Mutagenic Analysis of the Destruction Signal of Mitotic Cyclins and Structural Characterization of Ubiquitinated Intermediates

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Mitotic cyclins are abruptly degraded at the end of mitosis by a cell-cycle-regulated ubiquitin-dependent proteolytic system. To understand how cyclin is recognized for ubiquitin conjugation, we have performed a mutagenic analysis of the destruction signal of mitotic cyclins. We demonstrate that an N-terminal cyclin B segment as short as 27 residues, containing the 9-amino-acid destruction box, is sufficient to destabilize a heterologous protein in mitotic *Xenopus* extracts. Each of the three highly conserved residues of the cyclin B destruction box is essential for ubiquitination and subsequent degradation. Although an intact destruction box is essential for the degradation of both A- and B-type cyclins, we find that the *Xenopus* cyclin A1 destruction box cannot functionally substitute for its B-type counterpart, because it does not contain the highly conserved asparagine necessary for cyclin B proteolysis. Physical analysis of ubiquitinated cyclin B intermediates demonstrates that multiple lysine residues function as ubiquitin acceptor sites, and mutagenic studies indicate that no single lysine residue is essential for cyclin B degradation. This study defines the key residues of the destruction box that target cyclin for ubiquitination and suggests there are important differences in the way in which A- and B-type cyclins are recognized by the cyclin ubiquitination machinery.

INTRODUCTION

Ubiquitin-mediated proteolysis gates progression through the eukaryotic cell cycle at several critical steps (for review, King *et al.*, 1994; Deshaies, 1995; Glotzer, 1995). This regulatory mechanism first emerged with the discovery of the mitotic cyclins, which accumulate during interphase and are rapidly degraded as cells exit mitosis (Evans *et al.*, 1983). Ectopic expression of nondegradable forms of cyclin A or cyclin B arrests the cell cycle at a late stage of mitosis, indicating that cyclin proteolysis is essential to return to interphase and enter the next cell cycle (Murray *et al.*, 1989; Ghiara *et al.*, 1991; Luca *et al.*, 1991; Gallant and Nigg, 1992; Holloway *et al.*, 1993; Surana *et al.*, 1993; Luo *et al.*, 1994; Rimmington *et al.*, 1994; Sigrist *et al.*, 1995). Two remarkable features of the cyclin degradation system include its temporal regu-

lation, because cyclin B is unstable only during late mitosis and early G1 (Hunt *et al.*, 1992; Amon *et al.*, 1994), and its substrate specificity, because the system is specific for cyclin and perhaps other inhibitors of anaphase (Holloway *et al.*, 1993); bulk proteolysis does not vary significantly during the cell cycle.

A key determinant of the specificity of the cyclin degradation system is the destruction box, a 9-amino-acid motif conserved among the N termini of A- and B-type cyclins (Glotzer *et al.*, 1991). Mutation of the conserved arginine within the destruction box stabilizes both A- and B-type cyclins in mitotic *Xenopus* extracts (Glotzer *et al.*, 1991; Kobayashi *et al.*, 1992; Lorca *et al.*, 1992; Stewart *et al.*, 1994) and stabilizes the mitotic cyclin Clb2p in budding yeast (Amon *et al.*, 1994). The destruction box is believed to serve as a signal for ubiquitination, because mutation of the conserved arginine also inhibits the ubiquitination of cyclin N-terminal fragments or fusion proteins in crude *Xenopus* extracts (Glotzer *et al.*, 1991) or in semipuri-

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fied systems (King *et al.*, 1995; Sudakin *et al.*, 1995). However, apart from the conserved arginine, the functional role of other residues within the 9- or 10-amino-acid region of sequence conservation has not been defined.

It is of particular interest to compare the sequence requirements for the proteolysis of A- and B-type cyclins, because there are two important physiological differences in the timing and regulation of their degradation (Minshull *et al.*, 1990; Whitfield *et al.*, 1990; Pines and Hunter, 1991; Hunt *et al.*, 1992). Cyclin A is degraded at metaphase, whereas cyclin B proteolysis does not commence until anaphase, and only cyclin B is stabilized in response to agents such as nocodazole that disrupt the integrity of the mitotic spindle. The sequences within cyclin A and cyclin B responsible for their distinct proteolytic properties have not been defined but could involve the destruction box itself, which varies consistently between A- and B-type cyclins at several positions. Alternatively, sequences outside the destruction box may be important for the differential timing and regulation of A- and B-type cyclin destruction.

Although the destruction box is essential for ubiquitination and degradation of mitotic cyclins, it cannot be sufficient signal for ubiquitin-dependent proteolysis, because it does not contain a conserved lysine residue capable of accepting ubiquitin. This function is probably fulfilled by lysine residues found in the non-conserved regions neighboring the destruction box, but the sites of ubiquitination have not been identified. Therefore, it is unclear whether there is a strict requirement for a particular lysine residue to act as a ubiquitin acceptor site or whether multiple lysine residues can fulfill this function.

For certain subtypes of mitotic cyclins, there may be additional degradation determinants located outside the N terminus. Although an N-terminal fragment of sea urchin (*Arbacia punctulata*) cyclin B is ubiquitinated and degraded in mitotic *Xenopus* extracts (Holloway *et al.*, 1993; King *et al.*, 1995), fragments of similar length derived from *Xenopus* cyclins A1 or B2 are stable (van der Velden and Lohka, 1993; Stewart *et al.*, 1994). Furthermore, point mutations in *Xenopus* cyclins A1 or B2 that inhibit their binding to p34^{cdc2} also inhibit proteolysis, suggesting that these proteins must be bound to p34^{cdc2} to be recognized by the cyclin destruction machinery (Stewart *et al.*, 1994; van der Velden and Lohka, 1994). Such mutations do not generally interfere with ability of *Xenopus* cyclin B1 to be degraded (Stewart *et al.*, 1994). It remains unclear why only certain types of cyclins exhibit an apparent requirement for p34^{cdc2} binding for their proteolysis.

Many of the components involved in destruction box-dependent ubiquitination have recently been identified, including two unregulated ubiquitin-conjugating enzymes, UBC4 (King *et al.*, 1995) and UBCx,

which is a homologue of clam E2-C (Aristarkhov *et al.*, 1996; Yu *et al.*, 1996). Cyclin ubiquitination is regulated through the mitotic activation of a destruction box-dependent ubiquitin ligase (E3) activity that is associated with a large protein complex called the Anaphase-Promoting Complex (APC) (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995) or the cyclosome (Sudakin *et al.*, 1995). Together with the ubiquitin-activating enzyme E1, these components catalyze the polyubiquitination of cyclin B, which targets the protein for rapid proteolysis by the 26S proteasome. APC contains at least eight distinct proteins, two of which, Cdc16p and Cdc23p, are essential for cyclin proteolysis and ubiquitination in budding yeast (Irniger *et al.*, 1995; Zachariae and Nasmyth, 1996). However, the mechanism of cyclin ubiquitination remains elusive, as does the identity of the APC subunit(s) involved in substrate recognition. In addition to its role in cyclin ubiquitination, APC function is also required for anaphase (Irniger *et al.*, 1995; Tugendreich *et al.*, 1995), probably by catalyzing the ubiquitination of unidentified destruction box-containing proteins that regulate sister chromatid cohesion (Holloway *et al.*, 1993). Defining the sequences in cyclin necessary for its degradation may therefore aid in the identification of novel substrates of the APC-dependent ubiquitination pathway.

MATERIALS AND METHODS

Construction of Cyclin Derivatives

All constructs for the expression of cyclin fragments and fusion proteins were created in pET expression vectors (Novagen, Madison, WI) that contained a T7 RNA polymerase promoter. This enabled expression in vitro with T7 RNA polymerase or in a suitable *Escherichia coli* host that expresses T7 RNA polymerase, such as BL21 (DE3; Studier and Moffatt, 1986). Plasmids expressing the *Arbacia* cyclin B derivatives 13-91prA, 13-66prA, and 13-53prA were previously described (Glotzer *et al.*, 1991).

Derivatives of the *Arbacia* substrates 13-66prA and 13-110 were generated by using PCR-mediated mutagenesis and confirmed by automated DNA sequencing. Details of construction, including the sequences of oligonucleotides used for mutagenesis, can be obtained from R.W.K. Construct 40-66prA contains methionine and histidine residues before residue 40 of *Arbacia* cyclin. The CS1 derivative of 13-66prA was generated by PCR with a 3' oligo that inserted the amino acids IEGR at the junction between cyclin and protein A. The CS2 derivative of 13-66prA was generated by PCR mutagenesis that altered residues 54–58 of cyclin (TAQAG mutated to IEGR). The 13-110 fragment used in this study differs from that published previously (Holloway *et al.*, 1993), because it contains no leader peptide.

N-terminal fusions of *Xenopus* cyclin B1 or *Xenopus* cyclin A1 to protein A were constructed by polymerase chain reaction (PCR) amplifying the appropriate region from pGEM vectors carrying the cDNAs for the corresponding cyclins (gift of J. Minshull, University of California, San Francisco, CA). These fragments were cut with *Nsi*I and *Bss*HI and used to replace the corresponding fragment from 13-66prA. Constructs encoding the amino terminal 102 amino acids of *Xenopus* cyclin A1 or *Xenopus* cyclin B1 were created in a similar manner but subcloned into the pET 11c vector (Novagen). Each of these constructs contained a hexahistidine tag at the C terminus.

Expression of Substrates

For expression in reticulocyte lysate, supercoiled plasmid DNA was used to program TNT reticulocyte lysate (Promega, Madison, WI) in the presence of [35 S]-methionine (New England Nuclear, Boston, MA) according to the manufacturer's instructions. Proteins were translated for 2 h at 30°C. Cycloheximide was added to 100 μ g/ml, and aliquots were snap frozen in liquid nitrogen. Protein A fusions and cyclin N-terminal fragments were expressed in *E. coli* and purified and iodinated as described (Glötzer *et al.*, 1991; Holloway *et al.*, 1993).

Preparation of Extracts and Degradation Assays

Interphase *Xenopus* extracts were prepared as described (Murray, 1991), except that eggs were activated with the calcium ionophore A23187 (free acid form; Calbiochem, La Jolla, CA) at a concentration of 1 μ g/ml. Eggs were crushed by centrifugation 50 min after activation. Cycloheximide was added to 100 μ g/ml, and extracts were frozen in the presence of 200 mM sucrose. To activate cyclin degradation, we added bacterially expressed *Arbacia* cyclin B lacking its N-terminal 90 amino acids (Glötzer *et al.*, 1991) to thawed interphase extracts at a final concentration of 60 μ g/ml. Extracts were incubated at 23°C for 40 min to activate the cyclin degradation system and then placed on ice. We have found that cyclin degradation is more rapid and more reproducible in extracts that are prepared at later times after egg activation. In general, half-lives were reduced approximately twofold by using extracts prepared in this manner. Cyclin degradation remains dependent on addition of Δ 90 protein in these extracts. We have also found that a single cycle of freeze-thawing an extract increases the half-life of cyclin proteins approximately twofold.

For degradation assays, we mixed 2 μ l of TNT reticulocyte lysate with 18 μ l of interphase or mitotic *Xenopus* extract containing 0.5 mg/ml bovine ubiquitin. Aliquots of 2 μ l were quenched with 18 μ l of SDS-sample buffer (63 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 100 mM dithiothreitol [DTT]). Samples were boiled and separated on 12% SDS-polyacrylamide gels. Gels were dried and phosphorimaged overnight with a Fuji phosphor-imager. Half-lives were determined by fitting an exponential curve to the quantitated data. Degradation assays were repeated in a minimum of three independent experiments. The data reported are from a single experiment that demonstrated the fastest half-lives. We found that the degradation rates with 35 S-labeled substrates were comparable to those that used the bacterially expressed and iodinated counterparts (our unpublished results).

Preparation of Methylated Ubiquitin

Bovine ubiquitin (Sigma Chemical, St. Louis, MO) was reductively methylated as previously described (Hershko and Heller, 1985) and was dialyzed against XB buffer containing (in mM) 10 HEPES, pH 7.7, 100 KCl, 0.1 CaCl₂, 1 MgCl₂, and 50 sucrose. The protein was concentrated to 15 mg/ml with a Centricon 3 concentrator and stored at -70°C.

Purification of Hexahistidine-tagged Conjugates and Cleavage with Factor Xa Protease

Hexahistidine (his)-tagged ubiquitin was the gift of J. Callis (University of California, Davis, CA). To generate conjugates for cleavage, 60 ng (5 μ l) of iodinated substrate ($\sim$ 100 μ Ci/ μ g) was added to 25 μ l of mitotic *Xenopus* extract in the presence of 20 μ l of his-tagged ubiquitin (8 mg/ml). Reactions were incubated at room temperature for 20 min and quenched by addition of 1 ml of quench buffer (50 mM N-ethylmaleimide in 50 mM Tris, pH 8, and 100 mM NaCl). Nickel agarose beads were added (100 μ l), and the mixture was incubated for 15 min at room temperature with rotation. The beads were pelleted in a microcentrifuge and washed three times by adding 1 ml of quench buffer and washed twice in quench buffer

containing 500 mM NaCl but lacking N-ethylmaleimide. Conjugates were eluted by adding 200 μ l of elution buffer containing (in mM) 50 Tris pH 8.0, 100 NaCl, and 50 EDTA and incubating for 15 min at room temperature. The beads were pelleted, and the supernatant was retained. Approximately 70% of the counts eluted from the beads under these conditions. Acetylated bovine serum albumin (BSA) was added to the eluted conjugates, and the proteins were dialyzed into cleavage buffer containing (in mM) 20 Tris, pH 8, 100 NaCl, and 2 CaCl₂. Cleavage reactions were performed in a total of 40 μ l and contained 0, 100 ng, 500 ng, or 1.5 μ g of protease and $\sim$ 7200 cpm of conjugates. Reactions were incubated for 3 h at room temperature and quenched by addition of an equal volume of SDS-sample buffer. Samples were analyzed on 5–15% gradient polyacrylamide gels, followed by autoradiography.

Isolation of Ubiquitin Conjugates and Cleavage by Cyanogen Bromide

For cleavage of conjugates with cyanogen bromide, a mutant version of 13-66prA was used in which alanine 57 of the cyclin sequence was mutated to methionine. The single methionine present in protein A was mutated to alanine so that the only internal methionine was provided by the newly engineered methionine. To generate conjugates, the iodinated protein (0.1 μ g, $\sim$ 100 μ Ci/ μ g) was incubated with 225 μ l of concentrated high-speed supernatant derived from a mitotic extract in the presence of 1 mg/ml bovine ubiquitin. The reaction was incubated 20 min at room temperature, and the reaction was quenched with 3.2 ml of SDS-sample buffer. The sample was boiled, and 880 μ l was loaded on each of four 11 $\times$ 14 cm 5–15% polyacrylamide gels in a single gel-width lane. After electrophoresis, the sample was transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) with a semi-dry blotter (BRL, Bethesda, MD). After drying, the filters were exposed to X-ray film for 4 h at room temperature. Using the autoradiograph as a guide, we obtained slices of filter that corresponded to nonubiquitinated, monoubiquitinated, diubiquitinated, and higher molecular mass cyclin-ubiquitin conjugates. To elute conjugates, nitrocellulose filter strips were incubated for 24 h at 37°C with 1 ml of 1.8 M DTT in the presence of 1% Triton X-100 in a 1.5-ml tube. This procedure elutes $\sim$ 50% of the counts and reduces methionine sulfoxides generated during oxidative iodination and electrophoresis. This reduction step is essential for efficient cleavage by cyanogen bromide. BSA (50 μ g) was added, and the sample was dialyzed against H₂O. The sample was then concentrated to 100 μ l with a Centricon 10 (Amicon, Beverly, MA) ultrafiltration unit. The sample was resuspended in 2 ml H₂O and reconcentrated to 100 μ l. Fifty microliters of this solution were then incubated with either 70% formic acid or 70% formic acid plus 30 mg/ml cyanogen bromide (Sigma). The sample was incubated overnight in the dark. The sample was then evaporated under vacuum, resuspended in 1 ml H₂O, and evaporated again to remove residual acid. The residue was resuspended in SDS-sample buffer, boiled, and analyzed by electrophoresis on 5–15% gradient gels followed by autoradiography.

RESULTS

To examine the sequence requirements for mitotic cyclin destruction, we have used the N-terminal domains of both *Xenopus* and sea urchin (*Arbacia punctulata*) cyclin B. The latter proved advantageous for mutagenic studies, because it contained a lower content of lysine residues (see below). Previous work has demonstrated that the N terminus of *Arbacia* cyclin B is sufficient to confer cell-cycle-specific destruction to a heterologous protein, *Staphylococcus aureus* protein A, in mitotic *Xenopus* extracts (Glötzer *et al.*, 1991).

Furthermore, the N terminus of *Arbacia* cyclin B is a sufficient substrate when expressed independently (Holloway *et al.*, 1993). We have avoided the use of full-length cyclins in this study, because point mutations outside the N terminus sometimes interfere with the ability of full-length cyclins to be degraded (Stewart *et al.*, 1994; van der Velden and Lohka, 1994).

We first tested whether the N termini of *Xenopus* cyclins A1 and B1 contain a destruction signal that is sufficient for mitosis-specific degradation in *Xenopus* extracts. [³⁵S]-methionine-labeled substrates were synthesized in reticulocyte lysate, and degradation rates were measured after addition of a ninefold excess of interphase or mitotic *Xenopus* extract. To compare with previous experiments, we also tested the degradation of the *Arbacia* substrates in these extracts. Figure 1A demonstrates that the N-terminal 13-91 amino acid of *Arbacia* cyclin B was sufficient to target protein A for destruction specifically in mitotic extracts; a similar result was obtained when the independently expressed fragment containing residues of 13-110 was tested. The half-life of each of these proteins was <5 min, similar to the degradation rate of full-length cyclins observed in vivo (Hunt *et al.*, 1992). Figure 1B shows that the behavior of the *Xenopus* B1 N terminus was indistinguishable from that of the *Arbacia* N terminus, because residues 3-86 of *Xenopus* cyclin B1 could efficiently target protein A for destruction in mitosis; a fragment consisting of the N-terminal 102 residues of *Xenopus* cyclin B1 was degraded in a similar manner. Each of these proteins was stable in an interphase extract (Figure 1B). However, a fusion protein containing residues 3-91 of *Xenopus* cyclin A1 was stable in both mitotic and interphase extracts (Figure 1C). The same result was obtained with an independently expressed fragment containing the N-terminal 102 residues of cyclin A. This finding confirms previous work showing that an N-terminal fragment of *Xenopus* cyclin A1 is not degraded in mitotic *Xenopus* extracts (Stewart *et al.*, 1994). Because full-length cyclin A is unstable in these extracts (our unpublished results), the defect in cyclin A1 proteolysis is not due to lack of a functional cyclin A degradation system. Instead, we conclude that the N terminus of *Xenopus* cyclin A1 either does not have a sufficient destruction signal or that the protein folds abnormally and therefore cannot be recognized by the degradation machinery.

Previous work has shown that an N-terminal fragment consisting of the first 89 residues of *Xenopus* cyclin B2 is not degraded in mitotic extracts (van der Velden and Lohka, 1993). We tested the degradation of a similar fragment consisting of the first 102 residues of *Xenopus* cyclin B2. We found that this protein was degraded with a half-life of ~20 min in mitotic extracts but was stable in interphase extracts (our unpublished results). Because *Xenopus* cyclin B1 and cy-

Figure 1. The N terminus of cyclin B is sufficient for degradation, whereas the N terminus of cyclin A is not. Constructs encoding N-terminal fragments of cyclin or N-terminal fusion proteins with protein A (prA) were expressed in reticulocyte lysate and labeled with [³⁵S]-methionine. Lysates were then mixed with a ninefold volume excess of interphase or mitotic *Xenopus* extract. Aliquots were taken at 0, 5, 10, 20, and 40 min after addition of substrate and analyzed by gel electrophoresis and phosphorimaging. (A) *Arbacia punctulata* cyclin B derivatives. (B) *Xenopus laevis* cyclin B1 derivatives. (C) *Xenopus laevis* cyclin A1 derivatives. (D) Alignment of the first 120 amino acids of *Arbacia* cyclin B (A.p. B), *Xenopus* cyclin B1 (X.l. B1), and *Xenopus* cyclin A1 (X.l. A1). The destruction box is indicated in black, and lysine residues are shown in bold type.

clin B2 have very similar destruction boxes (Figure 3), we conclude that the relative defect in cyclin B2 proteolysis stems from differences in sequences outside the destruction box.

Deletion Analysis of the Cyclin B N Terminus

To define a minimal sequence element in cyclin B sufficient to target a heterologous protein for destruction in mitosis, we made a series of deletion mutations in the *Arbacia* cyclin B-protein A substrates. We first analyzed the requirement for sequences C-terminal to the destruction box, which spans residues 42-50. Deletion of residues 67-91 (yielding substrate 13-66prA)

increased the half-life from <5 to 10 min. Further deletion of residues 54–67 (yielding substrate 13-53prA) increased half-life to 25 min. We next tested the effect of removing sequences N-terminal to the destruction box. Deletion of residues 13–39 from 13-66prA (yielding 40-66prA) increased half-life from 10 to 20 min. In contrast, deletion of the destruction box from 13-66prA increased the half-life to >60 min (Figure 2A). All of the above derivatives were stable in interphase extracts (our unpublished results). These experiments indicate that a portion of cyclin B as small as 27 amino acids that contains the destruction box is sufficient to destabilize a heterologous protein. However, physiological rates of destruction seem to require the larger N-terminal domain.

Similar results were obtained when the N terminus of *Xenopus* cyclin B1 was analyzed in analogous manner (Figure 2B). A substrate containing residues 3–86 of cyclin B1 fused to protein A was degraded with a half-life of <5 min in mitotic extracts. As expected, this substrate (and those discussed below) was stable in interphase extracts (our unpublished results). A fusion protein containing residues 31–67 of cyclin B1 was degraded with a half-life of 10 min (the destruction box spans residues 36 through 44 in *Xenopus* cyclin B1), suggesting that residues upstream of the destruction box are not essential. However, residues 31–55 of *Xenopus* cyclin B1 were not sufficient to target protein A for destruction (Figure 2B). Deletion of a similar region (residues 54–66) from the *Arbacia* substrate also resulted in significant, although incomplete, stabilization. Although there is no strictly conserved sequence element in this region, this sequence is rich in lysine residues that may act as ubiquitin acceptor sites. Alternatively, these sequences may be essential for proper recognition of the destruction box by the components of the cyclin ubiquitination system. We distinguish between these possibilities below.

The Cyclin A Destruction Box Is Not Efficiently Recognized in the Context of the Cyclin B N Terminus

The experiments presented in Figure 1 indicate that the N terminus of *Xenopus* cyclin A1 does not contain a signal that is sufficient for destruction in mitosis. Although A-type cyclins contain a conserved destruction box that is essential for proteolysis (Kobayashi *et al.*, 1992; Lorca *et al.*, 1992; Stewart *et al.*, 1994), A-type destruction boxes differ from B-type destruction boxes at several positions, suggesting that such sequence variation could result in distinct proteolytic properties. Figure 3 shows an alignment of the destruction box sequences from mitotic cyclins that span a wide phylogenetic distance. Although A- and B-type cyclins share a conserved arginine and leucine at positions one and four of the destruction box, respectively, the

Figure 2. Identification of a minimal destruction element in *Arbacia* cyclin B and *Xenopus* cyclin B1. Constructs encoding N-terminal fusion proteins of either (A) *Arbacia* cyclin B or (B) *Xenopus* cyclin B1 with protein A were tested for their ability to be degraded in mitotic *Xenopus* extracts. A schematic diagram of each construct is shown; the gray rectangle represents the destruction box, whereas the black rectangle represents protein A. Only the cyclin portion is drawn at proper scale; protein A is, in fact, fourfold larger than the cyclin sequence. Lysine residues within the cyclin portion of the protein are indicated with black dots. Proteins were expressed in reticulocyte lysate and mixed with a ninefold excess of mitotic extract. Samples were taken at 0, 10, 20, 40, and 60 min and analyzed by gel electrophoresis and phosphorimaging. Half-lives were determined by quantitative phosphorimager analysis.

C-terminal half of the destruction box differs consistently. B-type cyclins contain asparagine, aspartic acid, or glutamic acid at position six, whereas valine and threonine predominate in A-type cyclins. Furthermore, asparagine at position nine is highly conserved only among B-type cyclins. In some cases, those A-type cyclins that do not contain asparagine at position nine contain asparagine at position 10 or 11. Thus, whereas B-type destruction boxes seem to consist of nine residues, the A-type destruction box may extend to 10 or more residues.

Alternatively, sequences outside the destruction box could be important determinants of differences in recognition of A- and B-type cyclins by the destruction machinery. For example, the N termini of B-type cyclins are enriched in lysine residues compared with the N termini of A-type cyclins. Thus the defect in the ability of cyclin A N-terminal fragments to be degraded could stem from a lack of a proper ubiquitin acceptor site.

To test whether the destruction box itself contributes to the defect in the ability of the cyclin A N-terminal fragment to be degraded, we have made a series of chimeric proteins in which the destruction box of the

B-type cyclins	1	2	3	4	5	6	7	8	9	10	11	POS.
<i>S. pombe</i> cdc13	R	H	A	L	D	D	V	S	N			59
<i>S. cerevisiae</i> Clb2	R	L	A	L	N	N	V	T	N			25
<i>A. nidulans</i> nimE	R	A	A	L	G	D	V	S	N			52
<i>A. thaliana</i> cyc1	R	Q	V	L	G	D	I	G	N			38
<i>D. melanogaster</i> B	R	A	A	L	G	D	L	Q	N			37
<i>A. punctulata</i> B	R	A	A	L	G	N	I	S	N			42
<i>S. solidissima</i> B	R	N	T	L	G	D	I	G	N			40
<i>X. laevis</i> B1	R	T	A	L	G	D	I	G	N			36
<i>X. laevis</i> B2	R	A	A	L	G	E	I	G	N			30
<i>G. gallus</i> B2	R	A	V	L	E	E	I	G	N			32
<i>G. gallus</i> B3	R	S	A	F	G	D	I	T	N			51
<i>H. sapiens</i> B1	R	T	A	L	G	D	I	G	N			42

A-type cyclins	1	2	3	4	5	6	7	8	9	10	11	POS.
<i>D. melanogaster</i> A	R	S	I	L	G	V	I	Q	S			160
<i>S. solidissima</i> A	R	A	A	L	G	V	I	T	N			33
<i>X. laevis</i> A1	R	T	V	L	G	V	I	G	D	N		41
<i>X. laevis</i> A2	R	T	V	L	G	V	L	Q	E	N		26
<i>G. gallus</i> A	R	A	A	L	G	T	V	G	E			93
<i>M. musculus</i> A1	R	T	V	L	G	V	L	T	E	N		37
<i>H. sapiens</i> A	R	A	A	L	A	V	L	K	S	G	N	47

Figure 3. Alignment of the destruction boxes of mitotic cyclins. The sequences of putative destruction boxes from A- and B-type cyclins are shown. The position of the first residue of the destruction box is also indicated. B-type destruction boxes show the highest degree of conservation at positions one, four, and nine. A-type destruction boxes show strong conservation only at positions one and four. A- and B-type cyclins differ consistently at position six: asparagine, aspartate, and glutamate predominate in B-type cyclins, whereas this residue is valine or threonine in A-type cyclins.

Arbacia cyclin B substrate 13-66prA has been replaced with the destruction box of other cyclins. We first tested the ability of other B-type destruction boxes to substitute for the *Arbacia* destruction box. Figure 4 indicates that the *Xenopus* cyclin B1 destruction box could substitute efficiently for the *Arbacia* version, because the resulting protein was degraded with a half-life of 10 min. We next tested whether the destruction box of a more distantly related cyclin, that of *S. cerevisiae* Clb2p, could substitute for the *Arbacia* destruction box. Figure 4 shows that the hybrid protein was degraded as rapidly as the *Arbacia* protein in mitotic extracts, suggesting that the components that recognize the cyclin B destruction box are functionally conserved between *Xenopus* and budding yeast.

These experiments demonstrate that *Xenopus* extracts can efficiently degrade substrates containing a variety of B-type destruction boxes. We next tested whether the destruction box derived from *Xenopus* cyclin A1 could replace the *Arbacia* destruction box. As mentioned above, it is not clear whether the *Xenopus* A1 destruction box consists of 9 or 10 amino acids, so both substitutions were made. Figure 4 shows that neither the 9-amino-acid nor 10-amino-acid *Xenopus* A1 destruction boxes could functionally replace the *Arbacia* sequence. We performed further mutagenesis

of the *Arbacia* destruction box to determine which residues were responsible for this defect. There are three amino acid differences between the *Xenopus* B1 destruction box, which works efficiently in this context, and the *Xenopus* A1 destruction box, which does not. Position three is alanine in cyclin B1, but valine in cyclin A1. This is unlikely to cause the defect because valine is found at this position in other B-type cyclins, such as chicken cyclin B2 (Figure 3). Position six of the destruction box, however, differs consistently between A- and B-type cyclins. We therefore mutated the asparagine at position six of the *Arbacia* destruction box to valine, which is found in *Xenopus* cyclin A1. Figure 4 indicates that this point mutation increased the half-life of the protein from 10 to 25 min. However, the mutation was not completely stabilizing, in contrast to the protein containing the complete cyclin A1 destruction box. Position nine of the destruction box is a highly conserved asparagine in B-type cyclins; however, this position is not highly conserved among A-type cyclins. Figure 4 demonstrates that mutation of

Substrate	D-Box Sequence	Time (min)					Half-life (min)
		0	10	20	40	60	
13-66prA	RAALGNISN						10
XI B1 DB	RTALGDIGN						10
Clb2 DB	RLALNNVTN						10
XI A1 DB-9	RTVLGVIGD						>60
XI A1 DB-10	RTVLGVIGDN						>60
N47V	RAALGVISN						25
N50D	RAALGNISD						>60
N50A	RAALGNISA						>60
R42A	AAALGNISN						>60
L45A	RAAAGNISN						>60

Figure 4. Mutagenic analysis of the cyclin destruction box. A series of derivatives of the *Arbacia* substrate 13-66prA were constructed. The residues that differ from the sequence of wild-type *Arbacia* cyclin are underlined. Substrates XI B1 DB, Clb2 DB, and XI A1 DB contain precise replacements of the destruction box of 13-66prA with the corresponding sequences from *Xenopus* cyclin B1, *S. cerevisiae* Clb2p, or *Xenopus* cyclin A1, respectively. The numbering of substrates N47V, N50A, R42A, and L45A corresponds to their position in the *Arbacia* cyclin B sequence. Each of these substrates was expressed in reticulocyte lysate, mixed with mitotic extract, and analyzed by gel electrophoresis and phosphorimaging.

asparagine to alanine or to aspartate, which is found in *Xenopus* cyclin A1, alone is sufficient to stabilize a protein containing an otherwise wild-type cyclin B destruction box. These experiments suggest that differences at positions six and nine, but especially the latter, can account for the inability of the cyclin A1 destruction box to replace its B-type counterpart.

We also tested the effect of single-point mutations at the other highly conserved positions of the destruction box. Previous work has shown that conversion of arginine at position one of the destruction box to cysteine inhibits degradation (Glotzer *et al.*, 1991) as does conversion to alanine (Figure 4). Mutation of leucine at position four to alanine also stabilized the protein. It is interesting that a relatively conservative mutation at this position had such a strong effect on degradation. The presence of phenylalanine at this position in chicken cyclin B3 (Gallant and Nigg, 1994) indicates that perhaps only bulky hydrophobic residues are tolerated at this position.

Polyubiquitination Correlates with Degradation and Is Essential for Proteolysis

To establish that sequences in the destruction box function to target cyclin for ubiquitination, we tested a subset of the mutant proteins described above for their ability to be ubiquitinated in mitotic *Xenopus* extracts. The substrates were expressed in *E. coli*, purified with IgG-sepharose affinity chromatography, and radiolabeled with ^{125}I . This labeling method produces substrates of much higher specific activity than can be obtained by in vitro translation and therefore facilitates the detection of ubiquitinated intermediates. However, the formation of cyclin-ubiquitin conjugates is not an artefactual result of the oxidative iodination procedure, because high molecular mass conjugates of in vitro translated cyclin can also be observed by autoradiography after long exposure times (our unpublished results). Figure 5A shows that mutation of the conserved positions of the destruction box resulted in a decreased level of ubiquitination. The ubiquitination of the L45A and N50A mutants was almost completely abolished, consistent with their stability in mitotic extracts. The R42A mutant was ubiquitinated approximately fourfold less efficiently than wild type, with the defect preferentially affecting the formation of conjugates of high molecular mass. These data suggest that a threshold level of ubiquitination may be necessary for rapid proteolysis, because this mutant was stable in mitotic extracts (Figure 4). Mutation of asparagine to valine at position six of the destruction box (N47V) produced a similar defect in the formation of high molecular mass conjugates. However, this protein was still degraded, albeit at a slower rate than wild type. The *Arbacia* substrates containing the *Xenopus* B1 or yeast Clb2p destruction

Figure 5. Polyubiquitination correlates with degradation and is essential for proteolysis. (A) Ubiquitination of destruction box mutants. Derivatives of *Arbacia* 13-66prA were expressed in *E. coli*, purified, and radioiodinated. Labeled proteins were added to mitotic *Xenopus* extracts at a final concentration of 40 nM and incubated for 15 min before quenching with SDS-sample buffer. Samples were analyzed on 5–15% gradient polyacrylamide gels and phosphorimaged. The band labeled "contaminant" is a protein that copurifies with the protein A fusions. The amount of this contaminant varies from preparation to preparation. (B) Polyubiquitination is essential for cyclin degradation. Bacterially expressed and iodinated N-terminal fragment of *Arbacia* cyclin 13-110 was added to a mitotic *Xenopus* extract in the presence or absence of 120 μM methylated ubiquitin. Samples were taken at 0, 10, 20, and 30 min after substrate addition and analyzed by gel electrophoresis and autoradiography. A small amount of ubiquitin conjugation was present at the 0 min time point because of the brief delay between addition of the substrate and quenching of the reaction (~20 sec).

box were efficiently ubiquitinated; a protein containing the 9-amino-acid *Xenopus* cyclin A1 destruction box was not. The good correlation between the amount of high molecular mass conjugates and the rate of proteolysis of the mutants provides further evidence for the hypothesis that the primary function

of the destruction box is to target the protein for ubiquitination.

Hershko and colleagues have previously demonstrated that methylated ubiquitin, an inhibitor of polyubiquitin chain formation, can slow the degradation of both A- and B-type cyclins in clam embryo extracts (Hershko *et al.*, 1991). However, because of the labeling techniques used, ubiquitinated intermediates could not be observed. We therefore tested whether methylated ubiquitin could inhibit the proteolysis of the iodinated *Arbacia* N-terminal fragment 13-110. We analyzed the pattern of cyclin-ubiquitin conjugates that accumulated in the presence of 120 μ M methylated ubiquitin. In the absence of methylated ubiquitin, high molecular mass cyclin-ubiquitin conjugates were transiently observed during the course of degradation (Figure 5B). In the presence of methylated ubiquitin, the substrate instead accumulated as mono-, di-, and triubiquitinated species; higher molecular mass forms were not observed. The substrate was stabilized in the presence of methylated ubiquitin, indicating directly that polyubiquitin chain formation is essential for cyclin degradation. The appearance of di- and triubiquitinated species under these conditions suggests that ubiquitin is coupled to at least three distinct lysine residues within the cyclin N terminus.

Characterization of Ubiquitinated Cyclin B Intermediates

The ubiquitination of a substrate requires the presence of at least one lysine residue that can serve as a ubiquitin acceptor site (Bachmair and Varshavsky, 1989). We expected that sequences neighboring the destruction box in the cyclin N terminus could function as potential ubiquitin acceptor sites, especially because B-type cyclin N termini are relatively lysine-rich. To determine the sites of ubiquitin conjugation in the cyclin B N terminus, we used the *Arbacia* substrate 13-66prA as a model, because *Arbacia* cyclin B contains five fewer lysines in its N-terminal 100 residues than *Xenopus* cyclin B1 (Figure 1D). We constructed two derivatives of 13-66prA that contained a Factor Xa protease cleavage site either at the cyclin-protein A junction (Cleavage Substrate 1 [CS1]) or just downstream of the destruction box (Cleavage Substrate 2 [CS2]; Figure 6A). These mutations had no effect on the ability of these proteins to be degraded or ubiquitinated in mitotic extracts (our unpublished results). These fusion proteins were expressed in *E. coli* and purified with IgG-Sepharose affinity chromatography. Figure 6B shows that cleavage of unlabeled CS1 produced a C-terminal fragment of the expected size of 20 kDa, whereas cleavage of CS2 produced a slightly larger product, indicating that cleavage occurred at the introduced site. The wild-type protein and the two derivatives CS1 and CS2 were radioiodinated with

chloramine T. Because protein A is the only portion of these fusion proteins that contains tyrosine, 125 I is incorporated exclusively into protein A. Therefore, only the C-terminal fragment of CS1 and CS2 should retain the label after cleavage. We then tested whether cleavage of the ubiquitinated forms of these substrates resulted in the loss of ubiquitin from the substrate or whether the ubiquitin was retained on the labeled C-terminal fragment. We expected that if most of the ubiquitin were conjugated to one of the three N-terminal lysine residues, then protease cleavage of ubiquitin conjugates of either CS1 or CS2 should produce a uniform low molecular mass fragment that contained no ubiquitin. Alternatively, if all of the ubiquitin were conjugated to protein A, then cleavage of either ubiquitinated substrate should produce a smear of high molecular mass species, because the C-terminal fragment would retain ubiquitin. Finally, if ubiquitin were conjugated to the four lysines downstream of the destruction box, then cleavage of CS1 should produce a single fragment of low molecular mass, whereas cleavage of CS2 should produce a smear of high molecular mass fragments.

To isolate cyclin-ubiquitin conjugates, we added hexahistidine-tagged ubiquitin (Beers and Callis, 1993) to mitotic extracts in the presence of the iodinated 13-66prA derivatives. Ubiquitinated proteins were then purified from crude extracts with nickel affinity chromatography. Because cyclin is the only labeled protein present, only cyclin-ubiquitin conjugates are observed after autoradiography. This procedure allowed the isolation of ubiquitinated forms of cyclin with essentially no background of nonubiquitinated protein (Figure 6C). The purified conjugates were then cleaved by using varying amounts of Factor Xa protease. Because ubiquitin itself does not contain a Factor Xa cleavage site, the polyubiquitin chain and its linkage to the substrate should remain intact after treatment with protease (Chau *et al.*, 1989). This is illustrated in Figure 6C, which demonstrates that 13-66prA-ubiquitin conjugates that lacked a Factor Xa cleavage site were not efficiently cleaved by the protease. The small amount of 25- and 46-kDa bands produced probably results from nonspecific cleavage of cyclin at a site N-terminal to the CS2 cleavage site (as indicated by the fragment size). This is substantiated by the fact that these bands are not observed when conjugates of CS1 or CS2 are treated with protease. Therefore, this type of nonspecific cleavage should not interfere with the interpretation of the experiment.

Next, we cleaved ubiquitin conjugates of 13-66prA-CS1, the substrate that contains a Factor Xa site at the junction between cyclin and protein A. Cleavage produced two C-terminal fragments that correspond by molecular mass to nonubiquitinated protein A and monoubiquitinated protein A. Although a slight

amount of polyubiquitinated protein A is observed after cleavage, the bulk of the high molecular mass conjugates disappeared. This indicates that protease cleavage removed the majority of ubiquitin from the labeled C-terminal fragment, demonstrating that the major site of ubiquitination must lie N-terminal to the site of cleavage at the cyclin-protein A junction. However, this experiment demonstrates that a substantial proportion of the conjugates contain at least one ubiquitin molecule conjugated to protein A, as evidenced by the prominent monoubiquitinated C-terminal fragment that results from cleavage. Furthermore, the finding that a small amount of cleavage product retains its high molecular mass suggests that a small percentage of the substrate is polyubiquitinated on protein A.

A strikingly different result was obtained when conjugates of substrate 13-66prA-CS2 were cleaved with

protease (Figure 6C). In this case, the majority of cleaved conjugates retained a high molecular mass. The mass of the cleaved conjugates was shifted downward by ~5 kDa, equivalent to the mass of the N-terminal fragment removed. Cleavage was efficient, as demonstrated by the near-complete downward mobility shift of the monoubiquitinated species. This result indicates that the majority of the ubiquitin is conjugated to the C-terminal fragment that contains the lysine-rich region between the destruction box and protein A. These experiments using the two cleavable cyclin fusion proteins, when taken together, demonstrate that the majority of the ubiquitin must be linked to one or more of the four lysines that lies between the destruction box and protein A. Interestingly, cleavage of CS2 also yielded a product equivalent in size to the C-terminal fragment containing no ubiquitin. Because only ubiquitinated conjugates were used in the cleav-

Figure 6. Structural analysis of ubiquitinated intermediates with Factor Xa cleavage. (A) Schematic representation of cleavage derivatives of the *Arbacia* substrate 13-66prA. Each of these substrates is drawn to scale; the destruction box is indicated in black, lysine residues are indicated by black dots, and protein A is represented by the gray rectangle. The sites of cleavage in substrates CS1 and CS2 are indicated, along with the predicted sizes of the C-terminal cleavage fragments. (B) Cleavage of unlabeled CS1 (left panel) and CS2 (right panel). Substrates CS1 and CS2 were expressed in *E. coli*, purified by IgG-sepharose affinity chromatography, and treated with increasing amounts of Factor Xa protease for either 1.5 or 3 h. Samples were then separated by SDS-PAGE, and the gels were stained with Coomassie blue. The N-terminal fragment could not be detected on these gels, probably because it is not efficiently stained with Coomassie blue. (C) Proteolytic cleavage of cyclin-ubiquitin conjugates. *Arbacia* derivatives 13-66prA, 13-66prA-CS1, and 13-66prA-CS2 were expressed in *E. coli*, purified, and iodinated. These substrates were added to mitotic *Xenopus* extracts containing his-tagged ubiquitin. After a 20-min incubation, the reactions were quenched by addition of quench buffer containing *N*-ethylmaleimide that inactivates ubiquitin-conjugating enzymes and deubiquitinating enzymes. Conjugates were purified with nickel agarose beads, eluted, and cleaved for 3 h with varying amounts of Factor Xa protease. The C-terminal fragments produced by cleavage of substrate CS1 are indicated CTF-CS1, whereas those of substrate CS2 are indicated CTF-CS2.

age reaction, a minority of the conjugates must therefore contain ubiquitin exclusively N-terminal to the site of cleavage. Therefore, one or more of the three lysine residues N-terminal to the destruction box also functions as a ubiquitin acceptor site.

To confirm the finding that ubiquitin is conjugated to lysine residues both upstream and downstream of the destruction box and to characterize the distribution of ubiquitin moieties in intermediates that contained a discrete number of ubiquitins, we used an alternate method of purifying and cleaving conjugates (Sokolik and Cohen, 1991). We generated a mutant derivative of 13-66prA that replaced alanine with methionine at position 57 of the cyclin sequence. We also converted the single methionine present in protein A to alanine. This yielded a substrate that contained only a single internal methionine located between the destruction box and the cluster of four lysine residues in cyclin (Figure 7A). Ubiquitin itself does not contain an internal methionine and thus is not susceptible to cleavage by cyanogen bromide (Sokolik and Cohen, 1991). The mutant substrate was ubiquitinated and degraded with normal kinetics in mitotic extracts (our unpublished results). Cyclin-ubiquitin conjugates were generated by adding the iodinated substrate to mitotic extract. Individual conjugates were isolated by cutting out specific bands that had been resolved by gel electrophoresis (see MATERIALS AND METHODS). Conjugates were then cleaved with cyanogen bromide and analyzed by gel electrophoresis and autoradiography. Figure 7B demonstrates that cleavage of the unconjugated substrate was ~80% complete and produced a labeled C-terminal cleavage fragment of the expected size. Cleavage of the monoubiquitinated substrate generated products that corresponded in mass to the nonubiquitinated C-terminal fragment and a monoubiquitinated fragment. This suggests that ubiquitin must be present both N- and C-terminal to the site of cleavage. Cleavage of a diubiquitinated conjugate produced nonubiquitinated, monoubiquitinated, and diubiquitinated C-terminal fragments, again indicating that ubiquitin is dispersed to either side of the cleavage site. Cleavage of conjugates containing either four or six to seven ubiquitin molecules produced similar patterns. Although this experiment was not designed to be quantitative, the data suggest that 30–40% of the substrate contains ubiquitin conjugated solely to one of the N-terminal three lysines. In contrast, the protease cleavage experiments indicated that perhaps 10% of the substrate contained ubiquitin conjugated solely upstream of the destruction box. This discrepancy may result from the different manner in which the conjugates were isolated. Nevertheless, both mapping techniques suggest that ubiquitin can be conjugated to multiple lysine residues distributed throughout the cyclin N terminus.

Figure 7. Identification of ubiquitination sites by using cyanogen bromide cleavage. (A) Schematic diagram of a mutant version of 13-66prA containing a single internal methionine residue at position 57 of the cyclin sequence. The predicted cleavage product is diagrammed, as well. The destruction box is depicted in black, and lysine residues are indicated by black dots. (B) Cyanogen bromide cleavage of isolated cyclin-ubiquitin conjugates. The substrate containing a single internal methionine was expressed in *E. coli*, purified, iodinated, and added to mitotic *Xenopus* extracts. The ubiquitination reactions were quenched in SDS-sample buffer, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose. An autoradiograph of the filter was made, and pieces of filter corresponding to a conjugates of uniform mass were excised. Conjugates were eluted from the filter strips in the presence of DTT to reduce methionine sulfoxides generated during iodination or electrophoresis. The conjugates were dialyzed and then treated either with 70% formic acid (uncut lanes, U) or with formic acid plus cyanogen bromide (cut lanes, C), and the products were analyzed by electrophoresis and autoradiography. The filter fragments excised corresponded to nonubiquitinated cyclin (Cyc), monoubiquitinated cyclin (Cyc-Ub), diubiquitinated cyclin (Cyc-Ub₂), tetraubiquitinated cyclin (Cyc-Ub₄), and a mixture of higher mass forms (CycUb_{6,7}). The unmodified and ubiquitinated C-terminal cleavage fragments are indicated on the right (CTF).

No Single Lysine Residue Is Essential for Degradation

Deletion of sequences N-terminal or C-terminal to the destruction box of *Arbacia* cyclin B-protein A fusions resulted in partial stabilization (Figure 2). This stabilization could stem either from the loss of a ubiquitin acceptor site or from the deletion of a sequence necessary for recognition by the ubiquitination machinery. To distinguish between these possibilities, we mutated the lysine residues in the cyclin portion of *Arbacia* 13-66prA to nonubiquitinable arginine residues. We tested the ability of these mutant proteins, expressed in reticulocyte lysate, to be degraded in mitotic *Xenopus* extract. Figure 8A shows that a substrate lacking the three lysines N-terminal to the destruction box (13-66prA-R3) was still rapidly de-

Figure 8. Multiple lysine residues in the cyclin N terminus can serve as ubiquitin acceptor sites sufficient for rapid proteolysis. (A) Lysine residues in the cyclin portion of 13-66prA are not essential for degradation. Three mutants of the *Arbacia* substrate 13-66prA are shown. The destruction box extends from residues 42–50 and is depicted in gray; lysine residues are indicated by black dots. 13-66prA-R3 contains three lysine-to-arginine substitutions upstream of the destruction box. 13-66prA-R4 contains four lysine-to-arginine substitutions downstream of the destruction box. In construct 13-66prA-R7, all seven lysines in the cyclin portion of the fusion protein have been mutated to arginine. These substrates were expressed in reticulocyte lysate and tested for degradation in mitotic *Xenopus* extracts as described. (B) All 10 lysine residues in the *Arbacia* substrate 13-110 must be eliminated to stabilize the protein. The N-terminal fragment is drawn to scale with respect to the location of the destruction box (gray rectangle) and lysine residues (dots). Proteins were expressed in reticulocyte lysate, and degradation assays that used mitotic extracts were performed as described. The diffuse band present in all lanes is due to globin protein derived from the reticulocyte lysate.

graded in mitotic extracts. Surprisingly, a substrate lacking the four lysines downstream of the destruction box (13-66prA-R4) was degraded as rapidly as the wild-type protein. Even the elimination of all seven lysines in the cyclin portion of this fusion protein resulted in only a doubling of its half-life (13-66prA-R7; Figure 8A). These experiments are consistent with the mapping studies described above and indicate that

ubiquitin conjugation can occur at multiple sites throughout the substrate, including the protein A portion. Furthermore, conjugation at any of these sites seems sufficient to target the protein for destruction. The slower rate of proteolysis of 13-66prA-R7 is consistent with our finding that the majority of ubiquitin is normally conjugated to the cyclin portion of the fusion protein. Conjugation of ubiquitin to protein A in 13-66prA-R7 may not be so rapid as conjugation to cyclin, slowing the degradation of this mutant. Examination of the ubiquitination patterns of these mutants indicated that the R3 and R4 substrates were ubiquitinated as well as wild type, whereas the ubiquitination of the R7 mutant was only slightly diminished (our unpublished results).

To confirm the finding that no single lysine residue is essential for proteolysis, we measured the degradation rates of a series of lysine to arginine mutants constructed in the *Arbacia* cyclin N-terminal fragment 13-110. This substrate lacks the protein A moiety that could function as a heterologous ubiquitin acceptor site. This substrate contains 10 lysine residues (Figure 8B) that were converted to arginine in various combinations. Figure 8B demonstrates that the [35 S]-labeled wild-type substrate was rapidly degraded in mitotic extracts with a half-life of <5 min. A mutation of asparagine to alanine at position nine of the destruction box stabilized the protein, as was previously observed for the 13-66prA derivative. Independent elimination of the three N-terminal lysines (R3), the four lysines after the destruction box (R4), or the three C-terminal lysines (R3C) had no effect on half-life (Figure 8B). Similarly, elimination of the N-terminal seven lysines (R7), the C-terminal seven lysines (R7C), or the six external lysines (R6) had no effect on half-life, indicating that any single cluster of lysine residues was sufficient to support degradation (Figure 8B). Stabilization was achieved only when all 10 lysines were simultaneously converted to arginine (R10; Figure 8B). This experiment demonstrates that there is not a stringent requirement for a particular lysine residue to act as a ubiquitin acceptor site and is consistent with the finding that 13-66prA is ubiquitinated at multiple lysine residues distributed throughout the protein.

DISCUSSION

In this study we have examined the sequences in the N termini of mitotic cyclins that are required to target mitotic cyclins for ubiquitination and degradation during mitosis. In particular, we have demonstrated that a number of B-type cyclins have N termini that, when expressed independently or as fusion proteins, are subject to rapid, ubiquitin-mediated, cell-cycle-regulated proteolysis. We have used deletion analysis to define the sequences in the amino terminus that are

essential for polyubiquitination, and we have conducted a mutational analysis of the destruction box itself. Finally, we have localized the sites of attachment of ubiquitin to the cyclin fusion proteins.

Mutational Analysis of the Cyclin B Destruction Signal

Although the N-terminal 90 amino acids of *Xenopus* cyclin B1 and *Arbacia* cyclin B share only 37% percent sequence identity (with 25% of the identities consisting of lysine residues), each contains a degradation signal that is efficiently recognized in mitotic *Xenopus* extracts. The most highly conserved sequence feature in the cyclin B N terminus is the destruction box, which spans nine residues. Sequence comparison of cyclin B destruction boxes from diverse species indicates that only three residues of the destruction box—the arginine at position one, the leucine at position four, and the asparagine at position nine—are highly conserved. Our results show that each of these three positions is important for both the ubiquitination and degradation of cyclin–protein A fusion proteins. Although the degree of sequence conservation is much lower at other positions in the destruction box, certain trends can be identified. Position two is not highly conserved, and our experiments demonstrate that destruction boxes containing alanine, leucine, and threonine at this position are recognized equally well in *Xenopus* extracts. Position three is generally a small aliphatic residue such as alanine or valine; we did not specifically test the requirement for such a residue at this position. Glycine predominates at position five, but our data indicate that asparagine is also tolerated. The residues asparagine, aspartate, and glutamate dominate position six. Our results show that valine, which is often found at this position in A-type destruction boxes, does not efficiently support degradation in the context of a B-type destruction box. Position seven is frequently isoleucine or valine in both A- and B-type destruction boxes; both residues are recognized in *Xenopus* extracts. Position eight is only loosely conserved, and our data indicate that serine, glycine, and threonine are recognized equally well. Our experiments indicate there is no requirement for a specific phosphorylatable residue in the destruction box, because the *Xenopus* B1, *Arbacia*, and yeast Clb2p destruction boxes are all functional but do not contain a common phosphorylatable residue. It is therefore unlikely that phosphorylation of the D box is involved in the recognition process. This finding is consistent with previous work showing that phosphorylation of cyclin B is not essential for its degradation (Izumi and Maller, 1991; Li *et al.*, 1995).

A previous report showed that the *Xenopus* cyclin B2 N terminus was not degraded in *Xenopus* extracts (van der Velden and Lohka, 1993). We found that the *Xe-*

nopus B2 N terminus was degraded, albeit more slowly than the *Xenopus* cyclin B1 N terminus. Because the *Xenopus* cyclin B2 destruction box contains only two conservative changes from the *Xenopus* cyclin B1 destruction box (T to A at position two; D to E at position six), we find it unlikely that the destruction box of *Xenopus* cyclin B2 is responsible for its relative degradation defect. Instead, this may result from a deficiency in sequences outside the destruction box, although this remains to be tested directly.

Deletion analysis of the regions flanking the destruction box indicates that a cyclin B segment as short as 27 residues can target a heterologous protein for degradation in mitotic extracts. However, degradation rates for this short segment were fivefold slower than that observed for the entire N terminus. Although there are no highly conserved sequences outside the destruction box in the cyclin B N terminus, these experiments suggest there is a role for neighboring sequences, which perhaps serve to properly orient the destruction box for recognition by APC. Our mutational analysis of lysine residues in these regions suggests that the stabilization observed in the deletion mutants is unlikely to be a consequence of loss of ubiquitin acceptor sites.

Functional Differences between A- and B-Type Destruction Boxes

Whereas B-type cyclin N termini were generally unstable in mitotic *Xenopus* extracts, the *Xenopus* cyclin A1 N terminus was stable. We were surprised to find that the destruction box from *Xenopus* cyclin A1 was unable to substitute for the cyclin B destruction box, because an intact destruction box is required for the degradation of both types of cyclins (Glutzer *et al.*, 1991; Kobayashi *et al.*, 1992; Lorca *et al.*, 1992). Our experiments suggest that this defect stems primarily from a difference in the final position of the destruction box, which is asparagine in almost all B-type cyclins, yet it is very poorly conserved among A-type cyclins. Interestingly, it has been shown that an N-terminal fragment of clam cyclin A, which is one of the few A-type cyclins that contains asparagine at the final position (Figure 3), can target protein A for ubiquitination by components purified from clam extracts (Sudakin *et al.*, 1995). In contrast, our data indicate that an N-terminal fragment of *Xenopus* cyclin A1, which contains aspartate rather than asparagine at the final position, is not sufficient to target protein A for degradation or ubiquitination in mitotic *Xenopus* extracts.

Our results are surprising in light of the fact that A- and B-type cyclins seem to be ubiquitinated by the same components, because recombinant cyclin A will inhibit the degradation of both cyclins A and B in clam extracts (Sudakin *et al.*, 1995). Moreover, in clam extracts, the ubiquitin ligase that recognizes cyclin B

cofractionates with that for cyclin A (Sudakin *et al.*, 1995). How does one reconcile the fact that the cyclin A destruction box is necessary for degradation with the finding that it cannot always substitute for its B-type counterpart? One possibility is that the A-type destruction box is simply a weaker ubiquitination signal than the B-type destruction box and that full-length cyclins contain additional degradation signals that can compensate for the weak destruction box. If this were the case, one might expect that the A-type destruction box could support degradation of full-length cyclin B but not an N-terminal cyclin B fragment. This hypothesis was very recently tested by Klotzbücher *et al.* (1996), who found that the 9-amino-acid *Xenopus* A1 destruction box would not support degradation of full-length *Xenopus* cyclins B1 or B2. However, in contrast to our results, the authors found that the chimeric substrates continued to be ubiquitinated. It is possible that the cyclin A destruction box can promote a low level of ubiquitination when placed in the context of a full-length cyclin but that the level of polyubiquitin chain formation is insufficient to target the protein for proteolysis. Our experiments with methylated ubiquitin indicate that triubiquitinated cyclin fragments are stable in mitotic extracts, indicating that at least four ubiquitins, presumably in the form of a polyubiquitin chain, must be attached to cyclin to target the protein for degradation.

Multiple Lysine Residues Can Function as Ubiquitin Acceptor Sites

In addition to sharing a conserved destruction box, the N termini of B-type cyclins are enriched in lysine residues, especially in the region downstream of the destruction box, suggesting that this region might function as the primary ubiquitin acceptor site in cyclin B (Glötzer *et al.*, 1991). We were therefore surprised to find that every lysine residue had to be eliminated from the *Arbacia* N-terminal fragment to stabilize the protein. Biochemical analysis of the ubiquitinated intermediates of the fusion protein 13-66prA provided a structural explanation for these findings, because mapping experiments indicated that ubiquitin is conjugated to multiple lysine residues located both N- and C-terminal to the destruction box. Although our analysis indicated that the lysine cluster closest to the destruction box is the primary ubiquitin acceptor site in wild-type cyclin, the mutagenesis experiments indicate that any lysine residue in the cyclin N terminus can serve this purpose.

To our knowledge, this is the first physiologically relevant substrate of the ubiquitin-dependent proteolytic pathway for which the sites of ubiquitin attachment have been determined directly. Sites of ubiquitin conjugation have also been mapped on the engineered N-terminal extension used in studies of the N-end

rule, in which a single polyubiquitin chain is extended from one of two lysine residues at positions 15 or 17 (Bachmair and Varshavsky, 1989; Chau *et al.*, 1989). Mapping of ubiquitin conjugates of yeast cytochrome c indicates that ubiquitination occurs at a dilysine cluster near the N terminus, a region of the protein that is relatively unstructured (Sokolik and Cohen, 1991, 1992). c-Jun is another substrate of the ubiquitin pathway for which a "destruction domain" has been localized, which, incidentally, has no primary structural similarity with the cyclin destruction box. Although the sites of ubiquitination of c-Jun have not been mapped directly, mutation of individual lysine residues does not appreciably alter the rate of degradation or the pattern of ubiquitin conjugates (Treier *et al.*, 1994). The flexibility with which the site of ubiquitination is selected may therefore be a common feature of the ubiquitination reaction for substrates rich in lysine residues. Furthermore, because nuclear magnetic resonance analysis of the *Xenopus* cyclin B1 N terminus has failed to reveal any significant structure (H. Yu and M.W.K., unpublished data), the cyclin N terminus may adopt a flexible conformation facilitating ubiquitination at multiple sites.

Destruction Box-dependent Degradation of Noncyclin Proteins

Work in recent years has demonstrated that a diverse number of important cellular regulators are degraded by ubiquitin-dependent proteolysis. Examples include the tumor suppressor p53 (Scheffner *et al.*, 1993), the proto-oncogene c-jun (Treier *et al.*, 1994), the yeast transcriptional repressor Mata2 (Hochstrasser *et al.*, 1991), and the cyclin-dependent kinase inhibitor p40^{sup1} (Schwob *et al.*, 1994). However, the degradation signals in these proteins do not bear resemblance to the destruction box, and they are ubiquitinated by components distinct from APC. Recently, destruction box motifs have been implicated as degradation signals in several noncyclin proteins, including uracil permease (Galan *et al.*, 1994) and the ras exchange factor Cdc25p (Kaplon and Jacquet, 1995). However, their degradation does not seem to be cell-cycle-dependent, and it is not known whether the proteolysis of these proteins is ubiquitin mediated.

However, there is abundant circumstantial evidence for the mitotic degradation of a noncyclin protein through a destruction box-dependent mechanism. Addition of an N-terminal fragment of cyclin B to *Xenopus* extracts inhibits both cyclin B degradation and sister chromatid segregation, whereas addition of non-degradable cyclin B causes cell-cycle arrest in telophase (Holloway *et al.*, 1993). These findings suggest that anaphase is triggered by the degradation of destruction box-containing protein(s) distinct from cyclin. Furthermore, mutations in components of APC

such as Cdc16p and Cdc23p cause arrest in metaphase, suggesting that the putative anaphase inhibitor is a substrate of APC. An excellent candidate for such a protein has recently been identified in budding yeast, in which Pds1p is required for the proper execution of anaphase (Yamamoto *et al.*, 1996a). *Pds1* mutants are able to partially bypass the anaphase arrest of *cdc16* and *cdc23* mutants, suggesting the protein functions as an anaphase inhibitor (Yamamoto *et al.*, 1996b). Pds1p seems to be unstable only in G1-arrested cells (Yamamoto *et al.*, 1996a), as is the case for mitotic cyclins in yeast (Amon *et al.*, 1994). Although it is not known whether Pds1p stability is altered in *cdc16* or *cdc23* mutants, Pds1p contains a putative destruction box RLPLAAKDN (Yamamoto *et al.*, 1996a), which shares the asparagine conserved among B-type cyclins. However, it contains alanine at position six, which is more characteristic of A-type cyclins. Our data would suggest that such a substrate would be recognized in *Xenopus* extracts, although it might not be degraded quite so rapidly as B-type cyclins. Although the role of the destruction box in Pds1p stability remains to be tested, the phenotype of *pds1* mutants and genetic interactions with components of APC suggest it may be the first bona fide noncyclin substrate whose stability is regulated by APC-dependent ubiquitination. The complexity of APC composition suggests there may be additional destruction box-containing substrates awaiting discovery.

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