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Biochemistry

Cloning and characterization of a 20-kDa ubiquitin carrier protein from wheat that catalyzes multiubiquitin chain formation *in vitro*

(conjugation/protein degradation/ubiquitin mutant)

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ABSTRACT **Recent evidence indicates that the commitment to degrade cellular proteins by the ubiquitin proteolytic pathway is dependent on the covalent attachment of multiubiquitin chains to the target protein [Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. & Varshavsky, A. (1989) *Science* 243, 1576–1583]. We have isolated a 20-kDa ubiquitin carrier protein [E2(20 kDa)] from wheat by using ubiquitin covalent affinity chromatography and anion-exchange HPLC that catalyzes multiubiquitin chain formation *in vitro*. This reaction is blocked by the addition of a mutant ubiquitin in which arginine has been substituted for lysine at residue 48, demonstrating that the coupling of ubiquitin to ubiquitin is likely to be through an isopeptide linkage between the C-terminal glycine and Lys^{48} of ubiquitin. By immunoscreening a wheat cDNA expression library with anti-E2(20 kDa) antibodies, a cDNA encoding the complete protein was isolated. The clone (designated *UBC7*) was confirmed as encoding E2(20 kDa) by comparison of the derived amino acid sequence with peptide sequences of E2(20 kDa) tryptic fragments. The encoded protein contains a single cysteine at position 91, which is presumably the active site, and has regions of amino acid sequence similarity to other known E2s from plants and yeast. Expression of this cDNA in *Escherichia coli* produced an active E2 capable of catalyzing multiubiquitin chain formation *in vitro*. By virtue of its activity, E2(20 kDa) may have a pivotal role in protein degradation by the ubiquitin-dependent proteolytic pathway.**

Ubiquitin has several functions in eukaryotic cells that arise from its covalent attachment to other cellular proteins. The best characterized of these is its involvement in protein degradation, where ubiquitin conjugation serves to commit proteins to breakdown (1). Other processes mediated by the ubiquitin system include DNA repair (2), cell cycle progression (3, 4), cell surface recognition (5), and regulation of chromatin structure (6).

Conjugation of ubiquitin to protein substrates is accomplished by the sequential action of ubiquitin activating enzyme (E1), ubiquitin carrier proteins (E2s), and in some cases ubiquitin–protein ligase (E3) (1). E2s are responsible for the transfer of a thiol ester-linked ubiquitin from E1 to various cellular proteins (7). In this capacity, E2s are responsible in part for recognizing and tagging appropriate targets (8, 9). E2s exist as a family of enzymes that differ with respect to size, structure, and target protein specificity, at least *in vitro* (8, 9). A multitude of E2s exist in plants with wheat germ containing abundant species of 16, 20, 23, 25, and 26 kDa (10). Of the seven E2s identified in yeast (9), specific isoforms have been implicated in such diverse processes as degradation of abnormal and short-lived proteins (11), DNA repair (2), regulation of cell cycle (4), and response to stress (11).

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The structures of E2s characterized to date show several conserved features. They consist of a small (≈150 amino acids) conserved core to which various C- and/or N-terminal extensions may be appended (9). The core contains a highly conserved cysteine residue that functions as the active site for thiol ester bond formation with ubiquitin (12, 47). For several E2s, it has been proposed that substrate recognition may be dependent in part on the nature of their C-terminal extensions (2, 13). For example, *in vitro* conjugation of ubiquitin to histones by the yeast *RAD6* gene product and wheat E2(23 kDa) is abolished by deletion of their highly acidic C termini (13, 47). Moreover, the activity of wheat E2(23 kDa) can be conferred to another E2 upon transfer of the C-terminal extension (47).

The conjugation of ubiquitin to proteins occurs through the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and free lysine ε-amino groups of the target protein (1). Although it was originally proposed that single ubiquitins are attached at each site (14), evidence has now accumulated from both *in vitro* and *in vivo* studies that multiple ubiquitins can be attached, resulting in the formation of multiubiquitin chains (15, 16). By using a mutant ubiquitin where arginine has been substituted for Lys^{48} (termed [Arg^{48}]ubiquitin), Chau *et al.* (16) were able to block multiubiquitin chain formation *in vitro*, showing that Lys^{48} represents the primary site of isopeptide linkage between ubiquitins in the chain. This mutant also blocked ubiquitin-dependent protein degradation, demonstrating that the formation of multiubiquitin chains is required for the pathway's function *in vitro*. High concentrations of [Arg^{48}]-ubiquitin are cytotoxic in tobacco, implying that multiubiquitin chains are also essential *in vivo* (17).

Given that multiubiquitin chains appear to represent a necessary intermediate in the breakdown of proteins by the ubiquitin-dependent proteolytic pathway, we expect that the enzyme(s) responsible for multiubiquitin chain formation would play an essential role in eukaryotic cell physiology. Recently, a 25-kDa E2 has been identified from calf thymus that appears to serve this function, catalyzing the transfer of ubiquitin to ubiquitin *in vitro* (18). We report here the purification of a 20-kDa E2 from wheat that also catalyzes multiubiquitin chain formation *in vitro*, and the isolation of a cDNA that encodes this protein.* A model for the function of this enzyme in the ubiquitin proteolytic pathway is presented, based on its *in vitro* activity.

MATERIALS AND METHODS

Materials. Nontoasted wheat (*Triticum aestivum*) germ was a gift from General Mills (Minneapolis). Wheat (*T. aestivum* cultivar 'Augusta') seeds were provided by R.

Abbreviations: E1, ubiquitin activating enzyme; E2, ubiquitin carrier protein; r, recombinant.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M74077).

Forsberg (University of Wisconsin, Madison). Human ubiquitin and a mutant ubiquitin ([Arg^{48}]ubiquitin) expressed in *Escherichia coli* were purified by a modification of the method of Haas and Wilkinson (19). Both proteins (500 μg) were radiolabeled with carrier-free Na^{125}I (1.0 mCi; 1 Ci = 37 GBq; Amersham) by the chloramine-T method (20). Wheat germ E1 was obtained as described by Hatfield and Vierstra (21).

Purification and Immunodetection of E2(20 kDa). E2(20 kDa) was isolated from wheat germ using a modification of the ubiquitin covalent affinity procedure of Ciechanover *et al.* (22) and anion-exchange HPLC as described by Sullivan *et al.* (10). After elution from ubiquitin-Affi-Gel, proteins were separated by anion-exchange HPLC using a 75 × 7.5 mm DEAE column (TSK-DEAE-5PW; Bio-Rad) and a linear 30–330 mM NaCl gradient in 20 mM KH_2PO_4 (pH 6.9) containing 0.5 mM dithioerythritol. Immunoblot analysis employed anti-E2(20 kDa) sera in conjunction with alkaline phosphatase-labeled goat anti-mouse immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and the reagents nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Anti-E2(20 kDa) sera were generated by coupling 125 μg of purified native E2(20 kDa) to 75 μg of keyhole limpet hemocyanin with 0.08% glutaraldehyde and injecting the crosslinked protein mixed with complete Freund's adjuvant into 13-week-old BALB/c mice. A second injection of 65 μg of crosslinked protein in incomplete adjuvant was given 20 days later. Sera were collected 14 days after the second immunization. Samples were subjected to SDS/PAGE (23), and proteins were electroblotted onto poly(vinylidene difluoride) membrane (Millipore) prior to immunodetection.

Peptide Sequence Analysis of E2(20 kDa). Purified wheat E2(20 kDa) was digested with trypsin [100:1 (wt/wt)] in 0.5% $(NH_4)HCO_3$/1 mM $CaCl_2$/50 mM Tris·HCl, pH 8.1, at 23°C for 2 h. The reaction was terminated by the addition of glacial acetic acid to 0.1 M. Products were separated by reversed-phase HPLC using a 4.6 × 250 mm C_8 column (Vydac) and a 0–65% (vol/vol) linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Two peptide fractions eluting at ≈42 and ≈47% acetonitrile were subjected to Edman degradation followed by HPLC identification of the phenylthiohydantoin derivatives.

cDNA Cloning and Molecular Characterization of E2(20 kDa). An amplified λ-ZAP cDNA expression library prepared with poly(A)$^+$ RNA isolated from 48-hour-old etiolated wheat seedlings (24) was screened as described by Huynh *et al.* (25) with a 1:1000 dilution of anti-E2(20 kDa) sera. Immunopositive plaques were rescued to pBluescript phagemids in *E. coli* XL-1 Blue (26). Complete nucleic acid sequence of both strands of the full-length cDNA encoding E2(20 kDa) (designated *UBC7*) was obtained using the dideoxynucleotide chain-termination procedure with single-stranded DNA templates (27). Amino acid sequences were aligned using the PileUp program of the University of Wisconsin Genetics Computer Group Software Package (28).

Purification of E2(20 kDa) from *E. coli*. *UBC7* was inserted into the pET3a expression vector under control of the T7 promoter (29) and expressed in *E. coli* BL21(DE3). Logarithmic-phase cultures were induced with 1 mM isopropyl thiogalactoside for 3 h at 37°C and sonicated at ice temperatures in 50 mM Tris·HCl (pH 8.0) containing 1 mM Na_2EDTA, 14 mM 2-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride. The majority of the UBC7 protein product was soluble under these conditions. The resulting crude extract was applied to a DE-52 cellulose column, and the recombinant (r) E2(20 kDa) was eluted in a 100–150 mM KCl step in 50 mM Tris·HCl (pH 8.0). To separate the rE2(20 kDa) from several contaminating proteins, the DE-52 eluent was desalted by gel filtration and fractionated by HPLC using a 75 × 7.5 mm DEAE column (TSK-DEAE-5PW; Bio-Rad) and a linear 60–300 mM NaCl gradient in 20 mM KH_2PO_4 (pH 6.9).

Assays for Ubiquitin-E2 Thiol Ester Linkage and Ubiquitin Conjugate Formation. Thiol ester adduct formation between ubiquitin and E2(20 kDa) was assayed as described by Haas *et al.* (30). ^{125}I-labeled ubiquitin or [Arg^{48}]ubiquitin (1 μM) was incubated with E1 (10 nM), E2(20 kDa) (50–500 nM), and MgATP (2 mM) in 50 mM Tris·HCl (pH 8.0) at 30°C for 2 min. To assay for conjugate formation, the same reaction mixture was incubated at 37°C and allowed to proceed for various times. Products of these reactions were separated by SDS/PAGE and visualized by autoradiography.

RESULTS

Purification of E2(20 kDa) from Wheat Germ. Wheat germ contains several size classes of E2s that can be purified by ubiquitin covalent affinity chromatography (10), one of the most abundant being a 20-kDa species (Fig. 1). This species was purified to near homogeneity by anion-exchange HPLC, with the majority eluting as a peak at 110 mM NaCl. By this procedure, ≈250 μg of E2(20 kDa) was purified from 200 g of wheat germ. Similar-sized E2s also eluted at two other positions, one at ≈120 mM NaCl and the other coeluting with E1 at ≈135 mM NaCl. These minor fractions were shown to be structurally related to the major E2(20 kDa) fraction by their cross-reaction with antisera generated against the major 20-kDa protein (data not shown).

The major and minor E2(20 kDa) fractions migrated as a closely spaced doublet during SDS/PAGE (Fig. 1). A similar doublet was observed for the purified product of the E2(20 kDa) cDNA expressed in *E. coli* (see below). Anti-E2(20 kDa) sera, which failed to recognize any other size class of wheat E2s, recognized both species of the 20-kDa doublet. Rapid extraction of protein in SDS-containing buffer or incubation of the purified products with either wheat germ or *E. coli* extracts yielded the same migration pattern, suggest-

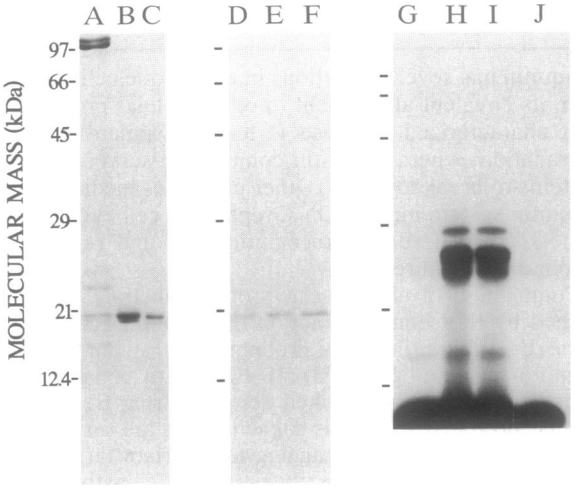

FIG. 1. Purification, immunodetection, and assay of E2(20 kDa) from wheat germ. Lanes A–C contain protein subjected to SDS/PAGE and stained with Coomassie blue. Lanes D–F show an immunoblot analysis of protein samples with anti-E2(20 kDa) serum. Lanes G–J show an assay for ubiquitin–E2 thiol ester formation. Lanes: A and D, proteins isolated by ubiquitin covalent affinity chromatography; B, E, and H, E2(20 kDa) protein fraction eluted during anion-exchange HPLC with 110 mM NaCl; C, F, and I, the *UBC7* gene product [rE2(20 kDa)] synthesized in *E. coli* under direction of a pET expression vector; G, thiol ester reaction lacking E2(20 kDa); J, thiol ester assay of E2(20 kDa) after heating the products in the presence of 2-mercaptoethanol.

ing that the size heterogeneity was not the result of posthomogenization modification (data not shown).

Enzymatic Activity *in Vitro*. The 20-kDa protein was determined to be an E2 by its ability to form a stable adduct with ^{125}I-labeled ubiquitin in an E1- and MgATP-dependent reaction (Fig. 1). The linkage formed was labile to 2-mercaptoethanol, consistent with its identity as a thiol ester bond (31). This adduct migrated as multiple species during SDS/PAGE, indicating the possibility that more than one ubiquitin may be bound per E2 (Fig. 1).

During extended incubations with only MgATP, E1, and ^{125}I-labeled ubiquitin, E2(20 kDa) converted ubiquitin into higher molecular mass conjugates with sizes consistent with the formation of multiubiquitin chains (for example, see Fig. 4). The lowest molecular mass species formed had approximately twice the molecular mass of ubiquitin. We concluded that this species represented a covalently bound ubiquitin dimer, as there was no other protein of sufficiently small size in the reaction to which ubiquitin could be conjugated. Higher-order species were consistent with the formation of tri-, tetra-, and higher-order ubiquitin chains, as well as a conjugate of ubiquitin to E2(20 kDa). These conjugates were the only products of this reaction, even in the presence of other wheat germ proteins, indicating that E2(20 kDa) is specific in its recognition of ubiquitin as a substrate. E2(20 kDa) showed no detectable activity for conjugating ubiquitin to purified wheat histones H2A/H2B (data not shown), which are *in vitro* substrates for several other E2s, including wheat E2(23 kDa), the yeast *RAD6* and *CDC34* gene products, and the 35-kDa E2 from reticulocytes (2, 4, 32, 33).

Cloning and Sequencing of a cDNA Encoding E2(20 kDa). With the observation that the 20-kDa E2 forms multiubiquitin chains *in vitro*, a molecular analysis of the protein was undertaken. By using anti-E2(20 kDa) sera, a cDNA encoding E2(20 kDa) was isolated from a wheat cDNA expression library. Approximately 80,000 recombinants were screened, resulting in the detection of four immunopositive phage. One of these contained an 851-base-pair cDNA (designated *UBC7*) with an open reading frame that would encode a polypeptide of 168 amino acids with a molecular weight of 18,840 and a pI value of 5.35 (Fig. 2). A poly(A) tract of 49 residues was present at the 3′ end.

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AATTATTCCTCCGCCCCGCCCGCCGCGAAAACCCTAGCCGCCGCCGATCCCATGGCCACC  60
                                                  M  A  T    3

GCTCCAGCCAGGCGAGCCTCCTCCTCCAGAAGCAGCTCCGAGATCTCGCGAACGACCCCG 120
 A  P  A  R  R  A  S  S  S  R  S  S  S  E  S  R  T  T  P  S   23

TCGATGGGTTTTCAGCTGGGCTTCGTCGACGACAGCAACGTCTTCGAGTGGCAGGTCACC 180
 M  G  F  Q  L  G  F  V  D  D  S  N  V  F  E  W  Q  V  T  I   43

ATCATCGGCCCGCCCGAGACCCTATATGATGGAGGCTATTTCAATGCAATTATGAGCTTC 240
 I  G  P  P  E  T  L  Y  D  G  G  Y  F  N  A  I  M  S  F  P   63

CCACAAAATTATCCGAACAGCCCTCCAACAGTCAGATTCACTTCTGAAATGTGGCATCCT 300
 Q  N  Y  P  N  S  P  P  T  V  R  F  T  S  E  M  W  H  P  N   83

AATGTTTATCCAGATGGGCGGGTATGCATTTCTATTCATCCACCTGGTGATGATCCAAAC 360
 V  Y  P  D  G  R  V  C  I  S  I  H  P  P  G  D  D  P  N  G  103
                    ▲
GGATATGAGCTCGCAAGTGAGCGTTGGACACCAGTCCATACGGTTGAGAGTATAGTATTA 420
 Y  E  L  A  S  E  R  W  T  P  V  H  T  V  E  S  I  V  L  S  123

AGCATCATTTCGATGCTTTCTAGTCCAAATGATGAATCACCGGCAAATATTGAAGCAGCT 480
 I  I  S  M  L  S  S  P  N  D  E  S  P  A  N  I  E  A  A  K  143

AAGGACTGGAGAGAGAAGCAGGATGAGTTCAAGAAGAAGGTCAGGCGCGCCGTACGGAAA 540
 D  W  R  E  K  Q  D  E  F  K  K  K  V  R  R  A  V  R  G  K  163

TCCCAGGAAATGCTCTGAATAAAATGGGAGTACTGGGGAAGTTAGCCATGCCATTTCAGG 600
 S  Q  E  M  L  *

TGTGGAGGGTTTTGCTGTTGATCTCCAAATGTCCATCCGACACTAGCAATTTCACTTCTC 660
CCCTGTATATTTTCCATTAGCCTGTCTCCTGTGGAGGTCTTGGTTTTGACTCTGAAAACG 720
CAACACATATTCTAAGGGTTTGGTTGTTGTAATTGTGTGGCTTCGGCTATCAATATTGAG 780
AAACTTGGGAGAGCTGCTACTT(A)49
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FIG. 2. Nucleotide and derived amino acid sequence of the *UBC7* cDNA encoding wheat E2(20 kDa). Underlined amino acids indicate the derived amino acid sequence of *UBC7* that corresponds with peptide sequence of two E2(20 kDa) tryptic fragments. The position of the putative active cysteine is indicated by an arrowhead. The double-underlined nucleotides indicate the position of a possible polyadenylylation signal (34).

Confirmation that *UBC7* encoded the authentic E2(20 kDa) protein came from comparison of the derived amino acid sequence with the sequence of two E2(20 kDa) tryptic peptides of 15 and 21 amino acids. These peptides were found to be adjacent cleavage products and matched the derived sequence at 35 of 36 positions with the peptide sequence specifying an additional lysine residue between Ile^{96} and His^{97} of the derived sequence (Fig. 2). E2(20 kDa) contained a single cysteine at position 91 that likely represents the active site for thiol ester formation with ubiquitin (12).

The derived amino acid sequence of *UBC7* showed moderate homology to that of a 16-kDa wheat E2, being 38% identical and 58% similar (47), and weak homology to a 23-kDa wheat E2 (32), being 24% identical and 43% similar (Fig. 3). Among all E2s characterized structurally to date, including yeast UBC1, UBC4, UBC5, and the *RAD6* and *CDC34* gene products (2, 4, 11, 35), the *Schizosaccharomyces pombe rhp6*$^+$ gene product (36), and a 17-kDa E2 isolated from human tissue (37), the derived amino acid sequence of *UBC7* showed greatest homology to the yeast *CDC34* gene product (43% identity, 67% similarity). Amino acid sequence alignment of E2(20 kDa) with the 16-kDa and the 23-kDa E2s from wheat revealed an insertion of ≈12 amino acids near the putative active site that is not present in the other wheat E2s (Fig. 3). E2(20 kDa) did not contain a C-terminal extension beyond the conserved E2 core.

Purification and Activity of rE2(20 kDa). To generate large amounts of protein for further study, *UBC7* was placed in a pET vector and expressed in *E. coli*. After induction of logarithmic-phase cultures, a 20-kDa protein accumulated to a maximum in 3 h. rE2(20 kDa) was purified to near homogeneity by anion-exchange HPLC, eluting at a position identical to the protein isolated from wheat. By this method, ≈2 mg of rE2(20 kDa) was purified from 1 liter of logarithmic-phase culture. rE2(20 kDa) was determined to be authentic full-length E2(20 kDa) by its comigration during SDS/PAGE with E2(20 kDa) isolated from wheat and its cross-reaction with antisera generated against the wheat protein (Fig. 1).

The enzymatic properties of rE2(20 kDa) were identical to those of the protein isolated from wheat. rE2(20 kDa) formed a thiol ester adduct with ubiquitin (Fig. 1) and produced similar amounts of multiubiquitin chains under identical reaction conditions (Fig. 4). The reaction appeared progressive, with di- and triubiquitin appearing first relative to the higher-order multiubiquitin chains, and proceeded slowly, such that after a 2-h incubation with 200 nM E2(20 kDa) only

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E2 20kDa    MATAPARRAS SSRSSSESRT TPSMGFQLGF VDDSNVFEWQ VTIIGPPETL   50
E2 16kDa    MSTPARKRLM RDFKRLQQDP PAGIS...GA PHDNNITLWN AVIFGPDDTP
E2 23kDa    MSSPSKRREM DLMKLMMSDY KVDMI..... ..NDGMHEFF VHFHGPKDSI
Consensus   MSTP--RR-M ---K----D- ---M----G- --D-N--EW- V-I-GP-DT-

                                                        ▼
E2 20kDa    YDGGYFNAIM SFPQNYPNSP PTVRFTSEMW HPNVYP.DGR VCISIHPPGD   99
E2 16kDa    WDGGTFKLTL QFTEDYPNKP PTVRFVSRMF HPNIYA.DGS ICLDILQ...
E2 23kDa    YQGGVWKVRV ELTEAYPYKS PSIGFTNKIY HPNVDEMSGS VCLDVIN...
Consensus   YDGG-FK--- -FTE-YPNKP PTVRFTS-M- HPNVY--DGS VCLDI-----

E2 20kDa    DPNGYELASE RWTPVHTVES IVLSIIS.ML SSPNDESPAN IEAAKDWREK  148
E2 16kDa    .........N QWSPIYDVAA ILTSIQS.LL CDPNPNSPAN SEAARMYSEN
E2 23kDa    .........Q TWSPMFDLVN IFEVFLPQLL LYPNPSDPLN GEAASLMMRD
Consensus   ---------- -WSP--DV-- I--SI-S-LL --PNP-SPAN -EAA----E-

E2 20kDa    QDEFKKKVRR AVRGKSQEML *......... ......... .........  168
E2 16kDa    KREYNRKVRE VVEQSWTAD* ......... ......... .........
E2 23kDa    KNAYENKVKE YCERYAKPED ISPEEEEEES DEELSDAEGY DSGDEAIMGH
Consensus   K-EY--KVRE -VE------- ---------- ---------- ----------

E2 20kDa    ...
E2 16kDa    ...
E2 23kDa    ADP*
Consensus   ---
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FIG. 3. Amino acid sequence comparison of E2(20 kDa) with wheat E2(23 kDa) (10) and wheat E2(16 kDa) (47). The putative active cysteine is marked with an arrowhead. A consensus was determined if two of the three corresponding amino acids were identical.

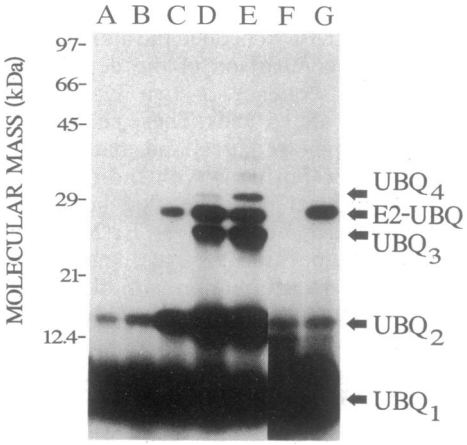

FIG. 4. Time course for multiubiquitin chain formation catalyzed by E2(20 kDa). Reaction mixtures contained 2 mM ATP, 10 nM E1, either 0 nM (lanes A and F) or 500 nM (lanes B–E and G) rE2(20 kDa), and either 1 μM ^{125}I-labeled ubiquitin (4700 cpm/pmol, lanes A–E) or 1 μM ^{125}I-labeled [Arg^{48}]ubiquitin (1600 cpm/pmol, lanes F and G). Reaction mixtures were incubated at 37°C for 0 h (lane B), 30 min (lane C), 2 h (lane D), or 8 h (lanes A, E–G). Samples were subjected to SDS/PAGE and autoradiography; lanes A–E were exposed for 16 h and lanes F and G were exposed for 64 h. Arrows to the right indicate positions of free ubiquitin monomer and multimers potentially containing two to four ubiquitin moieties. The putative E2(20 kDa)-ubiquitin (UBQ) conjugate is also indicated.

≈50% of free ^{125}I-labeled ubiquitin had been converted to higher-order structures. To define the nature of the ubiquitin–ubiquitin linkage, the activity of E2(20 kDa) was tested in the presence of [Arg^{48}]ubiquitin. Purified [Arg^{48}]ubiquitin was determined as active by its ability to form thiol ester adducts with E1 and E2(20 kDa) and by its formation of ubiquitin–histone H2A/H2B conjugates in the presence of wheat E2(23 kDa) (data not shown). When [Arg^{48}]ubiquitin was added to a conjugation reaction with E2(20 kDa), the formation of multiubiquitin chains was blocked; the only conjugate formed was that between [Arg^{48}]ubiquitin and E2(20 kDa). This suggests that rE2(20 kDa) forms multiubiquitin chains primarily by attaching ubiquitin to ubiquitin through Lys^{48}.

DISCUSSION

Here we report the purification and molecular characterization of a 20-kDa E2 from wheat that specifically recognizes ubiquitin as a substrate for further ubiquitin addition. Formation of multiubiquitin chains by this E2 is blocked by replacing ^{125}I-labeled ubiquitin with ^{125}I-labeled [Arg^{48}]-ubiquitin, indicating that concatenation of ubiquitin into chains is preferentially through Lys^{48}. This residue has been demonstrated to be the preferred linkage site for multiubiquitin chains in yeast, which serve as a strong signal for target protein degradation (16). In light of the importance of multiubiquitin chains, this enzyme may have an essential role in intracellular protein degradation. Wheat E2(20 kDa) is catalytically similar to a 25-kDa E2 characterized from calf thymus, which also forms Lys^{48}-linked multiubiquitin chains *in vitro* (18). No other wheat E2 we have tested [i.e., the 16-, 23-, 25-, and 26-kDa species (10)] will conjugate ubiquitin to ubiquitin (data not shown), suggesting that the 20-kDa species represents a functionally distinct E2 in wheat.

We observed that E2(20 kDa) eluted in multiple fractions during anion-exchange HPLC. Although the exact reason for this heterogeneity is unknown, these species are functional E2s, as judged by thiol ester assays, and are immunologically related, indicating that they may be modified forms of the same protein. Also unknown is the reason for the migration of E2(20 kDa) as a doublet during SDS/PAGE. Because the doublet is stable upon incubation in wheat germ or *E. coli* extracts (data not shown), it is probably not a result of posthomogenization modification. Furthermore, the recombinant protein also migrated as a doublet, suggesting that this observation may reflect an anomaly of the protein's migration during SDS/PAGE.

The E2(20 kDa)-ubiquitin thiol ester adduct migrated as multiple species during SDS/PAGE. Similar multiple thiol ester adducts have been observed for other E2s (32, 38, 47). Because the derived amino acid sequence of *UBC7* contains only one cysteine, this heterogeneity cannot result from multiple ubiquitins attached to more than one site on the E2. Neither is it due to the binding of ubiquitin dimers, trimers, etc., to the E2 since insufficient quantities of such multimers were produced during the 2-min thiol ester reactions. It could reflect the anomalous migration of the ubiquitin-E2(20 kDa) thiol ester adduct in conditions used for the SDS/PAGE assay (4°C in the absence of reducing agents).

Upon comparison of the derived amino acid sequence of *UBC7* with those of two other E2s from wheat, a strong homology is found surrounding the conserved cysteine residue [position 91 on E2(20 kDa); Fig. 3]. Replacement of this cysteine with serine by *in vitro* mutagenesis in the yeast *RAD6* gene product and the wheat 16-kDa and 23-kDa E2s results in proteins that are incapable of forming E2-ubiquitin thiol ester adducts (12, 47), indicating that this residue indeed constitutes the active site. Of special interest is a 12-amino acid internal insertion not present in the other E2s characterized from wheat. It is possible that this sequence may be responsible for the unique ability of E2(20 kDa) to form multiubiquitin chains. Similar short internal insertions after the active site are also present in two yeast E2s [*CDC34* and *UBC7* gene products (9)]. Of interest is the observation that the *CDC34*-encoded protein is also capable of a slow rate of multiubiquitin chain formation *in vitro* (39). Also apparent from amino acid sequence comparisons is the lack of a C-terminal extension in E2(20 kDa). The highly acidic C-terminal extension of the yeast *RAD6* gene product and the 23-kDa E2 from wheat appears responsible for the interaction of these E2s with histones *in vitro* (13, 47).

It was necessary to use E2(20 kDa) at high concentrations (≈500 nM) and long reaction times (>1 h) to convert substantial amounts of ubiquitin monomers into multiubiquitin chains (Fig. 4). This slow rate was similar to that reported for formation of multiubiquitin chains by the 25-kDa E2 from calf thymus (18). In contrast, conjugation of equivalent amounts of ubiquitin to histones by wheat E2(23 kDa) required far less enzyme (≈10 nM) under the same reaction conditions (32). The activity of E2(20 kDa) is not stimulated significantly by the addition of wheat germ extract, suggesting that the reaction is not dependent on E3 or any other cofactor. This low catalytic efficiency may explain why both wheat E2(20 kDa) and calf thymus E2(25 kDa) are present at high levels relative to other E2s in their respective tissues (this report and ref. 18).

The mechanism by which multiubiquitin chains are formed *in vivo* is still unknown, although at least two possibilities exist. Ubiquitin might be added sequentially to ubiquitins already conjugated to the target protein, progressively forming longer chains. In this case, the multiubiquitin chain-forming E2 would be required to decide which monoubiquitinated proteins are appropriate for chain elongation. However, given that some proteins remain monoubiquitinated [e.g., actin (40) and histone H2A (6, 41)] whereas others become multiubiquitinated, it is unlikely that a single or small group of E2s could be responsible for such a complex decision. Alternatively, as proposed by Bamezai *et al.* (42), multiubiquitin chains could be preassembled and subsequently attached as a unit to the target protein. This mech-

anism would require the existence of E2s that create free multiubiquitin chains. These chains would then have to be activated by E1, transferred to the appropriate E2s, and finally conjugated to proteins destined for degradation. Several observations support this model. (*i*) Wheat E2(20 kDa) and thymus E2(25 kDa) are capable of multiubiquitin chain formation *in vitro* (this report and ref. 18). (*ii*) Recent work by Chen and Pickart (18) shows that multiubiquitin chains can be activated by E1 and transferred to an E2. (*iii*) The pattern of ubiquitin conjugate formation for several proteins is indicative of a single multiubiquitin chain addition and not progressive addition of ubiquitin monomers [e.g., phytochrome (43) and cyclin (3)]. If such preassembled chains exist, their levels would be controlled not only by the rate of synthesis but also by the rate of disassembly by ubiquitin protein hydrolases (44). Measurements of ubiquitin pool dynamics in a variety of tissues indicate that most ubiquitin exists as conjugates (45). Although originally proposed to be ubiquitin–protein conjugates, they may also be free multiubiquitin chains awaiting attachment to the target proteins. In *Arabidopsis thaliana*, for example, several abundant ubiquitin conjugates exist with molecular masses consistent with that of multiubiquitin chains (46). The mechanism by which these chains are attached to target proteins remains to be elucidated. This may be a function of all E2s or just specific E2s, perhaps working in concert with ubiquitin–protein ligase (E3). Further enzymatic analysis of E2(20 kDa) and its use to generate multiubiquitin chains as substrates for other E2s may help to resolve these questions.

Note Added in Proof. Since the preparation of this manuscript, the isolation and characterization of a cDNA encoding a 25-kDa E2 from bovine thymus has been described that catalyzes multiubiquitin chain formation *in vitro* (48). This E2 exhibits little amino acid sequence similarity (25% identity and 49% similarity) to the wheat UBC7 protein described here indicating that more than one isoform of E2 can synthesize multiubiquitin chains.

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