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Cell Cycle-Dependent Expression of Mammalian E2-C Regulated by the Anaphase-Promoting Complex/Cyclosome

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Progression through mitosis requires the precisely timed ubiquitin-dependent degradation of specific substrates. E2-C is a ubiquitin-conjugating enzyme that plays a critical role with anaphase-promoting complex/cyclosome (APC/C) in progression of and exit from M phase. Here we report that mammalian E2-C is expressed in late G_2/M phase and is degraded as cells exit from M phase. The mammalian E2-C shows an autoubiquitinating activity leading to covalent conjugation to itself with several ubiquitins. The ubiquitination of E2-C is strongly enhanced by APC/C, resulting in the formation of a polyubiquitin chain. The polyubiquitination of mammalian E2-C occurs only when cells exit from M phase. Furthermore, mammalian E2-C contains two putative destruction boxes that are believed to act as recognition motifs for APC/C. The mutation of this motif reduced the polyubiquitination of mammalian E2-C, resulting in its stabilization. These results suggest that mammalian E2-C is itself a substrate of the APC/C-dependent proteolysis machinery, and that the periodic expression of mammalian E2-C may be a novel autoregulatory system for the control of the APC/C activity and its substrate specificity.

INTRODUCTION

The ubiquitin–proteasome pathway plays an important role in the selective and time-dependent degradation of short-lived regulatory proteins, such as cell cycle-related proteins (cyclins and cyclin-dependent kinase inhibitors), transcriptional regulators (p53, c-Jun, c-Fos, and c-Myc), and signal transducers (IκB and β-catenin), in eukaryotic cells (Weissman, 1997; Hershko and Ciechanover, 1998). Conjugation of ubiquitin (8 kDa) to the substrate protein is achieved by the action of three enzymes (Hershko *et al.*, 1983): a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). E1 catalyzes the ATP-dependent activation of ubiquitin, which involves the formation of a high-energy thiol ester bond between the COOH-terminus of ubiquitin and a cysteine residue of E1. The thiol ester-linked ubiquitin then is transferred from E1 to a cysteine residue in E2, which, either by itself or together with E3, subsequently transfers ubiquitin to a lysine residue of the substrate protein. E3 acts as a specificity factor that functions in substrate recognition. Polyubiquitinated proteins are recognized by the 26S proteasome complex (1500 kDa) and are rapidly degraded to short peptides (Coux *et al.*, 1996; Hershko and Ciechanover, 1998).

The highly ordered progression of the cell cycle is achieved by a series of elaborate mechanisms that control the periodic expression of many regulatory proteins. The ubiquitin–proteasome system is an important mediator of the rapid degradation of such proteins. Two major classes of E3 enzymes regulate cell cycle progression: the SCF complex and the anaphase-promoting complex/cyclosome (APC/C) (reviewed by Zachariae and Nasmyth, 1999). The SCF complex, which consists of the invariable components Skp1, Cullin, and the newly identified component Rbx1/ROC1, as well as a variable component known as an F-box protein, functions to destabilize G_1 cyclins and cyclin-dependent kinase inhibitors (Bai *et al.*, 1996; Skowyra *et al.*, 1997; Feldman *et al.*, 1997; Kamura *et al.*, 1999; Ohta *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999; Tan *et al.*, 1999). F-box proteins serve as intracellular receptors for the target proteins and thereby confer specificity to the SCF complex.

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In contrast, the APC/C is required for two events in mitosis: the separation of sister chromatids at anaphase and their exit from M phase into G_1 (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). The APC/C is a large (~1500 kDa) protein complex composed of 12 subunits in budding yeast and at least 10 subunits in mammals (Townsley and Ruderman, 1998; Zachariae and Nasmyth, 1999). It contributes to the ubiquitin-dependent proteolysis of mitotic cyclins, securins (Pds1/Cut2), Ase1, Geminin, Cdc20, Cdc5, and Polo-like kinase 1 (Plk1), most of which share a common nine-residue motif known as a destruction box (Glotzer *et al.*, 1991; Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995; Cohen-Fix *et al.*, 1996; Yamano *et al.*, 1996; Funabiki *et al.*, 1997; Juang *et al.*, 1997; McGarry and Kirschner, 1998; Prinz *et al.*, 1998; Shirayama *et al.*, 1998; Fang *et al.*, 1998b). Substrate specificity is determined, at least in part, by the regulated association of the APC/C with two classes of WD40 repeat-containing coactivator proteins: Cdc20 (or fizzy) and Hct1 (Cdh1 or fizzy-related) (Dawson *et al.*, 1995; Sigrist *et al.*, 1995; Schwab *et al.*, 1997; Sigrist and Lehner, 1997; Visintin *et al.*, 1997). In budding yeast, Cdc20 functions in the ubiquitination of proteins, such as anaphase inhibitor Pds1, the S-phase B-type cyclin Clb5, and the mitotic cyclin Clb3, whereas Hct1 contributes to the destruction of mitotic cyclin Clb2, the polo kinase Cdc5, and the spindle protein Ase1 (Townsley and Ruderman, 1998; Morgan, 1999; Zachariae and Nasmyth, 1999). In yeast and human cells, both Cdc20 and Hct1 bind to the APC/C, but the timing and regulation of their association are regulated differently. Cdc20 accumulates from late S phase through early mitosis; as cells exit from M phase, Cdc20 is ubiquitinated by the APC/C, resulting in its rapid degradation (Fang *et al.*, 1998b; Prinz *et al.*, 1998; Shirayama *et al.*, 1998). In contrast to Cdc20, Hct1 is present constitutively but binds to the APC/C only during exit from mitosis and G_1, stages of the cell cycle at which mitotic cyclins are unstable (Sigrist and Lehner, 1997; Fang *et al.*, 1998a; Kramer *et al.*, 1998).

Clam E2-C and its *Xenopus* homolog UbcX were identified as the E2 enzymes required for the destruction of mitotic cyclins (Aristarkhov *et al.*, 1996; Yu *et al.*, 1996). The mutation of the gene encoding the fission yeast APC/C subunit Cu+9p results in growth defect that can be rescued by the overexpression of the fission yeast E2-C homolog, UbcP4 (Osaka *et al.*, 1997). In addition, dominant negative versions of the human E2-C ortholog, designated hE2-C (or UbcH10), induce cell arrest at metaphase as well as inhibit sister chromatid separation and cyclin B degradation (Townsley *et al.*, 1997). Together, these observations suggest that this enzyme functions in the APC/C-dependent ubiquitination pathway, although the physical interaction between E2-C relatives and APC/C has not been described. The E2 enzyme Ubc4 in *Xenopus* also has been implicated in the ubiquitination of mitotic cyclins that are mediated by the APC/C (Yu *et al.*, 1996). The mechanisms by which E2-C and Ubc4 functionally and physically interact with the APC/C, the difference in substrate protein specificity between these E2 enzymes, and whether E2-C and Ubc4 are regulated in a cell cycle-dependent manner have remained unclear.

We now show that mammalian E2-C is expressed exclusively around mitosis, and is eliminated through ubiquitin-dependent proteolysis mediated by the APC/C as cells exit from mitosis. Our observations suggest that the abundance of mammalian E2-C is autoregulated through polyubiquitination by the APC/C, and that its degradation at early G_1 phase might contribute to a change in substrate specificity of the APC/C.

MATERIALS AND METHODS

Construction of Expression Plasmids and Mutagenesis

A search of a mouse expressed sequence tag (EST) database yielded a mouse clone (GenBank accession number, AA268341) that showed substantial homology to hE2-C. Sequencing of this clone revealed that it contained the full-length mE2-C cDNA. The entire coding region of mE2-C was subcloned into pBluescriptII SK+ (Stratagene, La Jolla, CA), pGEX-6P-1 (Amersham Pharmacia Biotech, Little Chalfont, UK) and pcDNA3 (Invitrogen, Carlsbad, CA). The pGEX-6P-1 plasmids encoding mE2-C(C114S), mE2-C(Dm1), or mE2-C(Dm2) were generated with the use of a mutagenesis kit (Stratagene). The mutant mE2-C(C114S) is identical to that described by Townsley *et al.* (1997).

Expression and Purification of Recombinant mE2-C Proteins

GST-tagged mE2-C proteins were expressed in *Escherichia coli* XL1-blue and were affinity-purified with glutathione-Sepharose CL-4B (Amersham Pharmacia Biotech), after which the GST tag was removed from mE2-C by cleavage with PreScission protease (Amersham Pharmacia Biotech). The concentration of the recombinant proteins was estimated by Coomassie blue staining.

Assay for Ubiquitin-E2 Thiol Ester Linkage

Assay of thiol ester linkage was performed as previously described (Jensen *et al.*, 1995) with minor modification. Equal amounts (0.5 μg) of mE2-C protein were mixed with 0.3 μg of recombinant rabbit E1 and 1 μl of ^{125}I-ubiquitin in a final volume of 15 μl containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM ATP, and 5 mM $MgCl_2$. After incubation for 15 min at 22°C, the reaction mixtures were heated at 95°C for 5 min in SDS sample buffer with or without 5% (vol/vol) 2-mercaptoethanol and then were subjected to SDS-PAGE on a 12% gel, autoradiography, and image analysis (BAS-2000, Fuji Film, Tokyo, Japan).

Antibodies

Rabbit polyclonal antibodies to mE2-C were generated in response to purified mE2-C protein as immunogen. Monoclonal antibodies to human CDC27 (AF3) (Yamano *et al.*, 1998) or to GST were kindly provided by H. Yamano (ICRF Clare Hall Laboratories, London, UK) and S. Tanaka (Hokkaido University, Sapporo, Japan), respectively. Monoclonal antibodies to ubiquitin (1B3), to human cyclin B1 (14541A), to Myc (9E10), or to human α-tubulin were obtained from MBL (Nagoya, Japan), PharMingen (Torrey Pines, CA), Boehringer Mannheim (Amsterdam, The Netherlands), and Zymed (South San Francisco, CA), respectively.

Cell Culture and Synchronization

NIH 3T3 and HeLa cells were cultured at 37°C under an atmosphere of 95% air–5% CO_2 in DMEM (Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) calf serum or fetal bovine serum (Life Technologies), respectively. For analysis of cell cycle progression from G_0 to G_1 phase, NIH 3T3 cells were arrested at the G_0–G_1 phase by subjecting them to contact inhibition during culture to confluence; they were then released from contact inhibition by replating at a density of ~30% of that at confluence (Shirane *et al.*, 1999). For analysis of the transition from M phase to G_1 phase, NIH

3T3 or HeLa cells were maintained in the presence of aphidicolin (1 μg/ml) for 18 h, were washed with phosphate-buffered saline, and then were incubated in aphidicolin-free medium for 3 to 4 h. The cells were subsequently incubated with nocodazole (100 ng/ml) for 18 h to induce arrest at M phase, after which culture dishes were shaken and floating cells were harvested for recovery of only those cells in M phase. After washing twice in phosphate-buffered saline, the cells were cultured in nocodazole-free medium for various times. The arrest of HeLa cells at the G_1–S boundary by aphidicolin was performed according to the method described by Fang *et al.* (1998a) with minor modification. Briefly, HeLa cells were incubated in the presence of aphidicolin (1 μg/ml) for 18 h, were washed with phosphate-buffered saline, and then were incubated in aphidicolin-free medium for 8 h. Thereafter, cells were incubated with aphidicolin (1 μg/ml) for 18 h and were harvested for experiments.

For cell cycle analysis, cells were exposed for 30 min to 10 μM bromodeoxyuridine. Harvested cells were fixed with 70% (vol/vol) ethanol, were treated with 2 M HCl containing 0.5% (vol/vol) Triton X-100, were neutralized with 0.1 M borax buffer (pH 8.5), were subjected to two-color staining with a fluorescein isothiocyanate-conjugated monoclonal antibody to bromodeoxyuridine (Becton Dickinson, Franklin Lakes, NJ) and propidium iodide (5 μg/ml), and were analyzed with a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson).

Transfection, Immunoprecipitation, and Immunoblot Analysis

Cells were transfected by the calcium phosphate method (Wigler *et al.*, 1977). Forty-eight hours after transfection, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.5% Triton-X 100, aprotinin (10 μg/ml), leupeptin (10 μg/ml), 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. Cell lysates (500 μl containing ~500 μg of protein) were incubated first for 1 h at 4°C with 50 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech) and then for 4 h at 4°C with 5 μg of the required antibodies together with protein G-Sepharose beads. For the detection of ubiquitinated proteins under denaturing conditions (Figure 4D), after extensive washing with lysis buffer the anti-Myc immunoprecipitates were boiled for 5 min with RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% [vol/vol] Nonidet P-40, 0.1% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS, 1 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate); the supernatants then were subjected to a second immunoprecipitation for 4 h at 4°C with 5 μg of ant-Myc and protein G-Sepharose beads.

Immunoprecipitates were washed four times with ice-cold lysis buffer, were fractionated by SDS-PAGE, and were subjected to immunoblot analysis with antibodies at a concentration of 1 μg/ml. Immune complexes were detected with horseradish peroxidase-conjugated polyclonal antibodies to mouse (Promega, Madison, WI) or rabbit (Southern Biotechnology Associates, Birmingham, AL) immunoglobulin G and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

In Vitro Ubiquitination Assay

HeLa cells were synchronized in G_1 phase by incubation for 2 h after the release from nocodazole-induced arrest and then were lysed in two volumes of hypotonic buffer (10 mM Tris-HCl [pH 7.5], 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 0.5 mM PMSF, 20 mM leupeptin) by freezing and thawing. The lysates were centrifuged for 4 h at 43,000 rpm (100,000 $\times$ *g*), and the resulting supernatants (S100) were used in the ubiquitination assay as described previously (Hatakeyama *et al.*, 1999; Kitagawa *et al.*, 1999). In brief, GST-mE2-C fusion protein (0.5 μg) was incubated for 60 min at 37°C with 20 μl of S100 supernatant at various concentrations. The GST-mE2-C protein was then immunoprecipitated with anti-GST, and its ubiquitination was detected by immunoblot analysis with antiubiquitin.

The APC/C-mediated ubiquitination assay was performed as described previously (Yamano *et al.*, 1998). In brief, the 20S APC/C was immunoaffinity purified from the S100 fraction of HeLa cells in G_1 with the use of anti-CDC27 (AF3)-coupled Affi-Prep protein A beads (Bio-Rad, Cambridge, MA). The 10-μl reaction mixture contained rabbit E1, mE2-C, immunopurified APC/C, bovine ubiquitin, and test substrate in a buffer of 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$,1 mM DTT, and 2 mM adenosine-5′-triphosphate (ATP). After incubation for 90 min at 37°C, Myc-tagged human cyclin B1, mE2-C, or GST-mE2-C as substrates were immunoprecipitated with anti-Myc, anti-mE2-C, or anti-GST, respectively. The resulting precipitates were subjected to SDS-PAGE on a 5–15% gradient gel (Bio-Rad), followed by immunoblot analysis with anti-cyclin B, antiubiquitin, or anti-mE2-C.

Pulse–Chase Experiments

Transfected HeLa cells were metabolically labeled with Trans^{35}S-label (ICN, Costa Messa, CA) at a concentration of 100 μCi/ml for 1 h. After incubation for various times in the absence of isotopes, the cells were lysed and subjected to immunoprecipitation with antibodies to Myc (9E10) and protein G-Sepharose. The immunoprecipitates were fractionated by SDS-PAGE and were subjected to analysis with the BAS-2000 image analyzer.

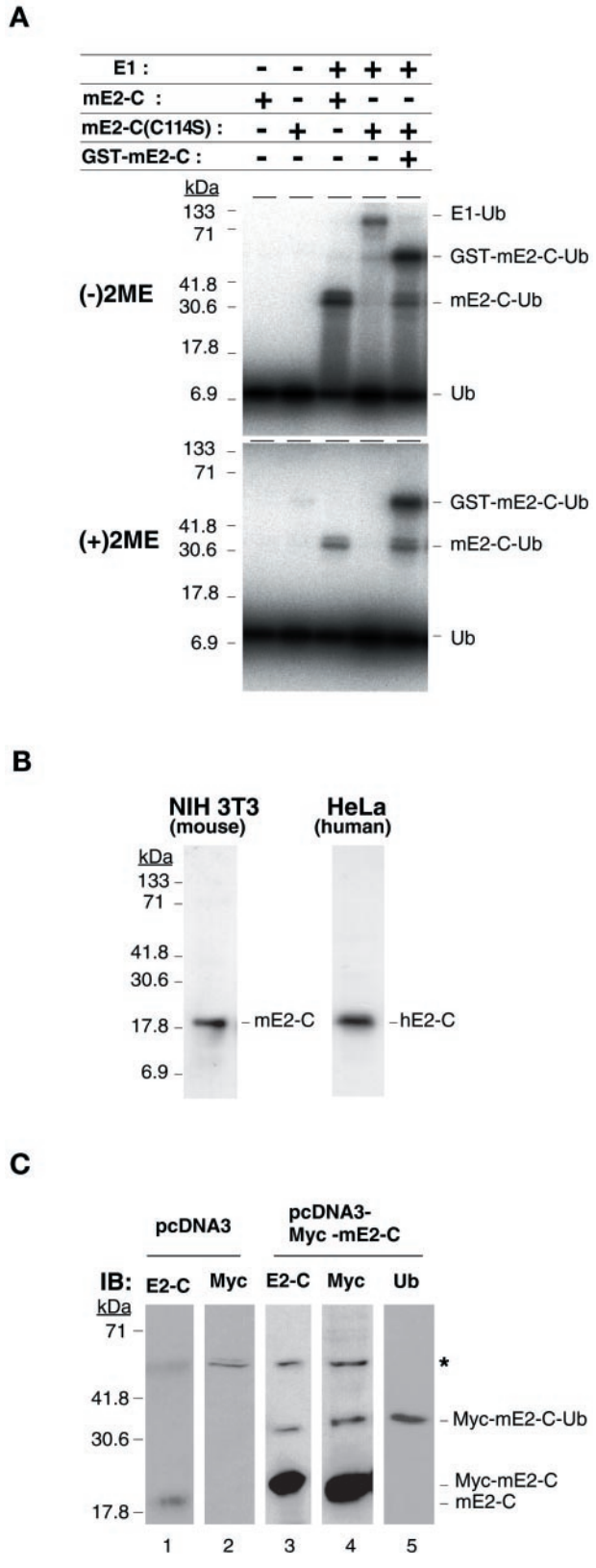
RESULTS

Autoubiquitination of Mammalian E2-C

We obtained a cDNA clone encoding mouse E2-C (mE2-C) through a search of an EST database. Recombinant mE2-C was produced in bacteria as a glutathione S-transferase (GST) fusion protein, which was purified with the use of glutathione beads and then was cleaved to remove the GST sequence. The recombinant mE2-C became conjugated with ^{125}I-labeled ubiquitin in vitro in the presence of, but not in the absence of, rabbit E1 (Figure 1A). The mutant mE2-C(C114S), in which Cys-114, the conserved putative active site of the enzyme, was replaced with Ser, did not become conjugated with ^{125}I-ubiquitin, indicating that Cys-114 is essential for the activity of mE2-C. In the presence of the reducing agent 2-mercaptoethanol, which dissociates thiol ester bonds, mE2-C still underwent conjugation with ^{125}I-ubiquitin, although to a lesser extent, suggesting that the residual ^{125}I-ubiquitin was attached by an isopeptide bond to a lysine residue of mE2-C. The fact that the mutant mE2-C(C114S) did not become conjugated with ^{125}I-ubiquitin in the absence or presence of 2-mercaptoethanol suggests that mE2-C undergoes autoubiquitination as a result of its own E2 activity. In contrast, the mutant mE2-C(C114S) was conjugated with ^{125}I-ubiquitin, when the wild-type mE2-C fused with GST also was present in this in vitro assay (Figure 1A, rightmost lane). These data suggest that the E2-C molecules can ubiquitinate one another, thereby the wild-type E2-C ubiquitinates the mutant mE2-C(C114S) protein.

Elimination of Mammalian E2-C Near the M-G_1 Transition

We prepared rabbit polyclonal antibodies to (anti-) mE2-C with the use of the recombinant mE2-C protein as immunogen. Immunoblot analysis of various cell lines with these antibodies revealed that mouse NIH 3T3 cells expressed a ~20-kDa immunoreactive protein (Figure 1B). The antibodies also showed cross-reactivity with hE2-C, which shares 96% sequence identity with mE2-C, in human HeLa cells.



The specificity of the antibodies was verified by transient transfection of NIH 3T3 cells with an expression plasmid encoding Myc-tagged mE2-C. A ~22-kDa protein was recognized by both anti-mE2-C and anti-Myc in the cells transfected with the Myc-mE2-C vector but not in those transfected with the empty vector (Figure 1C), indicating that anti-mE2-C react specifically with mE2-C. A larger immunoreactive protein (~30 kDa) was also detected by both anti-mE2-C and anti-Myc in the NIH 3T3 cells overexpressing Myc-mE2-C. This protein was recognized by antiubiquitin, suggesting that it was a monoubiquitinated form of Myc-tagged mE2-C. Given that such a monoubiquitinated protein was not detected when the mutant mE2-C(C114S) was similarly expressed in NIH 3T3 cells (our unpublished results), it is likely that the monoubiquitination of mE2-C in vivo is dependent on the autocatalytic activity of this enzyme.

We next examined whether mammalian E2-C expression is regulated in a cell cycle-dependent manner. The mE2-C protein was not detected in NIH 3T3 cells that had been arrested in G_1–S phase by aphidicolin treatment (Figure 2A). In contrast, mE2-C was abundant in cells arrested at metaphase by nocodazole treatment. To investigate further the changes in mE2-C expression during the cell cycle, we induced the arrest of cells at G_0–G_1 phase by contact inhibition and then allowed them to proceed through the cell cycle by replating. Flow cytometry revealed that the number of cells in S phase began to increase 12 h after release from arrest, reached a maximum at 21 h, and decreased thereafter, and that the number of cells in G_2–M phase was maximal after 21–24 h (Figure 2B). The expression of mE2-C was not detected during the 18 h immediately after the release of cells from arrest; it appeared at 21 h as the cells entered G_2–M phase (Figure 2B). The expression of mE2-C during M-G_1 progression was examined in detail by arresting cells in metaphase by nocodazole treatment and then allowing them to proceed into G_1 phase. The synchronized NIH 3T3 cells began to exit from mitosis ~1.5 h after their release from arrest and entered into G_1 phase within 6 h. The mE2-C protein was abundant during metaphase but disappeared as cells exited from mitosis (Figure 3A). Again, the timing of mE2-C down-regulation seemed to be relatively later than

Figure 1. Autoubiquitination activity of mE2-C. (A) Recombinant wild-type (wt) or mutant (C114S) mE2-C (20 kDa) was incubated with ^{125}I-ubiquitin in the absence or presence of E1 (116 kDa). The reaction mixtures then were boiled in SDS sample buffer, without (upper panel) or with (lower panel) 2-mercaptoethanol (2ME), and were subjected to SDS-PAGE and autoradiography. The positions of free ubiquitin (Ub), E1 conjugated with ubiquitin (E1-Ub), mE2-C conjugated with ubiquitin (mE2-C-Ub), and GST-mE2-C conjugated with ubiquitin (GST-mE2-C-Ub) are indicated on the right, and those of molecular size standards (in kilodaltons) are shown on the left. (B) Cell lysates prepared from mouse NIH 3T3 and human HeLa cells were subjected to immunoblot analysis with anti-mE2-C. The positions of the immunoreactive mE2-C and hE2-C proteins are indicated. (C) NIH 3T3 cells were transfected with either an expression plasmid encoding Myc-tagged mE2-C or the empty vector (pcDNA3). Cell lysates were subsequently prepared and subjected to immunoblot analysis (IB) with anti-mE2-C, anti-Myc, or antiubiquitin. The positions of mE2-C, Myc-mE2-C, and monoubiquitinated Myc-mE2-C (Myc-mE2-C-Ub) are shown. The asterisk indicates a nonspecific band. Because the exposure time was shorter for lane 3 than for lane 1, the endogenous mE2-C is not visible in lane 3.

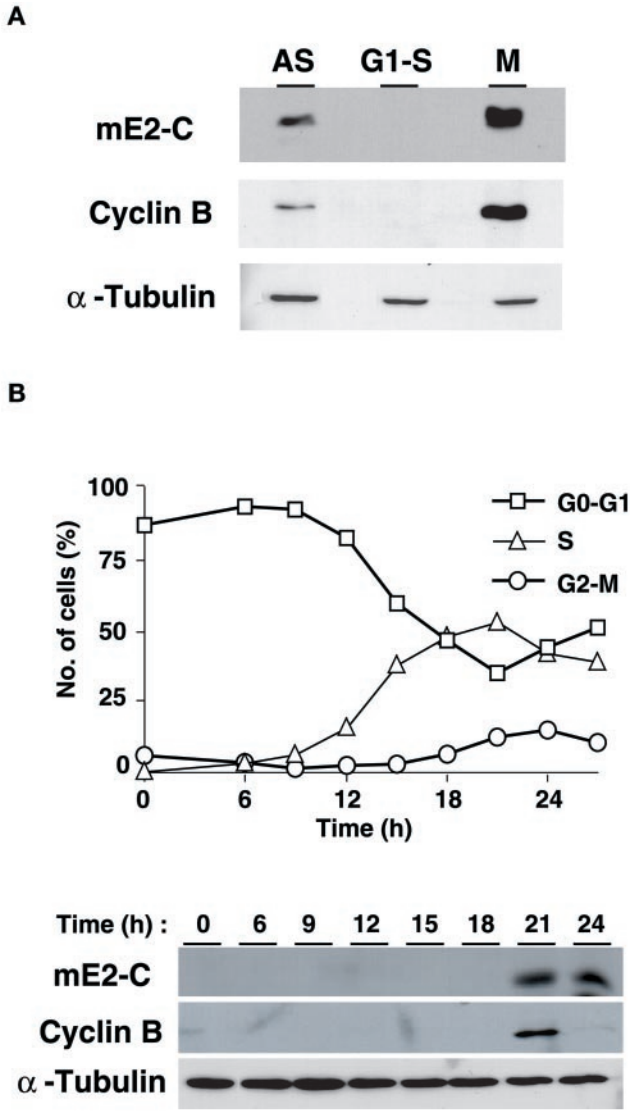


Figure 2. Expression of mammalian E2-C during G_2–M phase of the cell cycle. (A) Cell lysates were prepared from asynchronous (AS) NIH 3T3 cells or from cells synchronized at either G_1–S phase by aphidicolin treatment or M phase by nocodazole treatment and then were subjected to immunoblot analysis with anti-mE2-C (top panel), anti-cyclin B (middle panel), or anti-α-tubulin (bottom panel). (B) NIH 3T3 cells were arrested at G_0–G_1 phase by contact inhibition and then were induced to resume the cell cycle by replating. At the indicated times thereafter, the proportion of cells in G_0–G_1 phase (squares), S phase (triangles), or G_2–M phase (circles) was determined by flow cytometry and was expressed as a percentage of the total number of cells (upper panel). Cell lysates were also prepared, adjusted to equal protein concentrations, and subjected to immunoblot analysis (lower panel), as in (A).

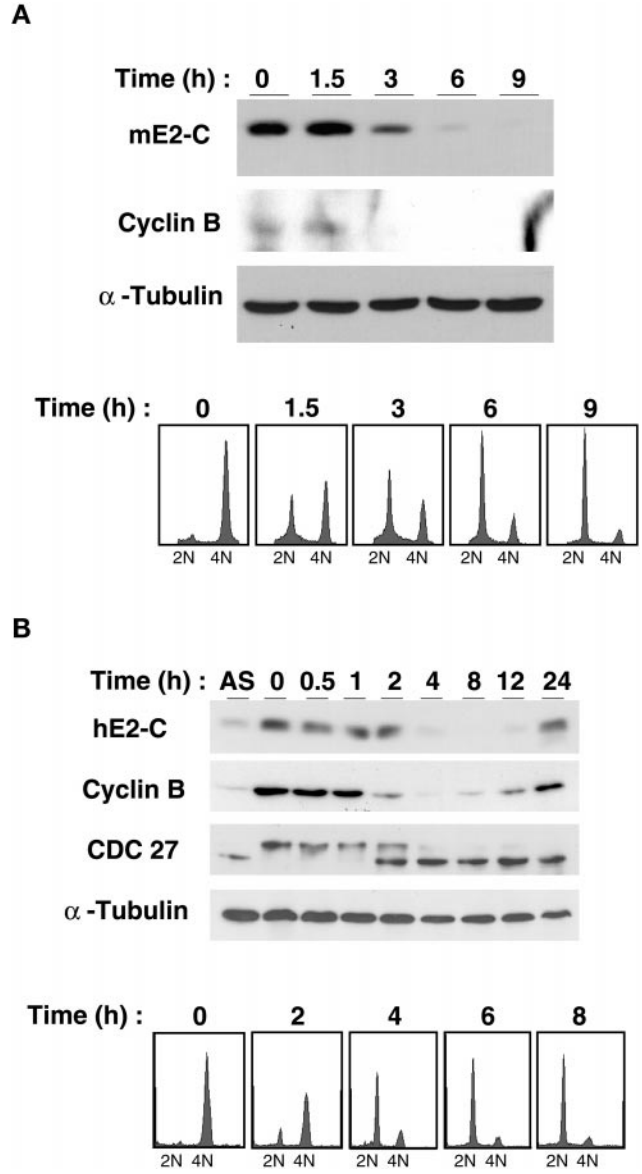


Figure 3. Down-regulation of mammalian E2-C at early G_1 phase. Mouse NIH 3T3 (A) and human HeLa (B) cells were arrested at metaphase by nocodazole treatment and then were incubated in nocodazole-free medium for the indicated times. Cell lysates were prepared from asynchronous (AS) or synchronized cells and then were subjected to immunoblot analysis with anti-mE2-C, anti-cyclin B, anti-CDC27, and anti-α-tubulin (upper panels), and the DNA content of cells was determined by flow cytometry (lower panels).

that of cyclin B disappearance. We also examined whether the expression of hE2-C oscillates in a similar manner during the cell cycle. HeLa cells were arrested at metaphase by nocodazole treatment and then were released to proceed synchronously through the cell cycle into G_1 phase. CDC27 (human APC3), a component of the APC/C, is present in a phosphorylated form (130 kDa) during mitosis and in a dephosphorylated form (90 kDa) during interphase (King *et al.*, 1995). We therefore used this change in mobility of CDC27 as an index of exit from mitosis and progression into G_1 phase. The mobility shift occurred 2 h after the release of HeLa cells from metaphase arrest (Figure 3B). The amount of cyclin B also decreased rapidly 2 h after the release of cells from arrest, which is indicative of its degradation at the end

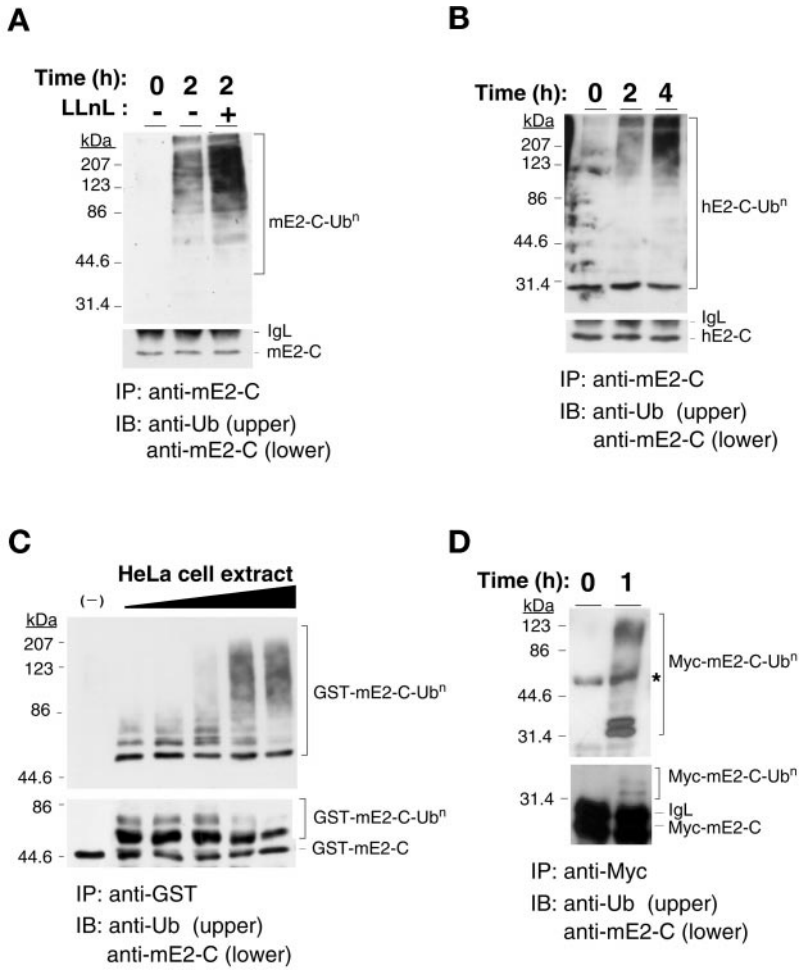


Figure 4. Cell cycle-dependent polyubiquitination of mammalian E2-C. (A) Mouse NIH 3T3 cells were arrested at metaphase by nocodazole treatment and then were incubated in nocodazole-free medium for the indicated times in the absence or presence of the proteasome inhibitor LLnL at a concentration of 250 μM. Cell lysates were prepared and subjected to immunoprecipitation (IP) with anti-mE2-C, and the resulting precipitates were subjected to immunoblot analysis (IB) with either antiubiquitin (upper panel) or anti-mE2-C (lower panel). The positions of mE2-C, polyubiquitinated mE2-C (mE2-C-Ub$_n$), and immunoglobulin light chain (IgL) are indicated. (B) Human HeLa cells were subjected to metaphase arrest, were incubated for the indicated times in nocodazole-free medium in the presence of LLnL, and were subjected to immunoprecipitation and immunoblot analysis, as in (A). The positions of hE2-C, monoubiquitinated hE2-C (hE2-C-Ub$_1$), polyubiquitinated hE2-C (hE2-C-Ub$_n$), and IgL are indicated. (C) A recombinant GST fusion protein of mE2-C was incubated for 1 h at 37°C in the absence (–) or presence of various concentrations (0.25 μg/μl, 0.5 μg/μl, 1.0 μg/μl, 2.5 μg/μl, and 5.0 μg/μl) of cell extract prepared from HeLa cells 2 h after release from nocodazole-induced arrest. The fusion protein then was immunoprecipitated with anti-GST and ws subjected to immunoblot analysis with antiubiquitin (upper panel) or anti-mE2-C (lower panel). (D) Cell lysates were prepared from NIH 3T3 cells expressing Myc-tagged mE2-C that had been arrested at metaphase by nocodazole treatment and incubated for 0 or 1 h in nocodazole-free medium. The lysates were subjected to immunoprecipitation with anti-Myc, and the resulting precipitates were boiled in SDS-containing buffer and were subjected to reimmunoprecipitation with anti-Myc. The final precipitates then were subjected to immunoblot analysis with antiubiquitin (upper panel) or anti-mE2-C (lower panel). The asterisk indicates a nonspecific band.

of mitosis. The hE2-C protein was expressed in metaphase but disappeared between 2 and 4 h after the release of HeLa cells from nocodazole block. These results with both mouse and human cells thus indicate that E2-C is expressed during mitosis and is eliminated as cells enter G_1 phase.

Degradation of Mammalian E2-C by the Ubiquitin–Proteasome Pathway

It was likely that the down-regulation of mammalian E2-C expression at early G_1 phase was due to an increased rate of proteolysis, given that the corresponding mRNA is constitutively expressed during the cell cycle (Arvand *et al.*, 1998). Thus, we examined whether mammalian E2-C is degraded by the ubiquitin–proteasome pathway. Cell lysates were prepared from NIH 3T3 cells that had been arrested at metaphase by nocodazole treatment and then incubated (or not) for 2 h in nocodazole-free medium in the absence or presence of the proteasome inhibitor *N*-acetyl-Leu-Leu-norleucinol (LLnL). The lysates then were subjected to immunoprecipitation with anti-mE2-C, and the resultant precipitates were subjected to immunoblot analysis with antiubiquitin. Whereas mE2-C showed only a low level of ubiquitination in cells at metaphase, it had undergone substantial polyubiquitination by 2 h after the release of cells from nocodazole block (Figure 4A). The polyubiquitination of mE2-C was further increased by the incubation of cells in the presence of LLnL. Similarly, hE2-C also was polyubiquitinated in HeLa cells 2 to 4 h after release from nocodazole block (Figure 4B).

We then investigated whether polyubiquitination of mammalian E2-C could be recapitulated in a cell-free system. A recombinant GST fusion protein of mE2-C was incubated in the presence of various concentrations of lysate prepared from HeLa cells that had been cultured for 2 h in nocodazole-free medium after nocodazole block. The reaction mixtures then were subjected to immunoprecipitation with anti-GST, and the resulting immunoprecipitates were subjected to immunoblot analysis with antiubiquitin. The cell lysate induced the polyubiquitination of GST-mE2-C in a concentration-dependent manner (Figure 4C). To exclude the possibility that another protein bound to mE2-C (not mE2-C itself) was ubiquitinated, we boiled Myc-mE2-C immunoprecipitates prepared from transfected NIH 3T3 cells in SDS-containing buffer, reimmunoprecipitated Myc-mE2-C, and subjected the second immunoprecipitates to immunoblot analysis with antiubiquitin. The ubiquitination

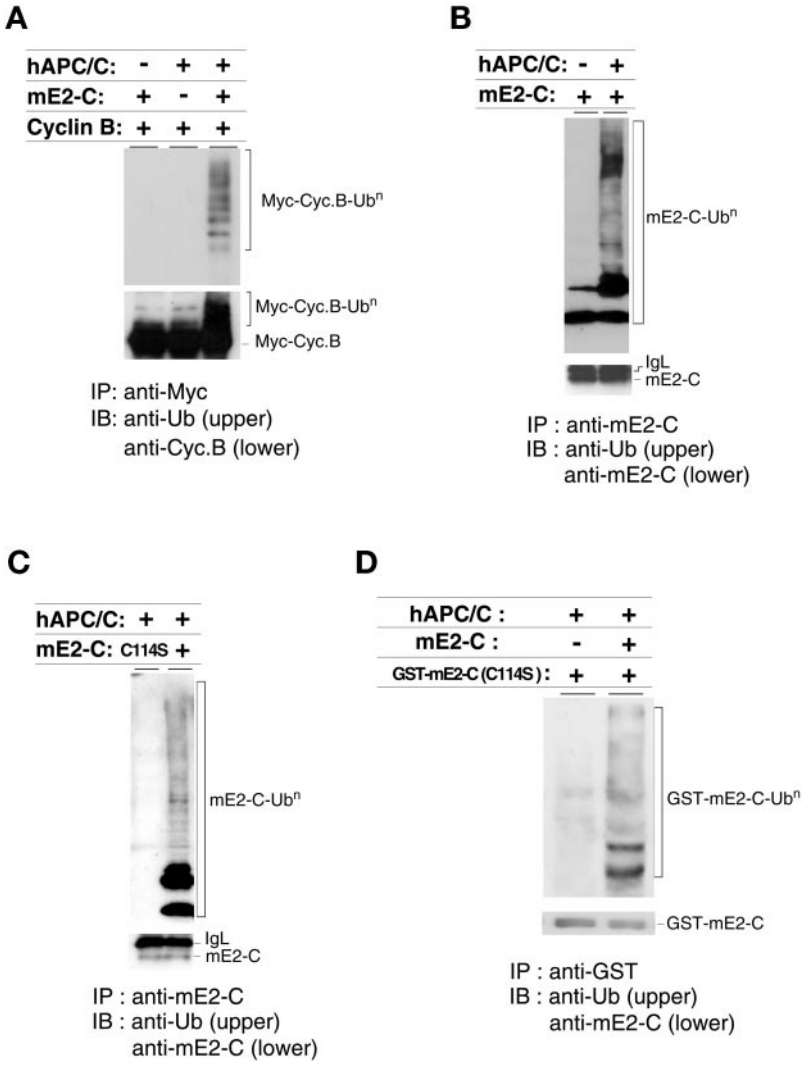


Figure 5. Polyubiquitination of mammalian E2-C by the APC/C. (A) Recombinant Myc-tagged cyclin B was subjected to an in vitro ubiquitination assay by incubation with rabbit E1, bovine ubiquitin, and either immunopurified human APC/C (hAPC/C) or recombinant mE2-C, as indicated. The reaction mixtures were subjected to immunoprecipitation with anti-Myc, and the resulting precipitates were subjected to immunoblot analysis with antiubiquitin (upper panel) or anti-cyclin B (lower panel). Cyc B, cyclin B. (B) Recombinant mE2-C was subjected to in vitro ubiquitination assay, as in (A), in the absence or presence of purified human APC/C. The reaction mixtures were subjected to immunoprecipitation with anti-mE2-C, and the resulting precipitates were subjected to immunoblot analysis with antiubiquitin (upper panel) or anti-mE2-C (lower panel). (C) Recombinant wild-type (wt) or mutant (C114S) mE2-C was subjected to in vitro ubiquitination assay in the presence of purified human APC/C and was processed as in (B). (D) Recombinant GST fusion protein of mutant (C114S) mE2-C was subjected to in vitro ubiquitination assay in the absence or presence of wild-type mE2-C, together with purified human APC/C and processed as in (B).

of mE2-C was still apparent under these stringent conditions (Figure 4D). These results demonstrate that the ubiquitinated protein is mE2-C and is not a protein associated with mE2-C. Thus, our in vivo and in vitro observations suggest that both mE2-C and hE2-C are polyubiquitinated by a factor (or factors) present in cells near the M–G_1 transition, and that they are subsequently degraded by the 26S proteasome.

Polyubiquitination of Mammalian E2-C by the APC/C

Many substrates of the APC/C are degraded during M and G_1 phases, and they contain destruction boxes (Glotzer *et al.*, 1991; Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1997; Juang *et al.*, 1997; McGarry and Kirschner, 1998; Shirayama *et al.*, 1998). Given that mammalian E2-C also is degraded at early G_1 phase and contains a pair of destruction boxes (see Figure 6A), this protein was also likely to be a substrate of the APC/C. To determine whether mE2-C is polyubiquitinated by the APC/C, it was incubated in a reaction mixture containing immunopurified human APC/C, rabbit E1, and bovine ubiquitin. The ubiquitin ligase activity of the purified human APC/C was confirmed in this in vitro assay with the use of Myc-tagged human cyclin B as substrate. Recombinant cyclin B underwent substantial ubiquitination only in the presence of both mE2-C and purified APC/C (Figure 5A), showing that the latter possesses E3 activity, but not E2 activity, and that mE2-C functionally cooperates with human APC/C. The mE2-C protein also underwent marked polyubiquitination in the presence of purified APC/C, whereas only mono- and diubiquitinated mE2-C were observed in the absence of APC/C (Figure 5B). These results suggest that APC/C participates in the polyubiquitination of mE2-C. The mutant mE2-C(C114S), which lacks E2 activity, was not ubiquitinated even in the presence of APC/C (Figure 5C), indicating that polyubiquitination of mE2-C requires the E2 activity of mE2-C. Given that two mE2-C molecules ubiquitinate one another (Figure 1A), we exam-

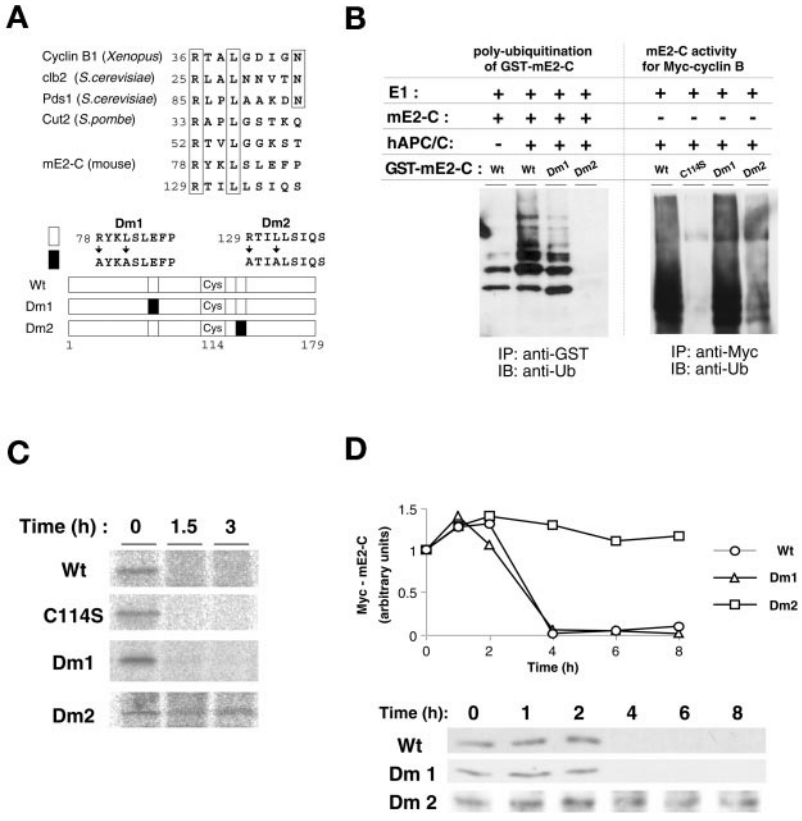


Figure 6. Destruction boxes are involved in the ubiquitination and stability of mammalian E2-C. (A) The amino acid sequences of the destruction boxes of cyclin B1 (*Xenopus laevis*), Clb2 (*Saccharomyces cerevisiae*), Pds1 (*S. cerevisiae*), Cut2 (*Schizosaccharomyces pombe*), and mE2-C (*Mus musculus*) are aligned in the upper panel. Amino acids conserved among these proteins are boxed. Residue numbers are shown at the beginning of each sequence. The mE2-C mutants used in the experiments in (B)–(D) are shown schematically in the lower panel. Open and closed boxes indicate wild-type and mutant destruction boxes, respectively. (B) Recombinant GST fusion proteins of wild-type and mutant (Dm1 and Dm2) mE2-C were subjected to in vitro ubiquitination assay in the absence or presence of purified human APC/C. The wild-type mE2-C also was added to each reaction. The reaction mixtures were subjected to immunoprecipitation with anti-GST, and the resulting precipitates were subjected to to immunoblot analysis with antiubiquitin (left panel). The E2 activity of recombinant GST-fused wild-type and mutant (C114S, Dm1, and Dm2) proteins was examined with purified human APC/C and Myc-cyclin B as a substrate as in Figure 5A (right panel). (C) HeLa cells transfected with vectors encoding the Myc-tagged wild-type and mutant (C114S, Dm1, and Dm2) mE2-C proteins were pulse labeled with [^{35}S]methionine and then were incubated in the absence of isotope for the indicated chase periods. Cell lysates then were subjected to immunoprecipitation with the monoclonal antibody to Myc, and the resulting precipitates were subjected to SDS-PAGE and autoradiography. (D) HeLa cells transfected with vectors encoding the Myc-tagged wild-type and mutant (Dm1 and Dm2) mE2-C proteins were arrested at metaphase by nocodazole treatment and then were incubated in nocodazole-free medium for the indicated times. Cell lysates then were subjected to immunoblot analysis with the monoclonal antibody to Myc.

ined whether the mutant mE2-C(C114S) is polyubiquitinated with APC/C in the presence or absence of wild-type mE2-C. Substantial polyubiquitination was apparent in the mutant protein, only when the wild-type mE2-C protein was present (Figure 5D). Thus, we conclude that the autocatalytic E2 activity of the mE2-C that undergoes polyubiquitination is not essential.

Requirement of the Destruction Box for Polyubiquitination and Degradation of Mammalian E2-C

The mE2-C protein contains two sequences that show similarity to the consensus sequence (RXXL) for destruction boxes identified in many APC/C substrates (mitotic cyclins, Pds1/Cut2, Cdc5, Cdc20, and Geminin) (Figure 6A). To determine whether these two sequences are required for APC/C-mediated polyubiquitination of mE2-C, we replaced the conserved arginines (Arg-78 and Arg-129) and leucines (Leu-81 and Leu-132) in the putative destruction boxes with alanine residues. The resultant mutant proteins containing substitutions in the first destruction box motif (R78A/L81A), or in the second motif (R129A/L132A) were designated Dm1 and Dm2, respectively (Figure 6A). GST fusion proteins of wild-type or mutant mE2-C were expressed in bacteria, purified, and subjected to the in vitro ubiquitination assay with purified APC/C (Figure 6B, left). In this assay, wild-type E2-C also was added to rule out the possibility that the mutations in the destruction boxes affect E2 enzymatic activity, given that this motif is located close to the active site of mE2-C. Compared with the extent of polyubiquitination of wild-type GST-mE2-C, that of GST-mE2-C(Dm1) was slightly reduced. In contrast, the ubiquitination of GST-mE2-C(Dm2) was markedly reduced. The E2 activity of these mutants was estimated with APC/C and Myc-cyclin B to examine whether the mutations in the destruction boxes affect E2 enzymatic activity. The E2 activity of mE2-C(Dm1) was almost comparable with that of wild-type protein (Figure 6B, right). Although the E2 activity of mE2-C(Dm2) was reduced compared with that of wild-type protein, the mutant protein harbored substantial E2 activity in comparison with the mutant mE2-C(C114S), which did not exhibit the E2 activity. Because mutations at both destruction boxes of mE2-C protein abolished the E2 activity, it was not possible to evaluate the effect of the double mutant without affecting E2 activity (our unpublished results). Thus, the destruction boxes are required for the APC/C-mediated polyubiquitination of mE2-C protein.

Thus, we examined whether the mE2-C mutants become more stable than wild-type protein using pulse–chase experiments. Compared with wild-type mE2-C, the Dm2 mutant protein exhibited a significantly extended half-life (Figure 6C), suggesting that this mutant escapes the ubiquitin-

dependent degradation mediated by APC/C. The Dm1 mutant seemed to be as unstable as wild-type protein. Consistent with the in vitro result that the mutant mE2-C(C114S) underwent the APC/C-mediated polyubiquitination with the wild-type mE2-C protein, the mutant protein was as unstable as the wild-type protein. This result suggests that E2 activity of the mE2-C may not be necessary for the degradation of the protein, when endogenous mE2-C is present. Rather, the destruction boxes as the motif recognized by APC/C seem to be important for the degradation of the mE2-C protein.

Furthermore, we examined the expression of wild-type mE2-C and the destruction box mutants at the M–G_1 transition (Figure 6D). The levels of wild-type and Dm1 mutant mE2-C proteins declined between 2 and 4 h after release from metaphase arrest. In contrast, the abundance of Dm2 mutant mE2-C was not reduced up to 8 h after release. These results suggest that mammalian E2-C, an E2 enzyme that functionally associates with APC/C, undergoes destruction box-dependent degradation at early G_1 phase.

DISCUSSION

E2-C has been thought to serve as a major E2 component that functionally associates with the APC/C ubiquitin ligase. The activity of E2-C, as well as that of the APC/C, is required for progression through and exit from M phase (Townsley *et al.*, 1997). It had also been thought that the APC/C (not the E1 and E2 enzymes) is the only ubiquitination component the activity of which oscillates during the cell cycle (Hershko and Ciechanover, 1998). However, we have now shown that the abundance of mammalian E2-C also varies during the cell cycle, being maximal at the peak of M phase and decreasing as cells exit from M phase. The disappearance of mammalian E2-C appears to be dependent on its polyubiquitination by the APC/C and subsequent degradation by the 26S proteasome. Thus, our results suggest that the degradation of mammalian E2-C may be a novel autoregulatory aspect of the APC/C system, functioning as a negative feedback loop or as a control of target specificity.

Mouse E2-C was identified as a protein that is up-regulated in NIH 3T3 cells transformed by a *EWS-FLI1* fusion gene that is associated with Ewing's sarcoma (Arvand *et al.*, 1998). The protein was shown to be expressed in G_2–M phase but not in G_0–G_1 phase, whereas its mRNA was detected throughout the cell cycle, suggesting that the abundance of mE2-C is controlled at the posttranslational level. We have now extended these observations by revealing the mechanism that underlies the periodic expression of mammalian E2-C protein and showing that mammalian E2-C is degraded at early G_1 phase. Various proteins that contribute to mitotic regulation, such as cyclin B and Cdc5, also undergo APC/C-mediated degradation at the end of mitosis (Glotzer *et al.*, 1991; Prinz *et al.*, 1998; Shirayama *et al.*, 1998). These proteins share a consensus motif known as the destruction box that is thought to function as a recognition motif for the APC/C. Because mammalian E2-C also contains the destruction box motif, it is a likely target of APC/C-mediated polyubiquitination.

The mE2-C protein, like its human ortholog, hE2-C, exhibited autoubiquitination activity in the presence of E1, and it became covalently conjugated with several ubiquitin molecules in the absence of an E3 enzyme. Autoubiquitination of E2 enzymes also has been described for Cdc34, Ubc4 (*Saccharomyses cerevisiae*), and Ubc1 (*Caenorhabditis elegans*), although the physiologic importance of such activity was unclear (Banerjee *et al.*, 1993; Gwozd *et al.*, 1995; Leggett and Candido, 1997). We have now shown that autoubiquitination of mammalian E2-C may not be critical for the autoregulatory system that underlies the periodic expression of this protein, given that the mE2-C(C114S) protein, a mutant lacking E2 activity, undergoes polyubiquitination in the presence of APC/C and wild-type mE2-C. To date, the abundance of other E2 enzymes has not been shown to oscillate during the cell cycle. We did not detect any substantial change in the amount of Ubc3, which functions with an SCF-type ubiquitin ligase at the G_1–S phase, during the cell cycle in NIH 3T3 cells (our unpublished results). In addition, it has been largely unclear whether the autoubiquitination activity of E2 enzyme is regulated in a cell cycle-dependent manner.

Overexpression of a dominant negative mutant of hE2-C results in the accumulation of mitotic cyclins and the inhibition of sister chromatid segregation (Townsley *et al.*, 1997). Thus, E2-C activity appears to be important for the metaphase–anaphase transition because of its role, together with the APC/C, in the ubiquitination of anaphase inhibitors. The expression of mammalian E2-C at the peak of M phase is, thus, consistent with its function. However, the APC/C continues to be active during G_1 phase, after mammalian E2-C has been degraded, suggesting that other E2 enzymes might function with the APC/C during G_1 (Amon *et al.*, 1994; Brandeis and Hunt, 1996). Thus, we hypothesize that the E2 enzyme that functionally associates with the APC/C changes at early G_1 phase from E2-C to another E2 enzyme. The degradation of mammalian E2-C mediated by the APC/C at this time may facilitate the exchange of the E2 enzymes. However, we did not observe any changes in the progression of the cell cycle when the mutant mE2-C (Dm2) was expressed in vivo (our unpublished results), suggesting that the degradation of mammalian E2-C protein may not be necessary for the cell cycle progression. The physiologic importance of the mammalian E2-C degradation remains to be determined in the future. In fission yeast, the abundance of the E2-C homolog UbcP4 does not oscillate during the cell cycle, suggesting that the regulation of mammalian APC/C is more complex than that of fission yeast APC/C (Osaka *et al.*, 1997).

The substrate specificity of the APC/C changes at late M phase from anaphase inhibitors to mitotic cyclins. In yeast, these different substrate specificities are thought to be conferred by coactivators of the APC/C, such as Cdc20 and Hct1 (reviewed by Townsley and Ruderman, 1998; Morgan, 1999; Zachariae and Nasmyth, 1999). Cdc20 is expressed in G_2–M phase and, together with the APC/C, regulates the progression from metaphase to anaphase by mediating the ubiquitination of anaphase inhibitors such as securins. Cdc20 itself is also ubiquitinated, probably by the APC/C, and is degraded during the exit from M phase (Prinz *et al.*, 1998; Shirayama *et al.*, 1998). Hct1 is activated at the M–G_1 transition, continues to be active until the G_1-S transition, and promotes ubiquitination and degradation of mitotic cyclins (Sigrist and Lehner, 1997; Fang *et al.*, 1998a; Kramer

et al., 1998). These results are consistent with the idea that the timing of late mitotic events results from the sequential activation of the APC/C by Cdc20 and Hct1. It is therefore possible that activated Hct1 binds to the APC/C and thereby facilitates the ubiquitination of Cdc20, Plk1, and E2-C. The possible switch of E2 enzymes associated with the APC/C may play an important role, together with APC/C coactivators, in conferring different substrate specificities on the APC/C.

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REFERENCES

Amon, A., Irniger, S., and Nasmyth, K. (1994). Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. Cell *77*, 1037–1050.

Aristarkhov, A., Eytan, E., Moghe, A., Admon, A., Hershko, A., and Ruderman, J.V. (1996). E2-C, a cyclin-selective ubiquitin carrier protein required for the destruction of mitotic cyclins. Proc. Natl. Acad. Sci. USA *93*, 4294–4299.

Arvand, A., Bastians, H., Welford, S.M., Thompson, A.D., Ruderman, J.V., and Denny, C.T. (1998). EWS/FLI1 up regulates mE2-C, a cyclin-selective ubiquitin conjugating enzyme involved in cyclin B destruction. Oncogene *17*, 2039–2045.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263–274.

Banerjee, A., Gregori, L., Xu, Y., and Chau, V. (1993). The bacterially expressed yeast CDC34 gene product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. J. Biol. Chem. *268*, 5668–5675.

Brandeis, M., and Hunt, T. (1996). The proteolysis of mitotic cyclins in mammalian cells persists from the end of mitosis until the onset of S phase. EMBO J. *15*, 5280–5289.

Cohen-Fix, O., Peters, J.M., Kirschner, M.W., and Koshland, D. (1996). Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev. *10*, 3081–3093.

Coux, O., Tanaka, K., and Goldberg, A.L. (1996). Structure and functions of the 20S and 26S proteasomes. Annu. Rev. Biochem. *65*, 801–847.

Dawson, I.A., Roth, S., and Artavanis-Tsakonas, S. (1995). The Drosophila cell cycle gene fizzy is required for normal degradation of cyclins A and B during mitosis and has homology to the CDC20 gene of *Saccharomyces cerevisiae*. J. Cell. Biol. *129*, 725–737.

Fang, G., Yu, H.T., and Kirschner, M.W. (1998a). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes Dev. *12*, 1871–1883.

Fang, G.W., Yu, H.T., and Kirschner, M.W. (1998b). Direct binding of Cdc20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol. Cell. *2*, 163–171.

Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221–230.

Funabiki, H., Yamano, H., Nagao, K., Tanaka, H., Yasuda, H., Hunt, T., and Yanagida, M. (1997). Fission yeast Cut2 required for anaphase has two destruction boxes. EMBO J. *16*, 5977–5987.

Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature *349*, 132–138.

Gwozd, C.S., Arnason, T.G., Cook, W.J., Chau, V., and Ellison, M.J. (1995). The yeast UBC4 ubiquitin conjugating enzyme monoubiquitinates itself in vivo: evidence for an E2–E2 homointeraction. Biochemistry *34*, 6296–6302.

Hatakeyama, S., Kitagawa, M., Nakayama, K., Shirane, M., Matsumoto, M., Hattori, K., Higashi, H., Nakano, H., Okumura, K., Onoe, K., Good, R.A., and Nakayama, K.-i. (1999). Ubiquitin-dependent degradation of IκBα is mediated by a ubiquitin ligase Skp1/Cul 1/F-box protein FWD1. Proc. Natl. Acad. Sci. USA *96*, 3859–3863.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system: resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. *258*, 8206–8214.

Irniger, S., Piatti, S., Michaelis, C., and Nasmyth, K. (1995). Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. Cell *81*, 269–278.

Jensen, J.P., Bates, P.W., Yang, M., Vierstra, R.D., and Weissman, A.M. (1995). Identification of a family of closely related human ubiquitin conjugating enzymes. J. Biol. Chem. *270*, 30408–30414.

Juang, Y.L., Huang, J., Peters, J.M., McLaughlin, M.E., Tai, C.Y., and Pellman, D. (1997). APC-mediated proteolysis of Ase1 and the morphogenesis of the mitotic spindle. Science *275*, 1311–1314.

Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G., Jr., Elledge, S.J., Conaway, R.C., Harper, J.W., and Conaway, J.W. (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657–661.

King, R.W., Peters, J.M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M.W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell *81*, 279–288.

Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., Nakayama, K.-i., and Nakayama, K. (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of β-catenin. EMBO J. *18*, 2401–2410.

Kramer, E.R., Gieffers, C., Holzl, G., Hengstschlager, M., and Peters, J.M. (1998). Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family. Curr. Biol. *8*, 1207–1210.

Leggett, D.S., and Candido, P.M. (1997). Biochemical characterization of *Caenorhabditis elegans* UBC-1: self-association and autoubiquitination of a RAD6-like ubiquitin-conjugating enzyme in vitro. Biochem. J. *327*, 357–361.

McGarry, T.J., and Kirschner, M.W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell *93*, 1043–1053.

Morgan, D.O. (1999). Regulation of the APC and the exit from mitosis. Nat. Cell. Biol. *1*, E47–E53.

Ohta, T., Michel, J.J., Schottelius, A.J., and Xiong, Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Mol. Cell *3*, 535–541.

Osaka, F., Seino, H., Seno, T., and Yamao, F. (1997). A ubiquitin-conjugating enzyme in fission yeast that is essential for the onset of anaphase in mitosis. Mol. Cell. Biol. *17*, 3388–3397.

Prinz, S., Hwang, E.S., Visintin, R., and Amon, A. (1998). The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. Curr. Biol. *8*, 750–760.

Schwab, M., Lutum, A.S., and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. Cell *90*, 683–693.

Seol, J.H., Feldman, R.M., Zachariae, W., Shevchenko, A., Correll, C.C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K., and Deshaies, R.J. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. *13*, 1614–1626.

Shirane, M., Harumiya, Y., Ishida, N., Hirai, A., Miyamoto, C., Hatakeyama, S., Nakayama, K.-i., and Kitagawa, M. (1999). Down-regulation of $p27^{Kip1}$ by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. J. Biol. Chem. *274*, 13886–13893.

Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. EMBO J. *17*, 1336–1349.

Sigrist, S., Jacobs, H., Stratmann, R., and Lehner, C.F. (1995). Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. EMBO J. *14*, 4827–4838.

Sigrist, S.J., and Lehner, C.F. (1997). Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. Cell *90*, 671–681.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209–219.

Skowyra, D., Koepp, D.M., Kamura, T., Conrad, M.N., Conaway, R.C., Conaway, J.W., Elledge, S.J., and Harper, J.W. (1999). Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. Science *284*, 662–665.

Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F.C., Ruderman, J.V., and Hershko, A. (1995). The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. Mol. Biol. Cell *6*, 185–197.

Tan, P., Fuchs, S.Y., Chen, A., Wu, K., Gomez, C., Ronai, Z., and Pan, Z.Q. (1999). Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of IκBα. Mol. Cell *3*, 527–533.

Townsley, F.M., Aristarkhov, A., Beck, S., Hershko, A., and Ruderman, J.V. (1997). Dominant-negative cyclin-selective ubiquitin carrier protein E2-C/UbcH10 blocks cells in metaphase. Proc. Natl. Acad. Sci. USA *94*, 2362–2367.

Townsley, F.M., and Ruderman, J.V. (1998). Proteolytic ratchets that control progression through mitosis. Trends Cell Biol. *8*, 238–244.

Visintin, R., Prinz, S., and Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. Science *278*, 460–463.

Weissman, A.M. (1997). Regulating protein degradation by ubiquitination. Immunol. Today *18*, 189–198.

Wigler, M., Silverstein, S., Lee, L.S., Pellicer, A., Cheng, Y., and Axel, R. (1977). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell *11*, 223–232.

Yamano, H., Gannon, J., and Hunt, T. (1996). The role of proteolysis in cell cycle progression in *Schizosaccharomyces pombe*. EMBO J. *15*, 5268–5279.

Yamano, H., Tsurumi, C., Gannon, J., and Hunt, T. (1998). The role of the destruction box and its neighboring lysine residues in cyclin B for anaphase ubiquitin-dependent proteolysis in fission yeast: defining the D-box receptor. EMBO J. *17*, 5670–5678.

Yu, H., King, R.W., Peters, J.M., and Kirschner, M.W. (1996). Identification of a novel ubiquitin-conjugating enzyme involved in mitotic cyclin degradation. Curr. Biol. *6*, 455–466.

Zachariae, W., and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. Genes Dev. *13*, 2039–2058.