Abstract. The mannose 6-phosphate (Man 6-P) receptor operates to transport both endogenous newly synthesized acid hydrolases and extracellular enzymes to the lysosomal compartment. In a previous study (Gabel, C. A., and S. A. Foster, 1986, J. Cell Biol., 103:1817-1827), we noted that β-glucuronidase molecules internalized by mouse L-cells via the Man 6-P receptor undergo a proteolytic cleavage and a limited dephosphorylation. In this report, we present evidence that indicates that the postendocytic alterations of the acid hydrolase molecules occur at a site through which the enzymes pass en route to the lysosomal compartment. Mouse L-cells incubated at 20°C with β-glucuronidase (isolated from mouse macrophage secretions) internalize the enzyme in a process that is inhibited by Man 6-P but unaffected by cycloheximide. As such, the linear accumulation of the ligand observed at 20°C appears to occur through the continued recycling of the cell surface Man 6-P receptor. The subcellular distribution of the internalized ligands was assessed after homogenization of the cells and fractionation of the extracts by density gradient centrifugation. In contrast to the accumulation of the ligand within lysosomes at 37°C, the β-glucuronidase molecules internalized by the L cells at 20°C accumulate within a population of vesicles that sediment at the same density as endocytic vesicles. Biochemical analysis of the internalized ligands indicates that: (a) the subunit molecular mass of both β-glucuronidase and β-galactosidase decrease upon cell association relative to the input form of the enzymes, and (b) the β-glucuronidase molecules experience a limited dephosphorylation such that high-mannose-type oligosaccharides containing two phosphomonoesters are converted to single phosphomonoester forms. The same two postendocytic alterations occur after the internalization of β-glucuronidase by human I-cell disease fibroblasts, despite the low acid hydrolase content of these cells. The results indicate, therefore, that acid hydrolases internalized via the Man 6-P receptor are processed within the endocytic compartment. In that endogenous newly synthesized acid hydrolases display similar alterations during their maturation, the results further suggest that the endosomal compartment is involved in the sorting of ligands transported via both the cell surface and intracellular Man 6-P receptor.

Newly synthesized acid hydrolases undergo several posttranslational modifications that affect both their protein and carbohydrate components during transit from the rough endoplasmic reticulum to lysosomes. Localization of the enzymes responsible for the processing reactions has been instrumental in delineating the intracellular trafficking pathway. The acid hydrolases are synthesized in the rough endoplasmic reticulum as proenzymes containing asparagine-linked, high-mannose-type oligosaccharides (10, 24, 41, 45). Within 20 min of their biogenesis, the hydrolases receive a specific recognition marker, mannose 6-phosphate (Man 6-P), which ultimately directs the enzymes to the lysosomal compartment (17, 29, 35, 46, 51). The two enzymes involved in the generation of the Man 6-P marker, UDP-N-acetylglucosamine:lysosomal enzyme phosphotransferase and α-N-acetylglucosamine phosphoglycosidase, are associated with the Golgi apparatus and are restricted to the cis and medial compartments of the organelle (18, 26, 38, 48, 49, 52). While in the Golgi apparatus, many of the nonphosphorylated high-mannose-type oligosaccharides on the newly synthesized acid hydrolases are processed to complex-type units containing galactose and sialic acid residues (25, 31). The oligosaccharide-processing enzymes responsible for the attachment of these residues are restricted to the trans face of the Golgi apparatus (9, 42, 43), indicating that acid hydrolases containing such structures migrate through the entire organelle during their maturation. Before exiting the Golgi apparatus (4, 15), or possibly within the trans-Golgi network (22), the acid hydrolases bind to a specific receptor (the Man 6-P receptor), which facilitates movement of the hydrolases to the lysosomal compartment; the transport of the receptor-bound ligands may involve coated vesicles (6, 36). Interestingly, two separate Man 6-P receptors have been identified, a 215-kD cation-independent and a 46-kD cation-dependent...
The actual site at which the Man 6-P receptors release their ligands is uncertain. The results from several morphologic studies that examined the disposition of the 215-kD Man 6-P receptor have failed to detect significant quantities of the receptor within lysosomes (4, 15, 54). Brown and coworkers (5) recently observed that the Man 6-P receptor accumulates within endosomal structures after treatment of rat hepatocytes with weak bases; these agents prevent receptor-ligand dissociation by elevating the pH of acidic vesicles (32, 37). From this observation, it was concluded that receptor uncoupling normally occurs within endosomes, and that the unoccupied receptor molecules recycle directly back to the Golgi apparatus while the released acid hydrolases move on to lysosomes (5). The endosomal compartment, therefore, may function in the sorting of endogenous newly synthesized proteins as well as proteins internalized via receptor-mediated endocytosis.

In a previous report, we examined the fate of the Man 6-P recognition marker attached to β-glucuronidase after endocytosis of the enzyme by mouse L-cells (12). The internalized enzyme molecules underwent a limited dephosphorylation such that diphasphorylated oligosaccharides were efficiently converted to monophosphorylated species but, for the most part, the monophosphorylated forms persisted within the cells. In addition, we presented evidence to suggest that the limited dephosphorylation occurred at a prelysosomal location. In this report, we examine the postendocytic processing of β-glucuronidase in more detail and show that the enzyme is altered in both its apparent molecular mass and state of phosphorylation within the endosomal compartment.

Materials and Methods

Cells

The 215-kD, Man 6-P receptor-positive, mouse L-cells and the P388D1 mouse macrophages were obtained from Dr. Stuart Kornfeld, Washington University, St. Louis, MO; the J774 mouse macrophages were obtained from Dr. Ira Tabas, Columbia University. Mucolipidosis type II fibroblasts (GM203A) were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ. All cells were maintained in α-minimal essential medium (MEM) containing 10% fetal calf serum.

Metabolic Labeling of Cells

Subconfluent monolayers of macrophages (either P388D1 or J774 on p-15 dishes) were grown in 60 ml of MEM, 10% fetal calf serum containing 3.5 μU of [3H]-labeled β-glucuronidase was added to confluent p-15 dish of L cells. After an overnight incubation the post-uptake supernatant was removed, and the cells were washed with 10 mM phosphate, pH 7.2, 150 mM NaCl, 2 mg/ml bovine serum albumin (PBS/BSA) to remove excess ligand, scraped from the dish into PBS/BSA using a rubber policeman, and collected by centrifugation. The cell pellets were extracted with 25 mM Hepes, pH 7, 100 mM NaCl, 5 mM phosphate, 0.15 trypsin inhibitory U/ml of Trypsol (Sigma Chemical Co., St. Louis, MO), 1% Triton X-100, and the extracts were clarified by centrifugation (10,000 g for 30 min). β-Glucuronidase was immunoprecipitated from the resulting supernatants and analyzed by SDS polyacrylamide gel electrophoresis as previously described (12). In several experiments, the samples were treated with endo-β-N-acetylactosaminidase H (endo H) prior to electrophoresis. In these instances, the immunoprecipitates were disaggregated in 50 mM Tris, pH 6.8, 1% SDS, 15 mM dithiothreitol, and the digested immunoprecipitate sample (0.035 ml) was diluted with 0.006 ml of 1 M sodium citrate, pH 5.5, and 0.01 ml of endo H (50 μU/ml) in citrate-phosphate, pH 5.6. The digests were incubated overnight at 37°C, 20 μg of hemoglobin was then added as carrier, and the proteins were precipitated with 10% trichloroacetic acid (TCA). The precipitates were washed with acetone, disaggregated in SDS sample buffer (30), and subjected to electrophoresis. Cells containing [3H]-labeled samples were soaked in Amplify (Amersham Corp.) prior to drying, and the [3H]-labeled samples were visualized using lightning plus intensifier screens (DuPont Instruments, Wilmington, DE).

Results in which the L cells were incubated at 20°C were performed by placing the petri dishes in an air-tight chamber with a room maintained at 20°C; the chamber was flushed with a 5% CO2-air mixture before sealing. For these experiments, ice-cold ligand solutions were added to the

Purification of P388D-secreted β-Glucuronidase

Mouse P388D1 cells (5 × 109) were grown to confluency in a spinner culture in MEM, 10% fetal calf serum. The cells were harvested by centrifugation, suspended in 1.5 liters of serum-free MEM containing 10 mM Hepes, pH 7, and replaced in spinner culture; after an initial 24 h of incubation, glucose was added to achieve a final concentration of 5 mM. After 2 d in culture the cells were removed by centrifugation and the medium was concentrated using a Minicon concentrator (Amicon Corp., Danvers, MA; the membrane filters had a cutoff of 10 kD); the resulting concentrate (70 ml) was dialyzed against 2 liters of 20 mM Tris, pH 8. The dialysate was applied to a column (1 × 11 cm) of DE-52 cellulose equilibrated in 20 mM Tris, pH 8, and the column was eluted with a linear gradient of NaCl in 20 mM Tris, pH 8, from 0 to 500 mM (10 ml of each). β-Glucuronidase activity eluted as a single, symmetrical peak at ~150 mM NaCl. Peak fractions were pooled (total volume of 22 ml) and dialyzed against 1 liter of 20 mM Tris, pH 8, and the enzyme activity was concentrated by adsorption to a 2-m1 column of DE-52 in 20 mM Tris and subsequent elution with 0.5 M NaCl. The concentrate was applied directly to a column of Sephacryl S-200 (1.5 × 45 cm) equilibrated in 20 mM Tris, pH 8. The enzyme activity eluted as a single peak near the void volume of the column. The active fractions were pooled and dialyzed against 0.5 liter of 50 mM imidazole, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, pH 7.

An affinity column was prepared by coupling the 215-kD, cation-independent Man 6-P receptor (1 mg) to 5 ml of Affi-gel 10 (BioRad Laboratories, Richmond, CA) as previously described (50). The Man 6-P receptor was purified from bovine liver acetone powder (27); Dr. Morris Slodki, U.S. Department of Agriculture, kindly provided the yeast phosphomannan required for the receptor purification (27). The β-glucuronidase preparation (180 ml) was applied to the Man 6-P receptor affinity column; 70% of the activity bound and was eluted with 5 mM Man 6-P. The activity that ran through the column (30%) failed to bind when recomнатographed, suggesting that the β-glucuronidase molecules in this fraction lack the Man 6-P recognition marker. The Man 6-P-labeled enzyme was dialyzed against 1 liter of 20 mM Tris, pH 8. Protein concentrations were determined using the Coomassie Blue dye binding assay (1). The purified enzyme was labeled with 3H using the chloramine-T method (21), and the iodinated enzyme was separated from free 3H by chromatography on a 10-m column of Sephadex G-25 equilibrated in 20 mM Tris, pH 8, 1 mg/ml of ovalbumin, 2 mg/ml KI; 65% of the input β-glucuronidase activity (2.2 mU) was routinely recovered from the G-25 column.

Receptor-mediated Endocytosis of β-Glucuronidase

The metabolically labeled macrophage secretions were applied to mouse L-cells and the cultures were incubated at 20 or 37°C. In a typical experiment, 20 ml of MEM, 10% fetal calf containing 3.5 μU of the 3H-labeled β-glucuronidase was added to a confluent p-15 dish of L cells. After an overnight incubation the post-uptake supernatant was removed, and the cells were washed with 10 mM phosphate, pH 7.2, 150 mM NaCl, 2 mg/ml bovine serum albumin (PBS/BSA) to remove excess ligand, scraped from the dish into PBS/BSA using a rubber policeman, and collected by centrifugation. The cell pellets were extracted with 25 mM Hepes, pH 7, 100 mM NaCl, 5 mM phosphate, 0.15 trypsin inhibitory U/ml of Trypsol (Sigma Chemical Co., St. Louis, MO), 1% Triton X-100, and the extracts were clarified by centrifugation (10,000 g for 30 min). β-Glucuronidase was immunoprecipitated from the resulting supernatants and analyzed by SDS polyacrylamide gel electrophoresis as previously described (12). In several experiments, the samples were treated with endo-β-N-acetylactosaminidase H (endo H) prior to electrophoresis. In these instances, the immunoprecipitates were disaggregated in 50 mM Tris, pH 6.8, 1% SDS, 15 mM dithiothreitol, and the digested immunoprecipitate sample (0.035 ml) was diluted with 0.006 ml of 1 M sodium citrate, pH 5.5, and 0.01 ml of endo H (50 μU/ml) in citrate-phosphate, pH 5.6. The digests were incubated overnight at 37°C, 20 μg of hemoglobin was then added as carrier, and the proteins were precipitated with 10% trichloroacetic acid (TCA). The precipitates were washed with acetone, disaggregated in SDS sample buffer (30), and subjected to electrophoresis. Cells containing [3H]-labeled samples were soaked in Amplify (Amersham Corp.) prior to drying, and the [3H]-labeled samples were visualized using lightning plus intensifier screens (DuPont Instruments, Wilmington, DE).

Experiments in which the L cells were incubated at 20°C were performed by placing the petri dishes in an air-tight chamber with a room maintained at 20°C; the chamber was flushed with a 5% CO2-air mixture before sealing. For these experiments, ice-cold ligand solutions were added to the

The Journal of Cell Biology, Volume 105, 1987 1562
L cells while the dishes rested on ice; the timing of the uptake was initiated at the moment the plates were transferred from ice to the 20°C chamber. Experiments utilizing 125I-β-glucuronidase or 125I-β-galactosidase were carried out essentially as described above for the metabolically labeled preparation. The absolute amount of ligand added to the L cells was less, however, due to the high specific radioactivity of the iodinated ligands. β-Galactosidase was immunoprecipitated using an antiserum kindly provided by Dr. Richard T. Swank, Roswell Park Memorial Institute, Buffalo, NY.

Subcellular Fractionation

L cells (growing as confluent monolayers in 18 ml of MEM, 10% newborn calf serum) were incubated with 2 × 10⁶ cpm of 125I-β-glucuronidase at either 20 or 37°C. After 4 h, the cells were either harvested or the post-uptake supernatant was replaced with 20 ml of new MEM, 10% newborn calf serum, and the cells were incubated overnight at the appropriate temperature. To harvest, the cells were washed twice with 10 ml of cold PBS/BSA and scraped from the dishes into PBS/BSA. At this point, unlabeled L cells derived from two similar p-15 dishes were combined with the 125I-β-glucuronidase-containing cells; the unlabeled cells had been maintained at the same temperature as the cells to which they were added. The cells were collected by centrifugation, resuspended in 10 ml of 0.25 M sucrose, and again collected by centrifugation. The cell pellet was suspended in 8 ml of 0.25 M sucrose, pH 7, containing 10 mM acetic acid, 10 mM diethylamine, and 1 mM EDTA. After a 10-min incubation on ice, the cell suspension was homogenized with 30 strokes of a type A pestle in a Dounce homogenizer. The lysate was centrifuged (800 g for 5 min), and 6 ml of the resulting postnuclear supernatant was layered on top of a 30-ml 10-30% (wt/vol) linear gradient of metrizamide in 0.25 M sucrose (the gradient was formed over a 3-ml cushion of saturated sucrose). The tube was centrifuged for 2.5 h in a VTi50 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 18,000 rpm, after which the gradient was collected into 20 2-ml fractions.

Aliquots of each fraction (including the postnuclear supernatant and nuclear pellet) were analyzed for β-N-acetylgalactosaminidase and β-glucuronidase using the appropriate p-NO2-phenyl substrate (23), and galactosyltransferase was measured using [3H]UDP-galactose and N-acetylglucosamine as substrates (2); the galactosyltransferase assay was modified as previously detailed to negate the interference caused by the 125I radioactivity in the fractions (18). In all cases, ≥75% of the total cell-associated activities were recovered in the postnuclear supernatants, and ≥80% of the activities applied to the gradients were recovered after centrifugation.

Oligosaccharide Analysis

Glycopeptides generated by pronase digestion of dried gel pieces containing [3H]mannose-labeled β-glucuronidase were fractionated on concanavalin A (Con A)-Sepharose as previously described (12). The high-mannose-type glycopeptides (recovered in the 0.1 M ο-methylmannoside eluate of the Con A-Sepharose column) were desalted on Sephadex G-25 and digested with endo H (50). The digests were diluted with 2 ml of 2 mM ammonium acetate, pH 5.3, and applied to 5 ml columns of QAE-Sephadex equilibrated in 2 mM ammonium acetate. Negatively charged oligosaccharides bound to the resin and were eluted with a linear gradient of ammonium acetate of 2–350 mM, pH 5.3 (100 ml of each) (50).

Results

Isolation of Man 6-P-bearing Ligands

Two separate preparations of β-glucuronidase were used for these studies. In each case the enzyme was isolated from the medium surrounding mouse macrophage cells growing in culture. The first preparation is simply an ammonium sulfate precipitate of the growth medium isolated after a 2–3-day labeling of the cells with either [3H]leucine or [3H]mannose. The second ligand preparation contains β-glucuronidase which was purified from P388D1 secretions and labeled with 125I. The purified enzyme has a specific activity of 36 U/mg, and consists of a single molecular mass species of 75 kDa as detected by SDS-polyacrylamide gel electrophoresis (not shown); the observed molecular mass is consistent with the reported value of the proform of mouse β-glucuronidase (45). Analysis of the 125I-labeled β-glucuronidase preparation by SDS gel electrophoresis and autoradiography revealed a single labeled component which comigrated with the β-glucuronidase subunits (not shown), although only 36% of the radioactivity was precipitable with an antiserum prepared against rat preputial β-glucuronidase.

Internalization of 125I-β-Glucuronidase at 37 and 20°C

The relative ability of mouse L-cells to internalize 125I-β-glucuronidase at 20 and 37°C is shown in Fig. 1A. At 37°C, 125I-β-glucuronidase rapidly associated with the cells and the uptake became nonlinear after 60 min of incubation. The amount of radioactivity associated with the cells after 4 h is...
equivalent to 44% of the total β-glucuronidase applied to the cells. At 20°C the increase in cell-associated radioactivity was slower than at 37°C and the process remained linear for longer periods of time as a result of the slower rate of ligand depletion from the medium. The initial lag in the uptake at the lower temperature presumably reflects the time necessary for the cultures to reach 20°C; the ligands were added to the cells on ice to ensure that the cultures never attained temperatures >20°C. Comparison of the initial regions of the curves shown in Fig. 1 A indicates that the 125I-β-glucuronidase molecules accumulate sixfold faster at 37°C than at 20°C. In all cases, the cellular association was blocked by the addition of 3 mM Man 6-P to the incubations.

Fig. 1 B shows the effect of 18 μM cycloheximide on the uptake of the iodinated ligand. At this concentration cycloheximide inhibited the incorporation of [3H]leucine into TCA-precipitable proteins by 76% during a 6-h incubation of the L cells at 20°C. The 125I-β-glucuronidase associated with the cells at the same rate in the presence of the protein synthesis inhibitor, however, indicating that newly synthesized Man 6-P receptor molecules are not involved in the internalization process. This is expected in that newly synthesized molecules should not reach the cell surface at 20°C even in the absence of cycloheximide (44). In contrast, the amount of 125I-β-glucuronidase associated with the L cells after a 60-min incubation at 20°C was <5% of that observed after 60 min at 20°C. Finally, the amount of 125I-β-glucuronidase accumulated by the L cells in 60 min at 37°C in the presence of 10 mM EDTA was 90% of that accumulated in the absence of the chelator (not shown). The cation independence of the accumulation suggests that the 215-kD Man 6-P receptor rather than the 46-kD cation-dependent form (27) is responsible for the internalization of β-glucuronidase by the L cells.

Localization of the Intracellular Sites of 125I-β-Glucuronidase Accumulation

L cells were incubated with 125I-β-glucuronidase for 4 h at 20 and 37°C, after which the cells were either harvested or incubated for an additional period of time in the absence of the labeled ligand. The cells were then lysed by homogenization and the subcellular organelles were separated on a linear gradient of metrizamide. Fig. 2 A shows the distribution of β-N-acetylglucosaminidase and β-glucuronidase (lysosomal markers), galactosyltransferase (a Golgi marker), and 125I-β-glucuronidase after a 4-h incubation at 37°C. As expected, the glycosidases show a bimodal distribution corresponding to a heavy (fractions 3–7) and light (fractions 8–11) population of lysosomal vesicles. The relative amount of the endogenous acid hydrolases recovered within the heavy and light lysosomes varied between different experiments (compare Fig. 2 A and B); the relationship between the two lysosomal populations is unclear but a similar bimodal distribution of acid hydrolase activity has been observed previously for cells grown in culture (11, 40). The bulk of the galactosyltransferase activity was recovered in fractions 14–16, although a second smaller peak of activity sedimented in the region of the light lysosomal vesicle population. The 125I-β-glucuronidase fractionated with both the heavy and light lysosomal populations and paralleled the distribution of the endogenous 4 acid hydrolases.

The distribution of 125I-β-glucuronidase recovered from the L cells after a 4-h incubation and subsequent 16-h chase at 37°C continued to parallel the distribution of the endogenous acid hydrolase activities (Fig. 2 B). The absolute distribution of acid hydrolase activity between the light and heavy peaks changed relative to the gradient shown in Fig. 2 A, and the 125I-β-glucuronidase showed a similar alteration. In contrast, the distribution of the 125I-β-glucuronidase recovered from L cells incubated at 20°C did not mirror the endogenous acid hydrolase profiles. After a 4-h incubation with the ligand at 20°C, the bulk of the 125I-β-glucuronidase sedimented with the light population of lysosomal vesicles (Fig. 2 C). The radioactivity at the top of the gradient represents enzyme molecules released from vesicles which were broken during the cell lysis and centrifugation procedures. It is worth noting that a higher percentage of the iodinated β-glucuronidase was recovered at the top of the gradient compared with that of the endogenous activity, suggesting that the two pools of β-glucuronidase are in separate vesicles and that the 125I-containing population is more susceptible to lysis. After a 4-h incubation and a 16-h chase at 20°C, the distribution of 125I-β-glucuronidase molecules remained distinct from
Figure 3. Colloidal silica density-gradient centrifugation of L-cell homogenates. ~251-13-Glucuronidase (4 x 10^5 cpm) was added to the mouse L-cells (growing in MEM, 10% newborn calf serum) to allow internalization of the radiolabeled ligand. After the incubation, the labeled cells from a single dish were harvested and combined with a similar number of cells collected from an unlabeled p-10 dish. The mixed cell suspension was centrifuged and the cell pellet was washed and lysed as in Fig. 2; the volume of lysis buffer in this case was decreased to 1.4 ml. 0.8 ml of each postnuclear supernatant was layered over 4.2 ml of colloidal silica mixture (Percol, adjusted to a density of 1.062 with 0.25 M sucrose) which was positioned over a 0.2-ml cushion of saturated sucrose. The gradients were centrifuged in a VTi80 rotor for 60 min at 18,000 rpm, and separated into 17 equal fractions. Aliquots of each fraction were assayed for 13-N-acetylglucosaminidase (•) and ~251 (○). The cell homogenates were derived from L cells incubated at (A) 37°C for 15 min, (B) 37°C for 4 h, (C) 20°C for 4 h, and (D) 20°C for 4 h followed by 37°C for 30 min.

cubation at 37°C sediments as a single peak that cofractionated with the light lysosome population on a colloidal silica-density gradient. It is well established that ligands internalized via the process of receptor-mediated endocytosis require ~30 min to reach the lysosomal compartment (19). As such, the bulk of the β-glucuronidase molecules accumulated by the L cells during the 15-min incubation at 37°C should reside within prelysosomal endocytic compartments. Importantly, the endosomal structures containing ~251-β-glucuronidase internalized at 37°C (Fig. 3 A) have the same apparent density as the vesicles containing ~251-β-glucuronidase internalized at 20°C (Fig. 3 C).

Fig. 3 also shows that the low temperature block is rapidly reversible. After a 4-h incubation with the labeled ligand at 20°C, the cells were shifted to 37°C for 30 min. As shown in Fig. 3 D, the distribution of the ~251-β-glucuronidase recovered from these cells paralleled the distribution of endogenous β-N-acetylglucosaminidase and was similar to the pattern observed after endocytosis at 37°C for 4 h (Fig. 3 B).

Proteolysis of the Internalized β-Glucuronidase Molecules

We observed previously that the P388D1-secreted β-glucuronidase molecules migrate faster on an SDS gel after association with the L cells at 37°C (12). As shown in Fig. 4, β-glucuronidase molecules internalized by the L cells at 20°C display a similar postendocytic maturation. L cells were incubated with [3H]leucine-labeled P388D1 secretions for 22 h at 20 or 37°C. After the incubation, β-glucuronidase was recovered by immunoprecipitation and analyzed by SDS gel electrophoresis; the immunoprecipitates were digested with endo H prior to electrophoresis to negate differ-
Maturation of β-galactosidase after internalization by L cells. A mixture of Man 6-P-bearing ligands was isolated from the J774 secretions by Man 6-P receptor affinity chromatography and labeled with $^{125}$I. Mouse L-cells were incubated with the radiolabeled ligands after which the cells were solubilized and β-galactosidase was recovered by immunoprecipitation and analyzed by SDS gel electrophoresis. The autoradiogram shows the β-galactosidase subunits recovered from the input ligand mixture (lane 1), and the L cells after a 4-h uptake at 37°C (lane 2), a 4-h uptake at 20°C (lane 3), a 4-h uptake and a 4-h chase at 20°C (lane 4), and a 4-h uptake and 8-h chase at 20°C (lane 5).

...continued from the processing of the asparagine-linked oligosaccharides. The autoradiogram of the gel shown in Fig. 4 indicates that the [3H]labeled β-glucuronidase molecules recovered from the L cells after the 20°C incubation (lane 6) comigrate with the enzyme recovered from cells incubated at 37°C (lane 7). Importantly, the cell-associated forms migrated faster than the input form of β-glucuronidase (lane 8), suggesting that the molecules underwent a limited proteolytic cleavage upon cell association. The postendocytic maturation lowers the apparent mass of the β-glucuronidase subunits by ~2 kD, a value comparable to the proteolytic cleavage upon cell association. The postendocytic maturation occurs at a prelysosomal site. To further examine this possibility, the kinetics of the maturation were examined at 37°C. L cells were incubated with $^{125}$I-β-glucuronidase at 7°C to allow binding of the ligand to cell surface Man 6-P receptors. The cells were subsequently washed free of excess ligand and shifted to 37°C by the addition of prewarmed medium. After various times at 37°C, the cells were harvested and solubilized with Triton X-100, and β-glucuronidase was recovered by immunoprecipitation; Fig. 6 shows the autoradiogram of the immunoprecipitates after SDS gel electrophoresis. After a 10-min incubation at 37°C, the cell-associated β-glucuronidase molecules (lane 3) migrated with the same apparent mobility as the input (lane 6) and the cell surface-bound molecules (lane 2). However, after 20 min at 37°C, many of the molecules had been processed as evidenced by the appearance of a faster migrating form of the iodinated enzyme (lane 4). After a 30-min incubation at 37°C, all of the cell-associated molecules migrated as the lower molecular mass species (lane 5). Therefore, between 10 and 30 min after internalization at 37°C, the apparent molecular mass of the $^{125}$I-β-glucuronidase subunits decreases; the kinetics of this processing are consistent with the maturation occurring at a prelysosomal site.

Dephosphorylation of the Internalized β-Glucuronidase Molecules

β-Glucuronidase molecules internalized by mouse L-cells at 37°C undergo a limited dephosphorylation such that: (a) high-mannose-type oligosaccharides with two phosphomonoesters are converted to a monoester form; (b) the majority of the oligosaccharides that possess a single phosphomonoester persist. Moreover, the phosphorylated oligosaccharides associated with a mixture of [H]mannose-labeled P388D1-secreted acid hydrolases underwent the “two to one” conversion when internalized by the L cells at 20°C. Together, these observations suggest that a limited dephosphorylation of the acid hydrolases occurs at a site through which the enzymes pass en route to the lysosomal compartment (12). To verify that β-glucuronidase molecules are dephosphorylated after endocytosis at 20°C, L cells were incubated with [H]mannose-labeled J774 secretions. After an overnight incubation, the cells were harvested, β-glucuronidase was recovered by immunoprecipitation, and the [H]-labeled high-mannose-type oligosaccharides were isolated from the immunoprecipitated enzyme and analyzed by QAE-Sephadex chromatography (11). The distribution of the radioactivity recovered from the QAE-Sephadex columns is summarized in Table I. The J774-secreted (input) enzyme contained three species of...
phosphorylated oligosaccharides; units with one phosphomonoester, units with two phosphomonoesters, and hybrid-type units with one phosphomonooester and one sialic acid residue. Interestingly, the relative abundance of the three species varied between different preparations of β-glucuronidase as did the overall percentage of phosphorylation; similar results have been observed for the enzyme secreted by P388D1 cells (12). The percentage of phosphorylated oligosaccharides associated with β-glucuronidase molecules recovered from cells incubated at 37°C was similar to the input enzyme (~25%), but the cell-associated enzyme contained fewer diphosphorylated oligosaccharides as evidenced by the fivefold decrease in the overall ratio of diphosphorylated to monophosphorylated units (1.7 vs. 0.32 for the input and cell-associated enzyme, respectively). Likewise, the [3H]mannose-labeled β-glucuronidase molecules recovered from L cells incubated at 20°C showed a similar maturation relative to the input enzyme; specifically, the extent of phosphorylation remained comparable (35% vs. 36%) but the ratio of diphosphorylated to monophosphorylated oligosaccharides declined from 3.4 (input) to 0.85 (cell-associated). Therefore, β-glucuronidase molecules internalized by the mouse L-cells at 20°C experience the same partial dephosphorylation as the molecules internalized at 37°C (12).

**Postendocytic Maturation of β-Glucuronidase within I-Cell Disease Fibroblasts**

Patients suffering from I-cell disease (mucolipidosis type II) lack the enzyme UDP-N-acetylglucosamine:lysosomal enzyme phosphotransferase (26, 39). As a result of this deficiency, newly synthesized acid hydrolases produced by the patient's fibroblasts are not targeted to lysosomes and the intracellular levels of the acid hydrolases fall far below normal levels (53). Despite the inability of the I-cell fibroblasts to target their acid hydrolases to lysosomes, the cells possess the Man 6-P receptor and they will internalize ligands bearing the Man 6-P recognition signal (20). To determine whether the generalized acid hydrolase deficiency would affect the maturation of an acid hydrolase internalized via receptor-mediated endocytosis, I-cell disease fibroblasts were incubated with metabolically-labeled P388D1 secretions. As shown in Fig. 1, β-glucuronidase recovered from the I cells after incubation of the cells with [3H]leucine-labeled P388D1 secretions migrated on an SDS gel with the same apparent mobility as the enzyme internalized by mouse L-cells at 37°C (compare lanes 5 and 7). The similar mobility indicates that the I-cell fibroblasts convert the precursor form of β-glucuronidase to its mature counterpart.

In addition to the proteolytic processing, β-glucuronidase internalized by I-cell fibroblasts is dephosphorylated. As shown in Table II, the major [3H]mannose-labeled phosphorylated oligosaccharide recovered from the I-cell-associated enzyme contained a single phosphomonooester whereas the input enzyme contained predominantly the diphosphorylated species. The I cells not only mediate the two-to-one conversion, but the percentage of phosphorylated oligosaccharides recovered from the I-cell-associated enzyme (5.6%) was much less than that associated with the input enzyme.

**Table I. Characterization of the Phosphorylated Oligosaccharides Isolated from [3H]Mannose-labeled β-Glucuronidase**

| Preparation of J774 secretions | Enzyme source | Counts per minute recovered as: | Percent phosphorylated | 2:1 ratio |
| --- | --- | --- | --- | --- |
| | | N | 1SA | 1PM | 1PM/1SA | 2PM | |
| I | Input | 3,081 | 40 | 216 | 188 | 1,388 | 35 | 3.4 |
| 20°C cell-associated | 2,287 | 60 | 664 | 64 | 616 | 36 | 0.85 |
| II | Input | 5,187 | 88 | 364 | 240 | 1,000 | 23 | 1.7 |
| 37°C cell-associated | 6,706 | 148 | 1,620 | 100 | 544 | 25 | 0.32 |

[3H]Mannose-labeled J774 secretions were incubated with mouse L-cells at either 20 or 37°C. Each p-10 dish of confluent cells received 5 mU of β-glucuronidase activity in a total volume of 10 ml of α-MEM, 10% fetal bovine serum. After 18 h at the indicated temperature, the cells were harvested and solubilized with Triton X-100, and β-glucuronidase was immunoprecipitated from the extracts; the enzyme was also immunoprecipitated from an aliquot of the two input fractions. After SDS gel electrophoresis and autoradiography, the regions of the dried gel containing the radiolabeled β-glucuronidase subunits were excised and the radioactivity was solubilized by pronase digestion. The glycopeptides were fractionated on Con A-Sepharose and the high-mannose-type units were recovered and digested with endo H. Each digest was applied to a 5-ml column of QAE-Sephadex and the negatively charged oligosaccharides were eluted with a linear gradient of ammonium acetate as previously described (50). The radioactivity eluting in the position of a neutral high-mannose oligosaccharide (N) or a negatively charged unit containing one sialic acid residue (1SA), one phosphomonooester (1PM), one phosphomonooester and one sialic acid residue (1PM/1SA), and two phosphomonooesters (2PM) was determined by counting an aliquot of each fraction in a liquid scintillation counter. The 2:1 ratio was determined by dividing the radioactivity recovered as the 2PM species by the sum of the radioactivity recovered in the 1PM and 1PM/1SA species.
Table II. I-Cell Disease Fibroblasts Dephosphorylate Internalized β-Glucuronidase

| Source of β-glucuronidase | Percentage of radioactivity bound to QAE-Sephadex | Distribution of radioactivity in charged species (percentage of total): |
|---------------------------|-----------------------------------------------|--------------------------------------------------|
|                           |                                              | 1SA 1PM 1PM/1SA 2PM                               |
| P388D, Secretions (input) | 29                                           | 0.7 8.7 7.4 12                                    |
| L-cell-associated         | 31                                           | 3 20 2.4 5.6                                     |
| I-cell-associated         | 15                                           | 9.6 5.6 0 0                                     |

[14]Mannose-labeled P388D, secretions were incubated with mouse L-cells or human I-cell disease fibroblasts at 37°C. For each p-15 dish of confluent cells, 8.7 mU of β-glucuronidase (total input of 1.9 × 10⁶ cpm) were incubated for 24 h in a total volume of 30 ml. After the incubation, the cells were harvested and solubilized by Triton X-100 extraction, and β-glucuronidase was recovered by immunoprecipitation. After SDS gel electrophoresis and autoradiography, the regions of the dried gel containing the radiolabeled β-glucuronidase subunits were excised and the radioactivity was solubilized by pronase digestion. From each dish of cells, ~5,000 cpm of β-glucuronidase (or 33% of the total input β-glucuronidase) was recovered. The solubilized glycopeptides were fractionated on Con A-Sepharose, and the high-mannose-type units were digested with endo H (12). The released oligosaccharides were applied to a 5-ml QAE-Sephadex column and negatively charged oligosaccharides were eluted with a linear gradient of ammonium acetate as previously described (50). The radioactivity eluting in the position expected for oligosaccharides containing one sialic acid residue (1SA), one phosphomonoester (1PM), one phosphomonoester and one sialic acid residue (1PM/1SA), and two phosphomonoesters (2PM) was quantitated by counting aliquots of each fraction in a liquid scintillation counter.

(28.1%), indicating that many oligosaccharides are completely dephosphorylated within the I-cell fibroblasts. In contrast to the β-glucuronidase internalized by L cells, the major negatively charged oligosaccharide recovered from the I-cell-associated enzyme contained one sialic acid residue. This oligosaccharide probably was derived from the one phosphomonoester–one sialic acid hybrid molecule that was associated with the input enzyme. The I-cell fibroblasts dephosphorylate the oligosaccharides, but because the cells are deficient in a lysosomal neuraminidase, the sialic acid residues persist.

Discussion

The transport of macromolecules via receptor-mediated endocytosis to the lysosomal compartment is a multistep process. Much of our knowledge of the endocytic pathway has been obtained through the treatment of cells with inhibitors such as low temperature which disrupt the process and cause the accumulation of ligand at various points along the pathway. For example, at very low temperatures (<10°C) ligands bind to cell surface receptors but the receptor–ligand complexes remain at the plasma membrane (35). At temperatures near 20°C, the receptor–ligand complexes enter the cell but the ligands fail to reach lysosomes (8, 33). The rat hepatocyte asialoglycoprotein receptor internalizes 125I-asialofetuin at 20°C, but the 125I-ligand is not catabolized within the lysosomal compartment. Rather, morphologic studies have indicated that the internalized asialoglycoproteins accumulate within endosomal structures (8). Interestingly, Mueller and Hubbard (34) have recently demonstrated that asialoorosomucoid accumulated by rat hepatocytes at 16°C resides within a population of peripheral-type endosomes which are distinct from the more internal multivesicular endosomes in both their morphology and the presence of the asialoglycoprotein receptor. Thus, through modulations of temperature it is possible to accumulate ligands at distinct locations along the endocytic pathway.

The Man 6-P receptor-mediated internalization of β-glucuronidase by mouse L-cells is also a temperature-sensitive process. The initial rate of accumulation of the enzyme at 20°C is sixfold lower than at 37°C, but the internalization process proceeds linearly throughout a 4-h incubation at the lower temperature. The absolute amount of β-glucuronidase accumulated at 20°C far exceeds the amount of enzyme that binds to the cell surface at 7°C; thus, the accumulation of the enzyme observed at 20°C represents an intracellular buildup and not merely binding of the ligand to cell surface receptors. In addition, the cellular accumulation is unaffected by cycloheximide, indicating that newly synthesized receptor molecules are not required for the continued uptake. The prolonged linear accumulation of β-glucuronidase observed at 20°C appears, therefore, to proceed through the recycling of the Man 6-P receptor. Because a low pH is required for dissociation of the receptor and its ligand (20), the receptor–ligand complexes internalized at 20°C must reach an acidic compartment from which the receptor can recycle to the cell surface after dissociation of its ligand.

Although the Man 6-P receptor operates normally (albeit more slowly) at 20°C, the internalized ligands do not achieve the same destination as ligands internalized at 37°C. Subcellular fractionation studies were employed to examine the intracellular sites of ligand accumulation. After endocytosis of 125I-β-glucuronidase at 37°C, the internalized radiolabeled molecules sedimented on a density gradient with the endogenous acid hydrolase activities; thus, the 125I-β-glucuronidase molecules appeared to accumulate within lysosomes. The distribution of the radiolabeled molecules internalized at 20°C, however, did not parallel the endogenous acid hydrolase activities. Rather, the molecules were associated with a population of vesicles that sedimented at a density of ~1.11 g/ml. Importantly, the density of the vesicles that contained the 125I-β-glucuronidase internalized at 20°C is comparable to the density of endocytic vesicles that contained 125I-β-glucuronidase internalized after a brief incubation of the L cells at 37°C. Therefore, the acid hydrolases internalized by the L cells at 20°C appear to accumulate within a prelysosomal endocytic compartment. The low temperature leads to a complete inhibition of the delivery of β-glucuronidase to the lysosomal compartment as the 125I-labeled molecules continued to sediment with the buoyant vesicles even after 16 h of cellular association.

Within the prelysosomal compartment the internalized acid hydrolases experienced at least two postendocytic modifications. First, the apparent molecular mass of the subunits of both β-glucuronidase and β-galactosidase decreased. The magnitude of the molecular mass changes (2 and 19 kD for β-glucuronidase and β-galactosidase, respectively) are comparable to the decrease in molecular masses observed during the conversion of the proforms of the two enzymes to their mature counterparts (45). This similarity suggests that the macrophages secrete the proforms of the acid hydrolases, and that the subunits are processed to their mature state after endocytosis. Importantly, the maturation of the acid hydrolases occurred after endocytosis at both 37 and 20°C, indicating that lysosomal entry is not required for the processing.
It is interesting to note that the proforms of several endogenous newly synthesized acid hydrolases have been shown to undergo proteolytic maturation within a light population of lysosomal vesicles (3, 16); this site may correspond to the compartment containing the hydrolases endocytosed at 20°C. Moreover, Diment and Stahl (7) have recently shown that endosomes contain a cathepsin D-like activity, and that degradative newly synthesized acid hydrolases have been shown to undergo a very rapid proteolytic processing after endocytosis of epidermal growth factor (47). The proteolytic maturation of acid hydrolases may, therefore, reflect a common modification incurred by ligands (and some receptors) transported through the endosomal compartment.

The second postendocytic modification of the acid hydrolases observed within the prelysosomal compartment corresponds to a partial dephosphorylation. The major species of phosphorylated oligosaccharide associated with macrophage-secreted β-glucuronidase contained two phosphomonoesters. Upon internalization by the L cells, the percentage of phosphorylated oligosaccharides associated with the β-glucuronidase subunits remained comparable to the input form of the enzyme, but the number of diphosphorylated oligosaccharides was greatly reduced with a corresponding increase in the monophosphorylated species. The mechanism by which this "two to one" conversion occurs is unknown; the removal of the phosphate appears to proceed randomly (12). We have observed previously that lysosomes isolated from the L cells at steady state contain endogenous acid hydrolases which retain their phosphorylated oligosaccharides (11). Despite the presence of a large number of diphosphorylated oligosaccharides on newly synthesized molecules, the oligosaccharides recovered from lysosomes contained primarily the monophosphorylated species (11). Thus, the phosphorylated oligosaccharides attached to the endogenous L-cell acid hydrolases also undergo the two-to-one conversion during their maturation.

The results indicate that exogenous β-glucuronidase molecules internalized by the mouse L-cells experience a series of biochemical modifications that are similar (if not identical) to the posttranslational modifications incurred by newly synthesized acid hydrolases en route to the lysosomal compartment. The postendocytic modifications are observed when acid hydrolases are internalized at 20°C, even though the internalized molecules do not accumulate within the lysosomal compartment. As such, the enzymes responsible for the modifications must be present within the endocytic compartment. The nonlysosomal nature of the processing enzymes is supported by the finding that β-glucuronidase molecules internalized by acid hydrolase-deficient, I-cell disease fibroblasts undergo the proteolytic maturation and the limited dephosphorylation. Our results do not preclude the possibility that the lysosomal compartment may also contain enzymes capable of performing such modifications. However, the similar maturation of both endogenous and exogenous acid hydrolases transported by the Man 6-P receptor suggests that the two ligand populations proceed through a common endocytic sorting compartment en route to lysosomes.

This investigation was supported by grant GM-33342, from the National Institutes of Health and by an award from the Hirshl-Monique Foundation to Dr. Gabel.

Received for publication 27 March 1987, and in revised form 19 June 1987.

References

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
2. Brez, R., and W. Staubli. 1977. Detergent influence on rat-liver galactosyltransferase activities towards different acceptors. Eur. J. Biochem. 77:181-192.
3. Brown, J. A., and R. T. Swank. 1983. Subcellular redistribution of newly synthesized macrophage lysosomal enzymes. J. Biol. Chem. 258:15323-15328.
4. Brown, W. J., and M. G. Farquhar. 1984. The mannose 6-phosphate receptor for lysosomal enzymes is concentrated in cis Golgi cisternae. Cell. 36:295-307.
5. Brown, W. J., J. Goodhouse, and M. G. Farquhar. 1986. Mannose 6-phosphate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. J. Cell Biol. 103:1235-1247.
6. Campbell, C. H., R. E. Fine, J. Squaciraini, and L. H. Rome. 1983. Coated vesicles from rat liver and calf brain contain cryptic mannose 6-phosphate receptors. J. Biol. Chem. 258:2628-2633.
7. Diment, S., and P. Stahl. 1985. Macrophage endosomes contain proteases which degrade endocytosed protein ligands. J. Biol. Chem. 260:5611-5617.
8. Dunn, W. A., A. L. Hubbard, and N. N. Aronson, Jr. 1980. Low temperature selectively inhibits fusion between pinocytotic vesicles and lysosomes during heterophagy of 125I-asialofetuin. J. Biol. Chem. 255:5971-5978.
9. Dunphy, W. G., and J. E. Rothman. 1983. Compartmentation of asparagine-linked oligosaccharide processing in the Golgi apparatus. J. Cell Biol. 97:270-275.
10. Erickson, A. H., and G. Blobel. 1979. Early events in the biosynthesis of the lysosomal enzyme cathepsin D. J. Biol. Chem. 254:11771-11774.
11. Gabel, C. A., and S. A. Foster. 1986. Lysosomal enzyme trafficking in mannose 6-phosphate receptor-positive L-cells: demonstration of a steady-state accumulation of phosphorylated acid hydrolases. J. Cell Biol. 102:943-950.
12. Gabel, C. A., and S. A. Foster. 1986. Mannose 6-phosphate receptor-mediated endocytosis of acid hydrolases: internalization of β-glucuronidase is accompanied by a limited dephosphorylation. J. Cell Biol. 103:1817-1827.
13. Gabel, C. A., D. E. Goldberg, and S. Kornfeld. 1982. Lysosomal enzyme oligosaccharide phosphorylation in mouse lymphoma cells: specificity and kinetics of binding to the mannose 6-phosphate receptor in vivo. J. Cell Biol. 95:536-542.
14. Gabel, C. A., D. E. Goldberg, and S. Kornfeld. 1983. Identification and characterization of cells deficient in the mannose 6-phosphate receptor: evidence for an alternate pathway for lysosomal enzyme targeting. Proc. Natl. Acad. Sci. USA. 80:775-779.
15. Geuze, H. J., I. L. Slot, G. J. A. M. Strous, A. Hasilik, and K. von Figura. 1984. Ultrastructural localization of the mannose 6-phosphate receptor in rat liver. J. Cell Biol. 98:2047-2054.
16. Gieselmann, V., R. Pohlmann, A. Hasilik, and K. von Figura. 1983. Bio-synthesis and transport of cathepsin D in cultured human fibroblasts. J. Cell Biol. 97:1-5.
17. Goldberg, D. E., and S. Kornfeld. 1981. The phosphorylation of β-glucuronidase oligosaccharides in mouse P388D, cells. J. Biol. Chem. 256:13060-13067.
18. Goldberg, D. E., and S. Kornfeld. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. J. Biol. Chem. 258:3159-3165.
19. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. 1:1-39.
20. Gonzalez-Noriega, A., J. H. Grubb, V. Talkad, and W. S. Sly. 1980. Low temperature selectively inhibits fusion between pinocytotic vesicles and lysosomes during heterophagy of 125I-asialofetuin. J. Biol. Chem. 255:4937-4942.
21. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of [14C]labeled human growth hormone of high specific radioactivity. Biochem. J. 89:114-123.
22. Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. Science (Wash. DC). 234:438-443.
23. Hall, C. W., I. Liebaers, P. D. Natale, and E. F. Neufeld. 1978. Enzymic diagnosis of the genetic mucopolysaccharide storage disorders. Methods Enzymol. 50:439-456.
24. Hasilik, A., and E. F. Neufeld. 1980. Biosynthesis of lysosomal enzymes in fibroblasts. J. Biol. Chem. 255:4937-4945.
25. Hasilik, A., and K. von Figura. 1981. Oligosaccharides in lysosomal en-
zymes. Eur. J. Biochem. 121:125-129.

Gabel and Foster Prelysosomal Processing of Acid Hydrolases
26. Hasilik, A., A. Waheed, and K. von Figura. 1981. Enzymatic phosphorylation of lysosomal enzymes in the presence of UDP-N-acetylglucosamine: absence of the activity in cell fibroblasts. Biochem. Biophys. Res. Commun. 98:761-767.

27. Hoflack, B., and S. Kornfeld. 1986. Purification and characterization of a cation-dependent mannose 6-phosphate receptor from murine P388D1 macrophages and bovine liver. Proc. Natl. Acad. Sci. USA. 82:761-767.

28. Jessup, W., and R. T. Dean. 1980. Spontaneous lysosomal enzyme secretion by a murine macrophage-like cell line. Biochem. J. 190:847-850.

29. Kaplan, A., D. T. Achord, and W. S. Sly. 1977. Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. Proc. Natl. Acad. Sci. USA. 74:2026-2030.

30. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.

31. LeDonne, N. C., J. L. Fairley, and C. C. Sweely. 1983. Biosynthesis of α-galactosidase A in cultured Chang liver cells. Arch. Biochem. Biophys. 224:186-195.

32. Maxfield, F. R. 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol. 95:676-681.

33. Miller, K., J. Beardmore, H. Kanety, J. Schlessinger, and C. R. Hopkins. 1986. Localization of the epidermal growth factor (EGF) receptor within the endosome of EGF-stimulated epidermoid carcinoma (A431) cells. J. Cell Biol. 102:500-509.

34. Mueller, S. C., and A. L. Hubbard. 1986. Receptor-mediated endocytosis of asialoglycoproteins by rat hepatocytes: receptor-positive and receptor-negative endosomes. J. Cell Biol. 102:932-942.

35. Natowicz, M. R., M. M.-Y. Chi, O. H. Lowry, and W. S. Sly. 1979. Enzymatic identification of mannose 6-phosphate on the recognition marker for receptor-mediated pinocytosis of β-glucuronidase by human fibroblasts. Proc. Natl. Acad. Sci. USA. 76:4322-4326.

36. Pearse, B. M. F. 1985. Assembly of the mannose 6-phosphate receptor into reconstituted clathrin coats. EMBO (Eur. Mol. Biol. Organ.) J. 4:2457-2460.

37. Poole, B., and S. Ohkuma. 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J. Cell Biol. 90:665-669.

38. Reitman, M. L., and S. Kornfeld. 1981. UDP-N-acetylglucosamine:glycero-protein N-acetylglucosamine-1-phosphotransferase. J. Biol. Chem. 256:4275-4281.

39. Reitman, M. L., A. Varki, and S. Kornfeld. 1981. Fibroblasts from patients with I-cell disease and pseudo-hurler polydystrophy are deficient in uridine 5'-diphosphate-N-acetylglucosamine:glycoprotein N-acetylglucosaminylphosphotransferase activity. J. Clin. Invest. 67:1574-1579.

40. Rome, L. H., A. J. Garvin, M. M. Allietta, and E. F. Neufeld. 1979. Two species of lysosomal organelles in cultured human fibroblasts. Cell. 17:143-153.

41. Rosenfeld, M. G., D. Kreibich, D. Popov, K. Kato, and D. D. Sahatcini. 1982. Biosynthesis of lysosomal hydrolases: their synthesis in bound polysomes and the role of co- and post-translational processing in determining their subcellular destinations. J. Cell Biol. 93:135-143.

42. Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyltransferase in Hela cells: codistribution with thiamine pyrophosphatase in trans-Golgi cisternae. J. Cell Biol. 93:223-229.

43. Roth, J., D. J. Taatjes, J. M. Lucocq, J. M. Weinstein, and J. C. Paulson. 1985. Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. Cell. 43:287-295.

44. Saraste, J., and E. Kuismannen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Virus virus membrane glycoproteins to the cell surface. Cell. 38:535-549.

45. Skadlarek, M. D., Novak, E. K., and Swank, R. T. 1984. Processing of lysosomal enzymes in macrophages and kidney. In Lysosomes in Biology and Pathology. J. T. Dingle, R. T. Dean, and W. S. Sly, editors. Vol. 7. Elsevier, Amsterdam. 17-43.

46. Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. J. Cell Biochem. 18:67-85.

47. Stoscheck, C. M., and G. Carpenter. 1984. Down regulation of epidermal growth factor receptors: direct demonstration of receptor degradation in human fibroblasts. J. Cell Biol. 98:1048-1053.

48. Varki, A., and S. Kornfeld. 1980. Identification of a rat liver α-N-acetylgalactosaminyl phosphodiesterase capable of removing 'blocking' α-N-acetylgalactosamine residues from phosphorylated high mannose oligosaccharides of lysosomal enzymes. J. Biol. Chem. 255:8398-8401.

49. Varki, A., and S. Kornfeld. 1981. Purification and characterization of rat liver α-N-acetylgalactosaminyl phosphodiesterase. J. Biol. Chem. 256:9937-9943.

50. Varki, A., and S. Kornfeld. 1983. The spectrum of anionic oligosaccharides released by endo-β-N-acetylgalactosaminidase H from glycoproteins. J. Biol. Chem. 258:2808-2818.

51. von Figura, K., and V. Klein. 1979. Isolation and characterization of phosphorylated oligosaccharides from α-N-acetylgalactosaminidase that are recognized by cell surface receptors. Eur. J. Biochem. 94:347-354.

52. Waheed, A., R. Pohlmann, A. Hasilik, and K. von Figura. 1981. Subcellular location of two enzymes involved in the synthesis of phosphorylated recognition markers in lysosomal enzymes. J. Biol. Chem. 256:4150-4152.

53. Wiesmann, U. N., and N. N. Herschkowitz. 1974. Studies on the pathogenetic mechanism of I-cell disease in cultured fibroblasts. Pediatr. Res. 8:865-870.

54. Willingham, M. C., I. H. Pastan, and G. G. Sahagian. 1983. Ultrastructural immunocytochemical localization of the phosphomannosyl receptor in Chinese hamster ovary cells. J. Cell Biol. 93:135-138.

55. Williams, M. C., I. H. Pastan, G. G. Sahagian, G. W. Jourdain, and E. F. Neufeld. 1981. Morphologic study of the internalization of a lysosomal enzyme by the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA. 78:6967-6971.