Processing, Transport, and Secretion of the Lysosomal Enzyme Acid Phosphatase in Dictyostelium discoideum*

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To explain the different secretion kinetics of lysosomal enzymes in Dictyostelium discoideum, previous investigators have hypothesized the existence of a heterogeneous population of lysosomes containing either the enzyme acid phosphatase or other hydrolase enzymes. The hypothesis is based on the observation that the percentage of radiolabeled acid phosphatase secreted from the endoplasmic reticulum to Golgi complex (half-time of 3 min) as measured by the acquisition of resistance to the enzyme endoglycosidase H. Furthermore, Percoll gradient fractionations indicated that radiolabeled forms of acid phosphatase reached dense lysosomal vesicles at about the same time as final processing was occurring. Proper sorting of acid phosphatase in D. discoideum apparently was not critically dependent on low intravacuolar pH since the addition of ammonium chloride did not stimulate the missorting and acidification of acid phosphatase. These results are very similar to previous observations concerning other Dictyostelium lysosomal enzymes. Consistent with the existence of a heterogeneous population of lysosomes, the percentage of radiolabeled acid phosphatase secreted 4 h into a chase period was 15-fold lower as compared with another lysosomal enzyme, β-glucosidase. However, acid phosphatase, α-mannosidase, and β-glucosidase were all predominantly colocalized as determined by indirect immunofluorescence, which for the first time demonstrates the homogeneous nature of the lysosomal system in D. discoideum. Taken together these results suggest that the processing and transport of acid phosphatase may be similar in nature to the glycosidases. However, the different kinetics of secretion of acid phosphatase versus the colocalized glycosidase enzymes suggests that an undefined mechanism operates to distinguish these classes of enzymes at a step after localization to lysosomes but prior to secretion.

The biosynthesis of lysosomal enzymes has been well characterized in certain mammalian systems such as the human fibroblast (1, 2). Lysosomal enzymes are synthesized on membrane-bound polysomes and cotranslationally inserted into the rough endoplasmic reticulum where they receive N-linked high-mannose oligosaccharide side chains. These enzymes are later transported to the Golgi complex where modification to their carbohydrate moieties generates phosphomannosyl residues which have been shown to serve as the recognition markers for mannose 6-phosphate receptors (1). These receptors mediate transport of lysosomal enzymes to endosomal or prelysosomal vesicles where acidic conditions facilitate receptor-ligand uncoupling and the recycling of these receptors back to the Golgi (2, 3). However, a mannose 6-phosphate receptor-independent pathway may exist, because in I-cell fibroblasts lysosomal enzymes are not phosphorylated but reach lysosomes nonetheless (4, 5).

Dictyostelium discoideum is a simple haploid eucaryotic organism which can be manipulated both genetically and biochemically and can therefore serve as a good model system for studying protein transport and targeting (6). Also, no detectable mannose 6-phosphate receptor has been identified in this organism, thus making this a useful system to define alternative mechanisms for lysosomal enzyme targeting (7, 8). Two of the better-characterized enzymes, α-mannosidase and β-glucosidase, are synthesized on membrane-bound polysomes, cotranslationally inserted into the endoplasmic reticulum and modified by the attachment of high-mannose oligosaccharides on asparagine residues (8, 9). These membrane-associated precursor forms of the enzymes are next transported to the Golgi complex where their carbohydrate side chains receive specific modifications including the addition of phosphate and sulfate groups (10). At this point the hydrolases can follow two distinct pathways (11, 12). A constitutive pathway leads to the secretion of 5–10% of the precursor forms of lysosomal enzymes to the extracellular medium while the second pathway involves the directed movement of the remaining 90% of the lysosomal enzyme precursors to lysosomal organelles (7, 12). Two separate proteolytic cleavage events have been shown to occur along this pathway (11, 13). The first cleavage which may be important for targeting (13) generates intermediate polypeptides from the precursor polypeptide and is followed by additional cleavages in lysosomes to generate the nature forms of the enzymes. Increasing the lysosomal/endosomal pH from 5.4 to 6.4 with ammonium chloride does not prevent the proper sorting of lysosomal hydrolases but does prevent the cleavage of the lysosomally localized intermediate forms of the enzymes (14). Finally, the regulated secretion of mature lysosomal enzymes occurs from lysosomal compartments (15, 16).

A previous study using developing Dictyostelium cells revealed that acid phosphatase is initially synthesized in a
precursor form as a glycoprotein which undergoes a rapid molecular weight shift during a pulse-chase to a mature form of the protein (17). However, the biosynthesis of this enzyme has not been well characterized in axenically growing cells. Furthermore, previous biochemical and genetic studies have suggested that acid phosphatase may be targeted to a completely separate population of lysosomal vesicles than the glycosidases (15, 16). For instance, the secretion kinetics for acid phosphatase differs from the glycosidase enzymes during starvation (15). Also, the phenotypic properties of various classes of secretory mutants of Dictyostelium can be explained most easily by proposing heterogeneity in the enzyme composition of vesicles (16).

Several key points concerning the biosynthesis of this enzyme need to be addressed including 1) the molecular nature of the precursor to mature polypeptide processing events, 2) the kinetics of transport of the enzyme to the various intracellular compartments, and 3) the actual enzyme composition of the lysosomal vacular system. Our results described here indicate that there is a rapid proteolytic conversion of the newly synthesized acid phosphatase precursor to a slightly lower molecular weight mature form. This enzyme like the glycosidases was also found to move rapidly from the endoplasmic reticulum to the Golgi complex, and then to lysosomes. Finally, indirect immunofluorescence studies revealed the lysosomal enzyme in wild type Dictyostelium mutants in nature; the glycosidase and acid phosphatase enzymes were found to be mainly colocalized in the same vesicles.

**MATERIALS AND METHODS**

**Organism.—** D. discoideum strains Ax3 (wild type) and GM1 were grown axenically in TM medium (18) in a rotary shaker waterbath at 200 rpm and 21 °C. GM1 is an α-mannosidase and β-glucosidase structural gene mutant and contains no detectable α-mannosidase and β-glucosidase polypeptides.

**Enzyme Assays—** All enzyme assays were done at 35 °C using the appropriate p-nitrophenyl substrates purchased from Sigma. The assay conditions for the enzymes α-glucosidase-2, acid phosphatase, and β-glucosidase have been previously reported (17, 19, 20).

**Radioactive Labeling—** Logarithmically growing cells were collected by centrifugation (1000 g for 3 min) and resuspended to a titer of 1.5 X 10⁷ cells/ml. TM medium containing 750 μCi/ml of [³⁵S]methionine (specific activity 1.12 Ci/mmol, Du Pont, New England Nuclear), dried under a vacuum, and exposed to Kodak XAR-5 x-ray film at ~90 °C. Densitometric analysis of the band intensities contained on the fluorographs was performed using a LKB 2202 Ultrascan Laser Densitometer (LKB Instrument Inc., Gathersburg, MD).

**Endoglycosidase H and Glycopeptidase F Digestions—** Immunoprecipitates were resuspended in 20 μl of a solution containing 2% SDS and 10% β-mercaptoethanol, heated at 80 °C for 3 min, and centrifuged (10,000 g for 3 min). For digestion with endo H (Boehringer Mannheim), 26 μl of 100 mM sodium citrate, pH 5.5, solution was added to the recovered supernatant followed by 2.5 μl of endo H (1 unit/ml) in 50 mM sodium phosphate buffer, pH 5.5). For digestion with PNGase F (Boehringer Mannheim), 20 μl of a 100 μM EDTA, 200 μM sodium phosphate, pH 6.1, solution was added to the recovered supernatants followed by the addition of 10 units of PNGase F. Digestions proceeded overnight at 37 °C. The samples were then mixed with 40 μl of 2 x gel buffer and subjected to SDS-PAGE followed by fluorography.

**Subcellular Fractionation on Percoll Gradients—** Radioactively labeled Ax3 cells were mixed with a 50-100-fold excess of GM1 cells to facilitate breakage and recovery. These cells were then resuspended to 3 x 10⁶ cells/ml in MESSES buffer (20 mM MOPS, pH 6.5, 1 mM Na₂ EDTA, and 0.25 mM sucrose) and broken at 4 °C using a tight fitting Dounce homogenizer. Homogenates were then centrifuged (5,000 g for 5 min) to remove whole cells and nuclei. The postnuclear supernatants were removed and the pellet resuspended in additional MESSES buffer, rebroken, and centrifuged again. The combined postnuclear supernatants (5 ml) were layered on 21 ml of 24% Percoll (Sigma) made up in MESSES buffer, and these gradients were centrifuged for 60 min at 17,500 rpm in a Beckman type 40.2 rotor. Gradients were then separated into 100 μl fractions (3300 pellets) and cells to be analyzed for enzyme activity were isolated from the bottom using a microcapillary pipette connected to a peristaltic pump. Before immunoprecipitation the fractions were cleared of Percoll by centrifugation in a Beckman type 50 rotor at 100,000 g for 60 min. Samples were adjusted to 0.5% Triton X-100 prior to immunoprecipitation. It was necessary to subject Percoll samples to two rounds of immunoprecipitation in order to precipitate all the acid phosphatase present as measured by enzymatic activity.

**Immunofluorescence—** Small volumes (less than 250 μl) of Ax3 cultures at titers between 1-4 X 10⁶ cells/ml were placed onto coverslips. Cells were allowed to settle out and then were fixed in a solution of 3.7% formaldehyde/PBS (phosphate-buffered saline, pH 7.2) at 21 °C for 1 h. Coverslips were washed in several changes of PBS followed by the addition of primary mouse monoclonal antibodies to the Dictyostelium lysosomal enzymes α-mannosidase (IgGα) or acid phosphatase (IgGβ). Antibodies were diluted 1:100 in PBS solution (2.5 mg/ml bovine serum albumin, 0.1% saponin in PBS) and were applied to the cells for 1 h at 4 °C. Coverslips were again washed and followed by the addition of goat anti-mouse isotype specific fluorescein isothiocyanate or rhodamine isothiocyanate antibodies (Southern Biotechnology Inc.) diluted 1:50 in PBS solution. Coverslips were washed after a 1-h incubation, mounted in a solution of glycerol with p-phenylenediamine to prevent fading (32), and observed and photographed using an Olympus model BH-2 microscope equipped with ultraviolet illumination and a Kodak TMAX-400 print film. Control experiments were performed to determine the signal spillover between the two fluorescence channels. Cells were fixed with formaldehyde, incubated with only one antibody specific for acid phosphatase or α-mannosidase and then secondarily stained with the appropriate isotype-specific antibody as previously stated. The cells were then examined and photographed in both fluorescence channels. The specificity of our secondary antibodies was also tested. Fixed cells were labeled with only one antibody to either acid phosphatase or α-mannosidase and then secondarily stained with the wrong isotype specific antibody. These cells were then observed under each fluorescence channel and photographed as previously stated.

**RESULTS**

**Newly Synthesized Acid Phosphatase Undergoes Proteolytic Processing—** Fig. 1A represents the results of an experiment in which axenically growing cells were pulse radiolabeled for 10 min with [³⁵S]methionine followed by a chase period in unlabeled medium of 0 or 90 min. Radiolabeled acid phosphatase was immunoprecipitated from detergent-treated cells and subjected to SDS-PAGE followed by fluorography. A comparison of lane 2 with lanes 1 and 3 reveals that newly synthesized acid phosphatase had a molecular mass of 56 kDa and follow-
tase antibody, and subjected to SDS-PAGE and fluorography.

Pulse phosphatase was immunoprecipitated from cell extracts and subjected to SDS-PAGE followed by fluorography. Panel B shows the kinetics of this processing. Cells were pulse labeled with [35S]methionine and chased for 2, 4, 6, 8, 10, and 20 min (lanes 1-6). Samples were taken at these points, solubilized, immunoprecipitated with acid phosphatase antibody, and subjected to SDS-PAGE and fluorography.

We conclude, therefore, that the shift in molecular mass during a chase of deglycosylated acid phosphatase from 42 to 41 kDa and similarly the shift of 56-55 kDa observed for fully glycosylated polypeptide was the result of a proteolytic cleavage event.

Kinetics of Transport of Acid Phosphatase from the ER to Golgi Complex—The rate of movement of glycoproteins from the ER to Golgi complex can be determined using the enzyme endo H which specifically cleaves N-linked high-mannose carbohydrate side chains between the two N-acetylglucosamine sugars (24). Oligosaccharide side chains modified by enzymes residing in the Golgi complex are resistant to cleavage by this enzyme; and hence the population of glycoproteins that have reached the Golgi compartment are distinct from those which have not based on size separation on SDS-polyacrylamide gels. Therefore, the half-time of acquiring resistance to endo H correlates with movement from the ER to the Golgi complex. Fig. 3A shows the result of an experiment in which cells were pulse labeled with [35S]methionine and chased for various lengths of time in unlabeled medium. Acid phosphatase was then immunoprecipitated, treated with endo H, and subjected to SDS-PAGE and fluorography.

Following pulse labeling, a deglycosylated polypeptide of 42.5 kDa was observed (lane 1 of Fig. 3). This polypeptide was converted during the chase to several endo H-resistant higher molecular mass polypeptides of 55, 53, and 51 kDa representing forms with zero, one, or two endo H-sensitive oligosaccharide side chains. The radiolabeled 53-kDa form exists after long chase periods implying that one side chain never becomes resistant to endo H cleavage (data not shown). Laser densitometric scans (Fig. 3B) revealed that 50% of the sensitive form of acid phosphatase was converted to the resistant forms after only 3 min of chase. This suggests that acid phosphatase was transported out of the ER to the Golgi complex at a very rapid rate.

Fig. 3B also indicates the rate at which the newly synthesized lysosomal enzymes α-mannosidase and β-glucosidase became resistant to endo H; the processing and transport of these lysosomal glycosidases have previously been described (6-9, 25). β-Glucosidase had approximately the same half-time of acquiring resistance to endo H as acid phosphatase and both of these enzymes differed from α-mannosidase whose half-time of movement to the Golgi complex was between 12 and 15 min.

Rate of Transport of Acid Phosphatase to Lysosomes—Panel A of Fig. 4 indicates the results of an experiment in which
FIG. 4. Movement of radiolabeled acid phosphatase and β-glucosidase to dense lysosomes. 10^6 Ax3 cells were collected, homogenized, and postnuclear supernatants were applied to 24% Percoll gradients. The gradient was separated into 20 distinct fractions and acid phosphatase (○-○), β-glucosidase (■-■), and α-glucosidase II (□-□) enzyme activities were determined, and the percentage of total enzyme activity for each enzyme was plotted versus the appropriate fraction number (panel A). In addition, cells were pulse labeled for either 10 or 30 min with [35S]methionine, homogenized, and subjected to fractionation on 24% Percoll gradients. Each gradient was separated into 20 fractions, and even-numbered fractions were centrifuged to remove Percoll. The Percoll-free samples were immunoprecipitated with either acid phosphatase (panel C) or β-glucosidase (panel B) antibody and subjected to SDS-PAGE followed by fluorography. The fractions were pooled, and the percentage of total radiolabeled enzyme in either the soluble (fractions 16, 18, and 20), ER/Golgi (fractions 12 and 14), light lysosomal (fractions 8 and 10) and/or dense lysosomal (fractions 2, 4, and 6) fractions was determined by laser densitometric scans.
postnuclear supernatants, prepared from growing cells, were fractionated on 24% Percoll gradients. Each fraction was assayed for the enzymes acid phosphatase, $\beta$-glucosidase, and $\alpha$-glucosidase-2; $\beta$-glucosidase and acid phosphatase are markers for lysosomes while $\alpha$-glucosidase-2 is a marker for the microsomal fraction which contains the ER/Golgi vesicles (13). Three distinct peaks containing both acid phosphatase and $\beta$-glucosidase activities were resolved on Percoll gradients. The major peak of enzyme activity was present in the dense bottom fractions (1–6) where secondary lysosomes should sedent. This peak was well separated from the peak of $\alpha$-glucosidase-2 activity between fractions 11–14 which contains the majority of the lighter ER/Golgi vesicles (13). Fractions 16–20 also contained acid phosphatase and $\beta$-glucosidase activities which represent soluble mature enzyme released from broken or ruptured vesicles. Finally fractions 9–13 consistently contain a peak of acid phosphatase activity twice that of $\beta$-glucosidase activity.

We next examined the intracellular movement of acid phosphatase to lysosomes using the Percoll gradient fractionation scheme just outlined. Cells were pulsed with [35S]methionine for either 10 or 30 min and following breakage, postnuclear supernatants were applied to 24% Percoll gradients and centrifuged. Gradients were fractionated, and radiolabeled acid phosphatase and $\beta$-glucosidase were immunoprecipitated and subjected to SDS-PAGE and fluorography. The results presented in Fig. 4, B and C indicate that a labeling time between 10–30 min was needed before the first wave of radiolabeled enzyme reached the dense lysosomal fractions 2, 4, and 6. For instance, after a 30-min pulse, 9 and 16% of the total radiolabeled forms of acid phosphatase and $\beta$-glucosidase, respectively, were present within these dense fractions. These data suggest that $\beta$-glucosidase moved to the lysosomes slightly faster than acid phosphatase.

The half-times for movement of newly synthesized enzymes to dense lysosomal fractions (2–6) were also determined. Cells were pulsed radiolabeled for 20 min and chased for 0, 20, 75, or 180 min in unlabeled medium. The percentages in fractions 2–6 of total radiolabeled forms of both acid phosphatase and $\beta$-glucosidase were obtained from a densitometric analysis of the fluorographs, and are plotted in Fig. 5 versus chase time in minutes. The half-times of movement of newly synthesized $\beta$-glucosidase and acid phosphatase to dense lysosomal vesicles were 22 and 30 min, respectively.

Acid Phosphatase and Glycosidase Enzymes Are Colocalized—As described above, acid phosphatase and the glycosidase activities overlapped on Percoll gradients suggesting these enzymes may reside in the same vesicles. To directly test this hypothesis, the intracellular locations of $\alpha$-mannosidase and acid phosphatase were determined by indirect immunofluorescence microscopy. Monoclonal antibodies to both enzymes were added simultaneously to formaldehyde-fixed cells. Following washes, isotype-specific goat anti-mouse antibodies coupled with fluorescein isothiocyanate and rhodamine isothiocyanate were added, and cells were visualized in a fluorescence microscope. As seen in Fig. 6, $\alpha$-mannosidase (panel B) and acid phosphatase (panel A) were colocalized in greater than 95% of the punctate vesicles. The remaining 5% of these vesicles contained acid phosphatase only (data not shown). In addition $\beta$-glucosidase and $\alpha$-mannosidase were also found to be colocalized to the same population of vesicles (data not shown). Control experiments (see "Materials and Methods") were also performed to determine the presence of signal spillover between the fluorescence channels as well as to test the specificity of the isotype specific secondary antibodies. The results from these experiments revealed that the secondary antibody staining was highly specific, and the amount of signal spillover between the red and green channels was not significant (data not shown).

Effects of Protease Inhibitors and Changes in Intravacuolar pH on Enzyme Secretion—The secretion kinetics of acid phosphatase activity differs from other lysosomal enzymes during starvation of cells (15) even though as described above, the enzymes reside in the same organelles. A radiolabeled pulse-chase protocol was used to compare the secretion of acid phosphatase and the glycosidases during growth conditions. In these experiments cells were pulse radiolabeled for 10 min with [35S]methionine and chased in unlabeled medium containing the chemicals listed in Table I. By 8 h of chase only 6% of radiolabeled acid phosphatase had been secreted.
and Poole (31) as detailed in Ref. 14. The percentage of radiolabeled \( \alpha \)-glucosidase secreted by 1.5 h macrophages (26) was determined by densitometric scans of x-ray films as described under "Materials and Methods." A vesicular \( \alpha \)-glucosidase was measured by a modified procedure of Ohkuma and Poole (31) as detailed in Ref. 14. The percentage of total radiolabeled acid phosphatase and \( \beta \)-glucosidase secreted was determined by densitometric scans of x-ray films as described under "Materials and Methods." under control conditions; in contrast, 70% of radiolabeled mature \( \beta \)-glucosidase had exited cells at this time. The small percentage of radiolabeled \( \beta \)-glucosidase secreted by 1.5 h represents precursor forms which escaped processing and targeting (7, 12). Raising the pH of the medium to 7.2 and adding NH\(_4\)Cl to 40 mM, results in an increase in intravacuolar pH from 5.4 to 6.4 (14). Under these conditions only 3% of acid phosphatase had been secreted by 8 h of chase, suggesting that maintenance of an acidic intravesicular environment was not essential for the intracellular retention of acid phosphatase. Also as previously observed (14) increases in intravesicular pH affected only a small increase (Table I) in the secretion of radiolabeled precursor forms of \( \beta \)-glucosidase (from 2.2 to 10.2%); under these conditions secretion of mature forms was slightly reduced (68 to 53%) by 8 h of chase.

The cysteine and serine protease inhibitors, leupeptin and antipain, have been shown to inhibit proteolysis of precursor forms of \( \alpha \)-mannosidase and \( \beta \)-glucosidase and boost secretion of these forms of the enzymes (13). The addition of leupeptin to cultures resulted in a stimulation in the missorting and secretion of labeled precursor forms of acid phosphatase and \( \beta \)-glucosidase after 1.5 h of chase. These results suggest that as observed for \( \alpha \)-mannosidase and \( \beta \)-glucosidase, proteolysis may be important in targeting of acid phosphatase to lysosomes. However, a significantly greater percentage of \( \beta \)-glucosidase precursor polypeptides were secreted as compared with acid phosphatase in cultures treated with leupeptin (Table I).

### DISCUSSION

This report examines the transport, processing, and secretion of the \textit{D. discoideum} lysosomal enzyme, acid phosphatase. Previous investigations had indicated that the secretion of this enzyme was differentially sensitive to the agents cycloheximide and chloroquine diphosphate and was kinetically distinct from that of lysosomal glycosidases. These observations led Dimond \textit{et al.} (15, 16)\(^7\) to propose that in \textit{Dictyostelium} the lysosomal enzyme system was heterogeneous in nature and included a separate population of vesicles enriched for acid phosphatase; heterogeneity in the population of lysosomal vesicles has also been proposed for kidney cells and macrophages (26). This model which predicts the existence of different sorting and secretion mechanisms for acid phosphatase versus the glycosidases is not novel since acid phosphatase may be sorted by a unique mechanism in other eucaryotic cells. For instance, in fibroblasts from 1-cell disease patients, the activities of most lysosomal enzymes are low while the activity of acid phosphatase is nearly normal. Therefore, a certain population of acid phosphatase may be transported by a mechanism distinct from the normal mannose 6-phosphate receptor pathway (27, 28).

Results presented here clearly demonstrate that the lysosomal system of \textit{Dictyostelium} is qualitatively homogenous in its vesicular content of lysosomal enzymes; this conclusion is based on two lines of evidence. First, the enzymes acid phosphatase, \( \beta \)-glucosidase, and \( \alpha \)-mannosidase distribute together upon fractionation of cell extracts on Percoll gradients. This is indirect evidence, however, since we could not eliminate the possibility that vesicles containing only acid phosphatase were similar in density to vesicles containing only the glycosidases. Use of indirect immunofluorescence provided direct evidence for the colocalization of both acid phosphatase and the glycosidase enzymes in numerous subcellular punctate vesicles. Our results do not eliminate the possibility of quantitative differences in vesicular enzyme content, but these data reveal that the majority of acid phosphatase is not sorted to a distinct lysosomal vesicle devoid of other hydrolases. Thus, the mechanism accounting for differential secretion properties of acid phosphatase cannot be based on differential sorting.

Results reported here demonstrate for the first time that the initial precursor form of acid phosphatase (56 kDa) is proteolytically processed within 20 min of synthesis to a mature form (55 kDa). Thus, all three of the well-characterized lysosomal enzymes in \textit{D. discoideum}, \( \alpha \)-mannosidase, \( \beta \)-glucosidase, and acid phosphatase (this report) are synthesized as precursor polypeptides which are proteolytically processed to mature forms. Our results are consistent with a previous study which showed that acid phosphatase was rapidly processed in \textit{Dictyostelium} (17). However, this earlier report did not directly address the question of proteolytic processing, and the results indicated that the molecular masses of the precursor and mature forms of acid phosphatase were 60 and 58 kDa, respectively, which are slightly different from our values of 56 and 55 kDa. Presently, we can not explain the minor discrepancies in these two reported molecular weights.

The subcellular transport of newly synthesized acid phosphatase from the ER to lysosomes required approximately 20 min as determined by Percoll gradient fractionation suggesting that the generation of the mature form may occur when the polypeptides arrive in lysosomes. The enzyme \( \beta \)-glucosidase behaves in a very similar manner which suggests that variable rates of processing and transport cannot explain secretion differences between acid phosphatase and the glycosidase enzymes.

As was found for \( \alpha \)-mannosidase and \( \beta \)-glucosidase (13), inhibition of proteolytic processing with the serine/cysteine proteinase inhibitor leupeptin led to the missorting and oversecretion of precursor forms of acid phosphatase. However, a greater percentage of newly synthesized acid phosphatase relative to \( \alpha \)-mannosidase and \( \beta \)-glucosidase was retained and processed to mature forms in treated cells (data not shown). This suggests that proteolytic processing \textit{per se} may not be as critical for the localization of acid phosphatase as for the glycosidases.

Secretion of acid phosphatase and \( \beta \)-glucosidase was also investigated using pulse-chase radiolabeling techniques. Over

### Table I

<table>
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<th>Hours of chase</th>
<th>Composition of media</th>
<th>Intravacuolar pH*</th>
<th>Percent of total secreted†</th>
<th>Acid phosphatase</th>
<th>( \beta )-Glucosidase</th>
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</table>

*Vesicular pH was measured by a modified procedure of Ohkuma and Poole (31) as detailed in Ref. 14.
†The percentage of total radiolabeled acid phosphatase and \( \beta \)-glucosidase secreted was determined by densitometric scans of x-ray films as described under "Materials and Methods."
a long chase period the enzyme acid phosphatase was secreted at a significantly slower rate compared with β-glucosidase. This result is consistent with previous studies analyzing the secretion of enzyme activity during starvation (15) and suggests that acid phosphatase may be differentially regulated in its secretion as pH compared to the glucosidase enzymes. Studies using weak bases were initiated to determine if intralysosomal/endoosomal pH might regulate the rate of secretion of lysosomal enzymes. Hohman and Bowers (29) have suggested that in Acanthamoeba differential association of lysosomal enzymes with membranes of vesicles recycling to the plasma membrane from lysosomes can account for different secretion kinetics. Furthermore, this association was found to be dependent on pH (30). Raising the lysosomal/endoosomal pH in Dictyostelium to near neutrality resulted in no increase in secretion of newly synthesized precursor forms of acid phosphatase and only a slight increase in the secretion of β-glucosidase precursor forms (Table I). Thus, as previously observed (14), maintenance of acidic lysosomal/endoosomal pH is not required for the proper sorting of acid phosphatase in Dictyostelium. Furthermore, increases in intravesicular pH caused a 50% decrease in the extent of secretion of mature acid phosphatase and only a 20% decrease in secretion of β-glucosidase by 8 h of chase. Thus, the mechanism regulating differential secretion of lysosomal enzymes may be partially dependent on pH consistent with previously reported results in Acanthamoeba (29, 30).

Taken together these results suggest that lysosomal enzymes in Dictyostelium may share the same lysosomal transport pathway and targeting signals, however, the mechanisms regulating secretion of these enzymes may be different. Several possible hypotheses may account for differential secretion. For instance, acid phosphatase may associate with internal membranes in a way distinct from the glucosidase enzymes. Previous results in Dictyostelium have shown that precursor forms of both α-mannosidase and β-glucosidase are membrane associated while the fully mature forms are soluble (7, 12). Similarly, preliminary results indicate that only precursor forms of acid phosphatase are membrane associated while mature forms appear to be soluble (data not shown). However, these in vitro conditions may not mimic the actual internal environment found in the lysosomal compartments and, therefore, preferential membrane association of mature acid phosphatase cannot be ruled out at this time. Acid phosphatase contains more carbohydrate side chains per mass of protein as compared with the glucosidases which also might account for preferential association with other protein molecules such as receptors, resulting in a low rate of secretion.

Further experiments will be needed to determine the specific signals used both for targeting of acid phosphatase to lysosomes and for regulating differential secretion. We have a collection of mutant strains which are defective in a number of aspects of lysosomal enzyme biosynthesis such as correct construction of N-linked carbohydrate structures, sorting of precursor polypeptides, and enzyme secretion. Biochemical analysis of these mutants may define the mechanisms critical for sorting and secretion of lysosomal enzymes. Finally, analysis of the cloned genes coding for these lysosomal enzymes may reveal protein domains or amino acid sequences responsible for sorting and secretion.

REFERENCES