Isolation of a cDNA Encoding a Mammalian Multiubiquitinating Enzyme (E225K) and Overexpression of the Functional Enzyme in Escherichia coli*

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The ubiquitin (Ub)-conjugating enzyme E225K catalyzes the synthesis of multi-Ub chains in which successive Ub units are linked by an isopeptide bond involving the ε-amino group of Lys-48 of Ub, and the COOH-terminal Gly residue of Ub, and the E2-terminal Gly residue of Ub, and the COOH-terminal Gly residue of Ub (Chen, Z., and Pickart, C. M. (1990) J. Biol. Chem., 265, 21835–21842). We now describe the polymerase chain reaction (PCR)-based cloning of an E225K-encoding cDNA from a bovine thymus library, using degenerate oligonucleotide primers based on the sequences of two E225K peptides. The cDNA encodes a 200-residue protein whose sequence bears similarities of 68 and 59%, respectively, to the sequences of the Ub-conjugating enzymes encoded by the UBCI and UBC4/UBC5 genes of the yeast Saccharomyces cerevisiae. These three yeast E2s play key roles in Ub-dependent proteolysis (Seufert, W., McGrath, J. P., and Jentsch, S. (1990) EMBO J. 9, 4535–4541). Comparison of the amino acid sequence of E225K with other known E2 sequences strongly suggests that Cys-92, one of two E225K Cys residues, forms the Ub thiol ester adduct that is an intermediate in E2-catalyzed multiubiquitination. The E225K-encoding cDNA was overexpressed in Escherichia coli, and the recombinant E225K protein was purified to electrophoretic homogeneity; enzymatic assays showed that its multiubiquitinating activity was quantitatively identical with that of the native protein. The availability of a cloned cDNA will allow us to assess the physiological role of E225K.

Ub, a highly conserved 76-amino acid polypeptide, functions as a covalent cofactor in energy-dependent intracellular proteolysis. The pathway through which Ub becomes attached to proteolytic substrates generally involves three enzymatic steps (1–3): 1) ATP-dependent activation of Ub by Ub-activating enzyme (E1), which ultimately results in the formation of a thiol ester bond between the COOH-terminal Gly residue of Ub and a sulfhydryl group of E1; 2) a transthiolation reaction, in which Ub is transferred from the thiol group of E1 to a thiol group on a Ub-conjugating enzyme (also known as an Ub carrier protein or E2); and 3) formation of an isopeptide bond between the COOH-terminal Gly residue of Ub, and the ε-amino group of an internal lysine residue of the target protein. Step 3 may require the participation of a Ub-protein ligase (E3) (1, 2). The target protein portion of the resulting Ub-protein conjugate is subsequently degraded by a large ATP-dependent protease complex (4, 5), and the Ub portion of the conjugate is regenerated by isopeptidases (2).

Proteins that are destined for degradation are frequently ligated to multiple molecules of Ub (6). Studies by Chau and co-workers (7, 8) have demonstrated that multiubiquitinated forms of a given target protein can be degraded much more rapidly than monoubiquitinated forms of the same substrate. Multiubiquitinated conjugates that undergo rapid degradation have a configuration in which one Ub is ligated to a specific Lys residue of the target and additional Ub molecules are ligated to Lys-48 residues of previously conjugated Ub molecules. This has been shown both by structural analysis of conjugates isolated from yeast (7) and by the inability of recombinant Ub molecules that lack a primary amino group at position 48 to support formation of multiubiquitinated conjugates (7, 8). Recent studies by Bachmair et al. (9) indicate that expression of a recombinant Ub bearing a Lys to Arg transition at position 48 leads to marked abnormalities in vascular tissue and necrotic lesions on leaves in the plant Nicotiana tabacum. These deleterious effects presumably result from partial inhibition of Ub-dependent proteolysis.

We have shown previously that one mammalian E2, E225K, exhibits a unique specificity for Ub itself as a conjugative target, catalyzing the formation of Lys-48-linked multi-Ub chains from Ub alone (10). E225K is a member of an E2 protein family that in mammals (11–14) and in yeast (15) includes multiple isoforms. In rabbit reticulocytes, only one of several E225K isoforms is known to function with high efficiency in E3-dependent ubiquitination, which leads to target protein degradation (11–13). E225K, E220K, E225K, and E220K all catalyze E3-independent ubiquitination of selected basic protein substrates such as histones (11, 14, 16, 17). In some cases this E2-catalyzed ubiquitination can lead to substrate degradation (18). The yeast Saccharomyces cerevisiae also possesses multiple E2s, and genes encoding five of these proteins have been cloned (15). The UBCI gene encodes a 215-residue protein whose amino acid sequence is 45% identical with the sequence of the two closely-related 16-kDa E2s encoded by the UBC4 and UBC5 genes (19, 20). These three E2s constitute
an essential subfamily of E2s that participate in protein turnover and whose functions partially overlap (19). The RAD6 gene encodes a 20-kDa E2 (21) that functions in DNA repair, sporulation, and DNA damage-induced mutagenesis (22). A fifth yeast E2, encoded by the CDC34 gene, is involved in the progression of the cell cycle from the G1 into the S phase (23). It is not yet clear whether the (putative) conjugative targets of the RAD6 and CDC34 proteins undergo Ubi-mediated degradation.

In this report, we describe the cloning of an E225K-encoding cDNA from bovine thymus and the expression of this cDNA in *Escherichia coli*. The cDNA encodes a 200-residue protein whose sequence shares some similarities with all known E2 sequences. However, the sequence of E225K most closely resembles the sequences of the UBC1, UBC4, and UBC5 proteins of *S. cerevisiae*, all of which function in Ub-mediated proteolysis (19, 20). This is the first mammalian E2 to be overexpressed in active form. The availability of the cDNA, and of large amounts of protein, will greatly facilitate studies on the biological role of E225K and on the structural basis of its multiquitinlinking activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—The bovine thymus cDNA library, in λ gt11, was purchased from Clontech Laboratories, Inc. Oligonucleotides (Table I) were synthesized in the microlab at SUNY/Buffalo, and were used directly after depurination. The PCR thermocycler and Taq polymerase were from Perkin-Elmer Cetus Instruments. Sequence (version 2.0) was from U. S. Biochemical Corp. E. coli strains TB-1 and NM5294/C1 were used as hosts for maintaining pUC19 and PotsNco-derived (24) plasmids, respectively. Initial database searching and sequence comparisons were performed with the programs FASTA (Pearson and Lipman algorithm (25)) and GAP (Needleman and Wunsch algorithm (26)), respectively. Both programs were part of the Wisconsin Genetics Computer Group package (27); additional programs in this package were used to manipulate and translate DNA sequences.

The preps of E1A, Ub, and 121Ub-We were described previously (10). Enzyme assays involving E225K were carried out as described (10). Protein concentrations were determined by the Bio-Rad dye reagent, using bovine serum albumin as a standard. Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs. Additional reagents were from Sigma unless stated otherwise. Drs. P. Sung and L. Prakash (University of Rochester) provided affinity-purified antibodies raised against a truncated RAD6 protein lacking the entire COOH-terminal polyubiquitination domain. A fifth yeast E2, encoded by the CDC34 gene, is involved in the progression of the cell cycle from the G1 into the S phase (23). It is not yet clear whether the (putative) conjugative targets of the RAD6 and CDC34 proteins undergo Ubi-mediated degradation.

**Peptide Sequences and Synthesis of Degenerate Oligonucleotides—**E225K was purified from bovine thymus (10). At the Harvard microchemistry lab, the purified protein (~50 μg) was digested in solution with Lys-C protease, and the resulting peptides were separated by reverse-phase high-pressure liquid chromatography. Three well-resolved peptides were sequenced by Edman degradation; the results are shown in Fig. 1A. Degenerate oligonucleotides (primers A1 and A2) were designed based on two segments of peptide sequence (Fig. 1A; Table I). The orientations of these oligonucleotides assumed that peptide 2 (Fig. 1A) represented the COOH terminus of E225K; this was based on the definite absence of Lys at the COOH terminus of this peptide. (Because of the length of peptide 1, its COOH-terminal sequence could not be determined unambiguously.)

PCR-based Cloning—Primer sequences are given in Table I (see "Results"). Fig. 1 shows a schematic illustration of the two-step cloning strategy, which is a modification of the PCR method of Frohman and co-workers (30, 31). Initially (Fig. 1A), recombinant phage (5 × 10^8 plaque-forming units) from the library were frozen and thawed twice to release DNA that then served as the template in a PCR reaction containing 1 × PCR buffer, 10 mm Tris-HCl (pH 8.3), 25°C, 50 mM KCl, 0.1% gelatin, 0.2 μm each of the four dNTPs, 25 units/ml Taq polymerase) and 2.5 mm MgCl₂; primers (1 μM each) were A1 and λa. After heating to 95°C for 2 min, the reaction mixture was subjected to 30 cycles of 95°C (1 min), 55°C (2 min), and 72°C (3 min) (Method 1). The temperature was then held at 72°C for 7 min and gradually cooled to ambient temperature.

An aliquot of the above PCR reaction product was diluted 100-fold with distilled water, and 10 μl of the diluted sample was used as the template for a second-stage PCR containing (100 μl) 1 × PCR buffer, 50 mm KCl, and 0.5 μM of primers A1 and A2. One cycle (95°C, 1 min) and 25 cycles (94°C, 1 min, 55°C 1 min, 72°C 1 min) were performed. This cycle yielded a PCR product of about 250 bp, which was then cloned into the Smal site of pUC19. The strand conditions were as above, except that annealing (55°C) and extension (72°C) periods were 1 min each (Method 2). With these conditions, a single PCR product of about 150 bp was generated, as seen by product analysis on agarose gels. This fragment was eluted from the gel slice (22) and cloned into the Smal site of pUC19. Both strands were sequenced by a modified Sanger procedure (33, 34), using A1 and A2 as sequencing primers. The deduced amino acid sequence of the 146-bp insert included the COOH-terminal portion of peptide 1 (GAPVPSPEY) and the NH₂-terminal Ser of peptide 2 (see Fig. 1A).

Two authentic primers (B1 and B2, Table I) were then synthesized, based on the nucleic sequence between primers A1 and A2. These primers were used in the second step to obtain a full-length E225K-encoding cDNA (Fig. 1B). To amplify the 5′-portion of the cDNA, recombinant phage (10^7 plaque-forming units) with 1.5 mm MgCl₂; primers (0.2 μM each) were B2 and λa. After heating to 95°C for 1 min, dNTPs (0.2 mm each) were added, and the reaction was subjected to 35 cycles of 94°C (1 min) and 65°C (4 min) (Method 3). Heating at 72°C (1 min) in the last cycle was gradually cooled to ambient temperature. Three major products were detected on agarose gels, with sizes of 1.1, 0.59, and 0.46 kb. Sequence analysis showed that 90% of the sequence of the largest product was unrelated to E225K. The 0.46-kb fragment was equivalent to the 0.59-kb fragment (below) but lacked 130 bp of the 5′ sequence, presumably as a result of incomplete extension during reverse transcription. Hence, only the 0.59-kb fragment (designated M-5′) was used in the rest of the cloning procedure. The 3′-portion of the cDNA (M-3′) was generated in a similar way, except that the primer pair was B1 and λC. The product of this latter reaction was 0.33-kb fragment.

The gel slices containing the fragments M-3′ and M-5′ were excised; each slice (containing ~0.5 μg of DNA) was boiled in 1 ml of distilled water for 5 min. Aliquots (5 μl) of each extract were combined in a reaction (100 μl) containing 1 × PCR buffer, 1.5 mm MgCl₂, and 0.2 μM each of λa and λC. After 35 cycles according to Method 2 (above), a single 0.83-kb product was obtained. This fragment was cloned into pUC19, and four independent clones were sequenced.

The Northern Blot—Total RNA, 2 μg, was isolated from 5 × 10⁶ murine erythrocyte/leukemic cells by the CelC/guanidinium thiocyanate procedure (35). Poly(A)+ RNA was obtained from total RNA by chromatography (36) on oligo(dT)-cellulose (New England Biolabs), 30 μg of total RNA, and poly(A)+ RNA (1–25 μg) were fractionated on a 1.2% agarose/formaldehyde gel and transferred to Zetaprobe filters (Bio-Rad) by capillary alkaline blotting, using 50 mm NaOH as transfer solvent (37). Filters were prehydrated (5 min, 45°C) in a solution containing 50% formamide, 0.25 mm NaHPO₄, (pH 7.2), 0.25 mm NaCl, 7% (w/v) SDS, and 1 mm EDTA. A 0.7-kb DNA fragment, generated from pUC19-E225K by PCR (Method 2; primers C1 and C2), was labeled with 32P by nick translation to a specific radioactivity of 10⁶ cpm/μg (38). This probe was denatured by boiling for 5 min and hybridized to the immobilized RNA in hybridization solution (2 × 10⁶ cpm/ml, 20 h, 43°C). Before autoradiography, blots were washed three times (15 min each, 26°C) in 0.1 × SSC, 0.1% SDS, once (15 min, 25°C) in 0.2 × SSC, 0.01% SDS, and once (30 min) in 0.2 × SSC, 0.1% SDS at 43°C.

Overexpression of E225K in *E. coli*—Two additional primers were synthesized that contained recognition sites for Ncol and BamHI (primers C1 and C2, respectively, in Table I). These primers were used to amplify the entire E225K coding sequence, starting from the (putative) initiator ATG and including 130 bp of 3′-untranslated sequence (as shown in Fig. 2, except that there was a C rather than a T at position 701 in the 3′-untranslated region). The reaction mixture (100 μl) contained 1 × PCR buffer, 1 μg of primers C1 and C2, and 10 ng of pUC19-E225K. After 30 cycles according to Method 2 (above), a 0.75-kb product was observed. This purified product was digested with Ncol and BamHI and then cloned into the expression vector PotsNco (24). The resulting plasmid, designated Pots-E225K, was used to transform E. coli strain AR120 (24). Transformsants were grown at 37°C in L broth containing 0.1% ampicillin. When λa had reached 0.4, nalidixic acid was added to a final concentration of 60 μg/ml. Growth was
continued for 8 h before harvesting cells by centrifugation. For the experiments shown in Fig. 5, E. coli harboring Pots-E2<sub>25K</sub> (with or without nalidixic acid induction) were harvested and resuspended in lysis buffer (4% of original culture volume) containing 50 mM Tris-HCl (5% base), 1 mM EDTA, 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 mM dithiothreitol, and 0.5 μg/ml lysozyme. After incubation at 25 °C for 20 min, deoxycholate (0.1% w/v) and DNPase I (10 μg/ml) were added, and the mixture was incubated at 37 °C for 30 min. The incubations were spun for 5 min at 15,000 × g, and an aliquot (2 μl) of each supernatant was assayed for Ub thiol ester formation and Ub synthesis (10).

Purification of Recombinant E2<sub>25K</sub>-E. coli expressing E2<sub>25K</sub> (1-liter culture) were harvested by centrifugation and resuspended in 12 ml of ice-cold buffer containing 50 mM Tris-HCl (5% base), 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. The suspension was passed through a French press with a precooled cell (American Instruments Inc.). All subsequent operations were carried out at 5 °C. The lysate was clarified by centrifuging for 60 min at 100,000 × g, and 0.2% protamine sulfate (pH 7.5) was added to the supernatant. After stirring for 30 min, the suspension was centrifuged at 12,000 × g for 10 min. The supernatant, containing 580 mg of protein, was applied to a 25-ml column of Pharmacia QA beads (Pharmacia LKB Biotechnology, Inc.) that had been equilibrated with buffer A (50 mM Tris-HCl (5% base), 1 mM EDTA, 1 mM dithiothreitol). The loaded column was washed with 75 ml of buffer A and then eluted with 85 ml of buffer A containing 0.2 M KCl. The latter eluate was subjected to ammonium sulfate fractionation; proteins precipitating between 50 and 65% saturation were collected by centrifugation, resuspended in buffer A, and dialyzed exhaustively against buffer A. An aliquot containing 5 mg of protein was loaded onto a fast protein liquid chromatography anion exchange column (Mono Q, Pharmacia LKB), eluted with a linear gradient of KCl as described previously (10). Fractions containing E2<sub>25K</sub> were pooled and further fractionated on a fast protein liquid chromatography gel filtration column (Superose 12, Pharmacia LKB). The peak of E2<sub>25K</sub> was electrophoretically homogeneous (data not shown). This protein was used for the single-turnover and mutabiquestimation experiments shown in Fig. 6.

RESULTS

Cloning and Sequencing of an E2<sub>25K</sub>-encoding cDNA—We used an efficient procedure to achieve rapid PCR amplification of a full-length E2<sub>25K</sub>-encoding cDNA (Fig. 1; see "Experimental Procedures," B, PCR amplification of full-length cDNA. Primers B1 and B2 (Fig. 2 and Table I) were based on the sequence of the initial 146-bp product, as indicated in Table 1.

The underlined sequences in Fig. 2 exactly match peptide sequences obtained by direct protein sequencing (Fig. 1A). This discrepancy is most likely to reflect the action of a carboxypeptidase on bovine E2<sub>25K</sub> prior to, or during, its isolation, since close scrutiny of the amino acid sequence data provided no evidence for any amino acid at this position of Peptide 2 (data not shown). Although the 5'-untranslated region is short, the coding region shown in Fig. 2 must be complete, since the encoded protein, when expressed in E. coli, co-migrated on SDS gels with authentic bovine E2<sub>25K</sub> (not shown) and, moreover, exhibited identical catalytic activities to the native enzyme (see Figs. 5 and 6, below). The amino acid composition predicted by the sequence shown in Fig. 2 agrees with that determined experimentally for bovine E2<sub>25K</sub> prior to, or during, its isolation.
The sequences of Peptides 1 and 2 are shown in Fig. 1A; for cDNA sequences, see Fig. 2.

Table I

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>A1</td>
<td>5'-CTGGTGGCGC, AT, CTCAC(TGTG, A, T, C)TA(T, C)G-3'</td>
<td>Based on Peptide 1 (LWAHVYA)</td>
</tr>
<tr>
<td>A2</td>
<td>3'-ACCCTGGA, AIC(TGTG, T, C)GGTGTG-5'</td>
<td>Based on Peptide 2 (WDVETATE)</td>
</tr>
<tr>
<td>A3</td>
<td>5'-GGTGGACGACTTCCCTGAACCGG-3'</td>
<td>Outer vector primer</td>
</tr>
<tr>
<td>A4</td>
<td>3'-GTAAATGCTCAACCAAGACCCAGTT-5'</td>
<td>Outer vector primer</td>
</tr>
<tr>
<td>B1</td>
<td>5'-GCCCACTTCTTCCAGAGATGGA-3'</td>
<td>Nucleotides 463-485, Fig. 2</td>
</tr>
<tr>
<td>B2</td>
<td>3'-GCCATTATCCCAGGAACACAGT-5'</td>
<td>Nucleotides 533-555, Fig. 2</td>
</tr>
<tr>
<td>C1</td>
<td>5'-GGGACCTTGGCAACATCG-3'</td>
<td>Underline = NcoI, nucleotides 7-7 to +13</td>
</tr>
<tr>
<td>C2</td>
<td>3'-GATCCCGTGTGAACACTT-5'</td>
<td>Underline = BamHI, nucleotides 725-744</td>
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</table>

Fig. 2. E225K cDNA and deduced amino acid sequences. The underlined amino acid sequences correspond to the Lys-C peptides shown in Fig. 1A, right. The 2 Cys residues are indicated in blocks; the wavy line in the 3'-untranslated region corresponds to a poly(A) addition signal.

Fig. 3. Homology of E225K amino acid sequence with sequences of other E2s. A, active site region. The arrowhead indicates the Cys residue most likely to be involved in Ub thiol ester formation (see text). Other E2 sequences are taken from the literature (19-21, 23; human 17K is an apparent human homolog of yeast RAD6 (44) and wheat 25K is a wheat germ E2 of unknown biological function (45). B, extended comparison with proteins encoded by UBC1 and UBC5 genes of S. cerevisiae (19, 20). Boxed residues are identical between two or more proteins; shaded residues are identical among all three proteins. Conservative substitutions are indicated by unboxed capital letters. Alignments and assignments of conservative substitutions were obtained by computer analysis using the program GAP (26, 27). The region between the arrowheads is discussed in the text.

Indeed, the amino acid sequence homology between E225K and other E2s extends beyond the region of the active site (Fig. 3B, below). We had anticipated that E225K would exhibit some similarity to yeast RAD6 at the amino acid sequence level,
since E2/subK reacts with several different anti-RAD6 antibodies (28). Computer analysis using the program GAP (26, 27) indicated that the amino acid sequences of RAD6 and E2/subK were 28% identical; taking into account conservative substitutions, the similarity was 51% (data not shown).

Among known E2s, however, the putative E2/subK active site most closely resembles the corresponding sites of the proteins encoded by the UBC1, UBC4, and UBC5 genes of the yeast S. cerevisiae (19, 20). As shown in Fig. 3A, E2/subK and UBC1 protein are 90% identical (18 of 20 residues) in the region surrounding the active site Cys. With the introduction of a single 1-residue gap, this region of the E2/subK sequence exhibits 84% and 79% identity, respectively, with the corresponding regions of the UBC5 and UBC4 proteins. The sequences of the latter two yeast E2s are nearly identical, and the proteins apparently have redundant functions (21).

The similarity between E2/subK, UBC1, and UBC5 extends well beyond the putative active sites of these proteins (Fig. 3B). There are 52 identical residues shared among all three proteins (Fig. 3B, shaded residues), with 80 identities between the UBC1 and E2/subK sequences and 66 identities between the UBC5 and E2/subK sequences. Taking into account conservative substitutions, E2/subK and UBC1 are 66% similar, and E2/subK and UBC5 are 59% similar (Fig. 3B). The similarity between E2/subK and UBC1 is greatest in a very extended region surrounding the putative active site Cys residue of each protein; in the 90-residue segment delimited by the arrowheads in Fig. 3B, E2/subK and UBC1 are 63% identical and 81% similar. E2/subK and UBC1 are most different in their COOH-terminal regions, where there are many fewer identical, and significantly fewer similar, residues (Fig. 3B). UBC1 also has a 22-residue COOH-terminal extension relative to E2/subK and UBC5 (200 residues) and UBC1 (215 residues) both have such extensions relative to the much shorter UBC4 and UBC5 proteins (148 residues).

Northern Analysis—Northern blot analysis was conducted using poly(A*) RNA isolated from murine erythroleukemic cells, since the RNA in commercially obtained bovine thymus was found to be largely degraded. Murine erythroleukemic cells are known to contain abundant E2/subK (29). As shown in lanes 1 and 2 of Fig. 4, murine erythroleukemic cells contain a prominent poly(A*) mRNA of ~1.2 kb that hybridized strongly with the E2/subK cDNA probe. A minor hybridizing band at 2.5 kb (with an intensity about 10% that of the 1.2-kb band) may represent a second E2/subK-encoding mRNA, a precursor of the 1.2-kb mRNA, or a cross-hybridizing mRNA that encodes a closely related E2. In support of the first two possibilities was the finding that the library contained at least two E2/subK-encoding cDNAs with identical coding sequences but with 3‘-translated regions of different lengths (see “Results,” above).

Overexpression of Functional E2/subK in E. coli—The E2/subK-encoding cDNA was subcloned into the expression vector PotsNco (24), and the resulting vector (Pots-E2/subK) was used to transform E. coli (see “Experimental Procedures”). When cells harboring this vector were induced with nalidixic acid (nald), as indicated. Cells were harvested, and crude lysates were prepared using deoxycholate as described under “Experimental Procedures.” Aliquots (2 μl) of lysates prepared from cells grown under the indicated conditions (± inducer) were incubated in a volume of 10 μl with added rabbit reticulocyte E1 (100 nM) and 125I-UB (0.5 μM; 9500 cpm/μM). Also present were (pH 7.3, 37°C): 50 mM Tris-ClH (20% base), 5 mM MgCl2, 2 mM ATP, 10 mM creatine phosphate, and 0.6 unit/ml each of creatine kinase and inorganic pyrophosphatase. Lanes 1 and 3, no other additions, assays were quenched at 3 min. Lanes 2 and 4, after 3 min, unlabeled Ub (12 μM) was added and the incubation was continued for 1 h more. Lanes 5 and 6, control in which 1 μM bovine E2/subK replaced the E. coli lysate. Lane 5, conditions as in lanes 1 and 3; lane 6, conditions as in lanes 2 and 4. All reactions were quenched with thiol ester sample buffer (10) prior to SDS-polyacrylamide gel electrophoresis on gels containing 12.5% acrylamide. Details of the assay are described in Ref. 10. (E1-UB, Ub adduct of added E1; 25K-UB, Ub adduct of E2/subK).

expressed E2/subK was in the soluble fraction of a crude E. coli lysate. As expected, production of E2/subK was observed only following induction with nalidixic acid (Fig. 5, lanes 1 and 2 versus lanes 3 and 4).

To test whether the expressed protein was fully active, we purified recombinant E2/subK to electrophoretic homogeneity (“Experimental Procedures”), and compared its kinetic properties to those of the native enzyme purified from bovine thymus (10). In the single-turnover experiments shown in Fig. 6A, the labile Ub adduct of E2/subK was formed during a
induction of *E. coli* harboring the vector Pots-\(E_{225K}\) with nalidixic acid, \(E_{225K}\) typically represented 5–10% of the soluble protein (data not shown). Purification of large quantities of recombinant \(E_{225K}\) should thus be straightforward, since the scheme that we used successfully on a small scale ("Experimental Procedures") avoids the use of covalent Ub affinity chromatography. The latter method, even though it is extremely powerful (1, 2), would require large quantities of \(E1\), and thus would limit the scale of \(E_{225K}\) purification.

**DISCUSSION**

We utilized a rapid and efficient procedure for cloning a bovine cDNA that encodes the mult ubiquitinating enzyme, \(E_{225K}\). The method, originally suggested by Frohman (31), avoids laborious screening of a cDNA library. It has a high probability of yielding a full-length cDNA, since at several stages there is an opportunity to select and proceed with larger cDNA fragments. The major disadvantage of this approach is its high error rate, which results from lack of proofreading by Taq polymerase (40). As described under "Results," this problem was overcome by sequencing several clones. Overall, this approach should be widely applicable for cDNA cloning whenever degenerate primers can be designed based on partial peptide sequences or consensus protein sequences.

The 200-residue protein encoded by the \(E_{225K}\) cDNA shares sequence homology with all known \(E2s\) (Fig. 3 and data not shown). The strong conservation of sequences surrounding the putative active site Cys residues of known \(E2s\) (Fig. 3A), including \(E_{225K}\), are indicative of a strong selective pressure. This may result in part from the need for multiple \(E2s\) to interact with common and conserved pathway component(s). For example, mammalian cells, as well as the yeast *S. cerevisiae*, appear to have only a single \(E1\)-encoding gene (41, 42). Since in such cells all \(E2s\) must form labile Ub adducts via interaction with the same \(E1\), there may well be a conserved \(E2\) domain that mediates interaction with \(E1\). This domain might include the region surrounding the \(E2\) Cys residue that accepts Ub from the \(E1\)-Thioester adduct. However, the apparent lack of high-affinity interactions between unactivated \(E1\) and unactivated \(E2s\) (see, for example, Refs. 2, 11, and 13) suggests that \(E1\)-\(E2\) interactions may be critically affected by the presence or absence of covalently bound Ub on one or both enzymes.

Both in the region of the active site Cys residue and overall, the sequence of \(E_{225K}\) is most closely related to the sequences of the \(E2s\) encoded by the *UBC1*, *UBC4*, and *UBC5* genes of the yeast *S. cerevisiae* (Fig. 3, A and B). The results of molecular genetic analyses indicate that the latter enzymes function in protein degradation (19, 20). The *UBC4* and *UBC5* genes encode a pair of closely related 16-kDa \(E2\) proteins; mutants in which both genes are disrupted fail to degrade a large fraction of short-lived and abnormal proteins and exhibit low Ub-protein conjugate levels under all conditions; they also show impaired survival under conditions of thermal and nutritional stress (20). Deletion of the *UBC1* gene results in a modest slow growth phenotype, with a more severe (but transient) impairment of growth following germination of spores (19). The latter result suggests that *UBC1* may have a predominant function in degradation of proteins in the early stages of growth following germination. Results of experiments in which multiple *UBC* genes were disrupted indicated that the functions of the *UBC1*, *UBC4*, and *UBC5* genes products partially overlap (19, 20). Thus, overexpression of *UBC1* in yeast cells lacking functional *UBC4* and *UBC5* partially restores rapid turnover of short-lived and abnormal

FIG. 6. Quantitative catalytic equivalence of native bovine and recombinant forms of \(E_{225K}\). The recombinant protein was purified as described under "Experimental Procedures"; the bovine protein was prepared as before (10). A, Ub\(_2\) synthesis (autoradiography). Separate pulse incubations for the recombinant and native proteins, conducted simultaneously (pH 7.3, 37 °C, 90 μl), contained 50 mM Tris-HCl (20% base), 0.8 μM \(E_{225K}\), 90 nM \(E1\), 0.4 μM \(^{125}\text{I}-\text{Ub}\), 5 mM MgCl\(_2\), 2 mM ATP, 10 mM creatine phosphate, and 0.6 unit/ml each of creatine kinase and inorganic pyrophosphatase. After 3 min, EDTA (10 mM, pH 7.0) was added to complex the Mg\(^{2+}\). Unlabeled Ub, 12 μM, was then added to initiate the chase. Aliquots (10 μl) were withdrawn at timed intervals and quenched with thiol ester sample buffer. Samples were electrophoresed in 12.5% gels, followed by autoradiography. Lanes 1–7, native protein; lanes 8–14, recombinant protein. Lanes 1 and 8, 0 min (before chase); lanes 2 and 9, 6 min; lanes 3 and 10, 20 min; lanes 4 and 11, 35 min; lanes 5 and 12, 60 min; lanes 6 and 13, 90 min; lanes 7 and 14, 120 min. A small amount of \(^{125}\text{I}-\text{Ub}\) does not get activated in the pulse incubation; a small amount of Ub\(_1\), faintly visible in lane 7, is formed during the chase, probably as a result of transfer to the Ub\(_1\) product (10). B, kinetic analysis. The bands corresponding to the \(E_{225K}\) adducts (panel A) were excised from the dried gels and counted. *Open circles*, recombinant protein; *filled circles*, native (bovine) protein. C, multi-Ub chain synthesis (autoradiography). Separate incubations for the native and recombinant proteins were carried out under the conditions described in A, except that 12 μM \(^{125}\text{I}-\text{Ub}\) (730 cpm/pmol) was continuously present, together with 95 nM \(E1\) and 1 μM \(E_{225K}\). Aliquots (16 μl) were withdrawn at timed intervals, quenched with 10 μl of normal SDS sample buffer (containing mercaptoethanol; Ref. 10), and heated to 100 °C prior to electrophoresis. The dried gel was autoradiographed. Lanes 1–5, native proteins; lanes 6–10, recombinant protein. Lanes 1 and 6, zero time; lanes 2 and 7, 30 min; lanes 3 and 8, 1 h; lanes 4 and 9, 2 h; lanes 5 and 10, 4 h.

brief pulse with highly labeled \(^{125}\text{I}-\text{Ub},\) MgATP, and \(E1\). EDTA was then added to prevent further adduct formation. Transfer of activated \(^{125}\text{I}-\text{Ub}\) to unlabeled Ub (12.2 μM) was monitored by SDS-polyacrylamide gel electrophoresis and autoradiography (10). The results obtained with native and recombinant \(E_{225K}\) were identical (Fig. 6, A and B), yielding \(k_d = 1280 \text{ M}^{-1} \text{ min}^{-1}\); this value is within a factor of 2 of that reported previously (10). The native and recombinant proteins also exhibited virtually identical kinetics in multi-Ub chain synthesis (Fig. 6C). Overall, the results of these quantitative analyses (Fig. 6) demonstrate that the cloned cDNA directs the synthesis of a 25-kDa \(E2\) that is structurally and functionally equivalent to bovine thymus \(E_{225K}\). Following
proteins (19). Mutants in which the UBC1 and UBC4 genes are disrupted are viable in mitotic growth but do not survive following sporulation and germination (19). Mutant cells in which all three of these genes have been disrupted are not viable under any conditions (19). The functional relationship between the mammalian E2 \( E_{225} \) and these three yeast E2s is not yet clear, although the multisubunitisquating activity of the \( E_{225} \) (10) is suggestive of a role in intracellular proteolysis in mammalian cells. Whether \( E_{225} \) can complement any of the phenotypes of yeast deletion mutants (above (19, 20)) remains to be determined; nor is it known whether any of the purified yeast E2s can synthesize multi-Ub chains from isolated Ub. Structural differences between \( E_{225} \) and UBC1 protein are most apparent in the COOH-terminal regions of the two proteins; these differences include the presence of a 22-residue COOH-terminal extension in UBC1 (Fig. 3B).

Recombinant \( E_{225} \) exhibits quantitatively identical properties with its native bovine counterpart in Ub synthesis and in multi-Ub chain synthesis (Fig. 6). Our likely ability to express and purify milligram quantities of \( E_{225} \) (see “Results”) will facilitate future investigations of the structural basis of the enzyme’s multisubunitisquating activity. In addition, the availability of large quantities of \( E_{225} \) will provide a ready enzymatic route to the production of large quantities of Lys-48-linked Ub oligomers. In preliminary experiments, we have used 100-μg quantities of recombinant \( E_{225} \) to synthesize 1-nmol quantities of Ub and Ubs. These products will be useful for structural characterization of the oligomers themselves; in addition, they may be used to probe the mechanisms and specificities of enzymes that bind to, or act on, such Ub oligomers. These enzymes include a number of isopeptidases (10, 43) and possibly the conjugate-specific protease (4, 5) as well.

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References

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