Expression and Accurate Processing of Yeast Penta-ubiquitin in *Escherichia coli*

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An expression vector (pSJyub-5) was constructed which contained five repeats of the "yeast ubiquitin gene" regulated by a heat-inducible λ P1, promoter. The vector, when expressed in *Escherichia coli*, produced a penta-ubiquitin of ~42 kDa. Purified penta-ubiquitin was found to be as active as the human mono-ubiquitin in the *in vitro* ATP/ubiquitin-dependent proteolytic assay of the reticulocyte lysate, indicating that the expressed gene product was recognized by the enzymes involved in the ATP/ubiquitin-dependent proteolytic pathway. The inability of penta-ubiquitin to act as a substrate in the pyrophosphate exchange reaction with the ubiquitin-activating enzyme $E_1$ suggested that it had a carboxyl-terminal Asn, in agreement with the nucleotide sequence. In *E. coli*, the expressed penta-ubiquitin was processed correctly to mono-ubiquitin. The fidelity of processing in *E. coli* was confirmed by the following observations. The amino acid compositions of the processed mono-ubiquitin and human ubiquitin were similar. The $^1$H NMR spectrum of peaks representing amide hydrogens of the carboxyl-terminal Arg-74, Gly-75, and Gly-76 of the processed mono-ubiquitin was identical with that of human ubiquitin. The immunoreactivity of processed mono-ubiquitin and human ubiquitin against polyclonal antibodies that recognized epitope(s) only on the carboxyl terminus of ubiquitin were similar. The human and processed mono-ubiquitin were equally active in degrading $^{13}$C-bovine serum albumin in the ATP/ubiquitin-dependent *in vitro* proteolytic assay with reticulocyte lysates. In the pyrophosphate exchange assay with isolated ubiquitin activating enzyme $E_1$, they were also equally reactive, confirming that the expressed and processed ubiquitin contained an intact carboxyl-terminal Gly-76. Purified penta-ubiquitin should prove to be a useful substrate for identifying and isolating processing enzyme(s) involved in the ATP/ubiquitin-dependent proteolytic pathway.

Ubiquitin is the most conserved protein known among eukaryotes (for review see Ref. 5). There are only three differences among yeasts, plants, and humans in the sequence of this 76-amino acid polypeptide (1–3). The crystal structure of human ubiquitin at 1.8-Å resolution shows clearly that the molecule has a compact structure containing three and one half turns of $\alpha$-helix surrounded by five strands of $\beta$-sheet. The carboxyl-terminal Leu-Arg-Gly-Gly residues protrude from the molecule; this portion has considerable freedom of motion and no hydrogen bonds or hydrophobic interactions with the rest of the molecule (4). It is probably this considerable mobility of the "tail" sequence that allows the carboxyl-terminal Gly of ubiquitin to attach covalently to a variety of nuclear, cytoplasmic, and cell surface proteins, including a growth factor, making ubiquitin a potential modulator of a number of cellular functions (5).

The sequence of ubiquitin in the eukaryotic genome is organized as a multiple head-to-tail, spacerless repeat of the genes encoding a precursor poly-ubiquitin. Although this unique organization of the ubiquitin gene is highly conserved, species-specific variations exist. The range of "ubiquitin gene repeats" found at a single locus varies from 3–9, 5–6, 6, >12, >16 in mammalian, chicken, yeast, *Xenopus*, *Drosophila*, and plants, respectively (6–8, 11, 9, 12, 13, 10, 14). While in yeast, only a single repeat of the poly-ubiquitin gene is transcribed (1, 9), multiple mRNA species observed in *Drosophila*, *Xenopus*, and human cells indicate that, in higher eukaryotes, a number of loci may be transcribed (8, 12–15). Interspecies variation is also found in the amino acid residue following the carboxyl-terminal Gly of the precursor poly-ubiquitin, which has been identified as Asn, Tyr, Lys, and Val in yeast, chicken, barley, and human cells, respectively (1, 11, 10, 8). The only exception to this single residue extension of poly-ubiquitin is found in *Xenopus laevis*, the gene of which ends with the carboxyl-terminal Gly-76 on the last repeat (12). The function of the single residue extension which blocks the terminal Gly-76 may be to prevent the precursor from being activated by $E_1$ (16). In *vitro* studies have shown that only mono-ubiquitin is capable of being conjugated to the ATP/ubiquitin-dependant proteolytic pathway (17, 27).

The intraspecies variations of poly-ubiquitin gene may imply selective activation of the precursors at different stages in the cell cycle (7). Deletion of the poly-ubiquitin gene in yeast renders the organism sensitive to high temperature, starvation, or other conditions that induce stress, suggesting that ubiquitin is an essential component of the stress response system (9). Ubiquitin is also encoded as "natural gene fusions." In these hybrid proteins, ubiquitin is fused to carboxyl-terminal amino acid sequence extensions (15, 18). The extensions, about 56–82 amino acids in length, are (70–80%) conserved between yeast and humans (18). A specific sequence motif of Cys-X$_2$-4-Cys-X$_2$-12-Cys-X$_2$-4-Cys, found in these fusion proteins, has a distinct similarity to the metal binding domains of the nucleic acid binding proteins involved in gene regulation (19–21). This unique organization of the ubiquitin gene raises the possibility that biologically active mono-ubiquitin found in the cell is essentially a cleavage product of the Gly-Met peptide bond of the precursor poly-ubiquitin and/or the Gly-X peptide bonds of the natural gene fusions or the single residue extension of the last repeat of poly-ubiquitin.

The enzyme that cleaves these peptide bonds would play a
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Critical role in the homeostatic control of ubiquitin and its conjugates in the cell. To test this hypothesis, we expressed a penta-ubiquitin gene in Escherichia coli and purified the gene product for use as a substrate for the enzyme(s) or the processes by which cells convert poly-ubiquitin to mono-ubiquitin. We report here the isolation of a functional penta-ubiquitin which supports the in vitro ATP-ubiquitin-dependent proteolysis in reticulocyte lysates and is a substrate for a processing enzyme. The data show, in addition, that E. coli can process the penta-ubiquitin gene product accurately, yielding biologically active mono-ubiquitin.

Materials and Methods

Restiction enzymes and a goat anti-rabbit antibody complexed with horseradish peroxidase were purchased from Bethesda Research Laboratories and Boehringer Mannheim (Indianapolis, IN). All other reagents and biochemicals were purchased from Sigma. Fast flow Mono Q resin for fast protein liquid chromatography was obtained from Pharmacia LKB Biotechnology Inc. Carrier-free Na\(^{22}P\)P was purchased from New England Nuclear.

Construction of Penta-ubiquitin Expression Vector

pNMhub is an expression vector that contains a single human ubiquitin gene regulated by a heat-inducible \(\beta\)-promoter (24). pNMhub contains two BglII restriction enzyme sites, one of which is at the second codon of the ubiquitin gene (Fig. 1). pUB-2 (Fig. 1) is a pBR322 derivative that contains a 2.4 kilobase pair insert which includes the yeast penta-ubiquitin gene (1). The specific sequence of the penta-ubiquitin gene terminating with a sequence encoding Asn and the plasmid containing this gene were kindly provided to us by Dr. A. Varshavsky (18). pUB-2 and pNMhub share the BglII site around the second codon of the ubiquitin gene. pUB-2 was digested with BglII and SmaI yielding an elution a 1.33 kilobase pair fragment containing the sequence for the penta-ubiquitin gene (Fig. 1). pNMhub was partially digested with BglII and then completely digested with HpaI to inactivate the single human ubiquitin gene. The appropriate BglII-HpaI digested vector was ligated to the BglII-HpaI digested pNMhub to generate a penta-ubiquitin E. coli expression vector pSJyub-5 (Fig. 1). As described for pNMhub, the penta-ubiquitin gene was regulated by a heat-inducible \(\beta\)-promoter (27). The construction and expression of the penta-ubiquitin gene expression vector were performed according to published procedures (28). The expression vector was transformed and propagated in E. coli AR58 cells (24).

Isolation of Mutant Gly-76-Ala, Human Mono- and Penta-ubiquitin

The synthesis and expression of a site-directed mutant in which the carboxyl-terminal Gly-76 was replaced with Ala were carried out according to published procedures (27). E. coli AR58 cells expressing the mutant Gly-76-Ala, human mono-ubiquitin, or poly-ubiquitin were grown in 15-liter cultures to obtain 100 g (wet weight) of cells. The cells were suspended in 50 mL of 0.05 M Tris, pH 8.0, 2 mM EDTA, 5% glycerol. The suspension was made 1 mM with dithiothreitol and after the addition of 160 mg of lysozyme was incubated at room temperature for 30 min. The suspension was sonicated in a Branson cell disruptor-200, for 10-15 min at maximum recommended bursts with constant stirring at 4 °C, and then centrifuged at 10,000 x g for 30 min. The supernatant was decanted, and the temperature of the solution was raised for 5 min with constant stirring after reaching 85-90 °C for the mutant Gly-76-Ala, or the human mono-ubiquitin expressing strain and 60-65 °C for the penta-ubiquitin expressing strain. The resulting solution was centrifuged at 10,000 x g for 30 min. The supernatant was precipitated with ammonium sulfate. Ammonium sulfate precipitates of the mutant Gly-76-Ala or of human mono-ubiquitin (45-80%) and penta-ubiquitin (0-85%) were dissolved and dialyzed against water before being subjected to fast flow Mono Q ion exchange chromatography (Pharmacia) on fast protein liquid chromatography. The column (2 x 45 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 8.6. The dialyzed ammonium sulfate solutions were made equivalent to the column buffer and loaded into the column in two parts. The column was washed with 5 column volumes of the buffer, and the adsorbed proteins were eluted by a two-step gradient, of 0-0.5 M KCl (shallow) followed by 0.3-1.0 M KCl (steep).

The shallow gradient eluted mutant Gly-76-Ala or human mono-ubiquitin at ~0.1 M KCl followed by fractions containing di-, tri-, tetra-, and penta-ubiquitin. (Fig. 2C) shows a Western transfer of the fractions, containing mono-, di-, tri-, tetra-, and penta-ubiquitin, that eluted immediately following the pure E. coli processed mono-ubiquitin. The steep gradient cleared the column of other proteins so that it could be reused. This step provides mutant Gly-76-Ala or human mono-ubiquitin of high purity. Fractions enriched in penta-ubiquitin were collected, dialyzed, and lyophilized before being loaded onto a column (1.5 x 100 cm) of Sephadex G-200. The fractions containing penta-ubiquitin were pooled, dialyzed, and lyophilized before rechromatography on the Mono Q ion exchanger.

When necessary, penta-ubiquitin from Mono-Q was purified further by preparative 18% PAGE.\(^1\) The yield of penta-ubiquitin was ~60% of the E. coli extracts. Concentrations of isolated E. coli processed or human mono- or penta-ubiquitin were estimated by measuring absorbance at 280 nm. Proteins were scanned from 240-290 nm to obtain a profile in this region; the peak of absorption maximum was 276 nm. The concentration of the protein of interest during the purification procedure was estimated after staining the polycrylamide gel with Coomassie Blue.

Preparation of Antibodies-1 and -2

Antibodies-1 and -2 were raised against two types of SDS-denatured ubiquitin. Antibody-1 recognized epitopes of ubiquitin including those surrounding the carboxyl terminal tail sequence. Antibody-2 recognized epitopes associated only with the tail sequence of ubiquitin. The preparation of these antigens was similar to the method published previously (30) modified as described below.

Preparation of Antigen-1—3.2 mg of pure human ubiquitin and 15 mg of bovine \(\gamma\)-globulin fraction was dissolved in 2.2 ml of water and cross-linked with 3% glutaraldehyde (80 \(\mu\)l); 20 \(\mu\)l were added every 10 min at room temperature and after the last addition the incubation continued for an additional 30 min.

Preparation of Antigen-2—3.2 mg of pure human ubiquitin without bovine \(\gamma\)-globulin was dissolved in 2.2 ml of water and cross-linked as described above. Antigens-1 and -2 were dialyzed overnight against 2 liters of PBS with two changes. The dialyzed antigens were made

\(^1\) The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
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2% in SDS and heated at 90 °C for 10 min before being diluted at least five times in PBS. They were stored at -20 °C in aliquots for injection into rabbits (New Zealand females 5-6 lb). Antigens (0.2 mg) were initially injected in complete Freund's adjuvant. The rabbits were boosted every 3-4 weeks with 0.12 mg of antigen in 1:10 complement Freud's adjuvant to maintain high titers of the antibodies. Blood was collected from the marginal ear vein 10 days after the previous boost with antigen-1 or -2. The crude antisera containing antibodies-1 or -2 were affinity-purified on ~10 ml ubiquitin-Sepharose columns (~3 mg of ubiquitin/ml of Sepharose). The serum was precipitated with 50% ammonium sulfate. The precipitate was dissolved and dialyzed against PBS. The solution was loaded onto the column, at a flow rate of 0.2 ml/min, and the procedure was repeated twice for better adsorption.

The column was washed with PBS (~5 column volumes or until the absorption at 280 nm was nearly 0) and then eluted by 100 mM glycine-HCl, pH 2.8. Fractions containing the peak of absorbance were collected as the affinity-purified antibody-1 or -2. The proteins were immediately neutralized with Tris base and stored in aliquots at -70 °C. Aliquots of the antibody were used once after being thawed.

Preparation of Rabbit Reticulocyte Lysate and Assay Conditions for in Vitro Proteolysis

The reticulocyte lysate fraction that supported the in vitro ATP/ubiquitin-dependent proteolysis of an exogenous substrate (45P-BSA) was purified according to a previously published procedure (17). Reticulocyte-enriched blood was lysed in a buffered hypotonic medium and loaded onto a DEAE-column. Proteins adsorbed onto the column were eluted with a gradient of KCl. Fractions collected contained the enzymes involved in the ATP/ubiquitin-dependent proteolytic pathway. The activities of these enzymes were followed by incubating tryptically labeled substrate (BSA used in this study) and measuring the ATP/ubiquitin-dependent trichloroacetic acid-soluble radioactivity released during the 2 h of incubation with ~200 μg of the reticulocyte lysate, at 37 °C.

Pyrophosphate Exchange Assay with Ubiquitin Activating Enzyme E1

Ubiquitin activating enzyme E1 was purified to apparent homogeneity by covalent affinity chromatography from the reticulocyte lysate as described previously (16). The incorporation of 32P into ATP was measured after the adsorption of the reaction mixture onto acid-washed activated charcoal as described previously (16). The assays were performed at 37 °C in 50-μl reaction volumes and were stopped by the addition of 0.5 ml of 5% (w/v) trichloroacetic acid followed by 0.3 ml of 10% (w/v) trichloroacetic acid and 0.2 ml of 10% (w/v) trichloroacetic acid. After four washes of the charcoal with 1 ml of 2% trichloroacetic acid containing 4 mM PPi, the radioactivity remaining bound was determined by Cerenkov counting.

RESULTS

Expression and Processing of Penta-ubiquitin in E. coli—To isolate the penta-ubiquitin gene product in large quantities, E. coli AR58 cells were transformed with expression vector pSJyub-5. The cells were grown at 30 °C and induced by raising the temperature to 40 °C for 0, 1, 2, and 3 h. Proteins induced in cells containing expression vectors having no inserts, single human ubiquitin genes, or penta-ubiquitin genes were analyzed by 18% PAGE (Fig. 2A). No proteins were induced in E. coli containing control plasmid. Ubiquitin was induced in cells containing the plasmid with a single ubiquitin gene (Fig. 2A) and a 42-kDa protein was induced in cells containing the vector pSJyub-5 (Fig. 2A). That the 42-kDa protein was the precursor penta-ubiquitin was confirmed by immunoblot analysis using antibody-1, which cross-reacts with penta-ubiquitin (Fig. 2B). Antibody-2, which reacts with the carboxyl-terminal region of ubiquitin, also cross-reacts with penta-ubiquitin (data not given). These results showed that penta-ubiquitin was expressed in moderate quantities. Antibody-1 also cross-reacted with proteins smaller than 42 kDa that were induced within 3 h and corresponded in size to tetra- and tri-ubiquitin (Fig. 2B), suggesting that antibody-1 may react better with progressively higher oligomers of ubiquitin. This observation also indicated that penta-ubiquitin may have been processed to smaller species in E. coli. To better demonstrate the existence of mono-, di-, tri-, and tetra-ubiquitin species from the penta-ubiquitin expressing strains of E. coli, the bacterial extract was partially purified (Fig. 2C). The pattern of fractions enriched in various ubiquitins (Western blot) obtained from fast flow Mono Q column chromatography are shown (Fig. 2C). Purified penta-ubiquitin from E. coli is shown in Fig. 2D following the purification procedure given under “Materials and Methods.” To confirm that penta-ubiquitin and E. coli-processed mono-ubiquitin were chemically equivalent and biologically active, a series of biophysical and biochemical studies were performed. The E. coli-processed mono-ubiquitin and E. coli-expressed penta-ubiquitin were purified according to the procedure described under “Materials and Methods.” Both the E. coli-processed mono- and human ubiquitin proteins had similar amino acid compositions (Table I). The table shows that there was no reduction of the levels of glycine when compared between the two ubiquitin molecules indicating that there was no loss of Gly-Gly due to tryptic cleavage. This observation suggests that there may be hydrolysis of a specific bond. The amino acid composition reflected the changes expected for the differences between human and yeast ubiquitin.

Immunodetection of Accurately Processed Mono-ubiquitin—The accurate processing of the penta-ubiquitin in E. coli was further substantiated with the antibodies which recognized the carboxyl terminus of ubiquitin. Antibody-1 recognized epitope(s) of the ubiquitin molecule including the carboxyl terminus portion, and antibody-2 recognized epitope(s) only on the carboxyl-terminal Arg-Gly-Gly portion of ubiquitin. E. coli-processed mono-ubiquitin, human ubiquitin, and the site-directed ubiquitin mutant Gly-76-Ala, in which the terminal Gly-76 was replaced by Ala (27), were analyzed by 18% PAGE (Fig. 3). The immunoblots of the proteins with antibody-1 or -2 indicated that antibody-1 reacted with all three ubiquitin proteins. Antibody-2 did not cross-react with the Gly-76-Ala mutant ubiquitin, confirming its specificity; however, it did cross react equally well with human and E. coli-processed mono-ubiquitin. Thus, the processing of penta-ubiquitin in E. coli was not random, and the epitopes associated with the tail residues of ubiquitin were present on the processed mono-ubiquitin.

Proton Nuclear Magnetic Resonance of the Amide Hydrogens of Ubiquitin—Evidence that the cleavage occurred at one particular bond during the processing of the penta-ubiquitin in E. Coli was obtained by isolating the E. coli-processed mono-ubiquitin from cells expressing penta-ubiquitin (see “Materials and Methods”). The processed mono-ubiquitin and mutant ubiquitin Gly-76-Ala were subjected to 1H NMR (500 MHz) (Fig. 4). The technique is sufficiently sensitive to detect small changes in the sequence or structure of the macromolecule (25, 26). The expanded region (Fig. 4) shows proton resonances of amide and aromatic residues. In the two-dimensional NMR spectrum, this region is magnified to facilitate the assignment of the amide hydrogen resonance peaks of the amino acids of ubiquitin. The resonance spectrum of E. coli-processed mono-ubiquitin was the same as that of human ubiquitin, but differed from that of the mutant Gly-76-Ala, in which the resonance peak corresponding to that of Gly-76 was altered to that corresponding to Ala-76.

These results indicate that E. coli-processed mono-ubiquitin appears to be identical with human ubiquitin. Even the
FIG. 2. Regulated expression and processing of mono- and penta-ubiquitin in *E. coli* and analysis of the proteins by Western transfer. *E. coli* AR58 cells containing either control plasmid with no insert (pNM-4), plasmid expressing mono-ubiquitin (pNHub), or plasmid expressing penta-ubiquitin (pSUUB-5) were grown at 30°C and induced at 42°C for 0, 1, 2, and 3 h. Aliquots of the cell suspension were analyzed by 18% PAGE as described under "Materials and Methods." A, Coomassie-stained gel representing the cell proteins from *E. coli* containing control plasmid with no insert, plasmid with mono-ubiquitin, and plasmid with penta-ubiquitin. B, a duplicate gel was transferred onto a nitrocellulose paper (BA83, Schleicher & Schuell, 0.2 μm). The blot was probed with anti-ubiquitin antibody-1 and stained according to the manufacturer's specifications with anti-goat antibodies complexed with horseradish peroxidase, described under "Materials and Methods." C, processing of the penta-ubiquitin, in *E. coli*, to bands corresponding to mobility to mono-, di-, tri-, and tetra-ubiquitin is seen in this Western transfer probed with ubiquitin antibody-1. The pattern of the *E. coli* extract, enriched in various ubiquitins, is shown after partial purification on a fast flow Mono Q column. D, 3 μg of pure penta-ubiquitin was subjected to 18% SDS-PAGE (protein). A similar gel was run, and the Western transfer probed with antibody-1 is shown (Western blot).
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TABLE I
Amino acid analysis of E. coli-processed mono-ubiquitin and human ubiquitin

Proteins were hydrolyzed according to standard procedure with 6 N HCl, in vacuum, and the results were not corrected for the loss of serine during hydrolyses. Minimum residue analyses were done compared to tyrosine.

| Amino acids                           | Human ubiquitin | Processed mono-ubiquitin |
|---------------------------------------|----------------|--------------------------|
| Asparagine or aspartic acid           | 18.0           | 18.9                     |
| Threonine                             | 7.3            | 7.8                      |
| Serine                                | 4.3            | 3.6                      |
| Glutamine or glutamic acid            | 13.0           | 13.8                     |
| Glycine                               | 7.4            | 7.3                      |
| Alanine                               | 2.6            | 2.9                      |
| Valine                                | 3.7            | 4.4                      |
| Isoleucine                            | 7.1            | 7.9                      |
| Leucine                               | 10.2           | 10.4                     |
| Tyrosine                              | 1.0            | 1.0                      |
| Phenylalanine                         | 2.0            | 2.3                      |
| Histidine                             | 1.0            | 1.0                      |
| Lysine                                | 7.6            | 8.0                      |
| Arginine                              | 6.5            | 6.0                      |
| Proline                               | 2.1            | 3.2                      |

secondary structure of the mutant Gly-76-Ala ubiquitin was similar to that of human ubiquitin; however, the purified protein was inactive in assays involving the ubiquitin activating enzyme and in the in vitro ATP/ubiquitin-dependent proteolysis of the reticulocyte lysate (27).

In Vitro Proteolytic Activities with Human Ubiquitin, E. coli Processed Mono-ubiquitin, and Penta-ubiquitin—To demonstrate that the E. coli-processed mono-ubiquitin and penta-ubiquitin were biologically active, we tested their function in the in vitro ubiquitin-mediated, ATP-dependent proteolytic assay. A crude preparation of enzymes which support ATP/ubiquitin-dependent conjugation and simultaneous degradation of exogenous substrates was purified according to the procedure described under "Materials and Methods." 125I-BSA was added as the exogenous substrate, and its degradation in the presence of human ubiquitin, penta-ubiquitin, and E. coli-processed mono-ubiquitin was monitored (Fig. 5). The three ubiquitin molecules were quantitatively similar in supporting 125I-BSA degradation (Fig. 5). These results suggest that the reticulocyte lysates contained an active processing enzyme which was able to cleave the terminal Asp and/or Gly-Met peptide bond of the penta-ubiquitin to produce a biologically active ubiquitin. The observation that 125I-penta-ubiquitin was hydrolyzed by the reticulocyte lysate to a protein that migrates on 18% PAGE at a position equivalent to human ubiquitin (data not shown) indicates that the processing enzyme(s) in the reticulocyte lysate may have hydrolyzed the penta-ubiquitin to mono-ubiquitin. However, it is unclear whether the terminal Asn was removed before the release of...
to test whether the unprocessed penta-ubiquitin, which is processing. Moreover, the reaction also offers an opportunity capped with Asn, can enter the ubiquitin pathway. In the pyrophosphate exchange assay using El, human ubiquitin and

The ubiquitin activating enzyme El activates the carboxyl-terminal Gly-76, as demonstrated by site-directed mutagenesis studies (27). A conservative substitution of Gly-76 with an Ala (27), or loss of the terminal Gly-Gly-76 residues (23) renders the molecule inactive in this reaction. Thus, the El reaction is an excellent measure of the accuracy of ubiquitin processing. Moreover, the reaction also offers an opportunity to test whether the unprocessed penta-ubiquitin, which is capped with Asn, can enter the ubiquitin pathway. In the pyrophosphate exchange assay using El, human ubiquitin and E. coli-processed mono-ubiquitin were equally active (Fig. 6), mono-ubiquitin from the substrate or whether processed penta-ubiquitin was itself conjugated to the protein before being processed to mono-ubiquitin. These results, however, do confirm that the E. coli-processed mono-ubiquitin was biologically active and was capable of being recognized by the enzymes in the ATP/ubiquitin proteolytic pathway.

Recognition of Human Ubiquitin, E. coli-processed Mono-ubiquitin, and Penta-ubiquitin by Ubiquitin-activating Enzyme—The ubiquitin activating enzyme El activates the carboxyl-terminal Gly of ubiquitin in the presence of ATP (16). This enzyme has a strict requirement for an intact carboxyl-terminal Gly-76, as demonstrated by site-directed mutagenic studies (27). A conservative substitution of Gly-76 with an Ala (27), or loss of the terminal Gly-Gly-76 residues (23) renders the molecule inactive in this reaction. Thus, the El reaction is an excellent measure of the accuracy of ubiquitin processing. Moreover, the reaction also offers an opportunity to test whether the unprocessed penta-ubiquitin, which is capped with Asn, can enter the ubiquitin pathway. In the pyrophosphate exchange assay using El, human ubiquitin and E. coli-processed mono-ubiquitin were equally active (Fig. 6), clearly demonstrating that the processing observed in E. coli was complete and accurate. In contrast, unprocessed penta-ubiquitin did not promote pyrophosphate exchange in the presence of ATP and Ei. This result suggests that penta-ubiquitin capped with Asn was not a substrate for the enzyme and that there was no processing activity associated with the isolated ubiquitin activating enzyme Ei. Incubation of penta-ubiquitin (0.2 mg/ml) with lysozyme (15-275 µg/ml) for 1 h did not result in any proteolytic cleavage of the penta-ubiquitin. However, although similar incubation of penta-ubiquitin with trypsin (10-20 µg/ml) resulted in the appearance of molecules corresponding to their mobilities on SDS-PAGE to mono-, di-, tri-, and tetra-ubiquitin in addition to others, pyrophosphate exchange assays using the tryptic digest with purified El showed no activity, indicating that trypsin had inaccurately processed penta-ubiquitin (data not shown).

DISCUSSION

In the past, the lack of an appropriate substrate has hindered the search for processing enzyme(s). The expression and purification of a eukaryotic penta-ubiquitin which is capable of being cleaved by a processing enzyme from reticulocyte lysate isolated from E. coli should greatly enhance the understanding of the ubiquitin processing enzyme and its role in regulating the ATP/ubiquitin pathway (9, 17). The results reported in this paper demonstrate that the expression vector pSJ-yub-5, propagated in E. coli cells, produced a 42-kDa protein, shown to be penta-ubiquitin by immunoactivity with antibody-1. The vector was expressed in E. coli cells because they contain no ubiquitin or ubiquitin-dependent proteolysis systems as observed in eukaryotes. The properties of penta-ubiquitin were similar to those of mono-ubiquitin with respect to resistance to heat denaturation or behavior during purification (see "Materials and Methods"). Isolated penta-ubiquitin (Fig. 2D) was shown to support ATP/ubiquitin-dependent proteolysis of 125I-BSA (Fig. 5). However, when the purified gene product was incubated with purified ubiquitin-activating enzyme El, the molecule was incapable of supporting pyrophosphate exchange (Fig. 6), suggesting that the terminal asparagine may have to be removed before the molecule is recognized by El.

An interesting finding of this study is that E. coli can process penta-ubiquitin accurately to a biologically active mono-ubiquitin. The conformation of ubiquitin in solution can be monitored by the chemical shifts of the NMR spectral peaks, which reflect the changes in the environment of particular residues (25, 26). In a constant magnetic field strength, the resonance frequency of a particular nucleus depends on its chemical environment. Every resonance line in one-dimensional NMR spectrum of the ubiquitin molecule has previously been assigned to its respective nucleus, so we were able to monitor the environment of any residue of interest (29). The spectrum of the E. coli-processed mono-ubiquitin was identical with that of human ubiquitin, including the carboxyl-terminal residues Leu-Arg-Gly-Gly (Fig. 4). The sensitivity of this technique was confirmed by examination of the mutant protein Gly-76-Ala.

The environments of all the residues were the same except that of residue-76. The spectral lines of Gly-76 were replaced by a set of new resonances that now represented Ala-76. The environments of all the residues were the same except that of residue-76. The spectral lines of Gly-76 were replaced by a set of new resonances that now represented Ala-76. The two-dimensional NMR spectra (Fig. 4) which resolved overlapping resonance spectra, thus enhancing sensitivity, provides information about the internuclear distances and data on the coupling constants which were too complex to be interpreted from a one-dimensional NMR spectrum (29).

Site-directed mutagenetic studies from this laboratory
showed that mutations in the tail region of ubiquitin completely abolished the ability of the molecule to participate in the proteolytic pathway (27). Even the most conservative substitution of the terminal Gly-76 with an Ala inactivated the mutant protein. These studies, along with the observations that brief exposure of ubiquitin to trypsin, which cleaved the carboxy-terminal Gly-Gly (23), show that inactivity of the mutant proteins is due to their inability to be activated by the ubiquitin activating enzyme E1. Our studies show that only E. coli-processed mono-ubiquitin was used as a substrate in the pyrophosphate exchange reaction with the ubiquitin activating enzyme E1. (Fig. 6). There are three potential explanations for the processing of the penta-ubiquitin product in E. coli. (a) The penta-ubiquitin may be so specifically folded in solution such that the Gly-Met peptide bonds between the pairs of monomers are strained and are cleaved nonspecifically by proteases. The conformational flexibility of the carboxyl-terminal tail of the ubiquitin molecule, determined both the x-ray crystallographic and NMR studies, permits at least 4 carboxyl-terminal residues, Leu-Arg-Gly-Gly, to be exposed in solution, while the rest of the molecule remains in a compact structure. Assuming a "beads-on-a-string" structure for the penta-ubiquitin gene product expressed by pSJyb-5 in E. coli, a few bonds are exposed and could be cleaved to give the smaller proteins corresponding in their mobilities in SDS-PAGE to tetra-, tri-, di-, and mono-ubiquitin (Fig. 2). Incubation of penta-ubiquitin with trypsin resulted in the appearance of molecules corresponding in their mobilities on SDS-PAGE to mono-, di-, tri-, and tetra-ubiquitin in addition to others. Pyrophosphate exchange assays using the tryptic digests with purified E1 showed no activity, indicating that trypsin had inaccurately processed penta-ubiquitin.

Thus, these results indicate that the processing observed in E. coli is due to specific and accurate cleavage of penta-ubiquitin to a biologically active ubiquitin and this cannot be reproduced by treatment with other proteases. (b) The gene product is autocatalytic and can cleave itself at the appropriate Gly-Met or Gly-Asn peptide bonds of the penta-ubiquitin. Consistent with this mechanism is a recent report demonstrating that ubiquitin, isolated without the heat step, has an intrinsic proteolytic activity (28). Ubiquitin was shown to cleave E. coli β-galactosidase and apomoglobin with an optimum at pH 8, and the activity was stimulated by calcium ions. Proteolysis was inhibited by monoclonal antibodies against ubiquitin and high concentrations of phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (28). In experiments performed with the penta-ubiquitin isolated by our procedure and incubated with 50 mM Tris, pH 7.4, for 1 h at 37 °C with or without an ATP regenerating system, we observed no autodegradation of the penta-ubiquitin (data not shown). (c) There is a processing enzyme in E. coli which may represent a primitive ubiquitin-like system in prokaryotes. If such a processing enzyme is present in E. coli, it must be inefficient because penta-ubiquitin was isolated from E. coli in reasonable quantities.

The conservation of the poly-ubiquitin genes in eukaryotes suggests that poly-ubiquitin is part of a system of defense against stress to which organisms are exposed as shown in yeast (9). The transcription of the poly-ubiquitin gene during the stress-response may efficiently provide the cell with amounts of ubiquitin required to conjugate the increased abnormal functional/structural proteins. Because a variety of proteins are known to be conjugated to ubiquitin, it is possible that amplification of ubiquitin is essential for the cell to modulate various housekeeping functions required to survive the conditions of stress. The study described here provides a method for isolating penta-ubiquitin in large quantities necessary to study these aspects of cellular regulation.

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