Postendocytic Maturation of Acid Hydrolases: Evidence of Prelysosomal Processing

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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 proenyzmes 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),[1] 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

1. *Abbreviations used in this paper*: endo H, endo-β-*N*-acetylglucosaminidase H; Man 6-P, mannose 6-phosphate.

form (27), but the raison d'être for the two receptors remains unclear. Within the lysosomal compartment, or just prior to entry, the proforms of the acid hydrolases are processed to their mature counterparts (3, 16, 45) and the Man 6-P recognition marker may be removed by a resident acid phosphatase (11, 13).

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 diphosphorylated 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 P388D$_1$ 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 (GM2013A) 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 P388D$_1$ or J774 on p-15 dishes) were grown in 60 ml of MEM, 4% fetal calf serum containing 28 μCi/ml of [2-^{3}H]mannose (American Radiochemical Corp., St. Louis, MO; 15 Ci/mmol) or 33 μCi/ml of [4,5-^{3}H]leucine (Amersham Corp., Arlington Heights, IL; 63 Ci/mmol). The cells were maintained for 2–3 d at 37°C; after each 24-h period, 0.4 ml of 0.5 M glucose was added to the dishes. At the end of the incubation, the medium containing secreted acid hydrolases (14, 28) was recovered and clarified in a clinical centrifuge. The supernatant was adjusted to 20 mM in Hepes, pH 7, and solid ammonium sulfate was added to achieve a final concentration of 80% saturation. After stirring on ice for 30 min, the precipitate was collected by centrifugation, dissolved in a minimum volume of 10 mM Hepes, pH 7, 150 mM NaCl, and dialyzed vs. Hepes-buffered saline. In a typical preparation, one p-15 dish of P388D$_1$ cells yielded ~20 mU of β-glucuronidase, where 1 U of activity as measured with *p*-NO$_2$-phenyl-β-glucuronide is equivalent to the formation of 1 μmol of *p*-NO$_2$-phenol/min.

Purification of P388D$_1$-secreted β-Glucuronidase

Mouse P388D$_1$ cells (5×10^8) 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 Minitam 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 (110 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-ml 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-glycerolphosphate, 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 (118 mU) 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 rechromatographed, suggesting that the β-glucuronidase molecules in this fraction lack the Man 6-P recognition marker. The Man 6-P-eluted 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 ^{125}I using the chloramine-T method (21), and the iodinated enzyme was separated from free ^{125}I by chromatography on a 10-ml 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 serum containing 3.5 mU of the ^{3}H-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 Trasylol (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*-acetylglucosaminidase 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 disaggregated 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 mU/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% tricholoroacetic acid (TCA). The precipitates were washed with acetone, disaggregated in SDS sample buffer (30), and subjected to electrophoresis. Gels containing ^{3}H-labeled samples were soaked in Amplify (Amersham Corp.) prior to drying, and the ^{125}I-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 within a room maintained at 20°C; the chamber was flushed with a 5% CO_2-air mixture before sealing. For these experiments, ice-cold ligand solutions were added to the

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 ^{125}I-β-glucuronidase or ^{125}I-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 × 10^6 cpm of ^{125}I-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 fresh 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 ^{125}I-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*-acetylglucosaminidase and β-glucuronidase using the appropriate *p*-NO_2-phenyl substrate (23), and galactosyltransferase was measured using [^{3}H]UDP-galactose and *N*-acetylglucosamine as substrates (2); the galactosyltransferase assay was modified as previously detailed to negate the interference caused by the ^{125}I 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 [^{3}H]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 α-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).

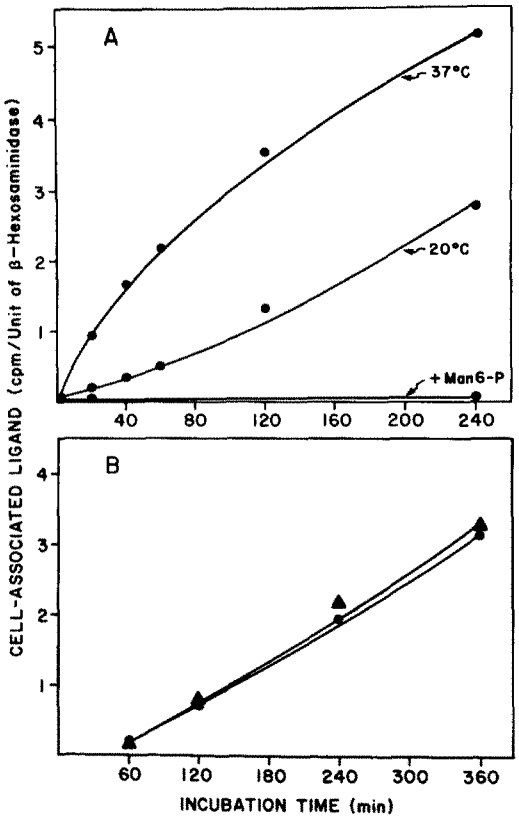

Figure 1. Accumulation of ^{125}I-β-glucuronidase by mouse L-cells. Confluent monolayers of L cells on p-6 dishes were incubated with 3.6 × 10^5 cpm of ^{125}I-β-glucuronidase in 3 ml of MEM, 10% FBS at either 20 or 37°C. At the indicated times, the post-uptake supernatants were discarded and the dishes were rinsed three times with 3 ml of PBS/BSA after which the attached cells were dislodged with a rubber policeman. The cell suspensions (in PBS/BSA) were centrifuged and the resulting cell pellets were frozen in a dry ice-ethanol bath. After all time points had been collected, each frozen pellet was suspended in 0.2 ml of 25 mM Hepes, pH 7, 5 mM PO_4, 0.1 M NaCl, 1% Triton X-100, 0.2% deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, and aliquots were counted in a gamma counter and assayed for β-*N*-acetylglucosaminidase. Where indicated, 3 mM Man 6-P was included in the incubation media; the inhibition by the monosaccharide was equivalent at both temperatures. (*A*) Comparison of the accumulation of ^{125}I-β-glucuronidase at 20 and 37°C; and (*B*) comparison of the accumulation of the ligand at 20°C in the presence (▲) and absence (●) of 18 μM cycloheximide.

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 [^{3}H]leucine or [^{3}H]mannose. The second ligand preparation contains β-glucuronidase which was purified from P388D_1 secretions and labeled with ^{125}I. The purified enzyme has a specific activity of 36 U/mg, and consists of a single molecular mass species of 75 kD 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 ^{125}I-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 ^{125}I-β-Glucuronidase at 37 and 20°C

The relative ability of mouse L-cells to internalize ^{125}I-β-glucuronidase at 20 and 37°C is shown in Fig. 1 *A*. At 37°C, ^{125}I-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 ^{125}I-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 [^{3}H]leucine into TCA-precipitable proteins by 76% during a 6-h incubation of the L cells at 20°C. The ^{125}I-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 ^{125}I-β-glucuronidase associated with the L cells after a 60-min incubation at 7°C was <5% of that observed after 60 min at 20°C. Finally, the amount of ^{125}I-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 ^{125}I-β-Glucuronidase Accumulation

L cells were incubated with ^{125}I-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 ^{125}I-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 ^{125}I-β-glucuronidase fractionated with both the heavy and light lysosomal populations and paralleled the distribution of the endogenous acid hydrolases.

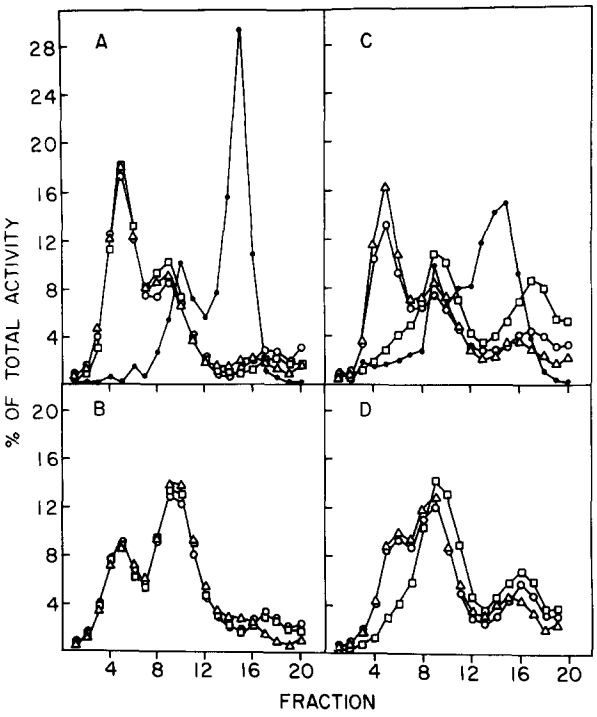

Figure 2. Metrizamide density-gradient centrifugation of L-cell homogenates. Confluent monolayers of L cells growing in MEM, 10% newborn calf serum on p-15 dishes were incubated for 4 h with ^{125}I-β-glucuronidase. After the incubation the medium was removed and the cells were either harvested immediately (*A* and *C*) or chased for an additional 16 h with fresh ligand-free medium (*B* and *D*). Homogenates of the cells were prepared and subjected to density-gradient centrifugation using a linear gradient of metrizamide, of 10–30% in 0.25 M sucrose. Aliquots of each gradient fraction were assayed for β-glucuronidase (○), β-*N*-acetylglucosaminidase (△), ^{125}I (□), and galactosyltransferase (●; *A* and *C* only). The cells in *A* and *B* were incubated at 37°C and those in *C* and *D* at 20°C. Fraction 1 corresponds to the bottom of the gradient.

The distribution of ^{125}I-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 ^{125}I-β-glucuronidase showed a similar alteration. In contrast, the distribution of the ^{125}I-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 ^{125}I-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 ^{125}I-containing population is more susceptible to lysis. After a 4-h incubation and a 16-h chase at 20°C, the distribution of ^{125}I-β-glucuronidase molecules remained distinct from

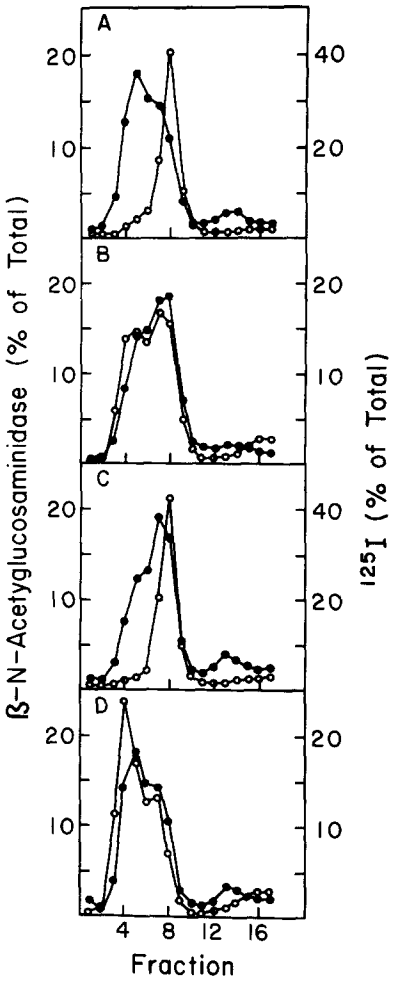

Figure 3. Colloidal silica density-gradient centrifugation of L-cell homogenates. ^{125}I-β-Glucuronidase (4 × 10^5 cpm) was added to the mouse L-cells (growing in MEM, 10% newborn calf serum) to allow internalization of the radiolabeled ligand. After the incubation, the labeled cells from a single dish 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 (Percoll, 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 β-*N*-acetylglucosaminidase (●) and ^{125}I (○). 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.

the distribution of the endogenous acid hydrolases (Fig. 2 *D*). Therefore, the metrizamide gradients indicate that ^{125}I-β-glucuronidase molecules internalized at 20°C do not achieve the same distribution within the lysosomal compartment as molecules internalized at 37°C.

The density of the intracellular vesicles containing β-glucuronidase internalized at 20°C coincides with the density of endosomal-like structures. As shown in Fig. 3 *A*, ^{125}I-β-glucuronidase internalized by the L cells during a 15-min incubation at 37°C sedimented 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 ^{125}I-β-glucuronidase internalized at 37°C (Fig. 3 *A*) have the same apparent density as the vesicles containing ^{125}I-β-glucuronidase internalized at 20°C (Fig. 3 *C*).

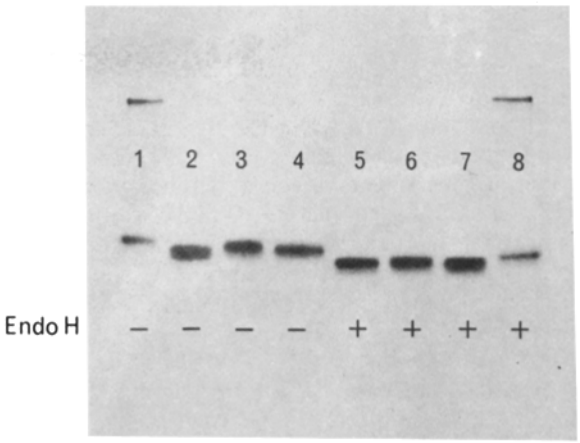

Figure 4. Comparison of cell-associated β-glucuronidase by SDS gel electrophoresis and autoradiography. [^{3}H]Leucine-labeled β-glucuronidase recovered by immunoprecipitation from various extracts was analyzed by SDS gel electrophoresis either before (lanes *1–4*) or after endo H digestion (lanes *5–8*). The region of the autoradiogram containing the β-glucuronidase subunits is shown. The enzyme was recovered from the input P388D$_1$ secretions (lanes *1* and *8*), and from cell extracts after an incubation of the labeled ligands with mouse L-cells at 37°C for 22 h (lanes *2* and *7*), mouse L-cells at 20°C for 22 h (lanes *3* and *6*)), and I-cell disease human fibroblasts at 37°C for 22 h (lanes *4* and *5*).

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 ^{125}I-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 P388D$_1$-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 [^{3}H]leucine-labeled P388D$_1$ 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-

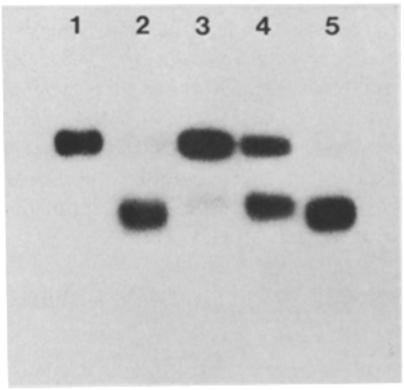

Figure 5. Maturation of β-galactosidase after internalization by L cells. A mixture of Man 6-P–bearing ligands was isolated from the J774 secretions by Man 6-P receptor affinity chromatography and labeled with ^{125}I. Mouse L-cells were incubated with the radiolabeled ligands after which the cells were 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*).

ences caused by the processing of the asparagine-linked oligosaccharides. The autoradiogram of the gel shown in Fig. 4 indicates that the [^{3}H]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 maturation reported for endogenous mouse β-glucuronidase molecules (45).

The macrophage secretions contain several acid hydrolases, including β-galactosidase. The subunits of mouse β-galactosidase are synthesized with an apparent molecular mass of 82 kD, and decrease by ~20 kD during the enzyme's maturation (45). To determine whether β-galactosidase subunits are "matured" after endocytosis, the fate of the enzyme was assessed as follows. Man 6-P–bearing acid hydrolases secreted from J774 macrophages were adsorbed to a 215-kD Man 6-P receptor affinity column; the bound enzymes were eluted with Man 6-P and labeled with ^{125}I. The mixture of ^{125}I-labeled Man 6-P–bearing acid hydrolases was applied to mouse L-cells and incubated at 20°C for 4 h. At the end of the incubation, the post-uptake supernatant was replaced with fresh medium and the cells were chased for various periods of time. Fig. 5 shows the autoradiogram of an SDS gel that contained the immunoprecipitated β-galactosidase recovered from the cell extracts; for comparison, lane *1* contains β-galactosidase immunoprecipitated from the input J774 secretions and lane *2* the β-galactosidase recovered from the L cells after endocytosis at 37°C. After a 4-h uptake at 20°C the cell-associated β-galactosidase molecules migrated with an apparent molecular mass of 81 kD and were indistinguishable from the input enzyme (compare lanes *1* and *3*). After 4 h of chase, however, many of the cell-associated β-galactosidase molecules had been processed to a lower molecular mass (lane *4*), and after 18 h of chase only the lower molecular mass species was detected (lane *5*). The migration of the lower molecular mass form of the cell-associated β-galactosidase molecules coincides with the molecules internalized at 37°C (compare lanes *2* and *5*). Thus, both β-galactosidase and β-glucuronidase undergo an apparent proteolytic maturation following internalization by the mouse L cells at either 37 or 20°C.

The similar maturation of β-glucuronidase observed at both temperatures coupled with the dissimilar location of the ^{125}I-labeled molecules within the L cells at the two temperatures (Fig. 2) suggested that the proteolytic processing occurs at a prelysosomal location. 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 [^{3}H]mannose-labeled P388D$_1$-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 [^{3}H]mannose-labeled J774 secretions. After an overnight incubation, the cells were harvested, β-glucuronidase was recovered by immunoprecipitation, and the ^{3}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

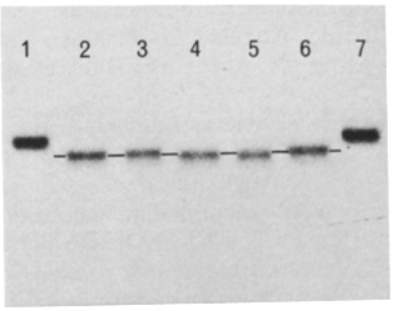

Figure 6. Kinetics of the postendocytic maturation of β-glucuronidase at 37°C. Mouse L-cells (confluent monolayers on p-15 dishes) were mixed with ^{125}I-β-glucuronidase (2.7×10^7 cpm in 12 ml of media containing 20 mM Hepes, pH 7) for 60 min at 7°C. The medium containing nonbound ligand was then replaced with fresh medium, prewarmed to 37°C, and the cells were incubated at 37°C. For each time point, a p-15 dish of cells was harvested, the cells were solubilized with Triton X-100, and β-glucuronidase was isolated by immunoprecipitation. The immunoprecipitates were digested with endo H and analyzed by SDS gel electrophoresis and radioautography. The autoradiogram shows the β-glucuronidase recovered after 0 (lane *2*), 10 (lane *3*), 20 (lane *4*), and 30 min (lane *5*) at 37°C. For comparison, the input β-glucuronidase molecules are shown with (lane *6*) and without (lanes *1* and *7*) endo H digestion.

phosphorylated oligosaccharides; units with one phosphomonoester, units with two phosphomonoesters, and hybrid-type units with one phosphomonoester 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 P388D$_1$ 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 [^{3}H]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 P388D$_1$ secretions. As shown in Fig. 4, β-glucuronidase recovered from the I cells after incubation of the cells with [^{3}H]leucine-labeled P388D$_1$ 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 [^{3}H]mannose-labeled phosphorylated oligosaccharide recovered from the I-cell–associated enzyme contained a single phosphomonoester 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 [^{3}H]Mannose-labeled β-Glucuronidase

Preparation of J774 secretions	Enzyme source	Counts per minute recovered as: N	1SA	1PM	1PM/1SA	2PM	Percent phosphorylated	2:1 ratio
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

[^{3}H]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 phosphomonoester (1PM), one phosphomonoester and one sialic acid residue (1PM/1SA), and two phosphomonoesters (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
$P388D_1$ 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

[^{3}H]Mannose-labeled $P388D_1$ 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 1.9×10^6 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 (55). 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 ^{125}I-asialofetuin at 20°C, but the ^{125}I-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 slower 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 ^{125}I-β-glucuronidase at 37°C, the internalized radiolabeled molecules sedimented on a density gradient with the endogenous acid hydrolase activities; thus, the ^{125}I-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 ^{125}I-β-glucuronidase internalized at 20°C is comparable to the density of endocytic vesicles that contained ^{125}I-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 ^{125}I-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 degradation of a ligand, mannosylated BSA, begins within the endosomal compartment of rabbit macrophages. Epidermal growth factor receptor also has 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 maturations 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 both 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.

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