR (*E′*). Cells in *F* were not preconditioned in serum-free medium. Rather, after loading with LY these cells were maintained for 16 h in the presence of serum; the biotinylated acid hydrolases also were introduced while the cells were bathed in serum. Notice that the distribution of the two fluorochromes is similar within these cells both in terms of relative intensity and number of fluorescent vesicles. Bar, 5 μm.

residue. After transport of the hydrolases to the Golgi compartment, a second phosphodiester group may be added to a mannose residue within the α1,6-branch of the β-linked mannose, and the phosphodiester groups subsequently are converted to their monoester counterparts (Lazzarino and Gabel, 1988, 1989). Likewise, many acid hydrolases are synthesized as proenzymes and the proteolytic conversion to the mature counterparts occurs beyond the Golgi apparatus within endosomal and lysosomal compartments (Brown and Swank, 1983; Gieselman et al., 1983). The Man 6-P–containing hydrolases isolated from the J774 secretions underwent protein-specific cleavages after endocytosis that also appeared to occur in separate compartments. β-Glucuronidase, for example, was processed rapidly after endocytosis from a 72-kD precursor to a 68-kD mature form. The rapidity of this processing is consistent with the proteolytic maturation occurring within a prelysosomal compartment (Gabel and Foster, 1987). In contrast, p38 was processed slowly to a 35-kD species; the kinetics of this maturation suggest a lysosomal event.

Degradation of the phosphorylated units also occurred in discrete steps that resulted, in part, from compartmentation of the processing phosphatase. We demonstrated previously that β-glucuronidase oligosaccharides were dephosphorylated within a prelysosomal compartment after endocytosis of the hydrolase via the cell surface CI Man 6-P receptor (Gabel and Foster, 1986*b*). This prelysosomal processing led to a qualitative change in the structure of the phosphorylated units as diphosphorylated oligosaccharides were converted to monophosphorylated species, but no change in the quantity of the phosphorylated units occurred. The mechanism by which the prelysosomal phosphatase is governed such that monophosphorylated units are not processed to nonphosphorylated species remains unknown. Endogenous β-glucuronidase produced by CI Man 6-P receptor-deficient J774 cells underwent a similar limited dephosphorylation during transport between the Golgi apparatus and the lysosomal compartment. β-Glucuronidase secreted by these cells contained predominantly diphosphorylated oligosaccharides as evidenced by the high ratio of diphosphorylated to monophosphorylated oligosaccharides (2:1 ratio >3). In contrast, after 3 h of chase, a time sufficient for the newly synthesized molecules to reach lysosomes (Brown and Swank, 1983; Gabel and Foster, 1987), the intracellular β-glucuronidase molecules contained a lower 2:1 ratio (0.65). If the oligosaccharides associated with the secreted molecules are assumed to be representative of those species attached to molecules that bind to the Golgi-associated Man 6-P receptor, then the diphosphorylated oligosaccharides attached to β-glucuronidase were converted to monophosphorylated species between the Golgi and lysosomal compartments. Thus, both endocytosed and newly synthesized β-glucuronidase undergo a limited dephosphorylation within a prelysosomal compartment. The intracellular location of this processing phosphatase remains unknown, but the enzyme apparently is a component of an endosomal compartment that is common to both the biosynthetic and endocytic pathways. Previous studies indicated that the two pathways converge within a late endosomal compartment (Griffiths et al., 1988). Moreover, since β-glucuronidase delivered by endocytosis to this compartment arrives via the CI Man 6-P receptor whereas newly synthesized β-glucuronidase produced by J774 cells arrives via the CD Man 6-P receptor (these cells lack the CI Man 6-P receptor), both receptors appear to deliver their ligands to a common phosphatase-positive prelysosomal compartment. The two receptors are known to coexist within intracellular compartments as antibodies prepared against the cytoplasmic tail of the CI Man 6-P receptor precipitate vesicles that also contain the CD receptor (Messner et al., 1989).

The subsequent fate of the phosphorylated oligosaccharides within the lysosomal compartment depends upon the cell type and the polypeptide. CHO and J774 cells display opposite extremes with respect to lysosomal dephosphorylation. CHO cells contain both the CI and CD Man 6-P receptors (Duncan and Kornfeld, 1988) and they efficiently dephosphorylate their endogenous acid hydrolases (Gabel et al., 1983). Likewise, these cells efficiently dephosphorylated the ^{125}I-labeled polypeptides introduced by endocytosis. CHO cell lysosomes, therefore, must contain a processing Man 6-Pase. In contrast, J774 cells which contain only the CD Man 6-P receptor did not dephosphorylate endogenous β-glucuronidase or ^{125}I-labeled molecules introduced by Fc receptor–mediated endocytosis within the lysosomal compartment. The J774 lysosomes, therefore, must contain an inactive form of the Man 6-Pase or lack the enzyme all together. This differential processing of the ^{125}I-labeled hydrolases indicates that the same enzymes can exist within lysosomes either in a phosphorylated or nonphosphorylated state.

Mouse CI Man 6-P receptor-positive L cells, on the other hand, display a complex and changeable dephosphorylation competence. ^{125}I-labeled polypeptides introduced into these cells by the CI Man 6-P receptor were dephosphorylated when the recipient cells were maintained at low density in the presence of serum. When maintained at a high cell density or in serum-free medium, however, the same ^{125}I-labeled species were not dephosphorylated (Einstein and Gabel, 1989). L^+ cells, therefore, change their dephosphorylation competence in response to extracellular components, and the transition does not require new protein synthesis (Einstein and Gabel, 1989). Surprisingly, the rate and extent to which individual ^{125}I-labeled polypeptides are dephosphorylated varies within the L^+ cells. β-Glucuronidase, for example, was dephosphorylated less rapidly and less extensively than p38 under any set of growth conditions. The differential dephosphorylation of β-glucuronidase relative to the other ^{125}I-labeled polypeptides was not observed after endocytosis of the same polypeptides into CHO cells. In the latter cells, dephosphorylation of β-glucuronidase paralleled the dephosphorylation of the other ^{125}I-labeled species. Thus, β-glucuronidase is a suitable substrate for the processing Man 6-Pase. The poor dephosphorylation of β-glucuronidase relative to p38 in the L^+ cells also did not result from the differential transport to lysosomes. The two polypeptides were transported to dense lysosome-like structures at a comparable rate and to a similar extent after endocytosis.

The serum requirement for the dephosphorylation reaction in L^+ cells may arise from a serum-dependent activation of a latent Man 6-Pase. Man 6-P–bearing acid hydrolases internalized by serum-deprived L^+ cells, however, were not distributed uniformly amongst all lysosomes. Rather, the acid hydrolases were restricted to a subset of LY-laden cellular lysosomes. In the presence of serum, on the other hand, the intracellular distributions of the two lysoso-

mal markers were similar. The differential distribution observed in the absence of serum thus indicates that the soluble content of the L^+ cell lysosomes can exist in a state of nonequilibrium. This behavior contrasts that observed previously in CHO cells; soluble lysosomal components within these cells rapidly exchange under the appropriate circumstances (Ferris et al., 1987). Interestingly, CHO cell lysosomes do not display a serum dependence in their ability to dephosphorylate endocytosed acid hydrolases (Einstein and Gabel, 1989). These cells, therefore, may not possess the capacity to segregate lysosomal contents. The L^+ cells, however, altered the extent of dephosphorylation in response to serum factors and they restricted the mixing of their lysosomal components. Based on the presence of distinct populations of Man 6-Pase–positive and Man 6-Pase–deficient lysosomes in CHO and J774 cells, respectively, the differential dephosphorylation of the ^{125}I-labeled polypeptides in L^+ cells may indicate that these cells contain both types of lysosomes, and that individual acid hydrolases distribute unequally between them. In this model, β-glucuronidase has a higher probability to be delivered to Man 6-Pase–deficient lysosomes than does p38, but the sorting efficiency is not absolute and is subject to change. Thus, in the absence of serum ligands are delivered primarily to Man 6-Pase–deficient lysosomes, but in the presence of serum the delivery mechanism favors Man 6-Pase–positive lysosomes. Moreover, the contents of the two lysosomal populations are not segregated permanently. Acid hydrolases internalized by cells in the dephosphorylation-incompetent state were dephosphorylated when the cells subsequently were exposed to serum. It is interesting to note that β-glucuronidase isolated from different human tissue sources varies greatly in terms of the percentage of steady-state molecules that remain phosphorylated. Platelet-derived β-glucuronidase, for example, contains a high percentage of phosphorylated enzyme while the same hydrolase isolated from liver is primarily dephosphorylated (Brot et al., 1974; Kaplan et al., 1977). Cells within an organism, therefore, must regulate the dephosphorylation of their acid hydrolases and the tissue culture cell lines recapitulate a natural processing variation. Further experimentation will be required to determine the mechanism by which the two lysosomal states of dephosphorylation competence are achieved and the significance of the differential processing.

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