Role of Acidic Intracellular Compartments in the Biosynthesis of Dictyostelium Lysosomal Enzymes

THE WEAK BASES AMMONIUM CHLORIDE AND CHLOROQUINE DIFFERENTIALLY AFFECT PROTEOLYTIC PROCESSING AND SORTING*

James A. Cardelli, Jan Richardson, and Debby Miears
From the Department of Microbiology and Immunology, Louisiana State University Medical Center, Shreveport, Louisiana 71130

Radiolabel pulse-chase and subcellular fractionation procedures were used to analyze the transport, proteolytic processing, and sorting of two lysosomal enzymes in Dictyostelium discoideum cells treated with the weak bases ammonium chloride and chloroquine. Dictyostelium lacks detectable cation-independent mannos-6-phosphate receptors and represents an excellent system to investigate alternative mechanisms for lysosomal enzyme targeting. Exposure of growing cells to ammonium chloride, which increased the pH in intracellular vacuoles from 5.4 to 5.8–6.1, slowed but did not prevent the proteolytic processing and correct localization of pulse-radiolabeled precursors to the lysosomal enzymes α-mannosidase and β-glucosidase. Additionally, ammonium chloride did not affect transport of the enzymes to the Golgi complex, as they acquired resistance to the enzyme endoglycosidase H at the same rate as in control cells. When the pH of lysosomal and endosomal organelles was raised to 6.4 with higher concentrations of ammonium chloride, the percentage of secreted (apparently mis-sorted) precursor polypeptides increased slightly, but proteolytic processing of intermediate forms of lysosomal enzymes to mature forms was greatly reduced. The intermediate and mature forms of α-mannosidase and β-glucosidase did, however, accumulate intracellularly in vesicles similar in density to lysosomes. In contrast, in cells exposed to low concentrations of chloroquine the intravacuolar pH increased only slightly (to 5.7); however, enzymes were inefficiently processed and, instead, rapidly secreted as precursor molecules. Experiments involving the addition of chloroquine at various times during the chase of pulse-radiolabeled cells demonstrated that this weak base acted on a distal Golgi or prelysosomal compartment to prevent the normal sorting of lysosomal enzymes. These results suggest that although acidic endosomal/lysosomal compartments may be important for the complete proteolytic processing of lysosomal enzymes in Dictyostelium, low pH is not essential for the proper targeting of precursor polypeptides. Furthermore, certain amines may induce mis-sorting of these enzymes by pH-independent mechanisms.

Virtually all lysosomal enzymes are synthesized as precursor molecules which are proteolytically processed to mature lysosomally localized forms (Cardelli and Dimond, 1988; Hasilik and Neufeld, 1980; Hasilik and von Figura, 1984; Richardson et al., 1988; Skudlarek et al., 1984). These polypeptides are first translocated into the lumen of the rough endoplasmic reticulum (RER)1 where they are modified by the attachment of asparagine-linked, manno-se-rich oligosaccharide side chains (Cardelli et al., 1986a; Erickson and Blobel, 1971; Hasilik and von Figura, 1984). Following transport to the Golgi complex, these glycoproteins are further modified by the addition of phosphate (Goldberg et al., 1984) and in many cases sulfate (Freeze, 1985) to carbohydrate side chains. In fibroblasts, membrane protein Man-6-P receptors2 have been described that bind mannose 6-phosphate residues on these lysosomal enzymes while they reside in the Golgi complex and sort these proteins from other proteins destined to be secreted from cells (Creek and Sly, 1984; von Figura and Hasilik, 1986). Upon arrival in acidic prelysosomal or endosomal compartments (Brown et al., 1986; Geuze et al., 1985; Mellman et al., 1986; von Figura and Hasilik, 1986), the enzymes are released from the receptors which then recycle back to the Golgi to bind newly arrived phosphorylated precursor polypeptides. A second Man-6-P receptor3 has been identified (Hoflack and Kornfeld, 1985), but what role this receptor may play in enzyme targeting remains to be elucidated.

Several lines of evidence suggest that lysosomal enzymes can be correctly sorted independently of the Man-6-P receptor-dependent pathway. For instance, lysosomal enzymes in fibroblast cells of patients with I cell disease are not phosphorylated (Goldberg et al., 1984; Hasilik and von Figura, 1984), and in the absence of the mannose 6-phosphate recognition marker these enzymes are secreted in precursor form (Hasilik and Neufeld, 1980). However, many differentiated cells in these patients such as leukocytes and hepatocytes contain nearly normal levels of lysosomal enzymes (Hasilik et al., 1981; Owada and Neufeld, 1982; von Figura and Hasilik, 1986). Furthermore, a variety of cells have been identified that lack any detectable Man-6-P receptors4 and yet they are able to correctly sort lysosomal enzymes (Gabel et al., 1983; Mainferme et al., 1985). Very little is known about the sorting mechanisms these cells use or the role of acidic pH compartment.

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4 To whom correspondence should be addressed.

1 The abbreviations used are: RER, rough endoplasmic reticulum; Man-6-P receptors, cation-independent mannose-6-phosphate receptors; Man-6-P receptors, cation-dependent mannose-6-phosphate receptors; FITC, fluorescein isothiocyanate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MES, N-morpholinoethanesulfonic acid.
ments in the targeting of enzymes to lysosomes by alternative pathways.

The cellular slime mold Dictyostelium discoideum is a useful organism in which to investigate lysosomal enzyme processing and targeting (reviewed in Cardelli and Dimond, 1988; Cardelli et al., 1987) because it can be easily manipulated biochemically and genetically. Furthermore, this organism lacks detectable Man-6-P receptors (Cardelli et al., 1986a; Cardelli et al., 1987) and may prove useful in defining the molecular nature of alternative targeting pathways. D. discoideum synthesizes at least two lysosomal enzymes, α-mannosidase and β-glucosidase, as precursor molecules that are proteolytically processed to mature enzymes (Cardelli et al., 1986a; Mierendorf et al., 1983; Pannell et al., 1982). The precursor polypeptides are synthesized on membrane-bound polysomes, cotranslationally translocated into the lumen of the RER, and N-glycosylated with typical mannose-rich carbohydrate side chains (Cardelli et al., 1986b; Cardelli et al., 1987). The glycosylated precursors are transported at non-uniform rates to the Golgi (Cardelli et al., 1986a; Mierendorf et al., 1985; Wood and Kaplan, 1986), where they are phosphorylated (Freeze and Wolgast, 1986) and sulfated on mannose residues (Freeze, 1985). A small percentage (<10%) of the precursor polypeptides escape further processing and are rapidly exported from cells along a constitutive secretory pathway (Cardelli et al., 1986a; Mierendorf et al., 1985). The remainder of the precursor polypeptides are proteolytically processed through intermediate forms to mature enzymes (Cardelli et al., 1986a; Pannell et al., 1982) that are targeted to lysosomes (Cardelli et al., 1986a; Mierendorf et al., 1983, 1985; Pannell et al., 1982). These mature, lysosomally localized enzymes are secreted at low rates during growth but are rapidly and extensively secreted when cells are starved for nutrients (Burns et al., 1981; Dimond et al., 1981; Mierendorf et al., 1986; Wood et al., 1983). Therefore, Dictyostelium lysosomal enzymes can be exported from cells following either a constitutive or regulated secretory pathway.

Our laboratory is attempting to define the molecular nature of the targeting signals and sorting mechanisms involved in the localization of lysosomal enzymes in D. discoideum. These studies include determining the roles of post-translational modifications to oligosaccharide side chains, proteolytic processing and acidic intracellular compartments in this localization process. In this report, we examine the effect of increases in intravascular pH, induced by ammonium chloride, on the intracellular processing and secretion of lysosomal enzymes in Dictyostelium. Our results clearly demonstrate that significant increases in intravascular pH do not alter the processing and sorting of at least two lysosomal enzymes. Furthermore, certain amines such as chloroquine can inhibit processing and cause mis-sorting of precursor polypeptides in an apparent pH-independent manner.

**EXPERIMENTAL PROCEDURES**

**Organism—**D. discoideum strains Ax3 (wild-type) and GM1 (carrying mutations in the α-mannosidase and β-glucosidase structural genes) were grown in axenic culture in TM medium (Free and Loomis, 1984) at 23 °C in shaking water baths rotating at 200 rpm.

**Measurement of Lysosomal/Endosomal pH**—The method of Ohkuma and Poole (1978) was used to measure lysosomal/endosomal pH. Exponentially growing cells were incubated for 5 or 18 h in the presence of 5 μg/ml FITC-dextran (M, 70,000, Sigma). To remove the extracellular FITC-dextran, cells were harvested by centrifugation (700 × g for 2 min) and resuspended in the same volume of TM medium; this procedure was repeated four times. The final cell pellet was resuspended in TM to a titer of 5 × 10^6 cells/ml. Two ml of the cell suspension were transferred to a 3.5-ml cuvette and adjusted to the desired concentration of ammonium chloride or chloroquine by the addition of the appropriate volumes of 1 M stock solutions. Fluorescence at 520 nm was measured at excitation wavelengths of 495 and 450 nm using a spectrophotofluorometer (SLM Aminco, SLM Instruments, Inc., Urbana, IL) with a high voltage setting of 500 mV and a gain setting of 10. The ratio of fluorescence at the two excitation wavelengths (495/450 nm ratio) was determined and compared to a pH standard curve to calculate the pH of the TM buffer. The pH standard curve was generated by adjusting TM medium containing 7 μg/ml FITC-dextran to various pH values and measuring the fluorescence ratio (495/450 nm ratio). Fluorescent microscopy of cells loaded with FITC-dextran revealed bright green vesicles and vacuoles ranging in size from 0.5 to 2 μm in diameter against a black (nonfluorescent) cytoplasmic background. This suggests that measured pH values truly reflect only the vacuolar space.

**Radioactive Labeling—**Exponentially growing cells (2–4 × 10^6 cells/ml) were collected by centrifugation (1000 × g for 2 min) and resuspended to a titer of 10^6 cells/ml in TM medium containing 750 μCi/ml [35S]methionine. After incubation for 10 min (pulse period) at 21 °C with shaking, cells were quickly collected by centrifugation and resuspended to 5 × 10^6 cells/ml in fresh TM medium (chase period) lacking isotope.

**Immunoprecipitation and Gel Electrophoresis—**Radiolabeled α-mannosidase and β-glucosidase polypeptides were immunoprecipitated from cell extracts and growth medium using an excess of monoclonal antibodies as described previously (Cardelli et al., 1986a; Mierendorf et al., 1985). The immunoprecipitated proteins were subjected to SDS-PAGE (7.5% acrylamide) according to the procedure of Laemmli (1970). Following electrophoresis, gels were prepared for fluorography as described previously using ENHANCE (Du Pont, New England Nuclear) and exposed to Kodak XAR-5 x-ray film at −90 °C. Band intensities were quantified by scanning fluorographs using an LKB 2202 Ultrascan Laser Densitometer (LKB Instrument Inc., Gaithersburg, MD) interfaced with an Apple II+ computer.

**Endoglycosidase H Digestion—**Immunoprecipitates were suspended in 25 μl of a solution containing 2% SDS and 10% β-mercaptoethanol. Following heating at 80 °C for 3 min, samples were centrifuged (19,000 × g for 3 min) and 25 μl of 100 mM sodium citrate, pH 5.5, was added to the recovered supernatant. After the addition of 2.5 μl of endoglycosidase H (1 unit/ml in 50 mM phosphate buffer, pH 5.5), samples were incubated at 37 °C overnight, mixed with an equal volume of 2 x gel sample buffer, and subjected to SDS-PAGE and fluorography.

**Subcellular Fractionation on Percoll Gradients—**Radioactively labeled Ax3 cells (1–5 × 10^6 cells) were mixed with a 50–100-fold excess of GM1 cells, collected by centrifugation (1000 × g for 5 min), and resuspended to 3 × 10^6 cells/ml in MESSE buffer (20 mM MES, pH 6.5, 1 mM EDTA, and 0.5 mM succrose). GM1 is an α-mannosidase and β-glucosidase structural gene mutant that lacks any detectable α-mannosidase and β-glucosidase polypeptides and is used to facilitate the recovery of subcellular fractions. Cells were broken with a tight fitting Dounce homogenizer (20 strokes) and resuspended (10,000 × g for 5 min) to remove unbroken cells and nuclei. Postnuclear supernatants (5 ml) were layered on 21 ml of 24% Percoll (Sigma) made up in MESSE buffer, and tubes were centrifuged at 17,800 rpm for 30 min in a Beckman type 40.2 rotor. Fractions (1.3 ml) were collected from the bottom using a microcapillary pipette connected to a polystylic pump. Prior to immunoprecipitation, samples were adjusted to 0.5% Triton X-100 and centrifuged at 100,000 × g for 60 min in a Beckman type 50 rotor to remove the Percoll. Acid phosphatase (Loomis and Kuspa, 1984) and α-glucosidase-II (Borts and Dimond, 1981) activities were measured in the presence of Percoll which did not interfere with the assays. Colored density marker beads (Pharmacia LKB Biotechnology Inc.) were included in a control gradient centrifuged in parallel to calibrate the density.

**RESULTS**

**Effects of Ammonium Chloride on Intravascular and Endosomal pH—**Weak bases like ammonium chloride accumulate in acidic intracellular compartments and raise the pH (Mellman et al., 1986; Ohkuma and Poole, 1978), which can profoundly affect the proper localization of lysosomal enzymes in many cell types. To determine if D. discoideum contained acidic compartments and to measure the effect of ammonium chloride on the pH of these compartments, cells were grown for one-half to two generations in the presence of...
the fluid phase marker FITC-dextran and then examined spectrophotometrically. The fluorescence of fluorescein is dependent only on the pH of the compartment in which it resides (Ohkuma and Poole, 1978), and, as indicated from the standard curve shown in Fig. 1, increasing the pH of a solution containing FITC-dextran from 4 to 7 resulted in a nearly linear increase in fluorescence (measured as the ratio of fluorescence at excitation wavelengths of 495 versus 450 nm). Thus, the measurement of this fluorescence ratio in cells loaded with FITC-dextran can be easily converted into pH values.

The pH of the lysosomal/endosomal compartments in exponentially growing cells which have accumulated FITC-dextran by pinocytosis over a period of 5 h was acidic and measured approximately 5.4 (Fig. 1). An identical pH was measured in cells incubated for 16 h suggesting that 5 h was sufficient time for the extracellular FITC-dextran to reach equilibrium in the endosomal/lysosomal system. The pH of intracellular compartments containing FITC-dextran increased to 5.8 within minutes when cells in growth medium at a pH of 6.4 were treated with ammonium chloride at a final concentration of 40 mM (Fig. 1). Increasing the concentration of ammonium chloride above 40 mM resulted in further increases in intravacuolar pH and at an ammonium chloride concentration of 100 mM, the pH reached 6.1. However, under these conditions protein synthesis was inhibited and significant amounts of lysosomal enzyme precursor polypeptides accumulated intracellularly (data not shown). Furthermore, this effect was not related to pH changes because NaCl at 100 mM concentrations also effectively reduced protein synthesis and lysosomal enzyme processing while having no effect on the pH (results not shown).

Therefore, in order to examine the potential effect of further increases in lysosomal/endosomal pH (greater than 5.8) on lysosomal enzyme biosynthesis while maintaining the concentration of ammonium chloride below 50 mM, cells were harvested from growth medium (normally at pH 6.4) and resuspended in fresh medium adjusted to a pH of 7.2. Under these conditions the cells remained viable for at least 8 h. More importantly, extracellular concentrations of ammonium chloride as low as 10 mM now raised the intravacuolar pH in cells to 6.1, while 40 mM concentrations resulted in a final pH of 6.4 (Fig. 1). This occurred because the extracellular concentration of the neutral ammonia molecule, considered the active chemical agent that rapidly crosses membranes and raises the intracellular pH (Dean et al., 1984), was dependent at any given ammonium chloride concentration on the solution pH; the higher the solution pH, the greater the ammonia concentration.

Effects of Ammonium Chloride on Proteolytic Processing and Secretion of α-Mannosidase and β-Glucosidase Precursors—The following series of experiments were performed to determine the effect of increases in the intravacuolar pH on the biosynthesis and secretion of lysosomal enzymes. Exponentially growing cells were pulse-labeled for 10 min with [35S]methionine, collected by centrifugation, and resuspended in unlabeled growth medium alone or in the presence of various concentrations of ammonium chloride to initiate the chase period. At various times, samples were collected and centrifuged to separate cells from growth medium. The radiolabeled α-mannosidase and β-glucosidase polypeptides were immunoprecipitated from cell extracts (prepared in 0.5% Triton X-100) and extracellular medium with specific monoclonal antibodies that were added simultaneously to each sample. The immunoprecipitates were then subjected to SDS-PAGE followed by fluorography. Fig. 2 indicates the results of an experiment in which the intravacuolar pH was increased to 5.8 with 40 mM ammonium chloride in growth medium at pH 6.4. As reported previously, in untreated control cells both α-mannosidase and β-glucosidase were synthesized as precursor polypeptides of molecular mass 140 kDa (Mierendorf et al., 1983; Pannell et al., 1982) and 105 kDa (Cardelli et al., 1986a; Cardelli et al., 1987), respectively, which were proteolytically processed through intermediated forms (80 kDa for α-mannosidase and 103 kDa for β-glucosidase) to mature enzymes. Exponentially growing cells were pulse-labeled for 10 min with [35S]methionine, harvested, and resuspended in fresh growth medium at pH 6.4. As reported previously, in untreated control cells both α-mannosidase and β-glucosidase were synthesized as precursor polypeptides of molecular mass 140 kDa (Mierendorf et al., 1983; Pannell et al., 1982) and 105 kDa (Cardelli et al., 1986a; Cardelli et al., 1987), respectively, which were proteolytically processed through intermediated forms (80 kDa for α-mannosidase and 103 kDa for β-glucosidase) to mature enzymes.

![Fig. 1. Fluorescent-based measurement of pH in cells treated with weak bases.](image)
forms with half-times of 35 and 20 min, respectively (Cardelli et al., 1986a; Table I). The β-glucosidase precursor polypeptide is transported more rapidly from the RER to lysosomes than the α-mannosidase precursor, thus accounting for the difference in their rate of proteolytic processing (Cardelli et al., 1986a). Also, as previously reported (Cardelli et al., 1986a; Mierendorf et al., 1985), approximately 10% of the α-mannosidase precursors and 2% of the β-glucosidase precursors escaped processing and were rapidly secreted from cells (Fig. 2).

Pulse-radiolabeled α-mannosidase and β-glucosidase precursors were processed more slowly in cells containing acidic compartments at a pH of 5.8 (Fig. 2, Table I). Interestingly, secretion of both precursor forms was reduced by greater than 80% in the presence of 40 mM ammonium chloride, although proteolytic processing was completed (Fig. 2 and Table I). Sodium chloride at a concentration of 40 mM had no effect on processing kinetics or secretion of lysosomal enzymes suggesting that the ammonium chloride effect was not salt-related. Finally, concentrations of ammonium chloride less than 40 mM had no effect on secretion or processing (results not shown).

We next investigated the effect of raising the pH of intracellular compartments to 6.1 and 6.4. Fig. 3 indicates that the rate of proteolytic processing of α-mannosidase in cells with acidic compartments at pH 6.1 was only slightly delayed compared to processing in control cells incubated in pH 7.2 growth medium. The rate of processing was more severely reduced, however, when the pH in the lysosomal/endoosomal organelles was raised to 6.4 with 40 mM ammonium chloride in growth medium at pH 7.2 (Fig. 3). For instance, after a chase period of 75 min, 60% of the intracellular α-mannosidase polypeptides were in precursor form (140 kDa), 30% were in intermediate form (80 kDa), and only 10% of the polypeptides were in the completely processed mature form (60 and 58 kDa). Longer chase periods resulted in the cleavage of greater than 90% of the precursor polypeptides and the very slow accumulation of mature forms (Table I). Fig. 3 also indicates that more than one α-mannosidase intermediate form transiently accumulated in these cells. In fact, the most abundant polypeptide present with a molecular mass of 84 kDa has never been observed as a processing intermediate in untreated cells. The processing of the α-mannosidase precursors to mature forms may, therefore, proceed through the generation of a family of short lived intermediate forms (80-84 kDa).

In contrast to the above, 100% of the newly synthesized intracellular β-glucosidase accumulated as a 103-kDa intermediate form in cells with intracellular compartments at a pH of 6.4 after a chase period of only 75 min (Fig. 3), although the rate of processing of the precursor polypeptides was reduced 2-fold (Table I). Consistent with the observations made for α-mannosidase, β-glucosidase intermediate forms were very slowly processed over a period of several hours to mature forms under these conditions. Thus, as summarized in Table I, increases in intravacuolar pH delayed but did not prevent precursor processing; however, continued increases in pH eventually resulted in an intracellular accumulation of intermediate forms of lysosomal enzymes that were very slowly processed to mature forms.

The half-time for the conversion during the chase period of the endoglycosidase H-sensitive radiolabeled precursor forms of α-mannosidase and β-glucosidase to resistant forms (an event that occurs in the Golgi) was 15 and 5 min, respectively, regardless of the intravacuolar pH (results not shown). This suggests that increases in pH did not effect the rate of transport of lysosomal enzyme precursors from the RER to the Golgi complex. Therefore, the delay in proteolytic processing was most likely due to an effect of ammonium chloride on a compartment in or beyond (distal to) the Golgi apparatus.

Table I also indicates the percentage of newly synthesized precursor polypeptides that escaped processing and were rapidly secreted from cells exposed to different concentrations of ammonium chloride. In control cells growing in medium at pH 6.4 (intravacuolar pH 5.4), 10% of the newly synthesized α-mannosidase precursors and 2% of the β-glucosidase precursors were not processed and instead were secreted within 25 min of initiation of the chase period (Cardelli et al., 1986a; Mierendorf et al., 1985). Increases in the pH of acidic compartments to 5.8 or 6.1 actually led initially to a reduction (particularly for α-mannosidase) in the secretion of unprocessed precursors, although all of the intracellular precursors were completely processed (Fig. 2; Table I). We have also observed a decrease under these conditions in the secretion of the entire spectrum of newly synthesized proteins that exit cells along a constitutive pathway (results not shown). Finally, when the intravacuolar pH was increased to 6.4, 15% of the α-mannosidase and 4.5% of β-glucosidase radiolabeled precursors were not processed and instead were exported from cells with the remaining intracellular precursors being cleaved to intermediate and mature forms. Therefore, although increases in intracellular pH led to first a reduction and then a small increase in secretion of precursor polypeptides compared to control cells, under the most alkaline conditions examined greater than 80% of the α-mannosidase precursors and 90% of the β-glucosidase precursors underwent proteolytic processing and were retained in intracellular compartments.

**Table I**

| Vesicular pH | α-Mannosidase | β-Glucosidase | α-Mannosidase | β-Glucosidase |
|--------------|---------------|---------------|---------------|---------------|
|              | Processing precursor forms | Formation of mature forms | Percent secretion of precursors |
|              | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 | 5.4 | 5.8 | 6.1 | 6.4 |
| Processing precursor forms | 28 | 32 | 37 | 30 | 10 | 17 | 17 | 17 | 35 | 38 | 47 | >180 | >180 | >180 | 10 | 2 | 2 | 0.5 | 3 | 2 |
| Formation of mature forms | 35 | 20 | 30 | 40 | 20 | 30 | 40 | 40 | 15 | 4.5 |
| Percent secretion of precursors | 10 | 2 | 2 | 0.5 | 3 | 2 | 4.5 |

* Vesicular pH was measured as described under "Materials and Methods."

a Half-times for processing were determined by densitometric scans of x-ray films as described under "Materials and Methods."

The percentage of total radiolabeled α-mannosidase and β-glucosidase secreted as precursor polypeptides following a 90-min chase was calculated based on laser densitometric scans.
Effects of Weak Bases on Lysosomal Enzyme Sorting

FIG. 3. Proteolytic processing of α-mannosidase and β-glucosidase precursors (PREC.) in cells exposed to high concentrations of ammonia. Cells were pulse-labeled for 10 min and chased in unlabeled growth medium adjusted to a pH of 7.2 in the presence of 10 or 40 mM ammonium chloride. At various times, samples were collected by centrifugation and α-mannosidase and β-glucosidase were immunoprecipitated from cell extracts and medium. Immunoprecipitates were subjected to SDS-PAGE followed by fluorography. ENDO.S/LYS., endosomal/lysosomal; INTER, intermediate.

Newly Synthesized and Proteolytically Processed Lysosomal Enzymes Are Correctly Targeted to Lysosomes in Cells Exposed to Ammonium Chloride—Precursor polypeptides in D. discoideum begin to be proteolytically processed in prelysosomal compartments (Richardson et al., 1988; Wood and Kaplan, 1985) followed by their transport to denser compartments where processing is completed (Richardson et al., 1988). Conceivably, increases in intracompartimental pH to 5.8 and 6.1 that do not inhibit complete proteolytic processing (Figs. 2 and 3) or lead to missorting and secretion (Table I) may block or prevent the mature forms of lysosomal enzymes from actually reaching lysosomes. To investigate this possibility, [35S]methionine pulse-labeled cells were chased in growth medium for 90 min in the presence of 40 mM ammonium chloride (raising the pH to 5.8). Cells were harvested by centrifugation, disrupted using a Dounce homogenizer, and the postnuclear supernatants fractionated by centrifugation. More importantly, a nearly identical percentage of the recovered radioactive α-mannosidase and β-glucosidase were localized to vesicles with a pH of 6.4. Cells were pulse-radiolabeled and chased for 100 min in fresh unlabeled medium at a pH of 7.2 containing 40 mM ammonium chloride. At this time, all of the intracellular β-glucosidase is an intermediate form while α-mannosidase is found as a mixture of precursor, intermediate, and mature forms (Table I). Significantly, analysis of cell extracts fractionated on Percoll gradients (results not shown) revealed that the radiolabeled intermediate and mature forms of α-mannosidase and β-glucosidase were localized to vesicles with a density of 1.10 g/ml equal to the density of vesicles which contained mature lysosomal enzymes (Fig. 4) while the remaining unprocessed α-mannosidase precursors were found at densities of 1.03–1.04 g/ml overlapping with the Golgi and endoplasmic reticulum membranes. Thus, although complete proteolytic processing of lysosomal precursor polypeptides was inhibited at near-neutral intravesicular pH values leading to an accumulation of intermediate forms, the majority of the

observed in other cells. The small amount of acid phosphatase activity at the top of the gradient represents enzyme released from lysosomes disrupted during homogenization. More importantly, a nearly identical percentage of the recovered radiolabeled mature α-mannosidase and β-glucosidase polypeptides co-sedimented with acid phosphatase activity near the bottom of the gradient. Identical results were obtained when the intravesicular pH was raised to 6.1 (results not shown). This suggests that with increases in vesicular pH to 5.8–6.1, cells were capable of processing and correctly targeting lysosomal enzymes.

We have also examined the movement and localization of newly synthesized enzymes in cells with acidic compartments at a pH of 6.4. Cells were pulse-radiolabeled and chased for 100 min in fresh unlabeled medium at a pH of 7.2 containing 40 mM ammonium chloride. At this time, all of the intracellular β-glucosidase is an intermediate form while α-mannosidase is found as a mixture of precursor, intermediate, and mature forms (Table I). Significantly, analysis of cell extracts fractionated on Percoll gradients (results not shown) revealed that the radiolabeled intermediate and mature forms of α-mannosidase and β-glucosidase were localized to vesicles with a density of 1.10 g/ml equal to the density of vesicles which contained mature lysosomal enzymes (Fig. 4) while the remaining unprocessed α-mannosidase precursors were found at densities of 1.03–1.04 g/ml overlapping with the Golgi and endoplasmic reticulum membranes. Thus, although complete proteolytic processing of lysosomal precursor polypeptides was inhibited at near-neutral intravesicular pH values leading to an accumulation of intermediate forms, the majority of the

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radiolabeled enzymes apparently were not mis-sorted and were instead correctly localized to lysosomes.

**Chloroquine Induces Mis-sorting of Newly Synthesized Lysosomal Enzyme Precursors without Substantially Altering Intravesicular pH—**To determine how general the effects of ammonium chloride were, we tested another commonly used amine, chloroquine. Surprisingly concentrations of chloroquine as high as 3 mM produced only a small increase in the intravesicular pH from 5.4 to 5.7 (Fig. 1); higher concentrations proved toxic to cells. More notably, chloroquine acted to dramatically reduce the extent of cellular processing of the α-mannosidase and β-glucosidase precursor polypeptides, and to stimulate secretion of these forms of the enzymes (Fig. 5). In the presence of 3 mM chloroquine greater than 90% of the pulse-labeled α-mannosidase and 70% of β-glucosidase precursor polypeptides escaped processing and were secreted from cells within the first 60 min of chase. As shown in the far right panel of Fig. 5 a much smaller percentage of radiolabeled precursors were secreted from untreated cells and cells exposed to ammonium chloride (which raised the intravesicular pH to 5.8; see Fig. 2). Lower concentrations of chloroquine were also effective in promoting the oversecretion of precursor polypeptides (Fig. 6). Unexpectedly, at all concentrations of chloroquine tested a greater percentage of newly synthesized β-glucosidase remained intracellular compared to α-mannosidase (Fig. 6). Finally, the radiolabeled α-mannosidase and β-glucosidase polypeptides that did remain in chloroquine-treated cells were eventually completely processed to mature forms.

**Chloroquine Acts on a Late Golgi or Prelysosomal Compartment to Cause Mis-sorting of Lysosomal Enzyme Precursors—**As described above, chloroquine prevented the proteolytic processing and stimulated the secretion of newly synthesized precursor forms of lysosomal enzymes by an apparent pH-independent mechanism. Conceivably, the precursor polypeptides did not actually exit cells via a constitutive secretory pathway but in fact reached lysosomes and in the presence of chloroquine were not cleaved to mature forms. In growing cells the secretion of mature lysosomal enzymes is a regulated process and proceeds at a very low rate unless cells are starved for nutrients (Burns et al., 1981; Dimond et al., 1983). However, the exocytosis pathway for mature lysosomal enzymes may no longer be tightly regulated in growing cells exposed to chloroquine; therefore, the contents of lysosomal vesicles which would include the unprocessed precursors would be efficiently and rapidly secreted. Thus, to determine if unprocessed precursors passed through lysosomes (i.e. were correctly targeted) prior to secretion from chloroquine-treated cells, cultures were pulsed with [35S]methionine and chased for 60 min in the absence of chloroquine. At this time, both of the newly synthesized α-mannosidase and β-glucosidase precursors had just been processed to mature forms and deposited in lysosomes (Cardelli et al., 1986a; Mierendorf et al., 1983). Chloroquine was then added to one flask at a final concentration of 3 mM, and cells were chased for an additional 5 h. Chloroquine was also added to a second flask after a chase period of 3 h. At the times shown in Fig. 7, α-mannosidase polypeptides were immunoprecipitated from cell extracts and medium, and subjected to SDS-PAGE. Panel A of Fig. 7 indicates that mature, radiolabeled α-mannosidase polypeptides began to be secreted from untreated cells into the medium by 4 h of chase, and by 6 h 20% of the labeled enzyme was extracellular. Significantly, no stimulation in the secretion of labeled mature α-mannosidase was observed in cells incubated in the presence of 3 mM chloroquine, when the weak base was added after 1 h of chase (Panel C) or added after 3 h of chase (Panel B). Identical results were found when the secretion of radiolabeled mature β-glucosidase was analyzed (data not shown). Thus, chloroquine did not induce the secretion of newly localized mature forms of lysosomal enzymes suggesting that this drug acted on the targeting pathway at or proximal to the proteolytic processing compartment to affect the mis-sorting of precursor polypeptides.

To more accurately determine the site along the transport pathway where chloroquine acted, cells were pulse-labeled with [35S]methionine for 10 min and chased in unlabeled growth medium. At various times during the chase (indicated in the legend to Fig. 8), chloroquine was added to individual flasks to a final concentration of 3 mM, and the chase was continued for a total of 90 min. Labeled α-mannosidase and β-glucosidase were immunoprecipitated from cells and medium and subjected to SDS-PAGE followed by fluorography. Because chloroquine penetrates cells very quickly (Dean et al., 1984), this experiment allowed a direct determination of the time at which addition of this weak base no longer effectively prevented the sorting and processing of lysosomal enzymes. Since we know the rate of movement of α-mannosidase and β-glucosidase between compartments along the

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**Fig. 5. Affects of chloroquine on precursor (Prec.) processing and secretion.** Exponentially growing cells were pulse-labeled for 10 min with [35S]methionine, harvested by centrifugation, and resuspended in fresh growth medium containing chloroquine at a final concentration of 3 mM. At the indicated times samples were collected, and α-mannosidase and β-glucosidase were immunoprecipitated. Immunoprecipitates were subjected to SDS-PAGE followed by fluorography. α-Mannosidase bands on the x-ray film for control cells (O—O, Fig. 2), ammonium chloride-treated cells (□—□, Fig. 2), and chloroquine (Chlor)-treated cells (○—○) were quantitated by scanning laser densitometry as detailed under "Materials and Methods," and the results are shown in the far right panel.
transport pathway (Cardelli et al., 1986a), measurements of this time can be used to approximate the compartment that chloroquine acts on. As indicated in Panels A and B of Fig. 8, delaying the addition of chloroquine during the chase period resulted in a progressively greater decrease in the amount of secreted unprocessed precursor and a corresponding accumulation in cells of processed mature forms of the enzymes. However, α-mannosidase and β-glucosidase responded with different kinetics to the addition of chloroquine. For instance, chloroquine added as late as 10 min into the chase period was almost as effective as when added at 0 min in preventing the processing and inducing the oversecretion of α-mannosidase precursors (Fig. 8, A and B). In contrast, a ten minute delay in the addition of chloroquine resulted in only one-half of the newly synthesized β-glucosidase precursor polypeptides being secreted while the other half was processed to the mature form. Furthermore, delaying the addition of chloroquine for 25 min resulted in an equal probability of secretion or processing of the α-mannosidase precursor. These two periods of time, 10 and 25 min, were slightly greater, respectively, than the times required for one-half of the newly synthesized β-glucosidase (5 min) and α-mannosidase precursors (15 min) to be transported from the RER to the Golgi complex (Cardelli et al., 1986a). In fact, these two times were more nearly identical to the half-lives for the processing of both of these precursors to intermediate forms (Fig. 2, Table I). Therefore, these results suggest that chloroquine acted directly or indirectly at or beyond a distal site in the Golgi complex, perhaps near the processing compartment, to prevent the normal sorting and cleavage of lysosomal enzyme precursors.

The labeled precursor polypeptides secreted from chloroquine-treated cells were as resistant to digestion with endoglycosidase H as polypeptides exported from untreated cells (results not shown). This suggests the enzymes have passed through the Golgi compartment where resistance to endoglycosidase H is acquired, lending further support to the hypothesis that chloroquine did not act at or before a proximal Golgi compartment.

DISCUSSION

It has become increasingly evident that in many cells acidic intracellular compartments which include coated vesicles (Leemansky et al., 1987), lysosomes (Ohkuma and Poole, 1978), endosomes (Maxfield, 1982), condensing vacuoles (Orci et al., 1987), and the distal Golgi stack (Anderson and Pathan, 1985) are required for the proper intracellular sorting of proteins from the secretory or endocytic pathway (Brown et al., 1985; Gonzalez-Noriega et al., 1980; Hasilik and Neufeld, 1980; Moore et al., 1983; Strons et al., 1985; Wagner et al., 1986; reviewed in Dean et al., 1984; Mellman et al., 1986; von Figura and Hasilik, 1986). The acidic conditions are thought to promote the separation of receptors and ligands allowing the receptors to return to the Golgi to bind newly arrived ligand. In contrast, very little is known about the transport and targeting pathways, and the role of acidic compartments in cells that package lysosomal enzymes without relying on the cation-independent phosphomannosyl receptors. This list of cells now includes leukocytes and hepatocytes in patients with I cell disease (Hasilik et al., 1981; Owada and Neufeld, 1982), Morris hepatoma 7777 cell lines (Mainferme et al., 1985), murine tumor cell lines derived from macrophages (Gabel et
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Fig. 8. The effect of delaying the addition of chloroquine (CHLOR.) to pulse-labeled and chased cells. Cells were pulse-labeled for 10 min and resuspended in unlabeled growth medium. To separate flasks, chloroquine was added to a final concentration of 3 mM at the times indicated in the figure. Following a chase period of 90 min, α-mannosidase and β-glucosidase were immunoprecipitated from cell extracts or medium and subjected to SDS-PAGE followed by fluorography. The intensities of the bands seen in Panel A were quantitated by laser scanning densitometry as described under “Materials and Methods,” and the data are plotted in Panel B. Prec., precursor.

al., 1983), and D. discoideum cells (Cardelli et al., 1986a; Cardelli et al., 1987).

We have examined the effects of two weak bases, ammonium chloride and chloroquine, on the proteolytic processing, secretion, and sorting of two lysosomal enzymes in D. discoideum. As reported here, Dictyostelium cells maintain lysosomal and endosomal compartments at an acidic pH of 5.4 as measured using the protocol of Ohkuma and Poole (Ohkuma and Poole, 1978). This value is higher than the range of pH values (4.5 to 4.8) reported for lysosomes in fibroblasts (Dean et al., 1984; Mellman et al., 1986; Ohkuma and Poole, 1978); however, pH measurements are an average of a large population of cells and reflect contributions from both endosomal and lysosomal compartments. Endosomal compartments for instance have been reported to have pH values close to 5.5 (Maxfield, 1982); therefore, if Dictyostelium contains an extensive endosomal system this might contribute to the higher measured pH. Furthermore, the measurement of pH involves only those compartments that contain the fluid phase marker FITC-dextran; therefore, acidic compartments (i.e., primary lysosomes) that are not connected with the endosomal system will not be averaged into the pH determination. It remains uncertain what significance (if any) can be attached to the more alkaline pH of Dictyostelium lysosomes and endosomes compared to other cells.

An increase in the pH of lysosomal/endosomal organelles from 5.4 to 6.4 (induced by high concentrations of ammonium chloride) resulted in an inhibition of proteolytic processing leading to an intracellular accumulation of intermediate forms of α-mannosidase and β-glucosidase, and a slight increase in the secretion of precursor polypeptides. The intermediate and completely processed mature forms were, however, packaged into organelles with the same density on Percoll gradients as mature lysosomes. Smaller ammonium chloride induced increases in pH (5.4 to 5.8–6.1) led to a reduction in the secretion of precursor polypeptides, but otherwise had little effect on the transport, processing, or localization of enzymes. This latter finding is not without precedent; other investigators have reported that ammonium chloride reduces the secretion of proteins following the constitutive secretory pathway (Oda et al., 1986), although this is not always observed (Thorens and Vassalli, 1986). Together, these results suggest that lysosomal enzymes in D. discoideum are correctly packaged into lysosomes in the absence of a substantial pH gradient.

Similar conclusions have been reached by other investigators studying cell lines which may target enzymes by mechanisms not dependent on the Man-6-P receptorC. For instance, von Figura and collaborators have determined that cathepsin C in Morris hepatoma 7777 cell lines (Mainferme et al., 1985) and cathepsin D in U937 monocytes (Braulke et al., 1987) may be targeted to lysosomes by pH-independent mechanisms. Similarly, Stromberg et al. (1985) and others (Hasilik et al., 1984) have concluded that pH-dependent mechanisms are not required for the sorting of myeloperoxidase to the azurophil granule (similar to lysosomes) in neutrophils. Finally, Waheed and Van Etten (1987) have reported that acid phosphatase is not mis-sorted in WI-38 fibroblasts treated with ammonium chloride. Together these reports suggest, in contrast to sorting pathways using the Man-6-P receptorC, that acidic compartments may not be as essential for the transport and sorting of lysosomal enzymes in cells that apparently use alternative sorting mechanisms.

At the present time, we can only speculate about the possible mechanism(s) involved in sorting of lysosomal enzymes by Man-6-P receptorC-independent pathways. If receptors do mediate sorting of lysosomal enzymes in these cells, they would have to be capable of releasing their ligands in prelyosomal compartments even under only slightly acidic conditions (i.e., pH 6.0–6.4), in order to recycle back to the sorting compartment in a ligand-free state. Conceivably, other factors...
more important than low pH may function to affect a release of ligands from receptors in these cells. We presently know nothing about these putative receptors. However, since lysosomal enzymes in cells that apparently do not utilize Man-6-P receptors usually contain mannose-6-phosphate moieties, it remains a formal possibility that these cells target the enzymes using the Man-6-P receptors.

Alternatively, sorting of lysosomal enzymes in these cells may not require receptors at all. For instance, specific alterations to lysosomal enzymes, such as covalent modifications or proteolytic cleavage (see below), may facilitate homor hetero-oligomeric interactions between the enzymes which might induce the budding of vesicles (containing these oligomers) from the sorting compartment (i.e. Golgi complex; Farquhar, 1985; Griffiths and Simons, 1986). These vesicles would then be delivered to the appropriate compartments to complete the packaging of these enzymes in lysosomes.

The accumulation of intermediate forms of lysosomal enzymes in ammonium chloride-treated cells may result from the inhibition of the processing proteinases by high pH. In fact, our laboratory (Richardson et al., 1988) as well as others (Hasilik, 1984; Oude, 1986; von Figura and Hasilik, 1986) have determined that acidic cysteine proteinases are most likely involved in the generation of mature subunits of lysosomal enzymes from intermediate forms. Surprisingly, this study revealed that in D. discoideum at least three intermediate polypeptide forms of α-mannosidase, molecular mass 80, 82, and 84 kDa, transiently accumulated in amine-treated cells. Only the 80-kDa polypeptide had previously been observed as a transient intermediate. This suggests that proteolysis of the α-mannosidase precursor to generate intermediate forms may involve several rapid cleavage events at closely spaced sites along the polypeptide backbone.

An unexpected result from this study was that under our experimental conditions chloroquine raised the endosomal/lysosomal pH only slightly (5.4–5.7), but greatly stimulated the secretion of unprocessed precursor polypeptides. These precursors passed through the Golgi complex (as evidenced by resistance to endoglycosidase H) but apparently never reached lysosomes and were not cleaved; no stimulation in the secretion of mature enzymes was observed. Our results do not rule out the possibility that chloroquine increased the pH of certain organelles such as primary lysosomes that are not connected to the endosomal system and therefore do not accumulate FITC-dextran. We can only conclude that within the limits of our measurements this weak base did not significantly raise the pH of organelles containing the fluorescent marker. At the present time we have no clear understanding on why chloroquine did not increase the pH of lysosomal/endosomal vesicles or how it acts in an apparent pH independent manner; we cannot of course eliminate an indirect effect of this amine (Dean et al., 1984). For instance, Sosa and Bertini (Sosa and Bertini, 1985) have reported that chloroquine can disrupt the interaction between lysosomal enzymes and the Man-6-P receptors. Our results suggest, however, that conclusions concerning the effects of weak bases on processing and sorting of proteins should be based on experiments using more then only one amine.

The following represents one possible explanation for the mechanism of chloroquine action. We have very recently determined that treatment of cells with the serine/cysteine proteinase inhibitors leupeptin and antipain prevents precursor processing and stimulates secretion of these uncleaved polypeptides (Richardson et al., 1988). This suggests that the initial proteolytic cleavage event may be important in the sorting process in Dictyostelium. Moreover, experiments described in this report involving the timed addition of chloroquine to radiolabeled cells indicated that chloroquine acted (directly or indirectly) at or near the compartment where precursors are initially cleaved. This information combined with the knowledge that chloroquine is a cysteine proteinase inhibitory in D. discoideum (North, 1985) suggests that this amine may act by the same mechanism as leupeptin, namely inhibition of precursor processing.

Many questions remain to be answered concerning the lysosomal system in Dictyostelium including: What is the nature of the proteolytic and sorting compartment(s) and how do these compartments function to ensure segregation of lysosomal enzymes from secretory proteins? Are carbohydrate side chains and their attendant modifications important in transport and targeting? What role(s) does proteolytic cleavage of precursor polypeptides play in the sorting process? Finally, what domains of the precursor polypeptides are essential for the generation or proper expression of lysosomal sorting signals? Biochemical and molecular genetic approaches (Cardelli and Dimond, 1986; Cardelli et al., 1986; Dimond et al., 1986; Liwi et al., 1985a; Woychick et al., 1986) and to dissect on a molecular level the transport and targeting pathways for lysosomal enzymes in D. discoideum.

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