Regulation of Lamp2a Levels in the Lysosomal Membrane

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The selective degradation of cytosolic proteins in lysosomes by chaperone-mediated autophagy depends, at least in part, on the levels of a substrate receptor at the lysosomal membrane. We have previously identified this receptor as the lysosome-associated membrane protein type 2a (lamp2a) and showed that levels of lamp2a at the lysosomal membrane directly correlate with the activity of the proteolytic pathway. Here we show that levels of lamp2a at the lysosomal membrane are mainly controlled by changes in its half-life and its distribution between the lysosomal membrane and the matrix. The lysosomal degradation of lamp2a requires the combined action of at least two different proteolytic activities at the lysosomal membrane. Lamp2a is released from the membrane by the action of these proteases, and then the truncated lamp2a is rapidly degraded within the lysosomal matrix. Membrane degradation of lamp2a is a regulated process that is inhibited in the presence of substrates for chaperone-mediated autophagy and under conditions that activate that type of autophagy. Uptake of substrate proteins also results in transport of some intact lamp2a from the lysosomal membrane into the matrix. This fraction of lamp2a can be reinserted back into the lysosomal membrane. The traffic of lamp2a through the lysosomal matrix is not mediated by vesicles, and lamp2a reinsertion requires the lysosomal membrane potential and protein components of the lysosomal membrane. The distribution of lamp2a between the lysosomal membrane and matrix is a dynamic process that contributes to the regulation of lysosomal membrane levels of lamp2a and consequently to the activity of the chaperone-mediated autophagic pathway.

Key words: Lysosomes, membrane protein, molecular chaperones, protein cleavage, protein degradation, protein trafficking, rat liver

The lysosome-associated glycoprotein of 96 kDa, also known as lysosome-associated membrane protein type 2 (lamp2), belongs to a family of highly glycosylated lysosomal membrane proteins that share common features (1). Lamp2s are type I membrane proteins inserted in the lysosomal membrane by a single transmembrane region of approximately 20 amino acids near their carboxyl terminus. The bulk of these proteins, located in the lysosomal matrix, are heavily glycosylated. Lamp2s also have a short carboxyl-terminal region of

12 amino acids facing the cytosolic surface of the lysosomal membrane. The different members of this family show high sequence identity for their matrix region, but more divergent transmembrane and cytosolic regions. The roles of these lysosomal membrane proteins are not clear. They have been proposed to protect the lysosomal membrane from attack by lysosomal proteases. In addition, a small percentage of these proteins resides on the plasma membrane where they may contribute to cellular adhesion properties (2–4).

Lamp2a acts as a receptor for a selective pathway of degradation of cytosolic proteins in lysosomes known as chaperone-mediated autophagy (5). In this pathway, specific cytosolic proteins are directly transported through the lysosomal membrane into the lysosomal matrix where they are degraded (6,7). Interaction of substrate proteins with the heat shock cognate protein of 73 kDa (hsc73) in the cytosol stimulates their targeting to lysosomes (8). The presence of hsc73 in the lysosomal matrix is also necessary for the complete transport of substrate proteins into lysosomes (9,10). Substrate proteins bind to the cytosolic tail of lamp2a at the lysosomal membrane before their transport into and degradation within the lysosomal matrix (5). Blocking of the substrate–lamp2a interaction in rat liver lysosomes with specific antibodies against the cytosolic region of the rat lamp2a or with synthetic peptides with the same amino acid sequence as that region completely abolishes protein uptake (5). The binding of the substrates to lamp2a at the lysosomal membrane can be rate-limiting in the degradation process since overexpression of human lamp2a in cultured cells results in increased activity of this pathway. There is a strong correlation between the levels of lamp2a at the lysosomal membrane and the activity of the chaperone-mediated autophagic pathway in different physiological and pathological conditions that modify lysosomal uptake rates (A.M. Cuervo and J.F. Dice, unpublished results). However, little is known about the normal metabolism of lamp2a or the mechanisms involved in the control of lamp2a levels at the lysosomal membrane.

Multiple mRNAs for lamp2s have been identified (11,12), which result from alternative splicing and encode lamp2 molecules with different transmembrane and cytosolic regions (13). The different forms of lamp2 are expressed in a tissue-specific manner (12), suggesting that they might have different cellular functions. In addition to these multiple forms of lamp2 coexisting at the lysosomal membrane, a portion of lamp2 is also located in the lysosomal matrix (14,15). The origin of this lysosomal matrix lamp2 is still unclear. A direct deinsertion from the lysosomal membrane after conformational change, as well as a release by proteolytic cleavage of the short transmembrane and cytosolic tail have been proposed (14). Whether or not the distribution of

lamp2 between the lysosomal membrane and matrix is a dynamic process, and whether or not changes in lysosomal distribution have physiological relevance is unclear. We now report that changes in lamp2a catabolism and distribution between lysosomal membrane and matrix both contribute to the regulation of the activity of the chaperone-mediated autophagic pathway.

Results

Turnover and subcellular distribution of lamp2a

Rates of chaperone-mediated autophagy are modulated by changes in the levels of lamp2a at the lysosomal membrane (A.M. Cuervo and J.F. Dice, submitted). To understand how those changes in lamp2a levels are brought about, we analyzed rates of lamp2a synthesis and degradation along with possible changes in its subcellular distribution during nutrient deprivation when lysosomal membrane levels of lamp2a increase. We can differentiate lamp2a from other forms of lamp2 using an antibody developed against the cytosolic tail of lamp2a (5) that does not recognize the cytosolic tails of the other variants (A.M. Cuervo and J.F. Dice, submitted).

Using the antibody against the cytosolic tail of lamp2a and an antibody against the matrix region of lamp2 that will recognize all forms of lamp2 together, we compared rates of incorporation of radioactivity in lamp2a and all lamp2s in cells cultured in the presence or in the absence of serum (Figure 1A). We extended the studies to 30 h rather than the typical 1 h assay to allow lamp2s to maximally reach the lysosome. In this way, we could quantify the final, mature protein since we did not know if the antibodies recognized all the precursor forms of lamp2s. We normalized the incorporation of radioactivity into the immunoprecipitated proteins (Figure 1A) to equal numbers of cells at each point. We found no effect of serum on rates of synthesis of lamp2a (Figure 1A, left) or lamp2s (Figure 1A, right).

When we measured the degradation rate of total cellular lamp2a in mouse fibroblasts cultured in the presence or absence of serum, we found that after serum removal the half-life of lamp2a increased slightly (1.6 times; Figure 1B, left). The stabilization in the degradation rate of lamp2a clearly contrasts with the increased degradation of other cellular proteins including some of the other forms of lamp2 that are degraded more rapidly after serum removal (Figure 1B, right). When instead of measuring the half-life of lamp2a in total cellular extracts, we analyzed the half-life of the lamp2a located only in lysosomes (Figure 1C), we found a significant increase in the half-life of lamp2a after serum removal (Figure 1C, left) but a decrease in the half-life of all lamp2 forms considered together (Figure 1C, right). The increase in half-life of lamp2a in lysosomes (2-fold) would thus contribute to the increase in lysosomal membrane levels of lamp2a during serum withdrawal. Those results suggested that the control of lysosomal content of lamp2a occurs, in part, due to alterations in the rate of lamp2a degradation.

We found no differences by immunofluorescence in the levels of lamp2a at the plasma membrane of cells grown in the presence or absence of serum (data not shown). Levels of lamp2a in isolated endosomes, the other described intracellular location of lamp2, were lower in starved than in fed rats. However, that decrease in the endosomal content was common for all forms of lamp2, since we found a similar decrease using the antibody that recognized the matrix region of all lamp2s (data not shown). Recent studies have shown that the abnormal lysosomal content of lamp2 in several lysosomal storage diseases results from modified traffic of lamp2 through the Golgi/endosomal system, re-
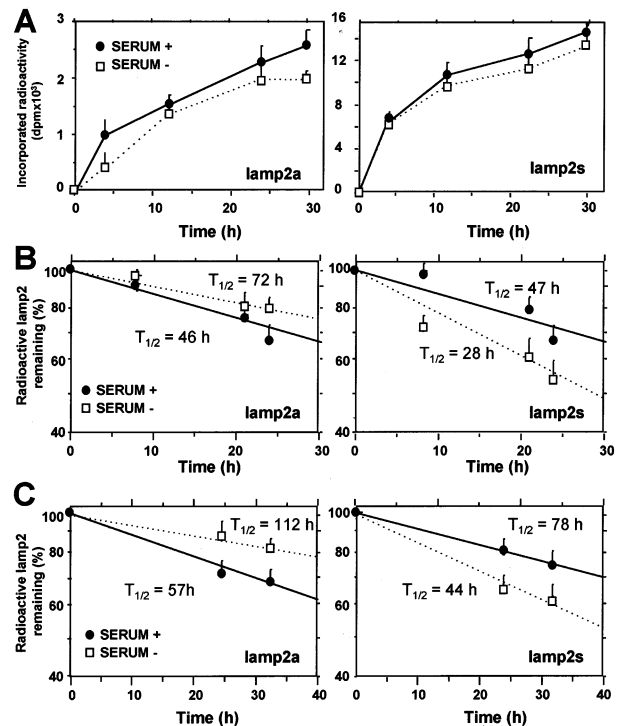

Figure 1: Effect of nutrient deprivation on lamp2a turnover. A. A mixture of [^{35}S]cysteine/methionine (200 µCi/ml) was added in the presence (serum +) or absence of serum (serum −) to confluent mouse fibroblasts. At the indicated times, the same number of cells were lysed and subjected to immunoprecipitation with specific antibodies against lamp2a (left) and lamp2s (right). Immunoprecipitates were resolved by SDS-PAGE, and quantified by exposure to a PhosphorImager screen. Values are means ± SE of three different experiments. B. Half-life of cellular lamp2: Mouse fibroblasts labeled as in A in the presence of serum for 2 days were then washed and maintained in fresh medium supplemented or not with serum. At the indicated times samples were processed as in A. The best exponential decay curve was calculated by linear regression analysis and the half-life of lamp2a or total lamp2s was calculated from five different experiments by the formula $t_{1/2} = \ln2/\text{degradation rate}$. C. Half-life of lysosomal lamp2: Mouse fibroblasts were labeled and chased as in B, but at the indicated times lysosomes were isolated, solubilized, and subjected to immunoprecipitation as in B. Half-lives were calculated from four different experiments as described in B.
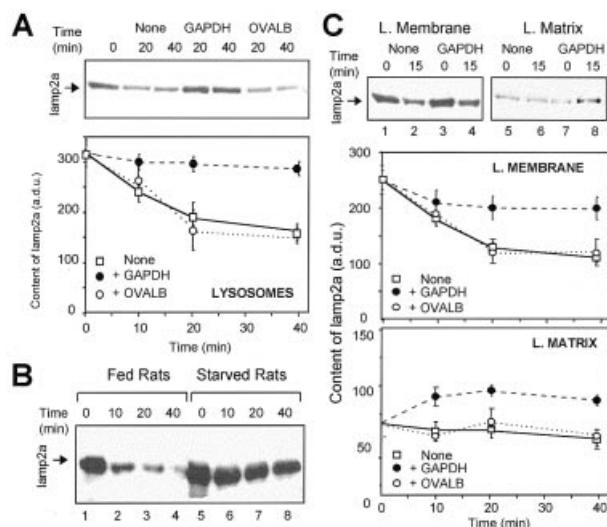
Figure 2: Effect of substrate proteins on the total content and distribution of lamp2a in rat liver lysosomes. A. Intact rat liver lysosomes were incubated at 37°C for 40 min without additions (NONE) or in the presence of 50 μg of GAPDH or ovalbumin (OVALB). At the indicated times of the incubation, lysosomes were sedimented and directly processed for SDS-PAGE and immunoblotted for lamp2a. Immunoblots were then quantified by densitometry. Values represent the average ± SE of eight different experiments, and are expressed in arbitrary densitometric units (a.d.u.). Values from different days were normalized according to the densitometric values of known amounts of lamp2a in the same immunoblot. B. Lysosomes isolated from fed (lanes 1–4) or 40 h starved rats (lanes 5–8) were incubated in the absence of substrates for the indicated times and then samples were processed as in A. A representative immunoblot of four different experiments is shown. C. Intact rat liver lysosomes incubated as described in A were recovered by centrifugation at the indicated times and then subjected to a hypotonic shock to separate lysosomal membranes (L. Membrane) and matrices (L. Matrix). The content of lamp2a in both fractions was analyzed as in A.

vealed as changes in the lamp2 glycosylation pattern (16). We did not find differences between the glycosylation state of lamp2a in total lysates from serum-supplemented or serum-deprived cells (data not shown).

Degradation of lamp2a in isolated lysosomes

Since the degradation of lamp2a in lysosomes contributes to regulation of its lysosomal levels, we next attempted to reproduce aspects of lamp2a degradation in isolated lysosomes. We measured changes in the lysosomal content of intact lamp2a using the antibody against its cytosolic region in isolated lysosomes incubated in the presence or absence of substrate proteins. As shown in Figure 2A, total levels of lysosomal lamp2a decreased with incubation time in the absence of substrate proteins. This decrease was at least partially blocked by a cocktail of protease inhibitors (data not shown). This decrease in lysosomal levels of lamp2a is substrate-dependent because, in the presence of a substrate, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), but

not in the presence of a non-substrate, ovalbumin (17,18), the decrease in total content of lamp2 was significantly reduced (Figure 2A). Addition of the extra-lysosomal medium from previously incubated lysosomes to isolated lysosomes did not change lamp2a loss (data not shown) suggesting that leakage of lysosomal proteases during the incubation does not contribute to the decrease in lamp2a levels.

Also supporting this relationship between the lysosomal degradation of lamp2a and the activity of chaperone-mediated autophagy, we found that the rate of degradation of lamp2a was higher in lysosomal membranes isolated from fed rats but decreased in response to starvation (2.8 and 0.53%/min in fed and 40 h starved rats, respectively) (Figure 2B). To avoid the protective effect on lamp2a that the higher content of membrane-bound substrate proteins might have in starved rats, lysosomal membranes were consecutively washed twice with 50 volumes of 0.5 M NaCl/MOPS buffer prior to the incubation. This wash procedure removed all immunodetectable GADPH from the lysosomal preparations. The decrease in lysosomal lamp2a degradation during nutrient deprivation also applies to lysosomes isolated from rat fibroblasts after serum removal (data not shown). Thus, under conditions of nutrient deprivation, a decrease in the lysosomal degradation of lamp2a might contribute to its increased concentrations in lysosomal membranes leading to activation of chaperone-mediated autophagy.

We have previously demonstrated that a portion of lamp2a can be detected in the lysosomal matrix as an intact protein (A.M. Cuervo and J.F. Dice, unpublished results). To determine if the matrix or membrane lamp2a was degraded in lysosomes incubated in the absence of substrate proteins, we separated the lysosomal membranes and matrices at each of the indicated times and analyzed their content of lamp2a. We found a decrease in the content of intact lamp2a containing the cytosolic tail at the lysosomal membrane (Figure 2C), but not in the matrix (Figure 2D). The loss of lamp2a at the lysosomal membrane was significantly inhibited in the presence of GAPDH, but not ovalbumin (Figure 2C). These results suggest that most of the lamp2a degraded in lysosomes corresponds to the membrane-associated protein.

Proteolytic cleavage of the lamp2 inserted in the lysosomal membrane

To identify the initial steps involved in the lysosomal degradation of lamp2a, we tried to slow the process by using isolated lysosomal membranes that lack the proteases located in the lysosomal matrix. The total amount of lamp2a in isolated lysosomal membranes decreased with the incubation time at similar rates to that described for intact lysosomes (Figure 3A, left; compare with Figure 2A,B). The decrease in the levels of intact lamp2a coincided with the appearance of one or two lower molecular weight forms of lamp2 that reacted with the antibody against the matrix region of the protein (Figure 3A, right), but not with the antibody against the cytosolic tail of lamp2a (Figure 3A, left). Cleavage of different forms of lamp2 at the lysosomal mem-
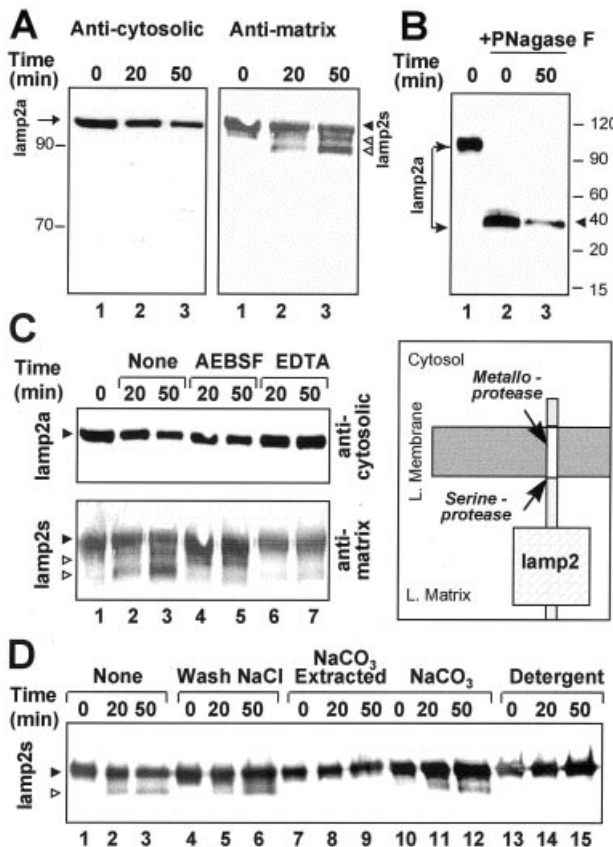
Figure 3: Proteolytic cleavage of lamp2a at the lysosomal membrane. A. Isolated rat liver lysosomal membranes were incubated at 37°C for the indicated times in MOPS buffer. At the end of the incubation samples were subjected to SDS-PAGE and immunoblotted with antibodies against lamp2a (anti-cytosolic; left) or all lamp2s (anti-matrix; right). Solid and open arrowheads indicate the position of intact lamp2 and lamp2 proteolytic fragments, respectively. B. Lysosomal membranes incubated as described in A were then denatured and treated with *N*-glycosidase F (PNagase F). At the end of the incubation, samples were processed for SDS-PAGE and immunoblotted with the antibody against lamp2a. Lane 1 contains lysosomal membranes not treated with glycosidase. C. Lysosomal membranes were incubated as in A without additions (None) or in the presence of AEBSF (1 μM) or EDTA (2 mM) for the indicated times. At the end of the incubation, samples were processed as in A. Arrowheads are as in A. A schematic model of lamp2a inserted at the lysosomal membrane and the hypothetical cleavage regions is shown on the right (see text for details). D. Lysosomal membranes were incubated for 1 h at 0°C in MOPS buffer (None), 0.5 M NaCl (Wash NaCl), 0.1 M NaCO₃ pH 11.3 (NaCO₃) or 0.2% Triton X-100 (Detergent). At the end of the incubation, membranes were collected by centrifugation, except for lanes 10–15, washed and resuspended in MOPS buffer. Resuspended membranes (lanes 1–9) and total samples (lanes 10–15) were incubated and processed as in A using the antibody against all lamp2s. For NaCO₃ 'Extracted' indicates that the membranes have been sedimented and resuspended in MOPS buffer before the second incubation. Arrowheads are as in A.

brane could contribute to the formation of some of those fragments, but the decrease in lamp2a levels indicated that some originate from partial cleavage of lamp2a. It is unlikely that the formation of the lower molecular weight species results from partial deglycosylation of lamp2a because the decrease in the amount of lamp2a was also evident after complete deglycosylation of the incubated membranes (Figure 3B). In addition, most of the partially deglycosylated forms are still recognized by the antibody against lamp2a (data not shown). Therefore, the intermediate forms of lamp2a must originate by cleavage of a portion of the protein containing the cytosolic region.

Using a battery of inhibitors specific for each of the four main groups of proteases, we found that a serine protease inhibitor, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), and also a metal chelating agent, ethylenediamine-tetraacetic acid (EDTA), modified lamp2 processing (Figure 3C). EDTA completely blocked the decrease of lamp2a levels (Figure 3C top, lanes 6 and 7) and formation of both intermediate fragments (Figure 3C bottom, lanes 6 and 7). In contrast, the serine protease inhibitor blocked only the formation of the smaller fragment, but not the larger one (Figure 3C bottom, lanes 4 and 5). In the presence of AEBSF, the amount of intact lamp2a decreased with the incubation time suggesting that the cytosolic tail was lost (Figure 3C top, lanes 4 and 5). In the absence of protease inhibitors the transition between the two smaller fragments of lamp2a is very fast, such that only the smaller form of lamp2a can be detected. After the incubation, we recovered the membranes by centrifugation and found that the smaller form of the truncated lamp2a remained in the supernatant (data not shown). Therefore, cleavage of lamp2a results in its release from the membrane into the matrix. Two different proteolytic activities acting sequentially might explain these results (Figure 3C, right). First, an EDTA-sensitive protease, presumably a metalloprotease, would cut the distal region of the protein containing the cytosolic tail; in a second step, a serine protease would cut the protein, probably in the transition from the transmembrane to the matrix region, resulting in the release of the lamp2 from the lysosomal membrane.

It is unlikely that the proteolytic cleavage of lamp2a results from cytosolic or lysosomal matrix contamination of the membrane fraction, because washing of the membranes with 0.5 M NaCl to eliminate weakly associated proteins did not modify rates of lamp2a processing (Figure 3D, lanes 4–6). These experiments also demonstrated that lamp2a cleavage is not energy dependent since no ATP was added in the incubation medium. Both proteases seem to strongly interact with membrane components since alkaline extraction was required to block lamp2a cleavage (Figure 3D, lanes 7–9). Notice that alkaline treatment *per se* did not have a direct effect on protease activity because membranes in which the alkali-extracted products were not removed by centrifugation still showed proteolytic cleavage of lamp2a (Figure 3D, lanes 10–12). Solubilization of the lysosomal membranes with Triton X-100 clearly decreased lamp2a processing (Figure 3D, lanes 13–15). Tween-20 and octylglu-
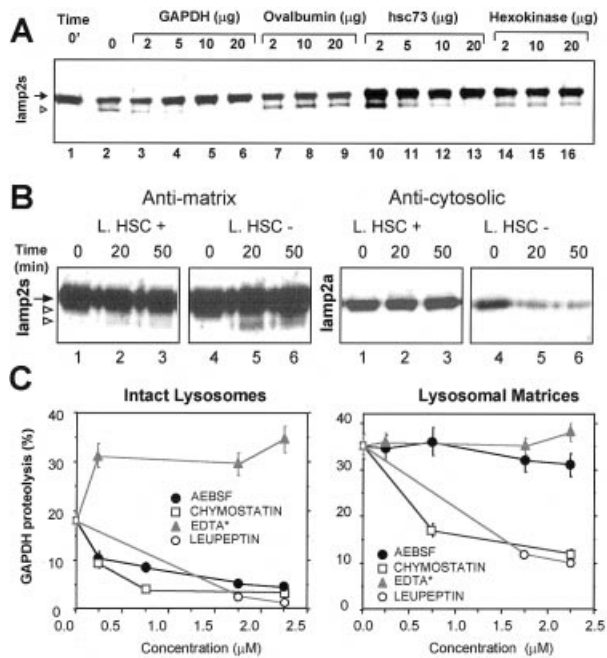
Figure 4: Relationship between the selective uptake of proteins into lysosomes and the cleavage of lamp2a at the lysosomal membrane. A. Lysosomal membranes were incubated under standard conditions without additions (lane 2) or in the presence of increasing amounts of GAPDH, ovalbumin, hsc73 or hexokinase as labeled. At the end of the incubation, samples were subjected to SDS-PAGE and immunoblotted with an antibody against lamp2s. Lane 1 shows nonincubated membranes. Open arrowhead indicates the lamp2 proteolytic fragment. B. Lysosomal membranes prepared from two lysosomal populations with different activity for chaperone-mediated autophagy were incubated for the indicated times under standard conditions. Samples were subjected to SDS-PAGE and immunoblotted for all lamp2s (anti-matrix; left) or for lamp2a (anti-cytosolic; right). Open arrowheads indicate lamp2 proteolytic fragments. C. Intact lysosomes (left) or lysosomal matrices (right) were preincubated for 10 min at 0°C with the indicated concentrations of EDTA, AEBSF, leupeptin or chymostatin. The ability of those lysosomes to take up and degrade radiolabeled GAPDH was then analyzed. Values are expressed as percentage of initial acid-precipitable GAPDH radioactivity converted to acid-soluble radioactivity at the end of the incubation, and are the average ± SE of five different experiments. (*) Note that values for EDTA correspond to mM rather than μM concentrations.

coside similarly decreased lamp2a cleavage (data not shown) suggesting that a close interaction between the protease(s) and lamp2a, and/or a lipid environment are necessary. Alternatively, the detergents may directly inactivate the proteases.

To determine whether or not this processing of lamp2a was somehow related with the transport of proteins to lysosomes by chaperone-mediated autophagy, we analyzed the effect of substrate and non-substrate proteins on lamp2a cleavage. Increasing amounts of GAPDH (Figure 4A, lanes 3–6) and hsc73 (lanes 10–13), both substrate proteins,

blocked the proteolytic processing of lamp2a. However, non-substrate proteins, such as ovalbumin (lanes 7–9) and hexokinase (lanes 14–16), did not inhibit lamp2a cleavage. Whether the substrate proteins directly block the protease recognition site at the cytosolic tail of lamp2a, or if the lamp2/substrate interaction induces a conformational change in lamp2a that makes it more resistant to the cleavage, need to be further analyzed.

Rates of lamp2 processing in the membranes of lysosomes active for chaperone-mediated autophagy were lower than in the membranes of lysosomes with low activity (Figure 4B). In addition, in conditions in which chaperone-mediated autophagy increases such as serum removal from cultured cells or prolonged starvation in rats, rates of lamp2 proteolytic processing decreased (data not shown).

We then blocked the lysosomal cleavage of lamp2a with EDTA in intact rat liver lysosomes and analyzed their ability to take up and degrade substrate proteins. As shown in Figure 4C preincubation of intact rat liver lysosomes with increasing concentrations of EDTA significantly increased GAPDH transport and degradation (Figure 4C, left). EDTA did not modify the degradation rates of GAPDH by isolated lysosomal matrices (Figure 4C, right), suggesting that the stimulatory effect of EDTA was mainly on the binding and uptake steps. No stimulatory effect, but a decrease in lysosomal GAPDH degradation was found when lysosomes were treated with protease inhibitors that did not modify lamp2a cleavage (Figure 4C, left). Those agents probably directly block the protease(s) responsible for GAPDH degradation within lysosomes (Figure 4C, right).

When we inhibited only the second step in the cleavage of lamp2 at the lysosomal membrane with increasing concentrations of AEBSF, we found a concentration-dependent decrease in the amount of GAPDH degraded by intact lysosomes (Figure 4C, left). We found a slight direct effect of AEBSF on GAPDH degradation by lysosomal matrix proteases only for the highest concentrations of the inhibitor (Figure 4C, right). The unexpected inhibitory effect of AEBSF on GAPDH uptake (Figure 4C, left) might be explained by the presence of truncated forms of lamp2 at the lysosomal membrane that could somehow interfere with the normal transport of substrate proteins. In the presence of AEBSF, we found an increase in the amount of substrate protein bound to intact lysosomes (data not shown), suggesting that the substrate protein binds to the other intact forms of lamp2a, but remains attached there because its internalization is blocked.

Together, these results suggest that the proteolytic cleavage of lamp2 at the lysosomal membrane might be the first step in its degradation, which probably takes place continuously in lysosomes under normal conditions. In the presence of substrate proteins, the blockage of this lamp2a processing by mechanisms still unknown, will result in an increase in the amount of receptor available at the lysosomal membrane

and, therefore, in an increase in the selective uptake of substrates.

Dynamics of the membrane and matrix forms of lamp2a

Interestingly, the total levels of lamp2a in isolated lysosomes incubated in the presence of substrate proteins did not change (Figure 2A), but we detected an initial decrease in the amount of lamp2a at the lysosomal membrane (Figure 2C) concomitant with an increase in lamp2a levels in the lysosomal matrix (Figure 2D). Since these preparations were isolated lysosomes, the lamp2 that appeared initially in the lysosomal matrix could only come from the lysosomal membrane.

Some of the conditions that modify the rates of chaperone-mediated autophagy (e.g. prolonged starvation) also show opposite changes in the levels of lamp2a at the lysosomal membrane and matrix (A.M. Cuervo and J.F. Dice, submitted). These observations provided the first hints that membrane and matrix forms of lamp2a may be in dynamic equilibrium.

We analyzed the effect of increasing concentrations of substrate proteins, GAPDH and ribonuclease A (RNase A), on membrane and matrix levels of intact lamp2a. We narrowed our study to the first 20 min of incubation when levels of lamp2 in both compartments are still changing (Figure 5C,D). The content of lamp2a in the matrix increased with the increase in substrate concentration, but then saturated at higher substrate concentrations (Figure 5A,B). Since the lysosomal uptake of substrate proteins is saturable (17,18), we could take into account the amount of substrate that was actually transported into lysosomes at each substrate concentration. The relationship between substrate protein transported and intact lamp2a internalized is linear (Figure 5A,B, insets). We have previously determined that the content of lamp2a at the lysosomal membrane is 5.8 μg per each 100 μg of lysosomal membrane protein (A.M. Cuervo and J.F. Dice, submitted). By densitometric analysis, we calculated the percentage of the initial amount of lamp2a at the lysosomal membrane that was internalized into the matrix for each concentration of substrate protein. With those values, and considering that GAPDH is transported as a monomer, we determined that for each 8–10 molecules of substrate protein transported into lysosomes, one molecule of intact lamp2a is also internalized.

As mentioned above, after the first 20 min of incubation with substrate proteins, levels of intact lamp2a in the lysosomal membrane and matrix are stable (Figure 2C). That stabilization could be explained by interruption of lamp2a internalization to the matrix, but also by traffic of part of the matrix lamp2a back to the membrane. When we rapidly removed the substrate proteins from the incubation medium after reaching this steady state phase, an increase of levels of lamp2a at the lysosomal membrane, along with a decrease in the lysosomal matrix occurred (Figure 5C). Those results

suggest that at least part of the intact lamp2a internalized could reinsert into the lysosomal membrane. We only detected this reinsertion in active lysosomes (lysosomes from starved rats) (Figure 5C), while lysosomes from fed rats reacted quite differently to the same incubation conditions (Figure 5D). According to these results, the reinsertion of lamp2a in the lysosomal membrane might be possible only when chaperone-mediated autophagy is activated. This reinsertion of part of the matrix lamp2a into the lysosomal membrane might also explain decrease in matrix levels of lamp2a concomitant with its increase in the lysosomal membrane after prolonged starvation (A.M. Cuervo and J.F. Dice, unpublished data).
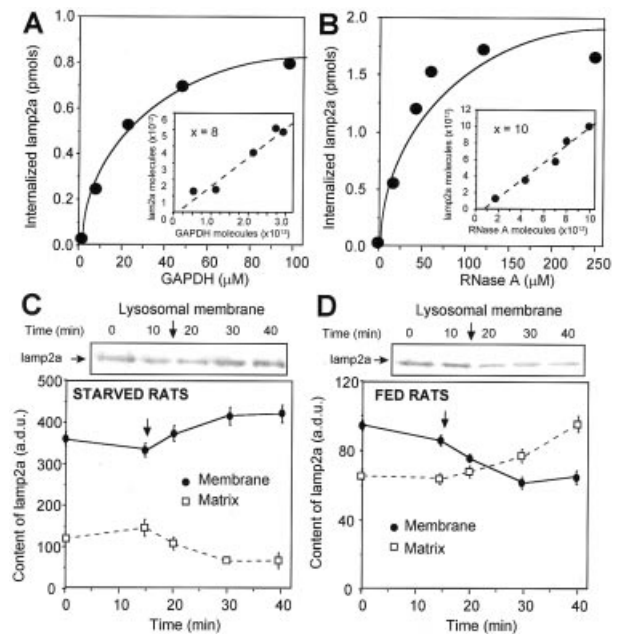

Figure 5: Substrate-dependent dynamics of lamp2a within the lysosomal membrane and matrix compartments. A, B. Isolated rat liver lysosomes were incubated for 20 min with increasing concentrations of GAPDH (A) or RNase A (B), as labeled. At the end of the incubation levels of lamp2a in lysosomal membranes and matrices were determined by immunoblotting. The increment of lamp2a in the matrix, expressed in pmols for increasing concentrations of substrate added, is shown. Insets correspond to the theoretical calculation of the number of molecules of lamp2a internalized in the matrix for each molecule of substrate transported. Results are the average value for eight different experiments and the SE for each value was less than 10% of the average. C, D. Lysosomes from starved (C) or fed (D) rats were incubated in the presence of 50 μg of GAPDH. After 15 min (arrow), lysosomes were sedimented, resuspended and incubated in the same buffer without GAPDH. At the end of the incubation, samples were processed as in A. Values shown are the mean value ± SE of the content of lamp2a in each fraction in four different experiments.
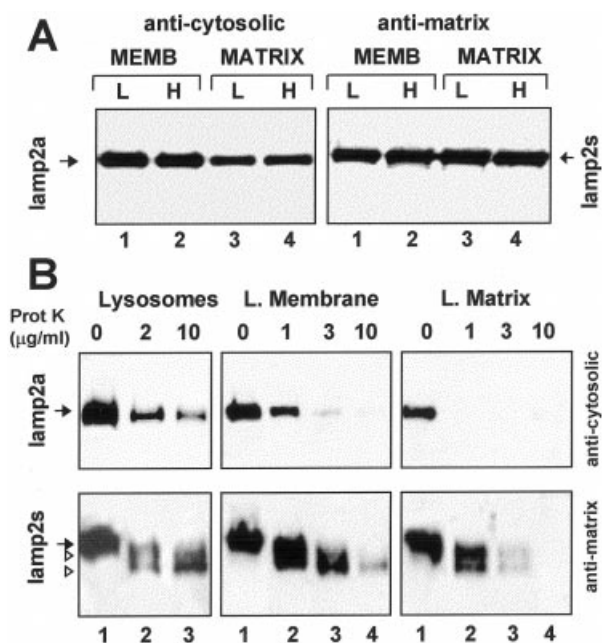
Figure 6: Characteristics of the matrix form of lamp2a. A. Rat liver lysosomes (100 µg protein) were subjected to hypotonic shock for 30 min at 0°C. After sedimentation at 105 000 × *g* for 30 min (low; L) or at 350 000 × *g* for 3 h (high; H), pellets and supernatants were subjected to SDS-PAGE and immunoblotted with specific antibodies against the cytosolic tail of lamp2a (left) or against a matrix region of the protein (right). B. Intact rat liver lysosomes (left), and lysosomal membranes (middle) and matrices (right) were incubated at 0°C for 10 min with the indicated concentrations of proteinase K (Prot K). At the end of the incubation, protease inhibitors were added and samples were subjected to SDS-PAGE and immunoblotted as in A. Arrow points to intact lamp2, and open arrowheads indicate two differently sized fragments from lamp2.

Characterization of the lamp2a internalized into rat liver lysosomes

We next asked whether the intact lamp2a was internalized into the lysosomal matrix associated with membranes or trapped in small vesicles. If the size of those vesicles was small enough, they might not be sedimented under the standard conditions in which we isolated the lysosomal matrix. We addressed this possibility in two different ways. First, we increased the speed and time of centrifugation to be able to sediment even very small vesicles. After centrifugation at 350 000 × *g* for 3 h (Figure 6A, lanes 2 and 4), levels of lamp2s in the matrix were similar to those obtained after standard centrifugation (105 000 × *g* for 30 min.) (Figure 6A, lanes 1 and 3). Second, we reasoned that if the lamp2a in the matrix was trapped in small vesicles, the protein must be, at least in part, protected from the attack of an exogenously added protease. In intact lysosomes, the cytosolic region of the lamp2a in the membrane is accessible to cleavage by proteinase K (Figure 6B, top left) and a lower molecular weight form of lamp2 lacking the cytosolic tail can now be detected with the antibody against the matrix side (Figure 6B, bottom left, lanes 1–3). A similar pattern was found in

isolated lysosomal membranes (Figure 6B, middle). Interestingly, in the lysosomal matrix the cytosolic tail of the lamp2 is still the most susceptible region to proteolysis by proteinase K (Figure 6B, right), but at higher protease concentrations the protein is completely degraded. Similar results were obtained using trypsin (data not shown). We have previously demonstrated that treatment of intact lysosomes with the indicated concentrations of proteinase K does not result in significant release of β-hexosaminidase or cathepsin L from the lysosomes into the incubation medium (5,17–19). In addition, we found no discontinuities in the membrane of treated lysosomes by electron microscopy, but we did find membrane gaps, using concentrations of proteinase K, five times those described here (A.M. Cuervo and E. Knecht, unpublished results). Therefore, it seems unlikely that the degradation of lamp2a in the lysosomal matrix results after the protease disrupts any membranous structure in which the lamp2a could be trapped. Taken together, these results suggest that the traffic of lamp2 from the lysosomal membrane into the matrix is not vesicle-mediated.

Recent studies have shown that some of the lamp2s in the lysosomal matrix associate with other lysosomal matrix proteins and lipids in large complexes that aggregate at the acidic intralysosomal pH (15). We found that the lamp2a recovered in the matrix fraction is not part of those large complexes. After sedimentation through a continuous sucrose density gradient, most of the matrix lamp2a migrated at the sucrose density expected for a monomer (Figure 7A). We found a small percentage of the lamp2a in the lysosomal matrix in a higher density region distinct from the higher density where the other lamp2s were recovered. In contrast with the complexes containing other lamp2s, the formation of the higher molecular weight complex of lamp2a was independent of pH (Figure 7A). Some of the lamp2s in the lysosomal matrix associate with specific lipids, and that association can be followed by floatation of those complexes in sucrose gradients (15). Using similar floatation assays, we found that part of lamp2a also floats through the gradient, but at a higher sucrose density than that of other lamp2s (Figure 7B). That floatation is abolished in the presence of 0.1% Triton X-100 (data not shown), suggesting that part of the lamp2a travels through the lysosomal matrix associated with lipids.

Requirements for the reinsertion of lamp2a into the lysosomal membrane

In similar experiments to those shown in Figure 5C, we found that the reinsertion of lamp2a into the lysosomal membrane is temperature dependent (Figure 8A). That fact allowed us to analyze some of the possible requirements of lamp2a reinsertion by preincubating the lysosomes with different compounds at 0°C before assaying for lamp2a reinsertion. Because we carried out these treatments after the incubation with the substrate protein, we could directly analyze their effect on lamp2a reinsertion independently of any possible effect on substrate uptake or lamp2a internalization. Addition of ATP to the incubation medium did not modify amounts of lamp2a membrane reinserted (Figure 8B). Rein-

sertion of lamp2a remained unchanged after eliminating any residual extralysosomal ATP by treatment with apyrase (Figure 8B). Recombinant HA-lamp2a was normally reinserted in

lysosomal membrane vesicles in which all soluble lysosomal content, including ATP, was eliminated (see below). These results suggest that the reinsertion of lamp2a into the lysosomal membrane is not an energy-dependent process. Complete disruption of the acidic intralysosomal pH with the Na^+/H^+ exchange ionophore, monensin (20), or with the specific inhibitor for the lysosomal membrane H^+-ATPase, bafilomycin A (20), did not affect lamp2a reinsertion (Figure 8B). We found a dramatic inhibition of lamp2a reinsertion when we collapsed the transmembrane potential with the K^+ ionophore, valinomycin (Figure 8B) (21). In the presence of valinomycin, the normally negative inside polarization of the lysosomal membrane, due to fixed negative charges (20), is neutralized. These results indicate that, as described for the insertion of proteins in the membrane of *Escherichia coli* (22), lamp2a reinsertion in the lysosomal membrane could be driven by the electrical potential across the membrane.

We then analyzed the reinsertion of lamp2a in lysosomal membranes subjected to different treatments. Insertion of purified recombinant HA-lamp2a into high salt-washed or $NaCO_3$-extracted membranes was similar to that in membranes washed with buffer (Figure 9A). Previous protease treatment of intact lysosomes, under conditions that do not affect membrane integrity (5,17–19), reduced HA-lamp2a insertion into their membranes (Figure 9A,B), suggesting that protein component(s) at the lysosomal membrane are required for lamp2a insertion. Blockage of the membrane-associated hsc73 with specific antibodies inhibited lamp2a reinsertion into the lysosomal membrane (Figure 9C), suggesting that the molecular chaperone may be required for this process. However, blockage of the cytosolic tail of the lamp2a already present in the lysosomal membrane with a specific antibody did not have any effect on lamp2a reinsertion (Figure 9C).

Discussion

The chaperone-mediated degradation of cytosolic proteins in lysosomes is a regulated process that allows cells to selectively discriminate between substrate and non-substrate proteins (6,7,23). Binding of substrate proteins to lamp2a, a receptor in the lysosomal membrane, is a rate-limiting step for their degradation (6, 7, AM Cuervo and JF Dice, unpublished result). Here we describe two different mechanisms that contribute to those changes in the lysosomal levels of lamp2a: the lysosomal degradation of lamp2a (Figures 1–4) and its dynamic distribution between the lysosomal membrane and matrix (Figures 5–9).

Figure 7: Association of matrix lamp2a to proteins and lipids. A. Lysosomal matrices were titrated at pH 5 (top) or pH 8 (bottom) and subjected to centrifugation through a 10–60% sucrose gradient as indicated in Materials and Methods. Fractions were collected and lamp2a and lamp2s were immunoblotted with specific antibodies. Values obtained by densitometric analysis are expressed as arbitrary densitometric units. B. Lysosomal matrices were subjected to floatation in a sucrose gradient as described in Materials and Methods. Collected fractions were processed as in A.
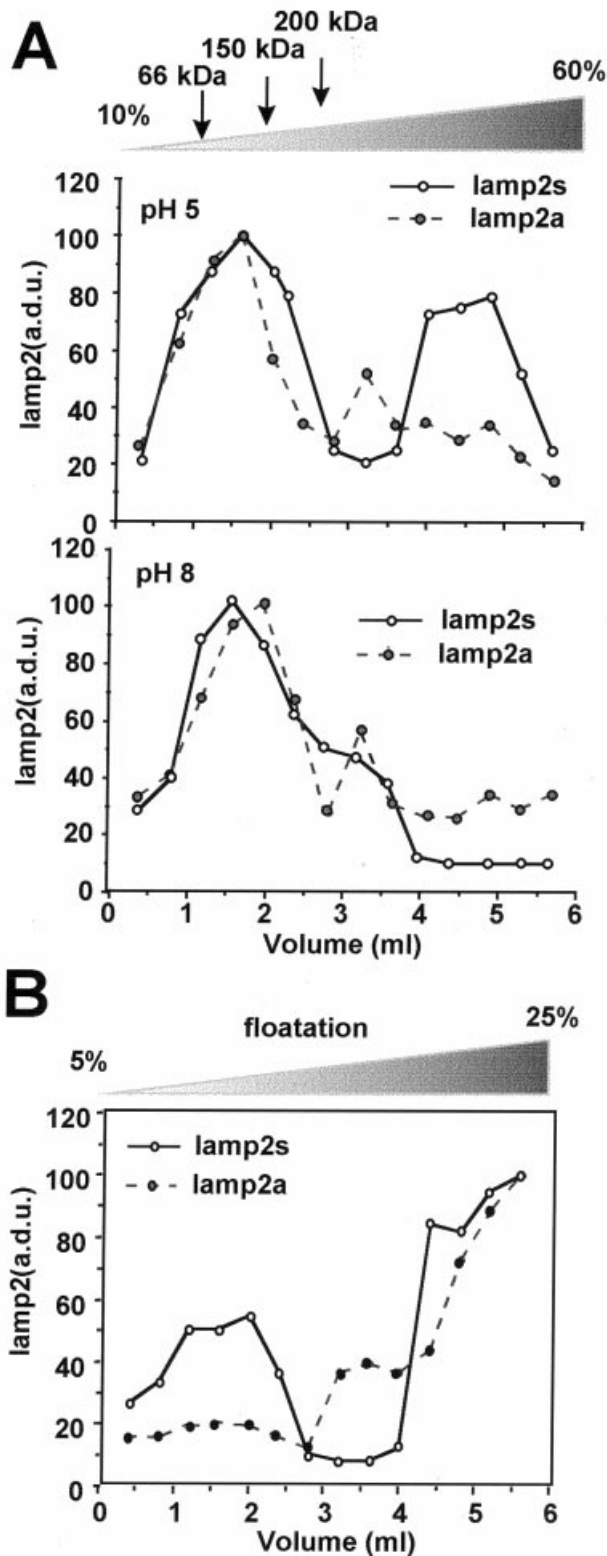
Previous studies have reported half-lives of lamp2s from 27 to 110 h, perhaps depending on the assay conditions and cell type (24,25). Our values of 28–112 h are almost identical to
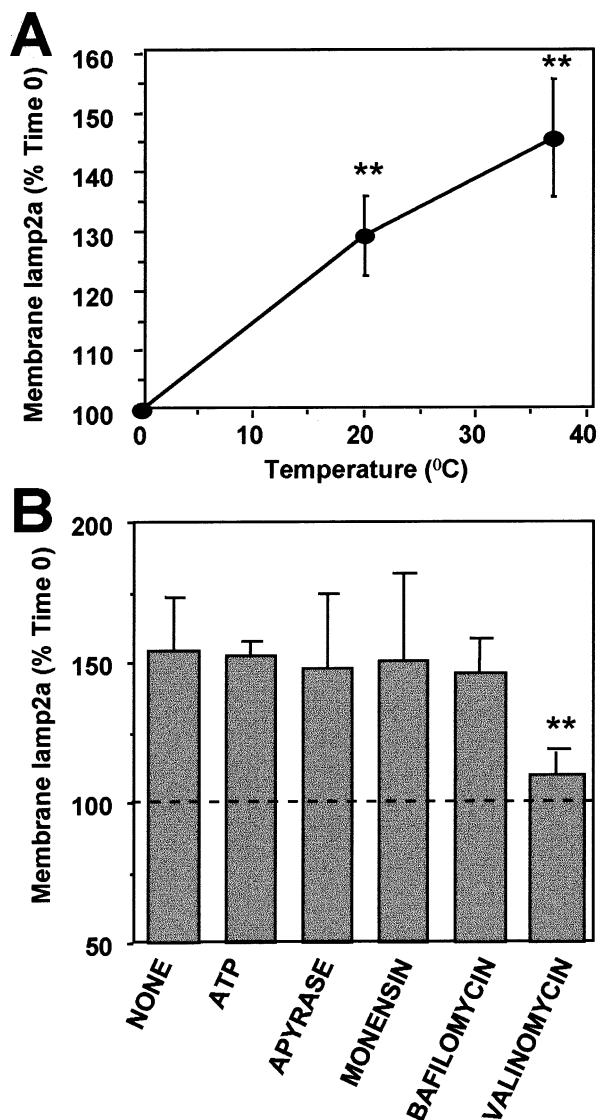

Figure 8: Requirements for the reinsertion of matrix lamp2a into the lysosomal membrane. A. Lysosomes were incubated with GAPDH and then sedimented and resuspended in buffer without GAPDH. Samples were then incubated for 15 min at 0, 20 or 37°C and lysosomal membranes were isolated and subjected to SDS-PAGE and immunoblot for lamp2a. Values are expressed as percentage of the lamp2a detected at lysosomal membrane at the end of the incubation with the substrate (100%) and are mean ± SE of four different experiments. B. Lysosomes were incubated with GAPDH as in A, sedimented and treated with the indicated compounds for 10 min at 0°C as described in Materials and Methods. After a 15 min incubation at 37°C in a GAPDH-free buffer, all samples were processed as in A. Values expressed as the percentage of the lamp2a detected at lysosomal membrane at the end of the incubation with the substrate (dashed line) are means ± SE of six different experiments.
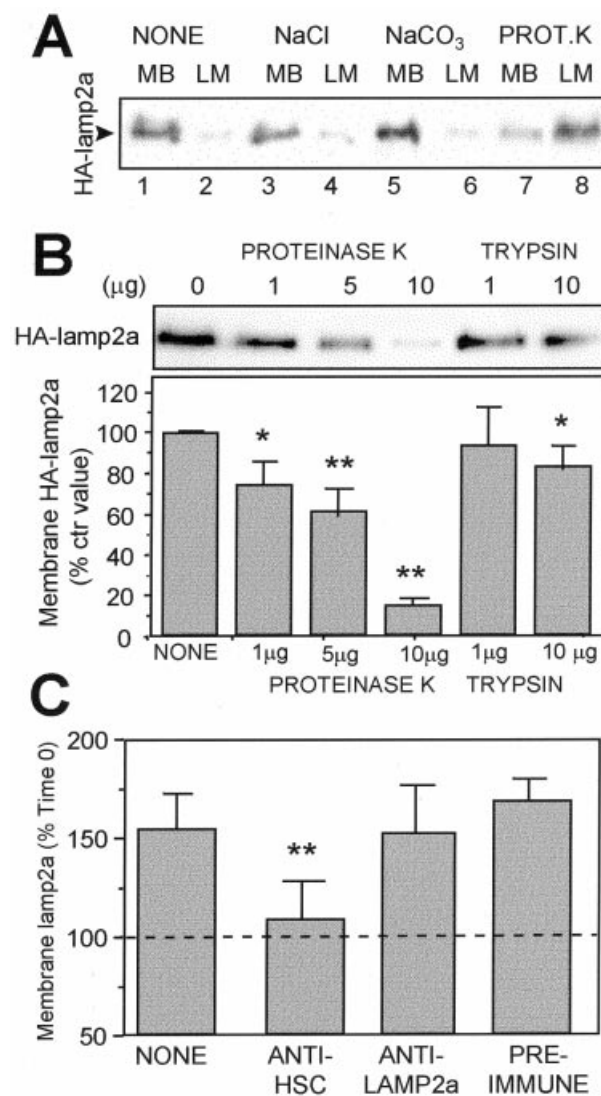
Figure 9: A membrane protein component required for lamp2a reinsertion in the lysosomal membrane. A, B. Rat liver lysosomal membranes were treated with 0.5 M NaCl, 0.1 M $NaCO_3$, or the indicated concentrations of proteinase K or trypsin as described in Materials and Methods. After two washes with hypotonic buffer (0.025 M sucrose/MOPS), membranes were recovered and resuspended in an isotonic buffer (0.25 M sucrose/MOPS) in the presence of 60 µg of purified human HA-lamp2a. Samples were incubated for 30 min at 37°C, recovered by centrifugation and subjected to a new hypotonic shock to separate membrane (MB) from lumen (LM). Fractions were subjected to SDS-PAGE and immunoblotted with a specific antibody against HA. Values shown are the means ± SE of the densitometric quantification of four similar experiments. C. Isolated lysosomes were incubated with GAPDH under standard conditions, sedimented and incubated without additions (none) or with specific antibodies against hsc73 (anti-HSC), against the cytosolic tail of lamp2a (anti-lamp2a), or with a preimmune serum (preimmune) for 10 min at 0°C. After incubation in a substrate-free medium, lysosomal membranes were isolated and subjected to SDS-PAGE and immunoblotted for lamp2a. Values are expressed as a percentage of the lamp2a detected at the lysosomal membrane at the end of the incubation with the substrate (dashed line) and are means ± SE of six different experiments.

this range, and we show that lamp2s as a group are degraded more rapidly during serum withdrawal (Figure 1B,C, right). The variability in lamp2s half-life may also be related to the complexity of the glycosylation pattern of the protein in each type of cell (25). However, it is unlikely that the increased degradation of lamp2s during serum deprivation, reported here, results from serum-dependent changes in lamp2a glycosylation since we did not find differences in the apparent size of the protein after serum removal. To our knowledge, the half-life of the lamp2a isoform has not been previously reported. The decreased degradation during nutrient deprivation applies to lamp2a but not to lamp2s in general. Those results support the idea proposed by several authors that the different forms of lamp2 might have different cellular functions (12).

In cultured cells, lamp2a degradation rates appear to differ for different subcellular compartments. For example, total cellular lamp2a degradation rates are faster than lysosomal lamp2a degradation rates (Figure 1B and Figure 1C, left). Those differences in degradation seem not to be related to differences between the total cellular lysosomal content and the lysosomes isolated in our preparations. Thus, the recovery of lysosomes (calculated as percentage of total β-hexosaminidase activity recovered in the lysosomal fraction) was similar for cells cultured in the presence or absence of serum. The differences in total cellular and lysosomal lamp2a half-lives might be related, at least in part, to lysosomal heterogeneity. We have previously described in rat liver two groups of lysosomes with very different activities for the selective transport of substrates (10). As we show here, less active lysosomes have higher lamp2a degradation rates *in vitro* than the active ones (Figure 4B). The lysosomal fraction isolated from cultured cells contain mainly active lysosomes (A.M. Cuervo, E. Knecht and J.F. Dice, unpublished results). The faster degradation rates of lamp2a in less active lysosomes can contribute to the shorter half-life of the protein in cellular extracts when compared with isolated lysosomes. In addition, part of the lamp2a can also be degraded in late endosomes or at the plasma membrane, its other main intracellular locations.

Different mechanisms have been described for the degradation of integral membrane proteins. In addition to the participation of cytosolic and lysosomal proteases in the degradation of some of those proteins (26–28), several examples of specific membrane-associated proteases responsible for the degradation of membrane proteins have been described (29–35). The coordinated action of two or more proteases in the release of proteins from different cellular membranes has been previously described for other integral membrane proteins such as the cholesterol-metabolism transcription factor (36) or the β-amyloid precursor protein (37). In both examples, intramembranous proteolysis of the substrate occurs. In addition to the release from membranes, the complete degradation of some intracellular proteins also requires the cooperative action of different proteolytic activities. For example, the degradation of the

vacuolar ABC transporter Ste6p in *Saccharomyces cerevisiae* involves coordinate cleavage by the proteasome, from the cytosolic side of the membrane, and the vacuolar proteases, from the vacuolar matrix (38).

We believe that the dual cleavage of lamp2a, described here for isolated lysosomal membranes, is relevant to the *in vivo* degradation pathway for lamp2a. Thus, fed animals have faster degradation rates for lamp2a (Figure 2B) and also increased processing of lamp2a (data not shown). The decreased degradation rates of lamp2a in the more active group of lysosomes are in concordance with their lower rates of lamp2a processing (Figure 4B). Finally, for fibroblasts cultured in the absence of serum, in which the degradation rate of lamp2a significantly decreases (Figure 1C), the amount of intermediate degradation forms of lamp2a in lysosomes is also lower than in lysosomes from serum-supplemented cells (data not shown).

The partial cleavage of lamp2a at the lysosomal membrane, such as shown when the second proteolytic activity is inhibited by increasing concentrations of AEBSF (Figure 4C, left), decreases the selective uptake of substrates. The truncated lamp2a might have a direct effect on the permeability of the lysosomal membrane (39). It is also possible that if the binding/transport of substrate proteins requires formation of multimeric complexes of lamp2a, the presence of partially cleaved lamp2a in those complexes might interfere with their normal multimerization. This finding might be especially relevant for understanding the malfunction of the chaperone-mediated autophagic pathway in different conditions such as aging.

Membrane levels of lamp2a are also modulated by changes in lamp2a distribution between membrane and matrix. Thus, the change in half-life of lamp2a in lysosomes (from 57 h in presence of serum to 112 h after serum removal) (Figure 1C, left) cannot explain by itself previously reported changes in the lysosomal content of lamp2a (e.g. in response to 20 h starvation) (A.M. Cuervo and J.F. Dice, unpublished results). Protein synthetic rates for lamp2a do not significantly change in the absence of serum (Figure 1A), so increased synthetic rates cannot account for this discrepancy. In some of the conditions previously analyzed (e.g. long starvation periods or different lysosomal populations), total lysosomal levels of lamp2a remained constant, but the distribution between membrane and matrix was changed (A.M. Cuervo and J.F. Dice, unpublished results). The reinsertion of part of the matrix lamp2a into the membrane during nutrient deprivation (Figure 5, left) may contribute to the increased lamp2a membrane levels. In the more active group of lysosomes the matrix lamp2a only amounts to 20% of total lysosomal lamp2a, but in less active lysosomes 40% of lamp2a is normally located in the matrix. Intact lamp2a can shuttle from the lysosomal membrane to the matrix (Figure 5A,B), and also in the opposite direction (Figure 5C).

It seems unlikely that lamp2a internalization is mediated by vesiculation of the lysosomal membrane since membrane invaginations or vesicles in lysosomes isolated from starved animals were rarely detected in previous electron microscopic studies (18,40,41). In addition, the lamp2a detected in the lysosomal matrix is not within sealed vesicles (Figures 6 and 7). The cytosolic region of the protein would be inside such vesicles, but this is the region most susceptible to proteolytic attack. The reasons for preferential cleavage of the cytosolic tail region are not known but may result from the heavy glycosylation of the rest of the molecule. The interaction of lamp2a with other proteins such as chaperones in the lysosomal matrix or lipids (Figure 7) might also protect the cytosolic and transmembrane region from proteolytic attack. Lipids associated with lamp2a (Figure 7B) may also play an important role in the membrane deinsertion or reinsertion of lamp2a.

Several other examples of membrane insertion/deinsertion of proteins have been recently described (42). SecA, a protein that participates in export of proteins across the bacterial plasma membrane, drives protein translocation by undergoing a cycle of membrane insertion and deinsertion (43,44). Membrane insertion of SecA requires interaction with acidic phospholipids in the membrane and ATP binding, while deinsertion is regulated by ATP hydrolysis (44) and the proton motive force (45). The membrane insertion of other proteins, such as thylakoid membrane proteins of chloroplasts, depends on GTP and a pH gradient (46). Finally, insertion into membranes independent of proteins, but probably driven by hydrophobic interactions between proteins and lipids, has been described for other thylakoid membrane proteins (47) and also for some imported mitochondrial proteins (48).

We have shown here that lamp2a membrane reinsertion is not ATP-dependent and does not depend on a pH gradient. However, it does require the normally negative inside/positive outside membrane potential (Figure 8B). High levels of sialic acid, polyphosphates and probably other negatively charged molecules have been described to negatively polarize lysosomes inside (20,49,50). This negative membrane potential is maintained without ATP and is insensitive to the blockage of the proton pump by bafilomycin (20). The negatively charged region has been identified structurally as a low electron density halo that separates the lysosomal membrane from the matrix contents (51). During the reinsertion process the cytosolic tail of lamp2a must completely cross the lysosomal membrane from the inside to the outside. For *E. coli* integral membrane proteins, the hydrophobic force generated from the transmembrane helix is enough to drive membrane insertion but charged residues flanking the hydrophobic anchor region determine their orientation in the membrane (22). In the cytosolic tail of lamp2a, the presence of four positive residues (KHHH) close to the transmembrane region could be responsible for its reinsertion in the right orientation.

Protease sensitive components in the lysosomal membrane are also required for lamp2a reinsertion (Figure 9). Of special interest for us is the role that the lysosomal membrane hsc73 might play in the regulation of lamp2a levels since blockage of the membrane-associated hsc73 interferes with lamp2a reinsertion (Figure 9B). The exact role of this molecular chaperone in promoting lamp2a reinsertion remains to be determined.

In summary, we present here two different mechanisms that contribute to the regulation of lamp2a levels in the lysosomal membrane and thereby to the regulation of chaperone-mediated autophagy. We propose that a portion of lamp2a is continuously cleaved from the lysosomal membrane following a two-step process that releases the truncated lamp2a into the lysosomal matrix where it is completely degraded. Under conditions of stress, such as nutrient deprivation, in which the activity of the pathway increases, the degradation of lamp2a is reduced resulting in an increased number of receptors at the lysosomal membrane. Transport of substrate proteins is coupled to the internalization of some of the lamp2a from the membrane into the lysosomal matrix. However, the levels of receptor at the lysosomal membrane remain elevated due to the reinsertion of lamp2a back into the lysosomal membrane.

Materials and Methods

Animals and cells
Adult male Wistar rats, weighing 200–250 g and fasted for 20 h before sacrifice, were used. Transformed rat embryo fibroblasts (Rat-2), mouse fibroblasts (NIH3T3) and human embryo kidney cells (HEK293) were from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified eagle medium (Sigma, St Louis, MO) in the presence of 10% newborn calf serum (NCS). To deprive cells of serum, plates were extensively washed with Hank's balanced salt solution (Life Technologies, Gaithersburg, MD) and medium without serum was added.

Chemicals
Sources of chemicals and antibodies were as described previously (5,17–19). The antibody against the cytosolic tail of rat lamp2a was raised in our laboratory (5). The monoclonal antibody against the matrix side of rat lamp2s was kindly supplied by Dr Michael Jadot (Facultes Universitaires Notre-Dame de la Paix, Namur, Belgium). The monoclonal antibody against hemaglutinin was a gift from Dr Anjana Rao (Department of Pathology, Harvard Medical School, Boston, MA). The monoclonal antibodies against the matrix region of the mouse lamp2s were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). *N*-Glycosidase F was from New England Biolabs (Beverly, MA), and valinomycin, monensin, bafilomycin A and apyrase were purchased from Sigma.

Isolation of rat liver subcellular fractions
Rat liver lysosomes were isolated from a light mitochondrial-lysosomal fraction in a discontinuous metrizamide density gradient (52) by the shorter method reported previously (40). After isolation, lysosomes were resuspended in 0.3 M sucrose/10 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.2 (MOPS

buffer). In some experiments, two separate lysosomal fractions with different activities for chaperone-mediated autophagy were isolated as described (10). Lysosomes from cultured cells were isolated as described (53). Integrity of the lysosomal membrane after isolation was systematically measured by β-hexosaminidase latency as previously described (17). Only preparations with more than 95% intact lysosomes were used. Lysosomal matrices and membranes were obtained as described by Oshumi (54). Briefly, lysosomal pellets were resuspended in 0.025 M sucrose and incubated for 30 min at 0°C. After hypotonic shock, lysosomal membranes were pelleted by centrifugation for 30 min at $105\,000 \times g$ (except where indicated) using a 1002 Ti rotor (Beckman Instruments, Palo Alto, CA). After removing the supernatants (lysosomal matrices), the pelleted membranes were washed once with MOPS buffer and resuspended in 0.3 M sucrose. Cytosolic fractions were obtained by centrifugation of the supernatant of the light mitochondrial-lysosomal fraction at $150\,000 \times g$ for 1 h at 4°C.

Sucrose density centrifugation and floatation assays

For sedimentation studies, matrices from lysosomes lysed in 5 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 10 mM 4-morpholineethanesulfonic acid were titrated to pH 5 or pH 8 and incubated for 30 min on ice. Samples were loaded on top of a 10–60% continuous sucrose gradient prepared in the same pH buffer and centrifuged at $250\,000 \times g$ for 20 h. Floatation assays of lysosomal matrices (pH 5) were performed as previously described (15) with the following modifications. Lysosomal matrices adjusted to 65% sucrose were overlaid with a continuous 10–60% sucrose density gradient adjusted to pH 5. The gradient was centrifuged for 16 h at $110\,000 \times g$.

Expression and purification of HA-lamp2a

Three consecutive HA epitope-tags were inserted in the hinge region of the cDNA for human lamp2a (2–4) and subcloned in the pCR3 mammalian expression vector (Invitrogen, San Diego, CA). Human embryo kidney cells transfected with that construct by the calcium phosphate method (55) were harvested 48 h post-transfection and solubilized in 1% octyl-glucoside in 50 mM Tris-HCl pH 8, 150 mM NaCl. HA-lamp2a in the solubilized fraction was recovered by affinity chromatography with anti-HA immobilized in a Sulfo-link gel (Pierce, Rockford, IL). The detergent in the purified fraction was removed by 24 h dialysis against MOPS buffer. No precipitation of HA-lamp2a was observed after detergent removal.

Synthesis rate and half-life of lamp2a

The biosynthetic rate of lamp2a was measured in mouse fibroblasts grown in 6-well plates. Three days after the cells reached confluence, they were incubated with 0.2 mCi/ml of an [^{35}S]methionine/cysteine mixture (Easy Tag-Express [^{35}S]; NEN™ Life Science Products, Boston, MA) in fresh medium supplemented with 10% NCS, or in the absence of serum but supplemented with the corresponding amount of methionine and cysteine present in 10% NCS. At the indicated times, cells were extensively washed and lysed in lysis buffer. Lysates were cleared by centrifugation and supernatants were incubated with a specific antibody against lamp2a or with an antibody that recognizes all forms of lamp2 previously conjugated to protein A-sepharose beads. After extensive washing with lysis buffer, the immunoprecipitate was subjected to SDS-PAGE. Dried gels were exposed to a PhosphorImager screen and quantified with a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). To determine degradation rates of cellular lamp2a mouse fibrob-

lasts at 60–70% confluency were radiolabeled with the same [^{35}S]methionine/cysteine mixture (0.2 mCi/ml) for 48 h in methionine/cysteine free medium (Sigma) supplemented with 10% NCS. After extensive washing, cells were maintained in the presence or absence of 10% NCS, and supplemented with a 2 mM mixture of cold methionine and cysteine. At increasing times, cells were recovered and processed as described above. The half-life of lamp2a was calculated after quantification in the PhosphorImager system from the formula $t_{1/2} = \ln2/\text{degradation rate}$. To determine the half-life of lamp2a in the lysosomal compartment, mouse fibroblasts grown in large square plates ($24.5 \times 24.5 \times 2$ cm) were labeled as above. At the indicated times of the chase period, cells were collected and lysosomes were isolated as described previously (53). Isolated lysosomes were then solubilized in lysis buffer and processed as described for the total cellular lysate.

Uptake and degradation of substrate proteins by isolated rat liver lysosomes

Substrate proteins were incubated with chymostatin-treated lysosomes as previously described (18,40). 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 GAPDH by isolated intact lysosomes or lysosomal matrices was measured as described (17). When protease inhibitors were added, the lysosomes (25 μg of protein) were first incubated with the indicated concentrations of inhibitors for 10 min at 0°C in a final volume of 50 μl of MOPS buffer containing 1 mM dithiothreitol and 5.4 μM cysteine. After that, 260 nM GAPDH radioactively labeled by reductive methylation ([^{14}C]GAPDH; (56)) was added and samples were incubated for 30 min at 37°C. 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) was collected on the filter. Radioactivity in the samples was converted to desintegrations per min 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. In every assay using intact lysosomes, the integrity of the lysosomal membrane at the end of the incubation was measured by β-hexosaminidase latency, and only experiments with > 93% intact lysosomes were considered.

Reinsertion of lamp2a in the lysosomal membrane

Intact rat liver lysosomes (25 μg protein) were incubated in the presence of GAPDH (50 μg) for 20 min at 37°C in MOPS buffer and then recovered by centrifugation ($26\,000 \times g$ for 5 min). Pellets were gently resuspended in MOPS buffer without GAPDH and incubated for 15 min under the same conditions. At the end of the incubation lysosomal membranes and matrices were separated by hypotonic shock as described above, and the content of lamp2a in each fraction was analyzed by immunoblotting. Where indicated, before the second incubation samples were incubated at 0°C for 10 min with ATP (5 mM), apyrase (10 μg/ml), monensin (10 μM)/Na-MOPS (25 mM), bafilomycin A (25 nM), valinomycin (1 μg/ml)/KCl (25 mM), anti-hsc73 (30 μg/ml), anti-lamp2a (50 μg/ml) or preimmune serum (50 μg/ml). For the membrane insertion of purified HA-lamp2a, vesicles formed by spontaneous resealing of sonicated lysosomal membranes were

used (54). Rat liver lysosomal membranes washed in MOPS buffer, 0.5 M NaCl or 0.1 M NaCO$_3$ were resuspended in MOPS buffer containing 30 μg of purified HA-lamp2a and sonicated three times for 10 s. Vesicles recovered by centrifugation at 185 000 × g for 15 min were then resuspended in MOPS buffer an incubated for 30 min at 37°C. At the end of the incubation, vesicles were pelleted and after hypotonic shock their membrane and lumen content of HA-lamp2a was analyzed by immunoblotting.

General methods

SDS-PAGE (57), immunoblotting (58) and fluorography (58) were performed by standard procedures. Protein concentration was determined by the Lowry method (59) using bovine serum albumin as a standard. Standard procedures were used for the determination of enzymatic activities as reported previously (17,18). Hsc73 was purified from rat liver cytosol by ATP-agarose affinity chromatography (60). 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.

Acknowledgments

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