In vitro proteolytic processing of a diubiquitin and a truncated diubiquitin formed from in vitro-generated mRNAs
(polyprotein/transpeptidation/isopeptidase/isoubiquitin/autoprotease)

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ABSTRACT Ubiquitin, a highly conserved protein of 76 amino acids found in all eukaryotes, is translated from mRNAs that either multiple, contiguous coding sequences of the protein or a single ubiquitin coding sequence fused to sequences coding for 52 or 76 amino acids. We describe here formation of monoubiquitin from in vitro translation of mRNAs containing either two complete sequences or one complete ubiquitin and 60% of a second ubiquitin. No diubiquitin precursor was found with the complete diubiquitin mRNA, but the truncated mRNA formed proteins with apparent molecular masses of 30, 24, 7, and 4 kDa. The latter two are the expected products from truncated ubiquitin mRNA. The 30-kDa protein was immunoprecipitated by anti-ubiquitin antibodies and was converted to ubiquitin and the 4-kDa form by a ubiquitin isopeptidase-like activity in wheat germ. Other data indicated that the 30-kDa protein had multiple ubiquitins, all linked by isopeptide bonds to the truncated ubiquitin. One of these was the radiolabeled translation product, which should have been linked to the truncated protein by a normal peptide bond. A model is proposed in which ubiquitin itself participates in a transpeptidase activity.

Ubiquitin is a 76-amino acid polypeptide found in all eukaryotic cells (1, 2). Recent studies of the genes encoding ubiquitin show that it is initially synthesized either as a polypeptide with multiple, contiguous stretches of the ubiquitin sequence or as a fusion protein in which ubiquitin forms the amino-terminal domain of a larger polypeptide (3). About half of the total ubiquitin in the cell is monoubiquitin, and the balance is present as ubiquitin conjugated in an isopeptide linkage to other cellular proteins (4–6). The most notable examples of the latter are histones H2A and H2B (7–9). Ubiquitin conjugation requires a complex of enzymes and ATP, which activate free ubiquitin and transfer it to protein (reviewed in refs. 10 and 11). Proteins with multiple ubiquitins are targeted for degradation by an ATP-dependent protease. Coupling of the two systems may account for the turnover of short-lived cellular proteins and abnormal proteins formed during stress such as heat shock (12–16). Another enzyme, ubiquitin isopeptidase, removes ubiquitin from protein conjugates without any subsequent degradation of ubiquitin or the protein (17–19).

Since monoubiquitin appears to be the substrate for the conjugation system, there must be proteolytic processing of polyubiquitin and ubiquitin fusion proteins synthesized from the mRNAs transcribed from the various ubiquitin genes (20). In a search for a polyubiquitin processing activity, we have inserted a cDNA encoding two ubiquitin sequences into a bacterial plasmid containing the bacteriophage SP6 promoter such that a diubiquitin mRNA could be transcribed and then translated in vitro. We subsequently modified this plasmid so that an mRNA coding for a truncated diubiquitin would be produced. The translation product of both mRNAs were processed in vitro to free ubiquitin, but the pathways appeared to be different. The possible mechanisms for the processing of the truncated diubiquitin suggest some unusual events in ubiquitin-mediated protein turnover.

MATERIALS AND METHODS

Materials. Restriction endonucleases and DNA-modifying enzymes were from New England Biolabs, Boehringer Mannheim, and Pharmacia P-L Biocemicals. The mRNA “cap” analogue m7G(5')ppp(5')G, ribonucleotides, and bovine serum albumin were from Pharmacia. RNase-free DNase was from Cooper Biomedical (Malvern, PA). In vitro translation kits were from Bethesda Research Laboratories and Promega Biotec (Madison, WI). Urea (enzyme grade) was from Bethesda Research Laboratories and NaDodSO4 was from Bio-Rad. Ubiquitin and all other reagents were from Sigma. Antibodies to ubiquitin and ubiquitin conjugates were from A. Haas (Medical College of Wisconsin, Milwaukee) and A. Ciechanover (Technion, Haifa, Israel). Preparations of ubiquitin aldehyde and ubiquitin hydrolase were from I. Rose (Institute for Cancer Research, Philadelphia). [35S]Methionine (>1000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. A synthetic peptide containing eight amino acids that span the proteolytic cleavage site between the multiubiquitin was from S. Adams (Monsanto). The wheat germ extract was from Bethesda Research Laboratories.

Construction of UB 2.0 and UB 1.6 Plasmids. A cDNA clone (21) that contains 12 base pairs of the second ubiquitin coding sequence plus a complete third and fourth ubiquitin coding sequence and 120 base pairs of the 3' untranslated region of the chicken polyubiquitin gene UB I (15) was inserted into the vector SP64 at the Pst I site in the polylinker (Fig. 1). This clone is designated UB 2.0. A second clone (designated UB 1.6) containing the one full repeat at the 5' portion of the cDNA and approximately 60% of the second repeat at the 3' portion (Fig. 1) was derived by partial Pvu II digestion of UB 2.0, followed by complete digestion with Smal I and treatment that favors blunt-end ligation. Restriction mapping was used to detect clones with the correct plasmids. In both of these clones there are no AUG codons preceding the authentic AUG of the first full ubiquitin coding sequence.

In Vitro Transcription and Translation. Plasmid DNA (5–10 μg) was linearized with EcoR I to generate a site for run-off transcription. The reaction was carried out for 1 hr at 40°C in a solution containing 40 mM Tris-HCl (pH 7.5), 6 mM MgCl2, 4 mM spermidine, 0.5 mM ribonucleotides, 1.5 mM m7G(5')ppp(5')G, 10 mM dithiothreitol, bovine serum albumin (100 ng/μl), and RNasin (1.3 units/μl), and SP6 polymerase (0.3 units/μl).

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RESULTS

Translation of mRNA from UB 2.0 DNA. Plasmid DNA containing two contiguous coding sequences for ubiquitin was transcribed to yield an mRNA that was translated by reticulocyte extracts to form monomeric ubiquitin, as indicated by the retention of a protein of 6- to 7-kDa on an anti-ubiquitin antibody column (Fig. 2A) or by immunoprecipitation (Fig. 2B, lanes 4–6). Ubiquitin levels increased with the time of the reaction, but no higher molecular mass material was detected, even after 5 min of translation, by using an antibody column, immunoprecipitation, or direct application of samples to a gel for NaDodSO₄/PAGE analysis. The affinity-purified antibody used for the reaction products shown in Fig. 2 (lane 5) does not detect ubiquitin molecules that have lost their carboxyl-terminal glycine residues (26); therefore, the products generated in vitro must have been complete molecules of ubiquitin.

These results indicate that ubiquitin was proteolytically processed very efficiently at the normal glycine-methionine sequence in the polyubiquitin molecule. However, ubiquitin has a methionine as the amino terminus, and it is possible that the internal AUG in the diubiquitin mRNA rather than the 5' AUG was selectively translated in vitro even though the 5' AUG is a good site for initiation of protein synthesis according to the scanning model of Kozak (27). To rule out internal initiation, we modified the UB 2.0 plasmid by deleting a region at the 3' portion, which led to a DNA coding for 1.6 ubiquitin molecules.

Translation of mRNA from UB 1.6 DNA. Like UB 2.0, UB 1.6 mRNA produced a protein of 6- to 7-kDa after a short incubation time, but several other proteins were also formed (Fig. 2B, lane 7). These included a 4-kDa protein and larger proteins with apparent molecular masses of 24 kDa and 30 kDa. The UB 1.6 mRNA should produce an intact molecule of about 14 kDa, and processing should give ubiquitin and a 4-kDa protein. Radioimmunoprecipitation showed that the 7-kDa protein was ubiquitin and that the 30-kDa protein contained ubiquitin (Fig. 2B, lanes 8 and 9). A 17-kDa band was also detected in the immunoprecipitate of the sample run in Fig. 2B, lane 9. The higher molecular mass proteins were unexpected, and we carried out the following experiment to determine if the 30-kDa protein, which contained radioactive ubiquitin, was processed to monoubiquitin and the 4-kDa protein.

Proteolytic Processing of the 30-kDa Protein. When the in vitro translation was stopped after 15 min by adding 1 mM cycloheximide and the mixture was allowed to incubate for 45 min at 23°C, all of the 30-kDa and 24-kDa proteins disappeared and increased amounts of ubiquitin appeared. However, only small amounts of the 4-kDa protein were detected (data not shown). When the reaction mixture was heated after 15 min of in vitro translation, little change occurred in the levels of the 30-kDa and 24-kDa proteins upon subsequent incubation (Fig. 3A, lane 6). If an extract of wheat germ was added after stopping translation by heating, levels of the high molecular mass proteins decreased, while the levels of both ubiquitin and the 4-kDa protein increased (Fig. 3A, lanes 2–4). Very little processing occurred if the wheat germ...
imide (1 mM), products by reticulocyte extract germ were 10 and 30 min incubated Lane 1: the reaction analyzed directly. (B) them samples (denoted to responding proteins, ubiquitin, of that the 30-kDa at 4-kDa of cleavage would be the translation in the almost all of the precursors 10 dependent

FIG. 3. Processing of the truncated diubiquitin mRNA translation products by a wheat germ extract. (A) In vitro translation in a reticulocyte extract was stopped after 15 min at 23°C by cycloheximide (1 mM), and the reaction mixtures were heated at 60°C for 3 min. A 5-μ sample was removed (lane 2), and 4 μl of a crude wheat germ extract was added to a 10-μl sample of the translation mixture. After 10 and 30 min (lanes 3 and 4, respectively) at 37°C, 7-μl samples were removed. Other samples (5 μl) of the 15-min translation mixture were incubated with 2 μl of wheat germ extract at 4°C (lane 5) or with 2 μl of 0.05 M phosphate buffer (pH 7.4) at 37°C (lane 6) for 45 min. Lane 1: 15-min translation reaction with no added mRNA and subsequent translation for 45 min with wheat germ extract. Samples from the reaction mixtures were mixed with gel loading buffer and were analyzed directly. (B) The radioactivity in the bands corresponding to the two high molecular mass and the two low molecular mass samples (denoted as □ and □, high molecular mass; ◆ and ◆, low molecular mass) was determined by excising the bands and counting them in a liquid scintillation counter. τ = 0 corresponds to lane 2 in A, and the 10- and 30-min samples are from lanes 3 and 4, respectively.

each extract was incubated at 4°C (Fig. 3A, lane 5). Quantitation of the changes in the amounts of the 30-kDa and 24-kDa proteins, ubiquitin, and the 4-kDa protein after 10 and 30 min at 37°C in the presence of the wheat germ extract indicated that the 30-kDa protein was a precursor for ubiquitin and the 4-kDa protein, but the kinetics were not those predicted for cleavage of a linear truncated diubiquitin molecule, which would be the translation product of the mRNA (Fig. 3B). Almost all of the ubiquitin produced over the 30-min period in the presence of the wheat germ extract occurred in the first 10 min, whereas the amount of the 4-kDa protein continued to increase throughout the 30-min incubation. At early times in the processing, the 24-kDa protein appeared to act as an intermediate in the degradation of the 30-kDa protein.

Properties of the Proteolytic Processing of Truncated Diubiquitin. The data cited above showed that conversion of precursors to products was inactivated by heating; it was dependent on addition of a cell extract and increased with time and temperature. To gain more information about a putative protease in the wheat germ extract, we tested a variety of inhibitors specific for various kinds of proteases. Surprisingly, except for N-ethylmaleimide, none of the standard inhibitors affected the processing activity (Table 1). Among the enzymatic activities known to process ubiquitin–protein conjugates, only the ubiquitin isopeptidases have properties similar to those found in our studies with the enzyme present in the wheat germ extract (28). Isopeptidases cleave between the carboxyl-terminal glycine of ubiquitin and the ε-amino group of lysine and generate free ubiquitin while leaving the polypeptide containing the lysine group to which the ubiquitin was attached intact. A preparation of ubiquitin carboxyl-terminal hydrolase (29) was inactive in this system. We also tested ubiquitin aldehyde (kindly supplied by I. Rose) and a synthetic octapeptide containing the amino acid sequence spanning the glycine-methionine site between contiguous ubiquitins. The former showed a 40% inhibition, but the peptide did not inhibit processing.

Structure of the Precursors Formed by Translation of the Truncated Diubiquitin mRNA. Based on their size, the kinetics of processing, and the properties of the processing activity, neither the 30-kDa nor the 24-kDa polypeptide were expected from in vitro translation of an mRNA that encodes a complete ubiquitin and truncated ubiquitin in a linear sequence. We hypothesized that the 30-kDa precursor consisted of an [35S]methionine-labeled intact ubiquitin formed during the in vitro translation plus two additional nonradioactive ubiquitins bound to an [35S]methionine-labeled truncated ubiquitin. The nonradioactive ubiquitins were postulated to be present in the reticulocyte extract and to be conjugated to lysines in the truncated ubiquitin by a ubiquitin conjugation system that could have been present in the reticulocyte extracts (30). The radiolabeled intact ubiquitin could be attached to the truncated protein through a normal peptide bond (Fig. 4, top structure) or in an isopeptide bond to a lysine of the truncated, labeled ubiquitin (Fig. 4, bottom structure).

We could distinguish between these two hypothetical structures by isolating the labeled 30-kDa precursor and determining its amino acid sequence after trypsin digestion. If this precursor had intact ubiquitin attached to the truncated ubiquitin of the protein in a normal peptide bond, trypsin would cleave at the arginine located three residues on the amino side of the internal methionine as well as at other lysine and arginine of the protein. Amino-terminal sequencing of this digest predicts that labeled methionine would appear equally in the first and third amino acids (see Fig. 4). If, Table 1. Effect of protease inhibitors on processing of products from in vitro translation of truncated diubiquitin mRNA

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% inhibition</th>
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<tbody>
<tr>
<td>Phenylmethanesulfonyl fluoride (1 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Tosyl-L-lysine chloromethyl ketone (100 μM)</td>
<td>0</td>
</tr>
<tr>
<td>Tosyl-L-phenylalanine chloromethyl ketone (100 μM)</td>
<td>0</td>
</tr>
<tr>
<td>Leupeptin (100 μg/ml)</td>
<td>0</td>
</tr>
<tr>
<td>EDTA (2 mM)</td>
<td>0</td>
</tr>
<tr>
<td>N-Ethylmaleimide (5 mM)</td>
<td>100</td>
</tr>
<tr>
<td>Ubiquitin aldehyde (2.5 μM)</td>
<td>40</td>
</tr>
<tr>
<td>Synthetic peptide LRGMQIF (100 μg/ml)</td>
<td>0</td>
</tr>
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Truncated diubiquitin mRNA was translated for 15 min at 23°C, and the reaction was stopped by adding cycloheximide (1 mM). The mixture was heated 3 min at 60°C and supplemented with 3 μl of a wheat germ extract (25 μg/ml) and 1 μl of inhibitor. Incubation was continued at 37°C, and samples were analyzed by NaDodSO4/PAGE after 15 min. The percent inhibition was measured by cutting out the 30-kDa band and measuring its radioactivity in a scintillation counter. A sample with no inhibitor was used to determine the extent of inhibition. The amino acid sequence of the synthetic peptide is represented by the single-letter code.
The tryptic digested truncated ubiquitin molecule. Two 

vitro terminus from gels.

However, the precursor contained all of its intact ubiquitins, including the one translated from the UB 1.6 mRNA and labeled with methionine, bound to the truncated portion of the protein in an isopeptide bond, all labeled methionine would appear in the first amino acid of the sequence. The results of sequencing showed that virtually all of the label in the tryptic digested precursor was in the amino terminus (Fig. 5B). Sequence of the undigested sample showed a similar distribution of label, which is the expected result. These data indicated clearly that the amino-terminal methionine of the truncated ubiquitin portion of the 30-kDa protein was free and that the intact labeled ubiquitin made from the UB 1.6 mRNA was linked by isopeptide bond to the truncated ubiquitin.

Studies of the Mechanism of Isoubiquitin Formation. Two pathways could lead to formation of an isoubiquitin from ubiquitin initially bound in normal peptide linkage to the truncated portion of ubiquitin. The first would be a proteolytic cleavage with release of the intact [35S]ubiquitin followed by its activation and conjugation utilizing ubiquitin-specific enzymes in reticulocyte extracts. If this were the pathway, then addition of free, unlabeled ubiquitin to the reaction mixture during in vitro translation should decrease the amount of radioactivity in the 30-kDa protein band. However, results of such an experiment in which we quantitated the levels of radioactive 30-kDa protein after separation in NaDodSO4/PAGE showed no decrease in the amount of radioactivity: there were 480 cpm in the 30-kDa protein made in the normal reaction and 520 cpm in the 30-kDa protein made from a reaction supplemented with unlabeled ubiquitin at 3 \( \mu \text{g}/\mu\text{l} \).

The second pathway invokes an intramolecular transpeptidation reaction in which the carboxyl terminus of intact ubiquitin is shifted from a normal peptide bond to an isopeptide bond of the truncated portion of the translation product. Transpeptidation could be mediated either by an enzyme recognizing the glycine-methionine sequence between the intact and partial ubiquitins, by the E3 enzyme of the ubiquitin conjugation pathway (which catalyzes the last step in conjugating ubiquitin), or by ubiquitin itself.

**DISCUSSION**

Our attempts to detect proteolytic processing of an in vitro generated polyubiquitin have led to some surprising results. Translation of an mRNA coding for two intact contiguous ubiquitins produced ubiquitin with the appropriate carboxy-terminal residues in what appeared to be a cotranslational proteolytic processing activity. No diubiquitin molecules were detected. We ruled out a possible internal initiation of translation at the 3' end of the ubiquitin mRNA by utilizing an altered form of the mRNA that contained the second start site but lacked a full intact ubiquitin. In vitro translation of the latter clearly produced a complete ubiquitin molecule as well as a smaller protein equivalent in size to the 3'-truncated form, and both proteins contained labeled methionine at the amino-terminal position. However, translation of the truncated diubiquitin mRNA produced several ubiquitin-containing proteins that were larger than the polypeptide corresponding to the mRNA sequence. These polypeptides were proteolytically processed to ubiquitin and a 4-kDa protein by an enzyme with properties of a ubiquitin isopeptidase. Partial chemical analysis showed that all of the radiolabeled methionine in the largest precursor was present as a free amino terminus. Three pieces of data (size of the precursors, pathway of processing, and distribution of radiolabel) suggest to us that there had been a transfer of the intact ubiquitin from its amino-terminal position to a lysine group in the truncated portion of the polyprotein during or shortly after translation of the UB 1.6 mRNA.

The addition to this postulated transpeptidation reaction, we propose that the truncated molecule has been conjugated with additional unlabeled ubiquitins and this accounts for the sizes of the in vitro translation products—they differ in values expected for ubiquitin. We do not have direct evidence for these additional ubiquitins: it was not feasible to add exogenously labeled ubiquitin and isolate it in the very small amounts of precursor generated in vitro since addition of a radioactive ubiquitin to reticulocyte extracts labels a number of endogenous proteins in the extract (30). However, two sets
of data strongly infer the presence of nonradioactive ubiquitins in the 30-kDa precursor. One of these is its molecular mass, which is consistent with a truncated protein plus three ubiquitins. Presumably the 24-kDa polypeptide has two isoubiquitins attached to the truncated protein. The second set of data are the kinetics of proteolytic processing, which showed that ubiquitin and the truncated product were not produced in equimolar amounts. This result can be explained by the presence of both labeled and unlabeled ubiquitins attached to a labeled truncated ubiquitin.

We have ruled out one mechanism for the putative transpeptidation reaction—namely, cleavage to free ubiquitin and reattachment by the ubiquitin conjugation system in the reticulocyte extract. Thus, transpeptidation appears to be an intramolecular reaction. This transfer could result from an activity of the E3 ubiquitin conjugation enzyme that normally transfers ubiquitin from an E2-ubiquitin complex to the ε-amino groups of lysines of a target protein. This E3 complex requires that the target protein have its amino terminus free, but ubiquitin’s presence as the amino terminus may preclude this specificity. Transfer might also be carried out by a ubiquitin transferase not previously described.

We are considering a different mechanism for transpeptidation, one that involves amino acids in the ubiquitin structure. In our model, the single histidine in ubiquitin, which interacts with solvent according to the crystallographic structure of the protein (31), and a hydroxyl amino acid in the sequence of ubiquitin are part of an active site (Fig. 6). One possible mechanism for destabilizing the peptide bond between glycine and methionine is shown, and the nitrogen in the ε-amino group of a lysine in the truncated ubiquitin part of the molecule is postulated to act as the nucleophile that leads to isopeptide bond formation. This model predicts an essential role for histidine in the transpeptidation activity. There are recent data indicating an essential role for histidine in ubiquitin (32).

We do not know if the transpeptidation activity postulated here for the truncated diubiquitin also accounts for the processing of the intact diubiquitin. Formation of the isoubiquitin from the UB 1.6 mRNA does lead to cleavage at the glycine-methionine bond between contiguous ubiquitins, and it could be the mechanism for polyubiquitin proteolytic processing. The immediate result would not be free ubiquitin but a polysubiquitin, which would then use a ubiquitin isopeptidase to produce individual ubiquitin molecules.

Another question raised by our results is whether a protein targeted for the ubiquitin degradation pathway obtains its isoubiquitins by initial attachment of ubiquitin to the amino-terminal residue of the target protein with subsequent transfer to lysines. It is clear from several recent papers (33–35) that the amino-terminal residue is extremely important in determining the degradation of the protein, but the precise mechanism of how the target protein is ubiquitinated has yet to be elucidated. Perhaps the results described here have application to this problem.

![Fig. 6. A hypothetical mechanism for ubiquitin's role as a transpeptidase.](image-url)