A Receptor for the Selective Uptake and Degradation of Proteins by Lysosomes

Ana Maria Cuervo and J. Fred Dice*

Multiple pathways of protein degradation operate within cells. A selective protein import pathway exists for the uptake and degradation of particular cytosolic proteins by lysosomes. Here, the lysosomal membrane glycoprotein LGP96 was identified as a receptor for the selective import and degradation of proteins within lysosomes. Specific substrates of this proteolytic pathway bound to the cytosolic tail of a 96-kilodalton lysosomal membrane protein in two different binding assays. Overexpression of human LGP96 in Chinese hamster ovary cells increased the activity of the selective lysosomal proteolytic pathway in vivo and in vitro.

Lysosomes are able to take up and degrade intracellular proteins by several mechanisms, including bulk vesicular processes such as macroautophagy and microautophagy and also by a selective molecule-by-molecule pathway (*1*). Certain cytosolic proteins, but not others, are substrates for the selective pathway of lysosomal proteolysis, and this pathway is especially active in cultured cells that are deprived of serum growth factors (*2, 3*) and in liver and other tissues of starved rats (*4, 5*). The selective pathway of lysosomal proteolysis has been reconstituted in vitro with the use of lysosomes from cultured human fibroblasts (*2, 3*) and from rat liver (*4, 5*). Two model proteins that have been used extensively in these assays (*5*) are ribonuclease A (RNase A) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The mechanistic features of this pathway of proteolysis resemble, in many respects, the transport of precursor proteins across other cellular membranes into organelles such as mitochondria or the endoplasmic reticulum (*6*). These similarities include specific binding to protein-containing components of the organelle membrane; kinetic intermediates in the transport of certain proteins; a requirement for the magnesium salt of adenosine triphosphate (Mg-ATP); and a molecular chaperone, the heat shock cognate protein of 73 kD (HSC73), in the cytosol (*6*). In addition, another member of the heat shock protein 70-kD (HSP70) family within the organelle is required for the efficient import of substrate proteins (*6*). Receptors for substrate proteins have been identified on the surface of mitochondria and other organelles (*6*). We now report that a known lysosomal glycoprotein of 96 kD (LGP96; also called LAMP2) is a receptor for the selective uptake of proteins into lysosomes for subsequent degradation.

Department of Physiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA.

*To whom correspondence should be addressed. E-mail: jdice@opal.tufts.edu

Rat liver lysosomal membranes were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and incubated in renaturation buffer containing RNase A (Fig. 1A) or [^{14}C]GAPDH (Fig. 1B). Both substrates bound to a 96-kD protein that was not a prominent stained protein band in the lysosomal membranes. No binding of RNase A occurred with mitochondrial membranes (*7*). The binding of [^{14}C]GAPDH encountered interference from nonradioactive GAPDH or RNase A but not from ovalbumin, a protein known not to be a substrate for the pathway (*5, 7*). The complex between radiolabeled GAPDH and radiolabeled RNase A and a 96-kD lysosomal membrane protein was also evident after molecular size chromatography (*7*). Finally, GAPDH was immobilized on agarose beads, and solubilized lysosomal membrane proteins were passed through this GAPDH affinity column. A 96-kD protein was eluted with low pH, and a 73-kD protein was eluted by a subsequent salt wash (Fig. 1C). The 73-kD protein was immunologically identified as HSC73 (*3, 7*).

The 96-kD band was sequenced after being eluted from the SDS-PAGE gel. The sequence obtained (Fig. 1D) matched exactly the NH_2-terminus of the mature rat lysosomal membrane protein LGP96 (*8*), which is an integral membrane protein whose function is uncertain. It has a predicted molecular weight of 45 kD, based on its cDNA sequence, but it is heavily glycosylated on the lumenal portion of the molecule. The cytosolic tail portion of the protein consists of 12 amino acids. A peptide corresponding to the cytosolic sequence GLKRHHTGYEQF (*8, 9*) inhibited the binding of RNase A to lysosomal membranes, whereas a peptide of the same amino acid composition, but in a randomized sequence, YRQEHLGHKGFT, had no effect (Fig. 2A). Similar results were obtained for the binding of [^{14}C]GAPDH (*7*). Consistently, preincubation of lysosomes with a specific polyclonal antibody to the cytosolic tail of LGP96 prepared in our laboratory also inhibited binding of RNase A (Fig. 2B) and GAPDH (*7*) to lysosomal membranes.

The binding of RNase A and GAPDH to LGP96 was stimulated by HSC73 added to immunoblots (*7*). Previous studies have shown that HSC73 binds RNase A near the KFERQ (*9*) sequence within the NH_2-terminal 20 amino acids, which are referred to as RNase S-peptide (*2, 3*), and that RNase S-peptide also competes for binding of RNase A to lysosomal membranes (*5*). We speculate that the interaction of HSC73 with the S-peptide region of RNase A somehow facilitates the interaction of this same region of RNase A with LGP96.

CHO cells express the selective lysosomal

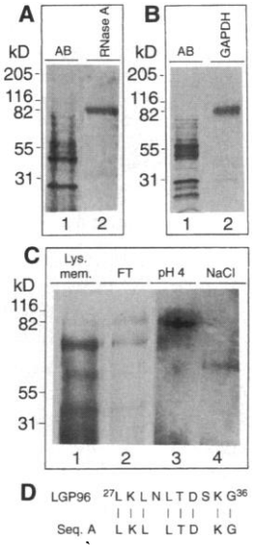


Fig. 1. Binding of substrates of the selective lysosomal degradation pathway to a lysosomal membrane protein of 96 kD. (**A** and **B**) Rat liver lysosomal membranes were immobilized in a nitrocellulose membrane after SDS-PAGE and incubated with RNase A or [^{14}C]GAPDH [lane 2 in (A) and (B), respectively] in a renaturation buffer (*16*). Bound protein was detected with a specific antibody to RNase A (*4, 5*) (A) or by direct exposure to a phosphor screen (B). Nitrocellulose-immobilized lysosomal membranes were stained with amido black (AB) (lane 1). (**C**) Rat liver lysosomal membrane proteins (Lys. mem.) were solubilized (lane 1) (*16*) and incubated with GAPDH immobilized in an Aminolink Plus gel column. Flow through (FT) the column was collected (lane 2), and after extensive washing, GAPDH-bound proteins were eluted with acetate buffer (pH 4.0) (lane 3) followed by elution with 1 M NaCl (lane 4). Solubilized membranes and collected fractions were subjected to SDS-PAGE, and the gel was stained with periodic acid and silver. (**D**) The LGP96 mature NH_2-terminal sequence (*8, 9*) and the sequence of the lysosomal membrane protein that binds GAPDH and RNAse A (Seq. A) were compared. Gaps indicate that the identity of the amino acid at that position was unclear.

pathway of proteolysis, but to a lesser degree than do human lung fibroblasts (*10*). We transfected CHO cells with a cDNA encoding human LGP96 (*11, 12*), and measured the amount of human LGP96 with antibodies to human LGP96 (*13*). The endogenous CHO LGP96 was recognized poorly by the human LGP96 antibody (Fig. 3A). However, using [^{14}C]GAPDH binding assays, we estimated that the CHO cell lines expressed two to three times more LGP96 than did the untransfected controls (*7*). The overexpressed human LGP96 appeared to be localized mainly in lysosomes, as more than 80% of the total LGP96 in homogenates was recovered in the lysosomal fraction (Fig. 3A), and most of it (>75%) was colocalized with cathepsin D (Fig. 3B).

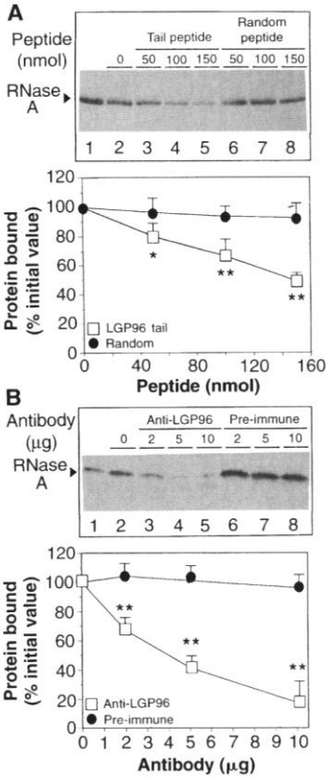
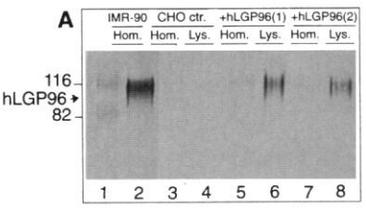


Fig. 2. Role of the LGP96 cytosolic tail in binding of RNase A to lysosomes. (**A**) Rat liver lysosomal membranes (100 μg of protein) were incubated in 10 mM Mops and 0.3 M sucrose (pH 7.3) with RNase A (50 μg) alone (lane 2) or with increasing concentrations (as labeled) of cytosolic tail peptide (lanes 3 to 5) or random peptide (lanes 6 to 8) for 20 min at 37°C. At the end of the incubation, membranes were pelleted, washed with buffer, and subjected to SDS-PAGE and immunoblot with a specific antibody to RNAse A (*4, 5*). (**B**) Rat liver lysosomal membranes (100 μg) were incubated alone (lane 2) or with increasing concentrations (as labeled) of a specific antibody to the cytosolic tail of LGP96 (anti-LGP96) (lanes 3 to 5) or of pre-immune antibody (lanes 6 to 8) for 20 min at 37°C. A second incubation with RNase A (50 μg) for 20 min at 37°C was performed, and samples were processed as in (A). RNase A (1 μg) is shown in lane 1 in (A) and (B). Graphs in (A) and (B) depict the densitometric analyses (mean ± SD) of six different experiments similar to those shown in the figure. Differences compared with control values were significant at $P < 0.01$ (*) and $P < 0.001$ (**).



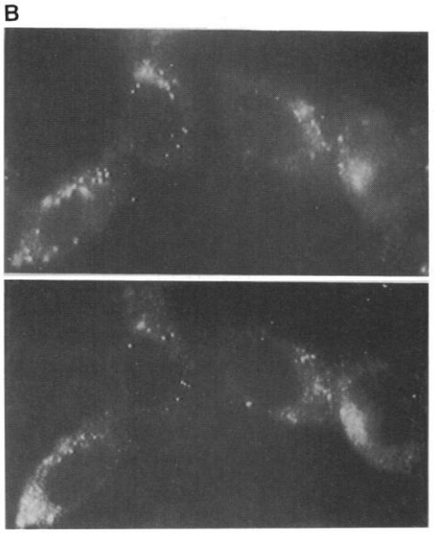
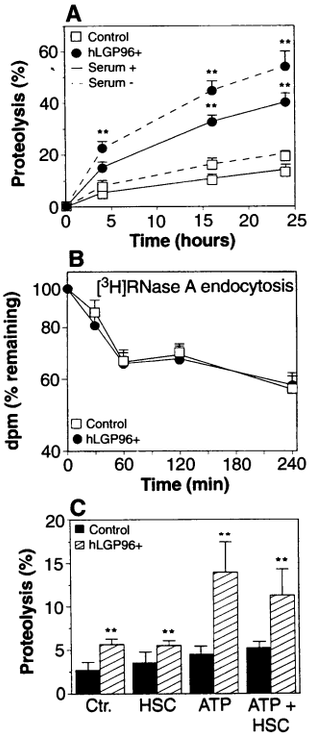


Fig. 3. Expression and distribution of human LGP96 in transfected CHO cells. (**A**) Nontransfected CHO cells (control, ctr.) (lanes 3 and 4) and two different single colonies of cells transfected with cDNA encoding human LGP96 [+hLGP96 (*1*) and +hLGP96 (*2*); lanes 5 to 8] were lysed by nitrogen cavitation, and lysosomes were isolated (*4, 5*). Protein (50 μg) from homogenates (Hom.) or lysosomes (Lys.) were subjected to SDS-PAGE and immunoblot with an antibody to human LGP96. Molecular size markers are shown on the left in kilodaltons. Molecular weight markers (lane 1) and homogenate from human fibroblasts (IMR-90) (lane 2) are for reference. Quantitation of human LGP96 amounts in lysosomes of transfected cells was determined by densitometric analyses of four different immunoblots. (**B**) Colocalization by immunofluorescence of human LGP96 and cathepsin D in CHO cells stably transfected with human LGP96 cDNA. Cells transfected by the standard calcium phosphate–mediated procedure were grown on cover slips and were fixed with 3% formaldehyde and incubated with a specific monoclonal antibody to human LGP96 (*13*) and a polyclonal antibody to cathepsin D (*18*). Secondary antibodies [Texas Red–labeled antibody to mouse and fluorescein isothiocyanate (FITC)–labeled antibody to rabbit (Jackson ImmunoResearch, West Grove, Pennsylvania)] were used, and fluorescence for Texas Red (human LGP96; top) or FITC (cathepsin D; bottom) was analyzed. Quantitation of colocalization was performed in 38 different microscopic fields and was expressed as a percentage of total dots labeled with both antibodies. No fluorescence was visible when secondary antibodies were used alone (*7*). Magnification, ×500.

Fig. 4. Lysosomal function in CHO cells transfected with human LGP96 cDNA. (**A**) Intracellular protein degradation is depicted. Confluent nontransfected CHO cells (control) and human LGP96–transfected cells were labeled with [^{3}H]leucine and [^{14}C]leucine, respectively (*17*), and after extensive washing, cells were replated at a 1:2 ratio of control (□) and transfected cells (●) in the same plate to reach confluence. Proteolysis was measured at the indicated times in cells in complete medium (serum +, solid lines) or cells in serum-deprived medium (serum −, dashed lines) (*17*). Values are mean ± SD of 12 different experiments, and differences between control cells and human LGP96–transfected cells were significant at $P < 0.001$ (**). (**B**) Endocytic activity is depicted. [^{3}H]RNase A was added to the medium of confluent nontransfected (control) or human LGP96–transfected CHO cells (hLGP96+). Aliquots of the medium were taken at the indicated times, and acid-soluble and -precipitable radioactivity was measured. Values are mean ± SD of nine different experiments. No significant differences were found between control and transfected cells. (**C**) Activity of isolated lysosomes is depicted. Freshly isolated lysosomes (*4, 5*) from nontransfected cells (control, solid bars) or human LGP96–transfected CHO cells (hLGP96+, hatched bars) were incubated with [^{14}C]GAPDH (230 nM) in the in vitro degradation system (*4, 5, 17*), without additions (control, indicated by ctr.) or with the indicated additions [bovine brain cytosolic HSC73 (*4, 5*) (HSC), Mg-ATP (ATP), or both (ATP + HSC)]. Proteolysis was measured as described (*17*). Values are mean ± SD of 20 different experiments. Differences between control and human LGP96-transfected cells were significant at $P < 0.001$ (**).



When confluent cells in culture are deprived of serum growth factors, the selective pathway of lysosomal proteolysis is activated (*2, 3*). To determine whether overexpression of human LGP96 affected this proteolytic pathway, we radiolabeled control CHO cells with [^{3}H]leucine and transfected cells with [^{14}C]leucine and then cultured both cell types on the same culture dishes to simultaneously follow degradation of ^{3}H- and ^{14}C-labeled proteins (*14*). Proteolysis was increased in the transfected cells, both in the presence and absence of serum, when compared with the control CHO cells (Fig. 4A). Similar results were obtained when control and transfected cells were analyzed in separate culture dishes (*7*). Cells that expressed half the amount of human LGP96 as the transfectants shown in Fig. 4A also showed half the increased protein degradation rates when compared to those in CHO cells, and cells transfected with plasmid containing no cDNA degraded proteins at rates similar to those of the untransfected control cells (*7*).

Several other aspects of the transfected cells were unaffected by overexpression of human LGP96. For example, the percentage of lysosomes broken during cell homogenization was similar in control and transfected cells (*7*), and [^{3}H]RNase A was taken up by cells and digested within endosomes and lysosomes at normal rates (Fig. 4B). In addition, the cells grew at normal rates and had unaltered rates of protein synthesis (*7*). Finally, lysosomes isolated from human LGP96–overexpressing cells were more active in the in vitro uptake and degradation of [^{14}C]GAPDH under all conditions tested (Fig. 4C).

Thus, overexpression of human LGP96 increased the activity of the selective lysosomal proteolytic pathway, and the level of LGP96 appears to be one rate-limiting component of the degradation machinery. It is not necessarily the only rate-limiting component, however, because overexpression of one protein may cause overexpression of interacting proteins (*15*). Whether LGP96 plays a role in the selective uptake of proteins by lysosomes, in addition to the binding of substrate proteins, remains to be studied.

REFERENCES AND NOTES

1. W. A. Dunn Jr., *Trends Cell Biol.* **4**, 139 (1994); S. A. Hayes and J. F. Dice, *J. Cell Biol.* **132**, 255 (1996).
2. H.-L. Chiang, S. R. Terlecky, C. P. Plant, J. F. Dice, *Science* **246**, 382 (1989); S. R. Terlecky, *Experientia (Basel)* **50**, 1021 (1994).
3. S. R. Terlecky and J. F. Dice, *J. Biol. Chem.* **268**, 23490 (1993).
4. F. Aniento, E. Roche, A. M. Cuervo, E. Knecht, *ibid.*, p. 10463; A. M. Cuervo, E. Knecht, S. R. Terlecky, J. F. Dice, *Am. J. Physiol.* **269**, C1200 (1995).
5. A. M. Cuervo, S. R. Terlecky, J. F. Dice, E. Knecht, *J. Biol. Chem.* **269**, 26374 (1994).
6. G. Schatz and B. Dobberstein, *Science* **271**, 1519 (1996).
7. A. M. Cuervo and J. F. Dice, unpublished results.
8. Y. Noguchu *et al.*, *Biochem. Biophys. Res. Commun.* **164**, 1113 (1989).
9. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
10. Not all cultured cells express this selective lysosomal pathway of proteolysis. For example, skin fibroblasts show less of this activity than do lung fibroblasts, and certain transformed cell lines (such as COS cells) have no detectable activity (L. Terlecky, S. R. Terlecky, J. F. Dice, unpublished results).
11. M. Fukuda, J. Viitala, J. Matteson, S. R. Carlsson, *J. Biol. Chem.* **263**, 18920 (1988).
12. CHO cells were transfected with human LGP96 cDNA in the plasmid designated pCR-3 (Invitrogen, San Diego, CA) and selected for stable transfectants by resistance to Geneticin.
13. Hybridoma H4B4 was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract NO1-HD-6-2915 from the National Institute of Child Health and Human Development.
14. J. S. Auteri, A. Okada, V. Bochaki, J. F. Dice, *J. Cell. Physiol.* **115**, 159 (1983).
15. S. Gratzer *et al.*, *J. Cell Biol.* **129**, 25 (1995).
16. Binding assays were performed with the following procedure. Rat liver lysosomal membranes (100 μg of protein) prepared as described (*4, 5*) were immobilized in a nitrocellulose membrane after SDS-PAGE and incubated with RNase A (382 mM) or [^{14}C]GAPDH (230 nM) in a renaturation buffer [50 mM tris-HCl (pH 7.5), 0.1 M potassium acetate, 0.15 M NaCl, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 1 mM EDTA, and 0.3% Tween 20] for 12 hours at 4°C. Bound protein was detected by ECL immunoblotting (Amersham International, Buckinghamshire, UK) with a specific antibody to RNase A (*4, 5*) or by direct exposure to a phosphor screen (Molecular Dynamics, Sunnyvale, CA). In other studies, rat liver lysosomal membrane proteins (2 mg) were solubilized in 20 mM tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100 and then centrifuged at 100,000*g* for 30 min. The supernatant was incubated with GAPDH immobilized in a 2-ml Aminolink Plus gel column (Pierce, Rockford, IL) for 2 hours at 25°C. Flow through the column was collected, and after extensive washing, GAPDH-bound proteins were eluted with 10 ml of acetate buffer (pH 4.0) [25 mM sodium acetate (pH 4.0), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.3% Tween 20] followed by 10 ml of 1 M NaCl in 0.1 M sodium phosphate buffer (pH 7.2).
17. Intracellular protein degradation in cultured fibroblasts was measured as described previously (*14*). Briefly, confluent cells were labeled with [^{3}H]leucine or [^{14}C]leucine for 20 hours at 37°C. After extensive washing, cells were trypsinized and replated in the presence of complete medium or serum-deprived medium. Aliquots of the medium were taken at the indicated times, and acid-soluble and -precipitable radioactivity was measured. Proteolysis was expressed as the percentage of precipitable radioactivity transformed to soluble radioactivity for each time. Proteolytic activity of isolated fibroblast lysosomes was measured by incubation of freshly isolated lysosomes (25 μg of protein) for 2 hours at 25°C with [^{14}C]GAPDH (230 nM) in the in vitro degradation system (*4, 5*). In some assays, bovine brain cytosolic HSC73 (5 μg/ml) or 5 mM ATP and 5 mM $MgCl_2$ or both were added. Proteolysis was expressed as the percentage of the initial acid-precipitable radioactivity converted to acid-soluble radioactivity.

18. We thank M. Berne for the peptide sequencing. The polyclonal antibody to cathepsin D was a gift of G. Sahagian, Department of Physiology, Tufts University, Boston, MA. This research was funded by a Fundacion Ramon Areces (Spain) and American Liver Foundation postdoctoral fellowships and by NIH grant AG06116.

17 April 1996; accepted 7 June 1996

Lymphocyte Apoptosis: Mediation by Increased Type 3 Inositol 1,4,5-Trisphosphate Receptor

Adil A. Khan, Mark J. Soloski, Alan H. Sharp, Gabriele Schilling, David M. Sabatini, Shi-Hua Li, Christopher A. Ross, Solomon H. Snyder*

B and T lymphocytes undergoing apoptosis in response to anti–immunoglobulin M antibodies and dexamethasone, respectively, were found to have increased amounts of messenger RNA for the inositol 1,4,5-trisphosphate receptor (IP_3R) and increased amounts of IP_3R protein. Immunohistochemical analysis revealed that the augmented receptor population was localized to the plasma membrane. Type 3 IP_3R (IP_3R3) was selectively increased during apoptosis, with no enhancement of type 1 IP_3R (IP_3R1). Expression of IP_3R3 antisense constructs in S49 T cells blocked dexamethasone-induced apoptosis, whereas IP_3R3 sense, IP_3R1 sense, or IP_3R1 antisense control constructs did not block cell death. Thus, the increases in IP_3R3 may be causally related to apoptosis.

Calcium entry into cells appears to be a critical early event in apoptosis (programmed cell death) (*1*). In thymocytes, apoptosis elicited by glucocorticoids is associated with a sustained increase in cytosolic calcium concentration, and depletion of calcium with chelating agents blocks apoptosis (*2, 3*). The endonuclease that causes characteristic apoptotic cleavage of chromatin is calcium-dependent (*2, 4*). Calcium entry also participates in cellular proliferation, especially in lymphocytes undergoing immune stimulation (*5*). The channels and mechanisms that account for the cellular

A. A. Khan and D. M. Sabatini, Department of Neuroscience; M. J. Soloski, Department of Medicine; A. H. Sharp, G. Schilling, and S.-H. Li, Department of Psychiatry; C. A. Ross, Departments of Neuroscience and Psychiatry; and S. H. Snyder, Departments of Neuroscience, Psychiatry, and Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

*To whom correspondence should be addressed.