Molecular cloning, expression and characterization of a ubiquitin conjugation enzyme (E2_{17kD}) highly expressed in rat testis

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Ubiquitin-conjugating enzymes (E2s) play a key role in ubiquitin-mediated proteolysis by catalysing the conjugation of ubiquitin to protein substrates. We have previously reported the cDNA cloning of a 14 kDa conjugating enzyme [E2_{14k}; Wing, Dumas and Banville (1992) *J. Biol. Chem.* 267, 6495–6501] that efficiently supported ubiquitination and protein degradation in reticulocyte extracts. Surprisingly, the structure of this E2 was markedly more similar to the *Saccharomyces cerevisiae* DNA repair gene RAD6, than to the *S. cerevisiae* UBC4/UBC5 genes which are required for the degradation of short-lived proteins and support much of the ubiquitination of yeast proteins. This suggested that mammalian homologues of UBC4/UBC5 remained to be identified. Using oligonucleotides derived from the *S. cerevisiae* UBC4 sequence as primers in a PCR reaction with rat muscle cDNA as a template, a 390 bp DNA fragment was amplified which predicted an amino acid sequence that was 83 % identical to yeast UBC4. Screening a rat testes cDNA library identified a family of cDNAs which predicted two very similar proteins with basic pIs

and molecular masses of approx. 16700 Da. Isoform 2E was expressed in *Escherichia coli* and purified to homogeneity. It supported ubiquitination to reticulocyte and testis proteins more rapidly *in vitro* and produced larger conjugates than E2_{14k}. Examination of RNA from different tissues indicated that this type of E2 was expressed in a broad spectrum of tissues but at particularly high levels in the testis. Fractionation of a testis extract by anion-exchange chromatography identified several putative ubiquitin protein ligase activities with which this E2 could interact in promoting conjugation of ubiquitin to proteins. One of these activities supported conjugation of ubiquitin to histone H2A, a substrate degraded in the ubiquitin system by a non-N-end rule mechanism. This paper reports the first cloning of an apparent mammalian homologue of *S. cerevisiae* UBC4/UBC5. Its high expression in testis and ability to efficiently support conjugation to testis proteins suggest that this family of E2s may play a role in the proteolysis that occurs during spermatogenesis.

INTRODUCTION

A major function of ubiquitin is its ability, when covalently bound to proteins via an isopeptide linkage, to target these proteins for recognition and degradation by the 26S proteasome [1]. This covalent linkage of ubiquitin occurs between the carboxy terminus of ubiquitin and the ϵ -amino groups of lysine residues in the target proteins. Proteins targeted for degradation are generally multi-ubiquitinated with many of the ubiquitin moieties actually linked to lysine residues of ubiquitin moieties already attached to the protein [2]. Ubiquitination of proteins occurs in a multi-step process [3]. Ubiquitin is first activated by ubiquitin-activating enzyme (E1) in a reaction which results in hydrolysis of ATP to AMP and pyrophosphate and in formation of a thiolester linkage between ubiquitin and E1. The activated ubiquitin is then transferred to a cysteine residue of one of a family of ubiquitin-conjugating enzymes (E2s; ubiquitin carrier proteins) [4] which then ligates the ubiquitin to the protein substrate either directly or with the requirement of a third protein, ubiquitin protein ligase (E3). E3, when required, appears to be important in recognition and binding of the protein substrate [5].

In this sequence of reactions, E2s appear to play key roles in determining the physiological consequences of ubiquitin conjugation. A diverse family of E2s exist (reviewed in [4]) which can be subclassified based on structural and functional differences. Structurally, all E2s share a conserved core domain of

approx. 16 kDa containing the cysteine residue required for the thiolester linkage with ubiquitin. Some E2s, designated class I, consist solely of this core domain and appear to require the presence of an E3 to support conjugation of ubiquitin. However, other E2s contain extensions at the carboxy (class II) or amino (class III) termini and these extensions have been suggested to play roles in substrate recognition [4] and can permit conjugation of ubiquitin to some proteins in an E3-independent manner [6,7]. Functionally, these enzymes show differences by both biochemical and genetic analysis. Biochemically, some such as rabbit reticulocyte E2_{14k} [8] and wheat germ E2_{15k} [9] can ubiquitinate endogenous proteins in their respective extracts. Other E2s, such as rabbit E2_{20k} and E2_{32k}, bovine E2_{25k}, wheat germ E2_{20k} and E2_{23k}, demonstrate more selective substrate specificity. Although unable to support efficient ubiquitination of endogenous proteins *in vitro*, E2_{20k}, E2_{32k} [6,7,10] and wheat germ E2_{23k} [11] are able to ubiquitinate histones. E2_{25k} [12] and wheat germ E2_{20k} [13] have the distinctive ability to form multi-ubiquitin chains *in vitro*.

Genetic analyses using *S. cerevisiae* have indicated a diverse array of functions for E2s. For example, loss of UBC2 (RAD6) function leads to defects in DNA repair, induced mutagenesis, and sporulation [14]. UBC3 (CDC34) is essential for the G1 to S transition in the cell cycle [15]. UBC10 is essential for peroxisome biogenesis [16]. The UBC1/UBC4/UBC5 family of genes plays an important role in selective proteolysis. Loss of UBC4/UBC5 genes results in impaired degradation of short-lived and abnormal

Abbreviations used: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin protein ligase; DTT, dithiothreitol; AMPNP, 5'-adenylyl imidodiphosphate.

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The sequences of clones 10A, 2E, and 4A reported in this paper have been submitted to Genbank under accession numbers U13175, U13176 and U13177 respectively.

proteins and a marked decrease in ubiquitination of cell proteins [17]. UBC1 deficiency leads to impaired growth after germination [18].

A clearer understanding of the functional and mechanistic aspects of the ubiquitin system can be obtained by integrating the genetic analyses in yeast with the biochemical analyses performed largely in plant and mammalian systems. Furthermore, such integration can permit development and testing of hypotheses regarding the physiological functions of these enzymes in mammalian systems. For example, we have been interested in identifying components of the mammalian ubiquitin-conjugating system that may be involved in the degradation of a broad spectrum of substrates such as that which occurs in tissues atrophying under catabolic conditions or in certain developmental processes characterized by marked loss of protein such as erythrocyte formation from reticulocytes or spermatogenesis. Reticulocyte extracts contain a highly active ubiquitin-dependent proteolytic system. Early studies of ubiquitin conjugation in this system have indicated that E2_{14k} best supports ubiquitination of reticulocyte proteins and proteolysis in this extract [8]. Because of this E2's apparent importance in the proteolytic functions of ubiquitin and its relatively broad substrate specificity, we have previously undertaken the molecular cloning of this enzyme [19]. Surprisingly, this E2 was structurally very similar (69% amino acid identity) [19] and functionally homologous to the yeast DNA repair gene UBC2 (RAD6) [20]. Interestingly, the primary structure was relatively dissimilar (39% amino acid identity) to the two yeast E2s, UBC4/UBC5, known to be required for degradation of short-lived proteins and for formation of a large part of the cell's steady-state pool of conjugates [17]. Since mammalian homologues of these two important yeast genes may have similar broad functions, it has been of significant interest to identify such homologues. We now report the molecular cloning and biochemical characterization of a family of rat E2 genes, designated E2_{17kB} (this enzyme has been designated as E2_{17kB} in keeping with the custom of naming mammalian E2s according to size and to distinguish it from another 17 kDa E2 previously identified in rabbit reticulocyte preparations but clearly distinct from E2_{17kB} as it does not support conjugation to endogenous proteins [21]) with strong sequence homology to UBC4/UBC5. These enzymes catalyse more efficient ubiquitination to reticulocyte proteins than E2_{14k}. Furthermore, these enzymes appear to be highly expressed in testis, one of the few tissues in the adult mammal demonstrating ongoing high rates of protein degradation, and can support ubiquitination of endogenous proteins in this tissue.

EXPERIMENTAL

Cloning of cDNAs encoding E2_{17kB}

RNA PCR was used to identify expression of a rat sequence potentially homologous to *S. cerevisiae* UBC4/UBC5. cDNA was synthesized from total rat muscle RNA (2 µg) in a 20 µl reaction using reverse transcriptase (Gibco-BRL Superscript Preamplification kit) according to the supplier's protocol and oligo dT (0.5 µg) as a primer. The cDNA (5 µl) was then used as template in a PCR reaction containing as primers, oligonucleotides 5'-GATCC(T/A)CCT(A/G)CTTCATGTTTCAGC(C/A)GG-3' corresponding to bases 49–75 and 5'-A(A/T)ACAGC(A/G)-TATTT(T/C)TT(A/T)GTCCA(T/C)TC-3' complementary to bases 421–445 of the UBC4 coding sequence [17]. Annealing was carried out at 45 °C, extension at 72 °C for 1 min and 25 cycles were performed.

The amplified DNA fragment was subcloned into pTZ18R (Pharmacia) and sequenced using the dideoxy chain termination

method and T7 polymerase (Pharmacia). Since the predicted protein sequence indicated marked similarity to UBC4/UBC5, the PCR-amplified DNA fragment was labelled with ³²P and used as a probe to screen a rat testis cDNA library in the λzapII vector (Stratagene). An aliquot containing 1 million recombinants was screened by transfer of plaques to nitrocellulose membranes and hybridization with the probe [22]. Purified positive phage were grown and the pBluescript plasmid containing the insert excised from the phage according to the manufacturer's instructions. The inserts in the plasmids were sequenced using the dideoxy chain termination method with either T7 (Pharmacia) or *Taq* polymerase (Promega). Sequence analyses were carried out using the PCGENE software package (Intelligenetics).

RNA blot analysis

Total RNA was prepared from tissues from male Sprague-Dawley rats (body wt. approx. 100 g; Charles River Laboratories) by the guanidium isothiocyanate/CsCl method [23]. RNA blots were prepared by electrophoresing 10 µg of RNA in 1% agarose containing 1.1% (v/v) formaldehyde followed by capillary transfer on to nylon membranes (Bio-Rad) and u.v. cross-linking of the RNA to the membrane. The membrane was hybridized at 65 °C with the ³²P-labelled PCR-amplified fragment in 50 mM Tris, pH 7.5/1 M NaCl/0.1% (w/v) sodium pyrophosphate/1% (w/v) SDS/0.2% polyvinylpyrrolidone (40000)/0.2% Ficoll (400000)/0.2% BSA/100 µg/ml salmon testes DNA. Washing of the membrane was conducted at the same temperature in 2 × SSC (SSC = 150 mM NaCl/15 mM sodium citrate)/0.1% SDS and the membrane was then subjected to autoradiography.

Expression of E2_{17kB} cDNA in *E. coli*

To characterize this family of E2s biochemically, one of the cloned cDNAs was expressed in *Escherichia coli* using the T7 promoter in the pET11D expression vector (Novagen). The insert for clone 2E was excised from the pBluescript plasmid with *Nco*I and *Eco*RV and ligated into the pET11D vector which had been digested with *Bam*HI, blunt-ended with Klenow polymerase, and then digested with *Nco*I. The plasmid was then transformed into the BL21 (DE3) host strain which is a lysogenic strain containing the bacteriophage T7 RNA polymerase gene under control of the *lac* repressor. The bacteria were grown at 37 °C in Luria broth with ampicillin to an A₆₆₀ of between 0.6 and 1.0. The culture was then cooled to 30 °C and induced with 1 mM isopropyl β-D-thiogalactopyranoside. The bacteria were harvested 3 h later by centrifugation at 1200 g for 20 min.

Purification of recombinant E2_{17kB}

Bacteria expressing E2_{17kB} from 800 ml of culture were prepared as described above. Subsequent procedures were carried out at 4 °C. Following washing of the cells with PBS, the cells were resuspended in 80 ml of 50 mM Tris, pH 7.5 (at 4 °C)/1 mM dithiothreitol (DTT)/5 mM EDTA and lysed by sonication. The lysate underwent centrifugation at 100000 g for 1 h. The supernatant was fractionated with (NH₄)₂SO₄ (ICN Ultrapure). The 40–75% fraction was resuspended in and dialysed against 50 mM Tris, pH 7.5/1 mM DTT. This was passed through a 5 ml DEAE-cellulose column (Whatman DE-52) equilibrated with 50 mM Tris, pH 7.5/1 mM DTT. The flow through was collected, made to 20 mM Mes with 0.5 M Mes, pH 6.2, and dialysed against

20 mM Mes, pH 6.2/1 mM DTT. The dialysed material was then passed through a 5 mm × 100 mm propylsulphonic acid cation-exchange column (Waters SP/HR15) equilibrated with 20 mM Mes, pH 6.2/1 mM DTT. Bound proteins were then eluted with the same buffer containing a gradient of NaCl (0–0.3 M, 10 mM/min). Fractions (1 ml) containing E2 activity were neutralized by making them to 50 mM Tris with 2 M Tris/HCl, pH 7.8, prior to freezing. This was found to prevent significant loss of activity from freeze-thawing.

Preparation of testis extracts

All procedures were carried out at 4 °C or on ice. Testes from Sprague-Dawley strain rats (body wt. 250 g; Charles River Laboratories) were sliced and homogenized in 5 vol. of ice-cold 50 mM Tris, pH 7.5 (at 4 °C)/1 mM DTT. Following centrifugation of the homogenate at 10000 *g* for 15 min, the supernatant was centrifuged at 100000 *g* for 1 h. Fraction II was prepared by passing the final supernatant over a DEAE-cellulose (Whatman DE-52) column (bed volume equal to volume of supernatant) equilibrated in the same buffer. Following washing with 2.5 column vol. of the equilibration buffer, bound protein was eluted with 3 column vol. of 50 mM Tris, pH 7.5/0.5 M NaCl/1 mM DTT. The eluate was dialysed against 50 mM Tris, pH 7.5/1 mM DTT/20% glycerol.

To identify potential E3 activities, testes were homogenized as above but in 50 mM Tris/Mes, pH 7.5/1 mM DTT. The 100000 *g* supernatant (24 mg) was applied to a 5 mm × 50 mm quaternary amine anion-exchange column (Pharmacia MonoQ) equilibrated with the homogenization buffer. Bound proteins were eluted with a 0–0.5 M NaCl gradient (10 mM/min in 50 mM Tris/Mes, pH 7.5/1 mM DTT) and fractions assayed for their ability to support E2_{17kB}-dependent conjugation as indicated below. The unbound material was adjusted to pH 6.5 with 0.5 M Mes and applied to a 5 mm × 100 mm propylsulphonic acid cation-exchange column (Waters SP/HR15). Bound proteins were eluted with a NaCl gradient as for the anion-exchange column but at pH 6.2. The unbound material was adjusted to pH 7.5 with 2 M Tris, concentrated to 0.6 ml and chromatographed on a gel filtration column (Pharmacia Superose 12), equilibrated and eluted with 50 mM Tris, pH 7.5, and 1 mM DTT.

Biochemical assays

Preparation and quantification of enzymes

E1 was purified from rabbit reticulocytes as previously described [21]. Recombinant E2_{14k} was produced and purified as previously reported [19]. The E1 and E2 enzymes were quantified by measuring the initial release of radioactive pyrophosphate following incubation in the presence of [γ -³²P]ATP and ubiquitin [24]. Fraction II (fraction of lysate-containing components of the ubiquitin system, but lacking ubiquitin) and the 0–30% (NH₄)₂SO₄ pellet of fraction II were prepared as previously described [21].

Iodination of proteins

Bovine ubiquitin (Sigma) was labelled with Na¹²⁵I to a specific radioactivity of 10000 c.p.m./pmol as described previously [25]. Histone H2A (Boehringer) was iodinated to a specific radioactivity of approx. 300000 c.p.m./μg by the chloramine-T method. Unincorporated iodine was removed by passing the reaction products over a 0.8 cm × 25 cm Sephadex G-25 column.

Thiolester assays

E2s covalently bound to ubiquitin in thiolester linkages were detected by incubating the enzymes in the presence of 50 mM Tris, pH 7.5/1 mM DTT/2 mM MgCl₂, 2 mM ATP/30–50 nM E1, and inorganic pyrophosphatase (20 units/ml). Following incubation at 37 °C for 1 min, the reaction was stopped with Laemmli sample buffer without 2-mercaptoethanol and electrophoresed at 4 °C on a 12.5% polyacrylamide/SDS gel. The gel was then dried and autoradiographed.

Conjugation assays

Assays were conducted in 50 mM Tris, pH 7.5/1 mM DTT/2 mM MgCl₂/2 mM 5'-adenylyl imidodiphosphate (AMPPNP)/30–50 nM E1/5 μM ¹²⁵I-ubiquitin. If radiolabelled substrates were used, then 25 μM unlabelled ubiquitin was present. AMPPNP was used in place of ATP to enhance the ability to visualize ubiquitin-protein conjugates since this analogue supported E1-mediated activation of ubiquitin which resulted in hydrolysis at the α-β phosphate, but did not support proteolysis which involves hydrolysis of ATP at the β-γ bond [26]. E2s were supplemented as indicated in the Figure legends. In some cases, 100 μM haemin, an inhibitor of conjugate degradation and isopeptidase activity [27] was also added. Fraction II, the 0–30% (NH₄)₂SO₄ fraction of fraction II previously shown to be devoid of E2 activity [21], and fractions from the MonoQ chromatography of the testes extracts were added as sources of E3 as indicated in the Figure legends. Reactions were incubated at 37 °C for 1 h unless otherwise indicated. Following quenching with Laemmli sample buffer and boiling, the samples were electrophoresed on polyacrylamide gels which were dried and autoradiographed. In some experiments, the ubiquitin incorporated was quantified by cutting the lanes of the gel and counting the radioactivity.

RESULTS

Identification of a sequence similar to *S. cerevisiae* UBC4/UBC5 expressed in rat muscle

To determine whether a UBC4/UBC5 homologue might be expressed in the rat, oligonucleotides derived from the UBC4 sequence (forward primer: residues 49–75; reverse primer: residues 421–445 of the coding sequence) were used in PCRs with rat muscle cDNA obtained by reverse transcription as template. Indeed, a fragment of 390 bp was amplified (Figure 1a) which, when sequenced (Figure 1b), contained an open reading frame which predicted an amino acid sequence with 83% identity to UBC4/UBC5.

Tissue expression of the putative UBC4/UBC5 homologue

In order to select an appropriate tissue cDNA library for use in screening for full length cDNAs, the levels of expression of this apparent UBC4/UBC5 homologue in various tissues were examined by probing an RNA blot prepared with total RNA from various rat tissues with the labelled DNA fragment (Figure 2). Major transcripts of 1.8 and 2.9 kb were detected. However, longer exposures revealed three other transcripts, 1.1, 1.4 and 3.9 kb in size. High levels of expression were seen in the testis, particularly of the 1.8 kb transcript. Low levels of expression were detectable in all tissues screened.

Cloning of cDNAs encoding rat UBC4/UBC5 homologue

To identify full-length cDNAs, the PCR-amplified DNA fragment was used as a probe to screen a cDNA library. A testis cDNA

Figure 1 PCR amplification of a rat DNA fragment with sequence similarity to *S. cerevisiae* UBC4

(a) Rat muscle RNA was reverse-transcribed and the cDNA was used as template in a PCR with oligonucleotides derived from two regions 346 bp apart in the *S. cerevisiae* UBC4 gene. The product was electrophoresed on a 2% agarose gel containing ethidium bromide and visualized with a u.v. transilluminator. (b) Sequence of the approx. 400 bp band identified in (a). Only the nucleotides between the two primers are shown. Translation of the sequence revealed an open reading frame, the sequence of which was 83% identical to the equivalent region of *S. cerevisiae* UBC4.

Figure 2 RNA blot of various rat tissues with a DNA fragment apparently homologous to *S. cerevisiae* UBC4

Aliquots (10 µg) of pools of RNA from the indicated tissues of five animals were electrophoresed on a 1% agarose gel and transferred to a nylon membrane. Following hybridization with the ³²P-labelled DNA fragment shown in Figure 1(a), the membrane was washed and autoradiographed.

library was chosen because of the high level of expression in this tissue. From 1 million recombinants screened, approx. 100 positive clones were identified. Subsequent screening and analysis by PCR and restriction enzymes revealed that these clones appeared to fall into three groups. The largest cDNA inserts in each group were sequenced (Figure 3). These inserts were 1.1 kb, 3 kb, and 2.5 kb in size for the 2E, 4A and 10A clones respectively. Open reading frames were identified. Because of the multiple cDNAs identified, complete sequencing only of the shortest cDNA clone, 2E was performed. For clones 4A and 10A, complete sequencing of the 5' ends were carried out. However, sequencing in the 3' direction was carried out only approx. 200 bp distal to the stop codon in the longest open reading frame. These open reading frames predicted amino acid sequences highly similar to that of clone 2E, indicating that these were

4A	TCGG	-201
4A	ATGCCGTTTCAGGTCGGAATCAGCAGGGTCTCTGAGCCTTGCGAGTGC	-151
4A	TGATCTGCTCGTTTGCAAAGGCCCTGTGTCTGCAGAGCCGGTGTGAGAA	-101
10A	-----	
2E	-----AATCCGCGCCGGCCCTTCCCGG	-51
4A	GAAGAGAGAATCCTTCCGCGCCGGGAGTAATCAAGAGTTTGGCCGGA	
10A	-----	
2E	TCCCTCCCCGTCCTCCGTCCTCCGCGCCGGCCAGCCGACCGTTTCCCAAC	-1
4A	CCTTTGAGTACACATCGGGATAGTGAAGACCCGGCGAAAAGCACAGACT	
10A	-----	
2E	ATGGCTCTGAAGAGAATCCACAAGGAATTGAATGACCTGGCTCGAGATCC	50
4A	-----AC-G--TA-T--C-T-G--TT--C--T--C--	
10A	-----	
2E	TCCAGCACAGTGTTCAGCAGTCTCTGTTGGAGATGATATGTTTCATTGGC	100
4A	-----A-----T-----C-----	
10A	-----	
2E	AGGCTACAATAATGGGGCCAAATGACAGCCCTATCAGGTTGGAGTATT	150
4A	-A--C-----T-----A--T-----A-----T-----C	
10A	-----	
2E	TTCTTGACTATTCAATTTCCCAACAGATTACCCCTTCAAACGCCCTAAGGT	200
4A	--T-----A-----T--T-----C-----A-----	
10A	-----	
2E	TGCATTTACAACAAGAATTTATCATCCAAATATTAACAGTAATGGCAGCA	250
4A	-----	
10A	-----	
2E	TTTGTCTTGATATTCTACGGTCACAGTGGTCTCCAGCACTAACTATTTC	300
4A	-----A-A-----T--TT-----T	
10A	-----	
2E	AAAGTACTTTTGTCCATCTGTTCTCTGTTGTGTGATCCCAATCCAGATGA	350
4A	-----T-----A-----T-----A--C-----A--C-----	
10A	-----	
2E	TCCTTTAGTGCCTGAGATTGCTCGGATCTACAAAACAGATAGAGAAAAGT	400
4A	C--CC-----A-----A-----T-----C-----C-----	
10A	-----	
2E	ACAACAGAATAGCTCGGAATGGACTCAGAAGTATGCCATGTAATTAAAG	450
4A	-----T-----G--GCTAC	
10A	-----	
2E	AAATTATTGGATAACCTCTACAAATAAGCTAGGAGAACTCTGAAGAGA	500
4A	CTAAAGTCAGATAAACCCTGATTATAGCTGGAATAAATTTAAATTACT	
10A	-----	
2E	GAAAGTCCTTTTGATTTCATTGTGCTCTCTATGAGCCACCGGCTCT	550
4A	TTTGAGTTTATAATCTTTATTGAGCTTCTGTACAGAAGGGGTGTGACA	
10A	GTTCTCTTTTGTATTCTTATCCGGCTCTCCCTATCAGACCTCATCT	
2E	ATCTTCCCCTGTGCACATGTTTACCTGACACAGCAGTGTCTGCTGTTGT	600
4A	TTTGCCCTATGGTGAGTTAGTCTGGATGCGTGTCTCAGCCGAATGAG	
10A	TTTTTGGTTTTTGTTTTTTGTTCCTCCCTCATTCAATTCATGCTCAT	
2E	ACATACTTGGAAACAACAATACTAGAAATCTGTAATCTGTACCAACATTG	650
4A	AAGTAAATCTTAAACAGACACAGAAATAGAAAGGACCTGGGGATCACT	
10A	CTGAGAAGACCTAAGTCTTCCAGCTTTGGACATAAATGCTTTTAGAAA	
2E	CCTCTAGCAGAGAAGTGTGTGTGAGAAGCCAGTCTCTCCGGCGCTAAC	700
4A	AGCCAGCCCATTTATTTTCATGGAGGAAATGAAACCAAGAAAGGAGAAC	
10A	CTGTAAAGTAGTTACAAGAAG	
2E	CTAGGCCTATTTTCGATGTAAGGGTGAACATTCATTTGATGGAACACATTG	750
4A	GAAT	
2E	TGTATATTTAAAGACCCAGTGCTCTCTCGAGCTTGTACGTTCAAGAA	800
2E	GATTAATCTGTGTAATAAACTGATTACTACAGTCATTACGG	841

Figure 3 Nucleotide sequences of clones identified by probing a rat testis cDNA library with the DNA fragment apparently homologous to *S. cerevisiae* UBC4

Clone 2E was sequenced completely and all nucleotides are shown. Clones 4A and 10A were sequenced from the 5' end to a region distal to the stop codon. Numbering is from the beginning of the coding sequence, determined as the first methionine in the open reading frame. For simplicity of presentation, nucleotides identical to the previous line are shown as a (—).

almost certainly the correct reading frames. Clones 4A and 10A showed identical nucleotide sequences in this coding region. However, the 5' non-coding region was longer in clone 4A. The 3' non-coding regions of these two clones were divergent beginning 56 bp distal to the stop codon. The nucleotide sequence of clone 2E was very similar to 4A and 10A in the coding regions (87% identity), but clearly divergent in both 5' and 3' non-coding regions.

When these open reading frames were translated (Figure 4) they encoded proteins of approx. 16700 Da and were relatively basic with predicted pIs of 7.85. The deduced sequence of clone 2E differed from those of clones 4A/10A by only four amino

E2 _{17k} (2E)	MAL---KRIHKELNDLARDPPAQCSAGVGDMPHMQATIMGPNDSPLYQG	47
E2 _{17k} (4A/10A)	-----N---S-----	47
UBCD1	-----N---Q---G-----L-----P-----	47
ATHUBC8	--S-----L---K---QK---TS-----AE-----AE---S---	47
UBC4	-SSS-----A---S---E---TS-----LY-----S---A---A---	48
E2 _{14k}	-STPARR-LMRDFKR-QE---VGV-GA-SENNIMQ-N-V-F--EGT-FED	50
E2 _{17k} (2E)	GVFFLTIIHFPTDYPFKPPKVAFTTTRIYHPNINSNGSICLDILRSQWSPAL	97
E2 _{17k} (4A/10A)	-----	97
UBCD1	-----	97
ATHUBC8	--LV---P-----R-KVF-----KE-----	97
UBC4	---S-----IS---K-----A-N-----KD-----	98
E2 _{14k}	-T-K-V-E-SEE--N---T-R-LSKMF--VYAD-----QNR---TY	100
RABE2F1	IE-N--AE-----DEK	
E2 _{17k} (2E)	TISKVLLSICSLCDPNPDPLVPEIARIYKTDREKYN-RIAREWTQKYA	146
E2 _{17k} (4A/10A)	-----D-----S-----	146
UBCD1	-----EL-----R---	146
ATHUBC8	-----T-----HM-----A-E-AT--N-----	146
UBC4	-L-----T-A-----H-----P-E-AT-----K---	147
E2 _{14k}	DV-SI-T--Q---DE--NS-ANSQA-QL-QENKRE-EKRVSAIVEQSWN	150
RABE2F1	TDQ-IQ-LIA-VN--Q-EH--R	
E2 _{17k} (2E)	M- 147 (100%/100%)	
E2 _{17k} (4A/10A)	-- 147 (97%/99%)	
UBCD1	-- 147 (95%/97%)	
ATHUBC8	-G 148 (80%/87%)	
UBC4	V- 148 (80%/88%)	
E2 _{14k}	DS 152 (38%/54%)	
RABE2F1	(52%/67%)	

Figure 4 Comparison of the deduced amino acid sequences for clones 2E, 4A and 10A with sequences of E2s from *Drosophila* (UBCD1) [29], *A. thaliana* (ATHUBC8) [28], *S. cerevisiae* (UBC4), and rabbit reticulocytes (E2_{14k} and RABE2F1) [19,30]

Residue numbers are shown at the right of each line. At the end of each sequence, in parentheses, are shown the percentage amino acid identity/similarity when compared with E2_{17k} clone 2E.

Figure 5 Recombinant E2_{17k} (a) expressed in *E. coli* forms a thiolester linkage with ubiquitin; (b) conjugates ubiquitin to reticulocyte proteins more efficiently and produces larger molecular mass conjugates than E2_{14k}

(a) The insert in clone 2E was expressed in *E. coli* under control of the T7 promoter. Following induction of expression, thiolester assays were performed on the lysates as described in the Experimental section. Lysate from the expressing bacteria (pET2E) was incubated with ATP and ¹²⁵I-ubiquitin in the absence (–E1) or presence (+E1) of E1, but the reaction was quenched with sample buffer containing 2-mercaptoethanol (+ME). As a further control, lysate from bacteria transformed with the expression vector alone (pET) was also assayed as above in the presence of E1 and without exposure to 2-mercaptoethanol. Reaction products were electrophoresed on 12.5% polyacrylamide gels and autoradiography was performed following drying of the gels. (b) Conjugation assays were performed as described in the Experimental section with ¹²⁵I-ubiquitin, E1, AMPPNP, haemin, and 0–30% (NH₄)₂SO₄ fraction (2.8 mg/ml) in the absence or presence of recombinant E2_{14k} or E2_{17k} (each at 40 nM). Incubation times were 2 min as the assay was linear in this range. Reaction products were resolved from free ubiquitin by electrophoresis on 10% polyacrylamide gels and the dried gels were autoradiographed.

acids. The predicted sequences were highly similar to *S. cerevisiae* UBC4/UBC5. When the three sequences were compared to UBC4, there was 80% amino acid identity and 85% similarity when conservative substitutions were included. When compared with other cloned E2s (Figure 4), marked similarity was found only with a family of conjugating enzymes from the plant *Arabidopsis thaliana* [28] and with the *Drosophila* UbcD1 gene [29]. The basic pI of the proteins would suggest that it would not bind to an anion-exchange column and therefore be localized in fraction I if expressed in reticulocytes. When compared with sequences obtained from peptides derived from a reticulocyte E2 recently found in fraction I [30] (Figure 4), there was strong sequence similarity only in the conserved region containing the cysteine previously shown to be involved in the thiolester linkage with ubiquitin [31]. If the three peptides were analysed together, there was only 52% amino acid identity. When compared with the previously cloned E2_{14k} [19], only 38% identity was observed.

Expression of E2_{17k} in *E. coli* and purification to homogeneity

To characterize biochemically this family of conjugating enzymes, one of the cDNAs (2E) was expressed in *E. coli* using the strong T7 bacteriophage promoter. Upon incubation of the crude lysate in the presence of ATP, purified reticulocyte E1 and ¹²⁵I-ubiquitin, a labelled band of approx. 23 kDa was detected in the extract derived from the expressing strain (Figure 5a). Such an activity was absent in the host strain transfected with plasmid vector lacking the E2 cDNA insert. This band disappeared upon treatment of the reaction with 2-mercaptoethanol (Figure 5a), indicating that the ubiquitin was linked to the protein via a thiolester linkage and therefore confirming that this protein had ubiquitin carrier activity. Subsequent studies (see below) demonstrated the ability of this protein to support ubiquitin conjugation, thereby confirming that the cloned cDNA did encode an E2.

Figure 6 Purification of recombinant E2_{17kB}

Aliquots of protein from lysate (20 μ g), following $(\text{NH}_4)_2\text{SO}_4$ precipitation (20 μ g), DEAE-cellulose anion-exchange chromatography (DEAE, 7.5 μ g) and propylsulphonic acid (Prop. sul.; 5 μ g) cation-exchange chromatography were electrophoresed on a 15% acrylamide gel and stained with Coomassie Blue.

Initial purification involved precipitating a large fraction of the expressed protein with a 40%–75% $(\text{NH}_4)_2\text{SO}_4$ precipitation (Figure 6). This step concentrated the lysate and also removed a large molecular mass contaminant. Further purification was then obtained by passing the resuspended and dialysed pellet through a DEAE-cellulose anion-exchange column. Most of the bacterial proteins were retained while the E2 remained in the flow through as expected based on the predicted basic charge of the protein. Final purification to homogeneity (Figure 6) was obtained by passing the unbound material through a propylsulphonic acid cation-exchange column. The E2 was observed to

Figure 7 E2_{17kB} supports conjugation of ubiquitin to testis proteins

Conjugation reactions were performed as described in the Experimental section. (a) Fraction II from testis (12 mg/ml) was incubated in the presence or absence of AMPPNP, ^{125}I -ubiquitin, and purified recombinant E2_{17kB} (200 nM). (b) The 0–30% $(\text{NH}_4)_2\text{SO}_4$ fraction (2 mg/ml) of testis fraction II was incubated with ^{125}I -ubiquitin, E1, AMPPNP, in the presence or absence of recombinant E2_{14k} or E2_{17kB} (22 nM). Incubation time was 5 min, during which the assay remained linear.

Figure 8 Identification of putative testis E3 activities with which E2_{17kB} interacts in promoting conjugation of ubiquitin to endogenous proteins

Testis extract was fractionated on a MonoQ column. Bound proteins were eluted with NaCl. Aliquots (10 μ l) of the fractions (1 ml) were incubated in 25 μ l reactions as described in the Experimental section with ^{125}I -ubiquitin, E1 and AMPPNP in the absence or presence of E2_{17kB}. Reactions were electrophoresed on 10% polyacrylamide gels and the dried gels were autoradiographed. Lanes were cut out and radioactivity counted. E2_{17kB}-dependent incorporation of ubiquitin was determined by subtracting the counts incorporated in the absence of E2_{17kB}. Reactions from the three major peak fractions are shown above. E2_{17kB}-dependent conjugation activity of the material eluted by the NaCl gradient is plotted below. T, top of resolving gel.

elute at a NaCl concentration of approx. 0.15 M. The final yield of protein was approx. 6 mg/l of bacterial culture.

E2_{17kB} supports ubiquitination of endogenous reticulocyte and testis proteins

To test whether this E2 can support ubiquitin conjugation to endogenous proteins, the purified protein was incubated in the presence of ATP, E1, ^{125}I -ubiquitin and the 0–30% $(\text{NH}_4)_2\text{SO}_4$ pellet of reticulocyte fraction II. This $(\text{NH}_4)_2\text{SO}_4$ fraction has previously been shown to be devoid of E2 activity [21]. Indeed, purified E2_{17kB} actively promoted the conjugation of ubiquitin into endogenous proteins of the extract (Figure 5b). In comparison with equivalent amounts of E2_{14k}, the E2_{17kB} was more efficient at promoting conjugation. Furthermore, the conjugates produced by E2_{17kB} were larger in molecular mass. Indeed, some of the conjugates were unable to enter the 4% acrylamide stacking gel.

Since E2_{17kB} appeared highly expressed in the testis, it was of interest to determine its ability to support conjugation in this tissue. Fraction II was prepared from testis and conjugation in this fraction could be demonstrated simply by adding ^{125}I -ubiquitin to the extract (Figure 7a). However, increased conjugation was demonstrated by supplementing the fraction with recombinant E2_{17kB} (Figure 7a). E2_{17kB} could also support conjugation in a 0–30% $(\text{NH}_4)_2\text{SO}_4$ pellet of fraction II (Figure 7b) which, as in reticulocytes [21], does not possess intrinsic

Figure 9 Conjugation of ubiquitin to histone H2A mediated by E2_{17kB} and a testis E3 activity

¹²⁵I-histone H2A (500 000 c.p.m.) was incubated in 25 μ l reaction with ubiquitin, E1 and AMPPNP, in the absence or presence of E2_{17kB} (2 μ M) and/or 10 μ l of the major peak fractions of putative E3 activity identified in Figure 8. The peak fractions were concentrated 4-fold and the salt diluted 20-fold prior to being used in the assay. Incubation time was 2 h at 30 °C.

conjugating activity. As in the reticulocyte preparation, E2_{17kB} was more active than E2_{14k} at promoting such conjugation and generated higher molecular mass conjugates (Figure 7b).

Identification of potential E3 activities in the testis

Since it has been proposed that small E2s without carboxy- or amino-terminal extensions, such as E2_{17kB} and E2_{14k}, require E3 activities to mediate conjugation, it was of considerable interest to try to identify E3 activities with which E2_{17kB} may interact. A testis extract was chromatographed over an anion-exchange column. Approx. 79% of the applied protein bound to the column, which was then eluted with a salt gradient. Fractions were assayed for the ability to promote conjugation of ubiquitin to endogenous proteins in the fraction in an E2_{17kB}-dependent manner (Figure 8). Three major peaks of activity were detected, eluting at approx. 0.2, 0.27, and 0.4 M NaCl concentrations.

When the unbound material was similarly assayed, no activity was detected. This does not appear to be due to the presence of inhibitors as further fractionation of this material on a cation-exchange or gel-filtration column did not reveal any other activities capable of supporting E2_{17kB}-mediated conjugation (results not shown).

To try to discriminate between whether the identified activities truly represented E3 activities as opposed to substrates to which E2_{17kB} could directly conjugate, the peak fractions were assayed for their ability to stimulate conjugation of ubiquitin to an exogenous substrate, histone H2A, previously shown to be degradable *in vitro* in a ubiquitin-dependent [32], but in a non-N-end rule, mechanism. It was observed that the peak eluting at approx. 0.4 M NaCl during MonoQ chromatography would support conjugation to this substrate in an E2_{17kB}-dependent manner (Figure 9). The two other major peaks were ineffective.

DISCUSSION

We have reported the molecular cloning and expression of a cDNA encoding a 17 kDa E2 possessing a number of distinctive features. Of all mammalian E2s cloned to date, this E2 appears

most active *in vitro* in conjugating ubiquitin to endogenous substrates. In comparison with E2_{14k} which also supports such ubiquitination, E2_{17kB} is more active and appears to produce larger molecular mass conjugates (Figures 5b and 7b). Furthermore, in contrast to most E2s, it possesses a net basic charge, and does not bind to anion-exchange resins. In these regards, this E2 is similar to an 18 kDa E2 recently purified from rabbit reticulocyte fraction I [30]. However, when sequences of peptide fragments of this 18 kDa E2 are aligned to similar regions of E2_{17kB} there is only 52% amino acid identity (Figure 4). Furthermore, this 18 kDa E2 can support conjugation and degradation of glyceraldehyde-3-phosphate dehydrogenase, actin and α -crystallin, activities that we are unable to demonstrate for E2_{17kB} (results not shown). Thus, there appears to be a family of E2s with net basic charge with two more members now described in this report.

These E2s_{17kB} are also distinctive in being the first mammalian E2s cloned with high sequence similarity to *S. cerevisiae* UBC4/UBC5. These genes serve vital functions in yeast. They are required for degradation of short-lived and abnormal proteins, are induced by heat shock, and appear to perform much of the ongoing ubiquitination of proteins in the yeast cell [17]. Such an apparently broad substrate specificity in yeast would be consistent with the ability of E2_{17kB} to actively conjugate ubiquitin to endogenous proteins (Figures 5b and 7). In addition, evidence exists that a mammalian homologue of UBC4/UBC5 can participate in turnover of short-lived proteins. Recently, an E2 from the plant *A. thaliana*, UBC8, has been shown to support conjugation of ubiquitin *in vitro* to the tumour suppressor gene p53 in the presence of the papillomavirus product E6 [33]. E2_{17kB} shares 80% amino acid identity with UBC8, indicating that it is the probable mammalian homologue of that plant E2. Indeed, our preliminary studies indicate that E2_{17kB} can support ubiquitination of p53 (S. S. Wing and G. Matlashewski, unpublished work). The recently described 18 kDa E2 from fraction I also supports such ubiquitination [34] suggesting that several mammalian E2s may be capable of this function. In contrast to UBC4/UBC5 though, our studies indicate that E2_{17kB} is not inducible by heat stress (results not shown).

Our studies indicate that E2_{17kB} is expressed in a broad spectrum of tissues (Figure 2), a finding consistent with the fundamental importance of UBC4/UBC5 in cellular protein degradation. However, strikingly, this E2 appears highly expressed in testis. This would be consistent with a proteolytic function for this E2 since this is one of the few tissues in the adult organism where there is rapid degradation of proteins occurring on an ongoing basis. During the formation of spermatids, most intracellular proteins are degraded (reviewed in [35]). Identification of an E2 highly expressed in testis is particularly intriguing as the ubiquitin system appears to be implicated in spermatogenesis. A Y chromosome gene homologous to E1 has been found to be specifically expressed in the mouse testis and genetically localized to the region containing the spermatogenesis gene, *Spy* [36,37]. Our studies are the first demonstration of conjugation activity in the testis. Furthermore, we have demonstrated that E2_{17kB} supports conjugation of ubiquitin to endogenous proteins in testis extracts (Figure 7). As in reticulocytes, E2_{17kB} appears to do this more efficiently than E2_{14k}, the only other cloned mammalian E2 with this catalytic activity. In addition, we have, by ion-exchange chromatography, identified several activities in the testis extract which appear to be likely to represent E3s (Figure 8). One of them (Figure 9) clearly supports conjugation of an exogenous substrate, histone H2A, in an E2_{17kB}-dependent manner, confirming it as an E3 activity. Since we have been unable to demonstrate direct conjugation of ubiquitin to a

protein in the presence of, as enzymes, only E2_{17kD} and E1, and because E2_{17kD} is structurally a class I E2 (see below), the other two peaks of activity are also likely to be E3s. At this time though, we cannot exclude the possibility that those particular fractions simply contain substrates which can be ubiquitinated directly by E2_{17kD}. Interestingly, histone H2A, like the vast majority of intracellular proteins, is an N-terminally blocked protein and thus we have also identified a mammalian E3 activity which supports conjugation to such non-N-end rule substrates. The only other known E3 activity with this feature is the E6-associated protein which, in conjunction with E6, can support conjugation of p53 [33]. The conjugation of histone H2A by only one of three potential E3 activities would also suggest that these E3s possess distinct substrate specificities. Further purification and characterization of these E3 activities may therefore lead to identification of natural substrates of this arm of the ubiquitin system in the testis.

By primary structure analysis, E2_{17kD} is classified as a class I E2 [4], since it does not possess either amino-terminal or carboxy-terminal extensions which have been suggested to permit substrate recognition without a requirement for an E3 [4]. Thus, it possesses only the conserved core domain found in all E2s. In this regard it is very similar to E2_{14kD}. However, the distinctive properties of the two enzymes make it quite clear that certain regions of this conserved core are different enough to determine these different functional properties. Biochemically, E2_{14kD} can mono-ubiquitinate histone H2A in the absence of E3 [6], whereas, E2_{17kD} requires an E3 activity to support any ubiquitination of this substrate (Figure 9). Furthermore, since E2_{14kD} can complement a UBC2 (RAD6) mutation in *S. cerevisiae* [20], it probably has different physiological functions from E2_{17kD}, which appears homologous to *S. cerevisiae* UBC4/UBC5. These E2s probably mediate their distinct functions through interactions with different E3s. Therefore, it is likely that distinctive sequences within this core region determine specificity for E3s. The cloning of these two enzymes along with the ability to mutagenize, express and rapidly purify them will now permit ready identification of these distinctive sequences.

Interestingly, a family of very similar E2s has been identified. These isoforms share 97% amino acid identity (Figure 4). The reason for the presence of multiple isoforms is unclear. This may simply serve a need for redundant enzymes to fulfill a critical cellular function. Alternatively duplication of a gene may permit different isoforms to be differentially regulated either in tissue or developmentally specific manners and in selective physiological contexts. For example, different cell types in the testis may express different isoforms whose genes are controlled in distinct mechanisms. Such a possibility can now be readily explored with the cloning of these different isoforms. It is possible that these different isoforms may interact preferentially with specific E3s. Although the high sequence similarity would argue against dramatic differences in specificity, this can now be explored carefully by recombinant production of each of these E2s.

We have also demonstrated, for the first time, rapid cloning of a mammalian homologue of a yeast ubiquitin system gene based on PCR amplification of mammalian cDNA using oligonucleotides derived from the yeast sequence (Figure 1). Since it is now clear that the ubiquitin system is highly conserved through evolution, such an approach may be applicable to other components of this system. The ability to rapidly identify homologues will permit quicker integration of yeast genetic data with biochemical and physiological information from studies in higher

eukaryotic systems. This will ultimately lead to a better understanding of the mechanisms and functions of the ubiquitin system in mammalian species.

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