SUPPLEMENTARY MATERIALS AND METHODS

Isolation of detergent resistant lysosomal membrane microdomains. Lysosomal membranes (150 µg protein) from rat liver or mouse cultured fibroblasts were incubated with 1% of non-ionic detergents in 150 mM NaCl, 50 mM Tris-HCl and 5 mM EDTA pH 7.4 (incubation buffer) on ice for 30 min. At the end of the incubation, samples were adjusted to 40% sucrose, sequentially overlayed with 500 µl step-wise discontinuous sucrose gradient (5-35%) and centrifuged at 200,000 g for 19 h in a SW-60Ti rotor (Beckman). Fifteen samples of 300 µl collected from the top of the gradient were subjected to acid precipitation with 10% trichloroacetic acid (TCA) and bovine serum albumin. Precipitates were washed with acetone, resuspended in electrophoresis sample buffer, and subjected to SDS-polyacrylamide electrophoresis and immunoblot. In some experiments, instead of the acid precipitation, the detergent-resistant fractions were diluted 10 times in incubation buffer and collected by centrifugation at 200,000g for 2h in a TLA-110 rotor (Beckman). To resolve LAMP-2A complexes, the detergent soluble and resistant fraction were adjusted to 0.1% NP-40, incubated on ice for 30 min and then loaded on top of a continuous density (10-80%) sucrose gradient for centrifugation at 140,000 g for 19 h in a TLA-SW-55 rotor (Cuervo and Dice, 2000). Aliquots of 500 µl taken from the top of the gradient were TCA precipitated and subjected to SDS-polyacrylamide electrophoresis and immunoblot.

Uptake and degradation of substrate proteins by isolated lysosomes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was radiolabeled with ^{14}C-formaldehyde by reductive methylation as described before (Jentoft and Dearborn, 1983). Cytosolic proteins from mouse fibroblasts in culture were metabolically radiolabeled by incubation with [^{14}C]leucine (2µCi/ml) at 37°C for 2 days (Cuervo et al., 1997). [^{14}C]GAPDH or a pool of radiolabeled cytosolic proteins [^{3}H]protein pool were incubated in MOPS buffer (10 mM 3-(N-morpholino) propanesulfonic acid) (MOPS) pH 7.3, 0.3 M sucrose, 1 mM DTT) with intact lysosomes for 30 min (Cuervo et al., 1995; Cuervo and Dice, 1996; Cuervo et al., 1997). Degradation of the radiolabeled substrates was measured after precipitation with TCA as described (Cuervo et al., 1995; Cuervo and Dice, 1996;

Cuervo et al., 1997). Proteolysis was expressed as the percentage of the initial acid-insoluble radioactivity (protein) transformed into acid-soluble radioactivity (amino acids and small peptides) at the end of the incubation. Where indicated, lysosomes were disrupted by a hypotonic shock before incubation with the substrates to assess changes in enzymatic proteolytic activity.

Intracellular protein turnover. To measure degradation of long-lived proteins, confluent cells were labeled with $[^3H]$leucine (2 μCi/ml) for 48 h at 37°C and then extensively washed and maintained in complete (10% NCS) or serum-deprived medium containing an excess of unlabeled leucine (2.8 mM), to prevent reutilization of radiolabeled leucine (Auteri et al., 1983). Aliquots of the medium taken at different times were precipitated with TCA and proteolysis was measured as above. Total radioactivity incorporated into cellular proteins was determined as the amount of acid-precipitable radioactivity in labeled cells immediately after washing. Where indicated 15 mM ammonium chloride and 100 μM leupeptine or 10 mM 3-methlyadenine were added in the culture medium during the chase. To deplete or load cells with cholesterol they were incubated with methybetacyclodextrin (25mM) or cholesterol (50μM) for 30 and 90 min, respectively.

Fluorescence and immunocytochemical staining. Cells grown on coverslips were fixed with a 3% formaldehyde solution, blocked, and then incubated with the primary and corresponding fluorescein isothiocyanate (FITC) or Cy5-conjugated secondary antibodies (Cuervo and Dice, 2000). Filipin staining was done after fixation and blocking by incubation with 0.5 mg/ml of Filipin (Sigma) for 1 h at room temperature to detect unesterified cholesterol. To visualize glycosphingolipid-enriched regions cells were incubated with Texas-Red-cholera toxin B subunit for 30 min at 37°C, to allow its internalization, followed by an antibody against cholera toxin B subunit for the same period of time to cluster the toxin labeled regions. Cells were then fixed and processed to immunofluorescence with other antibodies as descrbed before. Isolated lysosomes were placed on a 50 μl coverslip microchamber where they bound to the glass instantaneously and immunofluorescence was carried out following the same procedures as with whole cells. Images were acquired with an Axiovert 200 fluorescence microscope (Carl Zeiss Ltd., Thornwood, NY), subjected to deconvolution with the manufacture's software and prepared using Adobe Photoshop 6.0 software (Adobe Systems Inc., Mountain View, CA). Quantification was

performed using ImageJ software (NIH, MD) and the colocalization was calculated by JACoP plugin.

Electron microscopy and immunogold. Intact rat liver lysosomes and detergent-resistant regions of the lysosomal membrane floated in sucrose density gradients and collected by high speed centrifugation were negatively stained with 1% Uranyl Acetate. Immunogold labeling was performed as described previously (Cuervo et al., 1995) using the antibodies against LAMP-2A or LAMP-2B for 5 min, followed by gold secondary (GAR 1:100) for other 5 minutes. Samples were water rinsed and negatively stained with 1% Uranyl Acetate and viewed on a JEOL 100CX II transmission electron microscope at 80kV. Appropriate controls using only the gold-conjugated secondary antibodies were included. All grids were viewed on a JEOL 100CX II transmission electron microscope at 80kV. The amount of gold labeling per µm in the profiles of the intact lysosomes or the detergent resistant regions of the lysosomal membrane was quantified using Image J (NIH) in 15-20 different micrographies for each condition after thresholding.

Electrophoresis and immunoblot. After SDS-PAGE and immunoblotting (Laemmli, 1970), the proteins recognized by the specific antibodies were visualized by chemiluminescence methods (Renaissance^{R}, NEN-Life Science Products). For better resolution of the membrane proteins in two dimensional electrophoresis, lipids were solubilized and proteins were extracted and precipitated using the Clean-up kit^{R} (Bio-Rad). Isoelectrofocusing was carried out in 3-10 nonlinear IPG strips (Bio-Rad), and the second dimension in standard 10% SDS-PAGE gels. Proteins were visualized in a U.V. transilluminator after methanol/acetic fixation by staining with SyproRuby^{R} for 10 h at room temperature. Densitometric quantification of the immunoblotted membranes and stained gels was done with an Image Analyzer System (Inotech S-100, Sunnyvale, CA).

References

Auteri, J., Okada, A., Bochaki, V. and Dice, J. (1983) Regulation of intracellular protein degradation in IMR- 90 human diploid fibroblasts. *J. Cell. Physiol.*, **115**, 159-166

Cuervo, A., Dice, J. and Knecht, E. (1997) A lysosomal population responsible for the hsc73-mediated degradation of cytosolic proteins in lysosomes. *J Biol Chem*, **272**, 5606-5615.

Cuervo, A. and Dice, J. (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. *Science*, **273**, 501-503.

Cuervo, A., Knecht, E., Terlecky, S. and Dice, J. (1995) Activation of a selective pathway of lysosomal proteolysis in rat liver by prolonged starvation. *Am J Physiol*, **269**, C1200-C1208.

Cuervo, A. and Dice, J. (2000b) Unique properties of lamp2a compared to other lamp2 isoforms. *J Cell Sci*, **113**, 4441-4450.

Jentoft, N. and Dearborn, D. (1983) Protein labeling by reductive alkylation. *Methods Enzymol*, **91**, 570-579.

Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **227**, 680-685.