Transfer RNA is an essential component of the ubiquitin- and ATP-dependent proteolytic system

(protein degradation/\text{tRNA}^\text{His}/autoantibodies)

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ABSTRACT Protein degradation via the nonlysosomal ATP-dependent pathway in rabbit reticulocytes involves a number of components. In the initial event, ubiquitin, an abundant 76-residue polypeptide, becomes covalently linked to the protein substrate in an ATP-requiring reaction. Once marked in this way, the conjugated protein is proteolyzed in a reaction that also requires ATP. Ubiquitin-marking appears to be important to the progression of cells from one stage to another of the cell cycle; it may also be involved in gene activation. Here we show that tRNA is another essential component of the system. Ribonucleases strongly inhibit the ubiquitin- and ATP-dependent degradation of \textsuperscript{125}I-labeled bovine serum albumin in the reticulocyte system \textit{in vitro}. RNAs extracted from fractions of the reticulocyte extract or from mouse cells restore proteolytic activity. When the RNA is fractionated by gel electrophoresis, only the tRNA fraction is active in restoring proteolysis. Furthermore, pure mouse tRNA\textsuperscript{His}, isolated by immunoprecipitation with patient autoimmune sera, restores the proteolytic activity. The possibility that the level of uncharged tRNA in mammalian cells regulates the ubiquitin- and ATP-dependent proteolytic system is discussed.

Intracellular protein degradation is important to basic cellular functions such as regulation of key enzymes in metabolic pathways, response to changing parameters of the extracellular environment, and preferential removal of defective proteins. One intriguing aspect of protein degradation in all organisms is its absolute requirement for metabolic energy. ATP dependence, which is not expected on thermodynamic grounds, apparently reflects a mechanism that endows high specificity or control (for reviews, see refs. 1–3).

There are two main pathways for intracellular protein degradation in mammalian cells, lysosomal and nonlysosomal. The degradation of endocytosed proteins and the degradation of intracellular proteins under conditions of nutritional deprivation occurs mostly in the lysosomes. Nonlysosomal systems are probably responsible for the highly selective turnover of intracellular proteins under basal metabolic conditions (for reviews see refs. 1–3).

A nonlysosomal ATP-dependent proteolytic system from rabbit reticulocytes previously has been characterized and partially purified. The system degrades short-lived proteins in reticulocytes and other mammalian cells (2–5). It consists of several required components, one of which is ubiquitin, a 76-residue polypeptide found in all eukaryotic cells. Ubiquitin is activated by ATP in a two-step mechanism (6) and then covalently linked to the proteolytic substrate via an isopeptide bond between its COOH-terminal glycine and free ε-amino groups of lysine residues in the substrate.

Formation of this ubiquitin-protein conjugate appears to be the initial event that marks the substrate for degradation by as yet uncharacterized proteases (2, 3). ATP is also required for the proteolytic degradation of the conjugates (7).

The ubiquitin- and ATP-dependent proteolytic system is likely to be involved in regulation of gene expression at the chromatin level. Ubiquitin is found conjugated to histone 2A (uH2A semihistone) via an isopeptide bond (to lysine-119) in a subset of nucleosomes (8). Because these are preferentially associated with transcribed genes (9), it is possible that a locus-specific, ubiquitin-mediated proteolysis of chromosomal proteins underlies gene activation. Moreover, a temperature-sensitive cell-cycle mutant (arrested at the S/G\textsubscript{2} boundary) that has been shown to contain a thermolabile ubiquitin-activating enzyme (4) fails to conjugate ubiquitin to proteolytic substrates and H2A and to degrade abnormal and short-lived proteins (4, 5). Hence, ubiquitin-marking of either chromatin proteins or other regulatory proteins also may be involved in the transition of cells from one stage of the cell cycle to another.

The mechanisms that regulate the ubiquitin- and ATP-dependent proteolysis pathway are not known. Here we show that tRNA is another essential component of the system and discuss its involvement in the control of protein degradation.

MATERIALS AND METHODS

Materials. Rabbit reticulocyte lysate and crude reticulocyte fraction II were prepared according to Ciechanover \textit{et al.} (10). Purified tRNA\textsuperscript{His} was prepared as described by Hershko \textit{et al.} (11). Na\textsuperscript{125}I was from Amersham. Ribonucleases were from Worthington. Thymidine 3',5'-bisphosphate (pT\textsubscript{P}) was from Calbiochem. All other materials were of analytical grade.

A cloned mouse tRNA\textsuperscript{His} gene in M13RF was kindly provided by J. Harding (Columbia University). It contains 5 base pairs of 5' flanking sequence and 160 base pairs of 3' flanking sequence as well as the entire tRNA\textsuperscript{His} coding region (12).

Proteolytic Assays. Proteolytic assays were carried out as described by Hershko \textit{et al.} (13), except that the reaction mixture contained 1 mM CaCl\textsubscript{2}. For RNase inhibition and RNA complementation assays, the reaction mixture was preincubated for 30 min with 1 \mu g of micrococcal nuclease followed by addition of 0.1 mM pT\textsubscript{P} or 1.5 mM EGT A and incubation for an additional 15 min. The reaction was initiated by addition of RNA, ubiquitin, and \textsuperscript{125}I-labeled bovine serum albumin (\textsuperscript{125}I-BSA) and allowed to continue for 2 hr.

Abbreviations: \textsuperscript{125}I-BSA, \textsuperscript{125}I-labeled bovine serum albumin; pT\textsubscript{P}, thymidine 3',5'-bisphosphate.

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Cells, Extracts, and Sera. NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium (DME; GIBCO) supplemented with 10% bovine calf serum, sodium pyruvate (110 μg/ml), penicillin (60 μg/ml), and streptomycin (100 μg/ml). Cells were labeled with [32P]P (50 μCi/ml; 1 Ci = 37 GBq) in phosphate-free DME for 12-16 hr. Cell sonicates (14) were prepared and subjected to either immunoprecipitation (15) or direct phenol-extraction and ethanol-precipitation.

Sera from patients with polymyositis or related autoimmune disorders were provided by J. Hardin (Yale University) and M. Reichlin (Oklahoma Medical Research Foundation).

RNA Preparation and Analysis. Phenol-extracted, ethanol-precipitated RNA (from either crude reticulocyte fraction II, cell sonicates, or immunoprecipitates) was fractionated on 10% polyacrylamide gels containing 7M urea/90 mM Tris borate, pH 8.3/2.5 mM EDTA. Two-dimensional gel electrophoresis was as described by Rosai et al. (16). Unlabeled RNA was stained with ethidium bromide (0.5 μg/ml). Individual bands or spots were excised and RNA was extracted by the "crush-and-soak" method (17). RNase T1, fingerprints of eluted [32P]-labeled RNAs, were prepared (18) using thin-layer homochromatography on polyethyleneimine (PEI) 300 (Brinkmann) for the second dimension (14).

Transfer of RNA from gels to diazobenzamidomethyl paper was carried out according to Alwine et al. (19), except that the transfer buffer was 50 mM sodium acetate, pH 4.5. Prior to transfer, the gel was soaked in transfer buffer (2 × 20 min). Quantitation of tRNA[H] detected after blotting was accomplished by densitometric analysis of individual bands.

RESULTS

Ubiquitin- and ATP-Dependent Proteolytic System Is Inhibited by Ribonucleases. Proteolysis assays were carried out with crude reticulocyte fraction II, which contains all the required components except ubiquitin and ATP (10). As shown in Fig. 1A, preincubation of fraction II with either bovine pancreatic RNase A or micrococcal nuclease at concentrations exceeding 2 μg/ml inhibits the ubiquitin- and ATP-dependent degradation of [125I]-BSA by 80-90%. The inhibition is specific to ribonucleases: arginine-rich and H1 histones, lysozyme, soybean trypsin inhibitor, myoglobin, cytochrome c, and insulin did not exhibit any inhibitory effect (data not shown). Conversely, other ribonucleases with different recognition specificities (snake venom endonucleases, RNases T1 and T2) all showed strong inhibition at low concentrations (100-200 units/ml; not shown). DNase I at high concentration (2000 units/ml) did not inhibit (not shown).

To determine whether the inhibitory effect of the RNases is indeed due to their enzymatic activities rather than some unusual common feature(s) of the protein molecules, we inhibited the enzymatic activity of the RNases before adding them to the proteolytic system. As can be seen in Fig. 1B, preincubation of 200 ng of RNase A with 60 units of human placental ribonuclease inhibitor (Promega Biotec, Madison, WI) completely abolished its inhibitory effect. Preincubation of 1 μg of micrococcal nuclease with 50 μM pTP, a specific inhibitor of the enzyme (20), also relieved the inhibition. Likewise, omission of Ca2+, which is essential for micrococcal nuclease activity (20), resulted in no inhibition of the proteolytic activity (data not shown).

Finally, we tested the possibility that the ribonuclease effect is indirect. For instance, nucleoside treatment of crude fraction II could release inhibitor(s) of the proteolytic activity. To address this possibility, we incubated crude fraction II with micrococcal nuclease and then inhibited the enzyme by adding pTP. Then, four volumes of the nuclease-treated crude fraction II were added to one volume of untreated extract. No inhibition of proteolysis was observed (not shown). This experiment rules out the possibility that either released nucleotides or any protein moiety freed by removal of RNA can inhibit the ATP- and ubiquitin-dependent proteolytic activity.

Total RNA from Crude Reticulocyte Fraction II Restores the Proteolytic Activity of the RNase-Inhibited Proteolytic System. To test directly the role of RNA in the ubiquitin- and ATP-dependent proteolytic system, we first treated the complete system with micrococcal nuclease and then added the Ca2+ chelator, EGTA (1.5 mM) to inhibit the nuclease. Subsequently, phenol-extracted total RNA from crude

Fig. 1. (A) Inhibition of the ubiquitin- and ATP-dependent proteolytic system by RNase A (●) or micrococcal nuclease (○). Crude reticulocyte fraction II was preincubated for 30 min at 37°C with various amounts of micrococcal nuclease or RNase A before initiation of the proteolytic reaction. (B) Effect of inhibitors of RNases on the inhibitory effect of the enzymes on the ubiquitin- and ATP-dependent proteolytic system. Micrococcal nuclease (1 μg; ●) or RNase A (0.2 μg; ○) were preincubated at 37°C for 30 min with the indicated amounts of pTP or human placental ribonuclease inhibitor (u, units), respectively, before addition to a complete proteolytic system. The inhibition obtained with untreated ribonucleases was 84%. The background of 16% was subtracted for all samples and the results were normalized to 100% (maximal proteolytic activity, measured in a system in which ribonuclease was not added). Proteolysis was measured as described in Materials and Methods.
Fig. 2. (A) Polyacrylamide gel electrophoresis of RNA (10 μg) extracted from NIH/3T3 cells (lane 1) or from crude reticulocyte fraction II (lane 2). RNA was electrophoresed and visualized as described in Materials and Methods. (B) Ability of purified RNA subfractions to restore activity to the RNase-inhibited ubiquitin- and ATP-dependent proteolytic system. Crude reticulocyte fraction II RNA (1 mg) was separated on a polyacrylamide gel and the bands were visualized, excised, extracted, and precipitated as described in Materials and Methods. Various amounts of the RNA fractions (see lane 2 in A) were added to the micrococcal nuclease-inhibited crude fraction II (after nuclease inactivation by EGTA). Results were calculated as described in the legend to Fig. 1, except that the inhibition obtained with the nuclease was 81%. ○, Total RNA; △, 75S RNA; ◇, 5.8 S RNA; ●, 5 S RNA; ▲, tRNA.

Reticulocyte fraction II was added and found to restore the proteolytic activity completely (Fig. 2B). RNA also restored proteolytic activity to a system that had been treated either with micrococcal nuclease followed by inhibition with pTp or with RNase A followed by inhibition with placental RNase inhibitor. However, when the ribonuclease was not inhibited before addition of fraction II RNA, no restoration of activity was observed (data not shown). [We determined that treatment of crude fraction II with either RNase A or micrococcal nuclease almost completely destroyed all endogenous RNA (data not shown).]

tRNA Is the Component Necessary for Reactivation of the RNase-Inhibited Proteolytic System. To determine whether only certain RNA species were able to restore proteolytic activity, total fraction II RNA was separated by gel electrophoresis (Fig. 2A, lane 2). Cytoplasmic RNAs such as 7SL, 5.8S, 5S, and transfer RNAs were most abundant, while nuclear RNAs U1, U2, U4, U5, and U6 (see Fig. 2A, lane 1) were not detected. RNAs were extracted from each band indicated in Fig. 2A, lane 2, and added separately to a nuclease-treated proteolytic system in which the micrococcal nuclease had been inhibited by EGTA (Fig. 2B). Only the tRNA-sized molecules restored the activity of the nuclease-treated system; equivalent amounts of 7SL, 5.8S, and 5S RNA had no stimulatory activity.

The absolute amount of RNA required to restore activity is of the same order of magnitude as that originally present in fraction II; whereas 4.7 μg of total RNA was extracted from 20 μl of crude reticulocyte fraction II, only 0.5 μg of total RNA or 0.1 μg of tRNA was sufficient to restore 50% of the proteolytic activity in a 50-μl reaction mixture containing 20 μl (240 μg of protein) of crude fraction II. Moreover, the amount of tRNA required to restore proteolytic activity was approximately one-fifth that of total RNA from fraction II (Fig. 2B); that is, the concentration of tRNA that gave 50% restoration was about 2 μg/ml, compared to 10 μg/ml of total RNA. The relative activity is therefore in proportion to the abundance of tRNA in the total RNA population.

tRNA from another mammalian source, mouse NIH/3T3 cells (Fig. 2A, lane 1), likewise restored proteolytic activity to an RNase-treated system (data not shown), whereas non-tRNA small RNAs isolated from the same cells by using antibodies directed against small ribonucleoproteins (Sm, Ro, and La [21]) did not (data not shown). Human DNA and poly(A)+ mRNA (from hepatoma HepG2 cells) likewise had no effect (data not shown), as was the case for the polyanions poly(I), poly(I,C), poly(U), poly(C), and heparin and for protamine sulfate (data not shown).

Ubiquitin- and ATP-Dependent Proteolytic Activity Can Be Restored by Addition of a Single tRNA Species. Since the active component comigrated with tRNA, we sought to determine whether any individual tRNA species might be sufficient to reconstitute proteolytic activity. Certain patients with autoimmune diseases such as systemic lupus erythematosus and polymyositis produce autoantibodies directed against subsets of tRNAs (21). We used sera from three such patients to isolate pure tRNA species for addition to the nuclease-treated proteolytic system.

RNAs precipitated from 32P-labeled NIH/3T3 cell extracts by patient sera were analyzed by two-dimensional polyacrylamide gel electrophoresis (16) (Fig. 3A). Serum MN, which is of the anti-Jo-1 specificity (16), precipitated a single RNA species previously identified by RNA sequence analysis as tRNAHis (16); the antigenic protein is histidyl-tRNA synthetase (22). The other two sera, LL and SU, precipitate several previously uncharacterized tRNA-sized molecules. To characterize these RNA species, all prominent spots in Fig. 3A were subjected to RNA fingerprint analysis (data not shown). The three major species (two of which appear to be related) precipitated by serum LL each contain many modified nucleotides characteristic of tRNAs. These tRNAs can be immunoprecipitated even after phenol extraction, suggesting that serum LL recognizes some common structural RNA feature [e.g., modified nucleotides(s)]. Serum SU precipitates five major species (as well as several minor ones): one of the most prominent spots is tRNAHis (indicated by arrow in Fig. 3A); the other tRNA species were not identified. Protein is required for immunoprecipitation, suggesting that serum SU might recognize a protein synthesis elongation factor or tRNA-modifying enzyme.

When tRNAs isolated from NIH/3T3 cell immunoprecipitates were added to the inactivated proteolytic extract (Fig. 3C), tRNAHis (precipitated by serum MN) was sufficient to restore >80% of the proteolytic activity. In addition, tRNAs precipitated by serum SU (which include
tRNA<sub>His</sub>) restored the protein degradation activity, but the tRNAs precipitated by serum LL had no effect (Fig. 3C).

To determine the relative activity of the immunoprecipitated tRNA<sub>His</sub> in restoring ubiquitin- and ATP-dependent proteolysis, we quantitated the exact amounts of tRNA<sub>His</sub> precipitated by the patient sera. Using extracts of equivalent numbers of mouse NIH/3T3 cells and sera MN, LL, and SU, we isolated RNAs from immunoprecipitates, supernatants, and total cell extract. A portion of each RNA sample was tested for its ability to restore proteolytic activity (Fig. 3C), and another portion (20%) was electrophoresed on a 10% polyacrylamide gel and transferred to diazobenzyl-oxymethyl paper (19). The transferred RNA then was probed with a nick-translated cloned mouse tRNA<sub>His</sub> gene (12) (Fig. 3B). Serum MN precipitated only about 10% of the total cellular tRNA<sub>His</sub> (compare lanes 3 and 4), and serum SU precipitated <5% (lanes 5 and 6). This is consistent with the data in Fig. 3C, which shows that the supernatants after precipitation with MN and SU have 15-fold and 23-fold higher activity, respectively, than their corresponding immunoprecipitates. The activity of total RNA extracted from an equivalent amount of cell extract is similar to that of the two supernatants. This result was repeated in another experiment: approximately 5% of the tRNA<sub>His</sub> was precipitated by serum MN, and the restoration activity of the total extract was 9- to 13-fold higher than that of the immunoprecipitate.

**DISCUSSION**

We have shown that tRNA is an essential component of the ubiquitin- and ATP-dependent proteolytic system in that it can restore activity to a system whose endogenous RNA has been destroyed previously by ribonuclease. The proteolytic system is specifically and strongly inhibited by several different ribonucleases, serving as the basis of our assay. Either total RNA extracted from crude reticulocyte fraction II or the tRNA subfraction restored proteolytic activity. Total RNA from mouse cells or tRNA from calf liver had the same effect (unpublished observations), indicating conservation among mammalian organisms. Conversely, no other RNAs, DNAs, polyamions, or polycations tested mimicked the restoration seen with tRNA. tRNA from *Escherichia coli* or yeast did not detectably stimulate the RNase-treated proteolytic system when added in the same amounts as mammalian tRNA (unpublished results).

tRNA<sub>His</sub> immunoprecipitated alone by serum MN or together with other unidentified tRNA species by serum SU stimulated the activity of the inhibited system to a level 80% that of control. Three unidentified tRNA species (precipitated by serum LL) did not restore activity (Fig. 3). This experiment argues against the possibility that some other type of RNA molecule that comigrates with tRNA is responsible for the restored activity.

Extracts derived from equivalent numbers of NIH/3T3 cells were precipitated with serum MN, SU, or LL. The precipitates (P; lanes 3, 5, 7), supernatants (S; lanes 4, 6, 8), and an equivalent amount of the original extract (lane 2) were electrophoresed on a 10% polyacrylamide gel. The RNAs were transferred to diazobenzyl-oxymethyl (DBM) paper and probed with nick-translated M13-RP containing a mouse tRNA<sub>His</sub> genomic clone (see Materials and Methods). Lane 1: 32P-labeled total cellular RNA, used as markers (M). (C) Effect of purified immunoprecipitated tRNAs on the ubiquitin- and ATP-dependent proteolytic system. The proteolytic system was treated with micrococcal nuclease and the nuclease was inhibited with pTP as described in the legend to Fig. 2. RNAs were immunoprecipitated from equal volumes of cell extracts. RNAs extracted from the immunoprecipitates [a (MN), b (SU), c (LL)], supernatants after immunoprecipitation [a (MN), d (SU), e (LL)], and an equivalent amount of original extract (f) were resuspended in equal volumes. Various volumes of these RNA fractions were added to the inhibited proteolytic system.
sible for reconstituting proteolytic activity. Although we do not know the identity of the protein antigen recognized by serum SU, it is probably not histidyl-tRNA synthetase, because several different tRNAs are immunoprecipitated; thus, any minor contaminant would have to be present in two tRNA subpopulations selected by virtue of their association with two different autoantigens. Moreover, since the amount of tRNA\textsuperscript{His} recovered in the immunoprecipitate is consistent with its activity relative to total RNA, it is possible that tRNA\textsuperscript{His} is the only tRNA species that stimulates the system. However, at this time we cannot be certain that one or two additional tRNA species do not also fulfill the requirement.

An interesting aspect of the RNA requirement of the ubiquitin- and ATP-dependent proteolytic system studied here is its apparent substrate specificity. Although degradation of \textsuperscript{125}I-BSA was almost completely inhibited by ribonuclease (Fig. 1), the degradation of \textsuperscript{3}H-labeled globin was inhibited by 40%, and the degradation of \textsuperscript{125}I-labeled lysozyme was inhibited only slightly (10–20%) (data not shown). Thus, the ubiquitin- and ATP-dependent pathway is a multienzyme cascade, composed of at least nine essential factors (refs. 2, 3, 6; A. Hershko, personal communication). Thus, it is possible that not all the factors are necessary for degradation of all substrates or that the degradation of different substrates requires different amounts of the participating factors. Accordingly, different tRNAs may be required for the degradation of different protein substrates and, moreover, may be differentially sensitive to ribonucleases when treated in cell extracts.

Further studies are required to elucidate the exact role of the tRNA component in stimulating the ubiquitin- and ATP-dependent proteolytic system. First, we do not know whether tRNA participates in the conjugation of ubiquitin to substrate proteins or in the degradation of the resulting conjugates. Second, the endogenous tRNA(s) in reticulocyte fraction II appear to be protein-bound since fraction II is prepared by chromatography of reticulocyte lysate on DEAE cellulose followed by ammonium sulfate precipitation (10), conditions under which free tRNA is not precipitable. On the other hand, the protein-tRNA association appears freely reversible, since conditions required to reconstitute small ribonucleoproteins (23) are not needed to recover proteolytic activity after tRNA addition. The protein component has yet to be identified but could be histidyl-tRNA synthetase. Third, it must be established whether modifications of tRNA\textsuperscript{His} (16) are essential and whether any other species of tRNA can substitute.

Why should tRNA be an essential component of an intracellular proteolytic system? It is now accepted that rates of protein breakdown in bacterial and animal cells are precisely regulated and vary under different physiological conditions (for reviews, see refs. 1 and 2). In \textit{E. coli}, degradation increases upon starvation for amino acids, nitrogen, or glucose or when growth slows and the cells enter stationary phase (24); the enhanced degradation is reversed when the cells are cultured in fresh medium or reinitiate growth. Here, the level of uncharged tRNA appears to serve as a trigger (for review, see ref. 1); the interaction of deacylated tRNA with polyribosomes generates guanosine 3'-diphosphate 5'-diphosphate (ppGpp) (25), whose levels correlate inversely with the rate of stable RNA synthesis and directly with rates of protein catabolism. In eukaryotic cells, growth and protein synthesis are also strongly and inversely related to the rate of protein degradation (for reviews, see refs. 1 and 2), but a mediator nucleotide has not been identified. However, a mutant Chinese hamster ovary (CHO) cell with a temperature-sensitive histidyl-tRNA synthetase shows increased levels of protein degradation at the nonpermissive temperature (26); and histidinol inhibition of charging of tRNA\textsuperscript{His} produces a similar effect on wild-type CHO cells (27). Deprivation of specific amino acids, in particular alanine (28) and histidine (29), has been shown to induce protein degradation in rat liver. Hence, it is possible that the level of one or more species of uncharged tRNA (which varies inversely with the rate of translation) regulates the rate of intracellular proteolysis in eukaryotic cells. Alternatively, tRNA might participate directly in some re-action of the ubiquitin pathway. Whether the role of tRNA is regulatory or direct can be tested in the \textit{in vitro} system.

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