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.

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 radiolabeled with the chloramine T procedure. Recombinant UBC5 (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 x 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 MgCl₂/2 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-m1 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 containing 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 Aquaapore 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 MgCl2, 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 μg ubiquitin, 1 μM ubiquitin aldehyde, 1–2 pmol of 125I-labeled cyclin B (13–91)/protein A (referred to as 125I-cyclin; 1–2 × 105 cpm), 1 pmol of E1, 1 μM okadaic acid, 10 μg of protein of M-phase fraction 1A (containing active E2-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)‘ clarym 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, TLEFSDYPYKPVV (primer P1, 5‘-GAYTAYCITAYAARCCACC-3‘, sense direction), a vector primer (λgt22a1, 5‘-CAGACACATGGCGGAAATTG-3‘), where Y is T or C, R is A or G, and I is inosine, substituting for A, C, G, or T, 2 × 108 plaque-forming units (pfu) were plated for each PCR. Resulting transformants were subjected to 125I-labeled cDNA filters and the protein of interest was eluted with SDS/PAGE, washed in wash buffer, and eluted from the nitrocellulose membrane using 30 ml of 20 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 thioester 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 fraction from the Mono S column. The enzyme was adsorbed 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 thioester transfer process.
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 thioesters (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 thioesters (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. 2A), coincident with the 27-kDa ubiquitin-thiolester band (Fig. 2B). It was partially separated from the 18-kDa E2-A-ubiquitin thiolester that eluted at a lower size during gel filtration (Fig. 2B). 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. 2A. A degenerate oligonucleotide primer corresponding to the second peptide was designed, and then with a λgt11 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. 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),

**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 125I-cyclin; Cyc, free 125I-cyclin. (B) Formation of 125I-ubiquitin–E2 thioesters was determined as described in ref. 5, with 5-µl samples of column fractions. Cont., contamination in preparation of 125I-ubiquitin; Ub, free 125I-ubiquitin; E1-Ub, E2-C-Ub, and E2-A-Ub, positions of corresponding adducts.
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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. 5B, 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. 5B, 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 ~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 G2 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 G1 arrest (16), most likely by their requirement in the pathway leading to proteolysis of the cyclin-dependent kinase inhibitor p40scl (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, E2-C, that resides in an ~2S 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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