Selective Binding and Uptake of Ribonuclease A and Glyceraldehyde-3-phosphate Dehydrogenase by Isolated Rat Liver Lysosomes*  

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Ribonuclease A (RNase A) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are selectively taken up and degraded by isolated rat liver lysosomes by very similar processes. The uptake and degradation of both of these proteins are stimulated by the heat shock cognate protein of 73 kDa and ATP/Mg*. Both binding and uptake of RNase A and GAPDH by lysosomes are saturable, and uptake of RNase A and GAPDH requires a protease-sensitive component within the lysosomal membrane. GAPDH competes for binding and uptake of RNase A by lysosomes and vice versa while another protein, ovalbumin, does not compete. RNase S-peptide (amino acids 1-20 of RNase A) also competes for RNase A binding and uptake by lysosomes, while RNase S-protein (amino acids 21-124 of RNase A) does not compete. The uptake of RNase A by lysosomes appears to involve an intermediate step in which approximately 2 kDa of the polypeptide's COOH terminus remains outside lysosomes while the remainder is inside the lysosomal lumen.

Both nonlysosomal and lysosomal pathways of proteolysis operate in eukaryotic cells (1-3). Lysosomes are especially important in the degradation of long-lived proteins, and there are a variety of pathways of delivery of intracellular proteins to lysosomes for subsequent digestion. These pathways include endocytosis (4, 5), crinophagy (6), microautophagy (7-9), macroautophagy (10-14), and direct protein transport mediated by the heat shock cognate protein of 73 kDa (hsc73)* (1, 15).  

Endocytosis is responsible for delivery of exogenous polypeptides and certain plasma membrane proteins to the lysosomes for degradation (4, 5). Crinophagy diverts secreted proteins to lysosomes for degradation when their secretion is reduced (6). Microautophagy and macroautophagy appear to internalize different cytosolic proteins at similar rates (16). Lysosomes are also able to take up and degrade proteins in a selective process that is mediated by hsc73 (15, 17). Microautophagy has been reported in isolated lysosomes (7), but macroautophagy has not. The selective uptake and degradation of ribonuclease A (RNase A) and ribonuclease S-peptide (RNase S-peptide; residues 1-20 of RNase A) has been achieved using isolated lysosomes from human fibroblasts (18-20), and the selective uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by isolated rat liver lysosomes have been reported (21). Here we show selective uptake and degradation of RNase A and GAPDH by isolated rat liver lysosomes and demonstrate that these processes are mechanistically similar. Furthermore, a transport intermediate in the uptake of RNase A by lysosomes is identified.  

MATERIALS AND METHODS  

Animals—Male Wistar rats weighing 200-250 g were fasted for 20 h prior to use.  

Chemicals—Sources of chemicals were as described previously (19-21).  

hsc73—This constitutive member of the heat shock protein 70-kDa (hsp70) family was purified from a bovine brain extract by ATP-agarose affinity chromatography as described previously (22).  

Antibodies—The primary antibodies used were a rabbit IgG against bovine pancreatic RNase A (Rockland Inc., Gilbertsville, PA) and a mouse IgG against GAPDH (Dr. Amelia Martinez-Ramón, Instituto de Investigaciones Citológicas, Valencia, Spain). Secondary antibodies were goat IgGs raised against rabbit or mouse IgG and conjugated to alkaline phosphatase (Promega, Madison, WI).  

Isolation of Lysosomes and Mitochondria—Lysosomes were isolated from a light mitochondrial fraction in a discontinuous metrizamide gradient density gradient by the shorter method reported previously (21). Rat liver mitochondria were prepared as described (23).  

Standard Incubation Conditions to Study the Uptake of RNase A and GAPDH into Lysosomes—Lysosomes (100 pg of protein) were incubated in a final volume of 30 pl with RNase A (25 μg for Western blots or 50 μg for Coomassie Brilliant Blue R-250 staining) for 20 min at 37 °C in 0.3 M sucrose and 10 mM MOPS buffer (pH 7.2). The integrity of the lysosomal membranes, as judged by the latency of the lysosomal enzymes β-hexosaminidase, measured as N-acetyl-β-glucosaminidase activity in the absence of detergent (12), was >93% at the end of all incubations. Additional experimental details can be found in the table and figure legends.  

Proteolysis Measurements—Lysosomes (25 μg of protein) were incubated in a final volume of 125 μl with protein substrates. The substrates used were GAPDH radiolabeled by reductive methylation (21) with [3H]Formaldehyde (Amersham International, Bucks, UK) to a specific radioactivity of 1.2 × 10⁶ dpm/nmol, [3H]RNase S-peptide radiolabeled by reductive methylation with NaB³H₄ (Dupont NEN (23)) to a specific radioactivity of 1.1 × 10⁶ dpm/nmol, and [3H]leucine-labeled soluble proteins from L-132 human cells (5,000 dpm/μg of protein). These soluble proteins were labeled and prepared as described previously (25).  

Incubations were stopped by the addition of trichloroacetic acid to a final concentration of 10% or, in the RNase S-peptide experiments, phosphotungstic acid in HCl to final concentrations of 3.25 and 5%.
Selective Uptake of Proteins by Isolated Rat Liver Lysosomes

Effect of an ATP-regenerating system and hsc73 on the degradation of RNase S-peptide, GAPDH, and L-132 cell proteins by rat liver lysosomes

Lysosomes (25 μg) were incubated for 2 h at 25 °C in MOPS buffer with 35 nM [3H]RNase S-peptide or for 30 min at 37 °C with 230 nM [14C]GAPDH or 0.2 mg/ml soluble protein from metabolically labeled L-132 cells ([3H]-L-132) without additions or with an ATP-regenerating system (ERS, defined in Ref. 18), 20 μg/ml hsc73 (HSC73), or ERS and hsc73 together. Results are the mean ± S.D. for the number of experiments indicated within parentheses. Results are significantly different from control values (no additions) at \( p < 0.01 \) (*), 0.002 (**), and 0.001 (***) respectively. The acid-soluble material was collected by filtration in the Multiscreen Assay System (Millipore Corp., Bedford, MA) using a 0.45-μm pore membrane. Proteolysis was expressed as the percentage of the initial acid-insoluble radioactivity converted to acid-soluble radioactivity. The energy-regenerating system used in some experiments was as described previously (18).

Protein Sequencing—Determination of the amino acid sequence of the low molecular weight RNase A-derived band was carried out after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to Immobilon-S in an automated protein sequencer (Applied Biosystems, Foster City, CA).

General Methods—SDS-PAGE (17% gels for studies of RNase A and 12% gels for studies of GAPDH) and immunoblotting procedures were carried out as described previously (21). Quantitation of the immunoblots was performed with an 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 RNase A and GAPDH as standards in the immunoblotting assays. Statistical analyses were performed by the Student's \( t \) test. Best straight lines were calculated by linear regression, and best curved lines and \( K_m \) values were calculated using Enzfitter software (Elsevier-Biosoft, Cambridge, UK).

Table I

| Addition          | [3H]RNase S-peptide | [14C]GAPDH | [3H]-L-132 |
|-------------------|---------------------|------------|------------|
| None              | 9.2 ± 0.1 (4)       | 25.7 ± 2.6 (4) | 10.0 ± 4.1 (11) |
| ERS               | 14.4 ± 1.0 (4)****  | 35.3 ± 6.1 (4)** | 32.6 ± 4.8 (8)**** |
| HSC73            | 15.4 ± 1.1 (4)****  | 35.4 ± 5.4 (4)** | 11.9 ± 3.2 (4) |
| ERS + HSC73      | 23.9 ± 0.6 (4)****  | 38.9 ± 4.4 (4)** | 49.7 ± 6.3 (4)**** |

Fig. 1. Saturable binding and uptake of RNase A by rat liver lysosomes. Lysosomes (100 μg of protein) were incubated under standard conditions with increasing amounts of RNase A for 20 min at 37 °C without (A) or with (B) chymostatin. Samples in B were treated with proteinase K. Lysosomes were centrifuged and subjected to SDS-PAGE, immunoblotting, and densitometric analysis. Values shown are means of six different experiments. Values for non-specific association of RNase A to the tubes were subtracted from experimental values. Insets show Scatchard plots for the data. Two representative gels are shown (C) that contributed to the results shown in A and B. RNase A concentrations were as indicated on the figure. Lanes 1 and 8 are RNase A (5 μg).
RESULTS

Uptake of RNase A by Rat Liver Lysosomes—RNase A was incubated with freshly isolated rat liver lysosomes under conditions similar to those described to study the selective uptake of GAPDH (21). They included (a) incubation for 20 min at 37 °C with or without chymostatin to inhibit lysosomal proteolysis, (b) treatment or not with proteinase K to remove the protein outside of lysosomes, and (c) collection of lysosomes and analysis of pellets and supernatants by SDS-PAGE followed by Coomassie staining and/or immunoblotting using anti-RNase A IgGs.

As was the case for GAPDH (21), a portion of RNase A was associated with the lysosomal pellets, and part of this RNase A was resistant to proteinase K digestion (not shown). The RNase A that was found associated with lysosomes in the absence of protease inhibitors represented primarily (>85%) RNase A bound to the external face of the lysosomal membrane since it was susceptible to digestion by proteinase K. In chymostatin-treated lysosomes, the RNase A that was associated with lysosomes represented both surface-bound and internalized molecules since only part of it was susceptible to digestion by proteinase K; the RNase A remaining after proteinase K treatment represents RNase A inside lysosomes. Several other experiments similar to those carried out with GAPDH (21) but using RNase A as a substrate yielded equivalent results (not shown): (a) chymostatin and NH₄Cl were the most effective protease inhibitors, (b) binding and uptake were time- and temperature-dependent with a 10-min lag in uptake compared with binding, and (c) some binding (one-third that seen for lysosomes) but no import could be detected using isolated mitochondria under similar incubation conditions.

We tested the effect of hsc73 and an energy-regenerating system on the proteolysis of RNase S-peptide (which is degraded by the same pathway as RNase A but is a better substrate (20)). Both the energy-regenerating system and hsc73 were stimulatory, and both components added together were most effective (Table I). A similar pattern of stimulation was evident for GAPDH except that the amount of stimulation was smaller, and the stimulation by addition of both components together was not statistically different from either one added alone (Table I). The stimulation of proteolysis by ATP was also evident using a mixture of metabolically labeled soluble pro-
Selective Uptake of Proteins by Isolated Rat Liver Lysosomes

Proteinases such as Proteinase K and trypsin were used to treat rat liver lysosomes. After treatment, the uptake of RNase A and GAPDH was measured. The binding and uptake of both proteins were saturable, with apparent dissociation constants (Kd) of 95 and 10 PM for RNase A and GAPDH, respectively. The apparent Michaelis-Menten constants (Km) for uptake of RNase A and GAPDH were 4 and 18 PM, respectively.

Protease treatment of lysosomes reduces the uptake of RNase A and GAPDH. When lysosomes were treated with proteinase K, the binding and uptake of both proteins were decreased. This effect was more pronounced with GAPDH, where the uptake was reduced by more than 50%.

Figure 3 illustrates the effect of trypsin pretreatment of rat liver lysosomes on the uptake of RNase A and GAPDH. The figure shows a decrease in the binding and uptake of both proteins with increasing amounts of trypsin.

Figure 4 shows the effect of GAPDH and ovalbumin on the binding and uptake of RNase A by rat liver lysosomes. The figure demonstrates that the uptake of RNase A is inhibited by GAPDH and ovalbumin, with IC50 values of 7 and 8 µM, respectively.

In conclusion, the uptake of RNase A and GAPDH by rat liver lysosomes is saturable and competitively inhibited by both proteins. The inhibition of RNase A uptake by GAPDH and ovalbumin suggests a possible role for these proteins in modulating lysosomal proteolysis.

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RNAse A and GAPDH were isolated from rat liver lysosomes and incubated with increasing amounts of trypsin. The binding and uptake of both proteins were measured by SDS-PAGE and immunoblot analysis. The Kd values for binding were 95 and 10 PM for RNase A and GAPDH, respectively. The Km values for uptake were 4 and 18 PM for RNase A and GAPDH, respectively.

It is interesting to note that after proteinase K treatment of lysosomes, in addition to the intact RNase A band, a lower molecular weight band is consistently evident. This immunoreactive species migrates at the position of RNase A-protein suggesting the removal of approximately 2 kDa from RNase A. This result is further addressed below.

Protease Treatment of Lysosomes Reduces Uptake of RNase A and GAPDH—Lysosomes were treated with different concentrations of trypsin or Proteinase K. The uptake of RNase A and GAPDH was measured by SDS-PAGE and immunoblot analysis. The inhibition of uptake was more pronounced with GAPDH, where the uptake was reduced by more than 50%.

Figure 3 illustrates the effect of trypsin pretreatment of rat liver lysosomes on the uptake of RNase A and GAPDH. The figure shows a decrease in the binding and uptake of both proteins with increasing amounts of trypsin.

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tions of trypsin before incubation with RNase A or GAPDH. The uptake of RNase A (Fig. 3A) and GAPDH (Fig. 3B) was reduced by trypsin in a dose-dependent manner. The integrity of the lysosomal membrane was not affected by treatment even at 250 μg/ml based on latency measurements for lysosomal enzymes as described under “Materials and Methods.” In addition, compared with untreated lysosomes, the trypsin-treated lysosomes did not release detectable proteolytic activity, showed normal morphology by electron microscopy, and exhibited unaltered SDS-PAGE protein banding patterns (not shown). Incubation with trypsin and a 2-fold molar excess of soybean trypsin inhibitor did not affect uptake of RNase A or GAPDH, excluding a competition by trypsin for the binding or uptake machinery. Finally, similar results were obtained when lysosmes were pretreated with proteinase K or elastase instead of trypsin (not shown).

**Competition between RNase A and ovalbumin for lysosomal binding and uptake**—Increasing amounts of GAPDH decreased the binding (Fig. 4A, lanes 2–6) and uptake (Fig. 4C, lanes 2–6) of RNase A while GAPDH itself entered lysosomes in progressively higher amounts (Fig. 4C, inset). The Kᵢ values, 7–8 μM (Fig. 4, B and D), were in the same range as the Kᵢ and Kᵢ values for GAPDH binding and uptake. Ovalbumin did not compete for RNase A binding (Fig. 4A, lanes 7–9) or uptake (Fig. 4C, lanes 7–9).

As expected, increasing amounts of RNase A also decreased the binding (Fig. 5A, lanes 2–6) and uptake (Fig. 5C, lanes 2–6) of GAPDH while ovalbumin had no effect (Fig. 5, A and C, lanes 7–10). In this case the Kᵢ values, 122–152 μM, are in the same range as the Kᵢ for RNase A binding but are higher than the Kᵢ for RNase A uptake.

We also tested the effects of increasing amounts of RNase S-protein (Fig. 6, A and B, lanes 2–6) and RNase S-peptide (Fig. 6, C and D, lanes 2–6) on the binding (Fig. 6, A, C, and E) and uptake (Fig. 6, B, D, and F) of RNase A. RNase S-protein had no effect on binding or uptake of RNase A. In contrast, increasing amounts of RNase S-peptide reduced the binding (Kᵢ = 205 μM) and uptake (Kᵢ = 160 μM) of RNase A by lysosomes.

**An intermediate in the transport of RNase A into rat liver lysosomes**—We examined further the possibility that the band smaller than RNase A produced by proteinase K treatment (Fig. 1C, lanes 9–14; Fig. 3A, lanes 2–6; Fig. 4C, lanes 2–9; Fig. 6, B and D, lanes 2–6) was a lysosomal import intermediate. This intermediate form represents 16 ± 8% of total RNase A taken up by lysosomes after a 20-min incubation at 37°C and is found only associated with lysosomes and not in the supernatant fractions.

The possibility that this smaller form is generated as an intermediate in the intralysosomal degradation of RNase A is unlikely since this form is never seen unless the lysosomes are treated with proteinase K. Also, such an intermediate is never detected when RNase A is incubated with broken lysosomes or when RNase A is incubated with proteinase K without lysosomes (not shown).

We compared the RNase A inside lysosomes immediately after incubation or after a second 5- or 10-min incubation in the absence of RNase A to allow complete entry of the RNase A. Fig. 7A shows Coomassie-stained gels while Fig. 7B shows two separate immunoblots. The clipped RNase A evident immediately after incubation was present at reduced levels after a 5-min chase and was absent when 10 min were allowed for import. A likely interpretation of this result is that a kinetic block exists in the import of RNase A such that approximately 2 kDa of the protein remains susceptible to proteolysis when the rest of the molecule is within lysosomes. Amino-terminal sequence analysis of the lower molecular weight band produced

**Fig. 5. Effect of RNase A and ovalbumin on the binding and uptake of GAPDH by rat liver lysosomes.** Experimental details are as presented in the legend to Fig. 4. GAPDH (10 μg) is in lane 1 of panels A and C.
by the proteinase K treatment revealed the mature RNase A sequence, KETAA. Therefore, the clipped portion of RNase A must be at the carboxyl terminus.

A model for RNase A import into lysosomes consistent with the results presented in this paper is shown in Fig. 7C. RNase A interacts with hsc73 in the cytosol (step 1) and then binds to a receptor or a polypeptide transporter on the lysosomal membrane (step 2). Most of the RNase A is then rapidly imported into the lysosome interior, but approximately 2 kDa of the COOH-terminal portion of the protein remains outside the lysosome for a short time (step 3). Finally, the entire molecule is transported into the lysosome (step 4) where it is rapidly degraded (step 5) unless lysosomal proteases are inhibited.

**DISCUSSION**

There are many similarities in the lysosomal uptake of GAPDH (21), RNase A (19, 20), and RNase S-peptide (19, 20) including their stimulation by hsc73 and ATP/Mg++ (Table 1). Lysosomal uptake of RNase A and GAPDH is inhibited by prior proteolytic treatment of the lysosomes (Fig. 3), and binding of RNase S-peptide to fibroblast lysosomes is also inhibited by prior trypsin treatment (20). These results suggest the involvement of a proteinaceous receptor or transporter. More direct evidence for common elements in the lysosomal uptake mechanisms of these polypeptides includes competition for binding and uptake of RNase A by GAPDH (Fig. 4), competition for binding and uptake of GAPDH by RNase A (Fig. 5), and competition for binding and uptake of RNase A by RNase S-peptide (Fig. 6).

Quantitation of the binding and uptake of RNase A and GAPDH by rat liver lysosomes (Figs. 1 and 2) indicates that GAPDH binds more avidly than does RNase A ($K_d$ values = 10
and 95 μm, respectively). However, uptake of RNase A by lysosomes saturates more readily than uptake of GAPDH ($K_d$ values = 4 and 18 μm, respectively). Therefore, GAPDH binding and uptake by lysosomes occur in the same concentration range, but an excess of binding sites compared with uptake sites exists for RNase A. The initial RNase A molecules that bind to lysosomes must somehow be very efficiently coupled to the uptake process.

Competition for binding and uptake of GAPDH by RNase A (Fig. 5) is in the 100 μm range consistent with its competition being through binding to the lysosomes. The lack of competition for GAPDH uptake by RNase A in the 4 μm range suggests that the uptake machinery is different for the two proteins. Alternatively, the uptake machinery may be the same, but the efficiency of RNase A uptake may be decreased in the presence of GAPDH.

The Scatchard analyses (Figs. 1 and 2, insets) indicate maximal binding per 100 μg of lysosomes, of protein of 408 pmol of RNase A or 63 pmol of GAPDH. Our unpublished results indicate that rat liver lysosomes have an average volume of 0.042 μm³ consistent with reports of others (26). Using the same criteria that have been used to calculate mitochondrial binding and import sites (27-29), we calculate that each lysosome has approximately 1,700 binding sites for GAPDH and 10,000 for RNase A. These values are in the range of receptor numbers for the import of mitochondrial proteins (27-29).

One of the most striking apparent differences between the rat liver lysosomes used in these studies and the fibroblast lysosomes used previously (18-20) is the affinity for binding of substrate proteins. The $K_d$ value for binding of RNase S-peptide for fibroblast lysosomes was found to be 100 nm (20) while binding of GAPDH and RNase A to rat liver lysosomes shows a $K_d$ value of 10-95 μm. This difference between fibroblast and rat liver lysosomes may be due to the different experimental procedures used to measure binding and uptake in these different studies. For example, binding of RNase S-peptide to fibroblast lysosomes was carried out at 0 °C (20) while the binding to rat liver lysosomes reported here was carried out at 97 °C. Another consideration for this apparent difference is that the rat liver lysosomes may not be optimally active when derived from rats fasted for 20 h. We find that fasting for 96 h yields lysosomes that are 2-4 times more active, but whether this greater activity corresponds to altered $K_m$ or $K_d$ values remains to be determined.

The selective uptake of RNase A and RNase S-peptide by lysosomes appears to require a peptide region biochemically related to KFERQ (15, 19). Consistent with this idea, binding and uptake of RNase A can be competed with RNase S-peptide that contains the KFERQ sequence and not by RNase S-protein that does not contain a KFERQ-like region (Fig. 6). In addition, ovalbumin does not contain a KFERQ-like peptide region and does not compete for binding or uptake of RNase A (Fig. 4) or GAPDH (Fig. 5). GAPDH does not contain an exact KFERQ motif as previously defined (15), but it does contain a closely related sequence beginning at amino acid 258 in the human, hamster, and mouse sequence KVVKK. The three-dimensional structure of GAPDH shows that this sequence is in a solvent-exposed α-helical region of the tetramer (50). Whether this sequence is actually required for selective lysosomal uptake remains to be determined. Alternatively, KFERQ-like sequences might be formed by the tertiary structure of GAPDH.

The mechanisms by which RNase A and GAPDH are selectively taken up and degraded by lysosomes are not likely to be through microautophagy or macroautophagy since these processes do not show substrate specificity (14, 16) and are not known to be stimulated by ATP and hsc73. The lysosomal uptake process appears to be very similar to pathways of uptake of precursor proteins into organelles such as the mitochondrion and endoplasmic reticulum. These similarities include stimulation by ATP/Mg²⁺ and a cytosolic hsps70 and the requirement for an hsp70 within the organelle to pull precursor proteins across the organelle membrane (31-35). In addition, the import process appears to occur in stages; we report an import intermediate with approximately 2 kDa of the COOH-terminal portion of RNase A still exposed outside the lysosome (Fig. 7). Approximately 2 kDa from the COOH terminus, there are three consecutive branched-chain amino acids, IVY, that may stall the uptake process. In any event, intermediates have also been reported for the import of proteins into the mitochondrion and the endoplasmic reticulum (31-35). If these similarities extend further, there may be receptors for substrate proteins and/or hsc73 on the lysosome membrane as well as polypeptide translocation channels through the membrane as there are for proteins translocated into mitochondria and endoplasmic reticulum.

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