Effect of acidotropic amines on the accumulation of newly synthesized membrane and luminal proteins in Chinese-hamster ovary (CHO) cell lysosomes

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Lysosomes were isolated by sequential gradient centrifugation [Madden, Wirt & Storrie (1987) *Arch. Biochem. Biophys.* **257**, 27–38] from control or acidotropic-amine-treated Chinese-hamster ovary (CHO) cells. By marker-enzyme analysis, the preparation from chloroquine or NH_4Cl -treated cells was about 25-fold enriched for lysosomes compared with the postnuclear supernatant and contained little or no marker activities for the plasma membrane, rough endoplasmic reticulum, Golgi apparatus, mitochondria, cytosol and peroxisomes. The yield of amine-treated lysosomes was about 60% relative to the postnuclear supernatant. Electron microscopy and cytochemistry demonstrated that the amine-treated preparation was highly purified. Cytochemical analyses after a short-term pulse of horseradish peroxidase revealed that endosomal contamination of the lysosomal preparation was <1%. Lysosomal polypeptides were biosynthetically labelled with [^{35}S]methionine and identified by SDS/polyacrylamide-gel electrophoresis. As expected, the bulk accumulation of luminal proteins into lysosomes was decreased. The bulk accumulation of membrane proteins was increased by acidotropic amine treatment. There were also several qualitative differences in each lysosomal compartment, with new species observed and other species absent. These data suggest that a low pH is not necessary for the normal accumulation of the bulk of membrane proteins in lysosomes and that membrane trafficking from Golgi apparatus to lysosomes occurs at a high rate in acidotropic-amine-treated CHO cells.

INTRODUCTION

Targeting of lysosomal hydrolases to the organelle lumen can be perturbed by both amine treatment and mutation [for reviews, see von Figura & Hasilik (1986) and Storrie (1988)]. In acidotropic-amine-treated cells, newly synthesized lysosomal hydrolases are secreted into the media, leading to a depletion of the lysosomal lumen of hydrolases (Wiesmann *et al.*, 1975; Hasilik & Neufeld, 1980). Increased compartmental pH in amine-treated cells blocks the acid pH-dependent dissociation of lysosomal enzymes containing mannose 6-phosphate residues from their receptors [for a review, see Mellman *et al.* (1986)]. The receptor-hydrolase complexes accumulate in what has been identified either as *trans*-Golgi reticulum (Geuze *et al.*, 1985) or endosomes (Brown *et al.*, 1986). In the absence of unoccupied receptor or the mannose 6-phosphate recognition signal, newly synthesized lysosomal hydrolases become part of the default secretory pathway (Wieland *et al.*, 1987). Fibroblasts from patients with I-cell disease (mucopolipidosis II) also have decreased amounts of many lysosomal luminal hydrolases [for a review, see Neufeld & McKusick (1983)]. In these cells, the mannose 6-phosphate recognition signal is not synthesized (Hasilik *et al.*, 1981; Reitmann *et al.*, 1981).

Lysosomal membrane proteins are targeted to lysosomes by a mechanism different from that which targets luminal enzymes [for a review, see Storrie (1988)]. The rat

lysosomal membrane proteins LIMP I, LIMP II and LIMP III are targeted to lysosomes in the absence of *N*-linked oligosaccharides (Barriocanal *et al.*, 1986). The mouse lysosomal membrane glycoproteins lgp110 and lgp120 do not contain phosphate residues (Green *et al.*, 1987). The activities of the membrane-bound enzymes β -glucocerebrosidase (β -glucosidase; Pentchev *et al.*, 1973) and acetyl-CoA: α -glucosaminide *N*-acetyltransferase are not decreased in I-cell-disease fibroblasts (Berman *et al.*, 1974; Klein *et al.*, 1978). The activity of β -glucosidase has been shown to increase in acidotropic-amine-treated Chinese-hamster ovary (CHO) cells (Fritsch *et al.*, 1988). However, recent experiments have demonstrated specific lysosomal membrane proteins may be depleted from the lysosomal compartment of amine-treated cells. The chicken lysosomal membrane protein LEP100 has been shown to redistribute from lysosomes to endosomes and the plasma membrane (Lippincott-Schwartz & Fambrough, 1987).

In the present work we have characterized the effects of acidotropic amines on the accumulation in CHO-cell lysosomes of newly synthesized membrane and luminal proteins. We have demonstrated that lysosomes can be isolated from acidotropic-amine-treated and control cells by the same sequential density-gradient-centrifugation procedure. We have found that growth of CHO cells in the presence of acidotropic amines resulted in a complex response in which, for both lysosomal luminal and membrane proteins, some species nearly disappeared,

Abbreviations used: CHO, Chinese-hamster ovary; α -MEM, Eagle's minimal essential medium, α -modification without ribonucleosides and deoxyribonucleosides; PNS, postnuclear supernatant; HRP, horseradish peroxidase; PAGE, polyacrylamide-gel electrophoresis.

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others increased and some stayed nearly constant. In general, luminal proteins decreased, while membrane proteins increased. These observations suggest multiple fates for the accumulation of individual lysosomal luminal and membrane proteins in the organelle, and that the delivery of the bulk of membrane proteins to lysosomes does not require a low pH.

EXPERIMENTAL

Materials

trans-³⁵S-labelled methionine was purchased from ICN (Costa Mesa, CA, U.S.A.). Chloroquine phosphate was purchased from K and K Laboratories (Plainview, NY, U.S.A.). Acrylamide and bisacrylamide were purchased from Mallinckrodt (Paris, KY, U.S.A.). The 4-methylumbelliferyl sugar derivatives were manufactured by Koch-Light Laboratories and purchased from Research Products International (Mount Prospect, IL, U.S.A.).

Cells and culture

CHO-S (C2) cells were grown in suspension culture in Eagle's minimal essential medium, α -modification without ribonucleosides or deoxyribonucleosides (α -MEM) supplemented with 10% (v/v) heat-inactivated fetal-bovine serum (Pool *et al.*, 1983). For amine treatment, exponential-phase cultures (2×10^5 cells/ml, initial concentration) were grown in the presence of 0.1 mM-chloroquine or 15 mM-NH₄Cl for 16–18 h. At these dosages, chloroquine or NH₄Cl maximally inhibit the degradation of internalized HRP in lysosomes, with little effect on cell growth (Fritsch *et al.*, 1988).

Labelling conditions

Cellular RNA was metabolically labelled by culturing CHO cells in the presence of [2-¹⁴C]uridine (0.01 μ Ci/ml, 51.2 mCi/mmol) for 18 h. Protein was metabolically labelled by culturing CHO cells in the presence of *trans*-³⁵S-labelled methionine (8–17 μ Ci/ml, 1087–1100 Ci/mmol) for 18 h in α -MEM containing one-tenth the normal amount of methionine, 10% dialysed fetal-bovine serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml).

Isolation of lysosomes

Control and amine-treated CHO cells were disrupted by nitrogen cavitation and homogenized by four strokes of a Potter–Elvehjem homogenizer (Rome *et al.*, 1979; Pool *et al.*, 1983). The postnuclear supernatant (PNS) was isolated by centrifugation (Madden & Storrie, 1987). Lysosomes were isolated from the PNS of control and amine-treated CHO cells by two consecutive discontinuous-gradient centrifugations in the same manner as described for untreated CHO cells (Madden *et al.*, 1987).

Analysis of organellar markers

The following organellar markers were assayed as previously described. Lysosomal: β -hexosaminidase (Pool *et al.*, 1983), β -galactosidase (Rome *et al.*, 1979), β -glucosidase (Fritsch *et al.*, 1988; Pool *et al.*, 1983); plasma membrane: alkaline phosphodiesterase I (Aronson & Touster, 1974); Golgi apparatus: α -mannosidase (Pool *et al.*, 1983; Tulsiani *et al.*, 1982); cytosolic: lactate

dehydrogenase (Decker, 1977); peroxisomal: catalase, (Leighton *et al.*, 1968); and mitochondrial: cytochrome *c* oxidase (Madden & Storrie, 1988). Rough endoplasmic reticulum was labelled by long-term incorporation of [¹⁴C]uridine into trichloroacetic acid-precipitable rRNA (Storrie & Attardi, 1973). Endosomes were labelled by a 10 min uptake of horseradish peroxidase (HRP, 1 mg/ml; Adams *et al.*, 1982). For amine-treated cells, chloroquine (0.1 mM) or NH₄Cl (15 mM) was included in the uptake media and all the washes. HRP was assayed as described by Pool *et al.* (1983). Lysosomes were extrinsically labelled by incubating CHO cells for 16 h with HRP (50 μ g/ml). In a long-term uptake, HRP accumulates in lysosomes (Adams *et al.*, 1982). Protein was measured fluorimetrically by the fluorescamine procedure (Böhlen *et al.*, 1973; Udenfriend *et al.*, 1972). Metrizamide (present in gradient fractions) was diluted to reaction mixture concentrations less than 0.5% to prevent inhibition of β -glucosidase activity and to less than 1.5% to prevent inhibition of the other glycosidase activities. Recoveries of all marker activities ranged from 80 to 113%. Fold enrichment of organellar marker activity was calculated by dividing the specific activity recovered from each gradient fraction by the specific activity in the PNS.

Electron microscopy, HRP and acid phosphatase cytochemistry

For morphological analysis, lysosomes isolated from chloroquine-treated CHO cells were fixed as previously described (Madden *et al.*, 1987). For HRP cytochemistry, organelles were fixed as described by Casey *et al.* (1986). After pelleting the organelles for 20 min at 53 000 *g* (at 4 °C) in a Beckman SW 27 rotor, cell fractions were processed for HRP cytochemistry with diaminobenzidine as a substrate (Storrie *et al.*, 1984). As a control, diaminobenzidine was omitted from the cytochemical incubation of lysosomes prepared from cells incubated with HRP for 16 h. Isolated lysosomes were processed for acid phosphatase cytochemistry as described by Madden *et al.* (1987). CHO cells have no detectable 5'-nucleotidase activity (B. Storrie, unpublished work). All preparations were dehydrated with ethanol and embedded in Spurr's low-viscosity resin. Sections for morphological analysis were post-stained with lead citrate and uranyl acetate. Sections for cytochemical studies were in general not post-stained. The sections were viewed in a Zeiss EM 10-CA electron microscope at an accelerating voltage of 60 kV.

Separation of membrane and soluble (luminal) proteins

Lysosomes, metabolically labelled with *trans*-³⁵S-labelled methionine as described above, were isolated from amine-treated and control CHO cells. The lysosomes were pelleted for 20 min at 53 000 *g* (20 000 rev./min) in a Beckman SW 27 rotor at 4 °C. The pellets were resuspended in 10 ml of 100 mM-sodium carbonate, pH 11.5. The lysosomes were separated into total, membrane and luminal fractions for SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) by a modification of the Fujiki *et al.* (1982) procedure (Madden *et al.*, 1987).

SDS/PAGE

The SDS/PAGE formulation of Dreyfuss *et al.* (1984) was followed. The resolving gel was 8% (w/v) acrylamide. The conditions for electrophoresis and method

for fluorography were described previously (Madden *et al.*, 1987).

RESULTS

Marker-enzyme characterization of the lysosomal preparation

Chloroquine causes changes in the equilibrium density of lysosomes in sucrose density gradients (Limet *et al.*, 1985). However, despite the fact that acidotropic-amine treatment typically causes swelling of the lysosomal compartment (Ohkuma & Poole, 1981), and that chloroquine treatment (18 h) resulted in the vacuolization of CHO-cell lysosomes *in situ* (results not shown), previous work from this laboratory (Fritsch *et al.*, 1988) had indicated that lysosomes from amine-treated CHO cells behaved normally in Percoll gradients. Hence, we decided to apply our standard hybrid Percoll/Metrizamide gradient procedure to the isolation of lysosomes from amine-treated CHO cells.

Postnuclear supernatants were isolated from control CHO-cell cultures and cultures treated with 0.1 mM-chloroquine or 15 mM-NH₄Cl for 18 h. Previous work (Fritsch *et al.*, 1988) had shown that 0.1 mM-chloroquine and 15 mM-NH₄Cl provide maximal inhibition of the degradation of endocytosed HRP with little inhibition of cell growth. The PNS contained about 70% of the homogenate protein and about 80% of the homogenate β -hexosaminidase activity. Chloroquine had no significant effect on these percentages.

As shown in Fig. 1(a), when a PNS from chloroquine-treated cells was centrifuged in a hybrid Percoll/Metrizamide gradient (Madden & Storrie, 1987), 50–60% of the lysosomal-marker-enzyme activity and about 8% of PNS protein were found in the Percoll/17% Metrizamide (P/17) fraction. After flotation of material from this fraction through a discontinuous Metrizamide gradient (Madden *et al.*, 1987), 55–60% of the lysosomal-marker activity was found in the 5%/17% Metrizamide (5/17) fraction (Fig. 1b). The fractionation pattern for NH₄Cl-treated cells was essentially the same (results not shown). For control cells, material at the 5/17 fraction has been previously identified as a highly enriched lysosomal preparation (Madden *et al.*, 1987).

In the lysosomal preparation from amine-treated CHO cells, the activity of luminal lysosomal enzymes such as β -hexosaminidase and β -galactosidase would be expected to be decreased, whereas the activity of β -glucosidase, a membrane-bound enzyme (Pentchev *et al.*, 1973), should be increased (Fritsch *et al.*, 1988). As shown in Table 1, slightly more than a 2-fold decrease was observed in the specific activities of β -hexosaminidase and β -galactosidase with chloroquine treatment. For α -mannosidase, a similar decrease in specific activity was observed. Even though much of the α -mannosidase activity in the lysosomal preparation is presumably organelle-resident, only a small fraction of the α -mannosidase activity in CHO cells is lysosomal (Pool *et al.*, 1983). For β -glucosidase a 1.6-fold increase was observed. The relatively low level of fluorescent product generated by β -glucosidase is consistent with previous reports (Pentchev *et al.*, 1973). Alkaline phosphodiesterase, which has been shown to be associated with the lysosomal membrane (about 10.9–14.3% of the homogenate activity) in culture rat fibroblasts (Draye *et al.*, 1987), was

Fig. 1. A typical distribution of protein and lysosomal-marker-enzyme activities from 0.1 mM-chloroquine-treated CHO cells in the two discontinuous gradients used for the isolation of lysosomes from untreated CHO cells

Yields are expressed relative to the PNS. (a) First discontinuous gradient, hybrid Percoll/Metrizamide. Fractions: top, air/0.25 M-sucrose; S/P, 0.25 M-sucrose/6% Percoll; P/17, 6% Percoll/17% Metrizamide; 17/35, 17% Metrizamide/35% Metrizamide. (b) Second discontinuous gradient, Metrizamide. Fractions: top, air/0.25 M-sucrose; S/5, 0.25 M-sucrose/5% Metrizamide; 5/17, 5% Metrizamide/17% Metrizamide; 17/35, 17% Metrizamide/35% Metrizamide. These data are from one of four similar experiments.

present at only trace amounts in the CHO-cell lysosomal preparations (Table 1; Madden *et al.*, 1987).

As expected the control lysosomal preparation was enriched about 40-fold relative to the PNS for the three lysosomal marker enzymes, namely β -hexosaminidase, β -galactosidase and β -glucosidase (Table 1). The lysosomal preparation from chloroquine-treated cells, although isolated in high yield, was only about 25-fold enriched in marker-enzyme activity. The lower enrichment for lysosomal enzymes found with amine treatment may be a reflection either of increased protein content per lysosome, presumably as a consequence of decreased proteolytic activity in the presence of amines (Wibo & Poole, 1974), or the occurrence of unidentified contaminating organelle(s) in the preparation. The data depicted in Fig. 1 and Table 1 are from a representative experiment. Four such experiments were conducted, all

Table 1. Lysosomal preparationAbbreviations used: *RFU*, relative fluorescence units; *A*, absorbance units; ND, not determined, NA, not applicable

Organelle marker	0.1 mM-Chloroquine ...	Specific activity (<i>RFU</i> /min per mg or <i>A</i> /min per mg)		Enrichment (relative to the PNS) (fold)		Recovery (relative to the PNS) (%)	
		–	+	–	+	–	+
β -Hexosaminidase		779.3	312.2	41.0	22.5	26.8	60.5
β -Galactosidase		1424.4	604.7	48.7	26.1	24.5	59.0
α -Mannosidase*		38.2	17.1	0.2	0.3	0.1	1.9
β -Glucosidase		2.0	3.2	37.0	23.8	22.4	54.7
Cytochrome <i>c</i> oxidase		1.3	ND	1.5	ND	0.8	ND
Alkaline phosphodiesterase I		0.0	0.0	0.5	0.2	0.3	0.7
Lactate dehydrogenase		ND	0.2	ND	0.0	ND	0.1
Catalase		ND	ND	ND	ND	ND	ND
RNA		ND	ND	ND	ND	ND	ND
Protein		NA	NA	NA	NA	0.6	3.1

* Most α -mannosidase activity in CHO cells is associated with the cytosol or Golgi apparatus (Pool *et al.*, 1983). Hence no enrichment in α -mannosidase activity would be expected in lysosomal preparations.

Fig. 2. Morphological appearance of the lysosomal preparation isolated from 0.1 mM chloroquine-treated CHO cells

The sections were post-stained with lead citrate and uranyl acetate. The arrows denote densely staining areas within the multivesicular bodies.

with similar results. The range of enrichments for β -hexosaminidase activity in the lysosomal preparations from chloroquine-treated cells was from about 20–25-fold relative to the PNS.

Morphological and cytochemical characterization of the lysosomal preparation

To determine the possible contribution of other morphologically identifiable organelles to the lysosomal preparation from acidotropic-amine-treated cells, material from the 5/17 fraction was processed for electron microscopy. As shown in Fig. 2, the chloroquine-treated

lysosomal preparation appeared homogeneous morphologically. Almost all of the organelle profiles appeared multivesicular and contained densely staining material within the vesicles (Fig. 2, arrows). No profiles in the preparation could be recognized as mitochondria or other non-lysosomal organelles.

HRP, which accumulates in lysosomes via fluid-phase pinocytosis in both control and amine-treated CHO cells (Fritsch *et al.*, 1988), was used as an exogenous cytochemical marker to investigate the purity of the preparation. As shown in Fig. 3(a), almost all organelle profiles in the chloroquine-treated lysosomal preparation from

Fig. 3. Cytochemical analysis of the lysosomal preparation isolated from 0.1 mM-chloroquine-treated CHO cells

Lysosomal preparations obtained from chloroquine-treated CHO cells that had internalized HRP for 16 h were incubated in the presence (a), or absence (b) of diaminobenzidine. The sections were not post-stained. Arrows denote putative electron-dense areas. Arrowheads denote cytochemical reaction product (oxidized diaminobenzidine). CHO cells do not contain detectable, endogenous peroxidase activity (Adams *et al.*, 1982). A separate lysosomal preparation was cytochemically stained for endogenous acid phosphatase activity (c). The sections were not post-stained. CHO cells do not contain detectable 5'-nucleotidase activity (B. Storrie, unpublished work). It should be noted that the mild fixation conditions used to maintain acid phosphatase activity do not give maximal preservation of organelle structure.

cells incubated with HRP for 16 h contained HRP cytochemical product. Greater than 50 % of the profiles were darkly stained for HRP. About 40 % were moderately stained, and in less than 10 % of the profiles HRP activity was restricted to small areas (Fig. 3a, arrowheads). Less than 1 % of the profiles contained no visible

HRP cytochemical product. In the absence of diaminobenzidine, the substrate for the HRP reaction, no cytochemical product was seen (Fig. 3b). When compared with lysosomes isolated from untreated CHO cells (Madden *et al.*, 1987), lysosomes isolated from chloroquine-treated CHO cells contained more cytochemical product.

This may be due to decreased breakdown of HRP in acidotropic-amine-treated lysosomes (Fritsch *et al.*, 1988). After acid phosphatase cytochemistry, almost all organelle profiles in the preparation were positive for this endogenous lysosomal marker (Fig. 3c). Control lysosomes have been previously established to be positive for acid phosphatase activity (Madden *et al.*, 1987).

Low level of endosomes in the lysosomal preparation

To investigate whether endosomes are present in the chloroquine-treated lysosomal preparation, CHO cells were incubated with HRP for 10 min. During a short pinocytic pulse, HRP accumulates in endosomes (Adams *et al.*, 1982; Storrie *et al.*, 1984). As shown in Fig. 4(a), biochemically the bulk of the endosomal pulse was found in the two lightest fractions of the first gradient for both control and chloroquine-treated cells. Relative to the PNS, about 4% of the total control HRP activity and

8% of the total amine-treated activity was found at the 5/17 fraction of the second gradient, the lysosomal preparation (Fig. 4b).

To determine whether the pulse-ingested HRP in the lysosomal preparation was uniformly spread through the entire organelle population or was restricted to a subset, electron-microscopic cytochemistry was performed. As shown in Fig. 5, less than 1.0% of the profiles in the chloroquine-treated lysosomal preparation were positive for HRP. Of these HRP-positive profiles, some with rimmed deposits resembled endosomes (E; see Pool *et al.*, 1983; Marsh *et al.*, 1987) and others where the deposits filled the multivesicular structure, appeared more like lysosomes (Ly; Madden *et al.*, 1987).

On the basis of enzymic, morphological and cytochemical analyses, we conclude that the lysosomal preparation from acidotropic-amine-treated CHO cells, like the previously characterized control preparation (Madden *et al.*, 1987), was essentially devoid of other organelles.

SDS/PAGE

Total, luminal and membrane proteins labelled by long-term incorporation of [³⁵S]methionine were separated by SDS/PAGE in order to determine the effects of acidotropic amines on the accumulation of newly synthesized cellular proteins in lysosomes. When equal amounts of radioactivity from control and chloroquine-treated lysosomal fractions were applied to the gel lanes, differences in relative polypeptide composition could be seen in all compartments (Fig. 6a). Several polypeptides were decreased in relative amount (arrowheads), some disappeared completely (minus signs) and others increased (arrows). Most of the electrophoretic species that decreased were luminal polypeptides, although occasional examples of luminal species that increased in amount were seen (Fig. 6a, lanes 3 versus 4). In contrast, most of the polypeptides that increased in amount were membrane proteins (Fig. 6a, lanes 5 versus 6). However, examples of membrane polypeptides that decreased in amount were observed at 110–125 kDa and at about 33 kDa. Lippincott-Schwartz & Fambrough (1987) previously reported that a 100 kDa membrane protein is depleted from lysosomes when chicken fibroblasts are cultured in the presence of chloroquine. The diffuse 110–125 kDa species which was labelled with [³H]-mannose and [³H]galactose appeared to be glycosylated.

When equal amounts of protein from each lysosomal fraction were applied to the gel, the effects of chloroquine on the bulk accumulation of newly synthesized cellular proteins in lysosomes could be seen (Fig. 6b). As a whole, the specific activity of incorporation into total lysosomal protein was decreased by about 18% by chloroquine treatment. This reduction in total labelled lysosomal protein was entirely due to depletion of incorporation into the luminal fraction, which constitutes the bulk of lysosomal protein in both control and amine-treated cells. The specific activity of the luminal fraction was reduced about 32% by chloroquine treatment. In striking contrast, the specific activity of the membrane preparation increased 85%. The general decrease in labelling in the luminal compartment is consistent with the decrease in the specific activities of the luminal marker enzymes, β -hexosaminidase and β -galactosidase (Table 1) observed with chloroquine treatment. Similarly, the general increase in labelling in the membrane fraction

Fig. 4. Effect of 0.1 mM-chloroquine on the distribution of endosomes in the two discontinuous density gradients

Endosomes were extrinsically labelled by pulsing control and chloroquine-treated CHO cells with HRP for 10 min. Yields are expressed relative to the PNS. (a) First discontinuous gradient, hybrid Percoll/Metrizamide. Fractions: top, air/0.25 M-sucrose; S/P, 0.25 M-sucrose/6% Percoll; P/17, 6% Percoll/17% Metrizamide; 17/35, 17% Metrizamide/35% Metrizamide. (b) Second discontinuous gradient, Metrizamide. Fractions: top, air/0.25 M-sucrose; S/5, 0.25 M-sucrose/5% Metrizamide; 5/17, 5% Metrizamide/17% Metrizamide; 17/35, 17% Metrizamide/35% Metrizamide.

Fig. 5. Cytochemical localization of short-term (10 min) internalized HRP in the lysosomal preparation obtained from chloroquine-treated CHO cells

Endosomes are the predominant species labelled by a 10 min pinocytic pulse of HRP. The sections were not post-stained. E, putative endosome. Ly, putative lysosome.

is consistent with the increase in the specific activity of the membrane marker enzyme, β -glucosidase.

DISCUSSION

These experiments were designed to determine the effect of acidotropic amines on the accumulation of the bulk of newly synthesized luminal and membrane proteins in animal-cell lysosomes. On the basis of previous genetic and biochemical studies of individual proteins, we expected to observe a quantitative decrease in luminal-protein labelling and a general increase in membrane-protein labelling. Although strong quantitative differences were expected, we expected to observe few qualitative differences. Consistent with these expectations, the predicted quantitative differences were in general observed. Although the control and amine-treated SDS/PAGE patterns were largely similar, several qualitative differences were also apparent. The membrane and luminal compartments of lysosomes isolated from amine-treated CHO cells appeared to be devoid of certain species and to contain new protein species. We conclude from these observations that, unlike lysosomal luminal proteins, the bulk of lysosomal membrane proteins are accumulated in lysosomes by step(s) that do not require a low pH and that membrane trafficking from Golgi apparatus to lysosomes continued to proceed in amine-treated CHO cells.

Fundamental to these observations was the ability to isolate highly purified lysosomal preparations from both control and amine-treated CHO cells. Lysosomes from control cells were isolated by sedimentation of a PNS

through a hybrid Percoll/Metrizamide gradient, followed by flotation of the lysosome-rich fraction through a Metrizamide gradient to remove mitochondrial contamination (Madden *et al.*, 1987). By using the same procedure, lysosomes from cells cultured in the presence of 0.1 mM-chloroquine or 15 mM- NH_4Cl for 15–18 h were isolated with a yield of about 60%. The amine-treated lysosomes were homogeneous multivesicular bodies by electron microscopy, stained for acid phosphatase and long-term internalized HRP, and were essentially devoid (less than 1%) of endosomal contamination. Amine-treated lysosomes were isolated in higher yield than were control lysosomes (about 60% versus about 25%), presumably as a consequence of lysosomal swelling induced by chloroquine and NH_4Cl . In contrast with this increased yield, the amine-treated lysosomal preparation was less enriched for lysosomal-marker-enzyme activities than the control preparation (about 25-fold versus about 45-fold), even for the membrane associated activity, β -glucosidase (Pentchev *et al.*, 1973), which increased in specific activity. We attribute this decrease in fold enrichment to an apparent 2-fold increase in the amount of protein in the lysosomal compartment in amine-treated cells. Much of this increased protein may be due to the decreased proteolysis brought about by amine treatment (Wibo & Poole, 1974).

As expected, growth of CHO cells in the presence of either chloroquine or NH_4Cl resulted in a greater than 50% decrease in the specific activities of the luminal lysosomal enzymes, β -hexosaminidase and β -galactosidase, and an approx. 32% decrease in incorporation of [^{32}S]methionine radioactivity into luminal proteins. As

Fig. 6. SDS/PAGE of [^{35}S]methionine-labelled total (T), luminal (L) and membrane (M) proteins isolated from control (C) and chloroquine-treated (+) lysosomes

(a) Fluorograph containing equal amounts of radioactivity ('c.p.m.)/lane; (b) fluorograph containing equal amounts of protein/lane. Lysosomes were isolated from control and 0.1 mM-chloroquine-treated (18 h) CHO cells that had been metabolically labelled with [^{35}S]methionine. Lysosomes were separated into luminal and membrane fractions by Na_2CO_3 fractionation. Arrowheads denote polypeptides whose accumulation was decreased by chloroquine treatment. Minus signs to the left of the first lane denote polypeptides whose accumulation was apparently totally inhibited by chloroquine treatment. Arrows denote luminal (L) or membrane lysosomal polypeptides whose accumulation was increased by chloroquine treatment. The apparent molecular masses are shown in the left margin.

reviewed previously (Storrie, 1988), many laboratories have shown that amine treatment results in secretion of newly synthesized lysosomal hydrolases and a concomitant decrease in many luminal hydrolases. Unexpectedly, new electrophoretic species were labelled in the luminal fraction of amine-treated lysosomes. Some of these species may be due to decreased proteolytic processing.

As expected, growth of CHO cells in the presence of either chloroquine or NH_4Cl resulted in an increase in the incorporation of methionine into the lysosomal membrane fraction. There were few qualitative differences in the electrophoretic pattern of the solubilized lysosomal membrane fraction from amine-treated cells when compared with the control. However, new species were present, and species present in the control were absent. LEP100, a chicken lysosomal membrane protein, redistributes to endosomes and the plasma membrane in amine-treated cells (Lippincott-Schwartz & Fambrough,

1987). Consistent with this, we observed that polypeptides of roughly the same molecular mass failed to accumulate in lysosomes with amine treatment. The high level of accumulation of most lysosomal membrane proteins in the amine-treated organelle suggests that membrane trafficking from Golgi apparatus to lysosomes was largely unaffected by amine treatment. Consistent with this apparently normal membrane trafficking, fluid-phase pinocytosis by CHO cells has been shown to be largely unperturbed by acidotropic amine treatment (Fritsch *et al.*, 1988).

The extent to which lysosomal proteins, especially membrane proteins, exhibit normal secondary modifications in amine-treated cells remains an open question. Preliminary two-dimensional polyacrylamide-gel-electrophoretic patterns of the luminal and membrane fractions of amine-treated and control lysosomes indicate further differences in electrophoretic pattern with amine treatment (E. A. Madden & B. Storrie, unpublished work). These differences in pattern may well be due to differences in secondary modification. Unfortunately, antibodies to CHO-cell lysosomal proteins, which are mandatory for understanding these differences in the two-dimensional gel patterns, are not presently available. The two-dimensional polyacrylamide-gel patterns obtained from chloroquine-treated and NH_4Cl -treated lysosomes were essentially identical, indicating that the effects of chloroquine on the bulk of lysosomal proteins reflect acidotropic-amine effects generally and not chloroquine effects uniquely.

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