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The Cyclosome, a Large Complex Containing Cyclin-Selective Ubiquitin Ligase Activity, Targets Cyclins for Destruction at the End of Mitosis

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The ubiquitin-mediated degradation of mitotic cyclins is required for cells to exit from mitosis. Previous work with cell-free systems has revealed four components required for cyclin-ubiquitin ligation and proteolysis: a nonspecific ubiquitin-activating enzyme E_1, a soluble fraction containing a ubiquitin carrier protein activity called E_2-C, a crude particulate fraction containing a ubiquitin ligase (E_3) activity that is activated during M-phase, and a constitutively active 26S proteasome that degrades ubiquitinated proteins. Here, we identify a novel ~1500-kDa complex, termed the cyclosome, which contains a cyclin-selective ubiquitin ligase activity, E_3-C. E_3-C is present but inactive during interphase; it can be activated in vitro by the addition of cdc2, enabling the transfer of ubiquitin from E_2-C to cyclin. The kinetics of E_3-C activation suggest the existence of one or more intermediates between cdc2 and E_3-C. Cyclosome-associated E_3-C acts on both cyclin A and B, and requires the presence of wild-type N-terminal destruction box motifs in each cyclin. Ubiquitinated cyclins are then rapidly recognized and degraded by the proteasome. These results identify the cyclosome-associated E_3-C as the component of the cyclin destruction machinery whose activity is ultimately regulated by cdc2 and, as such, the element directly responsible for setting mitotic cyclin levels during early embryonic cell cycles.

INTRODUCTION

The activation of cyclin B/cdc2 complexes at the end of G2 drives cells into mitosis and, after a short lag, triggers activation of the cyclin destruction machinery that is responsible for the selective ubiquitination and proteolysis of the A- and B-type mitotic cyclins (Murray *et al.*, 1989; Luca and Ruderman, 1989; Félix *et al.*, 1990; Glotzer *et al.*, 1991; Hershko *et al.*, 1991; Ghiara *et al.*, 1991; Luca *et al.*, 1991; Hunt *et al.*, 1992; Gallant and Nigg, 1992). Cyclin destruction results in the release of inactive monomeric cdc2 and exit from mitosis into interphase of the next cell cycle. Biochemical and genetic analyses of ubiquitin-dependent proteolytic pathways in reticulocytes and yeast have shown that ubiquitin-protein conjugate formation and degradation usually require the sequential action of four components: a ubiquitin-activating enzyme (E_1), a ubiquitin-carrier protein (E_2), a ubiquitin-protein ligase (E_3), and a 26S proteasome complex that recognizes and degrades ubiquitinated proteins (reviewed by Finley and Chau, 1991; Hershko and Ciechanover, 1992). E_1's do not appear to be specialized. Some specificity may be provided by individual E_2 enzymes, a family of 18- to 28-kDa proteins (reviewed by Jentsch, 1992). Some E_2's can transfer ubiquitin directly to certain proteins in vitro, whereas others are active only in the presence of an E_3 (reviewed by Hershko and Ciec-

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hanover, 1992). Higher specificity is apparently provided by E_3 enzymes (100–350 kDa), which contain binding sites for both a particular E_2 and an appropriate target protein (Hershko *et al.*, 1986; Reiss and Hershko, 1990; Heller and Hershko, 1990; Dohmen *et al.*, 1991; Scheffner *et al.*, 1993) and thus enable the E_2-dependent transfer of ubiquitin to the target protein. To date, there is no evidence that 26S proteasome activity is either regulated or provides any specificity during the cell cycle (Mahaffey *et al.*, 1993, and Refs. therein).

Because ligation to ubiquitin commits many proteins to degradation, the selectivity of protein degradation is apparently determined by the specificities of the ligation systems. Several cases of selective ubiquitin-dependent proteolysis of important regulatory proteins have been described, including the red light-induced degradation of the plant photoreceptor phytochrome (Shanklin *et al.*, 1987); the degradation of the tumor suppressor protein p53 (Scheffner *et al.*, 1993); the proto-oncogene products c-jun (Treier *et al.*, 1994), c-myc, c-fos and E1A (Ciechanover *et al.*, 1991), and c-mos (Nishisawa *et al.*, 1993); the cytokine-regulated proteolytic processing required for activation of the mammalian transcription factor NF-κB (Palombella *et al.*, 1994); and possibly, the cell cycle-regulated degradation of the cyclin-dependent kinase inhibitor $p40^{sic1}$ (Schwob *et al.*, 1994). It seems likely that at least some of these processes are carried out by specific E_3 enzymes that recognize specific features in appropriate proteins. However, a relevant E_3-like activity has only been identified for p53: in the case of human papillomavirus, the viral oncoprotein E6 and a cellular protein termed E6-associated protein form a complex that exhibits E_3 activity in vitro (Scheffner *et al.*, 1993).

The development of concentrated cell-free systems from clam and frog eggs that reproduce cyclin-dependent activation of cdc2 and subsequent cdc2-dependent activation of cyclin destruction have provided some insight into the mechanisms and cell-cycle regulation of cyclin degradation (Murray *et al.*, 1989; Luca and Ruderman 1989; Luca *et al.*, 1991; Glotzer *et al.*, 1991; Hershko *et al.*, 1991, 1994). Ubiquitin-dependent cyclin destruction utilizes the same four activities used to degrade other proteins in somatic cells, but so far only E_1 and the 26S proteasome have been identified definitively. Previous work with clam egg extracts has shown that two other crude fractions were required for cyclin ubiquitination. One was a soluble fraction that appeared to contain an E_2 activity (E_2-C) that worked with cyclin B but not most other cellular proteins; its activity was not regulated during the cell cycle. The other was a particulate fraction that was presumed to contain an E_3; this fraction was inactive during interphase, was activated near the end of M phase, and could be activated in vitro by cdc2 (Hershko *et al.*, 1994). In this work, we have been able to obtain a cyclin-selective E_3 activity (E_3-C) that, in M phase, is associated with a soluble 1500-kDa complex, which we call the cyclosome. Cyclosome-associated E_3-C, working in concert with partially purified E_2-C, ubiquitinates both mitotic cyclins A and B. The soluble interphase form of E_3-C can be activated in vitro by the addition of cdc2, demonstrating that components that regulate its activity during the cell cycle are retained. The kinetics of E_3-C activation by cdc2 suggest that at least one intermediate is involved. This work thus identifies the cyclosome-associated E_3-C as the component that is directly responsible for setting mitotic cyclin levels during the cell cycle. The large size of the cyclosome could indicate a role in the integration of checkpoints (Hartwell and Weinert, 1989) that ensure the completion of essential mitotic events before the onset of cyclin destruction and entry into the next cell cycle.

MATERIALS AND METHODS

Materials

Ubiquitin and rcm-BSA (reduced-carboxymethylated bovine serum albumin) were obtained from Sigma Chemical Co. (St. Louis, MO), and okadaic acid was obtained from Boehringer-Mannheim (Indianapolis, IN). Ubiquitin-aldehyde was prepared as described (Mayer and Wilkinson, 1989). E_1 was purified from human erythrocytes by affinity chromatography on ubiquitin-Sepharose (Hershko *et al.*, 1983).

Fractionation of Clam Oocyte Extracts

Extracts of M-phase or interphase clam oocytes were prepared as described (Hershko *et al.*, 1994). Both types of extracts were fractionated on DEAE-cellulose into the unadsorbed fraction (fraction 1) and a fraction that contains all proteins that adsorbed to the resin and was eluted with 0.5 M KCl (fraction 2) as described (Hershko *et al.*, 1994). Active cdc2 was purified from M-phase extracts by affinity chromatography on $p13^{suc1}$-Sepharose beads, followed by elution and gel filtration chromatography (Hershko *et al.*, 1994). One unit of cdc2 activity was defined as that carrying out the incorporation of 1 pmol of phosphate/min into histone H1.

Salt extraction and ammonium sulfate fractionation of fraction 1 was carried out at 0–4°C as follows. Fraction 1 (5–10 mg of protein per ml) was mixed with four volumes of a solution containing 20 mM HEPES-KOH (pH 7.2), 300 mM KCl, and 1 mM dithiothreitol (DTT). The sample was centrifuged at 100,000 × *g* for 30 min, the pellet was discarded, and the supernatant was concentrated by ultrafiltration with Centriprep-30 concentrators (Amicon, Beverly, MA). The salt extract contained 50–60% of the protein of fraction 1, and its protein concentration was around 20–30 mg/ml. For ammonium sulfate fractionation, the salt extract of fraction 1 was diluted to a concentration of 10 mg/ml with a buffer containing 20 mM Tris-HCl (pH 7.2) and 1 mM DTT (buffer A). Saturated ammonium sulfate was added to 45% saturation and the sample was kept at 0°C for 30 min. After centrifugation (8000 × *g* for 10 min), the supernatant was sequentially fractionated with ammonium sulfate at 55% and 85% saturation, by procedures similar to those described above. The material precipitated in ammonium sulfate fractions 0–45% (fraction 1A) and 55–85% (fraction 1B) were dissolved in a minimal amount of buffer A containing 20% glycerol and were dialyzed overnight against the same buffer. The material precipitated in the ammonium sulfate fraction 45–55% was discarded. Usually, around 30–40% of the protein of the salt extract was

recovered in fraction 1A and around 20–25% was recovered in fraction 1B.

Preparation of Cyclins

A truncated derivative, cyclin A[Δ1–60] was expressed in BL21(DE3), and bacteria were lysed as described (Luca *et al.*, 1991). A construct encoding full length cyclin A, pT7A, was made as follows. Cyclin A cDNA was amplified by polymerase chain reaction (PCR) from plasmid pAXH+ (Swenson *et al.*, 1986) using primers that created an *NdeI* site at the initiator methionine (primer A151: 5′-AGGCATATGTCGCAACCTTTTCG-3′) and a *Bam*HI site in the 3′-untranslated region (primer A152: 5′-GCGGATCCTTGTTGATCTGATCCGG-3′). PCR was carried out under standard conditions using *Taq* polymerase (Promega, Madison, WI). The 1395 nucleotide PCR product was inserted into the *NdeI*/*Bam*Hi site of the bacterial expression vector pT7–7, a gift from Stan Tabor (Harvard Medical School). Cyclins were recovered in inclusion bodies and were renatured as follows. The washed pellet fraction from a 400-ml culture was further washed with 10 ml of a solution containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Triton-X-100, and 1 mM PMSF; the fraction was then washed with 10 ml of the same buffer containing 1 M guanidine-HCl. The pellet was resuspended to a protein concentration of 1 mg/ml in 6 M guanidine-HCl in buffer B (25 mM HEPES-KOH (pH 7.6), 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% NP-40, 10% (vol/vol) glycerol, and 1 mM DTT). After incubation at room temperature for 30 min, insoluble material was removed by centrifugation. Guanidine-HCl was gradually removed by dialysis against 20 volumes of buffer B containing 2 M, 1 M, and 0.5 M guanidine-HCl, each for 8–16 h. The preparation was finally dialyzed for 24 h against two changes of buffer B, after which insoluble proteins were removed by centrifugation (15,000 × *g* for 30 min). The concentration of soluble protein in the final preparations was in the range of 0.3–0.7 mg/ml and in both preparations, the recombinant protein was about 70–80% pure. Both renatured full-length cyclin A and cyclin A[Δ1–60] activated histone H1 kinase activity interphase extracts, assayed as described previously (Luca *et al.*, 1991).

Sea urchin cyclin B derivatives, cyclin B(13–91)/protein A, cyclin B(13–66)/protein A (Glotzer *et al.*, 1991) and cyclin B-AARL(13–66)/protein A (Holloway *et al.*, 1993) in BL21(DE3) were generously provided by Michael Glotzer (University of California, San Francisco). The construct encoding clam cyclin A(8–137)/protein A was made as follows. Plasmid $pAXH^+$ containing the full-length clam cyclin A sequence was linearized with *Hin*dIII, and the fragment encoding amino acid residues 8–137 was amplified by PCR using the primers: 5′-GAGATGCATGACGGAGAGAACCAAATG and 5′-CTCGCGCGCAGATTCCATAGAATCCTC. These primers also contain restriction sites for *Nsi*I and *Bss*HII, respectively. The PCR product was treated with *Nsi*I and *Bss*H II. Clam cyclin A has an *Nsi*I site at the region encoding amino acid 69, so two fragments were obtained. These were ligated into a vector derived from the expression plasmid cyclin B[Δ13–66]/protein A (Glotzer *et al.*, 1991), from which the region encoding the N-terminal cyclin B fragment had been excised with *Nsi*I and *Bss*HII. A plasmid containing both fragments in the correct orientation was identified by restriction analysis and was verified by sequencing. This was transformed into BL21(DE3)pLys for expression. All N-terminal cyclin fragment/protein A fusion proteins were expressed, purified by affinity chromatography on IgG-Sepharose, and radioiodinated as described previously (Glotzer *et al.*, 1991).

Cyclin-Ubiquitin Ligation Assay

Unless otherwise stated, reaction mixtures were contained in a vol of 10 μl: 20 mM HEPES-KOH (pH 7.2), 1 mg/ml rcm-BSA, 1 mM DTT, 5 mM $MgCl_2$, 0.5 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine phosphokinase, 50 μM ubiquitin, 1 μM ubiquitin-aldehyde, 1 μM okadaic acid, 1 pmole E_1, 1–2 pmol ^{125}I-cyclin B(13–91) (≈10^5 cpm), and enzymes or fractions as specified. The addition of ubiquitin-aldehyde, an inhibitor of ubiquitin-C-terminal isopeptidases (Pickart and Rose, 1985; Hershko and Rose, 1987), was necessary to prevent the action of such enzymes on cyclin-ubiquitin conjugates in crude preparations (Hershko *et al.*, 1994). Okadaic acid was added to prevent the action of protein phosphatases that inactivate the cyclin degradation system (Lorca *et al.*, 1991), whereas rcm-BSA was added to prevent the nonspecific adsorption of the labeled cyclin substrate. After incubation at 18°C for 60 min, the samples were separated by electrophoresis on a 12.5% polyacrylamide-sodium dodecyl sulfate (SDS) gel. The results were quantitated with a Fuji phosphorimager. The amount of radioactivity in all cyclin-ubiquitin conjugates (bands of molecular weight higher than the free cyclin substrate) was expressed as the percentage of the total radioactivity in each lane and was corrected for contaminants in this region by subtraction of the value of a corresponding area in a parallel blank incubation without enzymes.

RESULTS

Fractionation of Oocyte Extracts and Dissociation from Particles of the cdc2-Regulated Component

G2-arrested oocytes were activated with KCl, allowed to proceed into the first meiotic M phase, and collected about 25 min later, about 10 min before the onset of cyclin destruction and the first meiotic division (Westendorf *et al.*, 1989). Supernatants (13,000 × *g*) prepared from these cells will advance to the cyclin destruction point in vitro (Hershko *et al.*, 1991). From these extracts, we had previously resolved three components of the system that ligates cyclin B to ubiquitin (Hershko *et al.*, 1994). These include the ubiquitin-activating enzyme E_1, a ubiquitin-carrier protein selective for this process (E_2-C), and a cdc2-regulated component associated with particulate material. This last component could not be extracted from particles by treatment with detergents such as Nonidet-P-40 or Brij 35 (unpublished observations), suggesting that it is not associated with membranes. On the other hand, the activity could be dissociated from particulate material by extraction with salt, such as 0.25 M KCl, allowing further fractionation as illustrated in Figure 1. Extracts were fractionated on DEAE-cellulose yielding fraction 1, the flow-through, and fraction 2, material bound to the resin. Fraction 2 contains the ubiquitin-activating enzyme E_1 (Hershko *et al.*, 1994) as well as the 26S proteasome (E. Eytan and A. Hershko, unpublished results). Fraction 1 can be resolved by centrifugation at 100,000 × *g* into a supernatant, containing E_2-C, and a pellet fraction, containing a cdc2-regulated component (Hershko *et al.*, 1994). If, however, fraction 1 was first salt extracted with 0.25 M KCl, both E_2-C and the cdc2-regulated component remained in the soluble fraction. The two activities were then separated by ammonium sulfate fractionation into a component precipitated at 0–45% saturation (Figure 1, fraction 1A) and another at 55–85% saturation (Figure 1, fraction 1B). These two fractions were assayed for cyclin-ubiq-

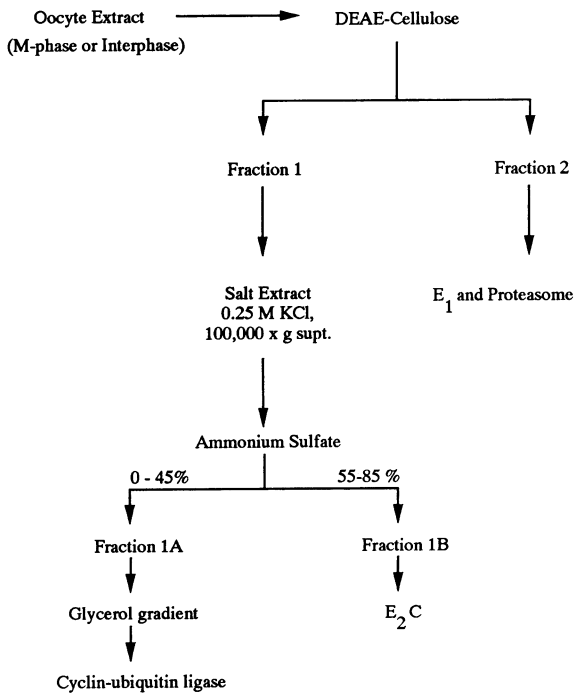

Figure 1. Scheme for fractionation of clam oocyte extracts. See text.

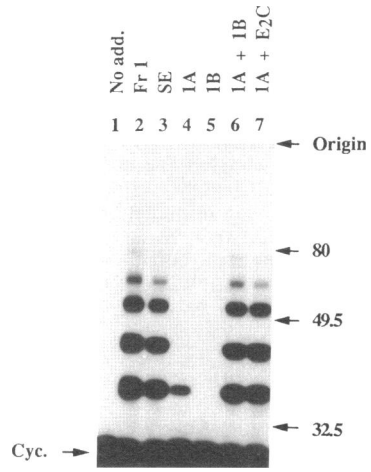

Figure 2. Cyclin-ubiquitin ligation activity in fractions from M-phase oocytes. The ligation of cyclin to ubiquitin was assayed in a mix containing ^{125}I-cyclin B(13–91), unlabeled ubiquitin, purified E1, MgATP, and okadaic acid, as described in MATERIALS AND METHODS. Where indicated, the various fractions from M-phase extracts were added in the following amounts: 10 μg fraction 1 (Fr 1); 10 μg salt extract of fraction 1 (SE); 5 μg fraction 1A (1A); and 10 μg fraction 1B (1B). Where indicated, 1 μl of partially purified E_2-C (Hershko *et al.*, 1994) was added. After incubation at 18°C for 90 min, the samples were subjected to gel electrophoresis followed by autoradiography. Cyc, ^{125}I-cyclin B(13–91). Numbers to the right indicate the position of molecular weight markers in kDa. Only part of the free cyclin band is shown.

uitin ligation activity in a mix containing an ^{125}I-labeled fusion protein consisting of an N-terminal fragment of cyclin B linked to bacterial protein A, unlabeled ubiquitin, E_1, MgATP, and okadaic acid, a phosphatase inhibitor that prevents inactivation of this system (Lorca *et al.*, 1991). ^{125}I-cyclin-ubiquitin conjugates appear as a ladder of higher molecular weight derivatives. The cyclin-ubiquitin ligation activity of the initial salt extract (Figure 2, lane 3) was comparable with that observed in fraction 1 from which it was derived (Figure 2, lane 2). Little activity was observed with fraction 1A by itself, and none with fraction 1B. However, reconstitution of cyclin-ubiquitin ligation activity was achieved by the combination of fractions 1A and 1B (Figure 2, lane 6). The extent of this reconstitution was full, but the recovery of activities was not. The apparent recovery of activities in fractions 1A and 1B, as compared with that in fraction 1, was around 65% and 30%, respectively.

The activity contributed by fraction 1B was identified as the cyclin-selective E_2-C because this fraction could be replaced by partially purified E_2-C (Figure 2, lane 7). The low activity observed with fraction 1A by itself may be due to a small amount of contaminating or weakly bound E_2-C, because it was not seen after further purification described below.

Fraction 1A Contains a Ubiquitin Ligase Activity that Is Activated by cdc2

In the experiment described above, the activities of the various fractions were assayed after a prolonged incubation that allowed considerable accumulation of cyclin-ubiquitin conjugates. A closer examination of the time course of conjugate accumulation revealed two phases: a lag of approximately 10 min, followed by a period of linear accumulation of cyclin-ubiquitin conjugates (Figure 3, open circles). These kinetics suggest the involvement of at least two different processes: the activation of a component(s) of the cyclin-ubiquitin ligation system during the initial lag period, followed by the action of the ligation system at a constant maximal rate. Because the intact cells used here and the unfractionated M-phase extracts derived from them require about 10–15 min to advance to a point where the cyclin destruction machinery is activated, the lag period in the activation of cyclin-ubiquitin ligation in the reconstituted system therefore probably reflects reactions occurring in intact oocytes.

To identify which component is activated during the lag phase, we used an approach employed earlier (Hershko *et al.*, 1994). When fraction 1B (or partially purified E_2-C) was preincubated with ATP and Mg^{2+} before the addition of fraction 1A and ^{125}I-cyclin, the kinetics of accumulation of cyclin-ubiquitin conjugates were not affected (unpublished observations). How-

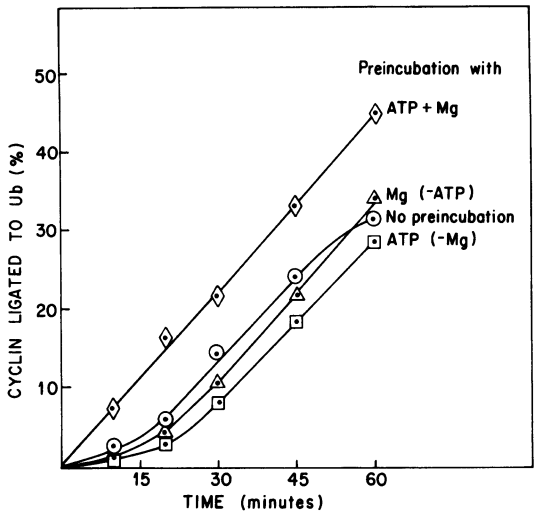

Figure 3. Kinetics of cyclin-ubiquitin ligation with fraction 1A from M-phase extracts, and effect of preincubation with MgATP. Reactions were prepared and incubated as described below. In all reactions, the zero minute point represents the time at which ^{125}I-cyclin was added. ○, no preincubation. The complete reaction containing fraction 1A, fraction 1B, ATP, and Mg^{2+} was assembled (with other components of the ligation reaction mix, including E1, ubiquitin, and okadaic acid, as described in MATERIALS AND METHODS). ^{125}I-cyclin(13–91) was added, the reaction was moved to 18°C, and samples were taken at the indicated times. ◇, ATP + Mg. Fraction 1A, ATP and Mg^{2+} were preincubated in ligation reaction mix at 18°C for 30 min. ^{125}I-cyclin B(13–91) and fraction 1B were added and samples were taken at the indicated times. △, Mg (-ATP). Fraction 1A and Mg^{2+} were preincubated in ligation reaction mix at 18°C for 30 min. ATP, fraction 1B, and ^{125}I-cyclin B(13–91) were added, and samples were taken at the indicated times. □, ATP (-Mg). Fraction 1A and ATP were preincubated in ligation reaction mix at 18°C for 30 min. Mg^{2+}, fraction 1B, and ^{125}I-cyclin B(13–91) were added and samples were taken at the indicated times. All incubations contained 5 μg protein of fraction 1A and 10 μg protein of fraction 1B, both from M-phase cells. Cyclin-ubiquitin (Ub) conjugate formation was analyzed by polyacrylamide gel electrophoresis and quantified with a phosphorimager.

ever, when fraction 1A was preincubated with ATP and Mg^{2+} and then mixed with E_2-C and labeled cyclin, the lag was abolished completely (Figure 3, diamonds). When either ATP or Mg^{2+} were omitted during preincubation of fraction 1A and added later, together with the labeled cyclin substrate and E_2-C, the lag was not abolished (Figure 3, triangles and squares, respectively). These findings indicate that fraction 1A contains both the cyclin-ubiquitin ligase and component(s) required for its activation. They further show that at least one step in the activation of fraction 1A requires MgATP.

Three considerations argue that this activation step involves the protein kinase cdc2. First, fraction 1A from M-phase oocytes contains high levels of cdc2 activity (unpublished observations). Second, in crude extracts of interphase cells, the addition of active cdc2 leads to activation of the cyclin destruction system after a lag (Murray and Kirschner, 1989; Félix *et al.*, 1990; Luca *et al.*, 1991). Third, the addition of cdc2 activates a component of the cyclin-ubiquitin system in the particulate fraction from interphase cells (Hershko *et al.*, 1994). It was therefore predicted that fraction 1A prepared from *interphase* cells, which lack active cdc2, would contain a similar component that could be activated by the addition of purified, active cdc2. To test this, fraction 1 from interphase cells was subjected to salt extraction and ammonium sulfate fractionation as outlined in Figure 1, and the resulting fractions were assayed for their ability to be activated by purified cdc2. As shown in Figure 4, addition of cdc2 to the combined fractions 1A and 1B from interphase cells caused the partial activation of cyclin-ubiquitin ligation after a lag of 20–30 min. Most importantly, preincubation of fraction 1A from interphase cells with cdc2 and MgATP abolished the lag and dramatically increased the initial rate of cyclin-ubiquitin ligation, whereas preincubation of fraction 1B under similar conditions did not (Figure 4).

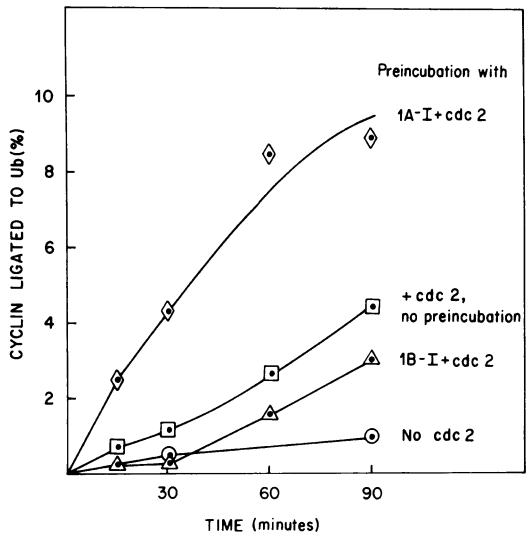

Figure 4. cdc2 activates a component in fraction 1A from interphase cells. □, + cdc2, no preincubation. Fraction 1A from interphase cells (1A-I), fraction 1B from interphase cells (1B-I), ^{125}I-cyclin B(13–91) (and a ligation reaction mix containing ATP, Mg^{2+}, E1, ubiquitin, okadaic acid, and other components as described in MATERIALS AND METHODS) were assembled, cdc2 was added, and samples were taken at the indicated times. ○, no cdc2 (same as above, but cdc2 was omitted). ◇, 1A-I + cdc2. Fraction 1A-I and cdc2 were preincubated in ligation reaction mix at 18°C for 60 min. ^{125}I-cyclin B(13–91) and fraction 1B-I were added, and samples were taken at the indicated times. △, 1B-I + cdc2. Fraction 1B-I and cdc2 were preincubated in ligation reaction mix at 18°C for 60 min. Fraction 1A-I and ^{125}I-cyclin B(13–91) were added, and samples were taken at the indicated times. All incubations contained 5 μg protein of fraction 1A-I and 10 μg protein of fraction 1B-I, both prepared from interphase cells. Where indicated, 400 units of cdc2 were added. Cyclin-ubiquitin conjugate formation was analyzed by polyacrylamide gel electrophoresis and quantified with a phosphorimager.

The activity obtained with cdc2-treated interphase fraction 1A was lower than that observed with M phase fraction 1A. This was not due to limitation by cdc2, because the additional cdc2 did not influence the kinetics or magnitude of the cyclin-ubiquitination activity.

Taken together, these results establish three points. First, the solubilized material of fraction 1A contains an activity that enables the cyclin-selective E_2-C to ubiquitinate cyclin. This component is required for E_2-C-dependent ubiquitination of cyclin, so it meets the definition of an E_3. Second, cyclin-ubiquitin ligase, which is inactive during most of the cell cycle, is activated during M phase. This is in contrast to E_2-C, which is active during both interphase and M phase. Third, the inactive interphase form of cyclin-ubiquitin ligase can be activated in vitro by the addition of cdc2. Although these results do not indicate whether the direct target of cdc2 is the ligase itself or some other component in fraction 1A, subsequent results favor the latter (see below).

The Cyclin-Ubiquitin Ligase Activated by cdc2 Is Part of a ~1500-kDa Complex

Solubilization of the cyclin-ubiquitin ligase activity allowed its further purification. Fraction 1A from early M-phase oocytes was converted to the active form by a 30-min incubation in the presence of MgATP and okadaic acid and then subjected to glycerol density gradient centrifugation. Gradient fractions were assayed for cyclin-ubiquitin ligation activity in the presence of ^{125}I-cyclin B, excess E_1, and partially purified E_2-C. A strong peak of cyclin-ubiquitin ligation activity was centered around fraction 8, corresponding to an apparent molecular mass of ~1500-kDa (Figure 5, A and B). We could not obtain a more precise size estimate because of the lack of suitable markers in this size range. By contrast, the bulk of the ubiquitin ligase activities directed at other endogenous protein targets appeared smaller (Figure 5C). This result indicates that the cyclin-ubiquitin ligase activity detected here is distinct from the majority of other ubiquitin ligases that act on the majority of cellular proteins. It is possible, however, that cyclin-ubiquitin ligase acts on certain other cell cycle-regulated proteins, which are also degraded near the end of mitosis (see DISCUSSION).

Cdc2 from M phase cells migrates as very high molecular weight aggregates under conditions of low ionic strength used in the glycerol gradients (data not shown), raising the possibility that the large size of the cyclin-ubiquitin ligation activity might be due to aggregation. We therefore repeated the size estimate at a higher ionic strength, with 250 mM KCl included in the gradient. Under these conditions, cdc2 is not aggregated and migrates as a ~100-kDa enzyme. However, the position of the cyclin-ubiquitin ligation activity remained unchanged (unpublished observations). We conclude that the apparent high molecular mass of cyclin-ubiquitin ligation activity is not an artifact caused by aggregation at low ionic strength. However, because the recovery of activity of cyclin-ubiquitin ligation activity was usually better at low ionic strength, most separations were carried out under these conditions.

When fraction 1A from interphase cells was incubated with cdc2 in the presence of MgATP and okadaic acid, and then fractionated on glycerol gradients, the formation of high molecular weight cyclin-ubiquitin ligase activity was observed (Figure 6A). No significant activity was found in a parallel incubation without cdc2 (Figure 6A, "Control"). The size of the active enzyme formed after incubation of interphase fraction 1A with cdc2 (Figure 6A) was similar to that observed after incubation of M-phase fraction 1A with MgATP (Figure 6B). In the latter case, incubation with MgATP greatly increased cyclin-ubiquitin ligase activity, but some low activity was also observed when M-phase fraction 1A without previous treatment was subjected to glycerol density gradient centrifugation (Figure 6B). The peak of that low activity seen in the untreated M-phase sample was somewhat larger than that of the activated enzyme. At present, we do not know the reason for this difference (see DISCUSSION). In spite of this unexplained point, the results show a good general correlation between (a) the experimental conditions required to abolish the lag in the accumulation of cyclin-ubiquitin conjugates in M-phase and interphase preparations (Figures 3 and 4) and (b) those required to turn on cyclin-ubiquitin ligase activity in interphase extracts (Figure 6). These correlations suggest that the lag represents a period in which active cdc2 leads to the activation of cyclin-ubiquitin ligase.

Cdc2-Activated Cyclin-Ubiquitin Ligase Acts on both Cyclin A and Cyclin B, and Specifically Requires Their N-terminal "Destruction Box" Regions

The cell cycle stage-specific degradation of the A- and B-type mitotic cyclins is a highly selective process (Evans *et al.*, 1983; Luca and Ruderman, 1989; Glotzer *et al.*, 1991). If the cyclin-ubiquitin ligase activity in fraction 1A is indeed responsible for the specificity and regulated timing of cyclin destruction, it should exhibit the appropriate substrate specificities. In the experiment shown in Figure 7, the activity of partially purified cyclin-ubiquitin ligase (peak glycerol gradient fractions of the active enzyme) on various cyclin derivatives was examined in the presence of purified E_2-C and E_1. Cyclin-ubiquitin ligation was examined in two different ways: ligation of various ^{125}I-labeled cyclin derivatives with unlabeled ubiquitin (Figure 7,

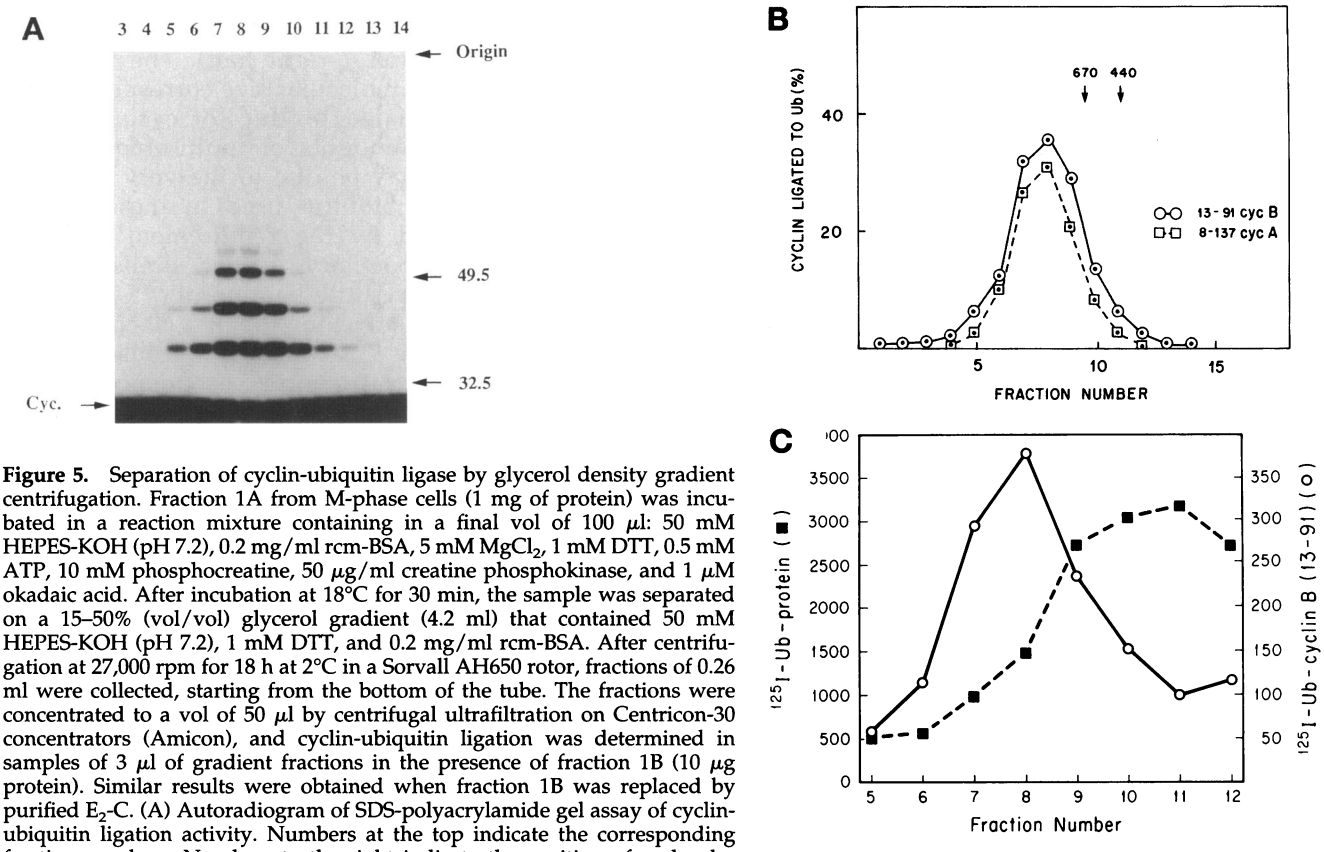

Figure 5. Separation of cyclin-ubiquitin ligase by glycerol density gradient centrifugation. Fraction 1A from M-phase cells (1 mg of protein) was incubated in a reaction mixture containing in a final vol of 100 μl: 50 mM HEPES-KOH (pH 7.2), 0.2 mg/ml rcm-BSA, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine phosphokinase, and 1 μM okadaic acid. After incubation at 18°C for 30 min, the sample was separated on a 15–50% (vol/vol) glycerol gradient (4.2 ml) that contained 50 mM HEPES-KOH (pH 7.2), 1 mM DTT, and 0.2 mg/ml rcm-BSA. After centrifugation at 27,000 rpm for 18 h at 2°C in a Sorvall AH650 rotor, fractions of 0.26 ml were collected, starting from the bottom of the tube. The fractions were concentrated to a vol of 50 μl by centrifugal ultrafiltration on Centricon-30 concentrators (Amicon), and cyclin-ubiquitin ligation was determined in samples of 3 μl of gradient fractions in the presence of fraction 1B (10 μg protein). Similar results were obtained when fraction 1B was replaced by purified E_2-C. (A) Autoradiogram of SDS-polyacrylamide gel assay of cyclin-ubiquitin ligation activity. Numbers at the top indicate the corresponding fraction numbers. Numbers to the right indicate the position of molecular weight markers in kDa. Cyc, position of free ^{125}I-cyclin B(13–91). Only part of the free cyclin band is shown. (B) Quantitation of cyclin-ubiquitin ligation activities in glycerol gradient fractions. ○, conjugation of ^{125}I-cyclin B(13–91) to ubiquitin (quantitation of the results in panel A). □, quantitative assay of the ligation of ^{125}I-cyclin A(8–137) to ubiquitin in fractions of the same glycerol gradient. Reaction conditions were similar to those described above, except for the different ^{125}I-cyclin substrate. Arrows indicate the migration position of marker proteins thyroglobulin (670 kDa) and apoferritin (440 kDa), separated on parallel gradients. (C) Comparison of cyclin-ubiquitin ligation and general protein-ubiquitin ligation activities in glycerol gradient fractions. Samples of 3 μl of glycerol gradient fractions were incubated with ^{125}I-ubiquitin (2.5×10^5 cpm, 50 pmol), in the presence or absence of 50 pmol unlabeled cyclin B(13–91) and 1 μl of a mixture containing E_2-C, E_2-A, and E_2-B (fractions 29–34 of the Superose 12 separation, see Hershko *et al.*, 1994). Other incubation conditions were as described in MATERIALS AND METHODS for the cyclin-ubiquitin ligase assay. The results were quantified by Phosphorimager analysis. The amount of radioactivity in total ubiquitin-protein conjugates (■) and cyclin-specific conjugates (○) (see Figure 7) is expressed in arbitrary units.

left half) or ligation of ^{125}I-ubiquitin with various unlabeled cyclin derivatives (Figure 7, right half). In the first type of incubation, cyclin-ubiquitin conjugates were assayed as higher molecular weight derivatives that appeared when cyclin-ubiquitin ligase and E_2-C were added; in the second type, all incubations contained those enzymes, and cyclin-ubiquitin conjugates were assayed as higher molecular weight derivatives that appeared when the appropriate unlabeled cyclin substrates were added. The experimental protocol of labeling with ^{125}I-ubiquitin was required to ensure that the high molecular weight derivatives of the various cyclins are indeed ubiquitin conjugates. In addition, incubation with ^{125}I-ubiquitin was required to detect conjugates of full-length cyclin, which could not be radioiodinated without loss of activity. It is noteworthy that the detection of conjugates of ^{125}I-ubiquitin with unlabeled cyclins became possible only after the glycerol gradient purification of fraction 1A, which separates cyclin-ubiquitin ligase from other, lower molecular weight ubiquitin-protein ligases (Figure 5C). Some contamination by other ubiquitin-protein ligases still persists in the partially purified cyclin-ubiquitin ligase preparation, but the remaining conjugates of ^{125}I-ubiquitin with other proteins can be easily distinguished from cyclin-selective derivatives (Figure 7, right part).

Cyclin-ubiquitin ligase recognizes fusion proteins containing amino acid residues 13–91 or 13–66 of cyclin B, regions that contain destruction box sequences that are required by the cyclin-selective, cell cycle-regulated degradation system (Glotzer *et al.*, 1991;

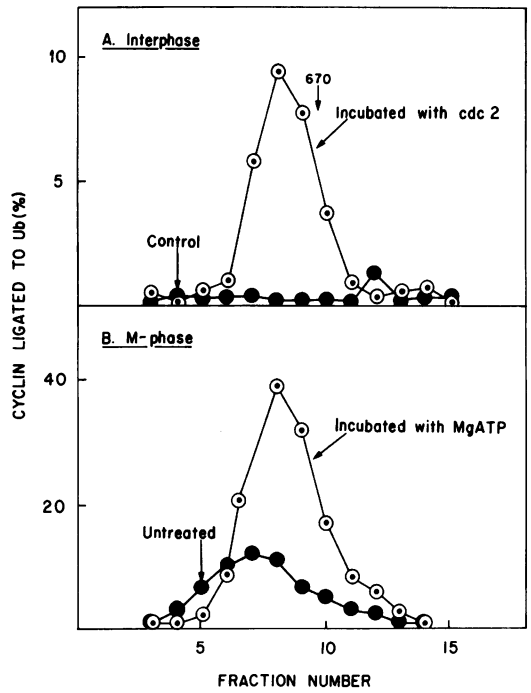

Figure 6. Formation of active, high molecular weight cyclin-ubiquitin ligase requires cdc2 and MgATP. (A) cdc2 activates the enzyme in interphase fraction 1A. Experimental conditions were similar to those described in Figure 5 except that fraction 1A from interphase (1 mg protein) was incubated at 18°C for 60 min in the presence (○) or absence (●) of 48,000 units of cdc2 before glycerol density gradient centrifugation. Cyclin-ubiquitin ligation was assayed in 3 μl samples of fraction of the gradient, in the presence of interphase fraction 1B (10 μg protein). (B) Incubation with MgATP activates the enzyme in M-phase fraction 1A. Samples of 1 mg of fraction 1A from M phase cells were incubated with ATP and $MgCl_2$ (○) or not (●) and were separated by glycerol density gradient centrifugation as described in Figure 5. Cyclin-ubiquitin ligation was determined under conditions similar to those described for Figure 5.

Luca *et al.*, 1991; Hershko *et al.*, 1994). By contrast, very little conjugate formation was observed with a mutated cyclin B(13–66) construct, in which the RAAL sequence in the destruction box had been changed to AARL (Holloway *et al.*, 1993). This result indicates that an intact destruction box recognition sequence in cyclin B is required for the action of the cyclin-ubiquitin ligase. The partially purified ligase also formed conjugates with a construct that contains residues 8–137 of clam cyclin A. In all cases, identical high molecular weight derivatives were formed with the corresponding cyclin constructs in the two types of labeling protocols, confirming that they are all cyclin-ubiquitin conjugates (Figure 7).

We were surprised to find that cyclin-ubiquitin ligase acts on N-terminal derivatives of both cyclin A and cyclin B, because cyclin A disappears before cyclin B, both in intact cells and in cell-free systems (Luca and Ruderman, 1989; Hunt *et al.*, 1992). The ligase also acts on bacterially expressed full-length clam cyclin A, as detected by its conjugation with ^{125}I-ubiquitin (Figure 7, right half). The predominant product had a molecular size corresponding to the mono-ubiquitinated product of cyclin A, and there were lower amounts of multi-ubiquitinated derivatives. This may be due to the very low concentration of ^{125}I-ubiquitin used in these experiments, necessitated by the requirement for very high specific radioactivity. Under similar conditions, there was very little ligation of ^{125}I-ubiquitin to truncated cyclin A lacking the first 60 N-terminal amino acids (Figure 7, Δ60 cycA). Because this region contains the destruction box sequence of clam cyclin A, the results again indicate the requirement of the ligase for the destruction box determinant.

It is possible that there are separate ubiquitin ligases that act on cyclin A and cyclin B, but these are not separated on the gradients used. To test this idea we compared the activities that ligate ubiquitin to the N-terminal fragments of cyclin A and B in different fractions of the glycerol gradient and found an exact coincidence between the two activities (Figure 5B). This result is compatible with the idea that the cyclosome contains a single ligase activity that acts on both cyclins, but it does not rule out the existence of two enzymes of similar size or that one large multi-enzyme complex contains separate sites for cyclin A and B. These possibilities were examined by testing the effects of unlabeled cyclin A derivatives on the conjugation to ubiquitin of a labeled cyclin B derivative in the presence of excess E_1 and E_2-C. If there are separate ligases or separate sites on a single ligase, then unlabeled cyclin A derivatives should not compete with labeled cyclin B. However, as shown in Figure 8, both full-length cyclin A and the N-terminal cyclin A fragment inhibited ligation of ^{125}I-cyclin B fragment [residues 13–91] to ubiquitin. Control incubations showed much less inhibition by cyclin A lacking the N-terminal destruction box domain or by the cyclin B[13–66] fragment containing the AARL cyclin box mutation. These results indicate a requirement for the destruction box in competing for a common site. It should be noted that the concentrations of the N-terminal fragments of either cyclin A or B required for effective inhibition of the conjugation of the ^{125}I-cyc B[13–91] were 10-fold higher than those of full-length cyclin A (see legend to Figure 8). This is presumably due to the low affinity of the system for the N-terminal cyclin fragments, as noted previously (van der Velden and Lohka, 1993; Hershko *et al.*, 1994).

In the above experiments, we used recombinant full-length cyclin A or its N-terminal fragment from the clam, whereas the recombinant cyclin B fragments were from the sea urchin. Though cyclin function is

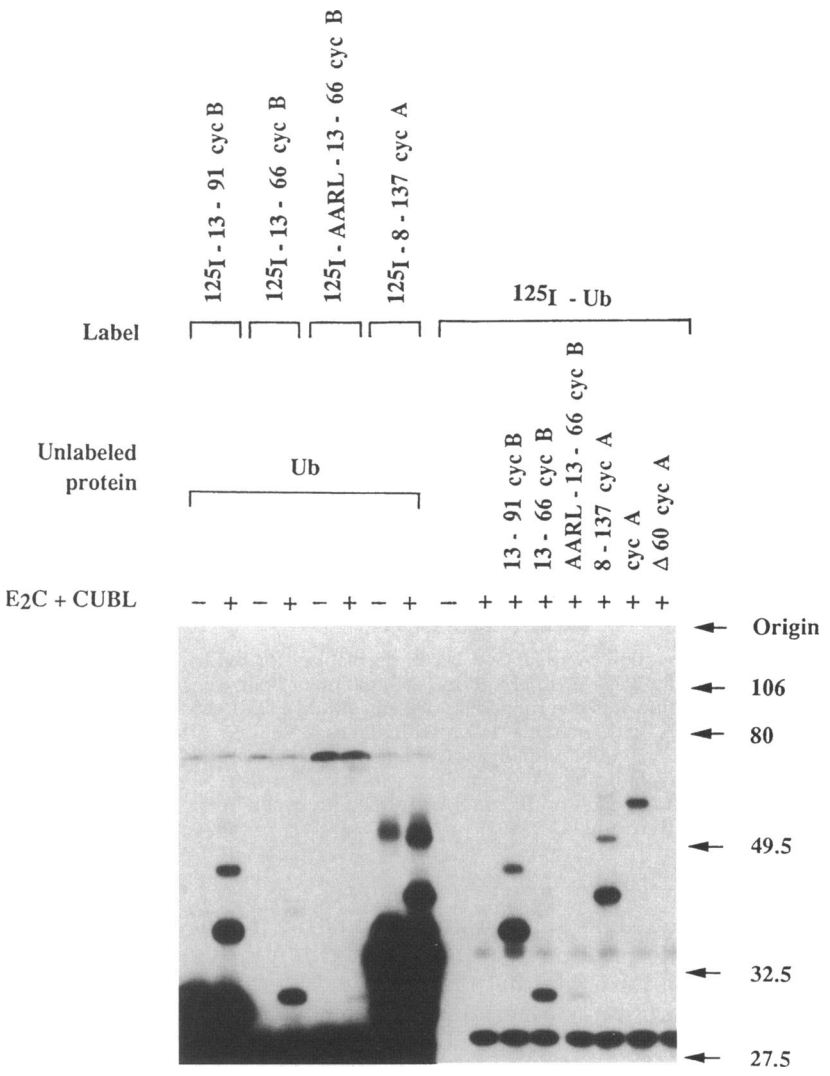

Figure 7. Selectivity of cyclin-ubiquitin ligase: requirement for N-terminal destruction box regions of cyclin A and cyclin B. The peak fractions from the glycerol gradient separation (fractions 7–9 in Figure 5) were pooled and used as the source of cyclin-ubiquitin ligase (CUBL). Where indicated, 3 μl of CUBL and 1 μl of E_2-C were added. In the left panel, the indicated ^{125}I-labeled cyclin derivatives (80–100 × 10^3 cpm, 3 pmol) were added in the presence of 500 pmol unlabeled ubiquitin. In the right panel, ^{125}I-ubiquitin (2.5 × 10^5 cpm, 50 pmol) was added in the presence of 50 pmol of all unlabeled N-terminal cyclin-protein A fusion proteins, or of 10 pmol of full-length cyclin A or cyclin A[Δ–60]. Other incubation conditions were as described in MATERIALS AND METHODS for the cyclin-ubiquitin ligation assay. After incubation at 18°C for 90 min, samples were separated on a 12.5% polyacrylamide gel. Numbers on the right indicate the position of molecular weight markers. In the case of the ^{125}I-labeled cyclin substrates (left part), all high molecular weight bands seen in the absence of E_2C and CUBL are contaminants present in the respective cyclin preparations. When ^{125}I-ubiquitin was used (right part), all higher molecular weight bands seen in the absence of unlabeled cyclin (but in the presence of E2C and CUBL) are conjugates with endogenous proteins.

usually strongly conserved and cyclins from different species are often interchangeable, it seemed possible that the clam ligase is specific for clam cyclin A, but it may erroneously bind N-terminal fragments of cyclin B from other species. Unfortunately, in spite of considerable efforts, we could not renature bacterially expressed full-length clam cyclin B, the protein needed to examine this question by direct methods. We therefore tested the effect of full length cyclin A on the degradation of native clam cyclin B and cyclin A. When early clam embryos are labeled with ^{35}S-methionine during interphase of mitosis 1, the two cyclins are labeled prominently; in extracts made from such embryos, they are degraded on schedule (Luca and Ruderman, 1989). As shown in Figure 9A ("Control"), ^{35}S-labeled endogenous cyclin A was degraded between 15 and 30 min, whereas cyclin B was degraded between 30 and 45 min of incubation. The addition of bacterially expressed cyclin A protein markedly slowed the degradation of both endogenous cyclin A and cyclin B. It is noteworthy that addition of cyclin A inhibited degradation of endogenous cyclin B more strongly than that of endogenous cyclin A: ^{35}S-cyclin A was almost completely degraded by 60 min, whereas a significant amount of ^{35}S-cyclin B remained even after 100 min of incubation (Figure 9B, "+Cyc A"). The addition of cyclin A lacking the destruction box caused much less inhibition, although it delayed slightly the degradation of both cyclin A and B (Figure 9C, "+Δ60 Cyc A"). This small delay could be due to the action of cyclin A/cdc2 kinase (formed with the cdc2 protein present in the extract), which delays cyclin degradation in other systems (Lorca *et al.*, 1992b). However, the much stronger inhibition caused by *full-length* cyclin A cannot be due merely to the formation of cyclin A/cdc2 kinase activity because the active kinase is formed with equal efficiency by both full-length and truncated cyclin A (data not

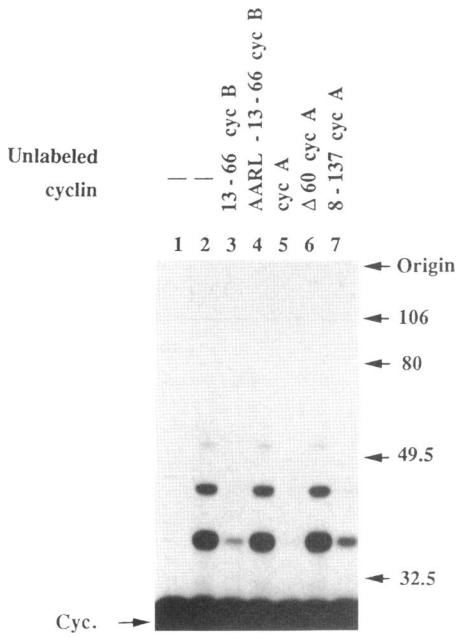

Figure 8. Competition by unlabeled cyclin derivatives for the ligation of ^{125}I-cyclin B(13–91) to ubiquitin. The ligation of ^{125}I-cyclin B(13–91) to ubiquitin was determined, as described in MATERIALS AND METHODS, without (lane 1) or with (lanes 2–7) 3 μl of partially purified cyclin-ubiquitin ligase (see legend to Figure 7) and 1 μl E_2-C. Where indicated, 100 pmol of various unlabeled N-terminal cyclin fragments, or 10 pmol of full-length cyclin A or cyclin A[Δ1–60] were added.

shown). These results suggest that most of the inhibition of cyclin destruction by full-length cyclin A is due to competition for a site that requires the N-terminal region. The addition of N-terminal fragments of cyclin B or cyclin A up to 20 μM had no significant influence on the degradation of endogenous cyclins (data not shown). This is presumably due to their low affinity, relative to that of native cyclins. These results suggest that cyclin A and cyclin B are degraded by a common system, and are compatible with the notion that they are subject to the action of a single cyclin-ubiquitin ligase.

Taken together, these results establish three points. First, cyclin-ubiquitin ligase activity can be distinguished from other ubiquitin ligase activities by its large size. Second, this ligase requires its target to contain a destruction box domain, and will not recognize cyclins containing slight mutations within this domain. Third, both cyclin A and cyclin B are recognized and ubiquitinated by a common ligase. In view of the large size of the complex containing cyclin-selective ubiquitin ligase activity, its cell-cycle stage-specific regulation by cdc2, and its central role in controlling mitotic cyclin levels and thus cell cycle progression, we call this complex the cyclosome.

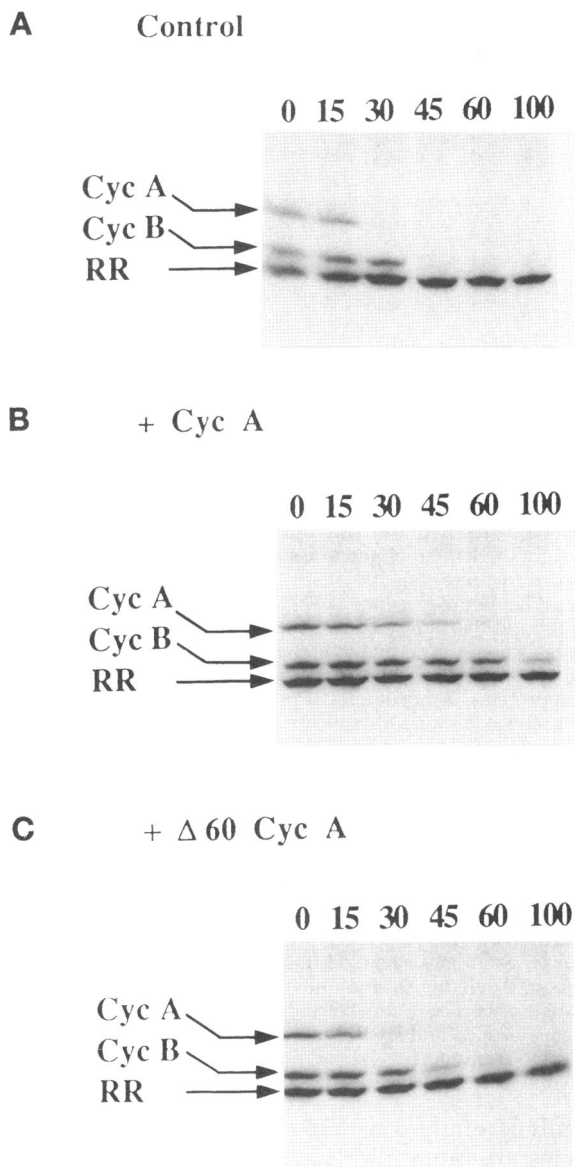

Figure 9. Recombinant cyclin A inhibits the degradation of endogenous cyclin A and cyclin B. Clam embryos were labeled with ^{35}S-methionine, and extracts were prepared as described previously (Luca and Ruderman, 1989), except that labeling was terminated in mid-interphase of the first mitotic cell cycle (74 min post-fertilization). ^{35}S-labeled extract was thawed on ice, diluted with an equal vol of buffer T (Hershko *et al.*, 1991), and supplemented with 1 mM ATP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and 0.5 mM DTT. Where indicated, recombinant cyclin A or cyclin A[Δ1–60] were added to a final concentration of 2 μM. The mixtures were incubated at 16°C, and at various time periods samples of 4 μl were taken, mixed with gel sample buffer, and boiled for 5 min. Samples were separated by electrophoresis on a 12.5% polyacrylamide-SDS gel and processed for fluorography (Amplify, Amersham). Control experiments showed that the addition of buffer B (in which recombinant cyclins are dissolved) at a volume similar to that of recombinant cyclins, had no significant influence on the degradation of cyclin A or B. Cyc A, cyclin A; Cyc B, cyclin B; RR, ribonucleotide reductase.

DISCUSSION

In this paper we describe a ~1500 kDa complex, the cyclosome, which contains a novel cyclin-ubiquitin ligase activity, E_3-C, with properties that indicate a central role in the regulated destruction of mitotic cyclins. These include its selectivity for cyclins containing N-terminal destruction box motifs, its inactivity during interphase, its activation during M phase, and the regulation of its activity by cdc2. By contrast, the cyclin-selective ubiquitin carrier protein E_2-C is active in both interphase and M phase (Hershko *et al.*, 1994). These findings suggest that the cdc2-regulated activation of E_3-C causes the sudden increase in the rate of cyclin ubiquitination near the end of M-phase. Cyclin-ubiquitin conjugates would then be rapidly recognized and degraded by the 26S protease complex, which appears to be constitutively active in the cell cycles of early frog (Mahaffey *et al.*, 1993) or clam (E. Eytan and A. Hershko, unpublished results) embryos.

Because enzymatic activity of this complex promotes the ligation of cyclin to ubiquitin in the presence of E_1 and E_2-C, it is formally homologous to other ubiquitin-protein ligases (E_3 enzymes) that ligate various proteins to ubiquitin in the presence of E_1 and an appropriate species of E_2 (reviewed by Hershko and Ciechanover, 1992). Of the few E_3's that have been identified so far, one of the best studied is $E_3\alpha$, a 350-kDa enzyme involved in the ligations of ubiquitin to proteins that have basic or hydrophobic N-terminal amino acid residues (Reiss *et al.*, 1988; Bartel *et al.*, 1990). $E_3\alpha$ has binding sites for its specific substrates (Reiss and Hershko, 1990) and for its specific E_2 partner (Reiss *et al.*, 1989), which may facilitate the transfer of activated ubiquitin from E_2 to target proteins. It remains to be seen whether cyclin-ubiquitin ligase has specific binding sites for cyclin and E_2-C and, if so, what specific features of cyclin structure are recognized by the enzyme. The destruction box is a likely recognition determinant for cyclin-ubiquitin ligase, but it may not be the only one. The observation that the affinity of N-terminal cyclin fragments is much lower than that of full-length cyclin (Figure 8) may indicate the involvement of additional regions of cyclin in binding to the enzyme.

E_3-C is distinguished from all other currently known E_3's by its large size and its cell cycle-regulated, kinase-dependent activation. The large size of the active form of E_3-C may reflect an association with other components that participate in the regulation of its activity, including components that sense and relay checkpoint information (Hartwell and Weinert, 1989). The nature of the particulate material with which the enzyme was originally associated also remains to be identified. Also unknown at this point is whether the cyclosome exists as a pre-formed, inactive complex during interphase or is assembled during mitosis. Preliminary experiments designed to discriminate between these two possibilities have not yet been informative. The prolonged lag in the activation of the interphase form of E_3-C by cdc2, which cannot be overcome by increased levels of cdc2 activity, suggests that E_3-C activation is not due to direct phosphorylation by cdc2, but this result is not inconsistent with either model for cyclosome activation. Obviously, only further purification of cyclosome-associated material will reveal the nature of these components and how they are regulated during the cell cycle.

Cyclin A disappears slightly before cyclin B, so we were surprised to find that a single cyclin-ubiquitin ligase acts on both cyclin A and cyclin B. This conclusion is based on the finding that the partially purified enzyme ligates ubiquitin to derivatives of both cyclin types (Figure 7), as well as on the exact coincidence of activities toward both cyclins in fractions of the glycerol gradient separation (Figure 5B), on the ability of cyclin A to compete with cyclin B for ubiquitin ligation (Figure 8), and on the ability of cyclin A to inhibit the degradation of both native, endogenous cyclin A and B (Figure 9). How, then, can cyclin A disappear before cyclin B? It has been pointed out (Lorca *et al.*, 1992a) that although the degradation of cyclin A is certainly *completed* before that of cyclin B, there are no data showing that cyclin A degradation is *initiated* earlier. It is possible, therefore, that degradation of both cyclins is initiated at the same time, and that cyclin A is degraded faster due to a kinetic advantage. The observation that exogenous cyclin A inhibits the degradation of endogenous cyclin B to a greater degree that that of endogenous cyclin A (Figure 9) may indicate that the affinity of cyclin A for E_3-C is higher than that of cyclin B. It is also possible that the delayed degradation of cyclin B is due to additional regulatory mechanisms. Consistent with this idea is the observation that, in the absence of a properly assembled mitotic spindle, cyclin A is destroyed on schedule but cyclin B is stabilized (Hunt *et al.*, 1992). In such cases, levels of regulation beyond activation of E_3-C may be involved, such as processes that affect the accessibility of the two cyclins to the ubiquitin ligation machinery.

Some observations remain to be explained. The E_3-C activity in fraction 1A from M phase cells is activated by preincubation with ATP and Mg^{2+} (Figures 3 and 6B). Whether this reflects the requirement for the continued action of cdc2 or of another, unidentified downstream kinase, is not yet known. Further purification of both the inactive and active forms of E_3-C, identification of other cyclosome-associated proteins, and their complete separation from cdc2 should resolve this problem. The same is true for the observation that the low activity form of cyclin-ubiquitin ligase in untreated, early M phase fraction 1A is larger than the activated E_3-C (Figure 6B). Activation of the

cyclosome could be accompanied by a conformational change. Alternatively, the larger peak of cyclin-ubiquitin ligase activity could be generated by a region of overlap, where the inactive ligase and a larger activator are incompletely separated.

Activation of the novel cyclin-ubiquitin ligase E_3-C identified here represents one of the final events regulated by cdc2 during M phase, namely, cyclin destruction and the release of inactive monomeric cdc2. These events are absolutely required for exit from M phase and entrance into the next cell cycle. The substrate recognition properties of E_2-C and E_3-C are "cyclin-selective" but do not rule out the possibility that they are responsible for the cell cycle stage-specific destruction of other proteins as well. For example, N-terminal fragments of cyclin B, which act as specific competitors of cyclin destruction and inhibit cdc2 inactivation, also block sister chromatid separation (Holloway *et al.*, 1993); methylated ubiquitin, which delays cyclin destruction and cdc2 inactivation by a completely different mechanism, also inhibits sister chromatid separation (Holloway *et al.*, 1993). These observations argue that the proteolytic machinery that destroys mitotic cyclins also recognizes proteins whose degradation is required for the sister chromatid separation. Although such proteins remain to be identified, it would not be surprising if future work shows that they too are targeted for ubiquitination, and thus for degradation, by the same E_2-C and E_3-C activities described here.

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