Altered dynamics of the lysosomal receptor for chaperone-mediated autophagy with age

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Summary

Rates of autophagy, the mechanism responsible for lysosomal clearance of cellular components, decrease with age. We have previously described an age-related decline in chaperone-mediated autophagy (CMA), a selective form of autophagy, by which particular cytosolic proteins are delivered to lysosomes after binding to the lysosome-associated membrane protein type 2A (LAMP-2A), a receptor for this pathway. Rates of CMA decrease with age because of a decrease in the levels of LAMP-2A. In this work we have investigated the reasons for the reduced levels of LAMP-2A with age. While transcriptional rates of LAMP-2A remain unchanged with age, the dynamics and stability of the receptor in the lysosomal compartment are altered. The mobilization of the lysosomal lumenal LAMP-2A to the membrane when CMA is activated is altered in lysosomes from old animals, leading to the presence of an unstable pool of lumenal LAMP-2A. By contrast, the regulated cleavage of LAMP-2A at the lysosomal membrane is reduced owing to altered association of the receptor and the protease responsible for its cleavage to particular membrane microdomain regions. We conclude that age-related changes at the lysosomal membrane are responsible for the altered turnover of the CMA receptor in old organisms and the consequent decline in this pathway.

Key words: Aging, Autophagy, Lysosomes, Lysosomal membrane proteins, Proteases, Lipid microdomains

Introduction

Autophagy is a conserved intracellular process responsible for the continuous clearance of cellular components in lysosomes (Cuervo, 2004; Levine and Klionsky, 2004; Mizushima, 2005; Yorimitsu and Klionsky, 2005). Three different types of autophagy have been described in mammals: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Cuervo, 2004). The three autophagic pathways differ in the way in which substrates are delivered to lysosomes, their molecular components and the mechanisms that regulate each of them. Macroautophagy and microautophagy involve 'in bulk' delivery of cytosolic components, soluble proteins and complete organelles, to lysosomes (Levine and Klionsky, 2004; Yorimitsu and Klionsky, 2005). Microautophagy is considered constitutively active whereas macroautophagy is activated in response to stress – although basal macroautophagy activity has also been described in different tissues (Levine and Klionsky, 2004; Mizushima, 2005). CMA is responsible for the lysosomal degradation of particular cytosolic proteins that are delivered one by one into the lysosomal lumen after crossing the lysosomal membrane (Massey et al., 2006a). Although basal CMA activity can be detected in many cells at low levels, like macroautophagy, CMA is maximally activated under stress conditions (prolonged starvation, oxidative stress and cellular exposure to toxic compounds) (Massey et al., 2006a). Both macroautophagy and CMA activity have been shown to decrease with age (Cuervo and Dice, 2000c; Dice, 1982; Donati et al., 2001; Terman, 1995).

Protein substrates for CMA are delivered to the surface of lysosomes by a cytosolic chaperone, the 70 kDa heat shock cognate protein (hsc70) (Chiang and Dice, 1988), which promotes the interaction of the substrates with a receptor protein at the lysosomal membrane, the lysosome-associated membrane protein type 2A (LAMP-2A) (Cuervo and Dice, 1996). Substrate proteins are unfolded (Salvador et al., 2000), assisted by the complex of chaperones and co-chaperones associated with the membrane (Agarraberes and Dice, 2001), and are then translocated into the lysosomal lumen with the help of a luminal-resident chaperone (lys-hsc70) (Agarraberes et al., 1997). Once in the lysosomal matrix, substrate proteins are rapidly degraded into their constitutive amino acids. Rates of CMA are directly dependent on the levels of LAMP-2A at the lysosomal membrane because binding of substrates to this receptor is the limiting step of this autophagic pathway (Cuervo and Dice, 2000a).

Activation of CMA during starvation follows activation of macroautophagy and it probably provides the amino acids and energy necessary for proper cellular functioning after prolonged starvation, when macroautophagy is no longer active (Mizushima, 2005). CMA is also activated as part of the cellular response to oxidative stress, and blockage of CMA renders cells susceptible to different stressors (Kiffin et al., 2004; Massey et al., 2006b). Impaired CMA has been proposed to underlie the pathogenesis of familial forms of Parkinson's disease and the renal hypertrophy associated with diabetes mellitus (Massey et al., 2006a). The described decrease in

CMA activity with age probably contributes to the worsening of these pathologies in aging (Cuervo and Dice, 2000c; Dice, 1982).

We have previously found that a decrease in the lysosomal levels of LAMP-2A is the main cause of the decline in CMA activity with aging, but the reasons for this decrease remain unknown (Cuervo and Dice, 2000c). LAMP-2A is a single span transmembrane protein with a heavily glycosylated luminal domain and a short (12 amino acids) cytosolic tail (Cuervo and Dice, 1996), where CMA substrates bind before translocation into the lumen (Eskelinen et al., 2003). Levels of LAMP-2A are regulated by both extralysosomal and lysosomal mechanisms. During oxidative stress, the observed increase in levels of LAMP-2A, and consequently higher CMA activity, is attained through transcriptional upregulation (Kiffin et al., 2004). However, during nutritional stress, the increase in LAMP-2A levels does not involve de novo synthesis of this receptor, but instead, is due to a decrease in the degradation rates of LAMP-2A at the lysosomal membrane and the mobilization of a fraction of the receptor normally resident in the lysosomal lumen toward the lysosomal membrane (Cuervo and Dice, 2000a). Dynamic association of LAMP-2A with discrete lipid microdomains in the lysosomal membrane plays a critical role in the regulation of its lysosomal levels (Kaushik et al., 2006). Because cathepsin A (Cuervo et al., 2003), the protease that initiates the selective cleavage of LAMP-2A, also localizes in these particular lipid regions, only the LAMP-2A sequestered in lipid microdomains is subjected to degradation.

Using a bitransgenic mouse model we have found that restoration of normal levels of LAMP-2A in old rodents preserves intact CMA activity and dramatically reduces the age-related intracellular accumulation of oxidized proteins (C.Z. and A.M.C., unpublished results). These results support the possibility of correcting CMA function in old organisms by preventing the age-dependent decrease of lysosomal LAMP-2A levels and justify our efforts to identify the reasons for this decrease.

In the present study we found that the decreased levels of LAMP-2A with age are not a consequence of transcriptional downregulation, but instead, originate primarily from changes in the lysosomal compartment which affect the normal turnover and dynamics of LAMP-2A. Age-related changes in the ability to incorporate the receptor into particular discrete membrane microdomains crucial for CMA regulation are behind the decreased levels of the CMA receptor in old organisms.

Results

Transcriptional regulation of LAMP-2A is preserved in old rodents

To analyze whether the decreased levels of LAMP-2A in rodents are a consequence of transcriptional downregulation of the *Lamp2* gene, we used quantitative real-time PCR to measure LAMP-2A mRNA levels in the livers of 4-, 12- and 22-month-old rats. As shown in Fig. 1A, we did not find significant differences in the levels of LAMP-2A mRNA with age, independently of whether CMA was maximally activated (starved animals) or not (fed animals). We obtained similar results using livers from two different strains of mice (data not shown), confirming that the observed decrease in the levels of the LAMP-2A protein cannot be explained by transcriptional downregulation. Transcriptional upregulation of LAMP-2A in response to mild oxidative stress is shown as a positive control (Kiffin et al., 2004).

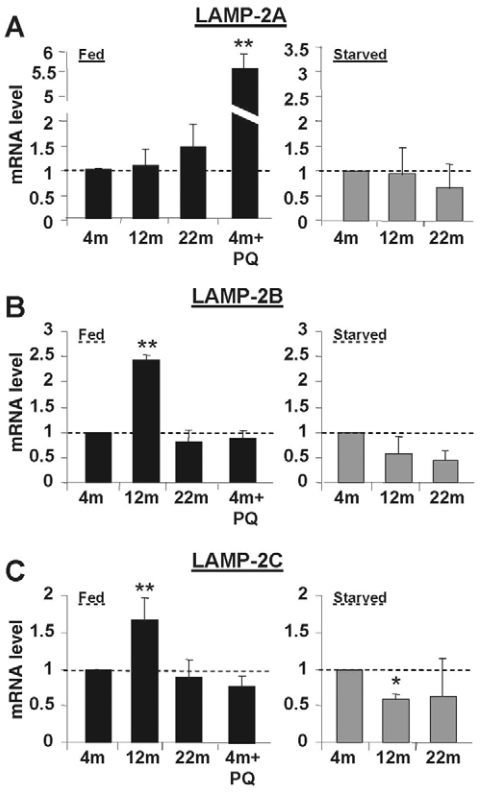

Fig. 1. Changes in the mRNA levels of the different LAMP-2 splicing variants with age. Semi-quantitative real-time PCR was used to compare mRNA expression levels for LAMP-2A (A), LAMP-2B (B) and LAMP-2C (C) in total mRNA isolated from the livers of normally fed (Fed) or 48-hour starved (Starved) 4-, 12- and 22-month-old rats. mRNA expression levels for the three isoforms in fed rats exposed to mild oxidative stress (treated with two injections of 40 mg/kg body weight of paraquat every 24 hours) are shown as a positive control for LAMP-2A transcriptional upregulation. Values were corrected for actin amplification in each sample and are expressed as fold increase compared with values for the different isoforms in 4-month-old rats (given an arbitrary value of 1). Values are mean + s.e.m. from four different experiments. Significant differences were observed compared with values from 4-month-old rats (*P<0.05, **P<0.01).

Lamp2 is a single gene that undergoes alternative splicing rendering three different mRNA species that encode the LAMP-2A, LAMP-2B and LAMP-2C variants of this protein (Gough et al., 1995). The three protein products have identical luminal regions (first eight or seven exons in human and rodents, respectively) but different transmembrane and cytosolic tail regions (last exon). The levels of the different isoforms vary from tissue to tissue and during embryogenesis (Licheter-Konecki et al., 1999). Although it is possible that all the isoforms share common functions, isoform-specific functions have also been described. LAMP-2A is the only isoform shown to participate in CMA (Cuervo and Dice, 1996), whereas mutations in LAMP-2B lead to major impairment in macroautophagy (Nishino et al., 2000). The function of LAMP-2C still remains unknown. Very little is known about

how the splicing is regulated and how changes in this regulation could affect the different forms of autophagy. To analyze possible changes in *Lamp2* splicing with age, we also measured mRNA levels of LAMP-2B and LAMP-2C in both fed and starved rats (Fig. 1B,C). In contrast to the levels of LAMP-2A which remained constant in both conditions, we found a consistent increase in the levels of LAMP-2B and LAMP-2C mRNA in livers of 12-month-old fed rats, but in both cases this increase was no longer evident in the oldest animals, or when the animals were previously starved (Fig. 1B,C). Although the relevance of the changes in LAMP-2B and LAMP-2C remains unknown, these results support that the observed decrease in LAMP-2A levels with age is not a consequence of transcriptional down-regulation or changes in the splicing of the *Lamp2* gene to favor expression of other LAMP-2 isoforms in the oldest animals.

Altered lysosomal dynamics of LAMP-2A in aging

We and other groups have reported the presence of intact molecules of LAMP-2A in the lysosomal lumen (Cuervo and Dice, 2000a; Cuervo and Dice, 2000b; Jadot et al., 1996). Part of this protein is redistributed to the lysosomal membrane in conditions that require maximal activation of CMA (Cuervo and Dice, 2000a). This dynamic distribution of LAMP-2A between the lysosomal membrane and lumen contributes to CMA regulation (Cuervo and Dice, 2000a). To determine possible changes in this regulatory mechanism with age, we first compared the percentage of LAMP-2A present in each compartment during CMA activation in lysosomes from livers of 4-, 12- and 22-month-old rats. Although the absolute amount of LAMP-2A decreases in both the membrane and the lumen (Cuervo and Dice, 2000c), we found a more pronounced effect on the membrane LAMP-2A. Thus the fraction of lysosomal LAMP-2A present at the lysosomal membrane decreased with age, increasing the percentage of LAMP-2A present in the lysosomal lumen. These results support the existence of possible alterations in the lysosomal dynamics of LAMP-2A with age (Fig. 2A).

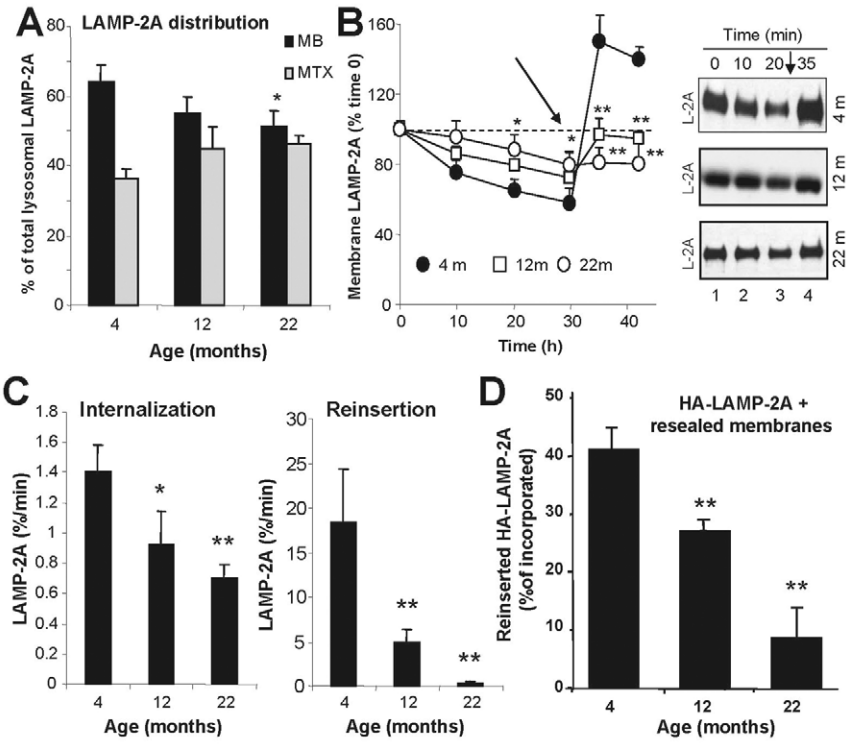

Fig. 2. Age-related changes in the dynamic distribution of LAMP-2A in lysosomes. (A) Distribution of LAMP-2A between lysosomal membrane and matrix. Membranes (MB) and matrices (MTX) from lysosomes isolated from livers of 4-, 12- and 22-month-old rats were subjected to SDS-PAGE and immunoblot against LAMP-2A. The percentage of total lysosomal LAMP-2A present in each compartment was calculated by densitometry of the immunoblots. Values are mean + s.e.m. of four to five different experiments. (B) Mobilization of LAMP-2A between the lysosomal membrane and matrix. Intact lysosomes from livers of 4-, 12- and 22-month-old rats were incubated in the presence of GAPDH (25 μg) for 15 minutes at 37°C and then the substrate was rapidly removed by centrifugation (arrows) and lysosomes were incubated in a substrate-free isotonic medium. At the indicated times, lysosomal membranes were isolated and subjected to immunoblot against LAMP-2A. Values are expressed as the percentage of LAMP-2A present at the lysosomal membrane at the beginning of the incubation and are the mean + s.e.m. of three different experiments. Right panels show representative immunoblots. (C) Rates of LAMP-2A internalization (left) and retrieval back to the lysosomal membrane after removal of GAPDH (right) were calculated from the data in B. Values are expressed as changes in the percentage of membrane LAMP-2A per minute. (D) Mobilization of recombinant LAMP-2A into lysosomal membranes. Purified recombinant HA-tagged LAMP-2A was placed into resealed lysosomal membranes isolated from 4-, 12- and 22-month-old rats as described under Materials and Methods. Vesicles were incubated at 37°C for 15 minutes and the amount of HA-LAMP-2A associated with the lysosomal membrane was quantified by immunoblot against HA of the membranes isolated by hypotonic shock and centrifugation (protease protection assay was used to determine that all the HA-LAMP-2A associated with the membrane had a cytosolic exposed C-terminus). Values are expressed as percentage of the total LAMP-2A incorporated in each group of resealed membranes and are mean + s.e.m. of three different experiments. Significant differences were observed compared with values from 4-month-old rats (*P<0.05; **P<0.01).

Although the molecular mechanisms involved in the retrieval of LAMP-2A to the lysosomal membrane when CMA is activated remain, for the most part, unknown, we have previously optimized an assay with isolated lysosomes that allows tracking of this process (Cuervo and Dice, 2000d). During active substrate translocation, we have found that a balance is continuously maintained between the amount of membrane LAMP-2A internalized with substrate proteins into the lumen and the amount of LAMP-2A retrieved back to the lysosomal membrane (Cuervo and Dice, 2000d). However, if during active uptake the substrate is suddenly removed (by centrifugation), internalization of LAMP-2A stops but retrieval back to the membrane persists for a longer time resulting in a net increase in the amount of LAMP-2A at the lysosomal membrane (Cuervo and Dice, 2000d). Thus, as shown in Fig.

2B, mobilization of LAMP-2A to the membrane can be determined in this assay comparing the membrane levels of LAMP-2A by immunoblot of lysosomes collected at different times. When lysosomes from livers of 4-month-old rats are incubated with CMA substrate proteins [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) shown here], there is an initial decrease in levels of LAMP-2A at the lysosomal membrane (about 30%; internalization step) resulting from the initiation of internalization of LAMP-2A associated to the substrate proteins. Right after the substrate is removed, LAMP-2A is no longer internalized but it is still retrieved to the membrane (reinsertion step), resulting in a net increase in LAMP-2A levels at the lysosomal membrane (up to 40% more than at time 0). By contrast, when we carried out similar experiments with intact lysosomes from 12- and 22-month-old rats, we found that internalization rates of LAMP-2A were gradually reduced (in agreement with the reduced rates of substrate uptake observed as animals age) (Fig. 2B), and that fewer molecules of LAMP-2A were retrieved to the lysosomal membrane, resulting in lower net amounts of the protein in this compartment after activation of CMA (Fig. 2B). The fact that the reduction in the rates of LAMP-2A reinsertion was more pronounced in both 12- and 22-month-old rats than in the rates of internalization (Fig. 2B,C), supports the concept that the decreased reinsertion of LAMP-2A is not merely the consequence of lower internalization, but that there is a primary and gradual defect in the ability of old lysosomes to mobilize LAMP-2A from the lysosomal lumen into the membrane.

To determine whether the poor rates of LAMP-2A mobilization to the lysosomal membrane in old rodents could result, at least in part, from age-related changes in the lysosomal membrane, or were solely the consequence of changes in the LAMP-2A molecule with age, we compared the reinsertion of recombinant LAMP-2A in lysosomal membranes isolated from rats of different ages. We have previously demonstrated that reinsertion can be reproduced with purified recombinant HA-tagged LAMP-2A and resealed lysosomal membranes lacking any lumenal content (Cuervo and Dice, 2000d). Reinsertion is independent of lumenal pH but it requires an intact membrane potential, which is restored once the membranes are resealed (Cuervo and Dice, 2000d). When isolated lysosomal membranes are sonicated on ice in the presence of recombinant LAMP-2A, they reseal, trapping LAMP-2A in their lumen. Incubation of the resealed vesicles at 37°C promotes reinsertion of part of the luminal LAMP-2A into the membrane. The cytosolic tail of LAMP-2A, but not its luminal (HA-containing) region, then becomes accessible to exogenously added proteases (Cuervo and Dice, 2000d). Using this assay we found a significant decrease in the amount of recombinant HA-LAMP-2A retrieved into the membranes with age (Fig. 2D). Because in all three assays the LAMP-2A utilized was the same and the only difference was the origin of the lysosomal membranes (4-, 12- or 22-month-old rats) we can conclude that the poor mobilization of LAMP-2A toward the membrane with age is due, at least in part, to changes in the lysosomal membrane.

Altered degradation of LAMP-2A in lysosomes with age

In addition to the dynamic distribution of LAMP-2A between the lysosomal membrane and matrix, changes in the degradation of LAMP-2A at the lysosomal membrane also contribute to regulating the amount of receptor available for substrate binding, and consequently CMA activity (Cuervo and Dice, 2000d). To determine possible changes with age in the degradation of LAMP-2A we compared its half-life in primary fibroblasts in culture at early and late passage. Although cell passage does not necessarily model all aspects of aging, we have previously found that senescent fibroblasts in culture display similar changes in CMA as those described in old rodents (decreased CMA activity and lower lysosomal levels of LAMP-2A when compared with early passage fibroblasts) (Cuervo and Dice, 2000c). Because we had found a marked decrease in the membrane levels of LAMP-2A with age, we expected to find accelerated rates of degradation for this protein. In fact, when we measured the degradation of LAMP-2A in primary fibroblasts in culture at early and late passage, after metabolic labeling and immunoprecipitation of LAMP-2A with an antibody specific for the cytosolic tail, we found considerably faster degradation rates for LAMP-2A in the late passage fibroblasts (Fig. 3A, insets). Quantification of the radioactivity associated with LAMP-2A at each time point allowed us to estimate the half-life of LAMP-2A to be about 38 hours under CMA resting conditions (supplemented with serum) in early passage fibroblasts, which increased to 77 hours when CMA was activated (by serum removal) (Fig. 3A). The half-life of LAMP-2A in late passage mouse fibroblasts, both in resting and CMA activated conditions, was significantly shorter (26 hours and 31 hours, respectively) (Fig. 3A). As in the early passage fibroblasts, almost all LAMP-2A degradation was still taking place in lysosomes, because it could be inhibited if the intralysosomal pH was raised with ammonium chloride (data not shown). These results indicated that the intracellular turnover of LAMP-2A increases with age.

To further characterize the reasons for this accelerated degradation of LAMP-2A we then compared the degradation of the membrane-associated and luminal LAMP-2A in lysosomes isolated from young and old rats. Using the antibody against the cytosolic tail of LAMP-2A, which allows us to track the disappearance of this tail as it gets cleaved off by the membrane proteases, we found that the rates of LAMP-2A cleavage in the membranes of lysosomes from older animals were significantly lower than in lysosomes from young animals (Fig. 3B, left). In fact, using an antibody against the luminal region of LAMP-2 to detect the truncated (lower molecular weight) form of the protein after the cytosolic tail and transmembrane region are cleaved (Fig. 3B, right top panel), we found that this truncated form of LAMP-2A was clearly visible in the lysosomes from young rats, but it was barely detectable in the lysosomes from old rats. By contrast, the same antibody recognized a wider immunoreactive band of lower molecular weight, which was observed only in the lumen of the lysosomes from the oldest animals (Fig. 3B, right bottom panel). This lower molecular weight form of the protein results from proteolytic cleavage rather than deglycosylation, as it is not observed in the presence of protease inhibitors (data not shown). The appearance of the matrix truncated form is not associated with a decrease in the cytosolic tail levels of LAMP-2A at the membrane, supporting the fact that it probably originates from degradation of the matrix-resident LAMP-2A (which is quite stable in the lysosomes from young animals) (Fig. 3B, bottom panel arrow). Thus, the accelerated

degradation of LAMP-2A in old rodents and senescent fibroblasts is probably a consequence of the observed instability of the protein in the lysosomal lumen with age, and not due to enhanced cleavage at the lysosomal membrane, which seems severely impaired with age.

Changes with age in the proteolytic complex responsible for LAMP-2A cleavage at the lysosomal membrane

We then analyzed the reasons for the inefficient cleavage of LAMP-2A at the lysosomal membrane with age. We have previously shown that protective protein/cathepsin A, a lysosomal serine carboxypeptidase, is the protease that triggers LAMP-2A lysosomal degradation (Cuervo et al., 2003). LAMP-2A undergoes two consecutive cuts in its cytosolic and transmembrane region that lead to the release of the truncated form of LAMP-2A into the lumen where it is completely degraded (Cuervo and Dice, 2000d). Although cleavage by a metalloprotease at the membrane precedes cleavage by cathepsin A, association of cathepsin A to the lysosomal membrane is limiting, as it is required to initiate LAMP-2A cleavage (Cuervo et al., 2003). In agreement with the lower degradation rates for LAMP-2A observed in the oldest animals, we found less cathepsin A associated with the lysosomal membranes isolated from 22-month-old rats compared with membranes from 4-month-old rats (Fig. 4A). The reduced levels of membrane-associated cathepsin A did not result from a decrease in the total amount of cellular or lysosomal cathepsin A, as they were comparable in both groups of animals or even slightly higher in the oldest animals (Fig. 4B). Total and specific carboxypeptidase activity of cathepsin A, required for LAMP-2A cleavage, also remained unchanged with age (Fig. 4C). However, the ability of cathepsin A to bind to the lysosomal membrane was severely impaired in the old animals. We have previously shown that binding of cathepsin A to the lysosomal membrane is a Ca^{2+}-dependent process (Cuervo et al., 2003). Incubation of intact liver lysosomes from 4- and 22-month-old rats with calcium, promoted the expected increase in the amount of membrane-associated cathepsin A in the lysosomes from the younger rats, but did not change or sometimes even lowered the levels of cathepsin A associated to the membrane of lysosomes from 22-month-old rats (Fig. 4D). These results support the fact that a primary defect in the ability of the protease that initiates the cleavage of LAMP-2A to associate with the lysosomal membrane may be the reason for the slower degradation of LAMP-2A at the lysosomal membrane with age. Paradoxically, we observed a progressive increase with age in the activity of the still unknown metalloprotease that also participates in LAMP-2A cleavage (Fig. 4E). Whether this increase in activity is a compensatory response to the reduced levels of cathepsin A at the lysosomal membrane, or whether it occurs independently of the changes in cathepsin A, is currently unknown.

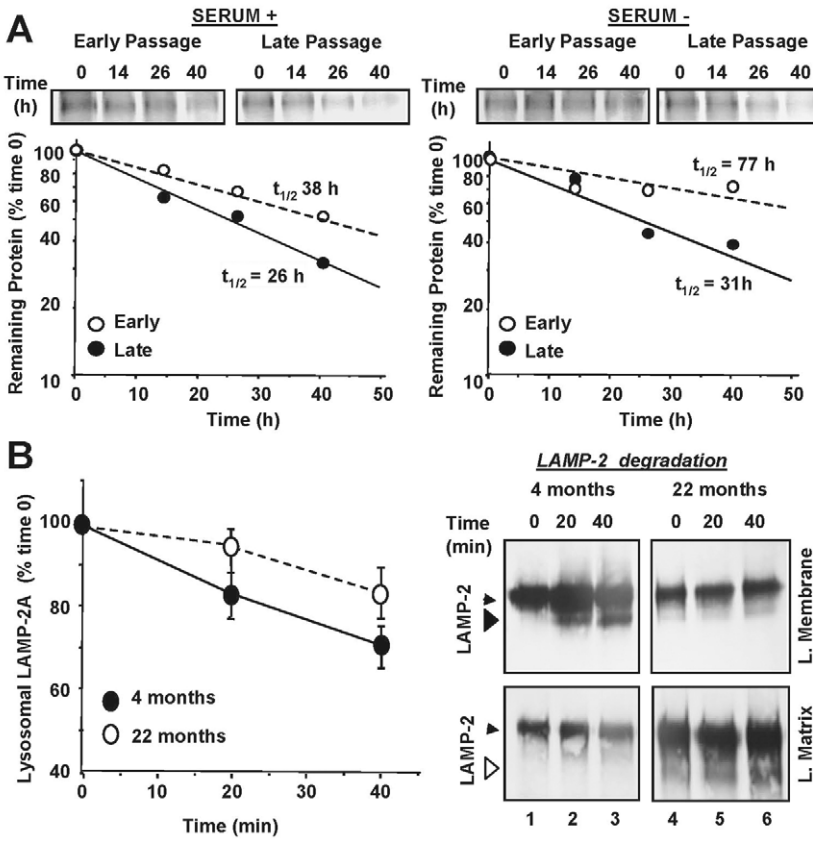

Fig. 3. Changes in the lysosomal degradation of LAMP-2A with age. (A) Half-life of LAMP-2A in cultured primary mouse fibroblasts. Early [population doubling level (PDL) 4-6] and late (PDL 12-15) passage primary mouse fibroblasts were labeled with [^{35}S]methionine:cysteine mixture and at the indicated times cells were lysed and subjected to immunoprecipitation with the antibody against the LAMP-2A cytosolic tail. Immunoprecipitates at different times from cells maintained in the presence (serum+) or absence (serum–) of serum were subjected to SDS-PAGE and fluorography, which were quantified by exposure to a PhosphorImager screen. Values are expressed as a percentage of radiolabeled LAMP-2A at time 0 and are mean of three different experiments. The best exponential decay curve was calculated by linear regression analysis and the half-life of LAMP-2A was calculated by the formula $t_{1/2}$=ln2/degradation rate. (B) Degradation of LAMP-2A in lysosomes. Liver lysosomes from 4- and 22-month-old rats were incubated at 37°C in an isotonic medium. At the indicated times lysosomes were pelleted and membranes and matrices were isolated after hypotonic shock by centrifugation. Samples were divided into two halves and subjected to SDS-PAGE and immunoblot with the antibody that recognizes the cytosolic tail of LAMP-2A or an antibody against the luminal region of LAMP-2, common to all spliced variants (shown here). The graph shows the changes in the amount of cytosolic tail at different times expressed as percentage of the amount present before the incubation. Values are mean ± s.e.m. of three different experiments. Filled arrowhead indicates the truncated form of LAMP-2A generated by cleavage of the protein at the lysosomal membrane, while open arrowhead indicates an unusual lower molecular weight LAMP-2A detected only in the lumen of lysosomes from the oldest animals.

Changes at the lysosomal membrane are responsible for the age-related changes in LAMP-2A levels with age

The inability of LAMP-2A to reinsert back into lysosomal membranes from old animals and the impaired binding of

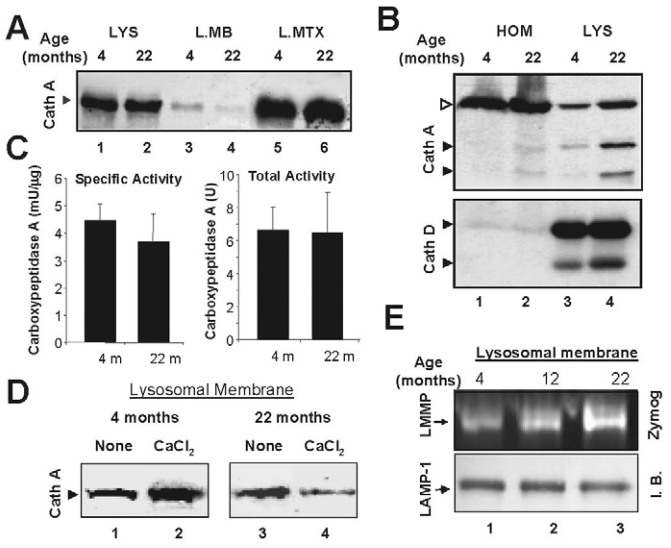

Fig. 4. Changes in the LAMP-2A-related protease complex at the lysosomal membrane with age. (A,B) Homogenates (HOM), Lysosomes (LYS), lysosomal membranes (L. MB) and matrices (L. MTX) (100 μg protein) from 4- and 22-month-old rats were subjected to SDS-PAGE and immunoblotted for cathepsin A or cathepsin D (as labeled). White arrowhead indicates the precursor protein while black arrowheads indicate the 32 and 22 kDa catalytic subunits. Only the 32 kDa catalytic subunit is shown in A. (C) Carboxypeptidase activity in lysosomes from 4- and 22-month-old rats was measured. Values are shown as total activity in the lysosomal fraction (right) or specific activity (corrected per protein amount; left) and are means + s.e.m. of three different experiments. (D) Intact lysosomes from 4- and 22-month-old rats were incubated in isotonic buffer supplemented or not with 1 mM $CaCl_2$ for 15 minutes. At the end of the incubation, lysosomal membranes were isolated after hypotonic shock and centrifugation and were subjected to SDS-PAGE and immunoblot for cathepsin A. (E) Lysosomal membranes from 4-, 12- and 22-month-old rats were subjected to zymography (zymog) in a gelatin-embedded gel and the areas of proteolytic cleavage were identified after staining with Amido Black. LMMP, lysosomal membrane metalloprotease. Immunoblot (I.B.) of the same membranes for LAMP-1 is shown in the bottom panel.

cathepsin A to the lysosomal membrane with age both point to the existence of age-dependent changes at the lysosomal membrane. Therefore, we analyzed in more detail the particular regions of the lysosomal membrane known to play a critical role in CMA regulation. We have recently found that LAMP-2A associates in a dynamic manner to discrete membrane microdomains where cathepsin A also localizes, thus becoming the regions where degradation of LAMP-2A takes place (Kaushik et al., 2006). We can isolate these LAMP-2A-containing membrane microdomains based on their resistance to extraction with the non-ionic detergent Triton X-114, followed by floatation in discontinuous sucrose density gradients (Kaushik et al., 2006). When we subjected lysosomal membranes isolated from livers of 4- and 22-month rats to these procedures and compared the distribution of LAMP-2A between the detergent-resistant regions and the regions solubilized by the detergent, we found a significant decrease with age in the percentage of LAMP-2A that localized into the discrete membrane microdomains under CMA resting conditions (when LAMP-2A is more actively degraded) (Fig. 5A, left). As we have previously reported, LAMP-2A was excluded from those regions in young animals when CMA was activated (Fig. 5A, right). Levels of flotillin-1, a protein resident in the microdomains often used as a marker for these regions, remained unchanged with age (Fig. 5B), suggesting that the decrease in the number of LAMP-2A molecules recovered in this fraction in the old animals was not due to a disruption of the membrane microdomains themselves (Fig. 5A). We obtained similar results when we used lysosomes from mice of different ages (data not shown). Because all the isolations were performed at 4°C and in the presence of protease inhibitors, it is unlikely that the lower amount of LAMP-2A in these regions results from a faster degradation in the old animals, but rather that there is a primary defect in the ability of LAMP-2A to reach the membrane microdomains.

We then compared the protein and lipid composition of the membrane microdomains isolated from rats of different ages. As shown in Fig. 5C, although the protein electrophoretic profile of the lipid microdomains was for the most part conserved with age, we found discrete age-dependent changes in the abundance of particular proteins, which could be the result of their inefficient targeting to those regions with age (the electrophoretic profile of the detergent-resistant regions is shown, and changing proteins are indicated with arrows). Regarding the lipid composition, we have previously shown that the LAMP-2A-containing membrane microdomains are particularly enriched in cholesterol. Interestingly, we found that in contrast to the gradual increase in total cellular cholesterol levels with age, lysosomes active for CMA isolated from the older animals contained significantly lower amounts of cholesterol in their membranes (Fig. 5D). We have previously shown that a decrease in the lysosomal cholesterol content, for example after treatment with cholesterol-depleting drugs such as methyl-β-cyclodextrin, reduced the amount of LAMP-2A that localizes in the lipid microdomains (Kaushik et al., 2006). Consequently, the observed reduction in lysosomal membrane cholesterol with age could explain, at least in part, the lower amount of LAMP-2A present in these regions in old rodents and, as a result, the impaired turnover of this receptor protein.

Discussion

Here we have shown that the decrease in levels of LAMP-2A that leads to declined CMA activity in aging does not result from an age-dependent transcriptional downregulation of this receptor (Fig. 1), but instead it originates from changes in the lysosomal membrane (Fig. 5), which alter LAMP-2A dynamic redistribution between the lysosomal membrane and the lumen (Fig. 2) and prevent its normal turnover (Fig. 3). Abnormal degradation of LAMP-2A in the lysosomal lumen replaces the regulated degradation of LAMP-2A, which is severely impaired in old rodents. The inability of LAMP-2A and cathepsin A, the selective protease responsible for the cleavage that triggers LAMP-2A degradation, to localize in discrete regions of the lysosomal membrane seems primarily responsible for the impaired processing of membrane LAMP-2A with age (Fig. 4). We propose that changes with age in the lysosomal membrane, rather than in the CMA receptor (Fig. 5), might be responsible for its reduced lysosomal levels in old cells and the consequent defective CMA with aging.

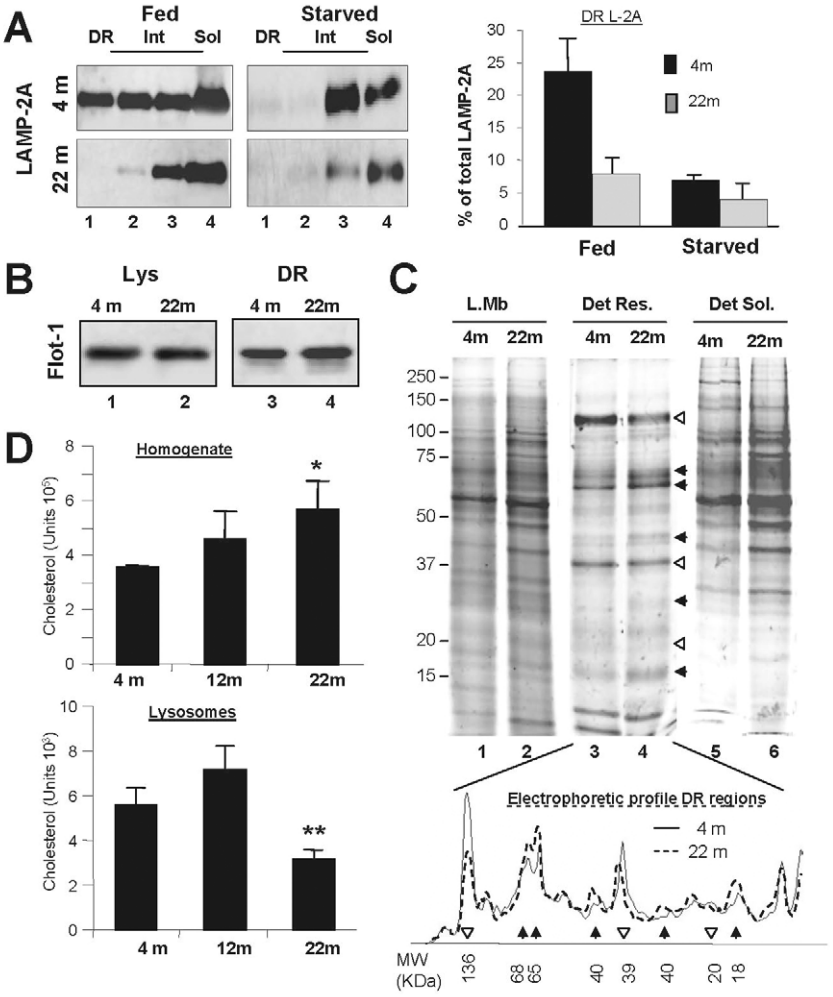

Fig. 5. Age-related changes in the lipid microdomains at the lysosomal membrane involved in CMA regulation. (A) Lysosomes from livers of fed or 48-hour-starved 4- and 22-month-old rats were extracted with 1% Triton X-114 and then subjected to sucrose gradient centrifugation. Aliquots collected from top to bottom were grouped as the detergent-resistant (DR), intermediate (Int) and detergent-soluble (Sol) fractions, which were then subjected to SDS-PAGE and immunoblotted for LAMP-2A. The graph on the right shows the mean value of the densitometric quantification of the LAMP-2A immunoblots from three experiments. Values are expressed as a percentage of the total LAMP-2A at the lysosomal membrane present in each region. (B) Flotillin-1 (Flot-1) content in lysosomes (Lys) and detergent-resistant regions (DR) isolated and processed as in A from livers of fed 4- and 22-month-old rats. Because Flotillin-1 localizes preferentially in the DR region, levels in the soluble fraction were undetectable. (C) Lysosomal membranes from 4- and 22-month-old rats and their corresponding Triton X-114-resistant (DR) and soluble (Sol) regions were subjected to SDS-PAGE and staining with Sypro Ruby. The densitometric electrophoretic profile of the detergent-resistant regions is shown (bottom). Arrowheads indicate decreased (white) or increased (black) levels compared with 4-month-old rats. (D) Quantification of cholesterol content in homogenates (Top) and lysosomes (bottom) from 4-, 12- and 22-month-old rat livers was carried out by the Amplex Red cholesterol assay. Values are expressed as total units in the fraction and are the mean + s.e.m. of triplicate samples in two different experiments. Significant differences were observed compared with values from 4-month-old rats (*P<0.05, **P<0.01).

Although our studies revealed no participation of changes in transcription of LAMP-2A in the decreased levels of this protein with age, some of the changes observed in the other spliced variants merit future attention. In particular, the significant transcriptional upregulation of the LAMP-2B and LAMP-2C variants in middle age rodents could offer some clues about the relation between the two stress-related autophagic pathways, CMA and macroautophagy. Thus, we have found that blockage of CMA induces constitutive activation of macroautophagy (Massey et al., 2006b), while CMA is upregulated in cells lacking essential macroautophagy components (S.K., N. Mizushima and A.M.C., unpublished data). Although both macroautophagy and CMA activities are reduced in old organisms, the chronological order of their decline is unknown. It is possible that the increase in the levels of the LAMP-2B isoform (a proposed macroautophagy player) observed at 12 months of age, when CMA activity is already reduced, conforms to a cellular attempt to compensate for the defective CMA by activating macroautophagy. Future time course studies of changes in both autophagic pathways with age are needed to test this hypothesis, and to determine the consequences of the observed upregulation of LAMP-2C at that age.

The decrease in LAMP-2A degradation at the lysosomal membrane with age was an unexpected finding, as we had anticipated that the lower membrane levels of the receptor in old rodents were probably a consequence of an accelerated turnover. The tightly regulated cleavage of LAMP-2A at the lysosomal membrane seems to be replaced by a less regulated degradation of this protein in the lysosomal lumen. We have previously shown that the LAMP-2A resident in the lysosomal lumen is quite stable, and that it has to be reinserted back into the lysosomal membrane to undergo regulated cleavage and degradation (Cuervo and Dice, 2000a). However, in lysosomes from old animals, a percentage of the protein present in the lumen is degraded probably through a different mechanism than the membrane protein (in fact, it shows a different proteolytic pattern). The degradation of LAMP-2A in the lysosomal lumen does not seem to be regulated in the same way as that taking place in the membrane. In fact, while in early passage fibroblasts, activation of CMA (by serum removal) slowed down LAMP-2A degradation, thus contributing to an increase in the amount of receptor available for the transport of substrate proteins, degradation rates of LAMP-2A remained unchanged after CMA activation in late passage fibroblasts. This loss in the ability to control levels of LAMP-2A by regulation of its degradation, is probably one of the main reasons for the inability of old organisms to upregulate CMA in response to nutritional stress (Cuervo and Dice, 2000c).

The reason(s) for the instability of LAMP-2A in the lumen

of lysosomes from old organisms remains unknown. Other authors have proposed that luminal lysosomal proteins organize in semi-aggregate complexes to avoid degradation (Jadot et al., 1996). It is possible that changes in the intralysosomal environment with age alter the ability of LAMP-2A to integrate into luminal complexes, or that the properties of these complexes themselves change with age. We cannot discount the idea that age-dependent changes in LAMP-2A – partial misfolding or covalent modifications such as oxidation or nitration – might render the protein more susceptible to degradation by the luminal proteases. The proteolytic pattern of the luminal LAMP-2A was different to that in the membrane, and in contrast to the membrane degradation of LAMP-2A, its luminal degradation was no longer inhibited by metalloprotease inhibitors, but instead it was blocked by cysteine and aspartic protease inhibitors (data not shown), Therefore, we conclude that the membrane protease complex associated with LAMP-2A (cathepsin A and an unidentified metalloprotease), is no longer responsible for the luminal cleavage, and that this is mediated instead by luminal resident cathepsins.

The role of the regulated reinsertion of LAMP-2A into the lysosomal membrane in the regulation of CMA activity has been shown in both cultured cells and in lysosomes from livers of rats and mice (Cuervo and Dice, 2000a). The diminished ability to mobilize LAMP-2A to the membrane with age could explain the higher percentage of LAMP-2A present in the lumen of lysosomes from older animals. We cannot discount the idea that modifications in LAMP-2A might be a result of its longer persistence in the matrix, which can alter the ability of the protein to reinsert into the lysosomal membrane. However, our study supports the notion that changes in the membrane with age clearly contribute to the low LAMP-2A retrieval. Membrane-associated hsc70 is the only membrane component identified so far to be necessary for reinsertion of LAMP-2A (Cuervo and Dice, 2000a). However, membranes of lysosomes from older animals have the same or sometimes even higher levels of hsc70 than membranes from younger animals (Cuervo and Dice, 2000c), suggesting that changes in membrane components other than hsc70 are responsible for the lower membrane retrieval of LAMP-2A.

The dynamic association of LAMP-2A to discrete membrane microdomains plays an important role in the regulation of CMA (Kaushik et al., 2006). We have previously shown that in conditions of CMA activation LAMP-2A is excluded from the lipid microdomains, thus promoting its organization into a functional multimeric complex. However, the lower levels of LAMP-2A in the microdomains of lysosomal membranes from old animals do not result in higher activity in these lysosomes, because the total amount of LAMP-2A present in the membrane is considerably lower. The association of LAMP-2A to the lipid microdomains promotes its degradation, because cathepsin A also binds selectively to these regions. The components that mediate the sequestration of both the receptor and the protease in these discrete membrane microdomains are not known, but levels of cholesterol at the lysosomal membrane and intralysosomal levels of Ca^{2+} are important for this process (Cuervo et al., 2003; Kaushik et al., 2006). Little is known about age-related changes in lysosomal Ca^{2+} content, but it is unlikely that it contributes to the reduced binding of cathepsin A to the membrane, because even in the presence of exogenously added Ca^{2+}, cathepsin A is still not able to bind to the lysosomal membranes from old rats. We have found changes in both the lipid and the protein composition of lysosomal membranes with age (Fig. 5). Total levels of cholesterol at the lysosomal membrane and levels of several proteins normally present in the LAMP-2A-containing lipid microdomains change with age. The nature of those proteins and whether they participate in the recruitment of LAMP-2A to those regions or are sequestered into the membrane microdomains in a way unrelated to CMA will require future investigation. We propose that intrinsic changes with age in the composition of the lysosomal membrane or in some targeting elements, yet to be identified, result in inefficient targeting of LAMP-2A to discrete areas of the lysosomal membrane, thus altering its dynamics in this compartment.

Although the primary cause of the changes at the lysosomal membrane that are responsible for the altered turnover of LAMP-2A and the consequent decrease in CMA remains unknown, failure of autophagy worsens these membrane changes and probably contributes to perpetuate the process. In a transgenic mouse model generated in our laboratory, we have found that maintenance of normal levels of LAMP-2A until advanced ages preserves normal LAMP-2A degradation at the lysosomal membrane and proper reinsertion from the lumen into the membrane (C.Z. and A.M.C., unpublished results). Thus it is possible that improper removal of CMA substrate proteins, including other oxidized protein products, further contribute to alter the lysosomal membrane and to aggravate the impairment of CMA. Comparative proteomics and lipidomics of the membranes of lysosomes from this mouse model and those of young and old wild-type mice, should allow us to discriminate changes in the lysosomal membrane secondary to CMA failure from those primarily responsible for the altered lysosomal dynamics of LAMP-2A with age.

Materials and Methods

Animals and cells

Male Fisher-344 rats aged 4, 12 and 22 months were obtained from the age-controlled pool of animals maintained by the National Institute of Aging. Where indicated, animals were starved for 24 or 48 hours before lysosomal isolation by completely removing food but maintaining water supply ad libitum, or treated with paraquat (40 mg/kg body weight two i.p. injections every 24 hours) to induce mild oxidative stress. Primary fibroblasts were cultured from E13.5-14.5 embryos as described (Yuspa et al., 1970). In our culture conditions these fibroblasts undergo a maximum of 15 cellular divisions. We considered as senescent, fibroblasts at any passage higher than 12, because after this division fibroblasts present phenotypic characteristics of senescent cells (slower replication times, greater size, increase of the nucleus to cytosol ratio, presence of autofluorescent lipofuscin-like granules and positive staining for β-galactosidase). Cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) in the presence of 10% newborn calf serum. To deprive cells of serum, plates were extensively washed with Hanks' balanced salts solution (Invitrogen, Carlsbad, CA) and fresh medium without serum was added.

Chemicals

Sources of chemicals and antibodies were as described previously (Cuervo and Dice, 1996; Cuervo and Dice, 2000a; Cuervo and Dice, 2000b; Cuervo and Dice, 2000c; Kaushik et al., 2006; Kiffin et al., 2004). The antibody against the cytosolic tail of rat and mouse LAMP-2A was prepared in our laboratory (Cuervo and Dice, 1996). The antibody against rat LAMP-1 (1D4B) was from the Developmental Studies Hybridoma Bank (Iowa University, Iowa City, IA), against flotillin-1 and hemagglutinin (HA) tag were from BD Transduction Laboratories (San Diego, CA), against cathepsin D was from Santa Cruz Biotechnology (Santa Cruz, CA), and against cathepsin A and the luminal region of LAMP-2 were generous gifts from Alessandra D'Azzo (St Jude Children Research Hospital, Memphis, TN) and Michael Jadot (University of Namur, Belgium), respectively.

Isolation of lysosomal fraction

Rat liver lysosomes were isolated from a light mitochondrial-lysosomal fraction in a discontinuous metrizamide density gradient (Wattiaux et al., 1978) modified as described (Cuervo et al., 1997). Preparations with more than 10% broken lysosomes, measured by β-hexosaminidase latency (Storrie and Madden, 1990), were discarded. Lysosomal matrices and membranes were isolated after hypotonic shock (Ohsumi et al., 1983).

Measurement of LAMP-2A degradation

To determine degradation rates of cellular LAMP-2A, mouse fibroblasts at 60-70% confluence were radiolabeled with 0.2 μCi/ml of a [^{35}S]methionine:cysteine mixture (Easy Tag-Express ^{35}S; NEN^{TM} Life Science Products, Boston, MA) for 48 hours in methionine:cysteine-free medium (Sigma) supplemented with 10% fetal bovine serum. After extensive washing, cells were maintained in the presence or absence of serum, and supplemented with a 2 mM mixture of unlabeled methionine and cysteine. At increasing times, cells were recovered and lysed using lysis buffer [50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS]. Lysates were cleared by centrifugation and supernatants were incubated with a specific antibody against LAMP-2A 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). The half-life of LAMP-2A was calculated from the formula $t_{1/2}$=ln2/degradation rate.

Rates of degradation of LAMP-2A in the isolated membranes were determined by immunoblot using a specific antibody against the cytosolic tail of LAMP-2A as previously described (Cuervo and Dice, 2000a). Briefly, isolated lysosomal membranes were incubated in MOPS buffer at 37°C and at different times aliquots were removed and subjected to SDS-PAGE and immunoblot for LAMP-2A.

Lysosomal dynamics of LAMP-2A

Reinsertion of LAMP-2A in isolated lysosomal membranes was carried out as previously described (Cuervo and Dice, 2000a). Briefly, freshly isolated intact rat liver lysosomes were incubated with substrate proteins for 10 minutes and then the substrate was rapidly removed from the incubation media by centrifugation. The lysosomal pellet was resuspended in substrate-free isoosmotic buffer and after a new incubation, membrane and matrix were separated and their LAMP-2A content was analyzed by immunoblot. For the membrane insertion of purified HA-LAMP-2A, vesicles formed by spontaneous resealing of sonicated lysosomal membranes were used (Cuervo and Dice, 2000a). Rat liver lysosomal membranes washed in MOPS buffer, 0.5 M NaCl or 0.1 M Na_2CO_3 were resuspended in MOPS buffer containing 30 μg of purified HA-LAMP-2A and sonicated three times for 10 seconds. Vesicles recovered by centrifugation at 185,000 g for 15 minutes were then resuspended in MOPS buffer and incubated for 30 minutes at 37°C. At the end of the incubation, vesicles were pelleted and following a hypotonic shock, their membrane and luminal HA-LAMP-2A content was determined by immunoblotting.

Isolation of detergent-resistant lysosomal membrane microdomains

Lysosomal membranes (150 μg protein) from rat liver were incubated with 1% of Triton X-114, in 150 mM NaCl, 50 mM Tris-HCl and 5 mM EDTA pH 7.4 (incubation buffer) on ice for 30 minutes and then subjected to centrifugation in a step-wise discontinuous sucrose gradient (5-40%). Gradient fractions, collected from top to bottom, were concentrated by precipitation in acid or by high speed centrifugation and then subjected to SDS-PAGE and immunoblot as described (Kaushik et al., 2006).

mRNA quantification

Total RNA was extracted from rat livers using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) following the manufacturer's indications, and stored at –80°C until use. The first strand cDNA was synthesized from 0.5 μg of the total RNA with the SuperScript II RNase H Reverse Transcriptase (Invitrogen) and oligo-$(dT)_{12\text{-}18}$ primers. Actin and a region of the exon 8 of LAMP-A, LAMP-B or LAMP-C were amplified with specific primers (LAMP-2A, 5′-GCAGTGCAGATGAAGACAAC-3′, 5′-AGTATGATGGCGCTTGAGAC-3′; LAMP-2B, 5′-GGTGCTGGTCTTT-CAGGCTTGATT-3′, 5′-ACCACCCAATCTAAGAGCAGGACT-3′; LAMP-2C, 5′-ATGTGCTGCTGACTCTGACCTCAA-3′, 5′-TGGAAGCACGAGACTGGC-TTGATT-3′; Actin, 5′-AAGGACTCCTATAGTGGGTGACGA-3′, 5′-ATCTT-CTCCATGTCGTCCCAGTTG-3′) using the SYBR green PCR kit (PE Biosystems, Warrington, UK). Amplification of the LAMP-2s and actin DNA products (120 and 108 bp, respectively) was measured in real time in a SmartCycler (Cepheid, Sunnyvale, CA). For both genes, the presence of a single amplified product was verified by agarose gel electrophoresis, and by analysis of the melting curves of the RT-PCR reaction. The expression levels of LAMP-2A in different samples were normalized with respect to those of actin in the same samples. Differences between samples were calculated based on the differences in the number of cycles required to reach a threshold fluorescence intensity level. We did not need to correct for fragment length because the size of the amplified fragments was very similar.

General methods

Protein concentration was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. Lysosomal enzymatic activities were measured as reported (Storrie and Madden, 1990). After SDS-PAGE (Laemmli, 1970) and immunoblotting (Towbin et al., 1979), the proteins recognized by the specific antibodies were visualized by chemiluminescence methods (Renaissance, NEN-Life Science Products, Boston, MA). Densitometric quantification of the immunoblotted membranes and stained gels was done with an Image Analyzer System (Inotech S1800, Sunnyvale, CA). Quantification of intracellular cholesterol was done using the Amplex Red Cholesterol Assay kit (Molecular Probes, Eugene, OR) as described elsewhere (Eskelinen et al., 2004). Zymography was carried out in gelatin containing gels as described before (Leber and Balkwill, 1997). In SDS-PAGE gels proteins were visualized after staining with SyproRuby (Bio-Rad, Hercules, CA) in a U.V. transilluminator. Student's *t* test was used for statistical analysis.

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