A Major Ubiquitin Conjugation System in Wheat Germ Extracts Involves a 15-kDa Ubiquitin-conjugating Enzyme (E2) Homologous to the Yeast UBC4/UBC5 Gene Products*

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In eukaryotes, conjugation of ubiquitin to proteins serves as a committed step for intracellular protein degradation. Formation of ubiquitin-protein conjugates involves the transfer of ubiquitin-conjugating enzyme (E2)-bound ubiquitin to the target proteins with or without the assistance of ubiquitin-protein ligase (E3). We report the isolation and characterization of an E2 purified from wheat germ that accounts for the majority of ubiquitin conjugation activity observed in vitro. This E2 is basic, has an apparent molecular mass of 15 kDa, and forms oligomers that dissociate upon treatment with sulfhydryl reducing agents. E215kDa will not work alone in vitro but requires an additional factor putatively identified as an E3 for substrate recognition. This E3 is distinct from E3α previously described to be required for N-terminal recognition of target proteins. Partial amino acid sequence analysis of E215kDa revealed a substantial identity (~80% in two peptide regions) with yeast E2s encoded by UBC4/UBC5 genes. This homology was confirmed by immunodetection of a 16-kDa yeast protein corresponding to the molecular mass of the UBC4/UBC5 proteins with E215kDa antisera. The products of yeast UBC4 and UBC5 genes along with that of UBC1 gene constitute a subfamily of functionally overlapping E2s that mediate the selective degradation of short-lived and abnormal proteins in vivo. Considering the high degree of functional and structural similarity of wheat E215kDa with that of yeast UBC4/UBC5, it is likely that yeast UBC4/UBC5 and their homologs from other eukaryotes exhibit the same E3 dependence in performing their roles in protein degradation.

Selective degradation of proteins is essential for controlling the levels of key enzymes and regulatory proteins as well as preventing the accumulation of abnormal proteins in cells. In eukaryotes, the ubiquitin-dependent proteolytic system plays an important role in this process (1–3). Mutational analysis has revealed that most abnormal and short-lived proteins are degraded by this mechanism (2, 4, 5). Examples of important cell regulatory proteins degraded, at least in part, by the ubiquitin pathway include phytochrome after photoconversion to the far red-light absorbing form (6), tumor suppressor p53 (7), cyclins (8), and MAF2 repressor (9). In this pathway, ubiquitin functions by becoming covalently attached to proteins, committing them for breakdown. Attachment is through an isopeptide linkage between the C-terminal glycine of ubiquitin and the ε-amino group of lysine residues within the target protein. Ubiquitin also becomes conjugated to itself forming multi-ubiquitin chains attached internally through ubiquitin residue Lys6 (10). The multi-ubiquitinated conjugates are then recognized by an ATP-requiring protease complex, which degrades the target proteins and releases ubiquitin in a free, functional form (10–12).

Ubiquitin ligation is accomplished by an ATP-dependent multi-step pathway consisting of ubiquitin-activating enzyme (E1) and members of the ubiquitin-conjugating enzyme (E2) family (1–3). Some E2s function alone, at least in vitro, in ligation ubiquitin, whereas others require the presence of a ubiquitin-protein ligase (E3) for protein substrate recognition. Multiple species of E2s have been isolated from yeast (2, 13), mammals (14, 15), and plants (16–19); some have been grouped into subfamilies of enzymes with related functions but potentially different specificities (2). The best-characterized subfamily includes yeast UBC1, UBC4, and UBC5, which are required for most of the ubiquitin-dependent degradation of abnormal and short-lived proteins in this organism (4, 5). Disruption of all three genes is lethal, demonstrating that these E2s serve an essential function in yeast. A gene from Drosophila encoding an E2 that is structurally and functionally equivalent to UBC4/UBC5, has recently been reported, suggesting that this E2 class is present in all eukaryotes (20).

Among the E2s characterized thus far, only the E2 encoded by RAD6 from yeast and its homolog from rabbit reticulocytes have been shown to be involved in E3-mediated ubiquitination and degradation of proteins in vitro and in vivo (21–23). These E2s work in concert with a specific E3, designated Elα (also known as E3 type I or UBR1 in yeast), in recognizing protein substrates bearing basic or bulky hydrophobic N-terminal residues (23–26). In contrast, yeast UBC1 does not function with Elα (2) and UBC4/UBC5 have been shown to be involved in degradation of protein substrates even in an ubr1 null mutant (27). Another E3, designated Elβ, also functions in N-terminal recognition, but its interaction with

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The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; PAGE, polyacrylamide gel electrophoresis; DTE, dithioerythritol; PMSF, phenylmethylsulfonyl fluoride; IgG, immunoglobulin G; HPLC, high performance liquid chromatography; PVDF, polyvinylidene difluoride.
specific E2s is currently unclear (28).

In the present study, we report the isolation of a 15-kDa E2 from wheat that accounts for most of the conjugating activity in wheat germ extracts. It depends on an activity we propose is an E3 and shows a high degree of sequence identity to the yeast UBC4 and UBC5 gene products (4, 5). Like the yeast enzymes, E2s, and E2 does not mediate E3-dependent ubiquitination of proteins in vitro, but functions with distinct E3s. This strengthens the hypothesis that individual E3s interact specifically with different E2s in recognizing various types of protein substrates.

**EXPERIMENTAL PROCEDURES**

Materials—Non-toasted wheat (Triticum aestivum) germ was purchased from General Mills (Minneapolis, MN). Purified human ubiquitin and purified human IgG (Calbiochem) were radiolabeled with Na<sup>35</sup>S as described (29). E1 was isolated from wheat germ extracts by covalent affinity chromatography with ubiquitin-Sepharose using the method described in Ref. 30 with the following modifications. E1 was eluted from the ubiquitin column with 2 mM AMP + 2 mM pyrophosphate (15). Arabidopsis thaliana E2<sub>15</sub> (E2<sub>15</sub>) (AMU: Ref. 19), and wheat E2<sub>115</sub> (E2<sub>115</sub>) (Table I) of Ref. 18) were partially purified by DEAE-cellulose chromatography from Escherichia coli cells harboring the respective cDNA cloned in pET3a expression vector. E3s preparations were obtained from rabbit reticulocytes as described (24) and were a generous gift from Dr. Avram Hershko.

**Preparation of E2**—Wheat germ (200 g) was ground in mortar and pestle at liquid N<sub>2</sub> temperatures and homogenized in 1 liter of 50 mM HEPES-KOH (pH 6.8) containing 2 mM DTE (Buffer A). The homogenate was centrifuged at 20,000 × g for 20 min at 4 °C, and the resulting supernatant was filtered through Whatman No. 3MM paper and centrifuged at 20,000 × g for another 20 min. The clarified supernatant was applied to a 50 ml S-Sepharose (Pharmacia) column equilibrated in Buffer A + 1 mM NaCl. The flow-through was concentrated to 8 ml by ultrafiltration using an Amicon cell and subjected to gel filtration chromatography using a 100 × 2-cm Sephadex G-75 (Pharmacia) column equilibrated in Buffer A + 1 mM NaCl and operated at a flow rate of 50 ml/h. The active fractions (as judged by their ability to restore ubiquitin-conjugating activity to the flow-through material from the S-Sepharose column) were pooled, dialyzed against Buffer A, and applied to a 10-ml S-Sepharose column. Proteins were eluted with a linear 0–75 mM NaCl gradient. Active fractions were pooled and dialyzed against Buffer A and proteins were eluted with 50 mM NaCl. The S-Sepharose eluate was made 1 M NaCl and passed through a 10-ml phenyl-Sepharose (Pharmacia) column equilibrated in Buffer A + 1 M NaCl. The flow-through was concentrated to 8 ml by ultrafiltration using an Amicon cell and subjected to gel filtration chromatography on a Spherogel TSK SEC400 size exclusion column (Bio-Rad) in Buffer A + 150 mM NaCl using a flow rate of 1 ml/min. The peak of activity was concentrated by ultrafiltration using a Centricon-10 microconcentrator (Amicon) and the buffer exchanged for Buffer B.

**Preparative SDS-PAGE**—E2<sub>15</sub> (500 μg of purified E2<sub>15</sub>) was separated by SDS-PAGE on a 16% polyacrylamide gel pre-electro-phoresed with 0.1% SDS in 0.1 M Tris-C<sub>1</sub> (pH 8.5) containing 10 mM DTE. The eluate was collected and analyzed using a Vertical PhastSystem (Pharmacia LKB Biotechnology Inc.) column equilibrated in Buffer A. Proteins were eluted with a 30-ml linear 0–750 mM NaCl gradient in Buffer A. Two ml fractions were collected and concentrated by ultrafiltration (Centricon) to a volume of 100 μl and the buffer exchanged for Buffer A. Two ml fractions were collected and concentrated by ultrafiltration (Centricon) to a volume of 100 μl and the buffer exchanged for Buffer A. The protein concentration was determined by Bradford assay. Two ml of each fraction was used in conjugation assays.

**Assay for Ubiquitin Thiol Ester and Conjugate Formation**—Formation of thiol ester adducts between <sup>125</sup>I-ubiquitin and E1 (0.2 μg) and the various E2s was carried out as described (30). The reactions were quenched by adding SDS-PAGE sample buffer with or without 2-mercaptoethanol and boiling the reaction mixtures for 3 min (30). Products of the reaction were separated by SDS-PAGE and visualized by autoradiography.

**Conjugation of <sup>125</sup>I-ubiquitin to proteins** was assayed in a 20-μl total reaction volume containing 0.35 μg of <sup>125</sup>I-ubiquitin, 10 mM creatine phosphate, 2 mM MgCl<sub>2</sub>, 1 mM ATP, 2 mM DTE, and 0.1 unit of phosphocreatine kinase in 50 mM HEPES-KOH (pH 6.8). For <sup>125</sup>I-labeled conjugation, reaction mixtures contained 5 μg of unlabeled ubiquitin (bovine) and 0.5 μg of <sup>125</sup>I-labeled enzyme. The source of ubiquitin-conjugating enzymes used in the different assays was as follows. (i) For the crude extract, 4 μl of clarified crude extract was used. (ii) The reconstituted system with E2<sub>115</sub> contained 4 μl of flow-through material and 1 μg of crude E2<sub>115</sub> IgG. (iii) The reconstituted system with E3 contained 0.2 μg of purified E1, 0.2–0.5 μg of the respective purified E2s, and partially purified E3 used at a volume equivalent to 190 μl of crude extract (see "Preparation of E3"). (iv) The reconstituted system with E3 contained 0.2 μg of purified E1, 0.2–0.5 μg of the respective purified E2s, and partially purified E3 used at a concentration of 1.6 micromoles (as defined in Ref. 28) per assay. Equivalent amounts of various E2s used in the assay were determined by using...
thiol ester activity. Reaction mixtures were incubated at 30 °C for 90 min, terminated by boiling in SDS-PAGE sample buffer containing 2-mercaptoethanol, and subjected to SDS-PAGE (34). The gels were stained with Coomassie Blue and dried between cellophane. Radioactive protein bands were visualized by autoradiography. For quantitative evaluation of conjugate formation using either 12SI-ubiquitin or 12SI-lysozyme, regions of the gels containing high molecular mass conjugates (>45 kDa) were excised and the radioactivity determined by scintillation spectroscopy.

RESULTS

Crude wheat germ extracts provide an active system for the ATP-dependent conjugation of ubiquitin to endogenous and purified substrates (30). However, upon fractionation of such crude extracts by cation-exchange chromatography using S-Sepharose, the conjugation activity of the unadsorbed material was severely impaired (Fig. 1), even though it contained E1 and a multitude of E2s (Ref. 16 and data not shown). Conjugation of ubiquitin to endogenous protein and to 12SI-lysozyme was reduced by 64 and 80%, respectively, indicating that a basic component essential for the in vitro ligation system (E2 or E3) was removed during this separation step (Fig. 3B).

We attempted to purify this component based on its ability to restore ubiquitin conjugation activity when added back to the flow-through material from crude extracts after passage through the S-Sepharose column. A combination of cation exchange and size exclusion chromatography, and covalent affinity purification using immobilized ubiquitin, resulted in the isolation of an active fraction containing a 15-kDa protein (Fig. 2A). Conditions for binding (requirement of E1 and ATP) and elution (DTE) from the ubiquitin affinity column suggested that this protein was an E2. However, protein recovery from the affinity column was uncharacteristically low for an E2, even if the unadsorbed material was again passed through the ubiquitin column, suggesting that binding to ubiquitin was weak and/or inefficient. Residual protein contaminants were removed from the ubiquitin affinity eluate by size exclusion chromatography. During this step, the 15-kDa protein eluted as a single species with a native molecular mass of ~28–30 kDa, suggesting that it was a homodimer. This subunit association required low ionic strength buffers (±150 mM NaCl) and was easily dissociated at higher salt concentrations. During SDS-PAGE under reducing conditions, the protein migrated as a 15-kDa monomer. But, during SDS-PAGE under nonreducing conditions (absence of 2-

mercaptoethanol in the SDS-PAGE sample buffer), oligomers of the protein up to pentamers were observed (Fig. 2C). This result suggested that denaturation in the absence of the reducing agent induces the formation of intermolecular disulfide bonds not present in the native enzyme.

The 15-kDa wheat protein was confirmed to be an E2 by its ability to form thiol-ester adducts with ubiquitin in an E1- and ATP-dependent reaction. Several adducts ranging in size from 27 to 34 kDa were observed following SDS-PAGE under nonreducing conditions (Fig. 2B). Under reducing conditions, the adducts were absent demonstrating that attachment of ubiquitin was via a thiol ester bond (data not shown). The multiple thiol ester adducts observed here for E215kDa were similar to those observed for several other E2s (16). Sullivan and Vierstra (17) showed that for wheat TuUBC1, these species probably result from artifactual migration of a single adduct in the nonreducing gel system used and not from the simultaneous attachment of multiple ubiquitin moieties.

When added back to the flow-through material of the S-Sepharose column, purified E215kDa restored its capacity to conjugate ubiquitin to endogenous protein substrates and added lysozyme. The size distribution of conjugates was nearly indistinguishable from that of the crude extract. However, the amount of conjugates formed exceeded that of the original crude extract (Fig. 3, A and B). With respect to endogenous substrates, a 2-fold stimulation was obtained, saturating at relatively low levels of added E215kDa. With respect to 12SI-lysozyme, a ~4-fold stimulation was obtained and was only saturable at very high levels of E215kDa (Fig. 3B and data not shown). No variation in the relative concentration of specific conjugates could be observed as E215kDa levels increased.

This result indicated that E215kDa was essential for most of the ubiquitin conjugation in wheat germ extracts. Additional confirmation was provided by using E215kDa antibodies to deplete the protein from crude extracts. Presence of E215kDa in the immunoadsorbed fraction was confirmed by E1-dependent autoconjugation of ubiquitin to the 15-kDa E2.

**Fig. 2. Purification and enzymatic analysis of E215kDa from wheat germ.** Panel A, SDS-PAGE of various fractions during the purification of E215kDa. Lane 1, crude extract; lane 2, proteins eluted from the preparative S-Sepharose column with 50 mM NaCl; lane 3, active fraction eluted from the second S-Sepharose column with a NaCl gradient; lane 4, DTE, pH 8.5, eluate from the ubiquitin affinity column; lane 5, active fraction collected from the size exclusion Spheroel TSK SEC400 column. Samples were subjected to SDS-PAGE on a 12% acrylamide gel and visualized by Coomassie Blue staining. Panel B, formation of thiol ester adducts between E215kDa and ubiquitin. 12SI-Ubiquitin, ATP, and 0.2 µg of E1 (lane 1) were incubated with 0.2 µg of the purified E215kDa (lane 2) or 0.5 µg of the purified A. thaliana UBQ4 gene product (lane 3) as described under “Experimental Procedures” and then subjected to SDS-PAGE in the absence of 2-mercaptoethanol. The positions of ubiquitin (UBQ) and E2-thiol ester (E2-UBQ) adducts are indicated. Panel C, detection of E215kDa oligomers with E215kDa antisera. One µg of E215kDa was denatured in the presence (lane 1) or absence (lane 2) of 2-mercaptoethanol and then subjected to SDS-PAGE, blotted onto PVDF membrane, and visualized using E215kDa antisera.
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Fig. 3. Ubiquitin conjugation in vitro is dependent on E215kDa. ATP-dependent conjugation of ubiquitin to [35S]lysozyme (LYSO) and [3H]ubiquitin (UBQ) to endogenous proteins were performed for 90 min at 30 °C. Conjugates were resolved by SDS-PAGE and detected by autoradiography. Panel A, reconstitution of ubiquitin conjugation activity by adding increasing amount of purified E215kDa to the flow-through material (FT) of crude extract (C) after passage through an S-Sepharose column. The amount of purified E215kDa added is indicated. Panel B, quantification of the incorporation of [35S]lysozyme (solid bars) and [3H]ubiquitin (shaded bars) into conjugates observed in panel A. Incorporation was quantified by scintillation spectroscopy of gel strips containing the various ubiquitin conjugates. Panel C, restoration of ubiquitin conjugation to [253]lysozyme in a crude extract depleted of E215kDa via immunoaffinity chromatography by adding back purified E215kDa. C, crude extract; UN, unadsorbed material from E215kDa immunoaffinity column. The quantities of purified E215kDa added are indicated.

Fig. 4. Homology of E215kDa with yeast E2s encoded by UBC1, UBC4, and UBC5 genes. Upper panel, comparison of amino acid sequences of two E215kDa peptides with the corresponding regions in the yeast UBC1, UBC4, and UBC5 gene products. Amino acids in E215kDa that are identical or chemically similar (D=E; R=K; S=T; I=L+V=M) to UBC1, UBC4, and UBC5 are boxed. Lower panel, cross-reaction of E215kDa antisera with yeast proteins. Lane 1, purified E215kDa; lane 2, yeast crude extract. Samples were subjected to SDS-PAGE, blotted onto PVDF membrane, and probed with a polyclonal antiserum raised against E215kDa.

munodepleted crude extracts lost the ability to ubiquitinate both endogenous and purified proteins with only a small fraction of the activity remaining in the unadsorbed material (Fig. 3C and data not shown). This activity could be restored by subsequent addition of purified E215kDa to the unadsorbed material.

In an attempt to identify the relationship of E215kDa with other known E2s, a partial amino acid sequence of the protein was determined. This was achieved for two peptides generated from E215kDa by S. aureus V8 protease digestion. Comparison of these sequences with those of all reported E2s revealed little homology with other wheat and Arabidopsis E2s sequenced to date (17–19), but a high homology with the yeast UBC4 and UBC5 gene products (Fig. 4, upper panel; Refs. 4 and 5). For the region encompassing the two peptides, the amino acid sequence of E215kDa was ~80% identical. A lesser

but significant homology was also observed with the yeast E2 encoded by UBC1 (~45% identity). The second peptide aligned with the C terminus of yeast UBC4 and 5, but had an additional glycine as its C-terminal residue. That wheat E215kDa is a homolog of yeast UBC4 and UBC5 E2s was further supported by immunoblotting yeast crude extracts with E215kDa antisera. The antisera cross-reacted with a 16–17-kDa protein corresponding to the molecular mass of UBC4 and UBC5 (Fig. 4, lower panel).

UBC4 and UBC5 are essential E2s for the ubiquitin-depent degradation of abnormal and short-lived proteins in yeast (4, 5). Although an E3 has also been proposed to be necessary for the breakdown of such proteolytic substrates (2), the cooperation of UBC4 and UBC5 with an E3 has not yet been demonstrated. With respect to E215kDa, we observed that whereas the crude extract reconstituted with E215kDa did conjugate lysozyme, purified E215kDa and E1 alone did not recognize lysozyme as a substrate, nor did it catalyze the

Fig. 5. Fractionation of the ubiquitin conjugation system requiring E215kDa. Panel A, failure of lysozyme conjugates and poly-ubiquitin chains to be formed by E1 and E215kDa alone. Conjugation reaction mixtures contained ATP, E1 (0.2 μg), E215kDa (1 μg), and [35S]lysozyme (LYSO) and native ubiquitin or [3H]ubiquitin (UBQ) and were incubated for the indicated time periods. Panel B, identification of a factor required for E215kDa-dependent conjugation. Crude wheat germ extract was fractionated by Q-Sepharose chromatography, and the bound proteins were eluted with a 0–750 mM linear NaCl gradient. The collected fractions were assayed for E1- and E215kDa-dependent conjugation of ubiquitin to [35S]lysozyme (LYSO) and [3H]ubiquitin (UBQ) to endogenous proteins. Conjugation activity in presence of E1 alone was subtracted from all results. Conjugates were separated by SDS-PAGE, the appropriate bands excised, and their radioactivity determined by scintillation spectroscopy. Panel C, reconstitution of conjugation activity with partially purified E3-like fraction S, Q1, and Q2 in presence of E1 and E215kDa. Conjugation was performed as in panels A and B.

Fig. 6. E3α-dependent ubiquitination of protein substrates by E215kDa and A. thaliana UBC1, respectively. E3, E1, and the indicated E2s were incubated with native ubiquitin and [35S]lysozyme (left five lanes) or [3H]ubiquitin alone (right five lanes). The conjugation assay contained ATP, 0.2 μg E1, 1.6 micromolars of E3α, and 0.5 μg of AUCBC1 or 0.2 μg of E215kDa. Conjugation was performed for 90 min at 30 °C, and the ubiquitin conjugates were resolved by SDS-PAGE and detected by autoradiography. LYSO, [35S]lysozyme; UBQ, [3H]ubiquitin. but significant homology was also observed with the yeast E2 encoded by UBC1 (~45% identity). The second peptide aligned with the C terminus of yeast UBC4 and 5, but had an additional glycine as its C-terminal residue. That wheat E215kDa is a homolog of yeast UBC4 and UBC5 E2s was further supported by immunoblotting yeast crude extracts with E215kDa antisera. The antisera cross-reacted with a 16–17-kDa protein corresponding to the molecular mass of UBC4 and UBC5 (Fig. 4, lower panel).

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formation of poly-ubiquitin chains (Fig. 5A). Only mono-ubiquitin conjugates of E1 and of E2$_{15\, kDa}$ were observed. This indicated that another factor was required for lysozyme recognition and ubiquitination, in addition to E1 and E2$_{15\, kDa}$.

To identify the additional component(s) of the E2$_{15\, kDa}$ system required for lysozyme conjugation, the crude extract was fractionated on S-Sepharose and Q-Sepharose columns. The recovered protein fractions were assayed for their ability to form ubiquitin conjugates with proteins endogenous to the fraction or with 125I-lysozyme in an E1- and E2$_{15\, kDa}$-dependent manner. S-Sepharose chromatography resolved one peak of activity (designated S) capable of conjugating both endogenous proteins and 125I-lysozyme (data not shown and Fig. 5C). Q-Sepharose chromatography identified two fractions competent to form conjugates with endogenous proteins and two fractions competent to form conjugates with 125I-lysozyme (designated Q1 and Q2). The second Q-Sepharose fraction of each profile (Q2) was coincident and contained the highest E2$_{15\, kDa}$-dependent specific activity (Fig. 5, B and C). The pattern of lysozyme conjugates formed by E1 and E2$_{15\, kDa}$ in cooperation with the fractions S, Q1, and Q2 resembled that of the crude extract except that the proportion of intermediate molecular mass products was increased relative to high molecular mass forms (Fig. 5C). Only the Q2 fraction effectively catalyzed the formation of high molecular mass conjugates of endogenous proteins in the presence of E2$_{15\, kDa}$ (Fig. 5C). This was particularly striking given that this fraction also had the lowest level of protein contaminants as potential substrates. Attempts to further purify the active factor in Q2 by size exclusion chromatography showed that it had a molecular mass in the 100–150-kDa range.

Based on the criteria used to define E3, i.e. a factor that participates with E1 and E2 in stimulating ubiquitin conjugation (3), it is possible that S, Q1, and Q2 contained an E3. The putative E3s could specifically interact with just E2$_{15\, kDa}$ or with additional members of the E2 family. To test the latter supposition, conjugation in the presence of S, Q1, and Q2 fractions was examined with various wheat and A. thaliana E3s including E2$_{16\, kDa}$ (AtUBC1; Ref. 17), E2$_{23\, kDa}$ (AtUBC4; Ref. 19), and E2$_{20\, kDa}$ (TaUBC7; Ref. 18); all were produced by expressing the corresponding cDNAs in E. coli. Equivalent amounts of E2 activity were used as determined by thiolester activity. In reactions containing lysozyme, all three fractions exhibited a strong preference for E2$_{15\, kDa}$; conjugation was ~10-fold higher than with any other E2 examined (data not shown).

The possibility that E2$_{15\, kDa}$ interacts with other E3(s) in addition to the putative E3s described above was tested by using E3x, isolated from rabbit reticulocytes (23–26). E3x and (its yeast counterpart UBR1) is required for recognition of substrate proteins based on the nature of their N-terminal residue (23–26). It works in concert with a 14-kDa reticulocyte E2 homologous to the yeast E2 encoded by RAD6 gene (21–23). When used in combination with E3x, E2$_{15\, kDa}$ was ~6-fold less effective in conjugating ubiquitin to lysozyme or to endogenous proteins (Fig. 6). In fact, many of the conjugates observed with endogenous proteins represented the E3x-independent conjugation of E1 and E2$_{15\, kDa}$ (compare reactions with or without E3x). Of all the remaining plant E2s, E3x functioned effectively only with A. thaliana E2$_{15\, kDa}$ encoded by the AtUBC1 gene. Conjugation was observed not only to 125I-lysozyme but also to endogenous proteins. Based on its E2 dependence, we conclude that the putative wheat E3s described here are distinct from E3x.

**DISCUSSION**

Ubiquitin-dependent proteolysis serves an essential role in eukaryotic intracellular protein degradation. The E2 enzymes encoded by the UBC1/4/5 gene family in yeast have been shown to be essential for eliminating abnormal and short-lived proteins by this pathway (2, 4, 5). We have identified a 15-kDa E2 from wheat that is closely related to yeast UBC4 and UBC5, based on peptide sequence homology and antibody cross-reactivity. This high degree of structural conservation suggests that E2$_{15\, kDa}$ has a similar essential cellular function in plants, i.e. mediating the degradation of short-lived and abnormal proteins. In agreement with this, we showed that E2$_{15\, kDa}$ is necessary for most of the ubiquitin conjugation to endogenous proteins and added lysozyme in wheat germ extracts.

E2$_{15\, kDa}$ is a basic protein that exists as a homodimer in low salt conditions. When denatured in the absence of reducing agents, the protein freely forms multimers. Conventional protocols for the purification of E2s currently rely on adsorption to and elution from DEAE-columns (termed Fraction I1; see Ref. 3). We find that E2$_{15\, kDa}$ does not purify with the rest of the wheat E2 family characterized to date by this method, implying that additional E2s may be present in eukaryotes that are not isolated with this conventional E2 purification strategy. One such candidate may be the factor identified by Gonen et al. (36, 37) as missing from Fraction I1 but essential for recognition and conjugation of N-acetylated proteins.

Previous studies have proposed that yeast UBC4 and UBC5 work in concert with an E3 (2–4). In the present study, we provide evidence that E2$_{15\, kDa}$ requires another factor for function that has E3-like activity. Three fractions containing such a factor(s) were partially purified from wheat germ by S- and Q-Sepharose chromatography (S, Q1, and Q2). They appear to be distinct from E3x (and its yeast counterpart UBR1; Refs. 23–26) previously identified in rabbit reticulocytes by their ability to work best with E2$_{15\, kDa}$ in vitro. They are also likely to be different from E3y because of their ability to ubiquitinate lysozyme (28). Based on these criteria, we designate the E3-like activity present in these fractions E3y. But the conclusion that these factors are indeed E3s will require a demonstration that they interact with target proteins (35). The functional and structural similarity of E2$_{15\, kDa}$ with UBC4 and UBC5 suggests that all E2$_{15\, kDa}$ homologs display E3 dependence. The E3y–E2$_{15\, kDa}$ pair is likely to be a major component in the ubiquitin-dependent proteolytic pathway, based on the requirement of UBC4- and UBC5-mediated proteolysis for the survival of yeast cells (4).

We do note that E2$_{15\, kDa}$ is also able to function in an E3-independent manner to catalyze the direct transfer of ubiquitin to a small group of basic hydrophobic wheat proteins (data not shown). Their molecular masses range from 23 to 27 kDa. They bind to S-Sepharose and elute along with E2$_{15\, kDa}$ in the 20–50 mM NaCl fraction, but they can be resolved from E2$_{15\, kDa}$ by phenyl-Sepharose chromatography. The identity of these substrates is unknown, but it remains possible that their direct recognition by E2$_{15\, kDa}$ only occurs in vitro.

Recognition of protein substrates by the ubiquitin pathway based on the nature of their N termini (N-End Rule) was recently shown to require the E2 encoded by RAD6 and E3x (or UBR1) (23, 25, 26). With respect to plant E2s, E3x does not function with E2$_{15\, kDa}$ but cooperates instead with another wheat E2, E2$_{20\, kDa}$ encoded by AtUBC1, to form ubiquitin conjugates. Previously reported failure of E2$_{15\, kDa}$ to substitute for the DNA repair function of RAD6 in rad6 null mutants (17) suggested to us that E2$_{15\, kDa}$ was not related to RAD6. However, the close relationship of E2$_{15\, kDa}$ and RAD6 at the
amino acid sequence level (63% similarity; Ref. 17) and the ability of E2_{16kDa} to functionally substitute for RAD6 with E3_{α} (shown here) suggest that these proteins share common functions including a role in recognizing and ubiquitinating substrates based on the nature of their N-terminal residue. Whether a homolog of E3_{α} exists in plants remains to be demonstrated.

A model in which the selectivity of the ubiquitin-dependent proteolytic pathway is controlled at the level of substrate recognition would imply the existence of multiple E3s, each preferentially recognizing specific substrates. In contrast, our results suggest that a wide variety of different proteins are recognized by a limited number of constitutive E2-E3 ligation systems. The data presented here suggest that at least two distinct ubiquitin ligation systems with different specificities exist in wheat germ, one requiring E2_{16kDa} and the another requiring E2_{6kDa}. Each in turn may require a distinct E3 activity, E3_{γ} or E3_{α} respectively, for substrate recognition.

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REFERENCES

1. Finley, D., and Chau, V. (1991) Annu. Rev. Cell Biol. 7, 25-69
2. Jentsch, S., Seufert, W., and Hauser, H.-P. (1991) Biochim. Biophys. Acta 1099, 127-138
3. Hersko, A. (1988) Cell 54, 11-12
4. Seufert, W., McGarth, J. P., and Jentsch, S. (1990) EMBO J. 9, 4535-4551
5. Seufert, W., and Jentsch, S. (1990) EMBO J. 9, 543-550
6. Shanklin, J., Jabben, M., and Vierstra, R. D. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 320-323
7. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. G., and Howley, P. M. (1990) Cell 63, 1129-1136
8. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) Nature 349, 132-138
9. Hochstrasser, M., Ellison, M. J., Chau, V., and Varshavsky, A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 4606-4610
10. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J.,戈da D. K., and Varshavsky, A. (1989) Science 243, 1576-1583
11. Gregori, L., Poosch, M. S., Cousens, G., and Chau, V. (1990) J. Biol. Chem. 265, 6354-6367
12. Matthews, W., Tanaka, K., Dricoll, J., Ichihara, A., and Goldberg, A. L. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 2597-2601
13. Qin, S., Nakajima, A., Nomura, M., and Arfin, S. (1991) J. Biol. Chem. 266, 15549-15554
14. Pickart, C. M., and Rose, J. A. (1988) J. Biol. Chem. 263, 1573-1581
15. Haas, A. L., and Bright, P. M. (1988) J. Biol. Chem. 263, 15268-15276
16. Sullivan, M. L., Callis, J., and Vierstra, R. D. (1990) Plant Physiol. 94, 710-716
17. Sullivan, M., and Vierstra, R. D. (1991) J. Biol. Chem. 266, 23878-23885
18. van Noeker, S., and Vierstra, R. D. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 10260-10261
19. Sullivan, M. L., and Vierstra, R. D. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 9661-9665
20. Treier, M., Seufert, W., and Jentsch, S. (1992) EMBO J. 11, 367-372
21. Jentsch, S., McGarth, J. P., and Varshavsky, A. (1987) Nature 329, 131-134
22. Wing, S. S., Dumas, F., and Banville, D. (1992) J. Biol. Chem. 267, 6495-6501
23. Dohmen, R. J., Madura, K., Bartel, B., and Varshavsky, A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 7351-7355
24. Reiss, Y., Kaim, D., and Hersko, A. (1988) J. Biol. Chem. 263, 2693-2698
25. Bartel, B., Wunning, L., and Varshavsky, A. (1990) EMBO J. 9, 3178-3184
26. Seng, P., Berleth, E., Pickart, C., Prakash, S., and Prakash, L. (1991) EMBO J. 10, 2187-2193
27. Johnson, E. S., Bartel, B., Seufert, W., and Varshavsky, A. (1992) EMBO J. 11, 497-500
28. Heller, H., and Hersko, A. (1990) J. Biol. Chem. 265, 6532-6535
29. Hersko, A., Ciechanover, A., Heller, H., Haas, A. L., and Rose, I. A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1763-1766
30. Hatfield, P. M., and Vierstra, R. D. (1989) Biochemistry 28, 735-742
31. Birckett, C. R., Foster, K. E., Johnson, L., and Gull, K. (1985) FEBS Lett. 187, 211-218
32. Rose, M., Winston, F., and Hieter, P. (1990) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U., and Greaves, M. F. (1982) J. Biol. Chem. 257, 10766-10769
34. Laemmli, U. K. (1970) Nature 227, 680-685
35. Reiss, Y., and Hersko, A. (1990) J. Biol. Chem. 265, 3685-3690
36. Gonen, H., Schwartz, A., and Ciechanover, A. (1991) J. Biol. Chem. 266, 19221-19231
37. Mayer, A., Siegel, N. R., Schwartz, A., and Ciechanover, A. (1990) Science 244, 1480-1483