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Biochemistry

Regulation of catabolism of microinjected ribonuclease A requires the amino-terminal 20 amino acids

(intracellular protein degradation/erythrocyte-mediated microinjection/human diploid fibroblasts/RNase S protein/RNase S peptide)

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ABSTRACT **RNase A introduced into the cytoplasm of IMR-90 human diploid fibroblasts by erythrocyte-mediated microinjection is degraded with a half-life of ≈75 hr in the presence of fetal bovine serum. In response to serum deprivation the degradative rate of microinjected RNase A is enhanced 2-fold. RNase S protein (amino acids 21–124) is degraded with a half-life similar to that of RNase A in the presence of serum, but its catabolism is not increased during serum withdrawal. Reconstitution of RNase S protein with RNase S peptide (amino acids 1–20) restored full enzymatic activity to the S protein as well as the ability of fibroblasts to increase its catabolism during serum deprivation. Finally, RNase S peptide microinjected alone shows the full 2-fold increase in degradative rate during serum withdrawal. These results show that recognition of RNase A for enhanced breakdown during serum deprivation is based on some feature of its amino-terminal 20 amino acids. Furthermore, our results indicate that the enhanced protein catabolism during serum deprivation can be highly selective.**

Average rates of intracellular protein degradation are enhanced in cultured cells that are deprived of serum, hormones, growth factors, or nutrients (1–15). This increased proteolysis is physiologically important because amino acids are used as an energy source under deprivation conditions (16, 17). It is now generally accepted that lysosomes are responsible for most of the enhanced degradation during withdrawal of serum, insulin, and amino acids (6, 14, 17, 18). However, the mechanisms by which cell proteins are delivered to lysosomes and whether such a process might be selective for certain cell proteins are still unresolved issues (14, 18–23).

The increased proteolysis during serum or hormone withdrawal appears to be somewhat selective in that it applies to long-lived proteins only. Short-lived proteins are affected little, if at all (2, 4, 6, 14, 24, 25). Degradation of two different molecular classes of long-lived proteins (small and basic) is increased during serum deprivation (26). We originally interpreted similar findings in tissues of diabetic and starved rats (27) as support for autophagy being a relatively nonspecific process superimposed on a more selective "basal" proteolysis (6, 27). Further consideration of our results (28) combined with additional data (25, 29) show that autophagy must, in fact, be selective for the more long-lived, small, and basic proteins. These results imply that deprived cells are able to recognize certain cellular proteins for enhanced degradation while sparing others.

To probe in more detail the mechanisms of selectivity in enhanced proteolysis, we have examined the degradation of bovine pancreatic RNase A (RNase A) using erythrocyte-mediated microinjection (30, 31). Previous studies in our laboratory have shown that small amounts of RNase A can be microinjected into confluent monolayer cultures of IMR-90 human lung fibroblasts by using polyethylene glycol (PEG) as the fusogen without altering cellular protein metabolism or growth rate (26). The microinjected RNase A is randomly distributed throughout the cytoplasm of the recipient fibroblasts, and its degradative rate is increased 2-fold upon withdrawal of serum, insulin, fibroblast growth factor, and dexamethasone.

RNase A was chosen as a model protein to analyze the mechanisms of enhanced degradation because its structure and those of several derivatives have been well characterized (32, 33). This paper focuses on the importance of the amino-terminal 20 amino acids of RNase A for the ability of fibroblasts to enhance the degradation of the total protein. The studies reported here also provide a striking example of selectivity in the degradation of different proteins during serum withdrawal.

MATERIALS AND METHODS

Growth of IMR-90 human diploid fibroblasts to confluent monolayers, iodination of proteins with lactoperoxidase-glucose oxidase, and loading of human erythrocyte ghosts with radiolabeled proteins have been described (26).

Reductive Methylation. Reductive methylation of proteins with NaB^3H_4 (7–9 Ci/mmol; 1 Ci = 3.7×10^{10} Bq; New England Nuclear) was performed by the method of Tack *et al.* (34). Typically, 0.5 ml of protein solution (20 mg/ml in 0.2 M borate buffer, pH 8.9) was mixed with sufficient 0.2 M formaldehyde to be in 4-fold molar excess of protein primary amino groups. To this mixture was added NaB^3H_4 such that the molar ratio of NaB^3H_4/HCHO was 0.4. The mixture was agitated and allowed to stand on ice for 10 min and then chromatographed over Sephadex G-10. Protein concentration of void volume fractions was assayed by the method of Lowry *et al.* (35) or Udenfriend *et al.* (36) by using RNase A as a standard, and the acid-soluble and acid-insoluble radioactivity was counted in a Packard Tri-Carb liquid scintillation counter. Acid-soluble radioactivity was always <1% of the acid-insoluble radioactivity.

Reconstitution of RNase A from RNase S Protein and RNase S Peptide. RNase S protein (amino acids 21–124) and RNase S peptide (amino acids 1–20), the two fragments produced by subtilisin treatment of RNase (see Fig. 1), were purchased from Sigma and used without further purification. Reconstitution was performed by the method of Richards and Vithayathil (37). S peptide was added in 5-fold molar excess to S protein in 100 mM phosphate buffer (pH 7.4). Reconstitution was complete upon vortexing, and the resulting preparation was assayed for RNase A activity by its ability to hydrolyze yeast RNA (Sigma) by the method of Kalnitsky *et al.* (38). We were typically able to reconstitute 90–100% of native RNase A enzyme activity using ^{125}I-labeled S protein and unlabeled S peptide, whereas

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Abbreviations: RNase A, bovine pancreatic RNase A; RNase S protein, the fragment (amino acids 21–124) produced by subtilisin treatment of RNase A; RNase S peptide, the fragment (amino acids 1–20) produced by subtilisin treatment of RNase A; PEG, polyethylene glycol.

both fragments were without activity prior to reconstitution. Similarly, 95% of RNase A activity resulted from reconstitution of [^{3}H]S peptide with unlabeled S protein. However, for unknown reasons, reconstitution was not possible when using [^{3}H]S protein and unlabeled S peptide. This reconstituted combination had only 20–40% of the specific activity of the native enzyme.

Microinjection. Our fusion procedure has been altered slightly from our original report (26). We now use Koch-Light PEG 1,000 from Research Products International (Elk Grove, IL) rather than PEG 1,000 from J. T. Baker. We established an optimal concentration for fusion of 40% PEG rather than the previously reported 10% for Baker PEG. The reasons for this difference are not known, but the 40% PEG has no effect on metabolism of cellular proteins labeled with [^{3}H]leucine (39), and degradative rates of microinjected RNase A are identical with 10% Baker PEG and 40% Koch-Light PEG (unpublished data).

Measurements of Degradation of Microinjected Proteins. Proteolysis was monitored by release of acid-soluble radioactivity into the medium. Complete precipitation of RNase A and RNase S protein was possible by using 3.25% phosphotungstate in 5% HCl (40). Quantitative precipitation of RNase S peptide required 24 hr at 4°C and carrier protein (RNase A or bovine serum albumin at a final concentration of 5 mg/ml). Aliquots (0.5 ml) were taken from cultures that originally contained 10 ml of medium. After correction for volume changes due to successive sampling, the following calculations allowed construction of degradation curves:

$$\left[\begin{array}{l}\text{cpm in monolayer}\\ \text{at end of experiment}\end{array}\right] + \left[\begin{array}{l}\text{total acid-soluble cpm}\\ \text{released into medium}\end{array}\right] = \left[\begin{array}{c}\text{total cpm microinjected}\\ \text{into cells}\end{array}\right]$$

$$\left[\frac{\text{total cpm microinjected} - \text{acid-soluble cpm in medium}}{\text{total cpm microinjected}}\right]_t \times 100 = \%\ \text{cpm remaining in cells at time } t.$$

RESULTS

Radioactive Labeling of Proteins. Our previous studies indicated that RNase A could be iodinated with up to 3 iodines per protein molecule without detectable effect on enzymatic activity or susceptibility to proteolytic attack *in vitro* (26). RNase S protein is ≈20 times more sensitive to trypsin and chymotrypsin than is RNase A, but the S protein sensitivity is also unaffected by iodination (data not shown).

RNase S peptide contains no tyrosine residues (Fig. 1), so we adopted reductive methylation (34) for some of our studies. We calculated the molar ratio of ^{3}H incorporation per enzyme molecule from the initial specific radioactivity in the reaction mixture and the resulting specific radioactivity of the labeled protein. Typically, the molar incorporation of —C^{3}H$_3$ per protein was <0.6. Because the methylation procedure produces primarily dimethyllysine (44), our calculations indicate that 30% of the protein molecules contain a single modified lysine.

[^{3}H]RNase A retained 80–100% of the activity of the unmodified enzyme and showed no increase in susceptibility to digestion by Pronase or chymotrypsin (data not shown). These results, together with the data in this paper showing that microinjected [^{3}H]RNase A has the same intracellular half-life as ^{125}I-labeled RNase A (^{125}I-RNase A), suggest that neither iodination nor reductive methylation has serious effects on RNase A structure.

Validation of Erythrocyte-Mediated Microinjection to Study Degradation of RNase A. We have previously reported (26) that: (*i*) erythrocyte ghosts do not appreciably degrade ^{125}I-RNase A, as measured by production of acid-soluble radioactivity or altered electrophoretic mobility in NaDodSO$_4$/polyacrylamide gels; (*ii*) microinjection of RNase A into fibroblasts does not affect rates of synthesis or degradation of cellular proteins; (*iii*) microinjected RNase A appears to be randomly distributed in the cytoplasm of recipient fibroblasts; and (*iv*) the major acid-soluble degradation product released into the medium is monoiodotyrosine, which cannot be utilized by the cell's protein synthesis machinery (45). Recent studies indicate that dimethyllysine or methyllysine is the major degradation product of microinjected methylated proteins (46). Because methyllysines are also not used by the cell's biosynthetic machinery (47), reutilization of the isotopes is not a concern in our degradation studies.

We have further determined that <1% of the microinjected radioactivity can be accounted for by ghosts that remain attached to the monolayer but not fused with cells. These experiments involved loading ghosts with a mixture of ^{125}I-RNase A and fluorescein-labeled RNase A. We counted ghosts attached to the monolayer but not fused with cells using fluorescence microscopy, and, in the same experiment, we monitored radioactivity microinjected into the monolayer.

A second possibility is that RNase A released from the loaded ghosts during the exposure to PEG might bind to the monolayer. To test this possibility, we placed free ^{125}I-RNase A on monolayers in the same volume as is used for loaded ghosts in a typical microinjection. Then we added PEG and washed the cells according to our usual fusion procedures. The amount of radioactive protein that adhered to the monolayer could account for no more than 10% of the radioactivity microinjected. Furthermore, <15% of this adhering radioactivity was degraded over a 48-hr period, and its degradation was the same in the presence or absence of fetal bovine serum.

Although RNase A and RNase S protein are not hydrolyzed by erythrocyte ghosts, the S peptide appears to undergo limited degradation by the ghosts during loading. Loading of [^{3}H]S peptide caused an increase in acid-soluble radioactivity of 6–10%, but no further increase occurred during incubation of loaded ghosts for 2 days at 37°C. This limited degradation was also unaffected by serum and therefore could not account for the degradation of microinjected RNase S peptide by fibroblasts.

Degradation of RNase A and RNase S Protein. In 26 separate experiments, ^{125}I-RNase A was degraded with a $t_{1/2}$ (±SD) of 75 ± 20 hr, and its $t_{1/2}$ (±SD) was decreased to 40 ± 14 hr during serum deprivation. In 10 separate experiments ^{125}I-labeled RNase S protein was degraded with a $t_{1/2}$ (±SD) of 61 ± 24 hr in the presence of serum and 65 ± 16 hr in the absence of serum. The half-lives of RNase A and RNase S protein were not significantly different in the presence of serum ($P > 0.05$). However, in the absence of serum the increased catabolism of RNase A was significant ($P < 0.005$), whereas no increase was evident for RNase S protein ($P > 0.40$) based on the two-tailed Student's *t* test.

To eliminate experiment-to-experiment variation in comparing protein half-lives, we coloaded and comicroinjected ^{125}I-RNase A and ^{131}I-labeled RNase (^{131}I-RNase) S protein into the same cells. Fig. 2 shows that both proteins have similar half-lives in media containing 10% fetal bovine serum. However, deg-

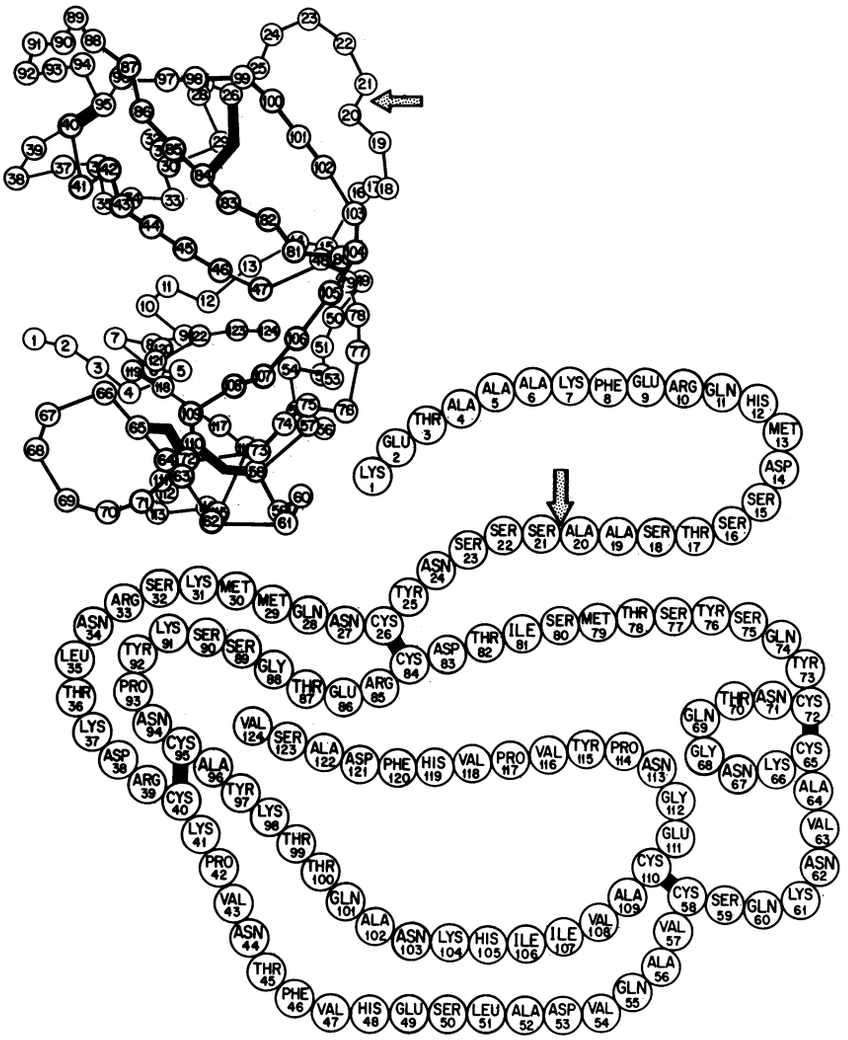

FIG. 1. Amino acid sequence and three-dimensional structure of RNase A. The amino acid sequence is as reported (41) but is arranged to reflect disulfide bonding (modified from ref. 42). The conformation is as shown by Dickerson and Geis (43). The arrows indicate the site of subtilisin cleavage to form S peptide (amino acids 1–20) and S protein (amino acids 21–124).

radation of RNase A was increased 2.4-fold, whereas catabolism of S protein was not changed when cells were deprived of serum. These results indicate that within the same cells, RNase A is recognized as a protein to be broken down more rapidly during serum deprivation, whereas RNase S protein is not.

To determine whether the loss of response to serum deprivation could be restored, we reconstituted ^{131}I-RNase S protein with unlabeled RNase S peptide. Reconstitution restored 96% of enzymatic activity to the S protein, and Fig. 3 shows that the degradation of coloaded and comicroinjected reconstituted ^{131}I-RNase S protein and ^{125}I-RNase A were similar both in the presence and in the absence of serum. Thus, reconstitution of RNase S protein with RNase S peptide restores the ability of fibroblasts to regulate the catabolism of S protein during serum withdrawal.

Degradation of [^{3}H]RNase A, [^{3}H]RNase S Protein, and [^{3}H]-RNase S Peptide. Fig. 4 shows that [^{3}H]RNase A has a half-life very similar to that of RNase A labeled with ^{125}I. Furthermore, fibroblasts were able to regulate the catabolism of [^{3}H]RNase A during serum withdrawal. [^{3}H]RNase S protein had a half-life similar to that of RNase A in the presence of 10% fetal bovine serum, but fibroblasts were unable to increase its catabolism during serum deprivation.

Fig. 4 also demonstrates that cells were able to fully regulate the breakdown of [^{3}H]RNase S peptide during serum withdrawal. These results suggest that fibroblasts increase the degradative rate of RNase A during serum deprivation in response to information in the amino-terminal 20 amino acids.

DISCUSSION

RNase A is a small, basic, nonglycosylated protein, and therefore we expected its breakdown to be regulated by serum and hormones (27–29). Furthermore, RNase A is a convenient choice for the study of the intracellular degradation of a particular protein because of the extensive literature regarding its structure (32, 33) and the commercial availability of well-defined fragments of the protein. The production of RNase S protein and S peptide by subtilisin treatment and the simple reconstitution of active RNase from the S protein and S peptide were first described by Richards and Vithayathil (37). We have made use of these fragments and their reconstitution products to study the role of the amino terminus of RNase A in directing the ability of fibroblasts to regulate its degradation during serum deprivation.

Our comicroinjection studies of ^{125}I-RNase A and ^{131}I-RNase S protein (Fig. 2) show the necessity of the S peptide for enhancement of degradation during serum withdrawal. The cellular degradative machinery can clearly distinguish between RNase A and RNase S protein. This selectivity is particularly striking because RNase S protein and RNase A differ only slightly

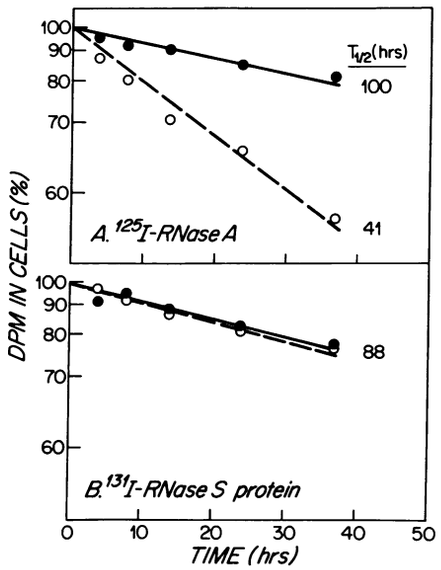

FIG. 2. Degradation of microinjected ^{125}I-RNase A and ^{131}I-RNase S protein. ^{125}I-RNase A (*A*) and ^{131}I-RNase S protein (*B*) were coloaded into human erythrocytes and then were comicroinjected into confluent monolayers of IMR-90 human diploid fibroblasts. Both ^{125}I and ^{131}I acid-soluble radioactivity released into the medium was monitored in a dual-channel Packard gamma counter. Degradative rates were determined both in the presence of 10% fetal bovine serum (●) and in the absence of serum (○). Each point represents the average value for four to six cultures; 15,000–20,000 dpm for each protein was initially microinjected into each culture. Similar results were obtained in two other experiments.

in physical properties and in overall conformation (32, 33). The fact that degradation of RNase A is unaffected by comicroinjection with RNase S protein rules out the possibility that the RNase

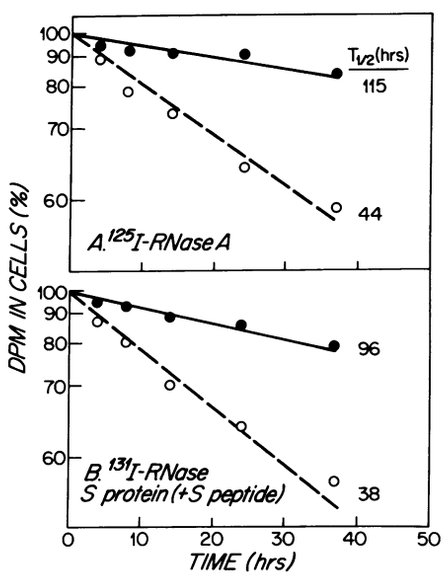

FIG. 3. Degradation of microinjected ^{125}I-RNase A (*A*) compared to ^{131}I-RNase S protein after reconstitution with RNase S peptide (*B*). Unlabeled S peptide was added to ^{131}I-labeled S protein in 5-fold molar excess and vortexed. This reconstituted RNase S protein was mixed with an equal amount of ^{125}I-labeled RNase A and was coloaded into human erythrocytes. The reconstitution restored full enzymatic activity to RNase S protein and also fully restored the ability of fibroblasts to regulate its catabolism during serum deprivation. ●, Presence of fetal bovine serum; ○, absence of serum. Other experimental details are as described in the legend to Fig. 2 and in the text.

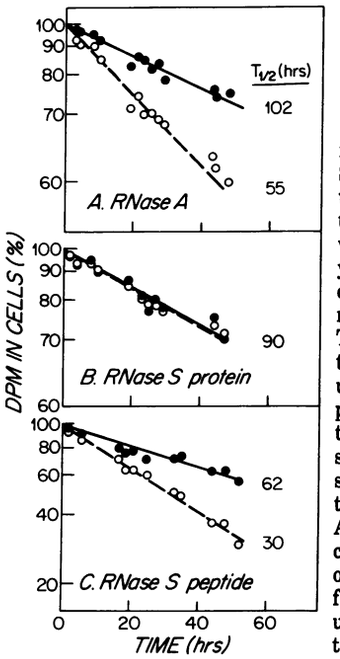

FIG. 4. Degradation of microinjected [^{3}H]RNase A, [^{3}H]RNase S protein, and [^{3}H]RNase S peptide. RNase A (*A*), RNase S protein (*B*), and RNase S peptide (*C*) were labeled by reductive methylation (34). Approximately 8,000 dpm for each protein was initially microinjected into cell cultures. The results shown are pooled from three different experiments that used four to six cultures for each protein in the presence of 10% fetal bovine serum (●) and four to six cultures in the absence of serum (○). Fibroblasts were able to regulate degradation of RNase A in the absence of serum (*A*) but could not increase the catabolism of RNase S protein (*B*). However, fibroblasts were able to fully regulate degradation of RNase S peptide (*C*).

S protein somehow alters the ability of the fibroblasts to respond to serum deprivation. Because we can restore enzymatic activity and serum-directed regulation of proteolysis by reconstitution of S protein with S peptide (Fig. 3), neither the enzymatic activity (37) nor the ability of fibroblasts to increase the degradative rate of the enzyme appears to be altered by the break between residues 20 and 21.

Our results shown in Fig. 4 indicate that [^{3}H]RNase A and [^{3}H]RNase S protein are degraded similarly to their iodine-labeled counterparts. Unfortunately, [^{3}H]S protein could not be reconstituted with S peptide to see if the serum responsiveness of the half-life could be restored. Most likely, the modification of lysine residues on the S protein alters the ability of the S peptide to reconstitute (32, 33).

The catabolism of [^{3}H]RNase S peptide is subject to full regulation by serum (Fig. 4*C*). We are not certain whether the information in the amino acid 1–20 region of RNase A that is responsible for its enhanced degradation is a function of primary sequence or secondary structure of the peptide. The amino acid 2–12 region of the S peptide is α helical when reconstituted with S protein, but the S peptide by itself has been reported to be entirely random coil (48) or partially α helical (49, 50) in aqueous solution. Furthermore, the secondary structure of the S peptide varies greatly depending upon tonicity and hydrophobicity of its environment (51). Therefore, it is difficult to predict to what extent the S peptide will show α-helical character once it has been microinjected into fibroblasts. Thus, cellular recognition of the S peptide for enhanced degradation during deprivation conditions may depend on amino acid sequence or secondary structure, or both. This recognition is perhaps analogous to other well-known biological systems in which a short peptide region directs the processing or intracellular location of an entire protein (52, 53).

It is also possible that the amino acids in the S peptide may be "tagged" by the fibroblast in some way that leads to enhanced breakdown during serum deprivation. Several chemical modifications of proteins have been proposed as rate-limiting steps in intracellular protein degradation. For example, disulfide interchange between free sulfhydryl groups of a potential substrate protein and those of membrane proteins have been implicated as a rate-limiting step in intracellular proteolysis (54, 55). The lack of cysteines in RNase S peptide rules out this model

as an explanation for the enhanced degradation of the S peptide during serum withdrawal. Another possible rate-limiting step in intracellular protein degradation has been proposed to be ubiquitin binding to lysine residues of the protein to be degraded (56). The S peptide has two lysine residues (at positions 1 and 7), and the possibility that ubiquitin binds preferentially to these lysines and then leads to the serum-responsive degradation of the S peptide is yet to be examined.

Our findings are important in that they show that enhanced degradation during serum deprivation can be highly selective, differentiating between two proteins with similar half-lives and physical characteristics. The increased degradation is thought to involve lysosomal pathways (6, 14, 18, 57), and studies in our laboratory using raffinose-labeled RNase A (58) suggest that the enhanced catabolism of RNase A during serum withdrawal is largely lysosomal (unpublished data). Several investigators have argued, often on the basis of electron micrographs showing lysosome-engulfed organelles, that lysosomal degradation must be relatively nonselective. However, other researchers have proposed models in which lysosomal catabolism might be able to display selectivity for particular cellular proteins (20, 23, 59). Although our studies show selectivity in the enhanced catabolism, it also seems likely that some portion of the autophagy under deprivation conditions is nonselective. For example, lysosomal sequestration of microinjected sucrose (59) and dextran (57) can be demonstrated in deprived cells, and random copolymers of poly(glutamate,tyrosine) are degraded more rapidly after serum withdrawal (26).

Further experimentation is necessary to determine which part of the RNase S peptide sequence is essential for its selection by cells for enhanced breakdown during serum withdrawal. Because the S peptide is hydrolyzed to a limited degree by the erythrocyte ghosts during the loading procedure, the entire 1- to 20-amino acid sequence is probably not required for regulation by serum. Possible approaches to localizing the sequences responsible for serum regulation include limited hydrolysis or chemical modification of specific residues and the production of synthetic variants of the primary sequence (32, 33, 60).

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