A Population of Rat Liver Lysosomes Responsible for the Selective Uptake and Degradation of Cytosolic Proteins*

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Two populations of rat liver lysosomes can be distinguished on the basis of their density. A major difference between these populations is that one contains the heat shock cognate protein of 73 kDa (hsc73) within the lysosomal lumen. The lysosomal fraction containing hsc73 exhibits much higher efficiencies in the in vitro uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase and ribonuclease A, two well-established substrates of the selective lysosomal pathway of intracellular protein degradation. Preloading of the lysosomal population that is devoid of luminal hsc73 with hsc73 isolated from cytosol activated the selective transport of substrate proteins into these lysosomes. Furthermore, treatment of animals with leupeptin, an inhibitor of lysosomal cathepsins, or 88 h of starvation also increased the amount of hsc73 within their lysosomal lumen, and these in vivo treatments also activated the selective transport of substrate proteins in vitro. Thus, the hsc73 located within lysosomes appears to be required for efficient uptake of cytosolic proteins by these organelles. The difference in hsc73 content between the lysosomal populations appears to be due to differences in their ability to take up hsc73 combined with differences in the intralysosomal degradation rates of hsc73. The increased stability of hsc73 in one population of lysosomes is primarily a consequence of this lysosomal population's more acidic pH.

In eukaryotic cells, lysosomes participate in intracellular protein degradation by a variety of pathways including macroautophagy, microautophagy, crinophagy, endocytosis, and a selective uptake of cytosolic proteins (1–4). The selective pathway of lysosomal proteolysis, first described in serum-deprived confluent cultured fibroblasts (5), is also operative in rat liver, especially after prolonged starvation (6, 7). The pathway has been reconstituted in vitro with lysosomes isolated from human fibroblasts (8, 9) and from rat liver (10, 11). Specific substrates of this pathway include: ribonuclease A (RNase A)1 and RNase S-peptide (residues 1–20 of RNase A), where the pentapeptide KFERQ appears to be required for their lysosomal uptake (12, 13), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Ref. 10).

The selective pathway of lysosomal proteolysis resembles in many respects the import of proteins synthesized on cytosolic ribosomes into the endoplasmic reticulum, mitochondria, peroxisomes, and nucleus. Thus, uptake of cytosolic proteins by lysosomes is saturable and time- and temperature-dependent (9–11). Substrates for lysosomal import compete with each other (11), and intermediates in the import process can be identified for certain protein substrates (11). In addition, uptake of selective cytosolic proteins by lysosomes is stimulated by ATP/MgCl2 and a cytosolic heat shock protein, the heat shock cognate protein of 73 kDa (hsc73; Ref. 8), and requires protein-containing components of the lysosomal membrane (9, 11). Recently, lgp96 has been identified as the protein responsible for the binding of RNase A and GAPDH to the lysosomal membrane (14). Furthermore, import of proteins into the endoplasmic reticulum (15, 16) and mitochondria (17, 18) requires an organellar form of a heat shock protein of 70 kDa (hsp70), and some hsc73, most probably a specific isoform, is localized to the lysosomal lumen and is required for the operation of this pathway of proteolysis (19, 20), most likely for the import of protein substrates into the lysosomal lumen.

It is well known that lysosomes are morphologically and biochemically heterogeneous (i.e. Refs. 21–24 and references cited therein). In our previous studies of the uptake of GAPDH and RNase A by rat liver lysosomes we noticed, by immunogold procedures, apparent differences among individual lysosomes in their ability to take up selective protein substrates (7, 10). We also observed that hsc73 was present within some, but not all, lysosomes, and that it colocalized with RNase A transported into the lysosomes (7). The amount of lysosomal hsc73 increased by 5–10-fold during prolonged starvation, and this increase was due to both an increase in the number of hsc73 molecules per lysosome and also to an increase in the percentage of lysosomes containing hsc73 (7).

Little is known about the mechanism(s) by which hsc73 reaches the lysosomal matrix, but hsc73, like many cytosolic proteins, can presumably enter lysosomes through macroautophagy and microautophagy. In addition, hsc73 may enter lysosomes by the selective import pathway that it stimulates, because two KFERQ motif peptides exist in hsc73 (13). Once in the lysosomal matrix hsc73 is relatively resistant to intralysosomal hydrolysis (19).

In this paper we characterize in rat liver the specific lysosomal population containing hsc73 to gain more insights into the role of intralysosomal hsc73 in the selective uptake of cytosolic lgp120, lysosomal glycoprotein of 120 kDa; GST, glutathione transferase; MOPS, 3-(N-morpholino) propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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1 The abbreviations used are: RNase A, ribonuclease A; RNase S-peptide, residues 1–20 of RNase A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsc73, heat shock cognate protein of 73 kDa; hsp70, heat shock protein of 70 kDa; FITC, fluorescein isothiocyanate;
proteins. We show that the hsc73 located within lysosomes is of paramount importance for the uptake of cytotoxic proteins by these organelles. Lysosomes that contain hsc73 have increased rates of uptake and decreased rates of intralysosomal proteolysis of hsc73, when compared with lysosomes that do not contain hsc73. Finally, the resistance of hsc73 to intralysosomal hydrolysis is explained, at least in part, by the slightly more acidic pH of this population of lysosomes.

EXPERIMENTAL PROCEDURES

Animals—Male Wistar rats weighing 200–250 g were fasted for 20 h prior to use. In some experiments fasting was prolonged for 88 h. For experiments with leupeptin, rats received the drug (2 mg/100 g of body weight by intraperitoneal injection) 1 h before sacrifice. All rats were fed ad libitum for at least 7 days before the experiments began.

Chemicals—Sources of chemicals and antibodies were as described previously (7, 9–11) with the following additions: fluorescein isothiocyanate-dextran (FITC-dextran) (average molecular weight, 67 kDa; 0.007 mol fluorescein/mol glucose), glutathione-agarose and bafilomycin A were from Sigma; Trans-S-Slabel™ was from ICN Pharmaceuticals Inc. (Costa Mesa, CA); antibodies against cathepsin L and against the cation-insensitive mannose 6-phosphate receptor were generous gifts from Dr. G. Sahagian (Tufts University, Boston, MA); and the antibody against the lysosomal glycoprotein of 120 kDa (IgP120) was from Dr. I. Mellman (Yale University, New Haven, CT); antibodies against hexokinase, aldolase, 3-phosphoglycerate kinase, and phosphoglycerate mutase were raised in rabbits against the purified proteins following standard procedures (25). Other reagents were of the best analytical quality available. Glutathione transferase (GST-hsc73), a fusion protein of hsc73 with GST at its amino terminus was obtained from a plasmid (pGX-2T, Pharmacia Fine Chemicals, Uppsala, Sweden) containing a human cDNA encoding hsc73 (26).

Isolation of Lysosomal Fractions—Lysosomes were isolated from a light mitochondrial fraction in a discontinuous metrizamide density gradient (27) by the shorter method previously reported (10). Fractions from the top layer (fraction 1) and the intermediate metrizamide interface (fraction 2) were collected separately, diluted five times with 0.3M sucrose from the top layer (fraction 1) and the 26.3/19.8% metrizamide interface (27) by the short term method previously reported (10). Fractions 1 and 2 were resuspended in 0.3M sucrose and centrifuged again (rotor SS-34; DuPont Instruments, Herts, UK). Lysosomes from fraction 2 were collected separately, diluted five times with 0.3M sucrose from the top layer (fraction 1) and the 26.3/19.8% metrizamide interface (27) by the short term method previously reported (10). Fractions 1 and 2 were resuspended in 0.3M sucrose and centrifuged again at 10,000 g for 10 min (10, 11). These observations suggested that different populations of lysosomes with distinct activities in the selective uptake of cytosolic proteins might be separable based on centrifugation. In most of our previous experiments we used a pool of two lysosomal fractions from a discontinuous metrizamide gradient (27) (10). Thus, the top layer and the 26.3/19.8% metrizamide interface (fraction 2) (10, 11). Therefore, in a first approximation to identify a lysosomal population active in selective protein uptake, the top layer and the 26.3/19.8% metrizamide interface were centrifuged separately and in succession at 10,000 × g for 5 min and at 37,000 × g for 10 min, thus giving a total of four different fractions (two pellets, P1 and P2, respectively) (33), immunoblotting (34), and fluorography (35) were carried out by standard procedures. Hsc73 was purified from bovine brain cytosol by ATP-agarose affinity chromatography (36). Densitometric analysis of the immunoblots was performed with an 2902 LKB Ultrascan laser densitometer (LKB-Pharmacia, Uppsala, Sweden) with a Hewlett-Packard (Palo Alto, CA) 3396 Series II integrator. The linearity of the method was established using different amounts of hsc73, GAPDH, or RNase A. Protein concentrations were measured by a modification of the Lowry et al. (37) method using bovine serum albumin as the standard. Enzymatic activities were measured by the standard procedures reported by Terlecky and Dice (9), Aniento et al. (10), Barrett and Kirschke (38), and Smith and Turk (39). The energy-regenerating system used in some experiments consisted of 10 mM MgCl2, 10 mM ATP, 2 mM phosphocreatine, and 50 μg/ml creatine phosphokinase. Statistical analyses were performed by the Student’s t test.

RESULTS

In previous experiments we found that uptake of RNase A by rat liver lysosomes was heterogeneous (7). In addition, we noticed that the uptake efficiency of GAPDH by lysosomes decreased when these organelles were collected by centrifugation at 10,000 × g for 5 min instead of the usual 37,000 × g for 10 min (10, 11). These observations suggested that different populations of lysosomes with distinct activities in the selective uptake of cytosolic proteins might be separable based on centrifugation.
Procedures. All values are relative to homogenates and are the means ± S.D. for n = 6.

**Table I**

| Enzymes                     | HSC+ lysosomes | HSC− lysosomes |
|-----------------------------|----------------|----------------|
| **Recovery**                | **Enrichment** | **Recovery**   | **Enrichment** |
| **Protein**                 | 0.08 ± 0.01    | 0.11 ± 0.02    | 0.08 ± 0.01    | 0.11 ± 0.02 |
| **Lyosomal enzymes**        |                |                |                |               |
| β-N-acetylglucosaminidase   | 3 ± 0.5        | 36 ± 17        | 3 ± 0.5        | 44 ± 15       |
| β-Hexosaminidase            | 3 ± 1          | 43 ± 11        | 7 ± 1          | 59 ± 10       |
| Cathepsin B                 | 3 ± 1          | 37 ± 12        | 4 ± 1          | 37 ± 13       |
| **Cytosolic enzymes**       |                |                |                |               |
| 1-lactate dehydrogenase     | 0.04 ± 0.01    | 0.5 ± 0.3      | 0.07 ± 0.02    | 0.6 ± 0.2     |
| GAPDH                       | 0.004 ± 0.000  | 0.1 ± 0.01     | 0.009 ± 0.000  | 0.1 ± 0.01    |
| **Mitochondrial enzymes**   |                |                |                |               |
| Succinate dehydrogenase     | 0.04 ± 0.003   | 0.5 ± 0.2      | 0.04 ± 0.001   | 0.4 ± 0.2     |
| Ornithine transcarbamoylase | 0.01 ± 0.006   | 0.1 ± 0.2      | 0.02 ± 0.008   | 0.1 ± 0.05    |

**Fig. 1. Levels of hsc73 in rat liver lysosomal populations.** Rat liver lysosomes, isolated from the top layer (fraction 1) and the first interface (fraction 2) of a discontinuous metrizamide gradient (see "Experimental Procedures"), were washed and centrifuged in succession at 4°C for 5 min at 10,000 × g and for 10 min at 37,000 × g to give two pellets (P1 and P2) and their supernatants (S1 and S2). Lanes 1 in A and B are hsc73 (1 µg). A, proteins (50 µg) from P1 (lane 2), P2 (lane 4), and S2 (lane 5) or 15 µg of protein from S1 (lane 3) were subjected to SDS-PAGE and immunoblot analysis with anti-hsc73. B, lysosomes from P1 and P2 were broken by freezing and thawing, and their matrix and membrane fractions were isolated (see "Experimental Procedures"). Proteins (50 µg) from each fraction were subjected to SDS-PAGE and immunoblot analysis with anti-hsc73.

and P2, and their supernatants, S1 and S2) to analyze. The protein content of these four fractions were as follows per rat liver: P1 = 1.5 ± 0.1 mg, P2 = 1.8 ± 0.2 mg, S1 = 0.14 ± 0.01 mg, and S2 = 0.44 ± 0.05 mg (38.7, 46.3, 3.6, and 11.4% of total lysosomal protein, respectively).

Immunoblot analysis of the levels of hsc73 reveals that one of these fractions, P2, was practically devoid of hsc73, whereas the remaining fractions had quite similar amounts of hsc73 when corrected for the amount of protein analyzed (Fig. 1A). The lysosome fractions referred to as P1 and P2 had similar low levels of broken lysosomes (<3% as determined by the latency of lysosomal enzymes (11)), but S1 and S2 contained higher levels of broken lysosomes (10%). S1 and S2 also contained more unidentified membrane fragments, as judged by electron microscopy, so the following experiments were carried out only with the P1 and P2 fractions, which represent 85% of the total lysosomal protein and which will be referred to hereafter as HSC+ and HSC− lysosomes, respectively.

Fig. 1B shows that hsc73 was present both in the matrix (about 65% of the total by densitometric analyses of similar immunoblots, when corrected for total protein) and in the membranes from HSC+ lysosomes. The small amount of hsc73 (20% or less of the hsc73 found in HSC+ lysosomes) occasionally observed in HSC− lysosomes (lane 3) was exclusively associated with the lysosomal membrane (lane 7). Proteinase K treatment of both lysosomal fractions confirmed that the major part of the hsc73 that is found associated with HSC+ lysosomes is in the lysosomal matrix, whereas all hsc73 associated with HSC− lysosomes is in the membrane (data not shown).

Other comparisons between the HSC+ and HSC− lysosomal populations revealed many similarities but also some differences. Thus, the study of the specific activities of different lysosomal markers (Table I) showed comparable activities of cathepsin B, β-N-acetylglucosaminidase, and β-hexosaminidase in both fractions. However, lysosomal enzymatic activities in HSC− lysosomes were always slightly but consistently higher than in HSC+ lysosomes. Recoveries of lysosomal enzymes (between 3–5% of total) were consistent with our previous studies working with the complete pool of lysosomes (5%) (10, 11). Also, contamination of any of the fractions with mitochondria (based on the activity of ornithine transcarbamoylase and succinate dehydrogenase) or cytosol (based on the activity of lactate dehydrogenase and GAPDH) was negligible. A typical late endosome marker, the cation-independent mannose 6-phosphate receptor, was not detected in either of the two lysosomal populations (data not shown), suggesting that both populations are mainly mature lysosomes. This conclusion is further supported by the high proteolytic activities (see below) and by the high activities of lysosomal enzymes (Table I) in both fractions. The SDS-PAGE pattern of bands was similar for total lysosomes and for lysosomal membrane and matrix fractions (Fig. 2A). However, some qualitative or quantitative increases in specific protein bands were evident in the membranes and matrix of HSC+ lysosomes (i.e.; at about 182, 87, 76, and 45 kDa for membranes and 70 and 47 kDa for matrix; marked by arrowheads in Fig. 2A). These differences were evident in each of 10 separate analyses, but the degree of difference varied from experiment to experiment, especially for the matrix proteins. The levels of lysosomal membrane (lpg120, Fig. 2B; lpg96, data not shown) and matrix (cathepsin L (CATH L), Fig. 2C) proteins were quite similar in HSC+ and HSC−lysosomes. Total proteolytic activities of broken HSC+ and HSC−lysosomes at different pHs against a pool of labeled proteins should be measured.
cytosolic proteins (10) or GAPDH were also indistinguishable (data not shown).

The amounts of several cytosolic proteins in HSC+ and HSC− lysosomes were quantitated using specific antibodies. Typical results for hexokinase and aldolase are shown in Fig. 2D. Hexokinase (and a presumed immunoreactive breakdown fragment) is preferentially localized in HSC− lysosomes, whereas aldolase is largely confined to HSC+ lysosomes. Quantitation of this and similar immunoblots indicate that HSC+ lysosomes are enriched for GAPDH, aldolase, and phosphoglycerate mutase. However, HSC− lysosomes were enriched for hexokinase, and both lysosomal populations contained equivalent amounts of phosphoglycerate kinase (Table II). Enrichment of HSC+ lysosomes in GAPDH and aldolase does not seem to be related to a lower proteolytical susceptibility of these enzymes once inside the lysosomal matrix, since equivalent rates of degradation were obtained when incubated with broken lysosomes from both populations (data not shown).

The ultrastructural appearance of the HSC+ and HSC− lysosomes is shown in Fig. 3 (A and B). Although there are not large differences, HSC+ lysosomes are in general smaller and more elongated than HSC− lysosomes. Morphometric analysis of 40 randomly selected lysosomal profiles from 10 different electron micrographs per fraction indicates an average area of 0.075 ± 0.017 μm² for HSC+ lysosomes and 0.102 ± 0.021 μm² for HSC− lysosomes. This difference is statistically significant (p < 0.02). Also, the form factor was 0.698 for HSC+ lysosomes and 0.907 for HSC− lysosomes (1.000 is a perfect circle).

We next investigated the uptake of RNase A and GAPDH by the two lysosomal populations. These proteins are two well established substrates of the selective lysosomal pathway of intracellular protein degradation (10, 11). As shown in Fig. 4, HSC+ lysosomes were much more efficient than the HSC− lysosomes in taking up both substrates. We also tested the
effect of exogenously added hsc73 plus ATP/MgCl2 and an energy regenerating system on the proteolysis of GAPDH and RNaseS-peptide by the two lysosomal populations (Fig. 5). We found that these compounds were stimulatory with both lysosomal populations as expected (10, 11) but that HSC1 lysosomes were more active under all conditions.

Then we studied whether HSC1 and HSC2 lysosomes exhibit differences in their abilities to take up hsc73. Fig. 6A shows that when intralysosomal proteolysis was inhibited and levels of hsc73 in matrix (after proteinase K treatment) were measured, HSC1 lysosomes were more effective in taking up exogenously added hsc73 (compare lanes 8 and 9), but some hsc73 was also incorporated into HSC2 lysosomes (Fig. 6A, lane 9), as was also the case with GAPDH and RNase A (Fig. 4). Although the amount of hsc73 associated to lysosomes after the proteinase K incubation is lower in Fig. 6 than in freshly isolated lysosomes (Fig. 1B), it should be noted that in these experiments lysosomes received additional treatments that may result in some intralysosomal hsc73 becoming accessible to the exogenously added protease. When RNase A was incubated with HSC− lysosomes that had incorporated hsc73 in a prior incubation (as in Fig. 6A), a portion of the RNase A was found associated with the lysosomal pellets (Fig. 6B, lane 5), and part of this RNase A was resistant to proteinase K digestion (Fig. 6B, lane 9). The uptake efficiency of these lysosomes were now at least as good as the original HSC+ lysosomes.

![Image](61x81 to 294x608)

![Image](326x431 to 546x608)

**FIG. 4.** Uptake of GAPDH and RNase A by lysosomal populations. RNase A and GAPDH (as labeled in the figure) were incubated under standard conditions and in the presence of chymostatin (see "Experimental Procedures”) with freshly isolated HSC+ or HSC− lysosomes. The lysosomes were incubated with proteinase K to degrade nontranslocated proteins, centrifuged, and subjected to SDS-PAGE and immunoblot analysis with anti-RNase A or anti-GAPDH antibodies. The insets show two representative immunoblots. The lower band in the RNase A immunoblot (left part of the figure) corresponds to an intermediate in the lysosomal transport of that protein (11). Histograms are means ± S.D. of densitometric analyses of similar immunoblots from four different experiments. Differences from HSC+ significant at $p < 0.01(*)$ and $p < 0.001(**).
FIG. 5. Effect of hsc73 and an ATP-regenerating system on the degradation of GAPDH and the RNase S-peptide by rat liver lysosomal populations. HSC+ and HSC− lysosomes (25 μg of protein) were incubated for 30 min at 37 °C in 0.3 M sucrose, 10 mM MOPS, pH 7.2, with 230 nM [14C]GAPDH (A) or 1 h at 25 °C, in the same buffer, with 35 nm [3H]RNase S-peptide (B) without additions (CTR) or with ATP/MgCl2 and an ATP-regenerating system and 10 μg/ml of hsc73 added (ATP+HSC73). The results are the means ± S.D. of six different experiments. The results are significantly different from HSC+ at p < 0.01 (*) and p < 0.001 (**).

(compare lanes 8 and 9). The presence of intralysosomal hsc73 was apparently sufficient to make the HSC− lysosomes competent for the selective uptake of cytosolic proteins.

We also increased the content of hsc73 in HSC− lysosomes by in vivo manipulations. In a first approach, rats were treated with leupeptin to inhibit lysosomal proteases prior to the isolation of the two lysosomal fractions. Immunoblot analysis with anti-hsc73 (Fig. 7A, upper panel) reveals an increase in hsc73 (lane 5) in the fraction corresponding to HSC− lysosomes. Although after leupeptin treatment the quantity of hsc73 increased in both lysosomal populations, this increase is considerably higher for HSC− lysosomes (ten times) than for HSC+ lysosomes (two times). The uptake efficiency of proteins (RNase A and GAPDH) (Fig. 7A, middle and lower panels) by the two lysosomal populations isolated from rats treated with leupeptin (lanes 4 and 5) was similar to the original HSC+ lysosomes (lane 2) and much higher than the HSC− lysosomes (lane 3).

Other in vivo evidence was provided by prolonged starvation (Fig. 7B). As reported previously (7), during prolonged starvation over the range 0−88 h, there is a progressive increase in the activity of the hsc73-dependent selective lysosomal proteolytic pathway coincident with an increase in lysosome-associated hsc73. The two lysosomal populations isolated from rats after 88 h of starvation were indistinguishable from those isolated after 20 h based on ultrastructural appearance and SDS-PAGE protein patterns (data not shown). However, after 88 h of starvation there was an increase in hsc73 in both lysosomal populations (Fig. 7B, upper panel), but the increase was most pronounced for the HSC− lysosomes. The uptake of two substrate proteins of this pathway, RNase A (Fig. 7B, middle panel) and GAPDH (Fig. 7B, lower panel) by these lysosomal populations showed a significant increase in the uptake efficiency of the original HSC− lysosomes after 88 h starvation (compare lanes 3 and 5). From these observations it appears again that the intralysosomal hsc73 is required for an efficient lysosomal uptake of proteins.

Hsc73 was taken up most avidly by HSC+ lysosomes (Fig. 6A, compare lanes 8 and 9), but this difference cannot completely account for the absence of hsc73 in the HSC− lysosomes. Because of the dramatic effect of leupeptin on the hsc73 content of HSC− lysosomes (Fig. 7A, upper panel), it seemed possible that hsc73 is also degraded more efficiently within the HSC− lysosomes than in the HSC+ lysosomes. In initial attempts to test this idea, we reductively methylated hsc73 but found that these preparations contained mostly inactive hsc73.
in that the protein no longer could bind to ATP (8). Therefore, we purified a metabolically labeled $^{35}$S-GST-hsc73 fusion protein and followed its degradation during incubation with freshly isolated HSC+ and HSC− lysosomes. $^{35}$S-GST-hsc73 is almost two times more effectively degraded by HSC− lysosomes than by HSC+ lysosomes (Fig. 8A). Because lower rates of hsc73 uptake were found for HSC− lysosomes (Fig. 6A) differences in efficiency on hsc73 degradation between both populations may take place in the lysosomal matrix. To verify this idea, after incubation of intact lysosomes with a large amount (2 $\mu$m) of $^{35}$S-GST-hsc73 so that a signal could be seen in the HSC− lysosomes (Fig. 8B, inset, lane 5), we reisolated the lysosomes that had incorporated a portion of the GST-hsc73 and subsequently measured the degradation of the fusion protein. HSC− lysosomes were again more effective in degrading the $^{35}$S-GST-hsc73 than were HSC+ lysosomes (Fig. 8B). Similar degradation rates and the same difference between HSC+ and HSC− lysosomes were obtained with the active fraction of hsc73 radiolabeled with $^{14}$C by reductive methylation that retained its ability to bind to ATP (data not shown). This result indicates that the $^{35}$S-GST-hsc73 fusion protein was valid for monitoring intralysosomal degradation of hsc73.

The differences between HSC+ and HSC− lysosomes in their degradation efficiency of hsc73 cannot be explained by differences in the proteolytic activity of broken lysosomes (see above). Therefore, we decided to measure the intralysosomal pH of both lysosomal populations using FITC-dextran. We found that the pH in HSC+ lysosomes was approximately 0.5 pH units lower than that of HSC− lysosomes (Fig. 9A). Supplying the lysosomal proton pump with ATP/MgCl$_2$ slightly increased this difference. That the lysosomal pH could be important in the degradation of intralysosomal hsc73 was apparent when HSC+ lysosomes were incubated for 30 min with or without ATP/MgCl$_2$ (Fig. 9B). Without ATP/MgCl$_2$, most intralysosomal hsc73 was degraded after 30 min of incubation of the HSC+ lysosomes, whereas in the presence of ATP/MgCl$_2$ when the lysosomes were slightly more acidified (Fig. 9A) the degradation of hsc73 was retarded. The effect of ATP/MgCl$_2$ was due to intralysosomal acidification and not due to some protective effect of ATP on the hsc73 because when the ATPase inhibitor bafilomycin A was added together with ATP/MgCl$_2$, the ATP-stimulated acidification of the HSC+ lysosomes was blocked (data not shown), and the lysosomal degradation of the endogenous hsc73 was increased (Fig. 9B, compare lanes 3 and 4).

We also investigated the proteolysis of hsc73 by HSC+ lysosomes treated with increasing amounts of NH$_4$Cl to increase their pH (Fig. 9C). Lysosomal pH was modified from 5.4 to 6.4 by NH$_4$Cl, and the proteolysis of hsc73 was found to be most effective in the range 5.75–6.20. Therefore, the difference in pH could explain why HSC− lysosomes are more effective than HSC+ lysosomes in degrading their endogenous hsc73.

**DISCUSSION**

We have identified two populations of rat liver lysosomes with very different abilities to selectively take up and degrade protein substrates such as GAPDH and RNase A (Figs. 4 and 5). These differences in uptake rates cannot be explained by
differences in the amount of lysosomes in both fractions, because only small differences in the activities of lysosomal enzymatic markers were found between both populations (Table I). In addition, analysis of RNase A uptake into individual lysosomes by immunogold and electron microscopy (7 and data not shown) confirmed that lysosomes with higher content of hsc73 incorporate a significantly larger amount of RNase A.

The two populations of lysosomes are similar in many respects, but they differ markedly in their content of hsc73 (Fig. 1). That the difference in lysosomal hsc73 in the lumens accounts for the different activities of HSC+ and HSC− lysosomes in their ability to selectively take up and degrade proteins could be shown experimentally by “loading” HSC− lysosomes with hsc73 in vitro (Fig. 6) and by two different in vivo treatments, leupeptin injection (Fig. 7A) and long term

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**Fig. 8.** Proteolysis of GST-hsc73 by HSC+ and HSC− lysosomes. A, [35S]GST-hsc73 (200 nM) was incubated with HSC+ and HSC− lysosomes (25 μg) at 25 °C for 60 min without additions (CTR) or with ATP/MgCl₂ (ATP). Proteolysis was measured as described under “Experimental Procedures.” The results are the means ± S.D. of 15 different experiments. The results are significantly different from HSC− at p < 0.001 (**). B, HSC+ and HSC− lysosomes (50 μg) were incubated with [35S]GST-hsc73 (2 μM) for 15 min at 37 °C. Lysosomes were collected by centrifugation, washed, and incubated again under the same conditions. At the indicated times, aliquots were taken, subjected to SDS-PAGE and fluorography. Values are means ± S.D. of densitometric analyses of five different experiments. Statistical significance between HSC+ and HSC− values: p < 0.01 (**). The inset shows a representative gel that contributed to the results shown. The arrowhead indicates the position of hsc73.

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**Fig. 9.** The pH of HSC+ and HSC− lysosomes and its effect on the intralysosomal degradation of hsc73. A, intralysosomal pH in HSC+ and HSC− lysosomal populations in the absence (CTR, control) or in the presence of 10 mM ATP/MgCl₂ (ATP). The lysosomal pH was measured using FITC-dextran as described under “Experimental Procedures.” The values are the means ± S.D. of five experiments and differences from HSC+ significant at p < 0.001 (**). B, degradation of hsc73 in HSC+ lysosomes treated or not with ATP. HSC+ lysosomes (100 μg of protein) (lane 1) were incubated, under standard conditions, for 30 min at 37 °C in the absence (NONE, lane 2) or in the presence of 1 mM ATP (ATP, lane 3) or 1 mM ATP plus 100 μM bafilomycin A (ATP+BAF, lane 4). At the indicated times, lysosomes were centrifuged and subjected to SDS-PAGE and fluorography. Values are means ± S.D. of densitometric analyses of six different experiments. Statistical significance between HSC+ and HSC− values: p < 0.01 (**). The inset shows a representative gel that contributed to the results shown. The arrowhead indicates the position of hsc73.
starvation (Fig. 7B). Importantly, these HSC– lysosomes loaded with hsc73 retained all other characteristics of HSC– lysosomes such as their more alkaline pH and their more rounded shapes and larger size (data not shown). In addition, membrane stability and proteolytic activity of HSC– lysosomes were not modified after long term starvation. These results suggest that the presence of lumenal hsc73 is the direct cause of the activation of the selective degradation pathway.

This interpretation is also consistent with results from cultured human fibroblasts in which intralysosomal hsc73 was neutralized in living cells by the endocytosis of an immunoprecipitating anti-hsc73 antibody (19). This treatment completely blocked the selective lysosomal degradation pathway without affecting other proteolytic pathways. These results together with the results presented here indicate that the intralysosomal hsc73 is required for the selective uptake of protein substrates into lysosomes. Similar roles for other hsp70 family members have been documented for the uptake of mitochondrial precursor proteins into mitochondria (15, 16) and for the uptake of endoplasmic reticulum or secreted proteins into the endoplasmic reticulum lumen (17, 18). Two main hypotheses have been presented about the mechanism of action of those lumenal chaperones (see Ref. 40 for review). In both of these models, a chaperone attached to the trans-side of the membrane interacts with the translocating polypeptide chain and prevents back movement of the protein (molecular ratchet model) or actively, by hydrolyzing ATP, pulls the protein across the membrane (translocation motor model). By analogy with these studies intralysosomal hsc73 could interact with the protein entering into the lysosomal matrix, providing the driving force to pull the import intermediate into the lysosome and preventing also the back movement of the protein to the cytosol. In this regard, a kinetic intermediate in the transport of RNase A through the lysosomal membrane has been identified (11).

Intralysosomal hsp70 appears to be hsc73 itself because the lysosomal hsp70 is recognized by two antibodies specific for hsc73 among all hsp70s tested, and the intralysosomal hsp70 co-migrates with one of the hsc73 isofoms in high resolution two-dimensional gels (19).2 We now provide additional support for this conclusion because cytosolic hsc73 can enter lysosomes in vitro, and this hsc73 functions as well as the lysosomal hsp70 in the selective uptake of proteins.

The preferential uptake of hsc73 by HSC+ lysosomes, evident when proteolysis is previously blocked (Fig. 6A, compare lanes 8 and 9), suggests that hsc73 can enter lysosomes, at least in part, through the selective pathway that it stimulates. Consistent with this finding is the presence of two KFERQ-like motifs in hsc73 and the ability of hsc73 to bind to other hsc73 molecules (15). Also, we repeatedly found a stimulation of the selective lysosomal uptake of protein substrates by low levels of hsc73 (Fig. 5) but competition by higher levels (data not shown) consistent with hsc73 entering lysosomes by the same pathway.

Combined with the reduced ability of HSC– lysosomes to take up hsc73, they also degrade intralysosomal hsc73 more rapidly than do HSC+ lysosomes (Fig. 8, A and B). This enhanced degradation in HSC– lysosomes appeared to be caused by the more alkaline pH in this lysosome population (Fig. 9A). Acidification of HSC+ lysosomes further reduced the degradation of intralysosomal hsc73 (Fig. 9B), and mild alkalinization using NH4Cl increased degradation of intralysosomal [35S]GST-hsc73 (Fig. 9C). In fact, HSC+ lysosomes degraded

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2 F. A. Agarraberes, S. R. Terlecky, and J. F. Dice, unpublished results.

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