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Cell Cycle–regulated Degradation of *Xenopus* Cyclin B2 Requires Binding to p34^{cdc2}

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The protein kinase activity of the cell cycle regulator p34^{cdc2} is inactivated when the mitotic cyclin to which it is bound is degraded. The amino (N)-terminus of mitotic cyclins includes a conserved "destruction box" sequence that is essential for degradation. Although the N-terminus of sea urchin cyclin B can confer cell cycle–regulated degradation to a fusion protein, a truncated protein containing only the N-terminus of *Xenopus* cyclin B2, including the destruction box, is stable under conditions where full length molecules are degraded. In an attempt to identify regions of cyclin B2, other than the destruction box, involved in degradation, the stability of proteins encoded by C-terminal deletion mutants of cyclin B2 was examined in *Xenopus* egg extracts. Truncated cyclin with only the first 90 amino acids was stable, but other C-terminal deletions lacking between 14 and 187 amino acids were unstable and were degraded by a mechanism that was neither cell cycle regulated nor dependent upon the destruction box. None of the C-terminal deletion mutants bound p34^{cdc2}. To investigate whether the binding of p34^{cdc2} is required for cell cycle–regulated degradation, the behavior of proteins encoded by a series of full length *Xenopus* cyclin B2 cDNA with point mutations in conserved amino acids in the p34^{cdc2}-binding domain was examined. All of the point mutants failed to form stable complexes with p34^{cdc2}, and their degradation was markedly reduced compared to wild-type cyclin. Similar results were obtained when the mutant cyclins were synthesized in reticulocyte lysates and when cyclin mRNA was translated directly in a *Xenopus* egg extract. These results indicate that mutations that interfere with p34^{cdc2} binding also interfere with cyclin destruction, suggesting that p34^{cdc2} binding is required for the cell cycle–regulated destruction of *Xenopus* cyclin B2.

INTRODUCTION

Cyclins are essential activators of cyclin dependent kinases (cdks), a family of protein kinases whose members trigger the principal transitions in the eukaryotic cell cycle, including the entry into mitosis, the transition from G1 to S phase, and the progression through START, a point of commitment to the cell cycle in the budding yeast (for recent reviews see Forsburg and Nurse 1991; Reed, 1991, 1992; Norbury and Nurse, 1992; Sherr, 1993). The levels of cdk proteins remain fairly constant throughout the cell cycle, but their protein kinase activity varies in a cell cycle–dependent manner, with highest activity usually just before the cell cycle transition they control. In all cases, the protein kinase activity of a cdk is low or absent until after it has bound its cyclin partner. Cyclins were named because of the oscillations in their abundance during the cell cycle (Evans *et al.*, 1983). A characteristic of cyclins is a loosely conserved sequence known as the "cyclin box" (Hunt, 1991; Nugent *et al.*, 1991) that is required to bind to its cdk partner (Kobayashi *et al.*, 1992; Lees and Harlow, 1993; Zheng and Ruderman, 1993). Although mitotic cyclins, whose levels are highest from late G2 through metaphase of mitosis, were the first to be described, another class of cyclins, whose levels peak in G1 or near the G1/S boundary, are known to be

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essential regulators of the progression through the cell cycle (Reed, 1991, 1992; Sherr, 1993). Although the exact mechanism by which G1 cyclins promote progression through the cell cycle has not been elucidated, it has been suggested that in mammalian cells they function as positive growth regulators, possibly through their ability to interact with the retinoblastoma gene product (Dowdy *et al.*, 1993; Ewan *et al.*, 1993) or by the ability of cyclin/cdk complexes to phosphorylate the retinoblastoma gene product (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1994) or other substrates essential for the initiation of S phase. The regulation of G1 cyclins appears to be under transcriptional control, and their levels decline once transcription stops. The instability of G1 cyclins may be caused by the presence of sequences rich in the amino acids P, E, S, and T, which are characteristic of rapidly degraded, short-lived proteins (for review see Rechsteiner, 1991). Deletion of a PEST-rich region of a G1 cyclin renders it resistant to proteolytic degradation (Ghiara *et al.*, 1991).

In contrast to G1 cyclins, mitotic cyclins reach their highest levels in late G2 phase, just before entry into mitosis (Evans *et al.*, 1983; Pines and Hunter, 1989, 1990; Hunt *et al.*, 1992). Cyclins A, B1, and B2 interact with $p34^{cdc2}$, a 34-kDa serine-threonine protein kinase that was the first cyclin-dependent kinase to be identified (for review see Norbury and Nurse, 1992; Pines, 1993). Mutations in the cdc2 gene of the fission yeast *Schizosaccharomyces pombe* and some alleles of the CDC28 gene, the cdc2 homologue in the budding yeast *Saccharomyces cerevisiae*, block entry into mitosis (Nurse and Bissett, 1981; Piggott *et al.*, 1982). Moreover, both cyclin B and $p34^{cdc2}$ are components of highly purified M-phase promoting factor (MPF), whose activity plays a central role in inducing entry into metaphase, both in meiotic and mitotic cells (Gautier *et al.*, 1988, 1990; Draetta *et al.*, 1989; Westendorf *et al.*, 1989). The activity of cyclin B/$p34^{cdc2}$ complexes is under transcriptional and posttranslational control. Although complexes between newly synthesized cyclin B and $p34^{cdc2}$ can be detected in early S-phase (Draetta and Beach, 1988; Pines and Hunter 1990), the binding of $p34^{cdc2}$ to cyclin B at this stage of the cell cycle is insufficient for the generation of active MPF (Solomon *et al.*, 1990; for review see Draetta, 1992). When part of a newly formed complex, $p34^{cdc2}$ is a substrate for phosphorylation on Thr14 and Tyr15, which inactivates its protein kinase activity (Gould and Nurse, 1989; Krek and Nigg, 1991), and on Thr161 (or 167) (Gould *et al.*, 1991; Krek and Nigg, 1991), which is essential for kinase activity (Solomon *et al.*, 1992, 1993; Fesquet *et al.*, 1993; Poon *et al.*, 1993). The phosphorylation-dephosphorylation of $p34^{cdc2}$ is a key posttranslational mechanism that ensures that its protein kinase activity is not activated until completion of DNA replication and the repair of any DNA damage.

A second level of posttranslational control of the cyclin/$p34^{cdc2}$ complex is the regulation of the levels of mitotic cyclins. Just as the synthesis of mitotic cyclins and their association with $p34^{cdc2}$ is essential for entry into mitosis, completion of mitosis and the start of a new cell cycle absolutely requires the inactivation of MPF activity, which is accomplished through the specific proteolysis of its cyclin subunit (Murray *et al.*, 1989). B-type cyclins are degraded during the transition from metaphase to anaphase of mitosis, trailing slightly the degradation of A-type cyclin (Luca and Ruderman, 1989; Minshull *et al.*, 1990; Whitfield *et al.*, 1990; Hunt *et al.*, 1992). The failure to degrade B-type cyclins arrests the cell cycle before the completion of mitosis, although the meta- to anaphase transition can still occur (Holloway *et al.*, 1993; Surana *et al.*, 1993). Cyclin degradation appears to be highly specific because few other proteins are known to be degraded only during this brief period in mitosis, and the levels of $p34^{cdc2}$ appear to be unchanged. It is not known what controls the exact timing of this event and what makes cyclin suddenly become a substrate for degradation. The addition of multiple ubiquitin molecules to lysine residues has been implicated in targeting cyclin for degradation, because ubiquitin has been found to be conjugated to sea urchin cyclin B in *Xenopus* egg extracts undergoing cyclin degradation (Glotzer *et al.*, 1991), and the addition of methylated ubiquitin, an inhibitor of polyubiquitination, delays cyclin degradation in in extracts of clam embryos (Hersko *et al.*, 1991) and *Xenopus* eggs (Holloway *et al.*, 1993). So far cyclin-specific ubiquitin-conjugating enzymes or ligases have not been identified, although they have been assumed to be activated during cyclin degradation. Recent genetic evidence also implicates the 26S ATP-dependent protease complex, which is involved in the degradation of other ubiquitin-conjugated proteins, in progression through mitosis in yeast (Ghislain *et al.*, 1993; Gordon *et al.*, 1993).

Sequences at the amino terminus (N-terminal) have been clearly demonstrated to be important for the degradation of mitotic cyclins. Truncated A- or B-type cyclins missing the first 60 to 100 amino acids are resistant to degradation (Murray *et al.*, 1989; Glotzer *et al.*, 1991; Luca *et al.*, 1991; Gallant and Nigg, 1992; Lorca *et al.*, 1992), and a truncated protein (B2Nt) containing only the first 89 amino acids of *Xenopus* cyclin B2 inhibited degradation of full length cyclin B2 (van der Velden and Lohka, 1993), presumably by binding a component(s) of the cyclin destruction pathway. The N-terminal sequence that is important for degradation is likely to be a nine amino acid–long sequence, known as the "destruction box", that is found in both A- and B-type cyclins, but not in G1 cyclins. Mutation of one or more highly conserved amino acids in this region prevents cyclin degradation (Glotzer *et al.*, 1991; Gallant and Nigg, 1992; Holloway *et al.*, 1993; Stewart *et al.*, 1994).

Surprisingly, despite the presence of a destruction box and the presence of ten lysine residues following the destruction box, B2Nt is stable under conditions where full length cyclin B2 is degraded. This suggests that other regions of the cyclin B molecule are essential for cell cycle–regulated degradation. In an attempt to determine which additional regions of cyclin B may be involved in its degradation during mitosis, we constructed a series of carboxyl-terminal (C-terminal) truncations and point mutations of conserved amino acids in the cyclin box of *Xenopus* cyclin B2 and examined the degradation of their translation products in cell-free extracts of *Xenopus* eggs. Our data indicate that mutations or C-terminal truncations of cyclin B2 that interfere with p34^{cdc2} binding also interfere with cyclin degradation, suggesting that cyclin is a substrate for cell cycle–regulated degradation only when it is bound to p34^{cdc2}.

MATERIALS AND METHODS

Construction of C-Terminal Deletions of Cyclin B2

cDNAs encoding truncated forms of *Xenopus* cyclin B2 lacking C-terminal 14, 68, 187, and 302 amino acids were prepared by polymerase chain reaction (PCR). Cyclin B2 cDNA that was excised by *Eco*RI from a full length clone of *Xenopus* cyclin B2 in pGEM1 (Izumi and Maller, 1991) served as a template. A common primer was used for the region encoding the N-terminus of cyclin B2 in combination with different primers complimentary to the desired position of the truncation. The sequence of the common primer is 5'GGCCGGGAATTCCGGCTAGATTTTATC3'. It contains an *Eco*RI restriction site flanked by six additional nucleotides at the 5' end and the sequence of cyclin B2 at the 3' end. The different 3' primers were specifically designed for each individual truncation to produce a stop codon at the desired position, followed by a *Bam*HI restriction site and four additional nucleotides. The sequence of each of the 3' primers are as follows:

(B2CΔ14) 5'GCGCGGATCCTTACTGAGGAAGGGTGCTGAT3'

(B2CΔ68) 5'CCGGGGATCCTTAGGTACCCCAGGTTCCCTG3'

(B2CΔ187) 5'GCCGGGATCCTTAAAGCTTACTGCGGGAGAC3'

(B2CΔ302) 5'CGGATCCTTAGGAAGGCACTTTGGGGGC3'.

PCR reactions were carried out in a DNA thermal cycler (Techne, Princeton, NJ) for 30 cycles each consisting of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. Each 100-μl reaction mixture contained 5 ng of template, 1 μM of each of the primers, 800 μM of each of the dNTPs, and 2 U of Vent DNA polymerase (New England Biolabs, Beverly, MA). The products of each reaction were cut out from an agarose gel and purified by using Geneclean (Bio 101, Vista, CA). After digestion with *Eco*RI and *Bam*HI, the fragments were ligated into pGEM 4.

Construction of Point Mutants

For the introduction of point mutations into the cyclin box or destruction box sequences of cyclin B2, full length cyclin B2 cDNA was excised from pGEM1 by *Eco*RI and cloned into M13mp18. To generate a destruction box mutation in B2CΔ14, the cyclin B2CΔ14 insert was excised from pGEM4 by *Eco*RI and *Bam*HI and ligated into M13mp18. Mutagenesis was performed with the BioRad Muta-Gene kit (Richmond, CA) based on the method described by Kunkel (1985). Double-strand fragments were cloned back into pGEM4, and all sequence changes were confirmed by sequencing with either the Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH) or the T7 Sequencing kit (Pharmacia, Piscataway, NJ).

Preparation of Capped mRNAs and Their Translation in a Rabbit Reticulocyte Lysate

mRNA encoding full length cyclin B2, cyclin B2 with C-terminal truncations, cyclin B2 with point mutations, or sea urchin Δ13 and Δ90 cyclin B was transcribed from the pGEM vector as described previously (van der Velden and Lohka, 1993). Briefly, plasmid DNA was linearized and used as a template for in vitro transcription by either SP6 or T7 RNA polymerase in the presence of 0.25 mM of the cap analogue m^{7}GpppG (Boehringer Mannheim, Indianapolis, IN). After removal of the DNA template by digestion with RNase-free DNase I (Promega, Madison, WI), incubations were stopped by the addition of an equal volume of 0.2 M tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8), 2% sodium dodecylsulfate (SDS), 20 mM EDTA (pH 8), 0.3 M NaCl and heated for 5 min at 65°C. Reactions were extracted twice each with phenol and chloroform, and RNA was recovered by ethanol precipitation. Final mRNA pellets were resuspended in H_2O at a concentration of ~1 mg/ml. To increase the efficiency of translation, 1–2 μl of RNA was heated for 10 min at 67°C before in vitro translation in 50 μl rabbit reticulocyte lysate (Promega) with 1 mCi/ml [^{35}S]methionine (Amersham, Arlington).

Destruction Assays for Cyclins Synthesized in Rabbit Reticulocyte Lysates

Cyclins synthesized in a rabbit reticulocyte lysate were tested for specific degradation in cytostatic factor (CSF)–arrested extracts of unfertilized *Xenopus* eggs. These extracts maintain the properties of metaphase II–arrested eggs from which they are derived and can be released from the metaphase block by the addition of Ca^{2+} ions (Lohka and Masui, 1984; Lohka and Maller, 1985; Murray and Kirschner, 1989). Endogenous and exogenously added cyclins are known to be specifically degraded during the release of the metaphase arrest. CSF extracts were prepared as described previously with minor modifications (van der Velden and Lohka, 1993). In tubes placed on ice reaction mixtures containing 70 μl CSF extract, between 0.5 and 4 μl of each reticulocyte translation product and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (20 mM HEPES pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 300 μM phenylmethylsulfonyl fluoride [PMSF], 5 μg/ml leupeptin) to a final volume of 100 μl were prepared. From each mixture 25-μl samples were either kept on ice for 30 min or incubated at 23°C in the absence or presence of Ca^{2+}, which was added to a nominal final concentration of 0.4 mM (2 μl of a 5 mM $CaCl_2$ solution added to 25 μl). After incubation, an equal volume of HEPES buffer was added, and samples were centrifuged at 4°C for 10 min at 16 000 × *g*. The clear supernatant was transferred to a clean tube and mixed with one-quarter of a volume of 5× Laemmli's sample buffer. Samples of 5 μl were heated for 2 min at 95°C and analyzed by SDS polyacrylamide gel electrophoresis (PAGE). The gels were soaked for 30 min in 1 M sodium salicylate (Chamberlain, 1979), dried, and exposed to Kodak XAR film (Rochester, NY). Autoradiographs were scanned with a Hewlett Packard Scan Jet IIC flatbed scanner (Palo Alto, CA) and analyzed by using the MacImage software program, version 1.31 (Wayne Rashband, National Institutes of Health, Research Services Branch, National Institute of Mental Health).

To ensure that nonspecific proteolysis stimulated by the addition of Ca^{2+} to 0.4 mM was not responsible for cyclin degradation in our assay, sea urchin cyclin BΔ90, which is resistant to cell cycle–regulated cyclin destruction but not to nonspecific proteolysis, was added to all reactions. The degradation of each of the test cyclin molecules was normalized to the degradation of sea urchin cyclin BΔ90, which in most experiments was <10%.

Destruction Assays of Cyclins Synthesized in Frog Egg Extracts

Xenopus full length cyclin B2, cyclin B2 with C-terminal truncations or point mutations, and sea urchin Δ13 and Δ90 cyclin B were also synthesized in freshly prepared CSF extracts essentially as described by Kobayashi *et al.* (1991). The degradation of the cyclins translated in CSF extracts was initiated by the addition of Ca^{2+} to a final concentration of 0.4 mM. A typical 10-μl reaction contained 7 μl extract, 1 μl [^{35}S]methionine (15 mCi/ml), 0.5 μl 1 mM amino acid mix minus methionine (Promega), 0.5 μl RNasin (4 U/μl, Promega), and 1 μl mRNA (heated for 10 min at 67°C). Reactions were incubated for 1 h at 23°C, at which time each reaction received 1 μl of a mixture of cycloheximide and emetine (final concentration 0.15 mM each) to block further protein synthesis and 1 μl Ca^{2+} (final concentration 0.4 mM) to start cyclin degradation. For analysis by SDS-PAGE and autoradiography, 3-μl samples were diluted with Laemmli's sample buffer until a final volume of 15 μl. Between 2 and 5 μl of each sample was loaded in each lane, depending upon the experiment and the amount of [^{35}S]methionine incorporated into cyclin. Autoradiographs of the gels were scanned as described above. Because the electrophoretic mobility of cyclin B2 and B2-Ala30/Arg32 synthesized from added mRNA was the same as cyclin B2 synthesized from mRNA endogenous to the extract, it was necessary to correct for the amount of cyclin B2 and B2-Ala30/Arg32 synthesized from exogenous mRNA by subtracting the amount of endogenous cyclin B2, which was determined from a control incubation that did not receive exogenous mRNA.

Antisera and Immunoprecipitations

Rabbit polyclonal antisera were raised against *Xenopus* cyclin B2 and against a carboxyl-terminal peptide of *Xenopus* p34^{cdc2}. To prepare antiserum to *Xenopus* cyclin B2, cyclin B2 expressed from a pET3A vector in *Escherichia coli* BL21(DE3) (Gautier *et al.*, 1990) was isolated, separated by SDS-PAGE, and eluted from polyacrylamide gels as previously described (van der Velden and Lohka, 1993). The rabbit was injected with 200 μg protein in complete Freund's adjuvant and received booster injections of 200 μg protein in incomplete Freund's on days 15, 34, 60, and 80. The serum used in experiments described here was collected on day 94. The serum recognized, on immunoblots, bacterially expressed cyclin B2 and a 48-kDa protein in CSF extracts of *Xenopus* eggs that decreased in abundance after Ca^{2+} addition. The sera could immunoprecipitate full length and C-terminal truncations of cyclin B2 protein that had been synthesized in reticulocyte lysates, as described in the text. To prepare antiserum to *Xenopus* p34^{cdc2}, a peptide with the sequence C-H-P-Y-F-D-D-L-D-K-S-S-L-P-D-N-Q-I-N, which corresponds to the C-terminal 19 amino acids of *Xenopus* p34^{cdc2} (Milarski *et al.*, 1991) and a cysteine residue for the covalent coupling of the peptide to other proteins, was chemically synthesized and conjugated to keyhole limpet hemocyanin (Biomolecular Resources, Colorado State University, Fort Collins, CO). The equivalent of 600 μg of peptide was injected with complete Freund's adjuvant into a rabbit, and booster injections of 600 μg in incomplete Freund's were injected on days 21, 38, 67, and 73. The serum used in the experiments described here was collected on day 77. The serum recognized a 34-kDa protein on immunoblots of oocytes, eggs, and embryos. The sera could immunoprecipitate histone H1 kinase activity from unfertilized *Xenopus* eggs and cyclin B2/p34^{cdc2} complexes as described in the text.

For immunoprecipitation each antiserum (3 μl) was incubated with protein A-agarose beads (Pierce, Rockford, IL) (20 μl of a 50% suspension in Tris-buffered saline) for 30 min at 4°C with gentle mixing. The loaded beads were washed several times with IP buffer: 20 mM HEPES (pH 7.5), 80 mM Na-β-glycerophosphate, 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, EGTA), 10 mM NaF, 1 mM DTT, 0.1% NP40, 300 μM PMSF, and 5 μg/ml leupeptin. Reticulocyte translation products (2.5 μl) of cyclin B2 mRNAs were incubated for 5 min at room temperature with an equal volume of CSF extract. Each sample was diluted to 400 μl with IP buffer and centrifuged at 4°C for 5 min at 16 000 × *g*. Subsequently, one-half of the clear supernatant was added to Protein A beads preloaded with cyclin B2-specific antibodies and the other half to beads preloaded with *Xenopus* cdc2-specific antibodies. Samples were gently rotated overnight at 4°C. After several washes in IP buffer, the bound proteins were eluted in Laemmli's sample buffer, heated for 2 min at 95°C, and analyzed by SDS-PAGE and autoradiography. Typically one-quarter of each sample was loaded per lane.

RESULTS

We have previously utilized an assay system similar to that described by Murray *et al.* (1989) to study *in vitro* the degradation of *Xenopus* mitotic cyclins during the transition from metaphase to interphase (van der Velden and Lohka, 1993). CSF extracts made from unfertilized *Xenopus* eggs, which are naturally arrested in metaphase II, retain a high level of MPF activity. On addition of Ca^{2+} to these extracts to a concentration of 0.4 mM, MPF activity is lost as a result of the degradation of internal cyclins, and the extracts are released from their metaphase arrest. Cyclins, such as *Xenopus* cyclin B2 or sea urchin cyclin BΔ13, synthesized in a rabbit reticulocyte lysate are relatively stable when added to fresh CSF extracts but are degraded rapidly after Ca^{2+} addition. In agreement with the results of Murray *et al.* (1989), sea urchin cyclin BΔ90, which is missing 90 amino acids at its N-terminus including the destruction box, is completely stable, confirming that the degradation observed in our hands was specific. Despite the presence of the destruction box, B2Nt, a C-terminal truncation of cyclin B2 that possesses the first 89 amino acids of *Xenopus* cyclin B2, was stable in this assay (van der Velden and Lohka, 1993), suggesting that other parts of the cyclin molecule are also required for the cell cycle–regulated degradation of mitotic cyclins.

In an effort to determine which regions of mitotic cyclins other than the destruction box are required for specific degradation, a series of deletion constructs that encode *Xenopus* cyclin B2 lacking 302, 187, 68, or 14 amino acids from their C-terminus were prepared. (These truncations are called B2CΔ302, B2CΔ187, B2CΔ68, and B2CΔ14, respectively.) B2CΔ302 is similar to B2Nt but has Ser90 and is lacking the phosphorylation site for cAMP-dependent protein kinase found in B2Nt. As shown in Figure 1A, all of the cyclin B2 truncations retained an intact destruction box, but only B2CΔ68 and B2CΔ14 contained a complete cyclin box, the region that shows the highest degree of homology between all cyclins and that is believed to be essential for interactions with cdk partners. Full length cyclin B2 and C-terminal deletion products of cyclin B2 that had been radiolabeled with [^{35}S]-methionine during translation in reticulocyte lysates were added to a CSF extract, and their stability was examined in both the absence and the presence of added Ca^{2+} (Figure 1B). In the ab-

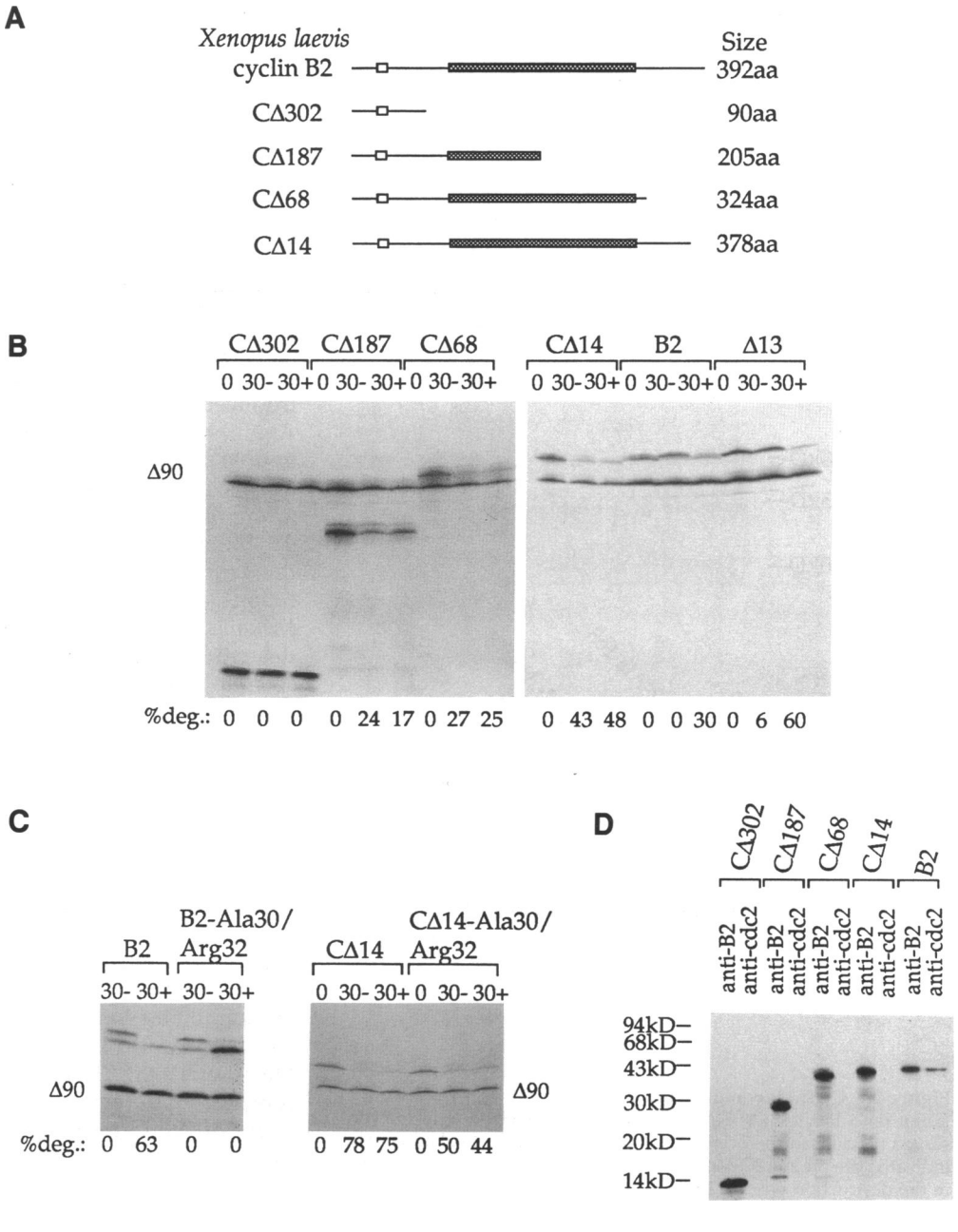

Figure 1. C-terminal deletion mutants of *Xenopus* cyclin B2. (A) Schematic representation of the truncated B2 cyclins CΔ302, CΔ187, CΔ68, and CΔ14. Both the destruction box (□) and the cyclin box (▩) are indicated for full length *Xenopus laevis* cyclin B2. (B) Destruction of truncated *Xenopus* B2 cyclins synthesized in rabbit reticulocyte lysates. [^{35}S]-labeled translation products of C-terminal deletion mutants of *Xenopus* cyclin B2, full length *Xenopus* cyclin B2, or sea urchin cyclin BΔ13 were added to a CSF extract. Each sample also received radiolabeled sea urchin cyclin BΔ90 as a negative control. At 0 min (0) or 30 min in the absence (30−) or presence (30+) of 0.4 mM Ca^{2+} reactions were stopped and samples were processed and analyzed on a SDS-15% polyacrylamide gel as described in MATERIALS AND METHODS. The percentage cyclin degradation (% deg.) is indicated under each lane. (C) Destruction of full length *Xenopus* cyclin B2, B2CΔ14, and their destruction box mutants B2-Ala30/Arg32 and CΔ14-Ala30/Arg32. The ^{35}S-labeled reticulocyte translation products of each of the constructs were incubated in a CSF extract to which radiolabeled sea urchin cyclin BΔ90 was also added. Samples were incubated for 0 min (0) or 30 min in the absence (30−) or presence (30+) of Ca^{2+}. Samples were electrophoresed on 10 (left) and 15% (right) polyacrylamide gels. (D) Inability of the C-terminal truncation of *Xenopus* cyclin B2 to bind to p34^{cdc2}. Radiolabeled reticulocyte-translated cyclin B2 and its truncated derivatives were mixed with an equal volume (2.5 μl) of CSF extract as a source for p34^{cdc2}. After 5 min at room temperature, mixtures were diluted until 400 μl and divided in half. Each half was used in an immunoprecipitation with either anti-*Xenopus* cyclin B2 or anti-*Xenopus* p34^{cdc2} polyclonal antiserum. Complexes were resolved on a SDS-15% polyacrylamide gel. Molecular weight markers are indicated on the left.

sence of Ca^{2+}, both full length cyclin B2 and sea urchin cyclin BΔ13 were completely stable during a 30-min incubation, and *Xenopus* cyclin B2 usually shifted to a form with a slightly lower electrophoretic mobility (see also Figures 1C and 2C), probably as a result of phosphorylation on Ser90. However, when Ca^{2+} was added to the extracts, typically between 30 and 70% of full length cyclin B2 or sea urchin cyclin BΔ13 was degraded. As expected, sea urchin cyclin BΔ90, which was added to all reactions as a negative control, was stable both in the absence and in the presence of Ca^{2+}. B2CΔ302 was also completely stable in the absence of Ca^{2+} and showed little or no degradation relative to sea urchin Δ90 cyclin B after Ca^{2+} addition. In contrast to the full

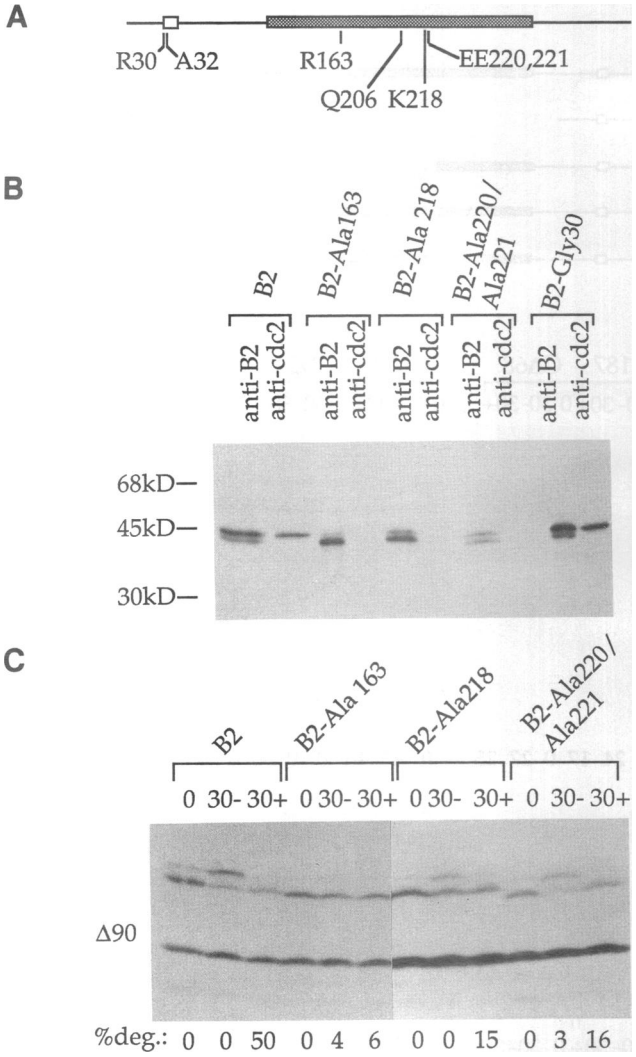

Figure 2. Cyclin box mutants of *Xenopus* cyclin B2 synthesized in rabbit reticulocytes. (A) Schematic showing the position of the conserved residues in the cyclin box that were mutated to alanine. Also indicated are arginine 30 and alanine 32 residues that were mutated in the destruction box. (B) Inability of the cyclin box mutants to bind $p34^{cdc2}$. The [^{35}S]-labeled reticulocyte translation products for normal cyclin B2 and cyclin B2 with mutations in the cyclin box or destruction box were incubated with CSF extract. The ability of the proteins to bind to $p34^{cdc2}$ was tested by immunoprecipitation as described in the legend to Figure 1D. The destruction box mutant B2-Gly30 was included as a control. Complexes were resolved on a SDS-10% polyacrylamide gel. Molecular weight markers are indicated on the left. (C) Stability of cyclin B2 with cyclin box mutations synthesized in rabbit reticulocyte lysates. [^{35}S]-labeled cyclin B2 or cyclin B2 with point mutations in the cyclin box was incubated in the CSF extract. Sea urchin cyclin BΔ90 was added to each incubation as a control. After 0 min (0) or 30 min in the absence (30−) or presence (30+) of 0.4 mM Ca^{2+}, samples were processed as described in MATERIALS AND METHODS and analyzed on a SDS-10% polyacrylamide gel. The percentage cyclin degradation (% deg.) is indicated under each lane.

length cyclins and B2CΔ302, the other C-terminal truncations were degraded even in the absence of added Ca^{2+}, with 24–78% of the C-terminal cyclin truncations being degraded, depending upon the molecule and the extract. The addition of Ca^{2+} was unable to stimulate any further degradation of these truncated molecules. These results are in agreement with the observations made recently by Stewart *et al.* (1994), who have shown that *Xenopus* cyclin B2CΔ24, which lacks the C-terminal 24 amino acids, is destroyed in a CSF extract at a similar rate in the absence or presence of Ca^{2+}.

To determine whether or not C-terminally truncated cyclin B2 is degraded by the same pathway as full length cyclins during mitosis, double point mutations were introduced into the destruction box region of B2CΔ14: Arg 30 to Ala (R30A) and Ala 32 to Arg (A32R). These double point mutations have been shown to inhibit the cell cycle–specific degradation of full length, mitotic sea urchin cyclin B (Holloway *et al.*, 1993). However, the double mutation in the destruction box did not inhibit the degradation of B2CΔ14, even though the same mutations completely blocked destruction of full length *Xenopus* cyclin B2 (Figure 1C). Thus, the C-terminally deleted B2 cyclins, B2CΔ187, B2CΔ68, and B2CΔ14, seem to be degraded by a pathway that is continuously active in CSF extracts and that is not dependent upon the destruction box sequences required for the degradation of full length mitotic cyclins. Apparently, the region of cyclin B2 between amino acids 90 and 205 is required for degradation by this pathway because B2CΔ302 is stable in CSF extracts, whereas B2CΔ187 is not.

All of the truncated B2 cyclins that were unstable in CSF extracts lack the extreme C-terminal region or this region and part of the cyclin box. Because both the C-terminus and cyclin box regions have been shown to be essential for binding of $p34^{cdc2}$ or $p32^{cdk2}$ to cyclin A and B1 (Kobayashi *et al.*, 1992; Lees and Harlow, 1993; Zheng and Ruderman, 1993), we attempted to determine whether the anomalous instability of the C-terminal truncations of cyclin B2 was because of the inability to bind to $p34^{cdc2}$. Immunoprecipitation experiments of [^{35}S]-labeled full length or C-terminally deleted cyclin B2 are shown in Figure 1D. Radiolabeled cyclins were incubated for 5 min with a CSF extract to allow binding of $p34^{cdc2}$, and one-half of each reaction mixture was immunoprecipitated with anti-cyclin B2 serum or with anti-*Xenopus* $p34^{cdc2}$ serum. Both full length cyclin B2 and its truncated derivatives could be immunoprecipitated by antiserum against *Xenopus* cyclin B2. However, only full length cyclin B2 was coimmunoprecipitated by antiserum against *Xenopus* $p34^{cdc2}$. These results demonstrate that, as is the case for cyclin A, none of the C-terminal truncations of cyclin B are able to bind $p34^{cdc2}$. With the exception of B2CΔ302, all of the

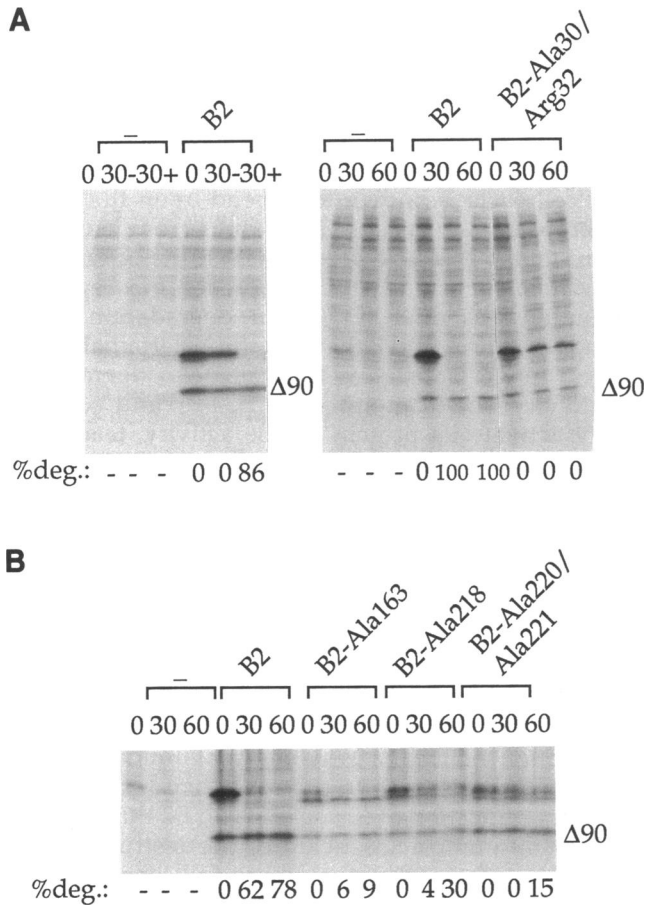

Figure 3. Degradation of normal cyclin B2 and cyclin B2 with mutations in the cyclin box after synthesis in a CSF extract. (A) Characterization of the destruction assay. mRNA for *Xenopus* cyclin B2, B2-Ala30/Arg32 (destruction box mutant), and sea urchin cyclin BΔ90 were translated in a CSF extract during a 1-h incubation at 23°C after which time protein synthesis was blocked by the addition of cycloheximide and emetine (0.15 mM each). (Left) Samples were divided into three equal portions; the first was kept on ice (0), and the second and third were incubated for 30 min at 23°C in the absence (30−) or presence (30+) of 0.4 mM Ca^{2+}. (Right) Ca^{2+} was added to a final concentration of 0.4 mM to CSF extracts with either no added mRNA (−), mRNA for cyclin B2 (B2), or mRNA for cyclin B2 Ala30/Arg32 (B2 Ala30/Arg32). Reactions were stopped before Ca^{2+} addition (0), or 30 and 60 min after Ca^{2+} addition. All incubations were electrophoresed on SDS-10% polyacrylamide gels. (B) Stability of *Xenopus* cyclin B2 with mutated cyclin box sequences after synthesis in a CSF extract. mRNAs for wild-type *Xenopus* cyclin B2 and for each of the cyclin box point mutants was translated in a CSF extract as described in the MATERIALS AND METHODS. Cyclin degradation was initiated by Ca^{2+} addition, and samples were incubated and processed as described for Figure 3A.

truncated cyclins that fail to bind p34^{cdc2} are also unstable in the CSF extracts in the absence of added Ca^{2+}. One interpretation of these results is that cyclins are inherently unstable but are stabilized by the binding of p34^{cdc2}. Alternatively, the C-terminal deletion products may be unstable for a reason unrelated to their inability to bind p34^{cdc2}, such as the presence of the region between amino acids 90 and 205. Kobayashi *et al.* (1992) have pointed out that cyclins with large C-terminal deletions may fail to bind p34^{cdc2} either because they lack a required amino acid sequence(s) or because the p34^{cdc2} binding region of cyclin is not accessible because of misfolding of the protein.

To examine more directly the role of p34^{cdc2} binding in the control of cyclin stability, point mutations that were anticipated to prevent the binding of p34^{cdc2} to *Xenopus* cyclin B2 were introduced in the cyclin box region. Figure 2A shows the sites at which highly conserved amino acids within the cyclin box were changed to alanine. As controls, other mutations were also made in the destruction box region of cyclin B2. Cyclin mRNA carrying the point mutations were translated in a reticulocyte lysate in the presence of [^{35}S]methionine. A radiolabeled product was obtained from all of the mutant cyclin mRNA with the exception of B2-Ala206. Therefore, our analysis of the effect of point mutations on cyclin stability does not include this mutation. When the radiolabeled cyclins were assayed for their ability to bind p34^{cdc2} by immunoprecipitation, none of the B2 cyclins with a mutation in the cyclin box were immunoprecipitated with the antiserum against p34^{cdc2} (Figure 2B). Therefore, none of these mutants was able to form a complex with p34^{cdc2} that is stable under the relatively gentle washing conditions applied. When immunoprecipitates were washed with lower salt and detergent concentrations (0.1 × IP buffer), a small, but detectable, fraction of each of the mutant cyclins coimmunoprecipitated bind p34^{cdc2}, indicating that any binding of mutant cyclins to p34^{cdc2} is much weaker than binding seen with wild-type cyclin B2. Radiolabeled cyclin box mutants of cyclin B2 were also tested as a substrate for degradation in CSF extracts (Figure 2C). As found in previous experiments, little or no cyclin degradation was observed for wild-type, full length cyclin in the absence of Ca^{2+}, but after the addition of Ca^{2+} to the extract 50% was degraded within 30 min. In contrast, cyclin box mutants of B2 were stable both in the absence and in the presence of added Ca^{2+}. After Ca^{2+} addition only 6–16% of the cyclins with the cyclin box mutations were degraded. Recently, Stewart *et al.* (1994) also showed that *Xenopus* cyclin B2-Ala 163 was stable in their cyclin degradation assay after Ca^{2+} addition. These results show that mutation of amino acids that are essential for binding to p34^{cdc2} inhibit cyclin degradation. Our observations are consistent with the hypothesis that p34^{cdc2} binding to cyclin is required for cyclin degradation during the transition from metaphase to interphase.

In our experience, cyclins that have been synthesized in rabbit reticulocyte lysates and used as substrates for degradation in a CSF extract are not de-

graded as efficiently as endogenous cyclins. Therefore, to confirm the importance of $p34^{cdc2}$ binding in cyclin degradation, the behavior of B2 cyclins with point mutations in the cyclin box was examined in another assay system that was recently described by Kobayashi *et al.* (1992). In this assay cyclin mRNA is first translated in a CSF extract in the presence of [^{35}S]methionine, new protein synthesis is blocked by addition of cycloheximide and emetine, and Ca^{2+} is added to trigger cyclin degradation. Figure 3A (left) shows that the mRNAs for *Xenopus* cyclin B2 and sea urchin cyclin BΔ90 are translated in a CSF extract. In the absence of Ca^{2+}, both products are stable, whereas after Ca^{2+} addition nearly complete degradation of *Xenopus* cyclin B2 was observed without significant degradation of sea urchin cyclin BΔ90. The right panel of Figure 3A shows that B2-Ala30/Arg32, the cyclin with the double point mutations in the destruction box, is also stable under conditions where the wild-type cyclin B2 was degraded. The slightly higher amount of radiolabeled product present at 0 min compared to 30 min and 60 min is likely the product of translation of endogenous cyclin B mRNA (see -RNA). This protein comigrates with cyclin B2 from exogenous mRNA and is degraded upon addition of Ca^{2+} to the extract (compare lanes -RNA, 0 and 30). When the relative amount of cyclin degradation was compared in the two assays, we observed that, depending on the extract, between 30 and 70% of cyclin B2 synthesized in reticulocyte lysates was degraded, whereas >75% of cyclin B2 synthesized in the CSF extract was usually degraded during the 30-min interval after Ca^{2+} addition. Thus, *Xenopus* cyclin B2 synthesized in a frog egg extract appears to be a better substrate for degradation than that synthesized in a reticulocyte lysate. Although the assay for the degradation of cyclins synthesized in CSF extracts is more sensitive than that used in Figures 1 and 2, in all cases the behavior of full length or mutant cyclins was qualitatively similar regardless of their source. In fact, with the exception of B2CΔ302, none of the C-terminal truncations that are unstable in CSF extracts could be detected when mRNA was translated directly in the CSF extracts, presumably because of their rapid degradation after synthesis.

When mRNAs that encode the cyclin box mutations were added to a CSF extract, translation products were obtained for B2-Ala163, B2-Ala218, and B2-Ala220/Ala221 mRNA but not for B2-Ala206 mRNA (Figure 3B). Within 30 min after Ca^{2+} addition >60% of wild-type cyclin B2 was degraded. In contrast, for each of the cyclin box mutants <10% degradation was detected during the first 30 min after Ca^{2+} addition, and most mutants were stable for the duration of the assay. These data are consistent with those obtained in the original destruction assay and support the hypothesis that the binding of $p34^{cdc2}$ to *Xenopus* cyclin B2 is required for cell cycle–specific degradation.

DISCUSSION

Progression through mitosis and the entry into the next cell cycle is known to depend upon the degradation of mitotic cyclins. The results from many different laboratories clearly show that the N-terminus of cyclins, particularly the destruction box region, is absolutely essential for cyclin degradation. Even though cyclins A and B that lack the N-terminal region are no longer substrates for the cyclin degradation machinery, they can still bind $p34^{cdc2}$ and continuously activate its protein kinase activity, leading to arrest of the cell cycle in mitosis. Although the N-terminus is necessary for cell cycle–regulated degradation, it does not always appear to be sufficient for targeting a protein for degradation during mitosis. On the one hand, the N-terminus of sea urchin cyclin B can confer specific degradation on *Staphylococcus aureus* protein A in a cyclin-protein A fusion protein (Glotzer *et al.*, 1991). The degradation of both full length cyclin and the degradation of the cyclin-protein A fusion protein is thought to be mediated by polyubiquination on lysine residues that follow the destruction box region. On the other hand, B2Nt, a C-terminal truncation of *Xenopus* cyclin B2 that contains the first 89 amino acids including the destruction box, is stable under conditions where full length cyclins are degraded, even though 10 lysines after the destruction box could serve as potential sites for polyubiquitination. The stability of B2Nt (van der Velden and Lohka, 1993) and the closely related B2CΔ302 described in this paper indicate that the first 90 amino acids of *Xenopus* cyclin B2 are not sufficient for degradation. Both proteins are stable when synthesized in bacteria (van der Velden and Lohka, 1993), reticulocyte lysates, or egg extracts. B2Nt can compete with full length cyclin B2 for a component(s) of the cyclin degradative pathway, and the addition of B2Nt to CSF extracts attenuates the degradation of endogenous cyclins normally seen after Ca^{2+} addition (van der Velden and Lohka, 1993). Furthermore, when added to *Xenopus* egg extracts capable of multiple cell cycles in vitro, B2Nt blocked progression through the first mitosis and prevented entrance into the next interphase, whereas extracts that did not receive B2Nt could support at least two additional cell cycles.

The apparent contradiction between the ability of N-terminal cyclin sequences to confer cell cycle–specific degradation to fusion proteins (Glotzer *et al.*, 1991; Stewart *et al.*, 1994) and the relative stability of B2Nt lead us to initiate the experiments presented here. These experiments were initially aimed at determining the regions of the cyclin B molecule, apart

from the destruction box, that influence cyclin degradation. Such sequences may have been supplied fortuitously by the protein A component of the cyclin-protein A fusion protein. To obtain information about the position of such regions, a series of cDNAs encoding C-terminal deletion mutants of cyclin B2, ranging in length from 90 (B2CΔ302) to 378 (B2CΔ14) amino acids, were constructed, and the proteins they encode were synthesized in reticulocyte lysates and assayed for degradation. We have found that only B2CΔ302 and full length cyclins were stable in CSF extracts that had not received additional Ca^{2+}. All of the other truncated B2 cyclins were rapidly degraded, independent of the addition of Ca^{2+} to the extract. A similar observation has been made for a *Xenopus* cyclin B2 deletion mutant, B2-CΔ24, which is also unstable both in the absence and presence of Ca^{2+} (Stewart *et al.*, 1994). These results indicate that the regions of the cyclin B2 molecule between amino acids 90 and 205 affect the stability of the cyclin molecule. Although we have not attempted to define this region more precisely, it is likely to lie between amino acids 145 and 205 because the stability of a protein encoding the first 145 amino acids of *Xenopus* cyclin B2 is the same as B2Nt (van der Velden, unpublished data). The reason that increasing the length of C-terminally truncated cyclins from 90 to 205 amino acids decreases their stability in metaphase-arrested CSF extracts is not clear. Because point mutations in the destruction box that prevent degradation of full length cyclins did not alter the stability of B2CΔ14 in CSF extracts, the degradation of the products of C-terminal deletion mutants probably proceeds by a mechanism different from that initially involved in the degradation of full length cyclin during mitosis. However, the possibility that the cell cycle–independent mechanism is involved in the late stages of cyclin degradation, after the cell cycle regulated step is completed, cannot be ruled out. Therefore, the extent to which the cell cycle–dependent and –independent mechanisms overlap remains to be determined.

Another possible explanation is that the mechanism responsible for the degradation of C-terminal deletion mutants of cyclin B2 is normally involved in the degradation of damaged or improperly folded proteins. Hence, full length cyclins would be stable because they can assume the properly folded conformation, whereas the products of most of the other C-terminal deletion mutants would be degraded because they cannot fold properly. It is tempting to speculate that B2CΔ302 is stable because it is not long enough to be misfolded. Again, one cannot yet rule out the possibility that such a mechanism also acts during cell cycle–regulated degradation of cyclins, after an initial cell cycle–dependent event. Interestingly, even though reticulocyte lysates are capable of protein degradation by the ubiquitin-dependent proteolytic pathway (for review Hersko and Ciechanover, 1992), the degradation of C-terminal deletion mutants of cyclin is more active in egg extracts than in reticulocyte lysates. The translation products of B2CΔ14, B2CΔ68, and B2CΔ187 never accumulated when mRNA was added to egg extracts but accumulated when mRNA was translated in reticulocyte lysates (van der Velden, unpublished data).

Previous studies have shown that the cyclin box and probably also the extreme C-terminal regions of cyclin A are essential for binding to p34^{cdc2} (Kobayashi *et al.*, 1992; Lees and Harlow, 1993). These regions were deleted from all of the C-terminal deletion mutants of cyclin B2 that were unstable in the CSF extract, and immunoprecipitation experiments confirmed that stable complexes between the C-terminal deletion mutants and p34^{cdc2} did not form. We, therefore, investigated the role of p34^{cdc2} binding to cyclin B2 in cyclin degradation by mutating highly conserved amino acids in the cyclin box to alanine, a replacement that is usually well tolerated without causing conformational changes. Immunoprecipitation experiments confirmed that these point mutants were unable to form stable complexes with p34^{cdc2}, although weak interactions may still occur. When added to CSF extracts, all of the full length cyclins with mutations in the cyclin box were stable in the absence of added Ca^{2+}, and after Ca^{2+} addition, their degradation was greatly reduced compared to full length wild-type cyclin B2. Although it is possible that the amino acids in the cyclin box that are important for p34^{cdc2} binding are also part of the recognition sites for cyclin degradation by a mechanism that is independent of p34^{cdc2} binding, we feel that it is more likely that p34^{cdc2} binding is required for cell cycle–regulated degradation of B2 cyclins. The small amount of degradation observed with the cyclin box mutants may have been due to the formation of unstable interactions between cyclins and p34^{cdc2} that markedly decreased the efficiency of degradation. The effect of cyclin box mutations on the stability of cyclin was also examined in a more sensitive assay in which cyclin mRNAs are translated directly in a CSF extract before the addition of Ca^{2+} initiates cyclin degradation (Kobayashi *et al.*, 1992). The results obtained in this assay were the same as those obtained for cyclin synthesized in reticulocyte lysates; all point mutants that were unable to bind p34^{cdc2} were stable for ≥30 min after Ca^{2+} addition. Only B2-Ala218 showed significant degradation after longer incubation times, but the kinetics of degradation were clearly different from those of full length, wild-type cyclin B2. The behavior of B2-Ala218 could be explained if the ability of this mutation to form complexes with p34^{cdc2} was intermediate to that of full length cyclin B2 and the other cyclin box mutations.

Our data indicate that the formation of a complex between *Xenopus* cyclin B2 and $p34^{cdc2}$ is essential for the cell cycle–regulated degradation of the cyclin subunit. The requirement for $p34^{cdc2}$ binding suggests that both cyclin B2 and $p34^{cdc2}$ are recognized simultaneously by one of the components of the cyclin degradative pathway or that a recognition site for degradation, such as the destruction box, is accessible only after cyclin B2 is bound to $p34^{cdc2}$. A precedent for the cyclin/$p34^{cdc2}$ complex serving as a substrate when the individual components are not substrates is found in the phosphorylation of $p34^{cdc2}$ by the protein kinase encoded by the wee1 gene. In this case, $p34^{cdc2}$ that is part of a complex with cyclin is phosphorylated on Tyr15, but unbound $p34^{cdc2}$ is not (Solomon *et al.*, 1990). Similarly, cyclin is part of the recognition site for cdc25 (Zheng and Ruderman, 1993), the phosphatase that dephosphorylates Tyr 15.

Still another explanation for the need for cyclin to bind $p34^{cdc2}$ before its degradation is that another component of the complex, such as a putative cyclin binding protein, must be phosphorylated by $p34^{cdc2}$ during one step of the degradative pathway. If this were the case, then phosphorylation within the complex is probably required because only low levels of cyclin degradation were seen when molecules with point mutations in the cyclin box were incubated in a CSF extract containing active, endogenous $p34^{cdc2}$/cyclin B2 complexes. It is unlikely that cyclin is the target for such phosphorylation because mutation of the major $p34^{cdc2}$ phosphorylation site in cyclins has no affect on their degradation (Izumi and Maller, 1991). However, the protein(s) that binds to B2Nt is a possible candidate for such a cyclin-binding protein. Moreover, given the evidence implicating ubiquitin conjugation in the cyclin degradation pathway, a cyclin-specific ubiquitin-ligase that needs to be phosphorylated to activate cyclin degradation is another possible candidate.

The findings that $p34^{cdc2}$ binding is necessary for the degradation of its cyclin partner are surprising, given that the N-terminal region is capable of conferring cell cycle–dependent degradation to a sea urchin cyclin B-protein A fusion protein, which, because it is lacking the cyclin box region, is unable to bind $p34^{cdc2}$ (Glotzer *et al.*, 1991; Stewart *et al.*, 1994). Recent studies by Stewart *et al.* (1994) have also found that *Xenopus* cyclin A requires $p34^{cdc2}$ binding for its degradation and that cyclin A proteins with point mutations in the cyclin box were relatively stable. They also found that a deletion mutant B2-CΔ24 and a cyclin box mutant B2-Ala 163 behaved similarly to the C-terminal deletion mutants and cyclin box mutants described here, but a B1-CΔ24 deletion mutant was still degraded in a cell cycle–regulated manner. Together these data strongly support the conclusion that both *Xenopus* cyclins A and B2 require $p34^{cdc2}$ binding for their specific degradation. As pointed out by Stewart *et al.* (1994), the degradation of the sea urchin cyclin B-protein A fusion protein may be more similar to that of *Xenopus* cyclin B1 than to either cyclin A or cyclin B2. Alternatively, if there is a requirement for $p34^{cdc2}$-mediated phosphorylation of a cyclin binding protein before degradation, as we speculate above, then this protein could still be efficiently phosphorylated by endogenous $p34^{cdc2}$/cyclin complexes when it is bound to the sea urchin B-protein A fusion protein. Only with a better understanding of cyclin degradation will we be able to explain these apparently contradictory results.

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