

Inhibition of Early but Not Late Proteolytic Processing Events Leads to the Missorting and Oversecretion of Precursor Forms of Lysosomal Enzymes in *Dictyostelium discoideum*

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Abstract. Lysosomal enzymes are initially synthesized as precursor polypeptides which are proteolytically cleaved to generate mature forms of the enzymatically active protein. The identification of the proteinases involved in this process and their intracellular location will be important initial steps in determining the role of proteolysis in the function and targeting of lysosomal enzymes. Toward this end, axenically growing *Dictyostelium discoideum* cells were pulse radiolabeled with [³⁵S]methionine and chased in fresh growth medium containing inhibitors of aspartic, metallo, serine, or cysteine proteinases. Cells exposed to the serine/cysteine proteinase inhibitors leupeptin and antipain and the cysteine proteinase inhibitor benzyloxycarbonyl-L-phenylalanyl-L-alanine-diazomethyl ketone (Z-Phe-AlaCHN₂) were unable to complete proteolytic processing of the newly synthesized lysosomal enzymes, α -mannosidase and β -glucosidase. Antipain and leupeptin treatment resulted in both a dramatic decrease in the efficiency of proteolytic processing, as well as a sevenfold increase in the secretion of α -mannosidase and β -glucosidase precursors. However, leupeptin and antipain did not stimulate secretion of lysosomally localized mature forms of the enzymes

suggesting that these inhibitors prevent the normal sorting of lysosomal enzyme precursors to lysosomes. In contrast to the results observed for cells treated with leupeptin or antipain, Z-Phe-AlaCHN₂ did not prevent the cleavage of precursor polypeptides to intermediate forms of the enzymes, but greatly inhibited the production of the mature enzymes. The accumulated intermediate forms of the enzymes, however, were localized to lysosomes. Finally, fractionation of cell extracts on Percoll gradients indicated that the processing of radiolabeled precursor forms of α -mannosidase and β -glucosidase to intermediate products began in cellular compartments intermediate in density between the Golgi complex and mature lysosomes. The generation of the mature forms, in contrast, was completed immediately upon or soon after arrival in lysosomes. Together these results suggest that different proteinases residing in separate intracellular compartments may be involved in generating intermediate and mature forms of lysosomal enzymes in *Dictyostelium discoideum*, and that the initial cleavage of the precursors may be critical for the proper localization of lysosomal enzymes.

ALL lysosomal enzymes examined to date are initially synthesized in the rough endoplasmic reticulum (RER)¹ as precursor polypeptides that are transported to the Golgi complex and subsequently proteolyti-

cally processed to generate lysosomally localized mature enzymes (9, 16, 30, 31, 47). Much remains to be learned about this process including the intracellular site(s) of proteolytic cleavage, the identity of the processing proteinases and the role of proteolysis in the activation, function, and targeting of lysosomal enzymes.

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1. *Abbreviations used in this paper:* endo H, endoglycosidase H; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; TLCK, *N*-p-tosyl-L-lysine chloromethyl ketone; Z-Gly-PheNH₂, carbobenzoxy-L-glycine-phenylalanine-amide; Z-Phe-AlaCHN₂, benzyloxycarbonyl-L-phenylalanyl-L-alanine-diazomethyl ketone.

The proteolytic processing of precursor polypeptides to mature forms of lysosomal enzymes usually occurs in several distinct steps. One or more intermediate forms are first generated from precursor polypeptides and in turn these polypeptides are cleaved to generate mature subunits. Both the kinetics of proteolytic processing of lysosomal enzymes and the compartments where cleavage occurs have been investigated (31, 47), however, the nature of the proteolytic en-

zymes is not well understood. Cysteine proteinases have been implicated in the generation of mature enzymes in several systems. For instance, proteolytic cleavage of intermediate forms of cathepsin D in fibroblasts to the mature form occurs in lysosomes, and is inhibited in the presence of cysteine proteinase inhibitors (24, 32). This suggests that cysteine proteinases operating in the acidic environment of lysosomes are responsible for the final proteolytic cleavage events. The nature and cellular location of the proteinases responsible for the generation of intermediate forms of lysosomal enzymes from the initially synthesized precursor are, however, less well defined than the lysosomally localized cysteine proteinases. Gieselmann et al. reported that the initial proteolytic processing of cathepsin D occurs in prelysosomal compartments (25) and is not inhibited by cysteine proteinase inhibitors, indicating another class of proteinase may be responsible for the primary proteolytic cleavage of lysosomal enzymes.

Dictyostelium discoideum is an excellent system to investigate the transport, processing, and localization of lysosomal enzymes because both genetic and molecular biology studies can be conducted with relative ease (9, 11). Two of the better characterized enzymes, α -mannosidase and β -glucosidase, are synthesized in the rough endoplasmic reticulum as polypeptides of 120 and 94 kD, respectively, that are cotranslationally modified by the addition of asparagine-linked mannose-rich oligosaccharide side chains to generate glycoproteins of 140 and 105 kD (11, 12). The α -mannosidase and β -glucosidase precursor polypeptides move from the RER to the Golgi complex at different rates (10, 40, 49) where they are further modified by the addition of sulfate (13, 19) and methyl-phosphate (20, 22) to mannose residues. After exiting the Golgi apparatus, 85–95% of the precursor molecules are transported to lysosomes where proteolytic processing to mature forms is completed (10, 40, 41, 49) while the remaining 5–15% of the molecules are secreted from the cell along a constitutive pathway as precursor polypeptides (10, 40). The proteolytically processed lysosomally localized enzymes are also secreted, but in a regulated manner (8). In this study we have used inhibitors for each of the four characterized classes of proteinases to learn more about the role of proteolysis in lysosomal enzymes biosynthesis. Our results suggest that more than one enzyme may be involved in the proteolytic processing of lysosomal enzymes and that the initial cleavage of precursor polypeptides may be important in the correct localization of lysosomal enzymes. Furthermore, processing of the precursor polypeptides may begin in a compartment intermediate in density between lysosomes and the Golgi complex.

Materials and Methods

Organism

The *Dictyostelium discoideum* wild-type strain, Ax3, was grown axenically in TM medium (18) at 21°–24°C on a rotary shaker. The α -mannosidase and β -glucosidase structural gene mutant, GM1, was used as carrier cells where indicated.

Proteinase Inhibitors Used

All inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO), with the exception of benzyloxycarbonyl-L-phenylalanyl-L-alanine-diazomethyl ketone (Z-Phe-AlaCHN₂) which was a kind gift of Dr. E. Shaw

(Friedrich Miescher-Institut, Basel, Switzerland). Stock solutions of E-64 (100 mM), leupeptin (25 mg/ml), and antipain (25 mg/ml) were made in water. Stock solutions of *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (10 mM) and chloroquine (1 M) were made in TM medium. L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), diazoacetyl-DL-norleucine methyl ester, 1,2-epoxy-3-(*p*-nitrophenoxy) propane, phenylmethylsulfonyl fluoride (PMSF), and pepstatin were solubilized in DMSO (50% wt/vol) and these stock solutions were immediately diluted in TM to the concentrations indicated in each experiment. Z-Phe-AlaCHN₂ was solubilized in DMSO to a final concentration of 1 M. The final concentration of DMSO did not exceed 1.0%; 1.0% DMSO alone had no effect on lysosomal enzyme processing.

Radioactive Labeling of Cells

Exponentially growing cells were harvested by centrifugation (1,000 $g \times 2$ min) and radioactively labeled by resuspension to a final concentration of 10⁷ cells/ml in fresh TM containing 0.75 mCi/ml [³⁵S]methionine (1,200 Ci/mmol; Amersham Corp., Arlington Heights, IL). After pulse labeling at 21°–24°C for the times indicated in Results, the cells and supernatants were collected by centrifugation (1,000 $g \times 2$ min) and resuspended to final concentration of 5 \times 10⁶ cells/ml in fresh TM medium without [³⁵S]methionine (plus inhibitors where indicated) to initiate the chase period. Upon completion of the chase period cells and supernatants were separated by centrifugation (1,000 $g \times 2$ min) and cell pellets were resuspended in 500 μ l of 0.5% Triton X-100.

Immunoprecipitation and Gel Electrophoresis

The α -mannosidase and β -glucosidase polypeptides were immunoprecipitated from cellular and secreted samples with the protein specific monoclonal antibodies 2H9 for α -mannosidase (39) and 2F5 for β -glucosidase (26). Detailed procedures for immunoprecipitation, subsequent 7.5% SDS-PAGE, and fluorographic treatment of the gels have been previously published (9, 40). Band intensities on fluorographs were quantitated using a laser densitometer (model 2202 Ultrascan; LKB Instruments, Inc., Gaithersburg, MD) and Omniscrite recorder (Houston Instrument, Austin, TX). Control experiments with known amounts of ¹⁴C-BSA demonstrated that band intensities were directly proportional to counts per minute of the sample over the ranges used in this study.

Endoglycosidase H Treatment

Immunoprecipitates containing radioactively labeled α -mannosidase or β -glucosidase polypeptides from cellular or supernatant samples were washed as described (11, 40), resuspended in 20 μ l of a 2% SDS, 10% β -mercaptoethanol solution, and heated at 90°C for 3 min. After centrifugation (10,000 $g \times 4$ min), 20 μ l of 50 mM sodium acetate, pH 5.5, was added to the recovered supernatant. Each sample was digested with 2.4 μ l (2.5 mU) of endoglycosidase H (endo H) (Miles Scientific Div., Miles Laboratories, Naperville, IL) for 16 h at 37°C. Control samples without endo H were incubated under the same conditions.

Percoll Gradient Fractionations

Cells were radiolabeled with [³⁵S]methionine according to the pulse-chase protocol previously outlined, and collected by centrifugation (1,000 $g \times 2$ min) and resuspended in 1 ml of MESES buffer (20 mM 2-(*N*-morpholino)ethanesulfonic acid, 1 mM disodium EDTA, 250 mM sucrose, pH 6.5). An approximate 100-fold excess of GM1 cells, an α -mannosidase and β -glucosidase structural gene mutant, was added to the radioactively labeled wild-type cells before fractionation, to facilitate handling. The GM1 and labeled wild-type cells were combined, broken on ice with 25 strokes of a Dounce homogenizer (Kontes Glass Co., Vineland, NJ), and centrifuged at 1,000 $g \times 5$ min to remove nuclei and unbroken cells. The unbroken cells were resuspended in 2 ml of MESES buffer and the homogenization cycle was repeated. 5 ml of the combined postnuclear supernatant was layered on 21 ml of 24% Percoll prepared in MESES buffer. Gradients were centrifuged for 1 h at 17,800 rpm in a rotor (model type 42.1; Beckman Instruments, Inc., Palo Alto, CA) at 4°C. After centrifugation, 20 1.3-ml fractions were collected from the bottom of the gradient using a peristaltic pump (ISCO, Inc., Lincoln, NE) and the fractions were adjusted to a final concentration of 0.5% Triton X-100. Samples were centrifuged for 45 min in a rotor (model type 50; Beckman Instruments, Inc.) at 40,000 rpm to remove the Percoll and were immunoprecipitated with antibodies specific for α -man-

nosidase and β -glucosidase, and subjected to 7.5% SDS-PAGE followed by fluorography. Marker enzyme assays were conducted on remaining samples for lysosome (acid phosphatase [37]) and endoplasmic reticulum (ER) membranes (α -glucosidase II [4]) in the presence of Percoll which did not interfere with the assay.

SDS-PAGE of Labeled Secreted Proteins

Cells were pulse radiolabeled and chased according to the protocol described previously. After harvesting by centrifugation ($10,000 g \times 30 s$), the cell samples were resuspended in $75 \mu l$ of $2\times$ gel sample buffer (36). To precipitate the supernatant proteins, samples of medium were adjusted to 5% TCA and $10 \mu g/ml$ tRNA. Samples were incubated on ice for 60 min and clarified at $10,000 g$ times 5 min. The supernatant was discarded and the pellets were resuspended in $75 \mu l$ of gel sample buffer. Cellular and supernatant samples were subject to SDS-PAGE and fluorography.

Results

Serine and Cysteine Proteinase Inhibitors, Antipain and Leupeptin, Prevent Proteolytic Processing and Induce Oversecretion of Lysosomal Enzyme Precursors

The majority of proteolytic enzymes characterized thus far, which are involved in the processing of precursor and intermediate forms of lysosomal enzymes and polypeptide hormones, are members of either the serine or cysteine class of proteinases (3, 17, 44). In fact, treatment of the purified *Dicystelium* α -mannosidase precursor with the serine proteinase trypsin resulted in the appearance of polypeptides identical in molecular weight to authentic cellular mature subunits (50). Therefore, the serine/cysteine proteinase inhibitors leupeptin and antipain were initially screened to determine if they would prevent proteolytic processing of lysosomal enzymes in vivo. Axenically growing cells were

labeled with [^{35}S]methionine for 10 min and chased in growth medium, with (Fig. 1, B) or without (A) leupeptin at a final concentration of 5 mg/ml. At the indicated times, cells were separated from the medium by centrifugation. Cellular and secreted α -mannosidase polypeptides were immunoprecipitated; the samples were subjected to SDS-PAGE followed by fluorography. Figure 1 B illustrates the effect of leupeptin with increasing chase times. For comparison, Fig. 1 A shows the control experiment where the pulse-chase was performed in the absence of inhibitor. There was an accumulation of the 80-kD intermediate form in leupeptin-treated cells throughout the chase period, concomitant with the absence of complete proteolytic processing to 58- and 60-kD mature forms. Comparison by laser densitometry of cellular mature forms present relative to the total amount of cellular α -mannosidase polypeptides present, revealed that the proteolytic processing of cellular α -mannosidase to mature forms was inhibited by 80% after a 1-h chase in the presence of leupeptin at 5 mg/ml. The 58-kD processed mature subunit appeared first, followed by the slower conversion of the 80-kD intermediate to the 60-kD mature form. While there was a considerable amount of 80-kD intermediate present early in the chase period (20 and 30 min) in untreated cellular samples, at later chase times the amount of 80-kD intermediate was small compared to the percentage of mature forms (<5%).

The percentage of secreted α -mannosidase relative to the total enzyme was also measured at each time point. These data, presented graphically in Fig. 1, show there was a dramatic increase in the amount of α -mannosidase precursor secreted in the presence of leupeptin. In the control samples, $\sim 10\%$ of the total radioactively labeled α -mannosidase was secreted, while cells treated with leupeptin secreted 75% of

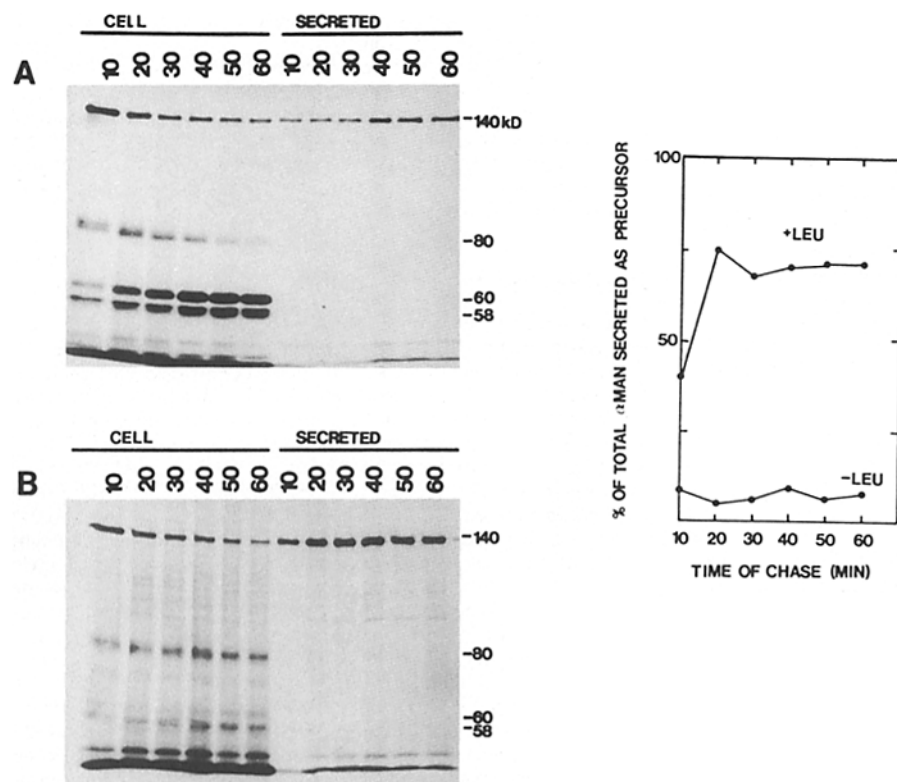


Figure 1. Pulse-chase experiments with normal and leupeptin-treated cells. Equivalent numbers of cells were radioactively labeled with [^{35}S]methionine for 10 min and chased in growth medium with (B) or without (A) leupeptin at a concentration of 5 mg/ml for 10, 20, 30, 40, 50, and 60 min. Cell extracts and secreted samples were immunoprecipitated with α -mannosidase-specific antibody and subjected to SDS-PAGE followed by fluorography. Band intensities were quantitated by laser densitometry.

the radioactively labeled α -mannosidase as precursor polypeptides.

As shown in Fig. 2, leupeptin also disrupted the normal proteolytic processing of another lysosomal enzyme, β -glucosidase. At the lowest concentration of leupeptin tested in this experiment (0.5 mg/ml), <5% of the newly synthesized 105-kD β -glucosidase was processed to the mature cellular form of 100 kD during the chase period; only the 103-kD intermediate form was observed intracellularly. Furthermore, incubation of cells with increasing concentrations of leupeptin also led to extensive secretion of the uncleaved β -glucosidase 105-kD precursor polypeptides. Thus, in cells exposed to leupeptin and antipain at a concentration of 5 mg/ml (results not shown for antipain), one observes an accumulation of intermediate forms of lysosomal enzymes and a six-fold increase in the percentage of labeled precursor polypeptides that escaped proteolytic processing and are rapidly secreted from cells.

Leupeptin May Act at an Intracellular Site on the Targeting Pathway Between the Golgi Complex and Lysosomes

To assess whether the precursor polypeptides secreted in the presence of leupeptin traversed the Golgi complex and were modified in a normal manner before exiting cells, α -mannosidase precursors, immunoprecipitated from medium of [³⁵S]methionine-labeled cells chased in the presence of leupeptin, were treated with endo H. This enzyme acts by cleaving mannose-rich oligosaccharide side chains at the chitobiose core, thus generating an α -mannosidase polypeptide of 120 kD from the 140-kD precursor. Under normal growth conditions, the 140-kD α -mannosidase precursor is transported from the RER through the Golgi apparatus, where the oligosaccharide side chains attached to this protein acquire resistance to cleavage by endo H (10). Resistance to endo H is detected by the appearance of a group of three polypeptides ranging in molecular mass from ~134 to 138 kD (Fig. 3, lanes 1 and 2). Similarly, the α -mannosidase precursors secreted from cells exposed to leupeptin were as

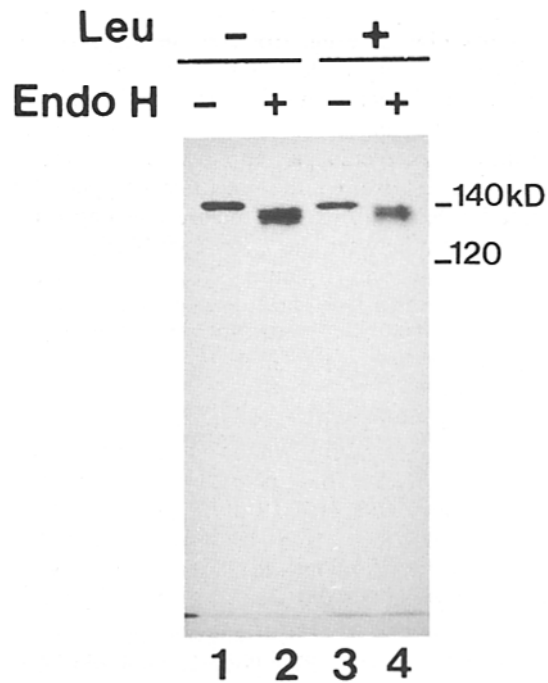


Figure 3. Endo H treatment of secreted precursor from cells grown in the presence of leupeptin. Cells were radioactively labeled with [³⁵S]methionine for 10 min and chased for 1 h in growth medium in the absence (lanes 1 and 2) or the presence of leupeptin at 5 mg/ml (lanes 3 and 4). After immunoprecipitation of secreted α -mannosidase, half of the samples were treated with endo H as described in Materials and Methods. Samples were subsequently subjected to SDS-PAGE followed by fluorography.

resistant as the control precursor to endo H digestion (Fig. 3, lanes 3 and 4) suggesting all of the precursors had passed through the Golgi complex and were modified correctly (becoming endo H resistant) before exiting the cell.

Conceivably, the precursor polypeptides are correctly sorted

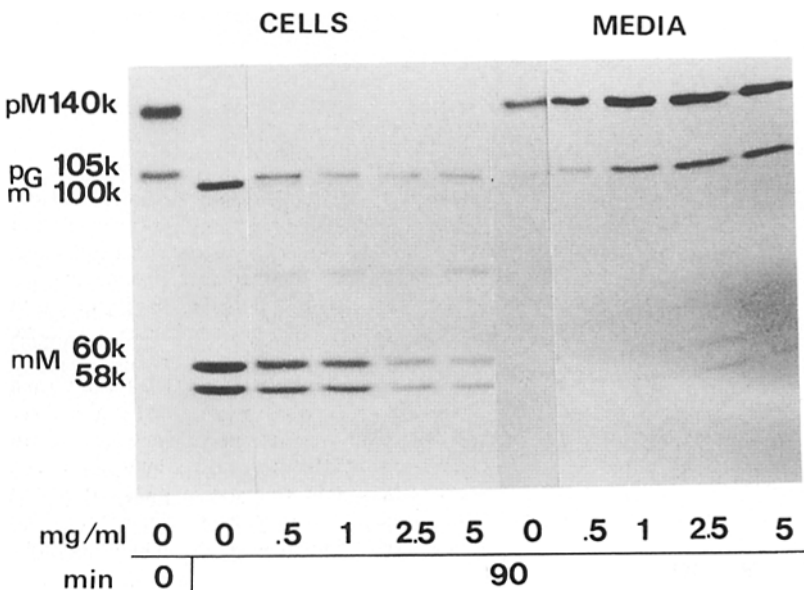


Figure 2. Effect of increasing concentrations of leupeptin on the processing and secretion of the lysosomal enzymes α -mannosidase and β -glucosidase. Cells were pulse labeled for 10 min and chased for 90 min in the presence of leupeptin at a final concentration of 0, 0.5, 1.0, 2.5, or 5.0 mg/ml. α -Mannosidase and β -glucosidase were immunoprecipitated from cellular and secreted samples and subjected to SDS-PAGE and fluorography. As a control, α -mannosidase and β -glucosidase were immunoprecipitated from cells collected immediately after the 10-min pulse-label period was complete (shown in the first lane).

to lysosomes in leupeptin-treated cells, but proteolytic processing is inhibited by the drug. However, secretion of the contents of lysosomes may no longer be regulated in these cells as it is in untreated cells, thus the uncleaved precursors quickly and efficiently exit cells. Alternatively, leupeptin may act at a site on the targeting pathway, near to the processing compartments, to misdirect the precursor polypeptides to the constitutive secretory pathway before reaching lysosomes. To distinguish between these two possibilities, cells were pulsed for 10 min with [³⁵S]methionine and chased for a total of 90 min. Leupeptin was added to a final concentration of 2.5 mg/ml to cells in separate flasks at various times after the initiation of the chase. Cellular and secreted samples were immunoprecipitated with antibodies specific for α -mannosidase and β -glucosidase, and subjected to SDS-PAGE and fluorography. Notably, when leupeptin was added to the cells 60 min into the chase period, the drug did not affect secretion of newly generated mature enzymes which had just reached lysosomes (Fig. 4, lanes 4, 5, 9, and 10), although addition at the initiation of the chase predictably inhibited processing and increased the percentage of precursors secreted into the media (lanes 1 and 6).

A 20-min delay in the addition of leupeptin (compared to addition at $t = 0$ min) during the chase period resulted in a 50% decrease in the percentage of the β -glucosidase precursors that were missorted and secreted from cells (Fig. 4, compare lanes 3 and 8 with 1 and 6). Moreover, 70% of the β -glucosidase remaining intracellular was completely proteolytically processed to the 100-kD mature subunit. In contrast, a 20-min delay in leupeptin addition resulted in only a 20% decrease in the percentage of missorted (secreted) α -mannosidase precursors. We attribute this result to the fact that β -glucosidase is transported to lysosomes at a faster rate than α -mannosidase (10) and therefore more quickly passes the site along the transport pathway where leupeptin presumably acts. However, regardless of when during the chase

leupeptin was added to cultures, only endo H-resistant precursor forms were secreted. This suggests that leupeptin's effect on inducing oversecretion is limited to uncleaved forms of the enzymes, which are located in a compartment distal to where the enzymes are modified in the Golgi.

Leupeptin Induces the Secretion of a Select Group of Intracellular Proteins

To determine the effect of leupeptin on the secretion of other cellular proteins, in addition to α -mannosidase and β -glucosidase, cells were pulsed for 10 min with [³⁵S]methionine and chased for the times indicated in Fig. 5, with or without the addition of leupeptin (final concentration 2.5 mg/ml). Samples were collected by centrifugation and total proteins in the supernatant samples were precipitated with 5% TCA. The precipitated samples were resuspended in gel buffer and subjected to SDS-PAGE followed by fluorography. Approximately 10 proteins, ranging in molecular mass from ~40 to 80 kD, appeared in the medium of untreated cells by 20 min of chase. In leupeptin-treated cells the secretion of each of these labeled proteins was greatly stimulated (Fig. 5, arrows). Very little change occurred in the extracellular protein profile of cultures treated with leupeptin after 20 min. In contrast, beginning at ~120 min, a new group of labeled proteins (Fig. 5, arrowheads) began to be secreted from untreated cells but not from leupeptin-treated cells. Therefore, leupeptin stimulated the oversecretion of many proteins early in the chase period; the proteins that appeared later in the medium of untreated cells may represent processed forms of the proteins that had already been secreted in leupeptin-treated cells. More than 50% of the proteins secreted from leupeptin-treated cells were precipitated with antibodies directed towards mannose-6-sulfate, an epitope found on all *Dictyostelium* lysosomal enzymes tested (35). This suggests that leupeptin may prevent the sorting of the majority of lysosomal hydrolases in *Dictyostelium*.

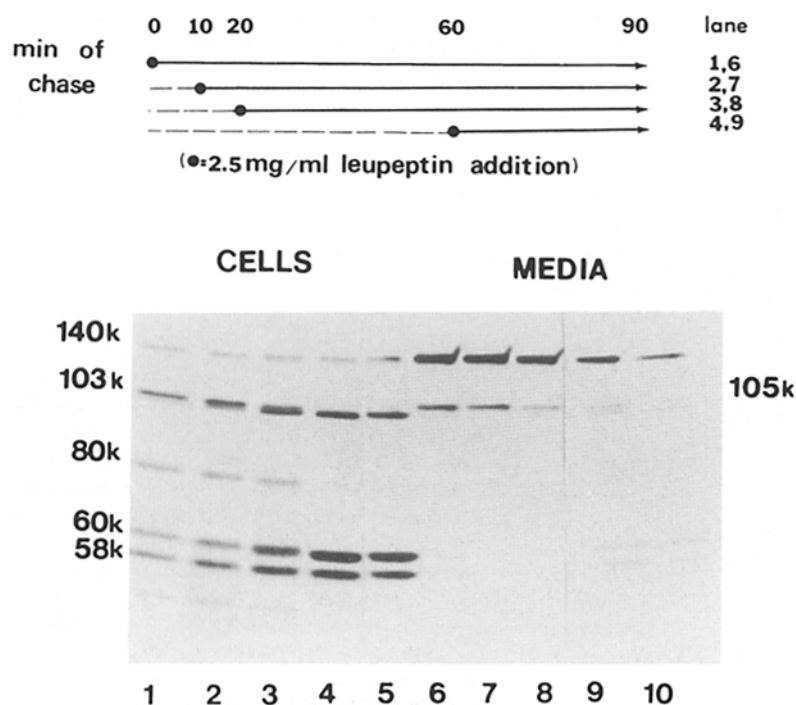


Figure 4. The effect of delaying the addition of leupeptin on proteolytic processing and secretion of newly synthesized α -mannosidase and β -glucosidase. Cells were pulse labeled for 10 min, collected by centrifugation, and resuspended in unlabeled growth medium. Leupeptin was added to separate flasks to a final concentration of 2.5 mg/ml, 0 (lanes 1 and 6), 10 (lanes 2 and 7), 20 (lanes 3 and 8), and 60 min (lanes 4 and 9) after the chase period had been initiated. Lanes 5 and 10 represent controls (without leupeptin). α -Mannosidase and β -glucosidase were immunoprecipitated from cellular (lanes 1-6) or secreted samples (lanes 7-10) after a 90-min chase, and subjected to SDS-PAGE, followed by fluorography.

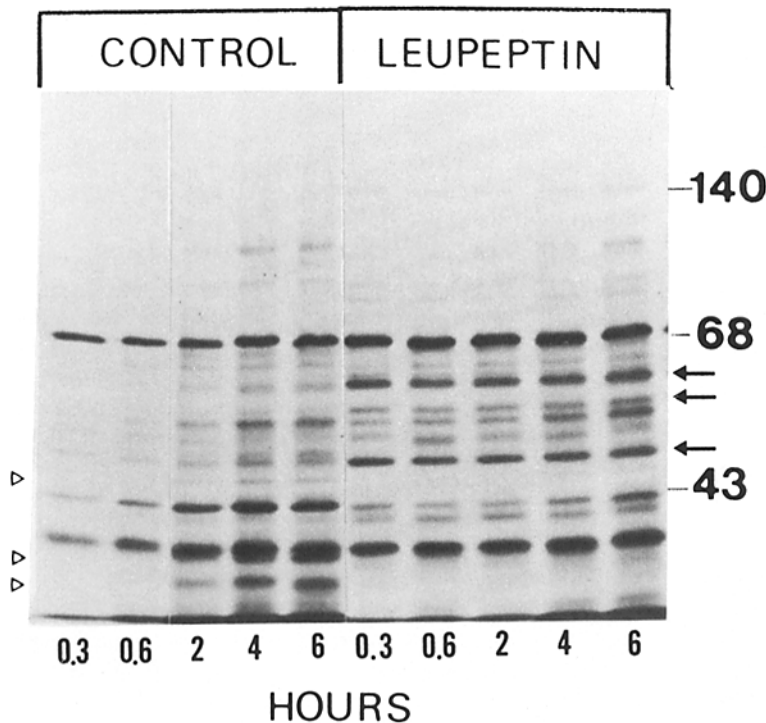


Figure 5. Effect of leupeptin on total protein secretion. Cells were pulse labeled with [³⁵S]methionine for 10 min and chased in unlabeled growth medium in the presence (2.5 mg/ml) or absence of leupeptin. Samples were collected at 0.3, 0.6, 2, 4, and 6 h. The radiolabeled proteins secreted from the cells were precipitated with TCA, resuspended in sample buffer, and subjected to SDS-PAGE and fluorography. The arrowheads indicate proteins unique to untreated cells, which appear late in the chase period. The arrows indicate proteins oversecreted in cells treated with leupeptin. Molecular weights were determined by ¹⁴C-molecular weight markers (Bethesda Research Laboratory, Gaithersburg, MD).

Serine, Metallo, and Aspartic Proteinase Inhibitors Do Not Prevent Proteolytic Processing or Alter Secretion of Lysosomal Enzymes

A variety of other proteinase inhibitors effective against serine, metallo, and aspartic proteinases were examined using our standard radiolabel pulse-chase protocol. Results using two of these agents, pepstatin and TLCK, are shown in Fig. 6, *A* and *B*, respectively. Pepstatin, an aspartic proteinase inhibitor, had no effect on the processing or secretion of either newly synthesized α -mannosidase or β -glucosidase. TLCK, a serine and cysteine proteinase inhibitor, had no effect on α -mannosidase processing but did prevent the complete processing of the 103-kD intermediate form of β -glucosidase to the 100-kD mature subunits at concentrations >2 mM. However, exposure of cells to concentrations of TLCK >2 mM resulted in a significant decrease in viability (measured by eosin dye exclusion), thus the effect of TLCK may be indirect. Additional pulse-chase experiments were conducted using the inhibitors diazoacetyl DL-norleucine methyl ester and 1,2-epoxy-3-(*p*-nitrophenoxy)propane, which inhibit aspartic proteinases and PMSF, a serine/cysteine proteinase inhibitor. None of these inhibitors had any effect on processing of α -mannosidase or β -glucosidase (inhibitor effects summarized in Fig. 7). Finally, no significant intra- or extracellular accumulation of precursor or intermediate forms of α -mannosidase or β -glucosidase was observed in cells treated for up to 8 h with TLCK and TPCK (serine/cysteine proteinase inhibitors) or the metallo proteinase inhibitors 1,10 phenanthroline and Z-Gly-PheNH₂ (data not shown).

Effect of Specific Cysteine Proteinase Inhibitors, E-64 and Z-Phe-AlaCHN₂, on Proteolysis and Targeting of Lysosomal Enzymes

Leupeptin and antipain are known to inhibit both serine and

cysteine proteinases. Therefore, to more accurately identify the processing proteinase(s) we subjected cells to a radiolabel pulse-chase protocol in the presence of the cysteine specific inhibitors (27) E-64 (Fig. 6, *C*) and Z-Phe-AlaCHN₂ (*D*). The results shown in Fig. 6 indicate that at the highest concentration of inhibitor used, cleavage of the precursor polypeptides of α -mannosidase and β -glucosidase was not significantly inhibited and no increase was observed in the secretion of these forms of the enzymes. However, significant amounts of the intermediate polypeptides of α -mannosidase (80 kD) and β -glucosidase (103 kD) accumulated intracellularly in a concentration dependent manner in Z-Phe-AlaCHN₂-treated cells (Fig. 6 *D*). In fact, at 2 mM Z-Phe-AlaCHN₂ $\sim 100\%$ of the intracellular population of β -glucosidase was the 103-kD intermediate form. Reduced amounts of the intermediate forms of α -mannosidase and β -glucosidase also accumulated in cells exposed to E-64 (Fig. 6 *C*).

Postnuclear supernatants prepared from [³⁵S]methionine-radiolabeled cells chased in the presence of Z-Phe-AlaCHN₂ were fractionated on Percoll gradients to determine the intracellular compartments in which the intermediate forms of lysosomal enzymes accumulated. Fig. 8 *A* shows the separation of enzyme markers on these gradients, which represent various intracellular organelles. α -Glucosidase-2 was used as the marker enzyme for the microsomal fraction (1, 7) and sulfated precursor, pulse labeled with ³⁵SO₄ for 15 min, was used as a marker for Golgi membranes. The typical Golgi marker enzyme activities galactosyl-, sialyl-, and fucosyl-transferase are not detectable in growing cells of *D. discoideum* (34, 49). The acid hydrolases, α -mannosidase, *N*-acetylglucosaminidase, and acid phosphatase, were used as marker enzymes for lysosomal membranes; all three enzymes cofractionated (data shown for α -mannosidase only). Using our method the Golgi and ER markers were contained within the same fractions (fractions 6–9, *d* = 1.05–1.06

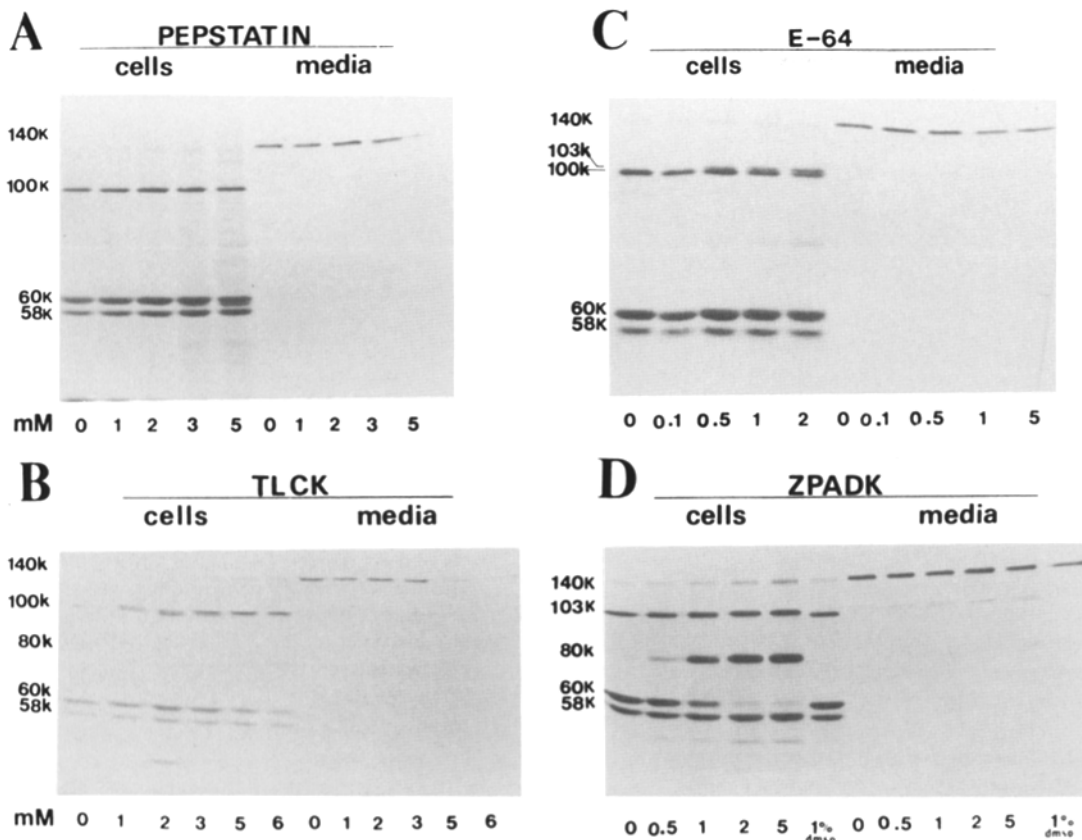


Figure 6. The effect of aspartic, serine, and cysteine proteinase inhibitors on processing and secretion of α -mannosidase and β -glucosidase. Cells were pulse labeled for 10 min and chased in the presence of various concentrations of pepstatin (A), TLCK (B), E-64 (C), or Z-Phe-AlaCHN₂ (D) in unlabeled growth medium for 90 min. α -Mannosidase and β -glucosidase were immunoprecipitated from cellular and supernatant samples and subjected to SDS-PAGE followed by fluorography.

g/ml). The lysosomal markers sedimented primarily in fractions 16 and 17 ($d = 1.075$ – 1.085 g/ml), well separated from the ER and Golgi membranes with a second minor peak of activity overlapping the Golgi and ER markers. The small amount of enzyme activity in the load zone (fractions 1–4) represents material released from ruptured vesicles during homogenization.

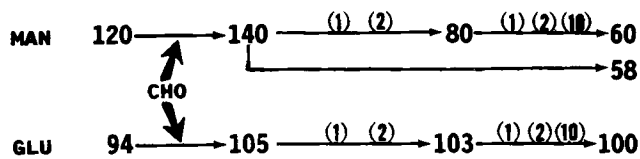
As indicated in Fig. 8 C, 60% of the radiolabeled intermediate forms of α -mannosidase and β -glucosidase in Z-Phe-AlaCHN₂-treated cells were found in a peak similar in density to the major peak (containing 60%) of labeled mature forms of the enzymes in untreated cells (Fig. 8 B). This dense peak corresponds in position to lysosomes as indicated in A. The second peak, containing 18% of the intermediate forms (Fig. 8 C) and 24% of the mature forms sedimented to the same position as the second smaller peak of lysosomal enzyme activity (Fig. 8 A). As described in the discussion, we believe these more buoyant lysosomal enzyme-containing vesicles may arise from the population of dense lysosomes (33). Thus, inhibition of cysteine proteinases apparently did not prevent intermediate forms of lysosomal enzymes from reaching mature lysosomes.

Compartments Involved in the Proteolytic Processing of Lysosomal Enzymes

Subcellular fractionation studies were conducted to deter-

mine the compartments where various proteolytically generated forms of α -mannosidase and β -glucosidase first appear. Cells were pulse labeled with [³⁵S]methionine for 30 min, followed by chase times of 0, 0.5, 1.5, and 4.0 h. The labeled cells were broken by Dounce homogenization in 0.25 M sucrose and membranes separated on Percoll density gradients. α -Mannosidase was immunoprecipitated from 18 fractions of equal volume, separated by SDS-PAGE, and visualized by fluorography.

After the 30-min pulse, the majority of the immunoprecipitated α -mannosidase was in the precursor form and was found primarily in fractions 6–9 (Figs. 9 and 10) corresponding to the Golgi/ER membrane peak (Fig. 8). Interestingly, the mature forms did not first appear in the Golgi fractions as suggested by Wood and Kaplan (49). Rather, the 80,000-mol-wt intermediate and the 58,000-mol-wt mature form first appeared predominantly in the middle portion of the gradient (fractions 10–14) between the Golgi marker and lysosomal membrane peaks (Fig. 8). The 60,000-mol-wt mature form was found predominantly in fractions 16–18. With longer chase times (0.5, 1.5, and 4.0 h) the precursor and intermediate polypeptides were chased completely to mature forms (Figs. 9 and 10, B–D). Additionally, at later chase times a second peak of mature radiolabeled forms of the enzymes appeared in the upper portion of the gradient (fractions 5–11) overlapping the ER/Golgi markers and small peak of lysosomal enzyme activity.



INHIBITOR	CLASS
1 LEUPEPTIN	SER, CYS
2 ANTIPAIN	SER, CYS
3 PMSF	SER, CYS
4 E-64	CYS
5 EPNP	ASP
6 DAN	ASP
7 TPCK	SER, CYS
8 PHENANTHROLINE	METALLO
9 PEPSTATIN	ASP
10 Z-PHE-ALACHN ₂	CYS
11 TLCK	SER, CYS
12 Z-GLY-PHENH ₂	METALLO

Figure 7. The effect of 12 proteinase inhibitors on proteolytic processing of α -mannosidase and β -glucosidase. The cotranslational addition of carbohydrate and subsequent processing of α -mannosidase and β -glucosidase is depicted in the top of the figure. The numbers correspond to the inhibitors listed below, and depict which inhibitors effectively inhibit processing, as well as at which step during proteolytic processing the cleavage is inhibited.

Discussion

The results reported in this paper demonstrate that *Dicystostelium discoideum* cells exposed to the cysteine/serine proteinase inhibitors leupeptin and antipain, as well as the cysteine proteinase inhibitor Z-Phe-AlaCHN₂, are defective in proteolytic processing of the lysosomal enzymes α -mannosidase and β -glucosidase. Specific inhibitors of the serine, metallo, and aspartic proteinase classes, however, had no effect on the proteolytic processing or secretion of lysosomal enzymes. In addition to inhibiting proteolytic processing of a large percentage of intracellular α -mannosidase and β -glucosidase precursors, antipain and leupeptin significantly enhanced (greater than sevenfold) the secretion of the unprocessed precursor forms. In contrast, Z-Phe-AlaCHN₂ did not inhibit the processing of precursor polypeptides but did prevent the cleavage of intermediate forms of the enzymes which were correctly localized to lysosomes. Using an improved Percoll subcellular fractionation method we have determined that proteolytic processing of lysosomal enzymes may initiate in previously undescribed vesicles with densities between those of the Golgi and lysosomes. Completion of proteolysis to generate mature forms of the enzymes occurs immediately upon or soon after arrival in dense lysosomes. We therefore propose that the initial cleavage of precursor polypeptides may be critical for the proper sorting of lysosomal enzymes and is catalyzed by a proteinase(s) active in a post-Golgi/prelysosomal compartment whose properties remain to be more fully elucidated. More-

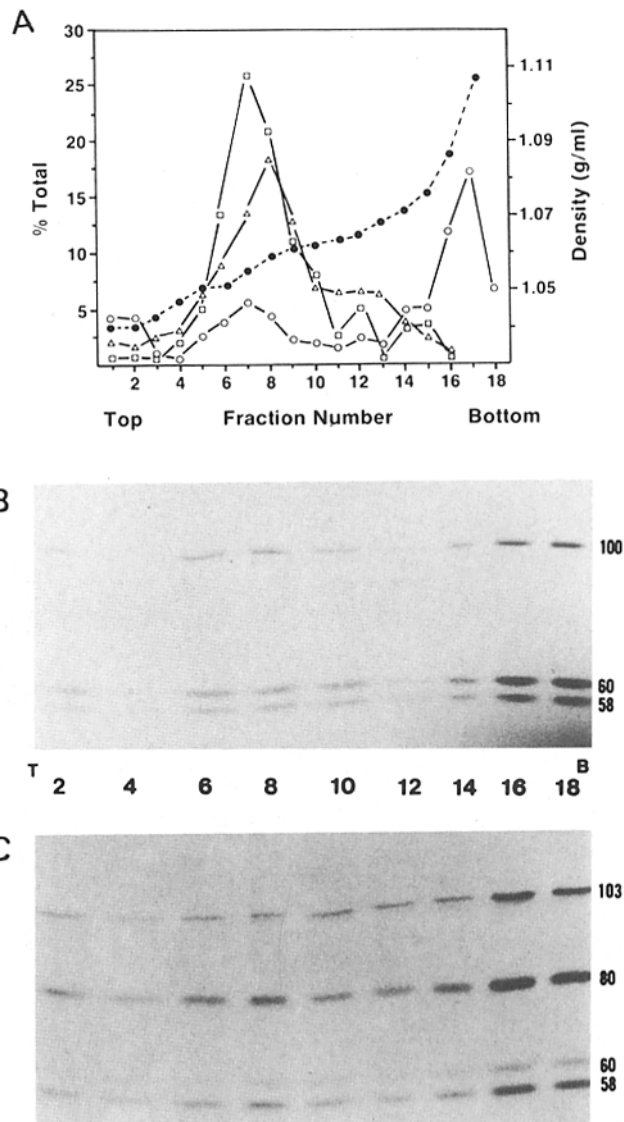


Figure 8. Subcellular fractionation of radiolabeled cells treated with Z-Phe-AlaCHN₂. Cells were pulse labeled for 10 min with [³⁵S]methionine and chased for 90 min in unlabeled growth medium, in the presence (C) or absence (B) of 5 mM Z-Phe-AlaCHN₂. Post-nuclear supernatants were fractionated on 24% Percoll gradients as described in Materials and Methods. 20 fractions were collected and α -mannosidase and β -glucosidase were immunoprecipitated from alternate fractions. Samples were subjected to SDS-PAGE and fluorography. A indicates the distribution of membrane markers in Percoll density gradients. AX3 cells were fractionated as described in Materials and Methods. The amount of each marker is expressed as the percentage of the total enzyme activity found on the gradient. (□) α -Glucosidase-2; (Δ) sulfated α -mannosidase precursor; (○) α -mannosidase; (●) density.

over, intermediate forms of lysosomal enzymes are processed to mature subunits by a cysteine proteinase(s) which may reside in lysosomes.

The intermediate forms of the lysosomal enzymes α -mannosidase and β -glucosidase in Z-Phe-AlaCHN₂-treated cells accumulated predominantly in intracellular compartments identical in density, on Percoll gradients, to mature lysosomes. This data suggests that proteolytic processing of in-

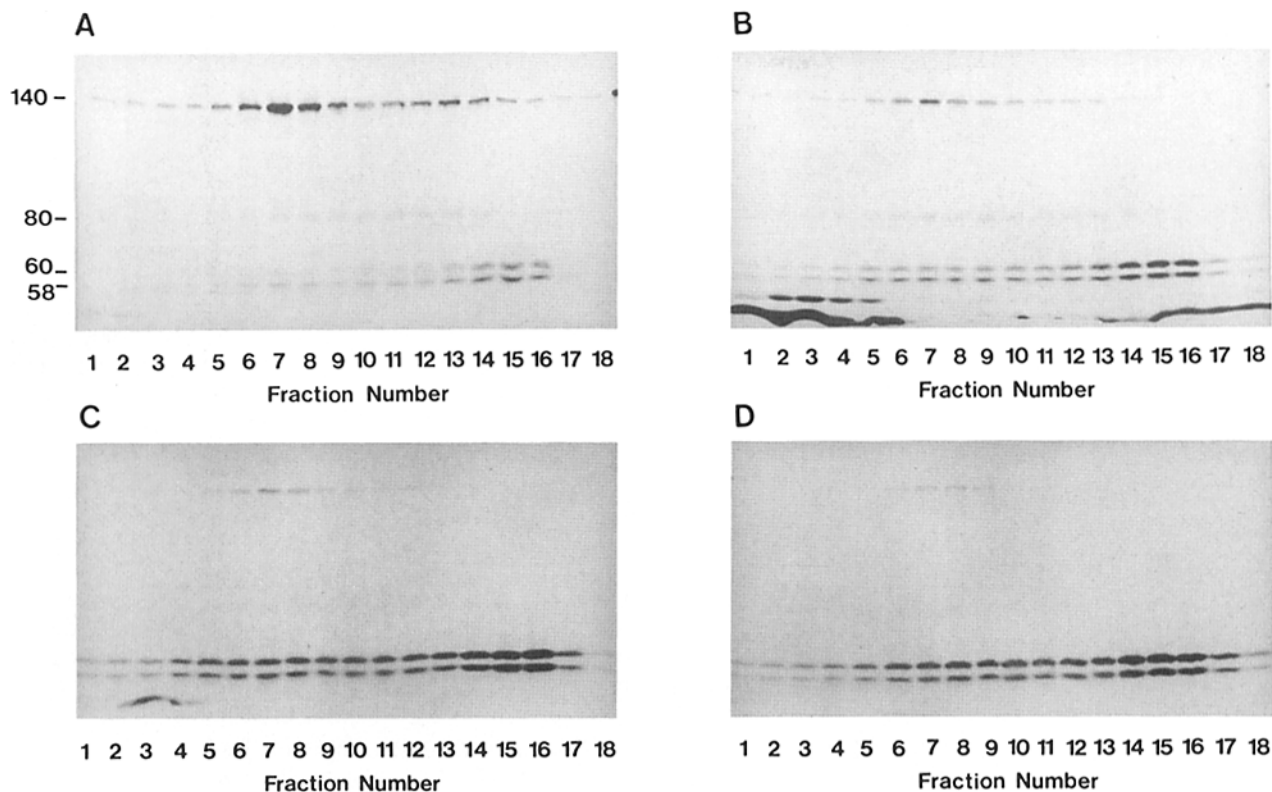


Figure 9. Intracellular transit of α -mannosidase in wild-type cells. Cells were labeled for 30 min and chased for 0 (A), 30 (B), 90 (C), or 240 (D) min. After labeling, cells were fractionated on Percoll density gradients and α -mannosidase was visualized as described in Materials and Methods. Fraction 1 corresponds to the top of the gradient.

intermediate forms of lysosomal enzymes is not required for sorting of enzymes to lysosomes or for the maturation of these organelles, and that a cysteine proteinase catalyzes the reaction, consistent with observations of others (21, 24, 32). A second peak of the intermediate forms was also observed in middle fractions of the gradient which overlapped with both a second peak of radiolabeled mature forms of the enzymes seen in untreated cells as well as a peak of lysosomal enzyme activity. The second lighter population of vesicles containing lysosomal enzymes has been observed in other cell types (46). The delayed appearance of the more buoyant second peak of radiolabeled mature forms of the enzymes during a pulse-chase of untreated cells suggests these lighter vesicles may be derived from the more dense secondary lysosomes. Consistent with the observations of Hohman and Bowers (33), these lighter vesicles may be recycling enzymes to the cell surface. Therefore, in cells treated with Z-Phe-AlaCHN₂, these lighter vesicles would also contain radiolabeled intermediate forms of the enzymes which have accumulated in secondary lysosomes.

Brown and Swank reported that processing of precursor β -glucuronidase and β -galactosidase to mature forms occurs just before or very soon after arrival of these proteins in lysosomes (5). Others have found, however, that processing may be initiated in a distal Golgi compartment(s) (25, 49). We report here that processing of α -mannosidase and β -glucosidase precursors may be initiated in a compartment with an apparent density on Percoll gradients intermediate to the Golgi complex and lysosomes. The initial processing com-

partment may include coated vesicles and elements of the endosomal system (23, 29, 48). This conclusion is tentative, however, because the Golgi complex in *Dictyostelium* is poorly defined as it lacks the enzymes commonly used as markers in mammalian cells. Therefore, the intermediate forms of the lysosomal enzymes may be localized to a part of the Golgi we can not assay for, such as the *trans*-Golgi network (28).

We favor the hypothesis that precursor forms are first cleaved in endosomes. For instance, Diment and Stahl found precursor forms of cathepsin D in endosomes (15) and endosomes have been proposed to be an intermediate compartment for lysosomal enzymes before reaching lysosomes (6, 29). Also, the effect of leupeptin can most easily be explained by hypothesizing it reaches endosomes by pinocytosis and inhibits the processing of precursor proteins in this compartment.

Of all the inhibitors examined only the serine/cysteine proteinase inhibitors leupeptin and antipain prevented the proteolytic processing of α -mannosidase and β -glucosidase precursors. Furthermore, we have recently observed that the weak base chloroquine, (which is also a proteinase inhibitor [2, 14, 38]), when used at concentrations that did not significantly raise the pH of the lysosomal and endosomal compartments, also prevented precursor processing and stimulated precursor secretion while not affecting the cleavage of intermediate forms of the lysosomal enzymes (Cardelli, J. A., J. M. Richardson, and D. Miers, manuscript submitted for publication). Therefore, the precursor-processing enzymes

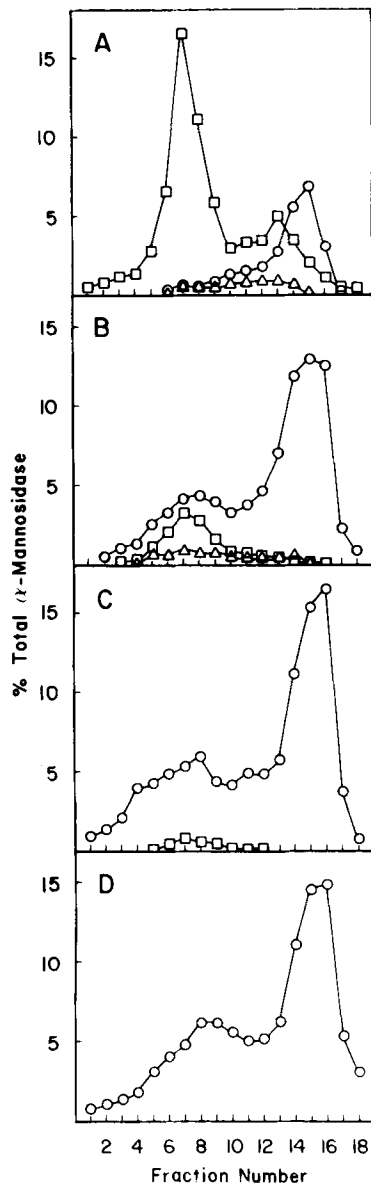


Figure 10. Densitometric analysis of the fluorograms presented in Fig. 9. The fluorograms shown in Fig. 9 were quantitated by laser densitometry. The graphs present the amount of each form as a percentage of the total labeled α -mannosidase present on the gradient. (□) α -Mannosidase precursor; (Δ) α -mannosidase intermediate; (○) sum of 58- and 60-kD mature form.

may differ both in cellular location and biochemical properties from the cysteine proteinases which generate mature subunits from intermediate forms. Presently, the precursor-cleaving proteinases cannot be clearly categorized into one of the four defined classes of proteinases.

Cells treated with leupeptin and antipain apparently mis-sorted the precursor polypeptides to the constitutive secretory pathway whereupon they were rapidly and efficiently exported to the extracellular medium. These results suggest that proteolytic processing may play an important role in targeting of lysosomal enzyme precursors to lysosomes. Conceivably, after delivery of newly synthesized lysosomal enzymes to the distal compartments of the Golgi complex such as the *trans*-Golgi network (28) or to a prelysosomal com-

partment where sorting occurs, cleavage of the precursor polypeptides to intermediate forms generates a sorting signal (embodied perhaps in a unique conformation) necessary to direct the enzymes to lysosomes where processing is completed. Leupeptin and antipain inhibit the cleavage event and polypeptides lacking the targeting signal are instead exported from cells along the constitutive secretory pathway.

One can envision alternative models to explain the over-secretion of unprocessed precursors in cells treated with leupeptin or antipain. For instance, in untreated cells the proteolytic processing of precursor polypeptides to intermediate and mature forms in a prelysosomal compartment may be necessary in order to release the polypeptides from association with membranes (40). The exact nature of the precursor membrane interaction remains to be determined but the precursor polypeptides are tightly associated with the vesicular membrane (9–11, 40). Furthermore, the proteolytic release of polypeptides in a prelysosomal compartment might allow potential sorting receptors to return to the Golgi complex to bind newly arrived precursors. Inhibiting proteolytic processing with leupeptin and antipain would result in the accumulation of receptors in the prelysosomal compartment, and their depletion from the Golgi complex. Therefore, newly arrived precursor polypeptides would no longer be sorted (via receptors) from the Golgi complex to lysosomes but would exit the cell along the constitutive pathway. Finally, we cannot exclude the possibility that leupeptin or antipain indirectly prevented normal targeting because of some nonspecific or indirect effect, however, these compounds do not possess highly reactive groups with the potential of interacting with constituents in the cell other than serine and cysteine proteinases (2).

Future objectives of this laboratory will include a biochemical definition of the compartments where processing occurs as well as an intensive characterization of the proteinases which catalyze the cleavage events. It will also be important to determine whether the amino acid sequences and/or protein conformation generated by the processing events facilitate the proper targeting of lysosomal enzymes. These data should allow us to more clearly define the targeting mechanisms operating in *Dictyostelium discoideum*.

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