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Biochemistry

E2-C, a cyclin-selective ubiquitin carrier protein required for the destruction of mitotic cyclins

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ABSTRACT **Ubiquitin-dependent proteolysis of the mitotic cyclins A and B is required for the completion of mitosis and entry into the next cell cycle. This process is catalyzed by the cyclosome, an ≈22S particle that contains a cyclin-selective ubiquitin ligase activity, E3-C, that requires a cyclin-selective ubiquitin carrier protein (UBC) E2-C. Here we report the purification and cloning of E2-C from clam oocytes. The deduced amino acid sequence of E2-C indicates that it is a new UBC family member. Bacterially expressed recombinant E2-C is active in *in vitro* cyclin ubiquitination assays, where it exhibits the same substrate specificities seen with native E2-C. These results demonstrate that E2-C is not a homolog of UBC4 or UBC9, proteins previously suggested to be involved in cyclin ubiquitination, but is a new UBC family member with unique properties.**

Mitotic entry and exit in most organisms is controlled by the synthesis and destruction of cyclin B, a positive regulatory subunit of the protein kinase cdc2, the catalytic component of mitosis-promoting factor (1, 2). Cyclins are marked for destruction by the covalent addition of ubiquitin at the end of mitosis (3–5). Ubiquitinated cyclins are then rapidly degraded by the 26S proteasome (5). Previous work with cell-free systems derived from clam eggs led to the discovery that this process is catalyzed by a cyclin-specific ubiquitin ligase, E3-C, which is part of an ≈22S particle, the cyclosome (6). Cyclosome activation is initiated by cdc2 (6, 7) and terminated by an okadaic acid-sensitive phosphatase (8). Subsequent work with other organisms has revealed that this particle contains homologs of two yeast proteins, cdc16 and cdc27 (9), proteins required for the destruction of cyclin B and the metaphase–anaphase transition (10, 11). Cyclosome-associated E3-C catalyzes cyclin ubiquitination using a specialized E2 (ubiquitin carrier protein; also called ubiquitin-conjugating enzyme or UBC) originally identified in clams as E2-C (5).

Multiple species of E2s were first found in animal cells (12) and at least 10 different UBCs have now been identified in yeast (13). While all of these are related structurally, genetic and molecular analysis has revealed that different UBCs have different cellular functions. Two closely related UBCs, UBC4 and UBC5, appear responsible for ubiquitin-dependent degradation of most short-lived and abnormal proteins (13). UBC2 (RAD6) is required for several functions, including DNA repair, sporulation (14), and N-end rule degradation (15). UBC3 (CDC34) is required for the G_1/S transition (16), where it appears to participate in the ubiquitin-dependent destruction of the G_1 cdk inhibitor, $p40^{sic1}$ (17). UBC9 is required for cell cycle progression in late G_2 or early M; both CLB5, an S-phase cyclin, and CLB2, an M-phase cyclin, are stable in UBC9 mutants, suggesting that UBC9 may be responsible for cyclin ubiquitination (18). In extracts of frog eggs, recombinant UBC4 protein, as well as an unidentified E2 species, can ubiquitinate cyclins (9). Furthermore, in this system, UBC9 did not support cyclin ubiquitination. These conflicting results raised questions about the identity and selectivity of the E2 responsible for degradation of mitotic cyclins. The data reported here establish that E2-C is a novel, cyclin-selective UBC family member.‖

MATERIALS AND METHODS

Materials. Ubiquitin aldehyde was prepared as described (19). E1 was purified from human erythrocytes by affinity chromatography on Ub-Sepharose (20). Sea urchin cyclin B (13–91)/protein A was expressed and purified as described (3). Proteins were radioiodinated by the chloramine T procedure. Recombinant UBCH5 (21) was generously provided by A. Ciechanover (Technion, Haifa, Israel).

Purification of E2-C. Extracts of M-phase clam oocytes were prepared and fractionated on DEAE-cellulose, as described (5). Fraction 1 (flowthrough) was centrifuged at 100,000 × *g* for 1 hr. The supernatant, previously shown to contain E2C (5), was used for purification of this protein. Fraction 1A, a subfraction containing active E3-C, was prepared by salt extraction and ammonium sulfate fractionation, as described (6). E2-C was first subjected to cation-exchange chromatography on Mono S. This was required to separate it from at least some of the many other E2 species present in this fraction (5) and from ubiquitin, which would interfere with the subsequent step of affinity chromatography on ubiquitin-Sepharose. Free ubiquitin does not bind to this column, whereas E2-C does. A sample of the high-speed supernatant of fraction 1 (5) (10 mg of protein) was applied to a Mono S HR 5/5 column (Pharmacia) equilibrated with 20 mM Hepes·KOH (pH 7.2) containing 1 mM dithiothreitol (DTT) (buffer A). The column was washed with 10 ml of buffer A and then subjected to a 40-ml gradient of 0–200 mM KCl in buffer A. Samples of 1 ml were collected at a flow rate of 1 ml/min into tubes containing 0.5 mg of carrier ovalbumin. Fractions were concentrated by centrifuge ultrafiltration with Centricon-10 concentrators (Amicon), and salt was removed with a 1:20 dilution with buffer A, followed by another ultrafiltration to a final vol of 100 μl. E2-C eluted at ≈70 mM KCl. It was incompletely separated from E2-A, an abundant low molecular weight E2 (5).

For covalent affinity purification, ubiquitin-Sepharose beads (≈20 mg of ubiquitin per ml of swollen gel) were prepared as described (20). Ubiquitin-Sepharose beads (1 ml) were washed twice with 10 vol of 20 mM Tris·HCl, pH 7.2/5 mM $MgCl_2$/2 mM ATP/0.1 mM DTT/0.2 mg of ovalbumin

Abbreviations: UBC, ubiquitin-conjugating enzyme; DTT, dithiothreitol.
†The first two authors made equal contributions to this work.
‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. U52949).

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per ml (buffer B). Beads were mixed with an equal volume of buffer B containing 3 nmol of E1 and were rotated at room temperature for 10 min. Subsequently, 300 μl of partially purified E2-C preparation following the Mono S step were added, and rotation was continued at 18°C for another 20 min. Beads were spun (500 rpm; 3 min) and the supernatant fraction (flowthrough) was collected for estimation of the enzyme not bound to ubiquitin-Sepharose. Beads were washed twice with 10 ml of 20 mM Tris·HCl, pH 7.2/1 M KCl/0.2 mg of ovalbumin per ml and then three times with 10-ml portions of 20 mM Tris·HCl, pH 7.2/0.3% octyl glucoside, a nonionic detergent that prevents nonspecific adsorption of proteins. Enzymes bound to ubiquitin-Sepharose were eluted by mixing the beads with 2 ml of 50 mM Tris·HCl, pH 9.0/5 mM DTT/0.3% octyl glucoside at room temperature for 5 min. The pH 9 eluate was neutralized by the addition of 0.1 M Tris·HCl at pH 7.2, the preparation was concentrated, and the solution was changed by a 1:20 dilution in a buffer consisting of 20 mM Tris·HCl, pH 7.2/0.1% octyl glucoside, followed by ultrafiltration to a final vol of 300 μl.

Microsequencing. Proteins were resolved by SDS/PAGE and stained with Coomassie blue, and the 21-kDa band was excised and subjected to trypsin (Promega) by the in-gel digestion procedure (22). Resulting peptides were separated by reverse-phase HPLC on RP-300 Aquapore column (Perkin–Elmer), with an acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. Peptides were sequenced with standard chemistry, on a model 476A protein-peptide sequencer (Perkin–Elmer).

Assay of E2-C Activity. This was determined by the cyclin-ubiquitin ligation assay (5), under conditions where E1 and E3-C were in excess while E2-C was limiting. Unless otherwise indicated, the reaction mixture contained, in a vol of 10 μl, 40 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 0.5 mM ATP, 10 mM phosphocreatine, 50 μg of creatine phosphokinase per ml, 1 mg of reduced carboxymethylated bovine serum albumin per ml, 50 μM ubiquitin, 1 μM ubiquitin aldehyde, 1–2 pmol of ^{125}I-labeled cyclin B-(13–91)/protein A (referred to as ^{125}I-cyclin; 1–2 $\times$ 10^5 cpm), 1 pmol of E1, 1 μM okadaic acid, 10 μg of protein of M-phase fraction 1A (containing active E3-C and essentially free of E2-C) (6), and E2 source as specified. After incubation at 18°C for 60 min, samples were separated by electrophoresis on SDS/12% polyacrylamide gel. Results were quantified by PhosphorImager analysis.

cDNA Library Screening. A poly(A)$^+$ clam ovary cDNA library, cloned in the phage vector λgt22 (Stratagene) and provided by R. Palazzo and G. Peng (University of Kansas) was screened by PCR. In this library, cDNA inserts are tailed at the 5′ end with *Sal* I and the 3′ end with *Not* I. The successful PCR primer pair consisted of a degenerate oligonucleotide primer encoding an E2-C peptide, TLEFPSDYPYKPPVV (primer P1, 5′-GAYTAYCCITAYAARCCACC-3′, sense direction), a vector primer (λgt22a1, 5′-CAGACCAACTGGTAATGG-TAGCG-3′), where Y is T or C, R is A or G, and I is inosine, substituting for A, C, G, or T; 2 $\times$ 10^6 plaque-forming units (pfu) were used in each PCR. Reaction mixtures contained 3 mM $MgCl_2$, 0.25 mM dNTP, 1$\times$ PCR buffer (Perkin–Elmer), 1.25 units of *Taq* polymerase (Perkin–Elmer), 200 pmol of primer P1, and 50 pmol of primer λgt22a1; reactions were carried out at 94°C for 45 sec, 56°C for 45 sec, and 72°C for 1 min for 30 cycles. A 900-bp reaction product was purified by agarose gel electrophoresis (23) and cloned into the plasmid vector pCRII vector (TA cloning kit; In Vitrogen, San Diego) using the manufacturer's protocols. The insert DNA was sequenced using pCRII vector primers (T3 and T7), and, subsequently, internal unique sequence primers CE24 (5′-CACCAGTAGTAAAGTTCACCACAC-3′, sense direction) and CE24R (5′-CATAGGAAGCAGTCCAATTCTC-3′, antisense direction) using protocols from the Sequenase 7-deaza-dGTP sequencing kit (United States Biochemical). The identification of two other E2-C peptide sequences within the cloned region (ILLSLQSLLG and ENWTASYDV) established it as a candidate E2-C clone. To screen for clones encoding full-length E2-C, 2.4 $\times$ 10^5 plaques of the library were plated onto top agar (20,000 pfu per plate) and replicas were taken onto Hybond-N membranes (Amersham). For screening, the 900-bp PCR fragment of the original cDNA clone was gel purified, labeled with [^{32}P]dCTP by random priming, and recovered after filtration on Sephadex G-50 (23). Membranes were hybridized with the labeled probe in SSC for 65°C for 14 hr; following high-stringency washes, positive plaques were cored and vortexed in SM buffer (100 nM NaCl/10 mM $MgSO_4$/50 mM Tris·HCl, pH 7.5/.01% gelatin) to release the phage. Cored plaques were plated onto 10 LB plates at a concentration of 500 plaques per plate and rescreened with the 900-bp insert; positive plaques were stored in SM buffer. To determine insert sizes, PCRs were performed using the library vector primers λgt22a1 and λgt22a2. Several plaques yielded inserts of 1.5 kb. This insert was gel purified, cloned into the pCRII vector, and sequenced using primers T7, CE24, and CE24R. This led to identification of a fourth E2-C peptide sequence (RTLLMSGDPGITAFPDGDNLFK). Matches between sequences of the peptides derived from purified E2-C protein and the protein sequence encoded by the cloned cDNA are indicated in Fig. 3*A*.

Production of Recombinant E2-C Protein. PCR product containing the 1.5-kb E2-C insert was diluted 1:1000 and a second PCR was performed with primers CE2Ful (5′-GGGCATATGTCGGGACAAAATATAGATC-3′, sense direction) and CE2Rev (5′-GGGAAGCTTCTATTTAT-CACTCTGAGCAG-3′, antisense direction), designed to create a 5′ *Nde* I site at the presumptive initiator methionine and a *Hin*dIII site at the 3′ end; the resulting product was subcloned into pT7-7 (24). The resulting construct was transformed into BL-21(DE3)pLysS cells (Novagen). Cells were grown in 100 ml of LB containing 50 μg of ampicillin per ml and 34 μg of chloramphenicol per ml to an OD of 0.6. Isopropyl β-D-thiogalactopyranoside was added to 1 mM, and cells were incubated at 37°C for 3 hr. Cell pellets were washed in cold PBS (140 mM NaCl/2.7 mM KCl/10 mM Na_2HPO_4/1.8 mM KH_2PO_4) and lysed in 3 ml of 1 mM EDTA/1 mM DTT/50 mM Tris·HCl, pH 7.6/10 μg of leupeptin per ml/10 μg of chymostatin per ml.

RESULTS

Purification of E2-C for Microsequencing. E2-C was partially purified by cation exchange chromatography on Mono S and then subjected to covalent affinity chromatography on ubiquitin-Sepharose. In the presence of E1 and MgATP, E2s bind to immobilized ubiquitin by thiolester linkage; ubiquitin-bound enzymes can then be eluted with high concentrations of DTT or by raising the pH (20). In the experiment shown in Fig. 1, ubiquitin-Sepharose beads were mixed with three kinds of mixtures. The complete mixture contained the peak of E2-C from the Mono S column, E1 purified from human erythrocytes and MgATP; the two others were controls, lacking either E1 or the source of E2-C. The fraction not adsorbed to ubiquitin-Sepharose (flowthrough) was collected and, following extensive washing of the beads, the enzymes bound to ubiquitin-Sepharose were eluted with pH 9 buffer containing 5 mM DTT. Quantitative assays of E2-C activity in these fractions (Fig. 1) showed that, in the complete mixture, virtually all E2-C activity was adsorbed to ubiquitin-Sepharose (removed from the flowthrough) and was recovered in the pH 9 eluate. By contrast, when E1 was omitted, there was no significant activity of E2-C in the pH 9 eluate, and most enzyme activity remained in the flowthrough. This result shows that binding of E2-C to ubiquitin-Sepharose required an E1-mediated thiolester transfer process.

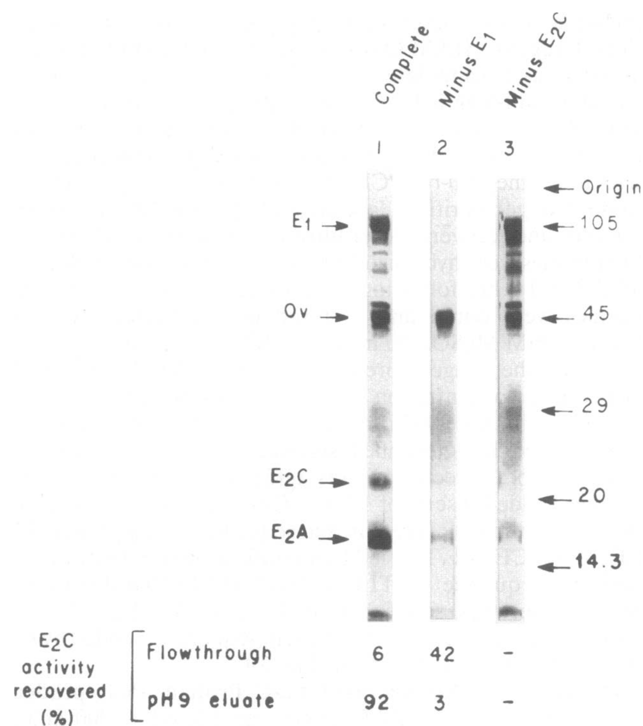

FIG. 1. Covalent affinity purification of clam oocyte E2-C. The pooled peak of the Mono S column containing E2-C activity was applied to ubiquitin-Sepharose as described. Lane 1, complete mixture; lane 2, similar mixture, but lacking E1; lane 3, similar mixture, but lacking the Mono S peak material. (*Upper*) Samples (20 μl) of the corresponding pH 9 eluates were separated on a 12.5% polyacrylamide gel and stained with silver reagent. Numbers on the right indicate position of molecular mass markers (kDa). (*Lower*) E2-C activity in fractions indicated was assayed as described and quantified by PhosphorImager analysis. Results are expressed as percentage of total E2-C activity applied to the ubiquitin-Sepharose beads.

The protein composition of the pH 9 eluates of these treatments was examined by SDS/PAGE and silver staining. As shown in Fig. 1, the pH 9 eluate of the complete reaction mixture (lane 1) contained several protein bands. These include an ≈105-kDa protein identified as E1 (which also binds to the ubiquitin column and is eluted at pH 9; see ref. 25), several bands in the range of 45–105 kDa that are cleavage products of E1 (25), and two bands at ≈21 and ≈16 kDa. The last two proteins were tentatively identified as E2-C and E2-A, respectively, based on the following considerations. First, both E2-C and E2-A are present in the partially purified preparation used for affinity purification, so both are expected to bind to the ubiquitin beads under the conditions used. Second, both proteins are absent from the pH 9 eluate of the control lacking E1 (lane 2), indicating that both are E2s. Third, they were also absent in the control containing E1, but lacking the source of E2-C (lane 3), indicating that the two low molecular weight bands are not derived from some contamination of the E1 preparation used for covalent affinity chromatography. On the other hand, the higher molecular weight bands in the region of 45–105 kDa are derived from E1 (compare lanes 2 and 3).

It should be noted that the expected molecular sizes of the adducts of E2-C and E2-A with ubiquitin (8.5 kDa) are 29.5 and 24.5, respectively; these are higher than those observed for their putative thiolesters (27 and 18 kDa; see ref. 5). This might be due to the well-known anomalous migration of proteins under the partially denaturing electrophoretic conditions required for detection of ubiquitin-E2 thiolesters (26, 27). To examine further the identity of putative E2-C, the pH 9 eluate of the preparation purified on ubiquitin-Sepharose was subjected to gel filtration on Superose-12. The activity of E2-C (determined by the cyclin–ubiquitin ligation assay) eluted mainly in fractions 33 and 34 (Fig. 2*A*), coincident with the 27-kDa ubiquitin-thiolester band (Fig. 2*B*). It was partially separated from the 18-kDa E2-A-ubiquitin thiolester that eluted at a lower size during gel filtration (Fig. 2*B*). We conclude that the anomalously migrating 27-kDa adduct is the ubiquitin thiolester of the 21-kDa E2-C protein.

Cloning of Clam E2-C. Based on this identification, the 21-kDa E2-C was chosen for microsequencing. Material originating from 100 ml of clam oocyte extract was processed by the Mono S and ubiquitin-Sepharose steps described above and the 21-kDa band was digested with trypsin as described. Sequences of four tryptic peptides were obtained, as shown in Fig. 3*A*. A degenerate oligonucleotide primer corresponding to the second peptide was designed, and then with a λgt22 primer to screen a clam ovary cDNA library using PCR, as described. A partial length cDNA clone containing sequences corresponding to three of the four peptides was obtained and used to select several candidate clones encoding full-length E2-C. In these, the first peptide sequence was identified in the N-terminal

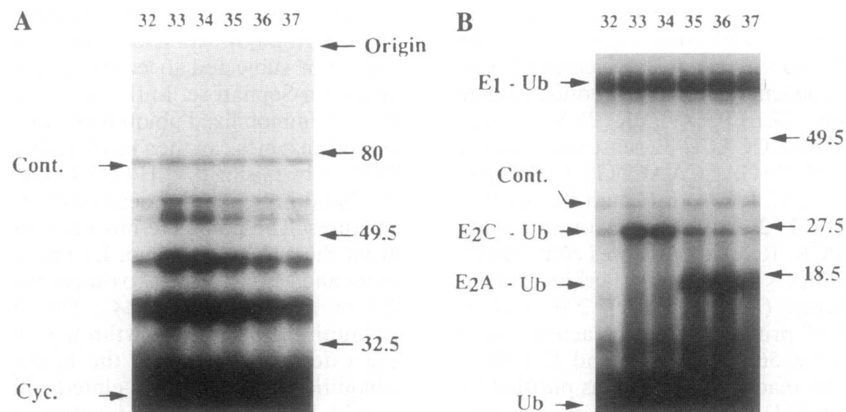

FIG. 2. Gel-filtration chromatography of affinity purified E2-C. A 200-μl sample of pH 9 eluate from ubiquitin-Sepharose affinity chromatography was applied to a column of Superose 12-HR 10/30 (Pharmacia) equilibrated with 50 mM Tris·HCl, pH 7.2/1 mM DTT/0.2 mg of horse heart cytochrome *c* (carrier) per ml/0.3% octyl glucoside. Fractions of 0.5 ml were collected at a flow rate of 0.4 ml/min. Each fraction was concentrated to a volume of 50 μl with Centricon-10 concentrators. Fraction numbers are indicated on the top, and positions of molecular markers are indicated on the right. (*A*) Activity of E2-C in cyclin–ubiquitin ligation was determined with samples of 0.5 μl of column fractions. Cont., contamination in preparation of ^{125}I-cyclin; Cyc, free ^{125}I-cyclin. (*B*) Formation of ^{125}I-ubiquitin–E2 thiolesters was determined as described in ref. 5, with 5-μl samples of column fractions. Cont., contamination in preparation of ^{125}I-ubiquitin; Ub, free ^{125}I-ubiquitin; E1-Ub, E2-C-Ub, and E2-A-Ub, positions of corresponding adducts.

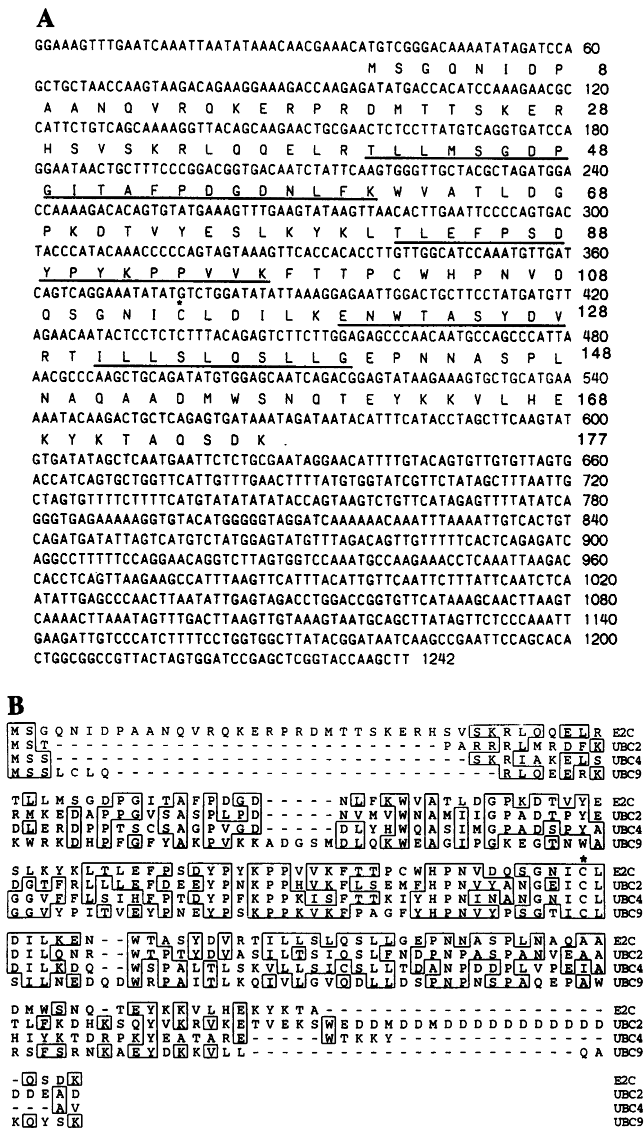

FIG. 3. Nucleotide sequence of clam E2-C cDNA and its deduced amino acid sequence. (*A*) Positions of nucleotide and amino acid residues are given on the right. Four peptides obtained by microsequencing are underlined. (*B*) Amino acid sequences were aligned using the BLAST program (28). *, Position of the catalytic cysteine residue used for thiolester formation with ubiquitin.

region (Fig. 3*A*). The same coding sequence was found in other independently isolated cDNA clones.

The sequence obtained contains only one long open reading frame, which is predicted to initiate at the first methionine codon. The size of the presumed translation product is 20 kDa, in good agreement with the size of purified E2-C observed by SDS/PAGE. The encoded protein is clearly an E2, as demonstrated by its extensive alignment with other cloned UBCs and an especially strong homology in the region of the domain containing the catalytic cysteine (Fig. 3*B*). Comparisons using the Lipman–Pearson alignment method indicated that the most closely related family members are yeast UBC2, *Saccharomyces cerevisiae* rad6, and *Schizosaccharomyces pombe* rhp6, which showed 42.6% and 41.4% similarity scores, respectively (data not shown). Clam E2-C does not appear to be a UBC2 homolog, since UBC2s from several different species show much higher conserved sequence similarities within the family (≈70%). The clam sequence contains a novel 30-amino acid N-terminal extension not found in any other UBC (13). Other unique regions include the adjacent sequence beginning at position 42, TLLMSGD, and a short C-terminal extension, KYKTAQSDK. These features indicate that E2-C represents a novel UBC.

Activities of Bacterially Expressed E2-C. To demonstrate conclusively that this novel UBC is actually E2-C, it was necessary to express recombinant protein and compare its properties with those of E2-C. The coding region was subcloned into the bacterial expression vector pT7–7, the protein was induced, and a crude lysate was assayed in two different ways. First, the ability of the recombinant protein to form thiolester adducts with ^{125}I-ubiquitin was examined (Fig. 4). For comparison, ubiquitin thiolesters of a mixture of natural E2-C and E2-A were separated on the same gel. It may be seen that the recombinant protein formed an adduct with ubiquitin and that the electrophoretic mobility of the ubiquitin thiolester of the recombinant E2 was identical to that of the 27-kDa adduct with native E2-C (Fig. 4, lanes 2 and 3). In addition, a minor species of a more rapidly migrating ubiquitin adduct of the recombinant protein (labeled *) was observed (lane 3). This may be a cleavage product or an incompletely denatured conformer of an E2-C/ubiquitin thiolester (26, 27). That both of these adducts are thiolesters is indicated by the observation that they are almost completely abolished by boiling with 2-mercaptoethanol (lanes 4 and 5). A small amount of higher molecular weight derivative persists after boiling with mercaptoethanol (lanes 4 and 5). This is presumably a product of self-ubiquitination (amide bond formation between ubiquitin and a lysine residue of the E2), previously observed *in vitro* with some E2s but not with others (29). It is noteworthy that similar autoubiquitination takes place with both natural and recombinant E2-C.

The ability of the recombinant E2 to promote cyclin–ubiquitin ligation was tested in the presence of activated, partially purified E3-C–cyclosome complexes. As shown in Fig. 5*A*, the recombinant E2 efficiently promoted this process, as compared to the action of natural E2-C. The recombinant E2 stimulated cyclin ubiquitination at remarkably low concentrations: half-maximal activation was obtained with 0.05 μM

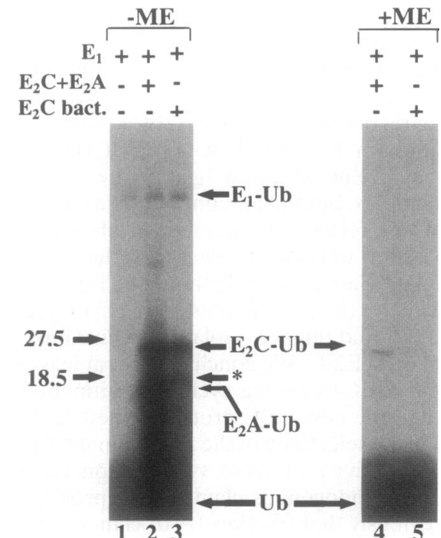

FIG. 4. Thiolester formation between ubiquitin and bacterially expressed E2-C. ^{125}I-ubiquitin–E2 thiolester formation was carried out as described in ref. 5. Where indicated, E1 (0.1 μM), 3 μl of partially purified E2-C following Mono S purification (labeled E2-C+E2A), or 0.5 μM bacterially expressed E2-C (E2-C bact.) was added. Samples were either boiled with 5% mercaptoethanol for 5 min (+ME) or were not treated (−ME) before electrophoresis. Numbers on the left indicate position of molecular mass marker proteins. E1-Ub, E2-C-Ub, and E2A-Ub indicate the positions of corresponding ^{125}I-ubiquitin–enzyme adducts; *, position of fast migrating adduct of E2-C with ^{125}I-ubiquitin.

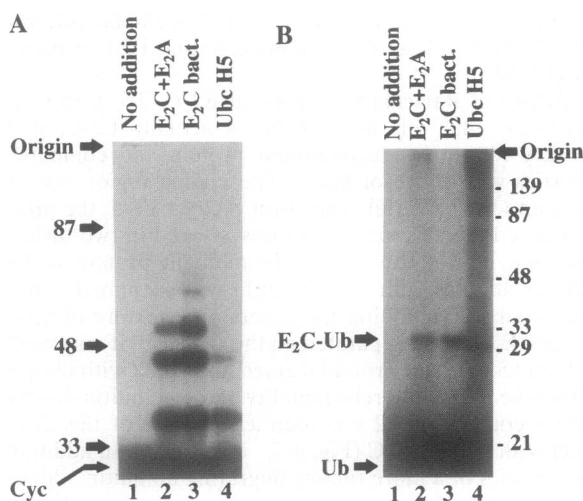

FIG. 5. Selectivity of action of bacterially expressed E2-C in cyclin–ubiquitin ligation: Comparison with a UBC 4 homolog. (*A*) Activity of different E2s in ligation of ^{125}I-cyclin to ubiquitin was determined as described, except that fraction 1A was replaced by a preparation of activated E3-C purified by gel filtration on Superose-6 (see ref. 8). Where indicated, 3 μl of a partially purified preparation of E3-C (following the Mono S step, labeled E3-C+E2A), 0.5 μM bacterially expressed E2-C (E2-C bact.), or 10 μM UBCH5 was added. Numbers on the left indicate position of molecular mass markers (kDa). Cyc., position of free ^{125}I-cyclin. (*B*) Ligation of ^{125}I-ubiquitin to proteins was determined in a reaction mixture containing, in a vol of 10 μl, 20 mM Hepes·KOH (pH 7.2), 5 mM $MgCl_2$, 0.5 mM ATP, 10 mM phosphocreatine, 50 mg of creatine phosphokinase per ml, 1 mM DTT, 1 mg of reduced carboxymethylated bovine serum albumin per ml, 0.1 μM E1, 2 μM UbAl, and 5 μM ^{125}I-ubiquitin ($\approx$150,000 cpm). All incubations contained 3 μl of a nonspecific clam oocyte E3 preparation (fractions 10 and 11 of the glycerol gradient shown in figure 5C of ref. 6). Where indicated, the different E2 preparations were added at concentrations similar to those described in *A*. E2-C–Ub denotes position of the autoubiquitination product of E2-C (see text). Numbers on the right indicate position of the molecular size marker proteins (kDa).

and maximal activation was obtained with 0.5 μM recombinant E2. Since it has been reported that UBC4 can support cyclin B ubiquitination in a *Xenopus* egg extract (9), we also tested the activity of a recombinant human UBC4 homolog, UBCH5 (21). As shown in Fig. 5*A* (lane 4), UBCH5 caused some stimulation of cyclin–ubiquitin ligation by the clam E3-C–cyclosome complex, but the amount of conjugates formed and their size (which reflects the number of ubiquitin molecules attached to cyclin) were much lower than those obtained with the recombinant clam protein. It should be further noted that, in this experiment, the recombinant UBCH5 protein had to be added at a 20-fold higher molar concentration than the recombinant clam E2-C. We conclude that, at least in the clam oocyte system, UBC4 supports cyclin ubiquitination much less efficiently than the new UBC protein cloned here.

To examine the selectivity of the recombinant clam E2-C, we compared the activity of these two E2s on the ligation of ^{125}I-ubiquitin to endogenous clam oocyte proteins. We have reported previously that fraction 1A of clam oocytes contains a "non-cyclin-specific" E3 that can be separated from the cyclin-selective E3-C–cyclosome complex by its smaller size. This nonspecific E3 ligates ^{125}I-ubiquitin to endogenous proteins in the presence of a mixture of clam E2s (6). The protein substrates for ubiquitin ligation are presumably clam oocyte proteins present in the partially purified preparation of the nonspecific E3. As shown in Fig. 5*B*, UBCH5 strongly stimulated the ligation of ^{125}I-ubiquitin to high molecular weight conjugates in the presence of nonspecific E3 from clam oocytes. This finding indicates that the human UBC4 homolog can act with an appropriate clam E3, as would be expected from the strong conservation of UBC4 homologs in evolution (13). Formation of the high molecular conjugates required addition of both UBCH5 and the nonspecific E3 (data not shown). By contrast, the recombinant clam E2-C had no significant influence on the formation of ubiquitin–protein conjugates by the nonspecific E3 (Fig. 5*B*, lane 3). The only stable adduct formed in the presence of the recombinant clam E2-C is a 30-kDa autoubiquitination product. The formation of this product does not require the presence of the nonspecific E3 (data not shown). The amount of the product is higher in Fig. 5 than in Fig. 4 due to the longer incubation time. Its apparent 30-kDa size in the denaturing conditions of gel electrophoresis is close to that expected for recombinant E2–ubiquitin adduct (29.5 kDa). A similar autoubiquitination product with native E2-C is seen with a mixture of natural E2-C and E2-A (Fig. 5*B*, lane 2). In this case, some formation of high molecular weight ubiquitin–protein conjugates is seen. This is presumably due to the action of E2-A, which had been found previously to coincide with a nonspecific ubiquitination activity (5). It thus seems that at least by the criterion of the lack of its action with a nonspecific E3, the recombinant clam E2-C is selective for the cyclin ubiquitination system, as indicated by previous studies on the partially purified natural enzyme (5). We thus conclude that the cDNA clone described here encodes the cyclin-selective E2-C that is responsible for the cell cycle stage-specific ubiquitination and destruction of mitotic cyclins A and B.

DISCUSSION

The selective ubiquitination and subsequent proteolysis of cyclins A and B near the end of mitosis is carried out by two components. First described in clam oocytes, these are a cyclin-selective ubiquitin carrier protein activity, E2-C, and a cyclin-selective ubiquitin ligase activity, E3-C, which catalyzes transfer of ubiquitin from E2-C to the target protein cyclin. Recent work has revealed that the ligase activity is part of an $\approx$22S complex, called the cyclosome in clams (6) and the APC in frogs, which contains homologs of at least two yeast cdc genes (9), but neither the identity of the ligase itself nor the basis of its substrate specificity is known. By contrast, several candidates have been suggested as cyclin-selective UBCs. In the budding yeast *S. cerevisiae*, repression of UBC9 synthesis blocks the cell cycle in either late G_2 or early M, and both the S phase cyclin CLB5 and the mitotic cyclin CLB2 fail to be degraded in these cells (18). These results suggested that both cyclin types are destroyed by a common pathway that requires UBC9. However, more recent work done with frog egg extracts reported that a *Xenopus* homolog of UBC9 did not cofractionate with the cyclin ubiquitinating activities. Instead, preliminary separation of endogenous UBC activities revealed that a fraction containing UBC4 could support cyclin ubiquitination and that recombinant UBC4 protein could substitute for this fraction (9). UBC4 involvement was somewhat surprising because of the lack of any demonstrable effect on cyclin destruction or cell cycle progression in yeast, where it clearly plays obvious roles in the turnover of abnormal proteins and the destruction of some rapidly turning-over normal proteins (13). These features are in striking contrast to those of UBC3 (cdc34) in which mutations cause a discrete G_1 arrest (16), most likely by their requirement in the pathway leading to proteolysis of the cyclin-dependent kinase inhibitor p40^{sic1} (17). Furthermore, a second UBC activity that also supported cyclin ubiquitination in frog egg extracts was noted (9).

In view of these disparate results, we took advantage of previous work in which we identified and partially purified a discrete UBC activity that was required for ubiquitination of mitotic cyclins in extracts of clam oocytes (5). This activity, called E2-C, was distinguished as the major cyclin-specific UBC activity on the basis of (*i*) its ability to ubiquitinate both

cyclin A and B, but not most other cellular proteins, (*ii*) the absolute dependence of this reaction on the presence of an intact unscrambled destruction box, and (*iii*) the corequirement for a cyclin-specific ubiquitin ligase activity, E3-C, that resides in an ≈22S particle, the cyclosome (5, 6). With purified E2-C in hand, it was possible to clone the corresponding cDNA from clam oocytes, confirm that the recombinant protein encoded by this cDNA did in fact exhibit the same properties as native endogenous E2-C in cyclin ubiquitination assays, and determine the identity of E2-C. By contrast, UBC4 stimulated cyclin ubiquitination only at very high concentrations, suggesting a low-affinity ubiquitin transfer from a nonspecific E2. These results clearly establish that E2-C is the specific UBC required for ubiquitination of the mitotic cyclins.

Comparisons of the sequence of E2-C with those of other cloned UBCs clearly indicates that it is a novel UBC. Recently, we have cloned a human E2-C (F. Townsley, S. Beck, A.H., and J.V.R., unpublished data). UBC-x, an E2 that can support cyclin ubiquitination in frog lysates, has also now been cloned (H. Yu, R, King, J. Peters, and M. Kirschner, personal communication). Sequence comparisons and enzymatic analyses indicate that the structure and function of all three enzymes are highly conserved. As the human gene is the 10th human E2 identified (S. Jentsch, personal communication), we refer to it as UBC-H10. The most distinctive features of this class are a 30-amino acid N-terminal extension, a short C-terminal extension, and a short stretch of amino acids just distal to the N-terminal extension. These domains are obvious candidates for mediating specific interactions with E3-C or other components of the cyclosome complex as well as other regulators of the cyclin destruction machinery. The availability of the *in vitro* cyclin ubiquitination assay and the high specific activity of recombinant E2-C protein should make a molecular investigation of these questions both feasible and straightforward.

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