INTRODUCTION

Lysosome-associated membrane proteins (lamps) are a group of lysosomal proteins with very similar structural characteristics but of mostly unknown function (Fukuda, 1991; Peters and von Figura, 1994). All of the lamps are type I integral glycosylated proteins with a large lumenal domain, a single transmembrane region of about 20 amino acids, and a short (10-12 amino acid) carboxyl terminus tail at the cytosolic side of the lysosomal membrane (Akasaki and Tsuji, 1998). Lamps are mainly localized to lysosomes but can also be detected in lower amounts in endosomes and at the plasma membrane (Furuno et al., 1989; Akasaki et al., 1993). Two different classes of lamps, lamp1 and lamp2, encoded by two different but evolutionarily-related genes have been described. The lamp2 gene undergoes alternative splicing resulting in at least three different mRNAs encoding different isoforms of lamp2 (Gough et al., 1995; Hatem et al., 1995; Konecki et al., 1995). The three lamp2 isoforms (a, b and c) identified so far show high amino acid sequence identity in their lumenal region, but different transmembrane and cytosolic regions (Gough et al., 1995). The lamp2 splice variants are expressed at different levels in different tissues (Konecki et al., 1995; Furuta et al., 1999) and have different distributions between the plasma membrane and lysosomes (Gough and Fambrough, 1997). Lamp2 is concentrated in tissues undergoing apoptosis during development, and the expression pattern for each lamp2 isoform becomes more tissue and cell-type specific as differentiation progresses (Licheter-Konecki et al., 1999).

Lamp2s are present in the lysosomal lumen as well as the lysosomal membrane (Jadot et al., 1996; Jadot et al., 1997). Part of this matrix form of lamp2 reversibly aggregates with other lysosomal enzymes in a pH-dependent manner (Jadot et al., 1997). The origin of the luminal form of lamp2a is still unclear, but a direct deinsertion from the lysosomal membrane after a conformational change, as well as a release by proteolytic cleavage of the short transmembrane and cytosolic tail have been proposed (Jadot et al., 1996). That kind of deinsertion has been described for other type I membrane proteins (Nishiyama et al., 1999), and cleavage has been described for other lysosomal membrane proteins such as acid phosphatase (Gottschalk et al., 1989) and lamp1 (Meikle et al., 1999). The high level of glycosylation of lamp2, in which the protein core accounts for only 40 kDa of the final glycosylated product of 96 kDa, and the small size of the transmembrane and cytosolic tail, make it difficult to identify the matrix forms of lamp2 as intact or truncated using conventional electrophoretic methods.

The function of most lamps remains unclear. They have been hypothesized to play a role in protecting the lysosomal membrane from its associated hydrolases (Fukuda, 1991). However, it has been shown recently that the complete elimination of lamp1 that constitutes almost 40% of the lysosomal membrane protein does not modify lysosomal stability (Andrejewski et al., 1999). A role for lamp2 in cell-cell or cell-extracellular matrix adhesion has been proposed (Lippincott-Schwartz and Fambrough, 1986; Carlsson et al., 1988; Saitoh et al., 1992; Licheter-Konecki et al., 1999). A role for lamp2 in maturation of autophagic vacuoles has also been proposed (Tanaka et al., 2000). By analogy with other alternatively spliced proteins (Ravetch and Perussia, 1989), the tissue-dependent expression of the different forms of lamp2...
(Konecki et al., 1995) suggests that they might have different cellular functions.

We identified the lamp type 2a (lamp2a) at the lysosomal membrane as a receptor for substrates of chaperone-mediated autophagy (Cuervo and Dice, 1996). In this pathway, specific cytosolic proteins are directly transported through the lysosomal membrane into the lysosomal matrix where they are degraded (Cuervo and Dice, 1998; Dice, 2000). Substrate proteins bind first to a constitutively expressed heat shock protein of 73 kDa (hsc73) in the cytosol that targets them to lysosomes (Chiang et al., 1989). A second chaperone located in the lumen of the lysosomes, the lysosomal hsc73, is required for the complete transport of substrate proteins into lysosomes (Agarraberes et al., 1997; Cuervo et al., 1997). We demonstrated that substrate proteins bind to the cytosolic tail of lamp2a at the lysosomal membrane before their transport and degradation in the lysosomal matrix (Cuervo and Dice, 1996). Inhibition of that binding with specific antibodies against the cytosolic tail of lamp2a or by competition with a synthetic peptide of the same amino acid sequence as the tail, results in blocking of substrate uptake and degradation in lysosomes (Cuervo and Dice, 1996). Interestingly, the overexpression of only lamp2a in cultured cells increased the rates of chaperone-mediated autophagy, suggesting that binding of the substrates to lamp2a at the lysosomal membrane might be a rate-limiting step in this pathway (Cuervo and Dice, 1996).

In the present study, using an antibody specific for lamp2a and another that recognizes all lamp2s we compare concentrations, biochemical properties, subcellular location, and lysosomal distribution of lamp2s in rat liver and fibroblasts in culture, the two systems in which chaperone-mediated autophagy has been well-characterized. We demonstrate that binding of substrate proteins to lamp2a is a rate-limiting step in chaperone-mediated autophagy under a variety of physiological and pathological conditions, while the other lysosomal forms of lamp2 or do not affect chaperone-mediated autophagy. Finally, we identify unique properties of lamp2a, such as the presence of a group of positive residues in its cytosolic tail essential for substrate binding/uptake.

MATERIALS AND METHODS

Animals and cells

Adult male Wistar rats weighing 200-250 g and fasted for 20 hours before sacrifice were used. Where indicated fasting was extended for longer times with free access to water. An age-controlled rat strain (Fischer 344) was used for the study of age-related changes, and 3- and 22-month old rats were compared. For some studies, 2,2,4-trimethylpentane, dissolved 1:1 in corn oil, was administered to the animals by gavage (1 g/kg of body weight) during 7 consecutive days. Human lung fibroblasts (IMR-90) were from the Coriell Cell Repositories (Camden, NJ), and Chinese Hamster ovary cells (CHO), human embryo kidney cells (HEK293), and rat lung fibroblasts (RFL-6) were from the American Type Culture Collection (Manassas, VA). Mouse skin fibroblasts were generously provided by Dr Alessandra D’Azzo (St Jude Children’s Research Hospital, Memphis, TN). Cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma, St Louis, MO) in the presence of 10% newborn calf serum (NCS), except for CHO cells that were grown in F-12 medium (Life Technologies, Gaithersburg, MD) with the same amount of serum. To deprive cells of serum, plates were extensively washed with Hanks’ balanced salts solution (Life Technologies, Gaithersburg, MD) and medium without serum was added.

Chemicals

Sources of chemicals and antibodies were as described previously (Terlecky and Dice, 1993; Cuervo et al., 1994; Cuervo et al., 1995; Cuervo and Dice, 1996). The antibody against the cytosolic tail of rat lamp2 was raised in our laboratory (Cuervo and Dice, 1996). The monoclonal antibodies against the luminal side of rat lamp2a, rat lamp1, human influenza hemaglutinin protein (HA) and cathepsin A were gifts from Dr Michael Jadot (Facultes Universitaires Notre-Dame de la Paix, Namur, Belgium), Dr Ira Mellman (Yale University School of Medicine, New Haven, CT), Dr Anjana Rao (Department of Pathology, Harvard Medical School, Boston, MA), and Dr Alessandra D’Azzo, respectively. The monoclonal antibodies against the matrix region of human, mouse, and hamster lamp2s, and human lamp1 were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Aminolink® and Sulfolink® gels and crosslinking agents were from Pierce (Rockford, IL).

Isolation of subcellular fractions

Rat liver lysosomes were isolated from a light mitochondrial-lysosomal fraction in a discontinuous metrizamide density gradient (Wattiaux et al., 1978) by the shorter method reported previously (Aniento et al., 1993). After isolation lysosomes were resuspended in MOPS buffer (0.3 M sucrose/10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.2). In some experiments, two separate lysosomal fractions with different levels of chaperone-mediated autophagy were isolated as described (Cuervo et al., 1997). Contamination of the lysosomal fraction with mitochondria (based on the activity of ornithine transcarbamoylase and succinate dehydrogenase) or cytosol (based on the activity of lactate dehydrogenase and GAPDH) could account for less than 0.5% of the lysosomal fraction. Lysosomes from cultured cells were isolated as described (Storrie and Madden, 1990). Integrity of the lysosomal membrane after isolation was measured by β-hexosaminidase latency as previously described (Terlecky and Dice, 1993). Only preparations with more than 95% intact lysosomes were used. Lysosomal matrices and membranes were obtained as described by Oshumi (Ohsumi et al., 1983).

Purification of lamp2a from rat liver lysosomes

Lysosomal membranes were solubilized in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS for 2 hours at 0°C. Lysates were cleared by centrifugation at 100,000 g for 30 minutes, and solubilized membrane proteins were recovered in the supernatant. Lysosomal membrane and matrix proteins were separately loaded in a column containing the antibody against the cytosolic tail of lamp2a immobilized in Aminolink® Plus gel previously equilibrated in lysis buffer. After extensive washing immobilized proteins were eluted with 100 mM glycine, pH 2.3, and collected in neutralizing Trizma base.

Uptake and degradation of substrate proteins by isolated rat liver lysosomes

Substrate proteins were incubated with chymostatin-treated lysosomes as previously described (Aniento et al., 1993; Cuervo et al., 1994). Transport was measured after proteinase K treatment of the samples, SDS-PAGE and immunoblot, as the amount of substrate resistant to the protease. Degradation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by isolated intact lysosomes was measured as described (Terlecky and Dice, 1993). Lysosomes (25 μg protein) were incubated in 10 mM MOPS, pH 7.2, 300 mM sucrose, 1 mM dithiothreitol, 5.4 μM cytochrome c for 30 minutes at 37°C with 260 nM GAPDH radioactively labeled with [14C] by reductive methylation (Jentoff and Dearborn, 1983). Reactions were stopped by the addition
of trichloroacetic acid to a final concentration of 10%. Acid-soluble material (amino acids and small peptides) was collected by filtration through a Millipore Multiscreen Assay System (Millipore, Bedford, MA) using a 0.45 μm pore filter, and the acid-precipitable material (protein and larger peptides) was collected on the filter. Radioactivity in the samples was converted to disintegrations per minute in a P2100TR Packard liquid scintillation analyzer by correcting for quenching using an external standard (Packard Instruments, Meriden, CT). Proteolysis was expressed as a percentage of the initial acid-insoluble radioactivity converted to acid-soluble radioactivity at the end of the incubation.

**Expression of lamp2a and HA-lamp2a**

The cDNAs for native (Carlsson et al., 1988; Fukuda, 1991) and mutated human lamp2a were subcloned in the pCR3 mammalian expression vector (Invitrogen, San Diego, CA), and CHO cells were transfected with these constructs by the calcium phosphate method (Maniatis et al., 1982). After Geneticin (Life Technologies) selection 5–10 different clones for each construct were isolated and assayed for their protein expression levels by immunoblot. Clones expressing similar levels of lamp2a were used for the protein degradation assays. For purification purposes three consecutive HA epitope-tags were inserted in the luminal hinge region of the cDNAs for native and mutated human lamp2a. Human embryo kidney cells were transfected with those constructs by the same method as above. After 48 hours cells were harvested and solubilized in 1% octyl-glucoside in 50 mM Tris-HCl, pH 8, 150 mM NaCl. Point mutations were performed with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturers instructions and were verified by DNA sequencing.

**Binding of proteins to lamp2a**

Specific binding of cytosolic proteins to lamp2a was analyzed as described (Cuervo and Dice, 1996). Briefly, lysosomal membranes were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. After blockage with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.2, 150 mM NaCl and 0.1% Tween-20, membranes were incubated with 14C-GAPDH (250 nM) in a renaturation buffer (50 mM Tris-HCl, 100 mM potassium acetate, 150 mM NaCl, 1 mM dithiothreitol, 5 mM MgCl2, 1 mM EDTA, and 0.3% Tween-20) for 12 hours at 4°C. Bound protein was detected after extensive washing by exposure to a PhosphorImager screen. For the binding of GAPDH to wild type and mutant recombinant HA-lamp2as the solubilized fraction from transiently transfected HEK293 cells was passed through an anti-HA Sulfolink® matrix and extensively washed with 50 volumes of 50 mM Tris-HCl, pH 8, 500 mM NaCl and 0.2% octyl-glucoside. The beads were resuspended in 10 volumes of 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.2% octyl-glucoside. For each binding assay 15 μl of the resuspended beads were incubated with rat liver cytosol (30 μg protein) in 200 μl of the above buffer for 45 minutes at 37°C. After two washes with 10 volumes of buffer, the beads were collected by centrifugation and subjected to SDS-PAGE and immunoblot for GAPDH. The amount of GAPDH bound to beads containing the anti-HA antibody but without lamp2a was subtracted from each experimental value. GAPDH binding was normalized to the amount of HA-lamp2a in each sample.

**Confocal microscopy**

Immunofluorescence staining of cultured cells was performed following conventional procedures. Cells were grown on coverslips until confluent and kept in the presence or absence of serum for 20 hours before fixing with a 3% formaldehyde solution. Coverslips were blocked and incubated with the primary and corresponding fluorescein isothiocyanate (FITC)- or Texas Red (TR)-conjugated secondary antibodies. Images were acquired as single scans on a Bio-Rad MRC-1024 confocal microscope (Bio-Rad Laboratories, Hercules, California, USA) equipped with an argon laser. Images were further analyzed and colocalization determined using MetaMorph (Universal Imaging). All digital microscopic images were prepared using Adobe Photoshop 5.0 software (Adobe Systems Inc., Mountain View, CA).

**Centrifugation in sucrose gradients**

Rat liver lysosomes were incubated with 1 mM dithiobis(succinimidylpropionate), a membrane-permeable reversible crosslinker, in MOPS buffer for 30 minutes at 25°C. The reaction was quenched with 50 mM Tris-HCl, pH 7.5, and lysosomes were recovered by centrifugation and separated into membrane and matrices as described above. Lysosomal membranes were solubilized in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% octyl-glucoside. Solubilized lysosomal membranes were loaded on the top of a continuous gradient of sucrose (20% to 60%) (5 ml total) in solubilization buffer. Samples were centrifuged at 140,000 g for 24 hours and aliquots of 200 μl were separately collected from the top to the bottom of the gradient. Thirty μl of each aliquot were used for SDS-PAGE and immunoblot with antibodies. A parallel sucrose gradient with molecular mass markers (Sigma) was included in each experiment.

**General methods**

Protein was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. Standard procedures were used for the determination of enzymatic activities as reported previously (Terlecky and Dice, 1993; Cuervo et al., 1994). CHO cells were stably transfected with the human lamp2a cDNA and mutant lamp2a cDNAs as described previously (Cuervo and Dice, 1996). Intracellular protein degradation in control and transfected CHO cells was measured as described (Auteri et al., 1983). Hsc73 was purified from rat liver cytosol by ATP-agarose affinity chromatography (Welch and Feramisco, 1985). Cellular fractions and isolated proteins were subjected to SDS-PAGE in slab gels (Laemmli, 1970). Gels were electrotransferred to nitrocellulose membranes using a Mini-Trans Blot SD wet transfer cell (Bio-Rad, Richmond, VA), and immunoblotting was performed following standard procedures (Towbin et al., 1979). Membranes were developed by chemiluminescence methods (Renaissance®, NEN-Life Science Products, Boston, MA). Densitometric quantification of the immunoblotted membranes was performed in Kodak, Scientific Imagin Film, using an Image Analyzer System (Inotech S-100, Sunnyvale, CA). Statistical analyses were carried out using the Student’s t-test.

**RESULTS**

**Characterization of the membrane and matrix forms of lamp2 in rat liver lysosomes**

The presence in rat liver of three different mRNA transcripts for lamp2 suggest that several forms of lamp2 proteins might coexist in that tissue (Konecki et al., 1995). We have previously developed an antibody against the twelve amino acids of the cytosolic region of rat lamp2a (Cuervo and Dice, 1996). This antibody does not recognize the cytosolic tail of the human lamp2a that only differs from the rat in two out of twelve amino acids (GLKHHHAGYEQF (human) vs GLKRHHHTGYEQF (rat)). The cytosolic regions of the other two predicted splice variants of lamp2 in rodent, lamp2b and lamp2c, differ from the a isoform in 8 and 9 out of 12 amino acids, respectively (Fig. 1A, shadowed residues). These large differences between the cytosolic regions of the predicted spliced variants of rodent lamp2 make it very unlikely that the antibody to the cytosolic tail of lamp2a also recognizes any of the other two variants.

To verify the specificity of the antibody, as well as to
determine the contribution of lamp2a to the total content of lamp2s in rat liver lysosomes, we subjected lysosomal membranes and matrices to affinity chromatography through a matrix containing the antibody raised against the cytosolic tail of lamp2a. We then analyzed the retained fraction and the flow through with the same antibody (Fig. 1B, left), and with an antibody raised against the luminal region common for all lamp2s (Fig. 1B, right). Fig. 1B, right, shows the presence of lamp2 isoforms that did not bind the antibody (lanes 3 and 4), and Fig. 1B, left, shows that the antibody against the cytosolic tail of lamp2a did not recognize these isoforms (lanes 3 and 4). We found similar electrophoretic mobility for the lamp2 in the retained and unretained membrane fractions after completely removing the carbohydrate groups with different glycosidases (data not shown). These results suggest that lamp2 not retained by the antibody against the lamp2a cytosolic tail corresponded to a complete form of the protein with a different cytosolic region and support the specificity of our antibody for lamp2a.

We were able to isolate lamp2a from both the membrane and, in lower amount, the matrix of lysosomes (Fig. 1B, left, lanes 1 and 2). The quantification of the purification of lamp2a by affinity chromatography revealed that lamp2a accounts for approximately 25% of the total lamp2s at the lysosomal membrane, and 10% of the total lamp2s in the lysosomal matrix. In both fractions some of the lamp2a was not recognized by the cytosolic tail antibody so did not contain the cytosolic region (truncated form) or contained a different one not recognized by the antibody (Fig. 1B, right, lanes 3-4). Other authors have suggested that the lamp2s detected in the lysosomal matrix might be truncated forms lacking the cytosolic and transmembrane regions (Jadot et al., 1996; Akasaki and Tsuji, 1998). However, at least for lamp2a, most of the protein detected in the matrix still contains the cytosolic tail. In addition, we found similar electrophoretic mobility for the membrane and matrix lamp2a after complete deglycosylation (data not shown). These results suggest that most of the lamp2a in the matrix corresponds to the intact protein.

For the purification studies of lamp2a (Fig. 1B) we started with the same amount of protein from lysosomal membranes and matrices (300 μg), but the matrix normally contributes 70% of the total lysosomal protein. When we analyzed the distribution of lamp2a in isolated liver lysosomes we found that 70-80% of lamp2a was located in the lysosomal membrane and 30-20% in the matrix (Fig. 1C, lanes 1 and 2). In contrast, the distribution of all the lamp2s considered together was 55% in membrane and 45% in matrix (Fig. 1C, lanes 3 and 4). The presence of another lysosomal membrane protein, lamp1, only in the lysosomal membrane fraction (Fig. 1C, lanes 5 and 6), and the predominantly matrix protease, cathepsin A, only in the matrix (Fig. 1C, lanes 7 and 8) demonstrate the purity of our membrane and matrix preparations.

Changes in levels of lamp2a at the lysosomal membrane correlate with changes in the rate of chaperone-mediated autophagy

We have previous evidence suggesting that lamp2a is the form of lamp2 contributing most to substrate binding. Thus, using a synthetic peptide with identical amino acid sequence as the cytosolic tail of lamp2a we blocked almost 80% of the binding of the substrates to lysosomes (Cuervo and Dice, 1996). In addition, overexpression of human lamp2a in CHO cells by itself increased the activity of chaperone-mediated autophagy (Cuervo and Dice, 1996).

We have used different approaches to analyze the effect that changes in the lysosomal content and distribution of lamp2a have on the activity of the chaperone-mediated autophagic pathway. Working with individual clones of CHO cells stably transfected with human lamp2a we found a very high correlation between the levels of human lamp2a expressed in each clone and rates of chaperone-mediated autophagy in those clones (Fig. 2A). In the clones overexpressing lamp2a the rates of proteolysis in the presence of serum were only slightly higher than in the control CHO cells. However, the increase in protein degradation after serum removal was significantly higher in clones 2 and 3 of the lamp2a-expressing cells. Fig. 2A shows the increase in the rate of proteolysis induced by serum deprivation for each clone corrected for their corresponding values in the presence of serum. The response to serum removal proportionally increased with the increase in the lysosomal content of human lamp2a. These results suggest that levels of lamp2a at the lysosomal membrane can be a rate-limiting step in the transport and degradation of substrate proteins in lysosomes.

To determine if the levels of lamp2a at the lysosomal membrane also correlate with the activity of the chaperone-mediated autophagic pathway under physiological conditions we isolated lysosomes from rats subjected to different periods
Lamp2s and chaperone-mediated autophagy of starvation. The lysosomal membrane levels of lamp2a increased with the starvation time through the entire starvation period analyzed (88 hours) (Fig. 2B, top), as did also the rate of chaperone-mediated autophagy (Cuervo et al., 1995). We found an increase in the lysosomal levels of lamp1 during the first 20 hours of starvation, but after that time lamp1 levels remained steady (Fig. 2B, bottom). Levels of lamp2a in the lysosomal matrix did not significantly change until the most prolonged periods of starvation, when they decreased (data not shown). We also found an increase in the lysosomal membrane content of lamp2a in rat fibroblasts in culture in response to serum deprivation, but no changes in the lysosomal membrane content of lamp1 (Fig. 2C, lanes 3-4). Levels of lamp2a in the lysosomal matrix remained unchanged after serum removal (Fig. 2B, lanes 5-6). Thus, rather than total lysosomal levels of lamp2a, levels of the protein at the lysosomal membrane correlate with the activity of the lysosomal pathway.

We then analyzed the lamp2a distribution in lysosomal populations with different rates of chaperone-mediated autophagy. Rat liver lysosomes can be separated based on density into two groups with a different content of hsc73 in their matrices and different activity for the direct transport of substrate proteins (Cuervo et al., 1997). As shown in Fig. 2D top (lanes 1 and 2) total lysosomal levels of lamp2a are only slightly higher in the more active population compared to the less active group. After separating lysosomal membranes and matrices we found that 78% of the lamp2a was present at the membrane and 22% at the matrix in the active population while the lamp2a distribution was 57% membrane and 43% matrix in the less active population (Fig. 2D top, lanes 3-6). These results support the idea that changes in the distribution of lysosomal lamp2a between the membrane and the matrix could contribute to the regulation of chaperone-mediated autophagy (Cuervo and Dice, 2000a). For unknown reasons lamp1 was enriched in the membrane of the less active group of lysosomes (Fig. 2D, bottom).

Other conditions in which we have recently identified increased chaperone-mediated autophagy in liver and kidney include a chemically-induced nephropathy (Cuervo et al., 1999). Chaperone-mediated autophagy is activated after exposure to gasoline additives and results in increased uptake of one substrate, alpha-2-microglobulin. In these studies we found higher levels of lamp2a in the membranes of lysosomes isolated from intoxicated animals than from the controls. We found no significant differences in matrix content of lamp2a or lysosomal levels of hsc73 (Cuervo et al., 1999).

A decrease in levels of lamp2a at the lysosomal membrane also occurs when the activity of the pathway decreases. Thus, the activity of the chaperone-mediated autophagic pathway
decreases in senescent fibroblasts in culture (Dice, 1993), and also in lysosomes isolated from livers of old rats (Cuervo and Dice, 2000b). Levels of lamp2a at the lysosomal membrane are significantly lower in old rats when compared with young rats. The content of lamp2a in the lysosomal matrix does not change or only decreases slightly with age.

The linear correlation between the lysosomal membrane levels of lamp2a and the rates of chaperone-mediated autophagy under the different conditions described in this section is shown in Fig. 3A, top. In contrast, we did not find a significant correlation between lysosomal membrane levels of lamp1 (Fig. 3B) or matrix levels of lamp2a (data not shown) and rates of chaperone-mediated autophagy.

**Contribution of the different forms of lamp2 to the binding of substrates for chaperone-mediated autophagy**

We also measured the lysosomal membrane levels of all forms of lamp2 considered together in the different conditions described for lamp2a. We did not find a significant correlation between the levels of all lamp2s at the lysosomal membrane and chaperone-mediated autophagy activity (Fig. 4A). This lack of correlation suggests that if other forms of lamp2 are

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**Fig. 3.** Correlation between lysosomal membrane levels of lamp2a and activity of chaperone-mediated autophagy. Lysosomal membranes from rat liver or cultured rat fibroblasts were prepared as described in Materials and Methods. Levels of lamp2a (A) and lamp1 (B) in those fractions were determined by densitometric analysis after immunoblotting. Lysosomal activity was measured as the ability of isolated lysosomes to degrade a radiolabeled substrate protein, 14C-GAPDH. The lysosomal activity for each condition analyzed is expressed relative to the activity of lysosomes isolated from fed rats or from cells maintained in the presence of serum that were given the arbitrary value of 1. Values are means ± s.e.m. of 5 to 10 different experiments. Lysosomes were isolated from: fed (FR) or starved rats (SR), 3-months old (YR) or 22-months old rats (OR), 2,2,4-trimethylpentane-treated rats to induce α2m globulin nephropathy (α2mGN), fibroblasts maintained in the presence (S+) or absence (S−) of serum, and CHO cells stably transfected with human lamp2a and expressing different levels (+, ++ lamp2a). Two groups of lysosomes with high (HSC+) and low (HSC−) activity for chaperone-mediated autophagy were isolated from rats starved for 20 hours. Best straight lines were calculated by linear regression.

**Fig. 4.** Lamp2 isoforms other than lamp2a and chaperone-mediated autophagy. (A) Lysosomal membranes from rat liver or cultured fibroblasts as described in Fig. 3 were subjected to SDS-PAGE and immunoblotted for all lamp2s. Total lamp2s values and chaperone-mediated activity were calculated as described in Fig. 3. Values are means ± s.e.m. of 5 to 10 different experiments for each of the conditions analyzed. (B) Rat liver lysosomal membranes (L.MB) were immunoprecipitated with the specific antibody against lamp2a. Lysosomal membranes, precipitates (IP) and supernatants (Post-IP) were subjected to SDS-PAGE and immunoblotted for lamp2a (top), all lamp2s (middle) or assayed for 14C-GAPDH binding (bottom) as described in Materials and Methods. Bound protein was detected using a phosphorImager screen.
involved in chaperone-mediated autophagy they are at least not rate-limiting components in that process.

To directly analyze the ability of the other forms of lamp2 to bind substrates we immunoprecipitated lamp2a from solubilized lysosomal membranes and then measured the binding of 14C-GAPDH to the remaining lamp2s. GAPDH only bound to the fractions containing lamp2a but not to fractions containing the remaining forms of lamp2s (Fig. 4B, lanes 1-3). These results confirm our previous hypothesis that lamp2a is the only isoform of lamp2 involved in binding of substrate proteins for chaperone-mediated autophagy.

Amino acid residues of lamp2a required for substrate binding

We have previously demonstrated that substrate proteins directly bind to the cytosolic region of lamp2a (Cuervo and Dice, 1996). To analyze which of the 12 amino acid residues of the cytosolic tail of lamp2a, if not all, were involved in substrate binding we performed site-directed mutagenesis of that area as shown in the diagram in Fig. 5A. We did not modify the GY dipeptide because it is conserved in all forms of lamp2s and it is required for targeting of lamp2a to lysosomes (Williams and Fukuda, 1990). The last hydrophobic amino acid (F) is also important for lamp2a targeting (Guarnieri et al., 1993), but since it is not conserved in the other forms of lamp2a we mutated it and analyzed its binding properties in vitro. Except for that mutation all the other lamp2a mutants were properly targeted to lysosomes (data not shown).

As we have previously shown for wild type lamp2a, cells overexpressing lamp2a/EQ or lamp2a/A mutants had higher rates of protein degradation after serum removal than control CHO cells (Fig. 5B). However, after replacement of the 4 positive charges in the cytosolic tail of lamp2a by alanines the rates of protein degradation during serum deprivation were indistinguishable from control cells (Fig. 5B) indicating that the lamp2a/4A mutation was not an active receptor.

Isolated lysosomes containing the lamp2a lacking the four positive residues showed lower ability for GAPDH binding, uptake, and degradation than the ones containing native or the other mutated forms of lamp2a (Fig. 5C). Using two different substrate binding assays to immobilized lysosomal membranes (data not shown) and to purified recombinant HA-lamp2a (Fig. 5D), we demonstrated that the decreased GAPDH degradation was a consequence of lower binding of substrate proteins to the lamp2a lacking the four positive residues. In this binding assay we found that elimination of a single positive residue or changes in residues distal to the GY dipeptide, including the hydrophobic terminal residue, did not modify lamp2a substrate binding ability (Fig. 5D).

Unique characteristics of lamp2a compared to other lamp2s

Besides the above described differences in lysosomal content and distribution between lysosomal membrane and matrix (Fig. 1), the membrane-associated form of lamp2a also differed from other forms of lamp2 in its sedimentation rates after cross-linking. We found that after centrifugation of detergent-solubilized lysosomal membranes through a continuous 20%-60% sucrose gradient lamp2a could be detected in four different regions of the gradient corresponding approximately to 100, 200, 400 kDa and 800 kDa (Fig. 6A). After reversing the crosslinking and analyzing the different fractions by SDS-PAGE and silver staining, we found that lamp2a was the only protein component of the 400 kDa complex (data not shown).
These results suggest that, though lamp2a could also associate with other proteins, at least part of the lamp2a at the lysosomal membrane is in the form of homomultimers. The other lamp2 forms were mainly located in the 100 to 200 kDa region (Fig. 6B). Therefore, the ability to form high molecular mass complexes at the lysosomal membrane seems more of a characteristic of lamp2a than the other isoforms.

When we analyzed the intracellular distribution of lamp2a compared to other isoforms of lamp2 (Fig. 7A, top) or to lamp1 (Fig. 7B, top) in mouse skin fibroblasts cultured under normal conditions we did not find significant differences. The three antibodies displayed a typical vesicular pattern distributed throughout the cytoplasm. Chaperone-mediated autophagy is activated in these cells after serum removal (Dice et al., 1986). Under those conditions lamp2a was preferentially found in lysosomes surrounding the nucleus (Fig. 7A and B.). A similar pattern of distribution has been previously observed for the lysosomes more active for chaperone-mediated autophagy based on their hsc73 content (F. Agarraberes and J. F. Dice, unpublished results). We did not find differences in the distribution of the other forms of lamp2 in the presence or absence of serum (Fig. 7A). The right panel shows the merged image of the staining with both antibodies (notice that the antibody against lamp2s recognizes lamp2a but with low affinity (Fig. 1A right, lane 1). Though to a lesser extent than for lamp2a, the staining for lamp1 also increased in the perinuclear region after removal of serum (Fig. 7B, bottom).

**DISCUSSION**

Chaperone-mediated autophagy is responsible for the selective degradation of cytosolic proteins in lysosomes during stress conditions (Cuervo and Dice, 1998; Dice, 2000). Here we show that the activity of chaperone-mediated autophagy is modulated under different physiological and pathological conditions by changes in the levels of lamp2a at the lysosomal membrane. Binding of substrate proteins to lamp2a is a rate-limiting step for their degradation (Fig. 2A), and there is a direct correlation between the levels of lamp2a at the lysosomal membrane and the rate of chaperone-mediated autophagy (Figs 2 and 3). Interestingly, in spite of the high similarity between different lamp2s, other isoforms of lamp2 do not participate in substrate binding (Fig. 4). The presence of a group of positive residues in the cytosolic tail of lamp2a, absent in the other forms of lamp2, is required for its ability to selectively bind cytosolic protein substrates for chaperone-mediated autophagy (Fig. 5). In addition, the unique ability of the lamp2a at the lysosomal membrane to multimerize may be of relevance for substrate transport into lysosomes (Fig. 6).

Different cellular functions for different forms of lamp2 have been proposed based on their tissue-specific expression (Konecki et al., 1995). However, a specific function for one of them but not for the others has not been described until now. We show here that only lamp2a is involved in the selective lysosomal binding of substrates for chaperone-mediated autophagy in rat liver and fibroblasts in culture (Fig. 4). We cannot discard the possibility that the antibody raised against the cytosolic region of lamp2a, absent in the other forms of lamp2, is required for its ability to selectively bind cytosolic protein substrates for chaperone-mediated autophagy (Fig. 5). In addition, the unique ability of the lamp2a at the lysosomal membrane to multimerize may be of relevance for substrate transport into lysosomes (Fig. 6).

![Fig. 6. Multimerization of lamp2a in the lysosomal membrane. Rat liver lysosomes were reversibly crosslinked and separated into membranes and matrices as described in Materials and Methods. Solubilized lysosomal membranes were subjected to sedimentation through a continuous sucrose gradient (20-60%) as described in Materials and Methods. Aliquots of each of the fractions collected from the top of the gradient were subjected to SDS-PAGE and immunoblotted for lamp2a (A) or for all lamp2s (B). The densitometric quantification of those immunoblots and the calculated molecular mass in kDa are shown.](image-url)
proteins to lysosomes (Figs 2 and 3), (3) the group of positive amino acids that participate in substrate binding are absent in the cytosolic tail of the other forms of lamp2 (Figs 1A, 5), (4) the multimerization of lamp2 at the lysosomal membrane may be important for the selective uptake of substrates in lysosomes (Cuervo and Dice, unpublished results), and is mainly detected for lamp2a (Fig. 6), and (5) the serum-related changes in the subcellular location of lamp2a are similar to the changes in cellular distribution of active lysosomes for chaperone-mediated autophagy (Fig. 7; F. Agarraberes and J. F. Dice, unpublished results).

The cytosolic protein substrates for chaperone-mediated autophagy all contain a pentapeptide motif biochemically related to KFERQ that targets them for lysosomal degradation (Chiang and Dice, 1988; Dice, 1990). That motif is not involved in the binding to the receptor at the lysosomal membrane (A. M. Cuervo and J. F. Dice, unpublished results). The requirement for the four positive residues in the cytosolic tail of lamp2a for substrate binding, described here (Fig. 5), suggests a charge-mediated substrate/receptor interaction.

Different explanations have been reasoned about the origin of the lamp2 detected in the lysosomal matrix such as its cleavage from the lysosomal membrane or its direct transport by vesicular fusion from the endosomal compartment (Jadot et al., 1996; Jadot et al., 1997). Since glycosylated proteins have hydrodynamic volumes that differ per unit of molecular mass from those of globular proteins used as molecular mass standards in our studies, we can not rule out the possibility that some of the matrix lamp2s not recognized by the antibody against the cytosolic tail correspond to truncated forms of lamp2a (Fig. 1A, right lane 4). Other authors have described formation of tetramers of truncated lamp2s, lacking a portion of its carboxy-terminal region (Akasaki and Tsuji, 1998). However, we demonstrate here that at least a part of the lamp2a present in the lysosomal matrix corresponds to the intact protein (Figs 1, 2, 4B). Thus, cleavage might explain the origin of some of the truncated forms of lamp2 in the matrix, but there must be other mechanisms by which intact forms of lamp2a appear in the lysosomal lumen (Cuervo and Dice, 2000a).

We now have evidence supporting the recruitment of part of the matrix lamp2a toward the lysosomal membrane under specific conditions that activate chaperone-mediated autophagy (Cuervo and Dice, 2000a). Interestingly, although matrix levels of lamp2a do not correlate in general with the rate of substrate degradation (Fig. 3B), in conditions such as prolonged starvation, we found an opposite change in membrane and matrix levels of lamp2a (Fig. 3A and B).

In addition to the transfer from the lysosomal membrane, it is still possible that part of the matrix lamp2a might originate from other vesicular structures after fusion with lysosomes. In this work we show heterogeneity in the lysosomal distribution of different forms of lamp2 (Fig. 2D) and also serum-dependent changes in the intracellular distribution of lamp2a (Fig. 7). Similar perinuclear distribution to the one shown here for lamp2a has been described for the lysosomes most active for chaperone-mediated autophagy using other markers such as hsc73 (F. Agarraberes and J. F. Dice, unpublished results). In addition, after prolonged starvation in rats, and based on the lysosomal hsc73 content, we have proposed the recruitment for chaperone-mediated autophagy of lysosomes normally less active for this pathway (Cuervo et al., 1997). It is thus possible that under extreme conditions, when chaperone-mediated autophagy reaches its maximum activity, a specific group of lysosomes receives lamp2a by fusion with other vesicles (endosomes or lysosomes) containing lamp2a.

Levels of lamp2a not only in the lysosomal membrane but also in the matrix are dynamic and independent of the changes of other lysosomal membrane proteins (Figs 2, 3 and 4; Cuervo and Dice, 2000a). Those changes in levels of lamp2a at the lysosomal membrane under different conditions have a direct effect on the rates of chaperone-mediated autophagy (Fig. 3). Further studies on the mechanisms that control levels of lamp2a at the lysosomal membrane might help us to understand how chaperone-mediated autophagy itself is regulated.

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