Journal of Cellular Biochemistry 34:81–100 (1987)
Proteases in Biological Control and Biotechnology 197–216

Regulation of the Ubiquitin-Mediated Proteolytic Pathway: Role of the Substrate α-NH_2 Group and of Transfer RNA

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Degradation of intracellular proteins via the ubiquitin pathway involves several steps. In the initial event, ubiquitin becomes covalently linked to the protein substrate in an ATP-requiring reaction. Following ubiquitin conjugation, the protein moiety of the adduct is selectively degraded with the release of free and reusable ubiquitin. Ubiquitin modification of a variety of protein targets in the cell plays a role in basic cellular functions. Modification of core nucleosomal histones is probably involved in regulation of gene expression at the level of chromatin structure. Ubiquitin attachment to cell surface proteins may play roles in processes of cell-cell interaction and adhesion, and conjugation of ubiquitin to other yet to be identified protein(s) could be involved in the progression of cells through the cell cycle. Despite the considerable progress that has been made in the elucidation of the mode of action and cellular roles of the ubiquitin pathway, many major problems remain unsolved. A problem of central importance is the specificity in the ubiquitin ligation system. Why are certain proteins conjugated and committed for degradation, whereas other proteins are not? A free α-NH_2 group is an important feature of the protein structure recognized by the ubiquitin conjugation system, and tRNA is required for the conjugation of ubiquitin to selective proteolytic substrates and for their subsequent degradation. These findings can shed light on some of the features of a substrate that render it susceptile to ubiquitin-mediated degradation.

Key words: ubiquitin, α-NH_2 group, transfer RNA, proteolytic pathway

Cellular proteins are in a state of constant turnover. The process is extensive and highly selective. Specific proteins are degraded within cells at widely different rates. Protein turnover is involved in basic cellular functions such as the regulation of the levels of key enzymes and regulatory proteins in metabolic pathways, the provision of amino acids under conditions of nutritional or hormonal deprivation, and the preferential disposal of defective proteins [1–10].

Received March 25, 1986; revised and accepted December 18, 1986.

Until recently, studies on protein turnover focused mainly on the description of its various phenomenological aspects, as opposed to the underlying mechanisms. Intracellular protein breakdown in all organisms has an absolute requirement for metabolic energy [2,3,9,10]. This ATP-dependence probably reflects unidentified mechanism that endow such system(s) with a high specificity toward their protein substrates.

Recent information indicates that in mammalian cells, separate lysosomal and nonlysosomal mechanisms may be involved in different aspects of protein degradation [11]. Endocytosed proteins (which enter the cell via receptor-mediated endocytosis) and pinocytosed proteins, are degraded in lysosomes. Lysosomal degradation of *intracellular* proteins occurs only under extreme stress conditions such as starvation [5,9]. On the other hand, nonlysosomal mechanisms are probably responsible for the highly selective turnover of intracellular proteins under basal metabolic conditions [9–12].

Some insight into the nonlysosomal (however, energy-requiring) mechanisms of protein breakdown was gained in our laboratory with the establishment of an ATP-dependent cell-free proteolytic system form reticulocytes. The degradative system is located in the cytosol and has an optimum pH of 7.6. It is stimulated specifically by Mg^{+2}ATP and degrades various exogenously added protein substrates [13]. The system is composed of several essential components. First, the system has been resolved on a DEAE ion exchange chromatography column into two complementing activities; unadsorbed material (fraction I) and high salt eluate (fraction II) [14]. The active principle of fraction I was purified to homogeneity and was found to be a polypeptide with a molecular weight of 8,500 daltons [15]. The protein was later identified as ubiquitin, a universally occuring polypeptide of hitherto unknown function [16]. Fraction II contains all the remaining enzymes necessary for the proteolytic activity [14].

An ATP-dependent conjugation of ubiquitin to substrates is crucial for their proteolysis in the cell-free reticulocyte system. Following incubation of purified [^{125}I] ubiquitin with fraction II in the presence of ATP, covalent binding of the polypeptide to endogenous and exogenously added substrates such as lysozyme and globin was observed, as analyzed by SDS-polyacrylamide gel electrophoresis [17]. Chemical analysis showed that ubiquitin is bound to the proteolytic substrate in an isopeptide bond, in which the COOH-terminal glycine of ubiquitin is bound to the ϵ-NH_2 group of internal lysines of the substrate [18,19]. Following ubiquitin conjugation, the substrate moiety of the conjugate is rapidly degraded with the release of free and reusable ubiquitin [18].

Using affinity chromatography on ubiquitin immobilized to Sepharose, we (Hershko, Ciechanover, and colleagues) partially purified and characterized the three enzymes that catalyze ubiquitin conjugation to the substrate [20–22]. The first enzyme, E_1 (ubiquitin-activating enzyme), activates ubiquitin in an ATP-dependent reaction to a high energy E_1 thiolester between the carboxy-terminal glycine of ubiquitin and a sulfhydryl group of the enzyme. The second enzyme, E_2, transfers the activated ubiquitin from E_1 to E_3, while E_3 catalyzes isopeptide bond formation between the activated ubiquitin and the protein substrate. E_3 is most probably the enzyme that participates in determining the specificity of the substrates that enter the ubiquitin pathway (see below). It should be noted that there is a remarkable similarity between the mechanism of activation of ubiquitin and that of amino acids, catalyzed by amino acyl-tRNA synthetases during protein synthesis (see below).

The enzymes involved in degradation of the conjugates have been characterized only partially. Four essential components that participate in the degradation of the conjugates have been isolated from the *unadsorbed* fraction of the ubiquitin affinity column. The study of the function of these factors is currently underway. It is known, however, that ATP is required not only for the formation of the conjugates but also for their degradation [23–26]. The known sequence of events in the ubiquitin proteolytic pathway is shown in Figure 1.

If ubiquitin protein conjugates are indeed intermediates in protein degradation, their levels should increase with the availability of rapidly degradable cellular proteins. In reticulocytes, a strong increase in the rate of degradation of newly synthesized proteins can be induced through the formation of abnormal proteins by using amino acid analogs. To address the possible involvement of the ubiquitin system in intracellular protein turnover in vivo, we used a polyclonal antibody that recognized ubiquitin-protein conjugates to isolate conjugates from cells in different metabolic conditions [27]. In cells labeled without the addition of analogs, about 0.5% of the total pulse-labeled proteins were immunoprecipitated. A tenfold increase in the level of labeled immunoprecipitable protein was observed under conditions of abnormal protein formation. Similar results were obtained in an analogous experiment carried out with Ehrlich ascites tumor cells [27]. Independent evidence in support of the notion that ubiquitin conjugation signals proteolysis came from a study by Chin et al [28]. They comicroinjected cells with [^{125}I] ubiquitin and hemoglobin. When the cells were treated with phenylhydrazine to denature the hemoglobin, a series of ubiquitin-globin conjugates was formed. The quantity of the conjugates was in direct proportion to the concentration of the denaturing agent and to the overall proteolysis of hemoglobin.

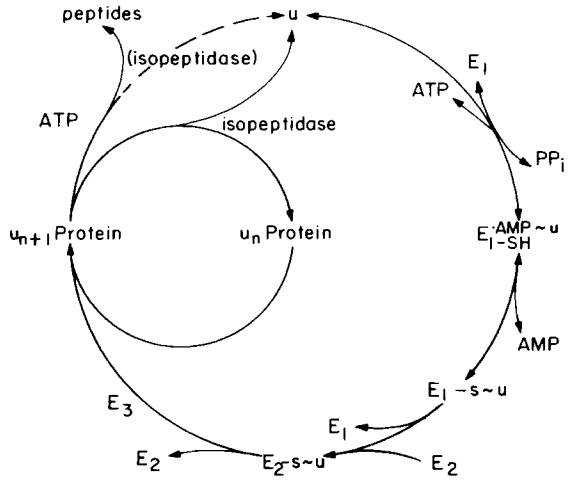


Fig. 1. Proposed sequence of events in the ubiquitin proteolytic pathway. A scheme modified from Hershko and Ciechanover [9] and Finley et al [31]. Ubiquitin (U) is activated in a two-step reaction to a high energy (~) thiol ester of the ubiquitin-activating enzyme (E_1). It is then transferred through an intermediate high energy thiol ester of E_2 to E_3, which catalyzes isopeptide bond formation with the proteolytic substrate. The dashed line on the left signifies a proposed (however, undocumented) role for isopeptidase activity. The pathway from ubiquitin conjugates to peptides is not understood in detail, but probably requires several protein factors (A. Hershko, personal communication) and ATP [23–26].

In another line of research, we found that the ubiquitin pathway is directly involved in the regulation of cell cycle and cell division using a cell cycle- arrested temperature-sensitive mutant cell (ts85) derived from the mouse mammary carcinoma wild-type cell line FM3A. At the nonpermissive temperature (39.5°C), the cells are arrested at the S/G_2 boundary and cannot proceed through mitosis, condense the chromatin or divide [29]. Remarkably, uH2A semihistone (the major ubiquitin conjugate in eukaryotic nuclei; in this protein, the COOH-terminal glycine of ubiquitin is bound to the ϵ-NH_2 group of internal lysine-119 of the histone H2A molecule via an isopeptide linkage) disappears from the chromatin at 39.5°C with a half-life of about 3hr [30]. In pulse chase experiments in vivo, we found that the disappearance of uH2A at the restrictive temperature was due to a reduced rate of ubiquitin-H2A conjugation. By affinity chromatography purification of the components of the ligase system on immobilized ubiquitin [21,22,31], the thermolabile component was identified as E_1 [31]. Subsequently, we found that while approximately 70% of pulse-labeled short-lived proteins were degraded within 4 hr in both the mutant and the wild-type cells at the permissive temperature (32°C), only 15% of the labeled proteins were degraded at 39.5°C in the mutant cells compared to 70% in the wild-type cells [32]. The ts85 mutant studies, together with the immunochemical studies and the microinjection experiments, provide extremely strong support for a model in which the modification of proteins by ubiquitin conjugation is a required step for their subsequent proteolysis. Furthermore, it implicates the involvement of the ubiquitin system in control of regulation of cell cycle and cell division. In addition, the ubiquitin system is probably involved also in regulation of gene expression at the level of chromatin structure. Varshavsky and his collegues found that approximately one in two nucleosomes of the transcribed *copia* and heat-shock 70 genes in *Drosophila melanogaster*-cultured cells contain uH2A, while less than one in 25 nucleosomes of the tandemly repeated, nontranscribed 1,688 satellite DNA contains uH2A, suggesting that most of the nucleosomal uH2A is associated with transcribed genes [33,34]. Later studies by the same group revealed a sriking enrichment of ubiquitinated nucleosomes at the 5′ end of the dihydrofolate reductase gene as compared with regions of the same gene downstream from the first ~400 base pairs [34–36]. The researchers suggested that site-specific ubiquitination could signal proteolytic removal of chromosomal proteins, which can be a necessary condition for transcription to occur. The data cannot exclude, however, a nonproteolytic function of ubiquitin modification of core nucleosomal histone molecules (such as a change in the three-dimensional structure of the nucleosome). It was recently reported that the nucleosomes of the active gene encoding the kappa light chain of the immunoglobulin molecule are not enriched with ubiquitin-modified histones [37]. It was further found that arrest of transcription of some other genes is not accompanied by quantitative changes in ubiquitinated histone molecules [38]. It seems, therefore, that while the role of ubiquitin in modifying proteolytic substrates is well established, its role in modification of core nucleosomal histone molecules is still obscure.

Recently, ubiquitin modification of the lymphocyte homing receptor, the platelet-derived growth factor receptor, and some other yet to be identified cell surface proteins was observed [39,40]. Assignment of a particular role for ubiquitination of cell surface protein can only be a matter of speculation at this stage. It could serve as a tag for surface proteins that are destined for degradation, and also play roles in processes such as cell-cell interaction and adhesion.

Powerful tools are now available to study the biological functions of the ubiquitin system, yet many major problems remain unsolved. Determination of the specificity of the ubiquitin ligation system for committment of a certain protein for degradation is a problem of central importance. A free α-NH_2 group of the substrate is an important feature recognized by the ubiquitin ligation system, and tRNA is necessary for the conjugation of ubiquitin and for the subsequent degradation of selective proteolytic substrates. These findings, which are described in detail below, may provide some insight into problems of specificity in the ubiquitin pathway.

RESULTS

Recognition of the Protein Structure by the Ubiquitin System: Role of the α-Amino Group

Intracellular protein breakdown is a highly selective process. Specific proteins are degraded at widely different rates. Since proteolysis per se is an exergonic reaction, one can rationalize for the energy requirement of intracellular protein breakdown only if the energy is used to obtain specificity. The question is which specific features of the protein structure are recognized by the ubiquitin conjugation system. Clearly, it cannot be the availability of ϵ-NH_2 groups of lysine residues, since most lysines are exposed on the surface of most native proteins.

Hershko et al [41] deduced a special role of the α-NH_2 group of the substrate when they examined the effects of selective modification of amino groups of proteins on their degradation by the ubiquitin system. They compared the effects of increasing degree of modification of amino groups of lysozyme by reductive methylation and carbamoylation at pH 6 and found that in the latter treatment the breakdown of lysozyme was inhibited at a much lower extent of amino group modification than in the case of reductive methylation [41]. Under the conditions employed for carbamoylation, a specific amino group of the protein which is important for ubiquitin conjugation may have been blocked selectively. That this may be the α-NH_2 group was suggested by the information that when carbamoylation is carried out at a slightly acidic pH, the α-NH_2 group reacts about 100-fold faster than the ϵ-NH_2 group because of the lower pK_a of the former. When selective methods were used to carbamoylate the terminal α-NH_2 groups of globin chains, degradation by the ubiquitin system was prevented even though practically all ϵ-NH_2 groups remained free [41].

The influence of selective blocking of α-NH_2 groups of proteins on their conjugation with ubiquitin was examined next. N^{α}-carbamoylation of globin or lysozyme greatly decreased their conjugation with [^{125}I]ubiquitin. The formation of high-molecular-weight conjugates containing multiple molecules of ubiquitin was drastically inhibited. It seems, therefore, that the requirement for a free α-NH_2 group is specific to a pathway leading to the formation of high-molecular-weight ubiquitin-protein conjugates committed for degradation. Formation of a monoubiquitin derivative such as that of histone H2A does not require a free α-NH_2 group, since this histone has a blocked NH_2-terminus [42].

The next question was whether proteins with blocked NH_2-termini can be made into substrates by creating new α-NH_2 groups. This was done by adding polyamino-acid side chains. In this process, the ϵ-NH_2 group of the lysine residue to which the polyamino acid is attached, is replaced by the α-NH_2 group of the side chain. Polyalanylation of lysozyme derivatives that had previously been carbamoylated at

their α-NH_2 groups restored to a large extent their susceptibility to degradation by the ubiquitin proteolytic system [41].

Further experiments indicated that a free α-NH_2 group in the absence of ϵ-NH_2 groups is sufficient for degradation by the ubiquitin system. This was examined in the case of guanidinated proteins, since guanidination with O-methylisourea blocks ϵ-NH_2 groups but not α-NH_2 groups. Rechsteiner et al [43] first observed that guanidinated proteins are degraded in reticulocyte lysates by an ATP-dependent process. Degradation of guanidinated proteins requires ubiquitin and the three ubiquitin-conjugating enzymes [41]. In crude reticulocyte fraction II, guanidinated lysozyme is degraded at about 25% of the rate of unmodified lysozyme. Conjugation of ubiquitin to ϵ-NH_2 groups may therefore serve to accelerate the rate or increase the affinity of substrates to some component of the proteolytic system.

In all the above experiments, chemically modified substrates were used to explore the role of the α-amino group. To examine whether naturally occurring modification of proteins' NH_2-termini has a similar effect, Hershko et al [41] determined the degradation of some N^{α}-acetylated proteins by the ubiquitin proteolytic system from reticulocytes. Many cellular proteins have acetylated NH_2-termini, but the function of N^{α}-acetylation is not known [44]. They found that N^{α}-acetylated cytochrome c and enolase from mammalian tissues are not degraded by the ubiquitin system, while their nonacetylated counterparts from yeast are good substrates [41] (Table I). None of the naturally occurring N^{α}-acetylated proteins tested were degraded by the ubiquitin system. On the other hand, aldolase and glyceraldehyde-3-P-dehydrogenase, which have free α-NH_2 termini, were not degraded by the ubiquitin system [41] (Table I). In these proteins, the unblocked α-NH_2 group might be buried and not available to the action of the ubiquitin system.

The cumulative evidence from the above experiments led to the suggestion [41] that the exposure of a terminal α-NH_2 group may be a required or necessary signal for protein degradation by the ubiquitin system. As opposed to ϵ-NH_2 groups of

TABLE I. Degradation of Natural N^{α}-Acetylated and Nonacetylated Proteins

		Degradation rate (%/hr)		
Protein	Source	−ATP (a)	+ATP, +Ub (b)	ATP+Ub-dependent (b − a)
N^{α}-Acetylated				
Enolase	Rabbit muscle	10.7	9.9	−0.8
Cytochrome c	Horse heart	24.4	26.4	2.0
Actin	Bovine muscle	1.9	2.2	0.3
LDH	Rabbit heart	0.8	2.0	1.2
Ovalbumin	Hen egg	2.2	2.0	−0.2
Carbonic anhydrase	Bovine erythrocytes	2.4	1.6	−0.8
Nonacetylated				
Enolase	Yeast	24.2	37.7	13.5
Cytochrome c	Yeast	21.8	43.6	21.8
Aldolase	Rabbit muscle	0	0.9	0.9
GAPDH	Rabbit muscle	0.6	0.6	0

All proteins were radiolabeled with [^{125}I]. LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-P dehydrogenase. (Adapted from Hershko et al [41].)

lysine residues that are mostly exposed, NH_2-termini in many native proteins are buried within the protein structure. The exposure of a buried NH_2-terminus may be the consequence of several types of alterations in the protein structure, such as denaturation or subunit dissociation. It is also possible that specific modulators or metabolites may control the rate of degradation of particular proteins by inducing conformational changes leading to the exposure of their α-NH_2-termini.

An interesting exception to the above generalizations has recently been reported by Gregori et al [45]. These investigations found that calmodulin from bovine brain is not degraded by the ubiquitin proteolytic system from reticulocytes, while calmodulin from *Dictyostelium discoideum* is degraded at a fairly rapid rate. Mammalian calmodulins are N^{α}-acetylated, but *Dictyostelium* calmodulin also has a blocked NH_2-terminus [46]; the nature of the blocking group has not been identified. Examination of the formation of conjugates of [^{125}I]ubiquitin with the various calmodulins showed that mammalian calmodulin is not conjugated, while *Dictyostelium* calmodulin forms a prominent monoubiquitin derivative. No higher molecular weight conjugates of *Dictyostelium* calmodulin were observed even in the presence of hemin, a regent that inhibits conjugate-degrading enzymes. It should be noted that two general "rules" that were observed for a variety of protein substrates (ie, requirement for a free α-NH_2 group and for the formation of high-molecular-weight conjugates) do not apply in the case of *Dictyostelium* calmodulin, suggesting a possible connection between the two requirements. It is possible that several ubiquitin ligation systems exist for various types of proteins, one that requires a free α-NH_2 group and the formation of high-molecular-weight conjugates, and another that does not.

If a free α-NH_2 group is an important feature of the protein substrate that is recognized by the ubiquitin ligation system, how are cellular N^{α}-acetylated proteins degraded? It was suggested by Jörnvall [47] that N^{α}-acetylation may protect proteins against degradation. On the other hand, Brown [48] reported that N^{α}-acetylated and nonacetylated proteins of cultured cells turn over at similar rates. Other reports indicate that some proteins blocked at their α-NH_2 groups, or at all amino groups, can be degraded in intact cells. Katznelson and Kulka [49] reported that proteins completely blocked at their amino groups are not degraded in reticulocyte extracts. However, these proteins are degraded in hepatoma cells, following microinjection, by an energy-dependent nonlysosomal system that differs from the ubiquitin pathway [49]. Other investigators also observed fairly rapid degradation of N^{α}-blocked proteins following microinjection, such as N^{α}-carbamoylated hemoglobin [50] or N^{α}-acetylated cytochrome c [51]. Tanaka et al [52] observed that although the modification of all amino groups of proteins reduced their rate of degradation in reticulocyte lysate, the residual degradation was still stimulated by ATP. It was suggested that ATP has two roles in protein breakdown, one requiring ubiquitin and another independent of ubiquitin [52]. It is possible that some α-NH_2–blocked proteins are degraded by a ubiquitin-independent (however, ATP-dependent) proteolytic system(s). It is also possible that N^{α}-acetylated proteins in cells are subject to deacetylation or even to a single endoproteolytic cleavage that would expose a new free α-NH_2 terminal and thus become subject to the action of the ubiquitin system.

While a free α-NH_2 group of proteins appears to be an important recognition determinant, it seems reasonable to assume that it is not the only one. Solubilized brain hexokinase is degraded rapidly by the ubiquitin proteolytic system from reticulocytes, while mitochondria-bound hexokinase is not [53]. Dissociation of hexokinase

from mitochondria could expose its NH_2-terminus or cause some other structural alteration. Reduction and thiol aklylation of bovine serum albumin increase its rate of degradation by the ubiquitin system [54]. This probably was not due to denaturation, since reduced albumin (without alkylation) is degraded at a much slower rate. It is not clear from this study which specific structural alteration (produced by reduction-alkylation) makes this protein more susceptible to the action of the ubiquitin system. No correlation was found between the charge, hydrophobicity, or aggregation of the various derivatives of serum albumin and their susceptibility to degradation [54].

Involvement of tRNA in Ubiquitin-Mediated Protein Breakdown

Recently we found that ubiquitin- and ATP-dependent degradation of labeled serum albumin (BSA) is strongly and specifically inhibited by ribonucleases [55]. Ribonuclease A from bovine pancreas and micrococcal nuclease at concentrations exceeding 2 μg/ml inhibit the degradation of [^{125}I]BSA by 80–90% (Fig. 2). The inhibition is specific to ribonucleases, since snake venom endonuclease and RNases T_1 and T_2 all showed strong inhibition at low concentrations. DNase I at high concentration did not inhibit the proteolytic system [55].

To determine whether the inhibitory effect of the RNases is indeed due to their enzymatic activities rather than to 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 Figure 3, incubation of RNase A with human placental ribonuclease inhibitor completely abolished its inhibitory effect. Pre-incubation of micrococcal nuclease with thymidine-3′, 5′-diphosphate (pTp), a specific inhibitor of the enzyme [56], also relieved the inhibition. Likewise, omission of Ca^{2+}, which is essential for micrococcal nuclease activity [56], resulted in no inhibition of the proteolytic activity [55] (data not shown).

To test directly the notion that RNA is required for the ubiquitin- and ATP-dependent proteolytic system (and that the inhibition of the system by the ribonucleases is due to the destruction of an essential endogenous RNA component), phenol-extracted total RNA from crude reticulocyte fraction II (Fig. 4A) was added to an RNA-depleted proteolytic system. Added RNA completely restored the inhibited proteolytic activity [55] (see Fig. 4B).

To determine whether only certain RNA species were able to restore preteolytic activity, total fraction II RNA was separated by gel electrophoresis (Fig. 4A, lane B). Cytoplasmic RNAs such as 7SL, 5.8S, 5S, and transfer RNAs were most abundant, while the nuclear RNAs U1, U2, U4, U5, and U6 were not detected (cf lanes A and B in Fig. 4A). RNAs were extracted from each band indicated in Figure 4A (lane B) and added separately to a nuclease-treated proteolytic system in which the micrococcal nuclease had been inhibited by EGTA (Fig. 4B). 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 [55].

tRNA from another mammalian source, mouse NIH/3T3 cells (Fig. 4A, lane A), likewise restored the 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 [57]) 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(IC), poly(U), poly(C), and heparin, and for protamine sulfate [55] (data not

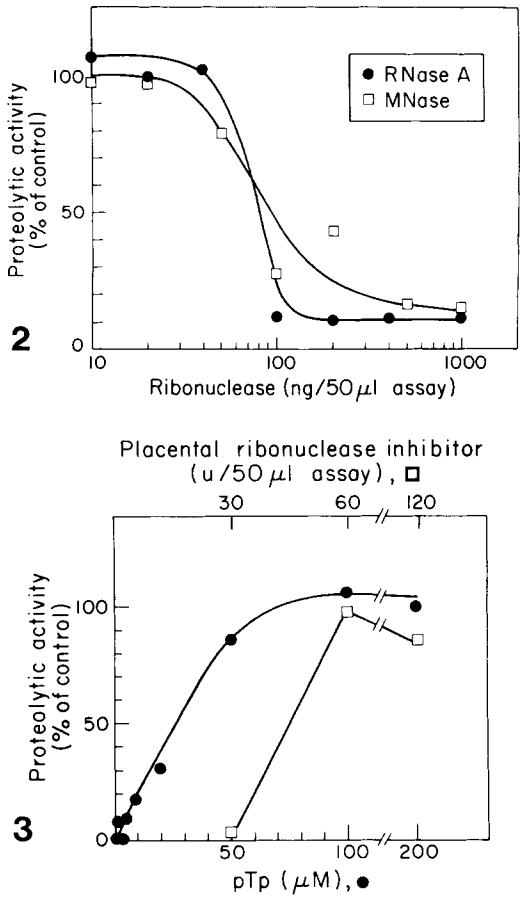
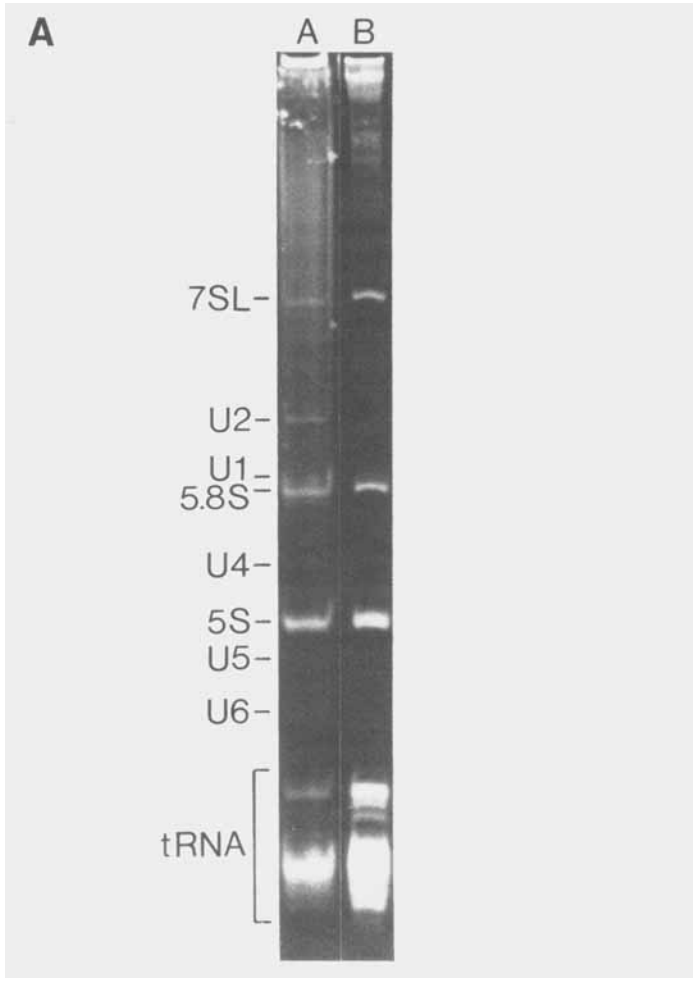


Fig. 2. Inhibition of the ubiquitin- and ATP-dependent proteolytic system by RNase A or micrococcal nuclease. Crude reticulocyte fraction II was preincubated with the indicated concentrations of the ribonucleases followed by initiation of the proteolytic reaction with the addition of [^{125}I]BSA, ubiquitin, and ATP. (Adapted from Ciechanover et al [55].)

Fig. 3. Effect of inhibitors of RNases on the inhibitory effect of the enzymes on the ubiquitin- and ATP-dependent proteolytic system. RNase A was preincubated with the indicated amounts of human placental ribonuclease inhibitor (□), and micrococcal nuclease was preincubated with the indicated amounts of pTp (●) prior to addition to a complete proteolytic system containing [^{125}I]BSA, crude reticulocyte fraction II, ubiquitin, and ATP. One hundred percent proteolytic activity was measured in a system to which RNase was not added. (Adapted from Ciechanover et al [55].)

shown). 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 disease such as systemic lupus erythematosus and polymyositis produce autoantibodies directed against subsets of tRNAs [57]. We used sera from three such patients to isolate pure tRNA species for addition to the nuclease-treated proteolytic system.

RNAs precipitated from [^{32}P]labeled NIH/3T3 cell extracts by patient sera were analyzed by two-dimensional polyacrylamide gel electrophoresis [58] (Fig. 5). Serum MN, which is of the anti-Jo-1 specificity [58], precipitated a single RNA species previously identified by RNA sequence analysis as $tRNA^{His}$ [58]; the antigenic protein



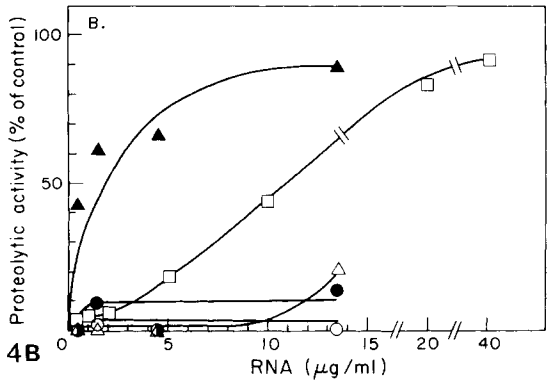


Fig. 4. **A**) Polyacrylamide gel electrophoresis of RNA extracted from NIH/3T3 cells (lane A) or from crude reticulocyte fraction II (lane B). **B**) Ability of purified RNA subfractions to restore activity to an RNase-inhibited ubiquitin- and ATP-dependent proteolytic system. RNA was separated on a polyacrylamide gel (lane B), and the bands were visualized, excised, extracted, and ethanol-precipitated. The indicated amounts of RNA fractions were added to a micrococcal nuclease-treated complete proteolytic system (following inhibition of the ribonuclease), and the degradation of [^{125}I]BSA was monitored. □, total RNA; ○, 7SL RNA; △, 5.8S RNA; ●, 5S RNA; ▲, tRNA. (Adapted from Ciechanover et al [55].)

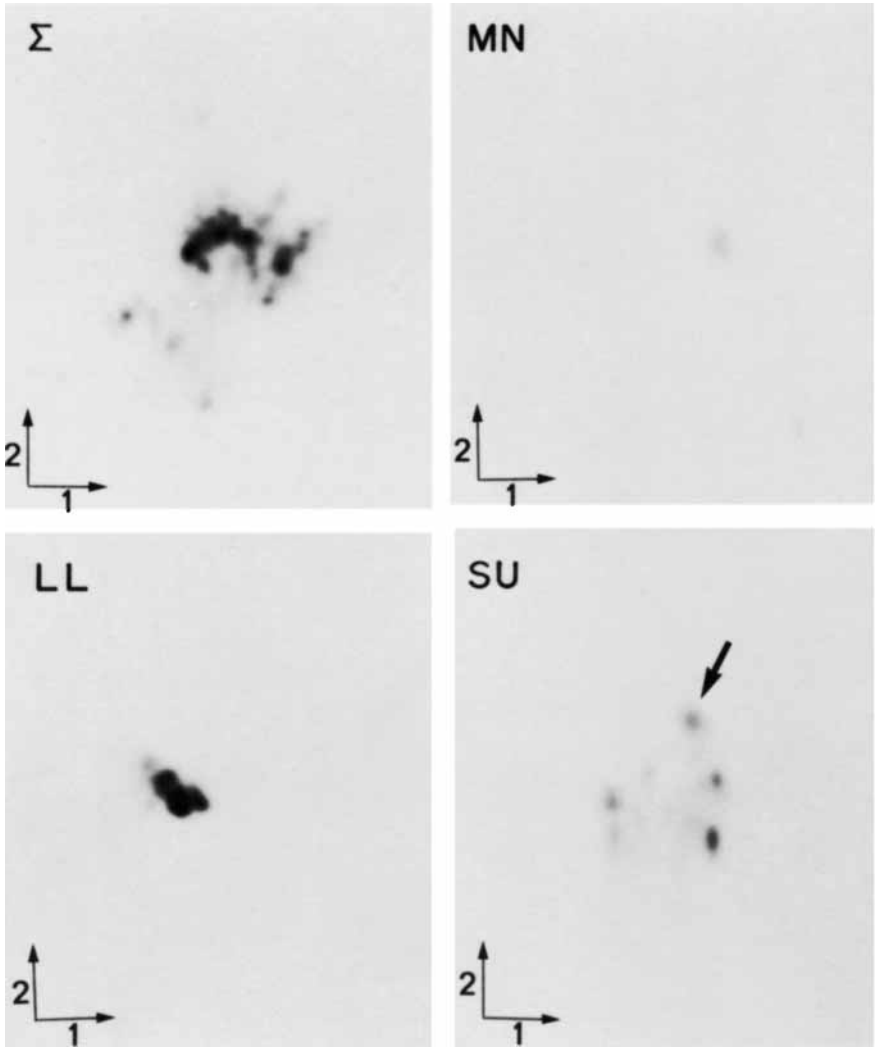


Fig. 5. Subsets of tRNAs present in immunoprecipitates of NIH/3T3 cell extracts. Cells were labeled with ^{32}P prior to RNA extraction and preparation of tRNA. Σ, total tRNA; MN, SU, LL, tRNAs precipitated using MN, SU, and LL autoimmune sera, respectively; arrow, $tRNA^{His}$. Arrows indicate order of the 2-D gel electrophoretic separation. (Adapted from Ciechanover et al [55].)

is histidyl-tRNA synthetase [59]. The other two sera, LL and SU, precipitated several previously uncharacterized tRNA-sized molecules. To characterize these RNA species, all prominent spots in Figure 5 were subjected to RNA fingerprint analysis (data not shown). Serum SU precipitates five major species (as well as several minor ones): one of the most prominent spots is $tRNA^{His}$ (Fig. 5, arrow); the other tRNA species were not identified. Protein is required for immunoprecipitation, suggesting that serum SU might recognize a protein synthesis elongation factor or a tRNA-modifying enzyme. Of the three major species precipitated by serum LL, none could be identified as $tRNA^{His}$ [55].

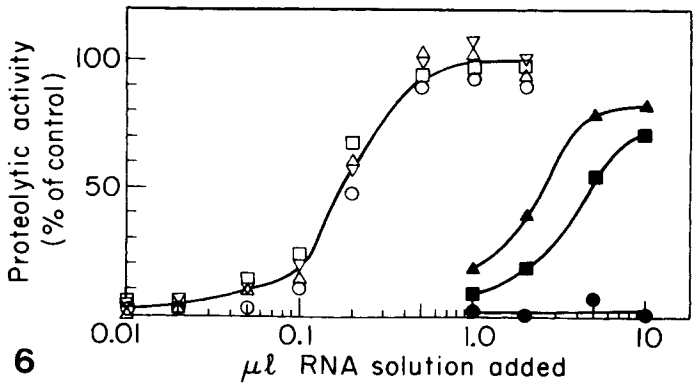
When tRNAs isolated from NIH/3T3 cell immunoprecipitates were added to the inactivated proteolytic extract (Fig. 6), $tRNA^{His}$ (precipitated by serum MN) was sufficient to restore >80% of the proteolytic activity. In addition, tRNAs precipitated by serum SU (which include $tRNA^{His}$) restored the protein degradation activity, but the tRNAs precipitated by serum LL had no effect [55] (Fig. 6).

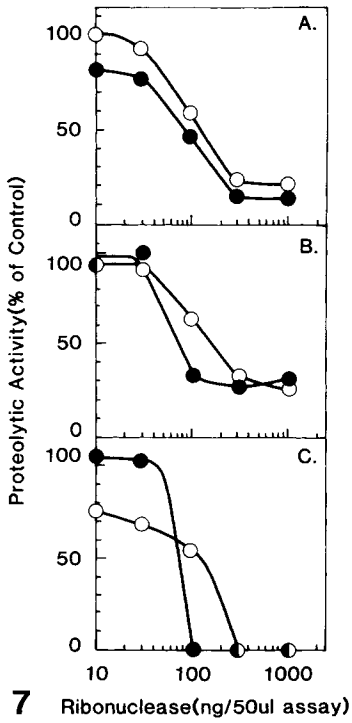
Further studies of the tRNA requirement for ubiquitin-mediated proteolysis revealed that while the degradation of [^{125}I]BSA was sensitive to ribonucleases and required tRNA, the degradation of [^{125}I]lysozyme was not affected by the ribonuclease treatment [55,60]. This finding indicated that the degradation of proteins in a ubiquitin-dependent mode probably occurs via two distinct pathways. We postulated that the two pathways should have some distinct enzymatic component(s), although other components of the system may be shared by both systems.

To confirm and further characterize these pathways, we sought to identify additional proteolytic substrates, the degradation of which is either sensitive or insensitive to ribonucleases. Classification of additional substrates was also important to rule out the possibility that the ribonuclease sensitivity of BSA degradation was not unique to this substrate and thus represents a more general feature of the system. The degradation of both α-lactalbumin and soybean trypsin inhibitor (STI) was found to be sensitive to treatment with ribonucleases (Fig. 7B,C, respectively) [60]. In addition, we showed that the degradation of reduced and carboxymethylated BSA (rcmBSA) is sensitive to ribonucleases as well (Fig. 7A) [60]. We chose rcmBSA not only because this substrate is degraded in a ubiquitin- and ATP-dependent mode, but also because intermediate ubiquitin-rcmBSA conjugates can be demonstrated [54] (see below). Native BSA is an effective substrate for the cell-free ubiquitin-dependent proteolytic pathway; however, ubiquitin-BSA conjugates could not be demonstrated using the native molecule (A. Ciechanover and A. Hershko, unpublished results). The kinetics of formation and degradation of ubiquitin-rcmBSA conjugates favors visualization of these conjugates, thus making rcmBSA a better substrate for analysis of the ribonuclease-sensitive step in the ubiquitin pathway (see below). RNase A was slightly more active in inhibiting the degradation of the three substrates (Fig. 7) [60]. This may reflect the fact that RNase A can more efficiently digest the tRNA component necessary for degradation of these substrates and that is contained in crude reticulocyte fraction II.

To test directly for the possibility that the inhibition of degradation of rcmBSA, α-lactalbumin, and STI was due to destruction of the tRNA component of the system by the ribonucleases, the system was treated with micrococcal nuclease followed by the addition of pTp or the Ca^{2+} chelator EGTA (see above) [55,56,60]. The subsequent addition of tRNA was found to restore the inhibited proteolytic activity completely [60] (Fig. 8).

In principle, tRNA can participate in either conjugation of ubiquitin to the proteolytic substrate or in degradation of the ubiquitin-protein conjugates. To test for either possibility, crude reticulocyte fraction II was incubated with [^{125}I]labeled ubiquitin and either rcmBSA, α-lactalbumin, or STI. Distinct conjugates were formed between ubiquitin and the added exogenous substrates (Fig. 9B) [60]. The exact structure of these ubiquitin-protein conjugates is not known. Previously [18], from the molecular weight of the conjugate, we tried to determine its structure and the stoichiometry of ubiquitin molecules attachment. It has been recognized recently that this is not possible anymore, as the system contains proteases that can cleave ubiqui-





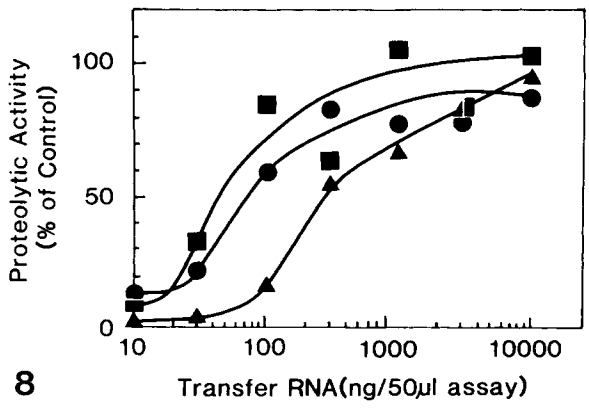
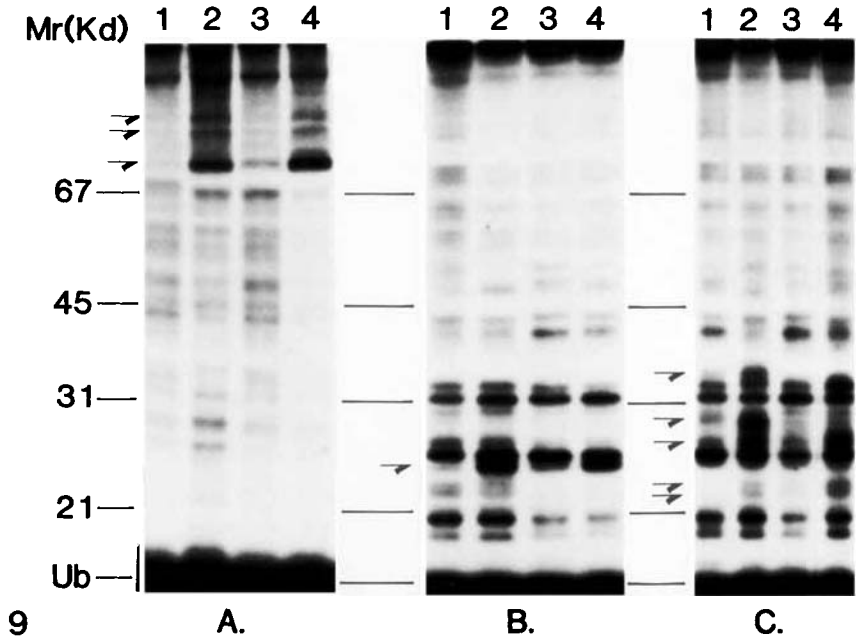


Fig. 6. Effect of purified immunoprecipitated tRNAs on the ubiquitin- and ATP-dependent proteolytic system. RNAs extracted from the immunoprecipitates (▲, MN; ■, SU; ●, LL) or from the supernatants after immunoprecipitation (△, MN; □, SU; ○, LL) were added to a micrococcal-inhibited ubiquitin system (following inhibition of the enzyme), and the degradation of [^{125}I]BSA was monitored. Serum MN precipitated only about 10% of the total cellular $tRNA^{His}$, and serum SU precipitated only <5%. The activity of the tRNA in the supernatant was therefore 9–23-fold higher than that of the tRNA extracted from the precipitate (in different experiments) and hence very similar to that of total tRNA prior to precipitation (▽) (Adapted from Ciechanover et al [55].)

Fig. 7. Effect of ribonucleases of the degradation of rcmBSA (**A**), α-lactalbumin (**B**), and STI (**C**). The degradation of the [^{125}I]labeled substrates was determined as described in Figure 2 in the presence of the indicated concentrations of RNase A (●) or micrococcal nuclease (□). (Adapted from Ferber and Ciechanover [60].)

Fig. 8. Effect of tRNA on the degradation of rcmBSA (■), α-lactalbumin (●), and STI (▲). Crude reticulocyte fraction II was preincubated with micrococcal nuclease. Following inhibition of the enzyme, ubiquitin, ATP, and tRNA (in the indicated concentrations) were added, and the degradation of the labeled proteins was monitored. (Adapted from Ferber and Ciechanover [60].)



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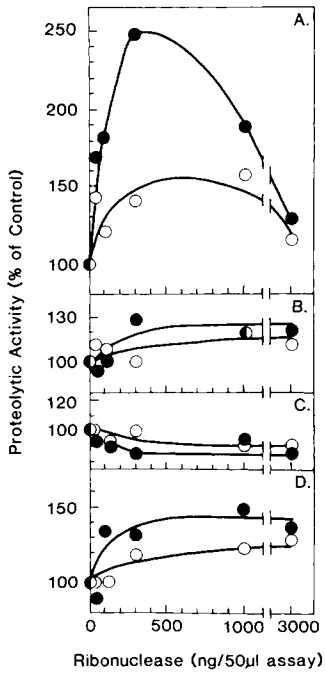

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Fig. 9. Effect of micrococcal nuclease and tRNA on the formation of [^{125}I]ubiquitin conjugates with rcmBSA (**A**), α-lactalbumin (**B**), and STI (**C**). Crude reticulocyte fraction II was preincubated with or without micrococcal nuclease, followed by inhibition of the nuclease and initiation of the reaction with the addition of [^{125}I]ubiquitin, ATP, and the appropriate substrate. The conjugates were resolved by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Lane 1, with no addition of substrate; lane 2, with added exogenous substrate; lane 3, treated with micrococcal nuclease; lane 4, as lane 3, but in addition tRNA was added following inhibition of the nuclease. Arrows on the left side of the panels indicate specific conjugates. (Adapted from Ferber and Ciechanover [60].)

Fig. 10. Effect of ribonucleases on the degradation of [^{125}I]radiolabeled oxidized RNase A (**A**), α-casein (**B**), β-lactoglobulin (**C**), and lysozyme (**D**). The degradation of the [^{125}I]radiolabeled substrates was determined in the presence of the indicated amounts of micrococcal nuclease as described in Figure 2. (Adapted from Ferber and Ciechanover [60].)

tin-protein conjugates rapidly [23]. Also, the occurrence of isopeptidase(s), which cleave the isopeptide bond between ubiquitin and proteins or peptides [23,61], and of polyubiquitin structures [62], also make analysis of ubiquitin-protein conjugates structure difficult.

Preincubation of crude reticulocyte fraction II with micrococcal nuclease prior to the addition of the substrate and labeled ubiquitin almost completely abolished the formation of conjugates between ubiquitin and the exogenous substrates (Fig. 9, lanes A3,B3,C3) [60]. Pretreatment of crude reticulocyte fraction II with RNase A resulted in a similar inhibition of specific conjugate formation (data not shown). As seen in Figure 9 (cf. lane 3 to lane 1 in all panels), the formation of conjugates between ubiquitin and most endogenous protein acceptors is not inhibited by nuclease treatment. However, some endogenous protein-ubiquitin conjugate formation is inhibited by nuclease treatment (Fig. 9, cf. bottom of lanes B1 and B2 to bottom of lane B3, and bottom of lanes C1 and C2 to bottom of lane C3) [60].

When tRNA is added to the micrococcal nuclease-treated system following inhibition of the nuclease, the specific conjugates are formed (Fig. 9, lanes A4, B4, C4) [60]. For nuclease-sensitive substrates, a strong correlation therefore exists between inhibition of conjugate formation and inhibition of degradation and between restoration of conjugation and restoration of degradation by exogenously added tRNA. The conjugation of ubiquitin to a nuclease-sensitive endogenous ubiquitin acceptor is also restored by tRNA (Fig. 9, lane C4). For an unknown reason, the restoration of formation of the same conjugate could not be demonstrated in the experiment shown in Figure 9B.

We have already noted that the degradation of lysozyme is not affected by nucleases [55,60]. Since it was also clear from the conjugation assays that the effect of micrococcal nuclease is highly selective and that most endogenous substrates are not affected by the nuclease treatment (Fig. 9), we sought to identify more ribonuclease-resistant proteolytic substrates. The degradation of [^{125}I]labeled oxidized RNase A, α-casein, β-lactoglobulin, and lysozyme was not inhibited by ribonucleases (Fig. 10). On the contrary, the degradation of oxidized RNase A, α-casein, and lysozyme was significantly stimulated when fraction II was preincubated with nucleases (Fig. 10 A,B,D, respectively).

To corroborate further the relationship between ubiquitin conjugation and degradation, crude reticulocyte fraction II was incubated with [^{125}I]labeled ubiquitin and unlabeled lysozyme, oxidized RNase A, and β-lactoglobulin. Distinct conjugates between ubiquitin and the proteolytic substrates were formed (Fig. 11, lanes 2). When the enzyme preparation was preincubated with micrococcal nuclease, no change in conjugate pattern was demonstrated (Fig. 11, lanes 3). Thus, for both nuclease sensitive and insensitive substrates, a strong correlation exists between ubiquitin conjugation to the substrate and its subsequent degradation.

DISCUSSION

What is the role of tRNA in the ubiquitin- and ATP-dependent proteolytic pathway? It is now accepted that protein breakdown rates in bacterial and animal cells are precisely regulated and vary under different physiological conditions (for reviews, see [2,3,9]). In *Escherichia coli*, degradation increases upon starvation for amino acids, nitorgen, or glucose, or when growth slows and the cells enter stationary phase

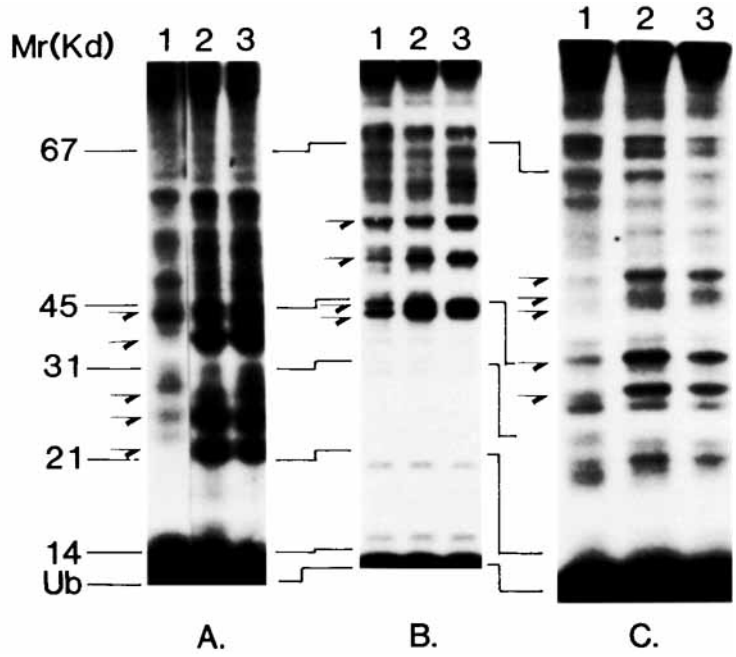

Fig. 11. Effect of micrococcal nuclease on the formation of [^{125}I]ubiquitin conjugates with lysozyme (**A**), oxidized RNase A (**B**), and β-lactoglobulin (**C**). Crude reticulocyte fraction II was preincubated with or without micrococcal nuclease, followed by inhibition of the enzyme (with pTp), and initiation of the reaction with the addition of [^{125}I]ubiquitin, ATP, and the appropriate substrate. The conjugates were resolved by SDS-polyacrylamide gel electrophoresis followed by auotradiography. Lane 1, with no addition of substrate; lane 2, with exogenous substrate; lane 3, pretreated with micrococcal nuclease. Arrows on the left side of the panels indicate specific conjugates. (Adapted from Ferber and Ciechanover [60].)

[63]; the enhanced degradation is reversed when the cells are cultured in fresh medium or reinitiate growth. There, the level of uncharged tRNA appears to serve as a trigger (for review, see [3]): The interaction of deacylated tRNA with polyribosomes generates a putative nucleotide mediator, guanosine 3′-diphosphate 5′-diphosphate (ppGpp) [64], 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 [2,3,9]), 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 [65]. Histidinol inhibition of charging of $tRNA^{His}$ produces a similar effect in wild-type CHO cells [66]. Deprivation of specific amino acids, in particular alanine [67] and histidine [68], 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), selectively regulates the rate of intracellular proteolysis in eukaryotic cells. Alternatively, tRNA might participate in some reaction of the ubiquitin pathway. For example, tRNA can play a role in the activation of ubiquitin in a mechanism similar to that involved in activation of amino acids.

It is also possible that tRNA participates in covalent modification of selective proteolytic substrates, a modification that increases their sensitivity to the ubiquitin

proteolytic system. Of note is that all three ribonuclease-sensitive substrates have an acidic NH_2-terminus (aspartic acid in STI [69] and in BSA [70] and glutamic acid in bovine α-lactalbumin [71]). Recent data from our laboratory indicate that an acidic NH_2-terminus can serve indeed as a recognition marker for the tRNA-dependent reaction. Other proteins with acidic NH_2-termini (such as the kappa light chain of the human immunoglobulin molecule [Bence-Jones protein][72]) are degraded via the ubiquitin pathway in a tRNA-dependent mode. The degradation of human α-lactalbumin, which has a 75% homology to the bovine molecule, however, has a lysine residue at the NH_2 terminus, is independent of tRNA (A. Ciechanover and S. Ferber, unpublished data). It is possible, for example, that a tRNA-dependent modification of the NH_2 terminus by addition of an amino acid residue, is required prior to recognition of the substrate by the ubiquitin ligase system. A tRNA-dependent posttranslational modification of acidic NH_2-termini of proteins by the addition of an arginine residue has been described [73]; however, its function has not been elucidated.

Recently, it was reported that the in vivo half-life of a protein is a function of its amino terminal residue. β-galactosidase with 16 different genetically engineered N-termini demonstrated half-lives that ranged from 2 min to more than 30 hr [74]. An N-end rule was proposed, according to which long-lived proteins have "stabilizing" amino termini, while short-lived proteins have "destabilizing" amino terminal residues. β-Galactosidase with glutamic or aspartic acid in its α-NH_2 position has a short half-life (10–30 min). It is possible that these residues are not "destabilizing" as such, but become so only through their ability to be conjugated (in a tRNA-dependent mode, for example) to other "destabilizing" residue. It is possible that either the native or a posttranslationally modified N-terminus is one important feature of the substrate, recognized by the ubiquitin ligation system prior to marking it with ubiquitin and further degrading it.

It is interesting to note that tRNA-dependent posttranslational addition of amino acids to proteins in vivo is increased under conditions of stress, for example, after physical injury to axons of nerve cells [75]. Although the relevance of this modification to proteolysis by the ubiquitin system is not known, it is interesting to note that the ubiquitin system is also activated under conditions of stress, such as heat shock [31,32,76]. It is possible that under these conditions, protein turnover is stimulated (to remove, for example, heat-denatured proteins). One can therefore reason that protein signalling, which precedes its recognition by the ubiquitin system, is increased as well.

Another possibility for the mechanism of action of tRNA is that it participates indirectly in some reaction of the ubiquitin pathway. For example, it can bind to the substrate and thus change its properties so that it is recognized by the ubiquitin ligation system. Of note is that the endogenous tRNA component of the ubiquition system appears to be protein bound, because fraction II prepared by chromatography on DEAE-cellulose followed by ammonium sulfate precipitation conditions under which free tRNA is not precipitable.

The degradation of oxidized RNAse A, α-casein and lysozyme was accelerated by ribonucleases. Ribonuclease treatment might inhibit the flow of endogenous substrates into the system in an early step of the pathway, thus preventing competition between exogenously added substrates and endogenous substrates on commonly shared enzymatic components. The two distinct pathways may therefore converge at one point, following which share common components of the pathway.

Why should there be two distinct pathways for ubiquitin marking of substrates? Not all ubiquitin-protein conjugates may be intermediates for proteolysis, and modification of proteins with ubiquitin may serve nonproteolytic function(s) as well. For example, ubiquitination of histones may alter their three-dimensional nucleosomal structure (without proteolytic removal of the histone molecules), marking specific regions of the chromosome for binding of yet to be identified factors involved in transcription or replication. The ubiquitination of cell surface proteins may also serve nonproteolytic functions. Thus, one pathway may be involved in ubiquitin marking for proteolysis, while the other serves other regulatory or structural function(s). Further purification of the in vitro system, identification of the tRNA protein carrier, and identification of ubiquitin substrates in vivo, will help to clarify this point.

CONCLUDING REMARKS

In the past few years considerable progress has been made in the elucidation of the enzymatic steps involved in the formation and degradation of ubiquitin conjugates. Powerful tools are now available to study the cellular roles of the ubiquitin system, including specific antibodies, a temperature-sensitive mutant, microinjection techniques, and cloned genes (not discussed; see, however, [77–79]). Still, many major problems remain unsolved, and the unknown greatly exceeds what we presently know of the ubiquitin system. What determines the specificity of the ubiquitin conjugation system for committment of a certain protein for degradation? It appears reasonable to assume that a free and specific α-NH_2 group is one of several features of the protein structure recognized by the ubiquitin ligation system. How does the system distinguish between ubiquitin ligation leading to protein breakdown and that involved in protein modification? What determines whether a particular protein is conjugated with a single ubiquitin or with multiple ubiquitin molecules? While we have no answers to these questions at present, available information suggests that there are several different ubiquitin-protein ligation systems and that these may act on different types of cellular proteins. Among other specific questions that still await elucidation are the intermediary reactions in the degradation of ubiquitin-conjugated proteins and the role of ATP in conjugates breakdown.

Concerning the cellular functions of the ubiquitin proteolytic pathway, available evidence is limited to its involvement in the breakdown of abnormal and rapidly turning-over normal proteins. It is now possible to examine the role of the ubiquitin system in the turnover of specific cellular proteins. In addition, the role of the ubiquitin system in a variety of basic cellular processes such as cell cycle-related events, gene expression and heat-shock response (not discussed; see, however, [31,32,76]) are of considerable interest. In some of these cases, possible nonproteolytic functions of ubiquitin conjugation have to be taken into account. It is quite possible that ubiquitination of histones and cell surface proteins are but few examples of ubiquitin function in protein modification, rather than breakdown. Using immunochemical [27] and microinjection [80] techniques, it was noted that a considerable fraction of cellular ubiquitin-protein conjugates are stable; these may represent modification products rather than degradation intermediates. The conjugation of a single or a few ubiquitin molecules may modulate enzyme activity, produce alterations in structural proteins, or change the activity of regulatory proteins. Evidently, much

more progress is required to elucidate the functions of ubiquitin in protein modification and breakdown.

ACKNOWLEDGMENTS

The author is indebted to Dr. Alan L. Schwartz for his very generous help, and thanks Ireta Ashby for her skillful help and patience in typing the manuscript. The author was supported by grant 85-00059/1 from the U.S.-Israel Binational Science Foundation (BSF), the Hedson Fund, and the Foundation for Promotion of Research at the Technion. The author is a Research Career Development Awardee of the Israel Cancer Research Fund, U.S.A.

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