Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10588–10592, November 1993
Biochemistry

The lysosomal proenzyme receptor that binds procathepsin L to microsomal membranes at pH 5 is a 43-kDa integral membrane protein

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Communicated by Günter Blobel, August 16, 1993

ABSTRACT **Two lysosomal proenzymes, procathepsins L and D, bind to mouse fibroblast microsomal membranes at acidic pH. This membrane association is independent of the mannose-6-phosphate receptors and requires the presence of the N-terminal propeptides of the enzymes. We have identified the protein that specifically binds procathepsin L at pH 5. A 43-kDa membrane protein coimmunoprecipitated with procathepsin L at pH 5 but not at pH 7 when cells were denatured with detergents. Similarly, a 43-kDa integral membrane protein bound procathepsin L in three kinds of ligand blots at pH 5 but not at pH 7. A synthetic peptide containing the 24 N-terminal residues of mouse procathepsin L blocked the binding of procathepsin L to this integral membrane protein on ligand blots. These results indicate that the 43-kDa integral membrane protein is a lysosomal proenzyme receptor that specifically binds the procathepsin L activation peptide at acidic pH.**

Phosphomannosyl residues added post-translationally mediate binding of lysosomal proenzymes to mannose-6-phosphate (M6P) receptors (MPRs) within the Golgi (1, 2). The MPRs deliver the ligands either to early endosomes (3) or directly to late endosomal compartments or prelysosomes, where the low pH causes the proenzymes to dissociate from the MPRs (4, 5). It is not clear how enzymes destined for lysosomes are sorted within prelysosomes from the MPRs, which are recycled back to the Golgi.

While MPRs play a major role in the intracellular transport of newly synthesized lysosomal enzymes in fibroblasts, additional proteins are probably involved in transporting the enzymes to lysosomes. For example, the transport of lysosomal enzymes in hepatocytes, Kupffer cells, and leukocytes is unaffected by a deficiency in the phosphotransferase enzyme, the protein that places phosphate residues on the high-mannose carbohydrate chains of lysosomal enzymes (6, 7). This suggests that there is an alternative M6P-independent intracellular pathway for lysosomal proteins in these cells.

MPR-independent membrane association has been reported for an increasing number of lysosomal proteins. Procathepsin D is membrane-associated in macrophages (8) and HepG2 cells (9, 10) in the presence of 10 mM M6P. Glucocerebrosidase and prosaposin are similarly bound to membranes in HepG2 cells (9), while procathepsin C is transiently membrane bound in Morris hepatoma 7777 cells (11), which lack the cation-independent MPR. It has not been established whether these proenzymes are interacting directly with membranes or with yet-unidentified receptor proteins embedded in the membranes. The role the membrane association plays in the intracellular transport of these proenzymes is also not known.

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We have demonstrated that at least two lysosomal proenzymes, procathepsins L and D, specifically bind to microsomal membranes at pH 5 in fibroblasts (12). Since the mature enzymatically active forms of these enzymes are soluble at acidic pH, membrane binding is probably mediated by the propeptides of the enzymes. The binding of procathepsin L is saturable and treatment of membranes with trypsin reduces the amount of procathepsin L bound by 50% (12), suggesting that the binding is mediated by a specific membrane protein or receptor. In the present study, we have used four methods to identify a 43-kDa integral membrane protein that mediates the binding of procathepsin L to microsomal membranes at pH 5. The physiological function of the membrane association is not known, but the pH dependence of the association suggests that the 43-kDa protein could play a role in sorting and/or activating procathepsin L in acidified vesicles.

MATERIALS AND METHODS

Materials. Reagents used include [^{35}S]methionine (Tran^{35}S-label; 850–950 Ci/mmol; 1 Ci = 37 GBq) from ICN; Dulbecco's modified Eagle's medium, Opti-MEM, and fetal bovine serum from GIBCO; phenylmethylsulfonyl fluoride, aprotinin, pepstatin, the cysteine protease inhibitor E-64, dithiothreitol, and Hepes from Boehringer Mannheim; ECL Western blotting detection reagents and donkey anti-rabbit serum conjugated to horseradish peroxidase from Amersham; the Hot Box system from Billups-Rothenberg (Del Mar, CA); Immobilon-P from Millipore; and 3,3′-dithiobis-(sulfosuccinimidylpropionate) (DTSSP) from Pierce. All other reagents were the highest quality available and were obtained from either Sigma or Fisher Scientific.

Polyclonal rabbit antiserum was prepared as described (13) against mouse procathepsin L expressed in bacterial cells as a fusion protein. The Kirsten sarcoma virus-transformed NIH 3T3 cell line (KNIH), a gift of C. Scher (University of Pennsylvania), was cultured and radiolabeled as described (12).

Coimmunoprecipitation. KNIH cells were labeled with [^{35}S]methionine in a Hot Box system and microsomal membranes were prepared and stripped of peripheral membrane proteins as described (12). The final membrane pellet was solubilized for immunoprecipitation with 500 μl of 50 mM sodium acetate (pH 5) containing 0.1% SDS and 2 mM EDTA. Samples were adjusted to contain 8 mM iodoacetamide, 4% (vol/vol) Triton X-100, and aprotinin (26 μg/ml). Polyclonal anti-procathepsin L serum (10 μl) was added and the sample was incubated at 37°C for 1 h and then at 4°C for 16 h. To remove material that binds nonspecifically to Sepharose at pH 5, samples were incubated for 3 h at room temperature with protein A-Sepharose [40 μl of a 1:1 (vol/vol) resin/water slurry per 10 μl of antiserum]. After removal

Abbreviations: M6P, mannose 6-phosphate; MPR, M6P receptor.

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of the Sepharose beads by centrifugation, affinity-purified anti-rabbit IgG-Sepharose (40 μl of 1:1 resin/water slurry per 10 μl of antiserum) was added and kept at room temperature for 3 h to bind the antigen–antibody complexes. The beads were washed three times with the same pH 5 buffer used to solubilize the membranes (12). Antigen–antibody complexes were eluted from the beads by treating at 100°C for 3 min in PAGE sample buffer containing 3.3% (wt/vol) SDS, 20% (wt/vol) sucrose, 0.008% bromophenol blue, 80 mM Tris·HCl (pH 7), 17 mM EDTA, and 17 mM dithiothreitol. Iodoacetamide (83 mM) was added to alkylate the reduced proteins. The immunoprecipitated proteins were resolved by SDS/PAGE on 12.5% gels (14) and visualized by fluorography (15).

Ligand Blots. KNIH integral membrane proteins were resolved on a 1.5-mm 12.5% polyacrylamide gel in the presence of SDS (14) and electrophoretically transferred to Immobilon-P in either 25 mM Tris·HCl (pH 7) containing 20% (vol/vol) methanol or in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) containing 10% methanol by using a semi-dry Western blot apparatus (Integrated Separation Systems, Hyde Park, MA) at 1 mA per cm^2 per h. Free binding sites on the blot were blocked by incubating the blot with 10 mM Tris·HCl (pH 7.4) containing 150 mM NaCl and 0.05% Tween 20 (Tween/saline) plus 0.5% nonfat dry milk for 2 h at room temperature. The blot was washed for three 15-min periods in Tween/saline and incubated for 2 h with constant agitation at 4°C with either PAGE-purified [^{35}S]methionine-labeled procathepsin L or secreted procathepsin L in KNIH-conditioned serum-free medium in buffer A [50 mM sodium acetate, pH 5/10 mM M6P/1 mM EDTA/1 mM EGTA/1 mM phenylmethylsulfonyl fluoride/60 μM E-64/1 mM pepstatin/bovine serum albumin (1 mg/ml)/0.05% Tween 20/0.1% SDS; 1 ml per 8 cm^2 of Immobilon-P]. A parallel blot was incubated with ligand in buffer B, which is identical to buffer A except that 50 mM Tris·HCl (pH 7.4) replaced the sodium acetate. After the procathepsin L binding steps, the blots were washed at room temperature for three 15-min periods with the appropriate binding buffer. When specified, a synthetic peptide (0.18 mM) containing the first 24 amino acid residues of mouse procathepsin L (which lacks the signal peptide but contains a 96-residue propeptide) plus alanylcysteine amide was included in the procathepsin L incubation mixture.

To be able to wash blots under more stringent conditions, bound proteins were cross-linked to the membrane proteins in one series of experiments. Parallel blots were incubated at 4°C for 2 h in buffer A or buffer B containing secreted procathepsin L in KNIH-conditioned serum-free medium and then washed for three 15-min periods in the same buffer minus ligand. Bound proteins were cross-linked to the membrane proteins by treatment of the blot with 1 mM 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) in phosphate-buffered saline (pH 7.2) for 30 min at room temperature. After the cross-linking step, the blot was washed for three 15-min periods with Tween/saline.

RESULTS AND DISCUSSION

Procathepsin L Is a Peripheral Membrane Protein at pH 5 in KNIH Cells. In KNIH mouse fibroblasts, 86% of the 36-kDa procathepsin L was membrane-bound when microsomal membranes were lysed at pH 5 (Fig. 1, lanes 1 and 2). Only 14% of the proenzyme was recovered in the soluble fraction. When a separate aliquot of the same membranes was lysed at pH 7 (Fig. 1, lanes 3 and 4) or pH 10.6 (Fig. 1, lanes 5 and 6), the procathepsin L was completely solubilized. Thus procathepsin L behaved like a peripheral membrane protein at acidic pH. These results are consistent with those obtained when a single aliquot of microsomal membranes was washed consecutively in buffers at various pH values (12). The

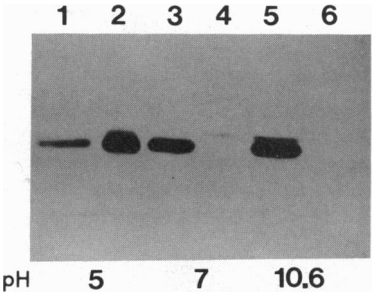

FIG. 1. Procathepsin L is a peripheral membrane protein at pH 5 in KNIH cells. Microsomes were prepared from one 85-mm plate of KNIH cells, divided into three equal parts, and then lysed at pH 5 (lanes 1 and 2), pH 7 (lanes 3 and 4), or pH 10.6 (lanes 5 and 6). The soluble proteins (lanes 1, 3, and 5) and membrane proteins (lanes 2, 4, and 6) generated from each wash were resolved on a SDS/polyacrylamide gel and electrophoretically transferred to Immobilon-P. Free binding sites on the blot were blocked by incubating the blot with Tween/saline plus 0.5% nonfat dry milk for 1 h at room temperature. The blot was incubated overnight at 4°C with anti-cathepsin L serum (1:15,000), washed three times with Tween/saline, and then incubated at room temperature for 30 min with donkey anti-rabbit serum conjugated to horseradish peroxidase (1:20,000). Procathepsin L was detected by chemiluminescence on exposure of the blot to x-ray film for 4 min. The amount of procathepsin L in each sample was determined by scanning the autoradiograph with a computing densitometer (Molecular Dynamics).

28-kDa single-chain and the 21-kDa heavy-chain forms of mature cathepsin L do not bind to microsomal membranes at pH 5 (12). Since EDTA, EGTA, and M6P were included in all buffers, membrane association is independent of divalent cations and not mediated by the MPRs (16, 17). Similar results were obtained when normal mouse fibroblast membranes were used (12), demonstrating that the pH-dependent membrane association of procathepsin L is not unique to the virally transformed KNIH cell line.

Procathepsin L Binds to a 43-kDa Integral Microsomal Membrane Protein. Four methods were used to identify the membrane protein that mediates binding of procathepsin L to microsomal membranes at pH 5. When procathepsin L antiserum was added to stripped microsomal membranes solubilized at pH 5 with 0.1% SDS, a single 43-kDa membrane protein coimmunoprecipitated with 36-kDa procathepsin L (Fig. 2). Immunoprecipitation of procathepsin L at pH 8 with 0.1% SDS produced numerous bands, presumably due to nonspecific adherence of proteins to the antigen–antibody complex, but a 43-kDa protein did not coprecipitate with procathepsin L (data not shown).

Theoretically, equivalent quantities of the ligand and receptor should be isolated by coimmunoprecipitation. In practice, less receptor is recovered because the noncovalent complex is washed several times in large volumes of buffer, which release ligand from the receptor. The antiserum can bind to both released and bound ligand but not to receptor that has lost its ligand during the wash steps. The stoichiometry of binding cannot be accurately determined until an antibody recognizing the 43-kDa protein is available.

The other three assays used to identify the membrane protein that binds procathepsin L were ligand blots, which utilized different techniques to detect native or denatured procathepsin L bound to an integral microsomal membrane protein. The membrane proteins resolved by SDS/PAGE are assumed to undergo partial renaturation during electroblotting to restore their capacity to bind ligands. Ligand blots have been employed to detect receptor proteins on nuclear membranes (18–20).

The first type of ligand blot detected the 43-kDa integral membrane protein by its ability to bind native nonradioactive

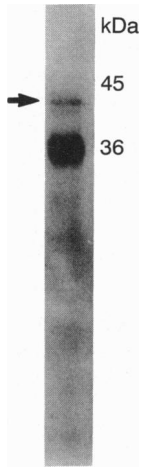

FIG. 2. Coimmunoprecipitation of a 43-kDa integral membrane protein with procathepsin L at pH 5. Microsomes were lysed at pH 5 to release soluble entrapped contents but leave procathepsin L bound to membranes. Procathepsin L was immunoprecipitated at pH 5 in 0.1% SDS and the immunoprecipitate was electrophoresed under reducing conditions on a SDS/12.5% polyacrylamide gel. A 43-kDa protein (arrow) coimmunoprecipitated with 36-kDa procathepsin L at pH 5. Immunoprecipitation of cathepsin L in buffer at pH 8 containing 0.1% SDS produced numerous bands, but no protein was evident at 43 kDa (data not shown).

procathepsin L secreted into KNIH-cell-conditioned culture medium (Fig. 3*A*, lane 1). Although this medium contains a number of secreted proteins, procathepsin L is the major excreted protein (21). The membrane protein–procathepsin L

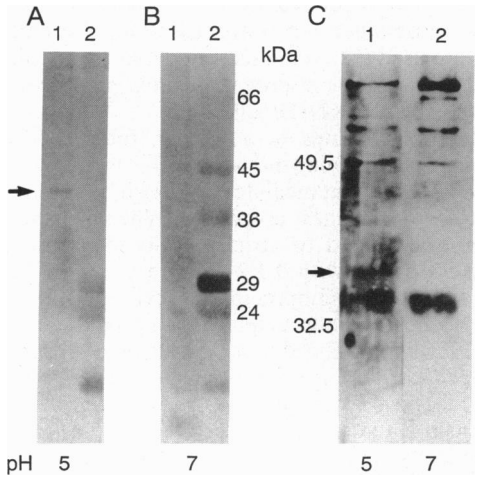

FIG. 3. Ligand-blot detection of a 43-kDa microsomal integral membrane protein that binds native procathepsin L at pH 5. (*A*) Microsomal integral membrane proteins (lane 1) and molecular mass markers (lane 2) were resolved under reducing conditions on SDS/polyacrylamide gels and transferred to Immobilon-P by electroblotting. The blots were incubated at pH 5 with KNIH-cell-conditioned culture medium containing secreted procathepsin L, M6P, EGTA, and EDTA. Procathepsin L bound to a 43-kDa integral membrane protein (arrow) was visualized, after addition of antiserum to procathepsin L and peroxidase-conjugated sheep anti-rabbit IgG serum, by a peroxidase reaction. (*B*) A parallel blot was treated as in *A*, except that incubation with medium containing procathepsin L was omitted, resulting in specific loss of labeling of the 43-kDa protein (arrow). (*C*) Microsomal integral membrane proteins were blotted as described in *A* except that bound procathepsin L was cross-linked with 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) to membrane proteins after incubating the blot in KNIH-cell-conditioned culture medium. Procathepsin L (arrow), detected by chemiluminescence, bound to a 43-kDa membrane protein at pH 5 (lane 1) but not at pH 7.4 (lane 2).

conjugates were visualized indirectly by a two-step immunodetection procedure utilizing horseradish peroxidase. The 43-kDa protein was not detected on a control blot exposed to the primary and secondary antibodies but not to procathepsin L (Fig. 3*B*, lane 1). Thus the band seen in the presence of procathepsin L is due to specific association of this proenzyme with the 43-kDa microsomal membrane protein.

Similar results were obtained when unlabeled procathepsin L secreted into KNIH-cell-conditioned medium was cross-linked to the proteins to which it bound on a ligand blot (Fig. 3*C*). Numerous bands were detected when the binding reaction was allowed to proceed either at pH 5 (lane 1) or pH 7 (lane 2), but only one membrane protein, having a molecular mass of 43 kDa (arrow), specifically bound procathepsin L at pH 5 but not at pH 7. The cross-linking step was added to reduce the loss of procathepsin L from the receptor during the numerous wash steps required by the double-antibody visualization step (Fig. 3). Increasing the number of wash steps prior to the cross-linking reaction decreased the background but also released procathepsin L from the 43-kDa protein (data not shown). Similarly, sodium chloride, normally added to buffers during Western blot preparation to reduce nonspecific background, was omitted during the binding and wash steps for this and all other ligand blots since salt interferes with the binding of procathepsin L to membranes (12).

A third type of ligand blot detected the 43-kDa membrane protein directly by its ability to bind denatured PAGE-purified [^{35}S]procathepsin L (Fig. 4). The denatured ligand bound to several proteins, but again only the 43-kDa membrane protein specifically bound the ligand at pH 5 (lane 1) but not at pH 8 (lane 2), even though the background bands were heavier at pH 8 than at pH 5. The fact that purified procathepsin L bound to the 43-kDa protein indicates that an additional protein, such as an activator (22, 23) or a protective protein (24), is not required to mediate the pH-dependent membrane association of the proenzyme.

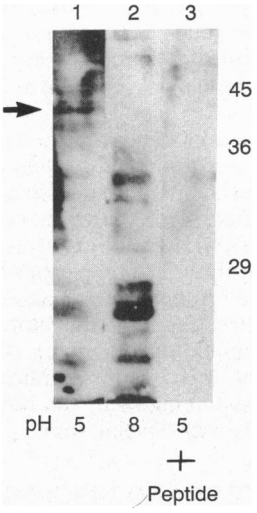

FIG. 4. Ligand-blot detection of a 43-kDa microsomal integral membrane protein that binds purified procathepsin L at pH 5 but not at pH 8. Microsomal integral membrane proteins were resolved under nonreducing conditions by SDS/PAGE and transferred to Immobilon-P by electroblotting. The filters were incubated at pH 5 (lane 1) or at pH 8 (lane 2) with denatured PAGE-purified [^{35}S]procathepsin L. After washing, procathepsin L was visualized by exposure of the blot to x-ray film. Procathepsin L bound to a 43-kDa microsomal membrane protein at pH 5 (lane 1, arrow) but did not bind to this protein at pH 8 (lane 2) or at pH 5 in the presence of a synthetic peptide (lane 3) consisting of the first 24 amino acid residues of mouse procathepsin L plus alanylcysteine amide. Molecular masses are indicated in kDa on right.

The ligand binding site is a linear sequence, not a conformational determinant, because denatured PAGE-purified procathepsin L retains its ability to bind to the integral membrane protein. Likewise, the 43-kDa protein does not bind to procathepsin L as a substrate because both native and denatured proenzyme bind to this protein, even in the presence of the competing cysteine protease inhibitor E-64. The 43-kDa protein was identified by ligand blots, whether or not the membrane proteins were reduced prior to electrophoresis, and by coimmunoprecipitation from detergent-treated microsomal membranes, suggesting that this protein is a monomer and is not disulfide-linked to another subunit.

A Synthetic Peptide Containing the 24 N-Terminal Residues of Procathepsin L Blocks Binding of Procathepsin L to the 43-kDa Membrane Protein. Binding of procathepsin L to microsomal membranes depends on the presence of the propeptide since the proenzyme binds to membranes but the mature biosynthetic forms do not (12). To establish the specificity of binding, ligand blots were carried out in the presence of a 26-residue synthetic peptide consisting of the first 24 amino acid residues of mouse procathepsin L after the signal peptide plus alanylcysteine amide [Thr^{18}–Gly^{41}, preprocathepsin L numbering (13)]. Binding of denatured procathepsin L to the membranes was blocked by the presence of 0.18 mM peptide (Fig. 4, lane 3), whether or not the peptide was alkylated (data not shown). A shorter synthetic peptide containing only 9 of the 24 N-terminal residues of the propeptide also inhibited the binding of procathepsin L to mouse fibroblast microsomal membranes (unpublished results). These results suggest that the pH-dependent binding of procathepsin L to membranes is a specific interaction mediated by a linear sequence in the N-terminal region of the procathepsin L propeptide.

Physiological Role. We have identified a 43-kDa integral membrane protein that binds procathepsin L at pH 5. The single binding protein was identified by ligand blots, whether or not the membrane proteins were reduced prior to electrophoresis, and by coimmunoprecipitation from detergent-treated microsomal membranes. These results suggest that the binding protein is a monomer and that ligand binding does not require the formation of intrasubunit disulfide bonds as has been reported for the cation-dependent MPR (25). Consistent with these results, preliminary data indicate that the membrane protein is capable of binding procathepsin L immediately after synthesis if the pH of microsomal membranes is adjusted to 5, making it difficult to distinguish at what point in its biosynthetic pathway the 43-kDa protein binds ligand *in vivo*.

This binding protein is not a MPR since 10 mM M6P was present in all wash buffers and this concentration of M6P not only inhibits the binding of M6P-bearing proteins to the MPRs but also dissociates MPR-bound proteins from the MPR (16, 17). Furthermore, the 43-kDa protein binds procathepsin L optimally at pH 5 (12), but the cation-dependent MPR binds M6P-bearing proteins optimally at pH 6.0–6.3 and the cation-independent MPR binds M6P-bearing proteins optimally at pH 6.0–7.4 (4, 26–28). In contrast to the 43-kDa protein, both MPRs demonstrate a sharp decrease in binding at pH values below 6.0 (27).

Our results indicate that the propeptide of the lysosomal cysteine proteinase cathepsin L not only regulates proteolytic activity but also mediates specific binding of the proenzyme to a 43-kDa integral microsomal membrane protein. Only the proenzyme, not the mature forms of the protein, shows pH-dependent membrane association (12). Consistent with this observation, a synthetic peptide based on the propeptide sequence blocks the binding of the proenzyme to the 43-kDa membrane protein.

The propeptides of yeast vacuolar and *Dictyostelium discoideum* lysosomal enzymes also play dual roles. The proregions of both carboxypeptidase Y (29–31) and proteinase A (32) mediate sorting to vacuoles in yeast, but a receptor that binds the propeptides has not been identified. Similarly, the N-terminal portion of β-hexosaminidase (amino acids 22–70) is required for correct targeting to vesicles of intermediate density in *Dictyostelium* (33). Proenzymes in *Dictyostelium* are membrane-bound (34), but the biochemical basis and physiological function of this association is not known.

The pH dependence of the membrane association of the mammalian lysosomal procathepsin L suggests that binding to the 43-kDa protein may occur in acidified vesicles, after release from the MPRs. The lysosomal proenzyme receptor may complement the MPR system by transporting lysosomal proenzymes released from MPRs to lysosomes.

Alternatively or in conjunction with a targeting role, the lysosomal proenzyme receptor could serve as an activator protein, aiding proteolytic removal of the propeptide in an acidic environment. Proteolytic activation of an inactive proprotein would cleave off the membrane-binding proregion and solubilize the active mature enzyme. It seems unlikely, however, that the 43-kDa protein is an activating protease itself, as the receptor–ligand complex is relatively stable, which is not common for an enzyme–substrate interaction at optimal pH.

We cannot eliminate the possibility that in some region of the Golgi the pH drops low enough that the receptor binds lysosomal proenzymes. Were this the case, the 43-kDa protein could potentially serve as an alternate pathway receptor. This seems unlikely, however, as we have detected the pH-dependent membrane association both in fibroblasts, which utilize a M6P-mediated sorting pathway, and in hepatoma cells, which presumably target lysosomal enzymes by an alternate mechanism (data not shown). Also, Conner (35) has demonstrated that the cathepsin D propeptide is not sufficient to target lactalbumin to lysosomes. Similarly, we have observed that the cathepsin L propeptide alone does not target invertase to lysosomes (G. D. Godbold and A.H.E., unpublished results).

This work was supported by American Cancer Society Research Grant BC-707 and by National Science Foundation Grant MCB-9204834.

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