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# Covalent linkage of ribonuclease S-peptide to microinjected proteins causes their intracellular degradation to be enhanced during serum withdrawal

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ABSTRACT **The amino-terminal 20 amino acids are required for microinjected ribonuclease A (RNase A) to be taken up by lysosomes and degraded at an enhanced rate during serum withdrawal. We used water-soluble carbodiimides to covalently attach the RNase S-peptide (residues 1–20) to [$^3$H]RNase S-protein (residues 21–124) at unspecified locations. We then measured catabolism of the [$^3$H]S-protein-S-peptide conjugate after its microinjection into human diploid fibroblasts. The attached S-peptide caused the degradation of S-protein to be enhanced 2-fold in the absence of serum. Control experiments showed that degradation of [$^3$H]RNase S-protein remained unresponsive to serum after conjugation with the inactive fragment, RNase S-peptide (residues 1–10). Covalent attachment of RNase S-peptide had a similar effect on the catabolism of two other proteins. Degradation rates of microinjected $^{125}$I-labeled lysozyme and $^{125}$I-labeled insulin A chain are normally unresponsive to serum withdrawal. However, breakdown rates of microinjected $^{125}$I-labeled lysozyme-S-peptide and $^{125}$I-labeled insulin A chain-S-peptide conjugates were increased 2-fold during serum deprivation. We suggest that RNase S-peptide acts as a "signal sequence" that directs cytosolic proteins to lysosomes through a pathway that is activated by deprivation conditions.**

Multiple pathways of intracellular protein degradation exist in mammalian cells (1–4), and the current challenge is to define the roles of each pathway in contributing to overall proteolysis. For example, cytosolic ATP-dependent pathways of protein catabolism are responsible for the degradation of abnormal proteins (2, 3, 5, 6) and perhaps certain short-lived normal proteins (7, 8), while lysosomes are responsible for most of the increased proteolysis in tissues of starved animals (9–12) and in cultured cells deprived of serum (1, 13, 14). Both lysosomal and nonlysosomal pathways probably contribute to the breakdown of intracellular proteins in optimally nourished tissues (2, 9–11) and in cultured cells supplemented with serum (1, 14).

Among the many advantages of erythrocyte-mediated microinjection (15–17) is the ability to modify proteins of interest prior to microinjection to probe effects of protein structure on rates and pathways of proteolysis. For example, several microinjection studies have addressed the role of ubiquitin in the rapid degradation of abnormal proteins by ATP-dependent pathways in the cytosol (6, 18–20).

We have concentrated on pathways of degradation of long-lived proteins whose catabolic rates are increased in cultured cells in response to serum withdrawal (21–24). Pancreatic ribonuclease A (RNase A) microinjected into human fibroblasts is degraded with a half-life of 80–100 hr. When serum is removed from the fibroblasts, its degradative rate is increased 2-fold (22). The S-peptide region (residues 1–20 of RNase A) contains the information required for recognition of the entire protein for enhanced catabolism during serum withdrawal. RNase S-protein (residues 21–124 of RNase A) is degraded at the same rate in the presence and absence of serum while catabolism of RNase S-peptide microinjected alone shows the full 2-fold increase in degradation after serum withdrawal (22).

The intracellular degradation of microinjected RNase A takes place within lysosomes since degradative products of microinjected RNase A that had been tagged with a radiolabeled inert sugar accumulated only within lysosomes (23). Furthermore, the increased degradation of RNase A during serum withdrawal results from enhanced lysosomal uptake of the microinjected protein from the cytosol (23). Microinjected RNase S-protein is also degraded within lysosomes, but its rate of uptake is not affected by serum. These results suggest that multiple pathways or rate-limiting steps exist for the transfer of cytosolic proteins to lysosomes for subsequent degradation.

We now show that covalent attachment of RNase S-peptide to other proteins prior to microinjection also causes their catabolism to be enhanced during serum withdrawal. These results suggest that information in the S-peptide can direct a variety of proteins from cytosol to lysosomes for enhanced degradation during serum deprivation.

## MATERIALS AND METHODS

**Materials.** All proteins and peptides were from Sigma except RNase S-peptide 1–10, which was prepared by limited trypsin digestion of RNase S-peptide as described (25).

**General Procedures.** Growth of IMR-90 human diploid fibroblasts to confluent monolayers, labeling of proteins by iodination or reductive methylation, loading of radiolabeled proteins into erythrocyte ghosts, and polyethylene glycol-mediated fusion of erythrocytes with fibroblasts were as described (17, 22, 23).

**Osmotic Lysis of Pinosomes.** This procedure for introducing proteins into the cytosol of cultured cells was modified from Okada and Rechsteiner (26) for optimal use with human fibroblasts (17). In brief, we found the hypotonic shock to be more effective in rupturing pinosomes if 40% (vol/vol) rather than 60% (vol/vol) culture medium was used. We also found it necessary to wait 24 hr after the osmotic lysis to obtain valid degradation measurements (17, 27). A more complete discussion of this technique as applied to protein degradation studies and comparisons with erythrocyte-mediated microinjection can be found elsewhere (17, 27).

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Abbreviations: RNase A, bovine pancreatic ribonuclease A; RNase S-protein, residues 21–124 of RNase A; RNase S-peptide, residues 1–20 of RNase A.

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**Measurement of Degradation of Microinjected Proteins.** Proteolysis was monitored by the release of acid-soluble radioactivity into the medium as described (22, 23). All proteins and peptides in this study were completely precipitated by 3.25% (wt/vol) phosphotungstate in 5% (vol/vol) HCl (23, 28). The acid-soluble radioactivity released into the medium was monoiodotyrosine for iodinated proteins and dimethyllysine for reductively methylated proteins (21, 22).

**Covalent Linkage of RNase S-Peptide to Proteins.** This procedure was performed essentially as described by Hoare and Koshland (29) using the water-soluble carbodiimide, 1-ethyl-3-dimethylaminopropylcarbodiimide (Sigma). Excess nonradioactive RNase S-peptide (15 mg) was added to 1 mg of radiolabeled protein dissolved in 2 ml of water. The carbodiimide was added to a final concentration of 0.4 M, and the pH was adjusted to 4.75. The formation of peptide and isopeptide bonds was monitored by carrying out the reaction in a Radiometer pH-stat (Copenhagen, Denmark) and recording the amount of dilute acid (0.01 M HCl) required to maintain the reaction at pH 4.75 (29). The coupling, which was complete within 12 hr, was terminated by addition of sodium acetate to 1 M followed by chromatography through Sephadex G-10 equilibrated with 5 mM sodium phosphate (pH 7.2) to remove the carbodiimide and the sodium acetate.

**Analysis of Proteins After Coupling with RNase S-Peptide.** The successful coupling of S-peptide to other proteins was demonstrated as an apparent increase in molecular weight of the radiolabeled protein using $NaDodSO_4$/polyacrylamide gel electrophoresis as described (30), except the acrylamide concentration was increased to 15%.

The presence of accessible S-peptide on each radioactive conjugate was shown by specific interaction with an RNase S-protein affinity column. The affinity column was prepared from 7 ml of swollen CNBr-activated agarose as described by the manufacturer (Sigma). A total of 40 mg of RNase S-protein was bound to 7 ml of agarose. Affinity chromatography was carried out by applying to the column 1 ml of radioactive protein or conjugate in phosphate-buffered saline (pH 7.2) containing an additional 0.5 M NaCl to reduce nonspecific binding. The same buffer (15 ml) was used to collect material that did not bind to the S-protein column. Bound radioactivity was eluted with 15 ml of 50% (vol/vol) glacial acetic acid (31, 32).

## RESULTS

**Characterization of the Protein Conjugates.** The successful attachment of nonradioactive RNase S-peptide to radiolabeled proteins resulted in an increased molecular weight of the recipient protein. We used $NaDodSO_4$/polyacrylamide gel electrophoresis (15% gels) under reducing conditions to compare migration positions of [$^3$H]RNase S-protein, $^{125}$I-labeled lysozyme, and $^{125}$I-labeled insulin A chain before and after conjugation with S-peptide. Each of the three radiolabeled proteins migrated more slowly after conjugation with RNase S-peptide (summarized in Table 1). The slower migration is unlikely to be caused by protein-protein cross-linking due to the 15-fold excess (by weight) of the S-peptide. S-peptide-S-peptide conjugates undoubtedly do occur but are not evident in these experiments since the RNase S-peptide is not radiolabeled.

The stoichiometry of RNase S-peptide linkage in the conjugates cannot be determined precisely since the migration of such protein conjugates in $NaDodSO_4$/polyacrylamide gel electrophoresis may not be strictly proportional to the logarithm of molecular weight. However, assuming such a relationship does exist, we estimate that each molecule of [$^3$H]S-protein had an average of one or two S-peptide molecules attached while $^{125}$I-labeled lysozyme had an average of two and $^{125}$I-labeled insulin A chain had an average of nine (see Table 1).

Table 1. Characterization of [$^3$H]RNase S-protein, $^{125}$I-labeled lysozyme, and $^{125}$I-labeled insulin before and after covalent attachment of RNase S-peptide.

| Protein | Apparent $M_r$ ($\times 10^{-3}$) | S-peptides attached, mol/mol | % bound to S-protein-agarose |
|---|---|---|---|
| [$^3$H]RNase S-protein | 19 ± 0.7 | | 2–5 |
| + S-peptide | 21 ± 1.0 | ≈1–2 | 95–98 |
| $^{125}$I-labeled lysozyme | 14 ± 0.9 | | 6–11 |
| + S-peptide | 18 ± 1.2 | ≈2 | 89–94 |
| $^{125}$I-labeled insulin A chain | <5 | | 5–17 |
| + S-peptide | 22 ± 2.1 | ≈9 | 83–95 |

Apparent molecular weights were determined after $NaDodSO_4$/polyacrylamide gel electrophoresis in 15% gels (30). Unlabeled proteins of known molecular weight were run in the same gels. Gels were stained with Amido Schwarz, destained, and the migration positions of the molecular weight standards were recorded. The gel was then frozen, sliced, and the $^3$H or $^{125}$I radioactivity was measured. The protein standards were bovine serum albumin (67,000), ovalbumin (45,000), lysozyme (14,500), and cytochrome c (11,500). Note that RNase S-protein migrates more slowly than expected for its molecular weight of 11,600. Similar results have been reported for RNase A (23). The molecular weight of the insulin A chain could not be precisely determined because the peptide comigrated with the bromphenol blue tracking dye. Where ± values are given, they refer to standard deviations of calculated molecular weights for three to five different gels. The number of S-peptides attached per protein molecule was calculated based on the molecular weight of RNase S-peptide (2160) and the increase in apparent molecular weight of the conjugate. The molecular weight of the oxidized insulin A chain was taken as the calculated value (2100). The column at the right refers to the percentage of radioactive sample that bound to RNase S-protein-agarose and could then be eluted with 50% (vol/vol) glacial acetic acid. The range of results for two determinations are shown.

The majority of [$^3$H]RNase S-protein-S-peptide, $^{125}$I-labeled lysozyme-S-peptide, and $^{125}$I-labeled insulin A chain-S-peptide had S-peptide attached in a way that left it free to interact with other macromolecules. RNase S-peptide interacts strongly with RNase S-protein (31, 33), and agarose-RNase S-protein has been used as an affinity column to isolate RNase S-peptide and its derivatives (32). The proteins prior to conjugation to S-peptide show little or no affinity for the RNase S-protein column, but after attachment of S-peptide they are largely retained (Table 1).

**Degradation of [$^3$H]RNase S-Protein-S-Peptide.** We previously reported that unlabeled RNase S-peptide added to $^{131}$I-labeled RNase S-protein prior to microinjection restored the recognition of the S-protein for enhanced degradation during serum withdrawal (22). Similar experiments using [$^3$H]RNase S-protein indicated that reconstitution with unlabeled S-peptide was not possible, presumably due to modification of lysines that are necessary for the reassociation (22). We took advantage of this latter result to covalently link S-peptide to [$^3$H]S-protein at locations other than its normal amino terminus. To remove any traces of S-peptide that may have associated with [$^3$H]S-protein noncovalently, we dialyzed the conjugate against 50% (vol/vol) acetic acid (32, 34). Subsequently, the acetic acid was removed by dialysis against 5 mM sodium phosphate (pH 7.2). Absence of detectable RNase A activity (35) confirmed that no reconstituted enzyme remained in the preparation (data not shown).

[$^3$H]RNase S-protein and [$^3$H]RNase S-protein-S-peptide were loaded into separate samples of erythrocyte ghosts and microinjected into IMR-90 fibroblasts. Fig. 1*A* shows that RNase S-protein is degraded after microinjection in a serum-independent manner as we reported (22). However, the

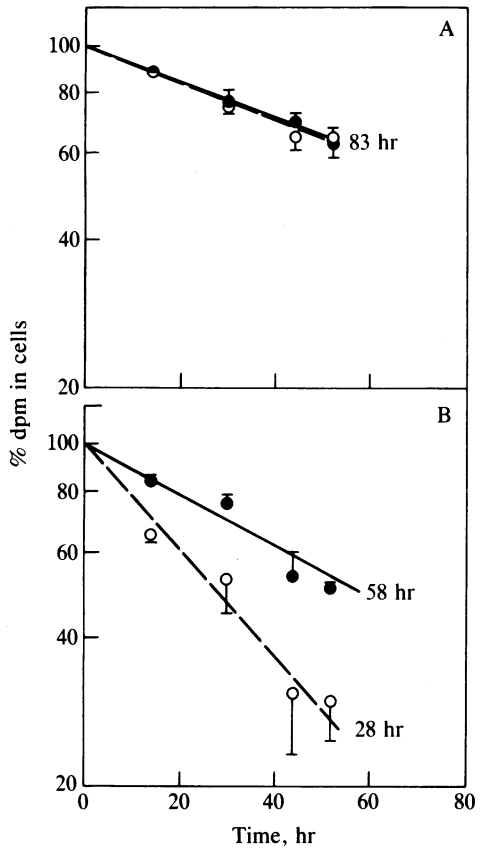


FIG. 1. Degradation of microinjected [$^3$H]RNase S-protein before (*A*) and after (*B*) conjugation with RNase S-peptide. RNase S-protein was reductively methylated using $NaB^3H_4$ as described (22). The specific radioactivity of [$^3$H]RNase S-protein was 2.1 $\mu$Ci/$\mu$g (1 Ci = 37 GBq) for the experiment shown. [$^3$H]RNase S-protein and [$^3$H]RNase S-protein-S-peptide were separately loaded into erythrocytes and microinjected into confluent cultures of fibroblasts. Approximately 60,000 dpm of radioactive RNase S-protein (*A*) or S-protein-S-peptide conjugate (*B*) was initially injected per plate of fibroblasts. Degradation was measured in the presence (●) and absence (○) of 10% (vol/vol) fetal bovine serum in the medium. Results shown are average ±1 SD for four plates of cells under each condition. The numbers are the half-lives in hr calculated from the best-fit line through the data points. Similar results were obtained in three additional experiments using erythrocyte-mediated microinjection and in one experiment using osmotic lysis of pinosomes to introduce proteins into fibroblasts.

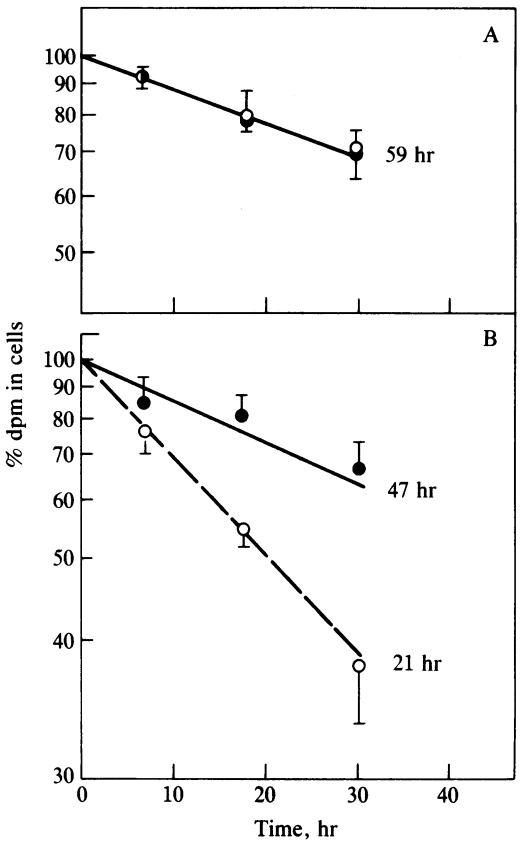


FIG. 2. Degradation of microinjected $^{125}$I-labeled lysozyme before (*A*) and after (*B*) conjugation with RNase S-peptide. The specific radioactivity of the $^{125}$I-labeled lysozyme was 0.9 $\mu$Ci/$\mu$g. Approximately 12,000 dpm of radioactive lysozyme (*A*) or lysozyme-S-peptide conjugate (*B*) were initially microinjected into each plate of fibroblasts. All other notations are as described in Fig. 1. Similar results were obtained in one additional experiment using erythrocyte-mediated microinjection to introduce the protein and conjugate into cells.

S-protein-S-peptide conjugate is degraded at an enhanced rate after serum withdrawal (Fig. 1*B*). Similar results applied to [$^3$H]RNase S-protein and [$^3$H]S-protein-S-peptide when they were introduced into fibroblasts by osmotic lysis of pinosomes (data not shown). These results suggest that the S-peptide can direct enhanced degradation during serum withdrawal even when it is not at its normal amino-terminal location.

To rule out the possibility that the carbodiimide treatment somehow altered RNase S-protein so that its degradation became serum responsive, we performed equivalent experiments in which the S-peptide fragment 1–10 was linked to [$^3$H]RNase S-protein. This fragment of S-peptide is not degraded in a serum-dependent fashion after microinjection because it lacks the essential glutamine-11 residue (27). This [$^3$H]S-protein-S-peptide 1–10 conjugate was degraded with a half-life of approximately 50 hr, and its degradation was not increased during serum deprivation (data not shown).

**Degradation of $^{125}$I-Labeled Lysozyme-S-Peptide and $^{125}$I-Labeled Insulin A Chain-S-Peptide.** Microinjection of lysozyme and the lysozyme-S-peptide conjugate was performed using erythrocyte-mediated fusion. $^{125}$I-labeled lysozyme was degraded with a half-life of 59 hr in this experiment (Fig. 2*A*). Attachment of S-peptide to lysozyme had little effect on its catabolism in the presence of serum, but the conjugate's degradative rate in the absence of serum was increased 2-fold (Fig. 2*B*).

Other preparations of iodinated lysozyme had half-lives ranging from 8 to 70 hr. We previously showed that lysozyme loses enzymatic activity and shows increased susceptibility to proteolytic attack *in vitro* after iodination (21) or reductive methylation (36). The variability in degradative rate of microinjected lysozyme probably reflects the degree to which its structure is disrupted by iodination.

The oxidized insulin A chain and its S-peptide conjugate were injected into fibroblasts using osmotic lysis of pinosomes. Our preliminary studies indicated that insulin A chain was internalized by fluid-phase pinocytosis since its rate of uptake was identical to that of [$^{14}$C]sucrose, a fluid-phase marker (27, 37). The insulin A chain-S-peptide conjugate was internalized at twice the fluid-phase rate, indicating some additional involvement of absorptive pathways. However, by beginning degradation measurements 24 hr after the osmotic lysis, the different modes of entry and other experimental problems associated with osmotic lysis were overcome (27, 37). The insulin A chain was degraded with a half-life of 100 hr in cells maintained in the presence or absence of serum (Fig. 3*A*). Attachment of S-peptide to the insulin A chain caused the degradative rate to double upon serum withdrawal (Fig. 3*B*).

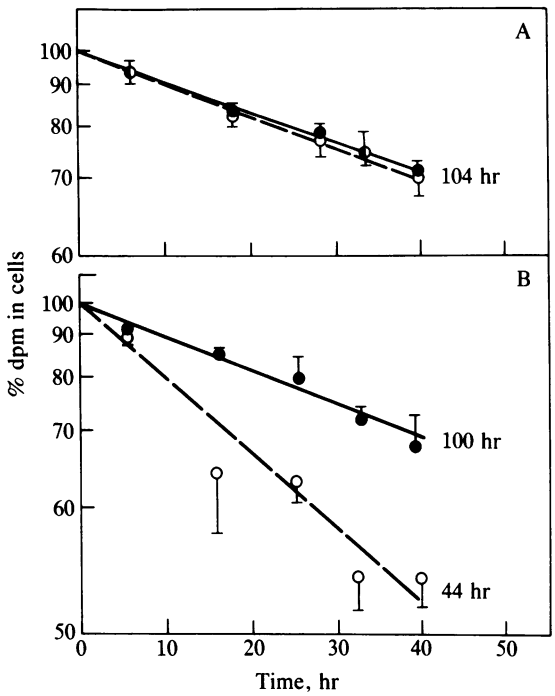


FIG. 3. Degradation of microinjected $^{125}$I-labeled insulin A chain before (*A*) and after (*B*) conjugation with RNase S-peptide. The specific radioactivity of the $^{125}$I-labeled insulin A chain was 0.6 $\mu$Ci/$\mu$g. Approximately 5000 dpm of the radioactive insulin A chain (*A*) or insulin A chain-S-peptide conjugate (*B*) were initially microinjected per plate of cells using osmotic lysis of pinosomes rather than erythrocyte-mediated fusion. All other notations are as described in the legend to Fig. 1. Similar results were obtained in two additional experiments using osmotic lysis of pinosomes.

## DISCUSSION

RNase S-peptide directs enhanced degradation of the entire RNase A molecule during serum withdrawal by increasing its rate of delivery to lysosomes. We have used this doubling of proteolytic rate during serum deprivation as an indication that S-peptide could direct conjugated proteins to this pathway of lysosomal degradation.

Our early results suggested that the position of attachment of RNase S-peptide was not important for its ability to direct enhanced catabolism during serum deprivation. Water-soluble carbodiimides catalyze the formation of peptide and isopeptide bonds between proteins (29). Since the RNase S-peptide and radiolabeled protein are mixed prior to addition of the carbodiimide, any accessible amino and carboxyl group can potentially be condensed. The conjugates should, therefore, consist of S-peptide linked to proteins in various ways and at different locations. Nevertheless, after one or two S-peptides were randomly attached to [$^3$H]RNase S-protein, the conjugate was degraded after serum withdrawal with single exponential decay kinetics (Fig. 1*B*).

To determine whether RNase S-peptide could also cause enhanced degradation of other proteins during serum withdrawal, we linked it to lysozyme and to oxidized insulin A chain. Enhanced degradation of $^{125}$I-labeled lysozyme-S-peptide was evident (Fig. 2*B*) with an average of only two S-peptides attached per lysozyme molecule. The linkage of S-peptide to $^{125}$I-labeled insulin A chain was more extensive, but the conjugate was also degraded in a serum-dependent manner (Fig. 3*B*). These results taken together suggest that a single S-peptide molecule is sufficient to cause a 2-fold increase in degradation rate of a protein after serum withdrawal. Additional S-peptide molecules attached to a protein do not appear to further increase the stimulation of catabolism. However, we cannot exclude the possibility that only one of the multiple S-peptides conjugated to insulin A chain was accessible for interaction with other macromolecules.

The ability of RNase S-peptide to cause enhanced degradation of a conjugated protein during serum withdrawal probably applies only to relatively long-lived proteins. Bovine serum albumin is degraded rapidly after microinjection (21, 38, 39), and its site of catabolism appears to be cytosolic (19, 23). Repeated conjugation experiments in which $^{125}$I-labeled bovine serum albumin-S-peptide was formed and then microinjected into cells indicated that S-peptide could not cause the catabolism of bovine serum albumin to be increased by serum withdrawal. The most likely explanation for these results is that rapidly degraded proteins cannot be effectively tagged for enhanced lysosomal proteolysis simply because they are degraded too rapidly by other proteolytic systems in the cell. Support for this idea includes the findings that short-lived proteins are not degraded more rapidly upon serum withdrawal (1, 40–42) and that many short-lived proteins are known to be degraded by cytosolic, ATP-dependent pathways (3, 8, 18). In addition, our unpublished results show that denatured RNase A (disulfide bonds "scrambled") (43) is degraded very rapidly after microinjection ($t_{1/2} < 8$ hr), and its catabolism is not increased by serum withdrawal despite the presence of the S-peptide region.

The mechanisms by which RNase S-peptide targets cytosolic proteins to lysosomes during serum withdrawal are unclear. We have established that the increased rate of degradation of RNase A upon serum withdrawal occurs due to enhanced delivery of the microinjected protein to lysosomes, its intracellular site of degradation (23). Selectivity in the uptake of cytosolic proteins by lysosomes has been proposed based on preferential binding to lysosomal membranes (44, 45) but might also be explained during serum withdrawal by preferential binding to regions of the endoplasmic reticulum (23, 46) or Golgi (47) that are forming autophagic vacuoles. The enhanced uptake of proteins during serum withdrawal might be due to unmasking membrane binding sites for RNase S-peptide. Alternatively, the S-peptide may be posttranslationally modified during serum deprivation so that its affinity to the binding site is increased.

A relatively nonspecific pathway of internalization of cytoplasm by lysosomes probably also exists. For example, microinjected [$^{14}$C]dextrans accumulate within lysosomes (48), and isolated lysosomes are capable of nonselective internalization of proteins (49–51). These results suggest that there are at least two different pathways by which cytosolic proteins can be taken into lysosomes for subsequent degradation.

We have identified the pentapeptide 7–11 of RNase S-peptide as containing the crucial information for the enhanced delivery of RNase A to lysosomes during serum withdrawal (27). If this pentapeptide (Lys-Phe-Glu-Arg-Gln) plays a general role in targeting cytosolic proteins to lysosomes during deprivation conditions, similar pentapeptides should occur in other cellular proteins. Two different lines of evidence confirm the presence of such pentapeptides.

We raised polyclonal antibodies to Lys-Phe-Glu-Arg-Gln-linked to bovine serum albumin and affinity purified the antibodies on an RNase S-peptide-agarose column. These antibodies were incubated with radiolabeled cytosolic proteins isolated from IMR-90 fibroblasts and then precipitated with a second antibody. At least 20–30% of the protein was specifically recognized by the antibody to S-peptide 7–11 (H.-L. Chiang and J.F.D., unpublished results).

We also conducted computer searches of the Protein Identification Resource data bank.‡ The exact pentapeptide

‡National Biomedical Research Foundation (1986) *Protein Sequence Database* (Georgetown University, Washington, DC), Tape Release 7.0.

is found only within the pancreatic ribonuclease family. However, when conservative substitutions of chemically related amino acids are allowed, related pentapeptides are found in 5 out of 5 proteins whose degradative rates are known to be enhanced during serum withdrawal. In contrast, related pentapeptides are evident in 0 out of 9 long-lived proteins whose degradative rates are known to be unaltered during serum withdrawal. Approximately 20% of mammalian intracellular proteins whose degradation characteristics are unknown also contain such pentapeptide regions, and these proteins occur preferentially in the cytosol. These results, which will be presented in full in a separate report (J.F.D., unpublished results), strongly suggest that the pathway of lysosomal degradation taken by microinjected RNase A will also apply to certain intracellular proteins.

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