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Biochemical Characterization of the Cyclin Ubiquitin Conjugation System

by

Randall W. King

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

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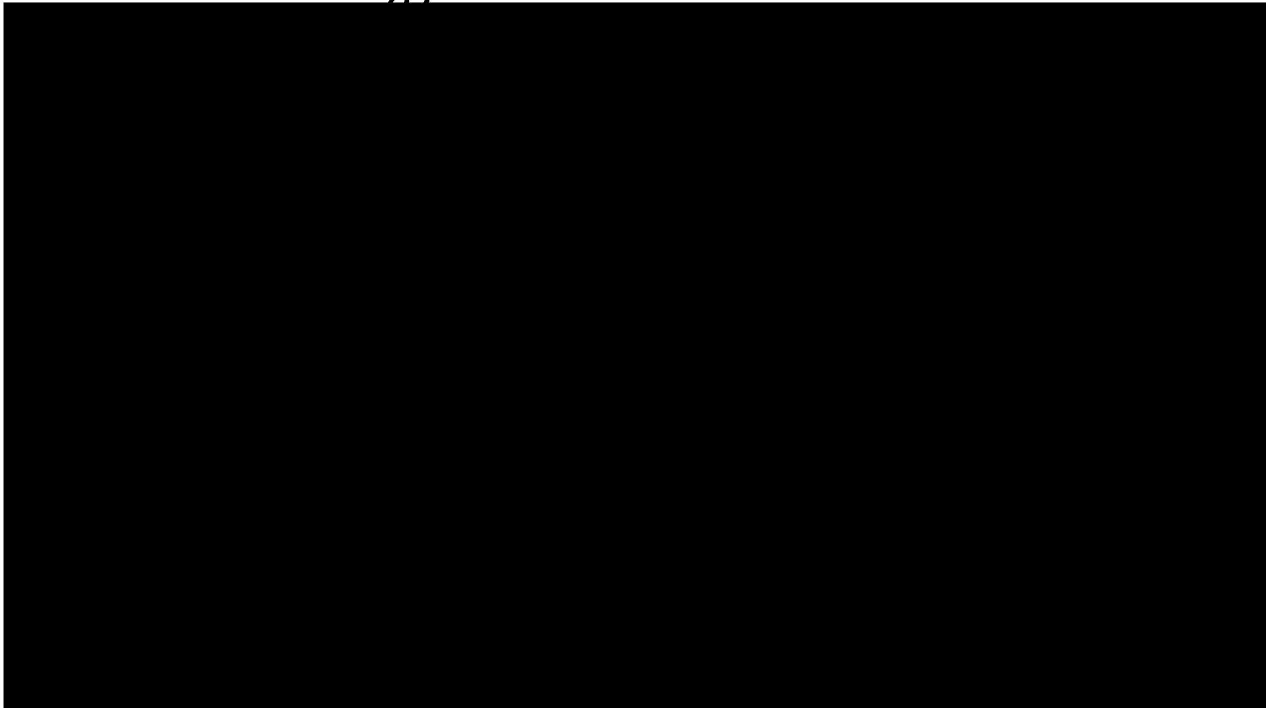

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To my wife and to my family,
for all they have done for me

Preface

There are many people whom I would like to thank for their input, support, and guidance over the past five years. I want to begin by acknowledging those people who contributed directly to the work presented in this thesis. Chapter 1 is adapted from a review article, entitled "Mitosis in Transition," written in collaboration with Peter Jackson and Marc Kirschner. The experiments presented in Chapter 2 are my own, but this work was done with the guidance of Michael Glotzer, who created several of the constructs used. The cloning and analysis of UBC9 and UBC4 is my own work, but the remainder of the experiments in Chapters 3 and 4 were done in close collaboration with Jan-Michael Peters. The purification of UBCX presented at the end of Chapter 3 was largely the work of Hongtao Yu, performed with my input. Certainly one lesson that I have learned over the past several years is the value of working together with highly talented, enthusiastic people.

There are several people who contributed materials that were essential for completing this work. I thank Jeremy Minshull (UCSF) for plasmids encoding the *Xenopus* mitotic cyclins; Marc Rolfe (Mitotix, Inc.) for recombinant ubiquitin-conjugating enzymes and antisera against human UBC4; Renee Yew (Harvard) for recombinant CDC34; Judy Callis (University of California, Davis) for his-tagged ubiquitin; Richard Vierstra (University of Wisconsin) for recombinant E1; and Stuart Tugendreich and Phil Hieter (Johns Hopkins) for antibodies against human CDC16 and CDC27.

Several other groups shared information prior to publication that played a crucial role in the experiments presented in this thesis. I thank Stephan Jentsch and Wolfgang Seufert for sharing the sequence of yeast UBC9 prior to publication. I would especially like to acknowledge Stefan Imiger and Kim Nasmyth, who found that CDC16 and CDC23 are essential for cyclin proteolysis in yeast, for

sharing their information prior to publication. I would also like to thank Kim for several stimulating discussions over the past several years.

There are many people who have contributed to my development as a scientist over the past five years, both at UCSF and at Harvard. I would like to thank the members of my thesis committee, Andrew Murray, David Morgan, and Mike Bishop, for their advice and support. Special thanks go to Andrew for initiating work on cyclin proteolysis and leaving the project in Marc's lab so that I could eventually work on it. I would also like to thank Mike for the wonderful tutelage during medical school and as a first-year graduate student. Few PIs take the time to participate in seminar courses for medical students or discuss papers weekly with rotation students. These were two of the most rewarding educational experiences I had as a student at UCSF, and I hope that I can be as interesting and stimulating a teacher someday.

Within the Kirschner lab, there are many people to thank. For technical assistance in pouring gels, doing plasmid preps, and keeping the lab sane, I wish to thank Teresita Bernal, without whom we would not have survived the move to Harvard. I would also like to acknowledge Louise Evans, whose sense of humor has made the Kirschner lab a great place to work. From the early days I would like to thank Ray Deshaies and Steve Doxsey for their humor, advice, insight, and friendship. The Kirschner lab has always been an exciting place to discuss new ideas, models, and experiments, and the cadre of talented post-docs in the lab had much to do with creating this environment. I also want to thank Michael Glotzer for showing me how to do experiments on cyclin degradation, and for many stimulating discussions about cyclin proteolysis. Michael taught me that just about anything can be made in a PCR machine, and instructed me in the fine art of iodination. Michael also taught me how to make *Xenopus* extracts and how to inject frogs; I hope the latter skill proves useful as I

return to medical school.

Since moving to Harvard, I have become especially indebted to three people in the lab. I wish to thank Peter Jackson for his input and friendship, but most of all for the discussions that we have had over the past three years, ranging from science to politics to books, or impromptu lectures given on any of a large number of topics. These discussions have been a highlight of my scientific training, and I hope they continue in the future. I thank Kevin Lustig for his friendship, creativity, optimism, intelligence, and particularly his enthusiasm for science and for people. He has a rare blend of talents that has enriched my scientific experience in the Kirschner lab immeasurably. I also wish to thank Kevin for helping me see reason when I was inclined to do otherwise. Finally, I wish to thank Jan-Michael Peters, whose arrival in the lab was instrumental to this work and to my development as a scientist. From our interactions I have truly learned the meaning of the word "colleague," and wish to thank Jan for his generosity, guidance, and friendship. This project simply would not have been accomplished without him, nor would I have found the science as rewarding in his absence. I hope that our collaborations will continue at some level in the future.

I would like to thank my advisor, Marc Kirschner, for everything that he has done, including inviting me to come with the lab during the move to Harvard. It's always hard to know whether you have made the right decision at every stage of your training, but in this case, I can't imagine that things could have gone any better, and for that I owe Marc a great deal of thanks. I thank Marc for creating such a wonderful environment for doing science, and for the freedom to make my own mistakes and to create my own successes. Marc's enthusiasm for creative science is contagious, and I hope that I can maintain a similar sense of excitement in my own lab in the future. I especially thank Marc for challenging me to think critically, creatively, and independently. These will be the most useful tools

that I will take with me when I leave. I also want to thank Marc for his friendship, and for elevating the practice of self-deprecating humor to a fine art.

Finally, I would like to thank a few additional people for the contributions they have made over the past five years and at earlier points in my life. I would like to thank my college chemistry professor, Jerry Mohrig, for introducing me to what great science is all about, and for encouraging me to pursue graduate training. He is also an outstanding teacher whom I one day hope to emulate. I would especially like to thank my parents, my first and best teachers, for their love and steadfast support, and especially my Dad, for the chemistry set, the time in the darkroom, and the myriad other experiences that stimulated my interest in science. I also thank my sister for her friendship and the time we were able to spend together in San Francisco. Last, but certainly not least, I thank my wife, Suzanne Bender, who has been at my side throughout graduate school. Her support and advice were crucial in many of the decisions that I have made during the past five years. I thank her for her confidence in me and in us, best exemplified by her willingness to move cross-country on short notice. Finally, I thank her for her love, which has made doing science all that much better.

Biochemical Characterization of the Cyclin Ubiquitin Conjugation System

Randall W. King

Abstract

The transition between interphase and mitosis is regulated by the cyclin-dependent kinase cdc2. Activation of cdc2 kinase requires the accumulation of a threshold level of cyclin B, whereas inactivation depends upon cyclin B proteolysis. In early *Xenopus* embryos, the periodic activation of the cyclin destruction system transforms continuous cyclin synthesis into alternating periods of interphase and mitosis.

Cyclin B is degraded by the ubiquitin pathway, which recognizes a short conserved sequence in the cyclin N-terminus called the destruction box. We demonstrate that each conserved position of the destruction box is essential for ubiquitination, but that non-conserved sequences within and outside the destruction box are not. Cyclin is ubiquitinated at multiple positions, and there is not a strict requirement for a specific lysine residue to act as a ubiquitin acceptor site.

We have biochemically fractionated *Xenopus* extracts to identify activities that can ubiquitinate cyclin B. We find that *Xenopus* extracts contain at least two distinct ubiquitin conjugating enzymes (E2s) that can support cyclin B ubiquitination. Neither E2 is cell cycle regulated. We have identified one of these E2s as a *Xenopus* homolog of *S. cerevisiae* UBC4, an enzyme implicated in the degradation of a wide variety of proteins. We have purified the second ubiquitin conjugating enzyme and find that it is a novel enzyme. A *Xenopus* homolog of UBC9, an E2 implicated in cyclin proteolysis in yeast, is not required for cyclin ubiquitination *in vitro*.

The mitotic specificity of cyclin degradation results from the activation of a 20S complex that contains a cyclin-ubiquitin ligase. We demonstrate that this complex contains homologs of yeast CDC16, CDC23, and CDC27, genes that

are essential for the transition from metaphase to anaphase. Purification of this complex, which we term the Anaphase-Promoting Complex (APC), from interphase or mitotic extracts indicates that it is composed of at least 8 distinct subunits, some of which become phosphorylated during mitosis. Peptide sequencing indicates that APC contains a *Xenopus* homolog of BimE, a protein essential for anaphase in *Aspergillus*; the remaining subunits are novel proteins.

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Chapter 1

Introduction

Transitions of the eukaryotic cell cycle

The formulation of the cell theory of Schleiden and Schwann was not completed until the 1850s when Rudolph Virchow proposed that all cells are the product of division of previously existing cells. During cell division, chromosomes must be replicated and segregated with great precision to preserve the integrity of the organism's genetic information. Different strategies have evolved to accomplish this feat. In prokaryotes, which contain a relatively simple genome, replication and chromosome segregation can occur simultaneously. In eukaryotes, which contain a complex genome distributed among multiple chromosomes, DNA replication is temporally dissociated from chromosome segregation, creating distinct cell cycle phases.

This thesis focuses on the biochemical mechanisms that orchestrate the events of the cell division cycle. The regulation of cell division occurs primarily at three transitions between different phases of the cell cycle. The first important transition occurs late during G1, just before the onset of DNA replication. This G1 regulatory transition has been called START in budding yeast, because it is the point at which yeast cells commit to undergoing a complete round of DNA replication and mitosis. In many cell types, this is the point at which growth is coordinated with cell division. For example, budding yeast must attain a critical mass to pass through the START transition and begin DNA replication. The existence of this control point can also be demonstrated by treating yeast with mating pheromones. Cells that have not passed START will undergo cell cycle arrest in G1; those that have passed START will continue to divide and arrest in the subsequent G1 phase. A similar G1 control point, called the restriction point, has been demonstrated in mammalian cells. Deprivation of growth factor prior to the restriction point will arrest cell division in G1; if removed after the restriction point, arrest will not occur until another round of division has ensued. In general, the

period during G1 prior to START is a time when the cell is responsive to external stimuli that can influence the decision of whether or not to divide.

Once the cell has passed START and has become committed to a round of cell division, DNA replication is initiated at multiple origins dispersed throughout the genome. Except in very special circumstances, the genome is replicated once, and cannot be replicated again until the cell has passed through mitosis. This illustrates another general feature of cell cycle control: certain cell cycle events are dependent upon the passage through a temporally distinct transition.

Another major transition governed by the cell cycle control circuitry is the transition from interphase to prophase. In some cell types, passage through this transition is influenced by controls that sense cell size. For example, the yeast *S. pombe* will pause during G2 and will not enter M phase until cells have attained a critical mass. In most cells, passage through this transition is also regulated by a checkpoint that monitors the status of the DNA and inhibits entrance into mitosis if the DNA has not been replicated or if there is DNA damage. This is important, as the condensation of incompletely replicated chromosomes can result in chromosome breakage or loss. Therefore, different types of signals converge to regulate the transition from interphase to mitosis.

The final important regulatory point in the cell cycle is the transition from metaphase to anaphase, the point at which sister chromatid segregation is initiated. The regulation of this transition takes two important forms. First, a minimal delay must be imposed between the onset of prophase and the onset of anaphase to allow time for chromosomes to be aligned at the metaphase plate. Second, a checkpoint mechanism operates to restrain the onset of anaphase if chromosomes are not attached to the spindle or aligned properly. This checkpoint ensures that chromosomes are accurately segregated to the spindle poles.

The fundamental cell cycle regulatory machinery is conserved

Our understanding of the molecular machinery that regulates these transitions has benefited greatly from the combined power of the genetic analysis of cell division in yeast, and a biochemical analysis in vertebrate embryonic systems. This approach has succeeded because the basic components that regulate cell division are conserved among all eukaryotic organisms. Here I briefly review the experiments that led to the discovery of the prototypic cell cycle regulator, the complex composed of cdc2 kinase and cyclin B. For more a more detailed account, see Murray and Kirschner (1989b) or Nurse (1990).

The genetic analysis of the problem of cell division began in the 1970s when screens were performed in the yeasts *S. cerevisiae* (Hartwell et al., 1970; Hartwell et al., 1974) and *S. pombe* (Nurse et al., 1976) to identify genes essential for cell cycle progression. Temperature-sensitive mutations that blocked cell division without interfering with cell growth were identified. Each of these mutants arrested at a specific cell cycle stage, suggesting that its function was required for a particular transition. Additional screens were performed that identified genes important for coordinating cell growth with cell division (Nurse, 1975). A great deal of attention has focused on the product of the *cdc2+* gene, a protein kinase whose mutant allele arrests at the G2/M transition. The finding that a human gene could complement the yeast *cdc2* mutation was the first indication that the cell cycle regulatory machinery is highly conserved (Lee and Nurse, 1987).

Meanwhile, separate studies had identified a cytoplasmic factor in unfertilized amphibian eggs that was capable of inducing oocytes to enter meiosis (Masui and Markert, 1971; Smith and Ecker, 1971). Maturation-promoting factor (MPF), as it was called, could also induce G2-arrested cells to enter mitosis (Miake-Lye et al., 1983). Further analysis revealed that MPF is transiently activated during mitosis (Wasserman and Smith, 1978; Gerhart et al., 1984), and can

be detected in a wide variety of cell types (Kishimoto and Kanatani, 1976; Sunkara et al., 1979; Kishimoto et al., 1982), suggesting that MPF is a universal regulator of the mitotic process.

How is MPF regulated so that it is only active during mitosis? The key insight into this problem came from analyzing the pattern of proteins translated during the early embryonic cell cycles of marine invertebrates. These studies identified a new class of proteins, called cyclins, that accumulate during interphase and are abruptly degraded during mitosis (Evans et al., 1983). This finding suggested a possible relationship between cyclin and MPF, because protein synthesis is required for entrance into mitosis (Hutlin, 1961; Kishimoto and Lieberman, 1964) and for activation of MPF (Gerhart et al., 1984; Wasserman and Masui, 1975), whereas activated MPF can induce mitosis in the absence of protein synthesis. Furthermore, cyclin could induce maturation and thus MPF activation when its RNA was injected into immature oocytes (Pines and Hunt, 1987; Swenson et al., 1986). The golden spike uniting the biochemical and genetic studies of cell division was hammered into place when MPF was purified and demonstrated to be composed of a heterodimer of cyclin B and a homolog of fission yeast *cdc2*$^+$ (Arion et al., 1988; Dunphy et al., 1988; Gautier et al., 1988; Labbé et al., 1988; Meijer et al., 1989; Labbé et al., 1989; Gautier et al., 1990;). This finding led to several predictions: first, cyclin synthesis should be necessary for activation of MPF and entry into mitosis. Second, the rapid disappearance of cyclin in mitosis suggested that its degradation might be the primary mechanism of inactivating the kinase.

The development of cell-free extracts that could perform events of the cell cycle *in vitro* (Blow and Laskey, 1986; Lohka and Masui, 1983; Murray and Kirschner, 1989a) was an important step that enabled these hypotheses to be tested. Specific ablation of cyclin B mRNA in *Xenopus* extracts inhibited entrance

into mitosis, indicating that cyclin synthesis is necessary for the transition from interphase to mitosis (Minshull et al., 1989). Strikingly, extracts treated with ribonuclease to destroy endogenous mRNA could undergo a complete cell cycle if a single mRNA, that encoding cyclin B, was added back (Murray and Kirschner, 1989a). These experiments proved that cyclin synthesis is essential for the G2/M transition, and that, at least in frog embryos, cyclin B can fulfill the protein synthesis requirement for entry into mitosis.

The importance of cyclin degradation in controlling cell cycle progression was also first demonstrated in *Xenopus* cell cycle extracts. Addition of mRNA encoding a mutant form of cyclin that lacked its N-terminal 90 amino acids caused extracts to arrest in a mitotic-like state with condensed chromosomes and elevated histone H1 kinase activity (Murray et al., 1989). Analysis of cyclin levels in these extracts demonstrated that the mutant cyclin was not degraded, indicating that cyclin degradation is necessary for inactivation of cdc2 kinase and exit from the mitotic state. This requirement appears universal, as overexpression of non-degradable forms of cyclin B produces a mitotic arrest in all organisms tested (Murray et al., 1989; Luca et al., 1991; Ghiara et al., 1991; Gallant and Nigg, 1992; Surana et al., 1993; Luo et al., 1994; Rimmington et al., 1994).

The cyclin B-cdc2 kinase provides a paradigm for understanding how the cell cycle is regulated by the cyclin-dependent kinases, and highlights the importance of protein phosphorylation and protein degradation in controlling cell cycle transitions. Families of related G1 cyclins and cyclin-dependent kinases have now been identified in yeast and in higher eukaryotes that are important for regulating other transitions of the cell cycle such as START and the onset of DNA replication (for review see Sherr, 1994). As I will discuss below, the activity of the G1 cyclins indirectly influences other cell cycle transitions because it plays an important role in regulating cyclin B stability. Although the START transition and

the G2/M transition appear to be controlled chiefly by the activity of a cyclin-dependent kinase, the metaphase-anaphase transition appears to be controlled by ubiquitin-dependent proteolysis. It is important to emphasize that a separate series of controls, called checkpoints, can intervene and inhibit passage through a particular transition (reviewed in Murray, 1992; 1995). Although the checkpoint pathways can modulate the activity of cell cycle regulators such as the cyclin-dependent kinases, they are not essential for orderly progression through the cell cycle. The remainder of this introduction discusses how the cyclin B/Cdc2 complex regulates the fundamental transitions of the cell cycle, and how the transition points in turn regulate the activity of the kinase and the cyclin degradation system.

The three fundamental transitions of the cell cycle were originally defined through physiological experiments or microscopic analysis of dividing cells. These transitions can also be defined in relation to the activity of state of MPF and the cyclin B degradation system (Figure 1-1). The G2/M transition, for example, is defined morphologically by events such as nuclear envelope breakdown, chromosome condensation, and spindle formation. During this transition, cyclin B accumulates and activates Cdc2 kinase. During the second transition, MPF activates a ubiquitin-dependent proteolytic system, causing both cyclin B destruction and the initiation of anaphase. In the final transition, defined physiologically as START, the cyclin degradation machinery is inactivated and the cell cycle is reset.

The principal challenge presented by the first transition is to understand the mechanisms that transform the continuous accumulation of cyclin into the rapid activation of MPF that occurs at the G2/M boundary (Figure 1-1A). The period preceding MPF activation can be divided into two parts: the time required for cyclin B to accumulate to threshold, and the subsequent period when cyclin is

Figure 1-1. Transitions of the mitotic cycle

A. The level of cyclin B oscillates in the cell cycle, driving repeated rounds of MPF activation and mitosis. Threshold represents the level of cyclin accumulation that is sufficient to activate MPF.

B. The first transition of mitosis involves the activation of MPF. The activation reaction is characterized by a lag phase, which can be separated into two steps. In the first step, cyclin accumulates to threshold. In the second step, MPF activation is delayed even though all components required for activation are present.

C. The second and third transitions of mitosis regulate cyclin B stability. The second transition, the activation of the cyclin degradation system, follows the activation of MPF by a lag period sufficient to allow the events of mitosis to occur. The third transition, the inactivation of the cyclin degradation system, occurs in response to controls external to the mitotic cell cycle, and resets the cell cycle to allow cyclin to reaccumulate.

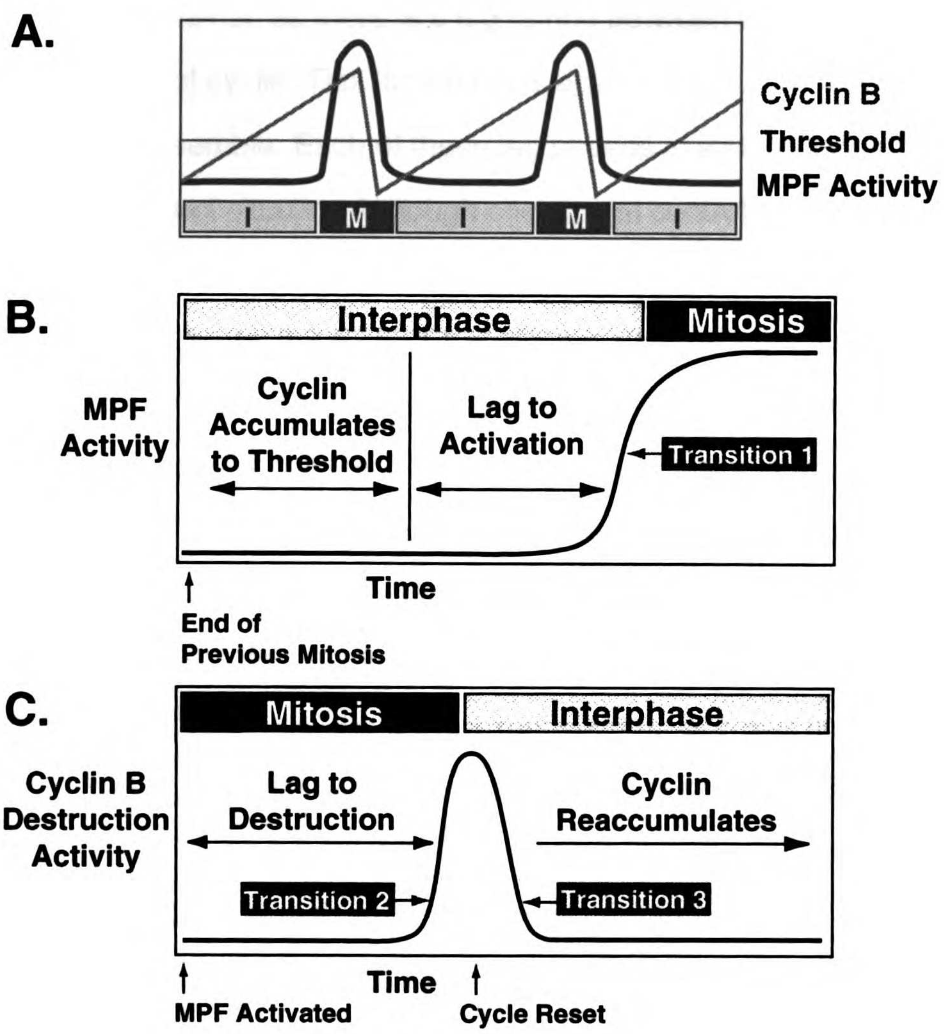


Figure 1-1

present at sufficient levels to activate MPF, yet the kinase remains inactive (Figure 1-1B). Although this second phase is difficult to observe during the rapid cell cycles of early embryos, it is prevalent in organisms such as *S. pombe* that coordinate growth with division during G2. The second transition of mitosis exhibits similar kinetic behavior, as there is a lag period between the activation of MPF and the destruction of cyclin. This lag phase is essential to allow time for the mitotic spindle to assemble. Each of these lag phases is an intrinsic characteristic of the mitotic control circuitry. Although checkpoint controls can intervene to lengthen these lag phases, such controls are not generally invoked during a normal cell cycle. Although the START transition is generally thought of as the beginning of the cell cycle, it is the last transition I will discuss here. It has only been recently appreciated that the START transition is the point at which the cyclin B degradation system is inactivated. In contrast to the previous two transitions, the decision to cross START is regulated each cell cycle by controls external to the mitotic regulatory machinery. The coordination of the activity of the cyclin degradation system with the START transition means that once a cell has restabilized cyclin B, it is committed to another round of cell division.

MPF activation and the transition from interphase to mitosis

As described above, cyclin B synthesis is essential for MPF activation and entrance into mitosis. In *Xenopus* egg extracts, activation of Cdc2 kinase requires addition of a threshold level of cyclin B (Solomon et al., 1990). This threshold can also be demonstrated in intact dividing embryos as the point after which cycloheximide treatment does not interfere with cell division. However, during the first mitotic division of *Xenopus* embryos, there is a lag between the point when cyclin has accumulated and the time that MPF is activated (Karsenti et al., 1987). This implies the existence of positive or negative regulators of the MPF activation

reaction. In some organisms, such as *Xenopus* embryos, the rate at which the cyclin threshold is reached can affect the rate at which the cell enters mitosis (Murray and Kirschner, 1989a; Solomon et al., 1990). In other organisms such as fission yeast, however, overexpression of cyclin B does not accelerate the G2/M transition, implying that some other reaction controlling MPF activity is rate-limiting for entrance into mitosis (Booher and Beach, 1987; Hagan et al., 1988).

There are both positive requirements and negative controls that influence MPF activation in yeast and vertebrate cells. To generate active MPF, Cdc2 must bind to cyclin B and be phosphorylated on threonine-167 (Gould et al., 1991). The kinase that phosphorylates this site is the Cdc2-activating kinase (CAK; Solomon et al., 1992), and its catalytic subunit is also a cyclin-dependent kinase, designated Cdk7 (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). CAK also requires a positive regulatory subunit similar to other cyclins, called cyclin H (Fisher and Morgan, 1994; Makela et al., 1994). This kinase complex may also play a role in regulating transcription and DNA repair, as it is also contained in purified preparations of the transcription factor TFIIH (Feaver et al., 1994; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). There appear to be two alternative mechanisms of generating active CAK. Phosphorylation of CAK on a site homologous to threonine 161 increases its ability to bind to cyclin H. However, assembly and activity can be obtained in the absence of Cdk7 phosphorylation if the p36 assembly factor is present (Fisher et al., 1995).

Since CAK activity does not vary significantly during the cell cycle (Fisher and Morgan, 1994), other regulators must restrain MPF activation until the G2/M transition. One mechanism through which MPF activity is muted involves inhibitory phosphorylation within the ATP-binding site of the kinase. During late S phase and G2, Cdc2 is phosphorylated on threonine-14 and tyrosine-15, maintaining the kinase in a catalytically inactive form (Draetta et al., 1988; Gould and

Nurse, 1989). In fission yeast and mammalian cells, mutation of these sites of inhibitory phosphorylation accelerates entrance into mitosis and cripples the ability of some types of cells to restrain mitotic entry in the presence of incompletely replicated DNA (Gould and Nurse, 1989; Krek and Nigg, 1991; Norbury et al., 1991).

In *S. pombe*, mutation of *wee1*, a tyrosine kinase that phosphorylates *cdc2*, causes cells to divide at a smaller size because they cannot restrain MPF activity during G2 (Featherstone and Russell, 1991; Russell and Nurse, 1987b). Simultaneous mutation of a closely related kinase, *mik1*, cause grossly premature entrance into mitosis that is lethal, indicating that inhibitory phosphorylation of *cdc2* is essential for normal cell cycle progression in *S. pombe* (Lundgren et al., 1991).

The activity of *wee1* is regulated by distinct mechanisms. In response to nutritional signals, another kinase, called *nim1* or *cdr1*, can phosphorylate *wee1* to inhibit its activity and promote entrance into mitosis (Coleman et al., 1993; Parker et al., 1993; Russell and Nurse, 1987a; Wu and Russell, 1993). This may be the major pathway through which growth and cell division is coordinated in *S. pombe*. During mitosis, *wee1* is phosphorylated at sites distinct from those hit by *nim1*, suggesting that other kinases can function during mitosis to inactivate *wee1*. This may be an important mechanism through which the cooperative activation of MPF is achieved (Tang et al., 1993; Figure 1-2).

Since tyrosine phosphorylation plays an important role in restraining MPF activity during the lag phase, the regulation of tyrosine dephosphorylation is a crucial step in MPF activation. Hence, much work has focused on the regulation of the tyrosine phosphatase, *cdc25*, which can dephosphorylate *cdc2* to activate it (Gautier et al., 1991; Kumagai and Dunphy, 1991; Russell and Nurse, 1986). Cdc25 activity is regulated by several complex pathways. The phosphatase

Figure 1-2. Cooperative mitotic phosphorylation and the activation of MPF

Following translation, cyclin B associates with monomeric, unphosphorylated Cdc2; Cdc2 is then activated by phosphorylation on threonine 161. In interphase, the activation of Cdc2 is restrained by tyrosine phosphorylation catalyzed by Wee1 or the product of the related gene, Mik1. The tyrosine phosphatase Cdc25 dephosphorylates the inactive, tyrosine phosphorylated Cdc2/cyclin B complex and activates the complex. This active cyclin B/Cdc2 complex constitutes Mitosis Promoting Factor (MPF). MPF can promote phosphorylation of a variety of mitotically active kinases, including forms of MAP kinase and the MPM-2 kinase. MAP kinase and MPM-2 kinase in turn can activate and stabilize the mitotic state and therefore MPF. Cdc25 can be activated by Cdc2 or the MPM-2 kinase, creating a positive feedback loop that contributes to the cooperative transition from G2 to mitosis. This feedback loop is amplified by the inhibition of negative regulators of mitosis: Wee1 is inhibited by mitotic phosphorylation, decreasing inhibitory tyrosine phosphorylation of Cdc2, and phosphatase 1 is inhibited, potentiating Cdc25 activation.

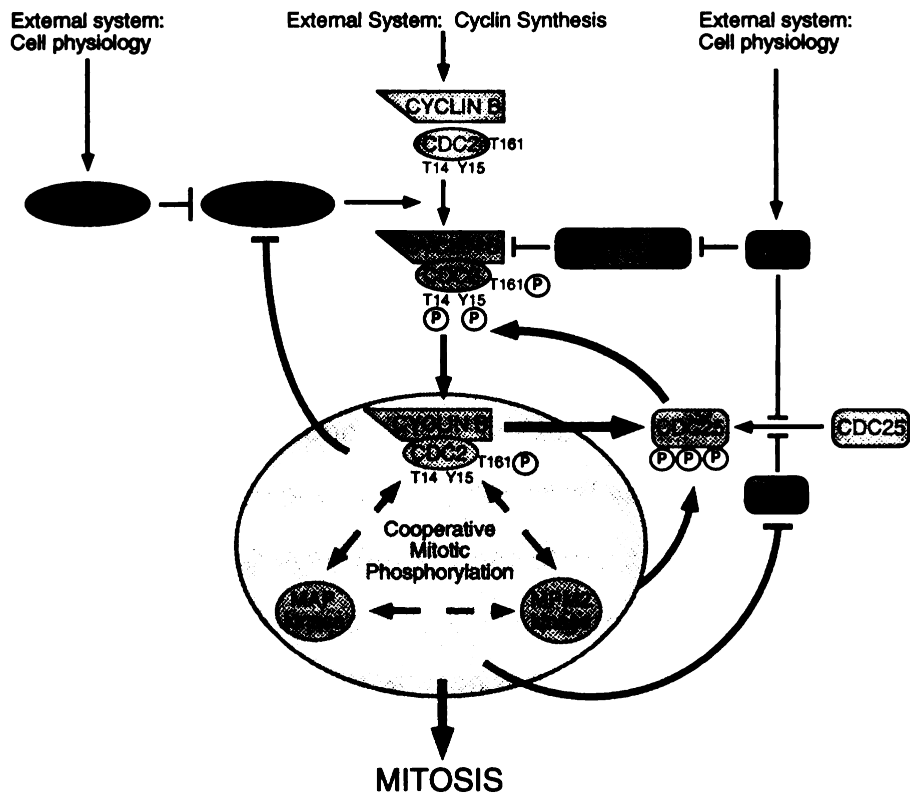


Figure 1-2

activity of Cdc25 increases during mitosis when the protein becomes phosphorylated (Izumi et al., 1992; Kumagai and Dunphy, 1992). Purified MPF can phosphorylate and activate Cdc25 *in vitro*, and mutation of these activating sites of phosphorylation decreases the ability of Cdc25 to promote mitosis (Izumi and Maller, 1993). These experiments suggest the possible existence of a positive feedback loop between MPF and Cdc25 activation (Figure 1-2). Additional cooperativity may be provided through the action of protein phosphatase 1 (PP1), which can antagonize the activity of Cdc25 by dephosphorylating it (Izumi et al., 1992). The activity of PP1 decreases as cells enter mitosis, probably as a result of inhibitory phosphorylation catalyzed by Cdc2 or other kinases activated during mitosis (Walker et al., 1992). A truncated form of PP1, believed to be insensitive to inhibition by mitotic phosphorylation, blocks mitotic progression in extracts, and this inhibition can be rescued by addition of excess Cdc25 (for a more complete description, see Maller (1994)). The activity of PP1 may also be regulated by a specific cellular inhibitor, called inhibitor 2, the levels of which peak during S phase and mitosis (Brautigan et al., 1990). Another phosphatase, PP2A, can also dephosphorylate Cdc25 *in vitro* (Izumi et al., 1992). Inhibition of PP2A by okadaic acid can activate mitosis in *Xenopus* extracts that contain subthreshold levels of cyclin B (Felix et al., 1990a). Although mutations of PP2A can be pleiotropic, some mutations cause premature mitosis in fission yeast (Kinoshita et al., 1993). Therefore, both PP1 and PP2A may be important regulators of the transition from interphase to mitosis, perhaps by regulating Cdc25 activity.

Novel mitotic kinases may also phosphorylate and activate Cdc25 to enhance the cooperative transition from interphase to mitosis. The monoclonal antibody MPM-2 recognizes a discrete subset of proteins that become phosphorylated during mitosis (Davis et al., 1983). The dephosphorylation of these epitopes coincides with MPF inactivation at the metaphase-anaphase transition

(Vandre and Borisy, 1989). Kuang and coworkers have purified from mature *Xenopus* oocytes two kinases capable of generating MPM-2 epitopes, neither of which contains Cdc2 (Kuang and Ashorn, 1993). One MPM-2 epitope kinase, which phosphorylates a very limited number of substrates, was identified as mitogen-activated protein (MAP) kinase. Though early studies failed to show evidence for MAP kinase activation during embryonic mitotic cycles (Ferrell et al., 1991), more recent work in *Xenopus* (Minshull et al., 1994) and mammalian cells (Heider et al., 1994) suggests that several kinases with substrate specificity similar to MAP kinase become transiently activated during mitosis. The second MPM-2 kinase, which phosphorylates the majority of MPM-2 epitopes, has not yet been identified. Both MPM-2 kinases are active only when Cdc2 kinase activity is present. The recent identification of Cdc25 as an MPM-2 epitope (Kuang et al., 1994) suggests that MPM-2 epitope kinases may also contribute to the cooperativity of MPF activation (Figure 1-2).

Although tyrosine phosphorylation ofCdc2 could provide a mechanism for the cooperative activation of MPF, this regulatory pathway does not appear to be universally important for controlling the transition from interphase to mitosis. In budding yeast, deletion of a homolog of Wee1 has no effect on mitotic progression unless the Cdc25 homolog is also deleted (Booher et al., 1993). Furthermore, elimination of inhibitory phosphorylation sites from the *S. cerevisiae* homolog of Cdc2 has no measurable effect on mitotic progression or the checkpoint that inhibits mitosis in the presence of unreplicated DNA (Amon et al., 1992; Sorger and Murray, 1992). In *Xenopus* extracts, addition of PP2A can lengthen the lag to MPF activation (Lee et al., 1994). This inhibition occurs even if a non-phosphorylatable mutant of Cdc2 kinase is used, indicating that PP2A can inhibit activation of MPF through mechanisms that must be independent of tyrosine phosphorylation.

These experiments suggest that while tyrosine phosphorylation can explain the cooperative activation of MPF in some situations, it cannot provide a complete explanation for the lag and threshold to mitotic activation in all systems. An interesting possibility for an inhibitory mechanisms independent of Cdc2 phosphorylation is suggested by analogy with the growing number of low molecular weight stoichiometric inhibitors of Cdk2 that have been described (for a recent review see Sherr and Roberts (1995)). Two of these inhibitors, the *rum1⁺* gene product of *S. pombe* (Moreno and Nurse, 1994), and the Sic1 gene product of *S. cerevisiae* (Mendenhall, 1993), can strongly inhibit the activity of Cdc2 when complexed with a mitotic cyclin. However, neither of these gene products is essential, suggesting that they may function primarily as checkpoint controls. A stoichiometric inhibitor could function to set a threshold for cyclin accumulation and thus contribute to the lag period. It could also explain the finding that MPF is rapidly inactivated when added to interphase *Xenopus* extracts (Lee et al., 1994).

Inactivation of MPF and the metaphase-anaphase transition

The activation of MPF induces the cell to divide by altering cell structure and function through mitotic phosphorylation (reviewed by Earnshaw and Pluta (1994) and Nigg (1993)). Such phosphorylation is catalyzed not only by Cdc2 kinase, but by other kinases, such as NimA (Ye et al., 1995) and the MPM-2 epitope kinases (Kuang and Ashorn, 1993) that become specifically activated during mitosis. After the cytoskeleton has been reorganized for division and the condensed chromosomes aligned on the metaphase plate, MPF activates the cyclin degradation system, thus inducing anaphase and its own demise. The remaining steps of cell division, such as cytokinesis, occur as mitotic phosphorylation is reversed after MPF inactivation. While it was natural to assume that anaphase was also triggered by dephosphorylation following MPF inactivation,

we now know that sister chromatid separation does not require MPF inactivation; instead, this transition depends on the activation of the destruction-box-dependent proteolytic system, which specifically degrades proteins such as cyclin B that contain a destruction box. Thus, while the metaphase-anaphase transition and cyclin degradation are mediated by the same cell cycle machinery, they are independent events.

The activation of the destruction box-dependent proteolytic system is therefore the biochemical correlate of the morphologically-defined metaphase-anaphase transition. Unlike the other structural changes that occur during mitosis, sister chromatid segregation and cyclin destruction are both irreversible events, making this proteolytic step the last possible target of checkpoint controls that block exit from mitosis in response to an incompletely assembled spindle. While the lag between the activation of MPF and the onset of cyclin destruction is difficult to observe in the rapid cell cycles of early embryos, the addition of purified MPF to interphase extracts of *Xenopus* eggs will activate the cyclin degradation system with a lag period corresponding roughly to the length of mitosis (Felix et al., 1990b). The dynamics of the activation of cyclin degradation raise questions similar to those explored for the activation of MPF itself: how is the activity of the degradation system suppressed while MPF is activated, and how is the degradation system activated in a concerted fashion after a lag period that allows the events of mitosis to occur?

As described earlier, our understanding of the mechanism of cyclin degradation has benefited greatly from the use of in vitro systems that recapitulate the regulated degradation of mitotic cyclins (Luca and Ruderman, 1989; Murray et al., 1989). The N-terminus of both A- and B-type cyclins contains a short conserved sequence, termed the destruction box, which when mutated or deleted will stabilize cyclin against mitosis-specific proteolysis (Glotzer et al., 1991; Lorca

et al., 1992; Luca et al., 1991). Mitotic cyclins can therefore be separated into two functional domains, the N-terminus, which targets the protein for degradation, and the C-terminus, which is required for binding and activation of Cdc2. The N-terminus of sea urchin cyclin B can direct the destruction of a heterologous protein, and when expressed independently, the N-terminal fragment is rapidly degraded in a mitosis-specific fashion (Glotzer et al., 1991; Holloway et al., 1993). However, mutations of *Xenopus* cyclins A and B2 that block binding to Cdc2 inhibit their degradation, suggesting that the requirements for recognition of these proteins by the cyclin destruction system may be more complex (Stewart et al., 1994; van der Velden and Lohka, 1994).

Several lines of evidence now suggest that cyclin is targeted for degradation by the ubiquitin pathway, which has been implicated in the turnover of other cellular regulators such as p53 (for review, see Ciechanover and Schwartz, (1994)). Cyclin B is ubiquitinated in mitotic, but not interphase *Xenopus* egg extracts, and ubiquitination depends upon the presence of an intact destruction box (Glotzer et al., 1991). Methylated ubiquitin, an inhibitor of ubiquitin-mediated proteolysis, blocks the degradation of cyclins A and B in clam embryo extracts (Hershko et al., 1991). Mutations in subunits of the yeast proteasome, a large complex required for the proteolysis of ubiquitinated proteins, arrest the cell cycle in in G2/M, in some cases with elevated levels of B-type cyclins and CDC2 kinase activity (Ghislain et al., 1993; Gordon et al., 1993). While these findings do not prove that a defect in cyclin B proteolysis is responsible for the cell cycle arrest, it is interesting that at least one yeast proteasome mutant can be suppressed by deletion of CLB2, the major yeast B-type cyclin (Friedman and Snyder, 1994)

The existence of non-degradable mutants of cyclin that retain the ability to activate cdc2 kinase made it possible to test the hypothesis that cyclin degrada-

tion and MPF inactivation are required to exit the mitotic state. Non-degradable mutants of cyclin A or B will arrest the cell cycle with elevated levels of kinase activity in embryonic extracts, in human cells, and in yeast (Murray et al., 1989; Ghiara et al., 1991; Luca et al., 1991; Gallant and Nigg, 1992) indicating that cyclin degradation is required for the inactivation of MPF. However, close examination of spindle morphology during mitotic arrest revealed that sister chromatid separation occurred normally, with arrest occurring in telophase (Holloway et al., 1993; Surana et al., 1993). Additionally, several mutant strains of *S. cerevisiae* complete anaphase without inactivating MPF (Surana et al., 1993). These data suggest that MPF inactivation is not required for the transition to anaphase, but rather that MPF must be inactivated for the cell to complete cytokinesis and return to the interphase state.

If MPF inactivation is not necessary for the initiation of anaphase, then what is the trigger for sister chromatid separation? When the cyclin N-terminus was added to mitotic *Xenopus* extracts at concentrations high enough to competitively block the degradation of endogenous cyclin, sister chromatid separation was also inhibited (Holloway et al., 1993). A similar fragment containing a mutated destruction box had no effect. This result suggests that destruction-box-dependent proteolysis is required for initiating anaphase. The key protein that is degraded is unknown, but it is unlikely to be a known cyclin, because the degradable cyclin fragment blocked anaphase onset in extracts that contained only exogenously added non-degradable cyclin. One attractive hypothesis is that this protein could play a physical role in holding sister chromatids together until it is degraded. There are examples of proteins other than cyclin that appear to be degraded at the end of mitosis. CENP-E is a kinesin-like protein that binds to kinetochores and may play a role in chromosome movement or spindle elongation (Yen et al., 1992). CENP-E is degraded at the end of mitosis, after cyclin B

degradation is initiated (Brown et al., 1994). Interestingly, this protein contains a sequence similar to the destruction box found in A-type cyclins, but whether this sequence targets the protein for degradation has not been tested.

Cyclin degradation could in principle be regulated by modifying the substrate, by activating the ubiquitination machinery, or through a combination of these mechanisms. Early characterization of the regulation of cyclin degradation in clam embryos suggested that degradation is controlled by the cell-cycle state of the extract rather than the cyclin (Luca and Ruderman, 1989). Recent biochemical fractionation of these extracts suggests that at least one component required for cyclin ubiquitination, which has not been identified, is mitotically regulated (Hershko et al., 1994). Other components, such as the ubiquitin-activating enzyme E1, and an unidentified ubiquitin-conjugating enzyme, appear to be constitutively active. Purified MPF can activate the mitotically-regulated fraction with a lag similar to that observed in the complete system, suggesting that the activation of this component by MPF is indirect. There is currently no positive evidence to suggest that phosphorylation of cyclin modifies its ability to be degraded. Furthermore, comparison of the N-termini and the destruction boxes of mitotic cyclins fails to show any conserved potential phosphorylation sites (Glotzer et al., 1991).

Interestingly, while cyclin A will induce some events of mitosis, it does not activate the degradation of itself or cyclin B when added to interphase extracts of clam or *Xenopus*, whereas cyclin B will initiate the degradation of both (Luca et al., 1991; Lorca et al., 1992). This difference may have evolved as mitotic cyclins were recruited to function in S phase. If A type cyclins were able to activate the degradation machinery, B type cyclins could not accumulate. Thus cyclin A, stripped of its ability to promote exit from mitosis, does not need to be subject to the same inhibitory modifications as cyclin B during the course of mitotic activa-

tion, and can be activated earlier in the cycle.

While the destruction-box-dependent proteolytic system for cyclins A and B may share some components, the degradation pathways may not be identical. For example, cyclin A is degraded during metaphase, in advance of cyclin B. Furthermore, cyclin B is stabilized by agents that block mitotic exit, such as microtubule inhibitors, while cyclin A is not (Whitfield et al., 1990; Minshull et al., 1990; Pines and Hunter, 1991; Hunt et al., 1992). One explanation for these findings is that some components of the cyclin degradation system may be activated early in mitosis to degrade cyclin A, while sister chromatid separation and cyclin B degradation might require additional components activated later in mitosis. This difference is supported by mutations in *S. cerevisiae* that arrest in telophase with elevated MPF activity and uncouple cyclin B degradation from the execution of anaphase (Surana et al., 1993; Toyn and Johnston, 1994).

The nature of the pathways leading to the activation of cyclin destruction are less well understood than the corresponding ones involved in activation of MPF. Phosphatases appear to play crucial roles in regulating both transitions. Type 2A phosphatases may be involved in inhibiting cyclin degradation (Lorca et al., 1991) while type 1 phosphatases may positively regulate degradation. Mutations of PP1 in *S. pombe* (Ohkura et al., 1989), *S. cerevisiae* (Hisamoto et al., 1994), *Drosophila* (Axton et al., 1990) and *Aspergillus* (Doonan and Morris, 1989) result in metaphase arrest. However, it is not clear that these phosphatases play a direct role in the activation of degradation; for they may be required for normal spindle assembly, and their inactivation may thus block exit from mitosis indirectly by activating a checkpoint control.

Resetting the mitotic cycle: the START transition

In embryonic systems, where cyclin degradation was first described, the

periods of cyclin instability are very brief (Figure 1-3A). In cycling clam embryos, cyclin is restabilized during telophase, allowing for rapid progression to the next mitosis (Hunt et al., 1992). Because cyclin restabilization coincided with MPF inactivation, the activity of the degradation system appeared dependent upon MPF activity. This suggested that MPF inactivation was sufficient to restabilize cyclin and allow the next cycle to occur.

Studies by Nasmyth and colleagues (Amon et al., 1994), however, suggest that the inactivation of MPF is not sufficient to inactivate cyclin degradation, since yeast arrested in G1 in the absence of MPF cannot accumulate cyclin B expressed from an inducible promoter. Instead, the protein is rapidly turned over with a half-life of less than one minute. Deletion of the destruction box increased stability 10-fold, but did not result in complete stabilization, suggesting that destruction-box independent mechanisms may also be active during this period. Perhaps most importantly, continued degradation during G1 did not require the continued presence of MPF activity. Thus, while MPF inactivation may be required to turn off the cyclin degradation system, the loss of MPF activity alone is insufficient to inactivate cyclin degradation and allow cyclin reaccumulation. Instead, it was found that expression of G1 cyclins is required to inactivate the cyclin B degradation system, a mechanism that ensures that G1 cyclins are produced in advance of B-type cyclins in the yeast cell cycle.

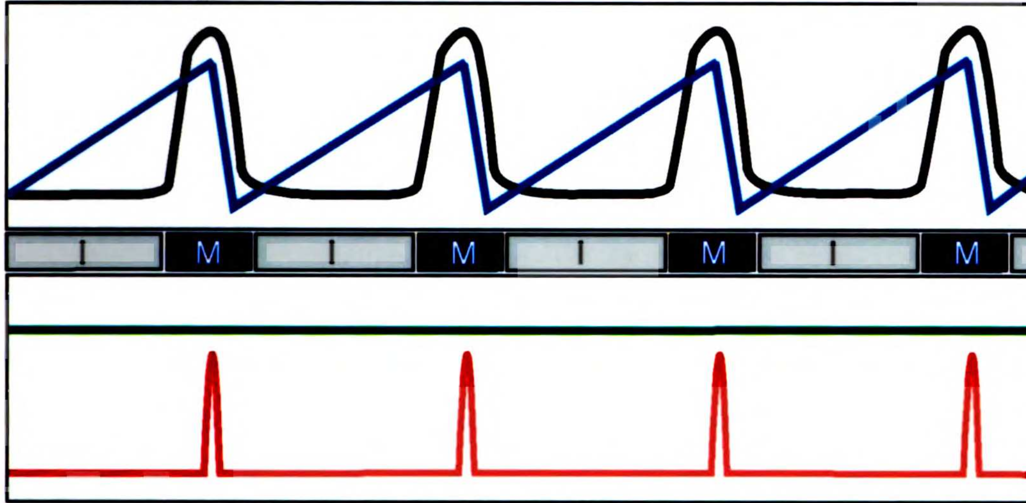
Although G1 cyclins have not been demonstrated to directly regulate cyclin B proteolysis in other eukaryotes, an interesting experiment suggests that this requirement may exist in mammalian cells. Okazaki et al. (1992) constructed a fusion protein composed of the N-terminus of *Xenopus* cyclin B2 and the oncogene mos to assess the function of mos during the G1 phase of the cell cycle. Interestingly, this fusion protein, expressed from a constitutive promoter in fibroblasts, is degraded in mitosis as expected, but does not reaccumulate until S

Figure 1-3. A model for the regulation of B-type cyclin proteolysis by G1 cyclins in embryos and somatic cells

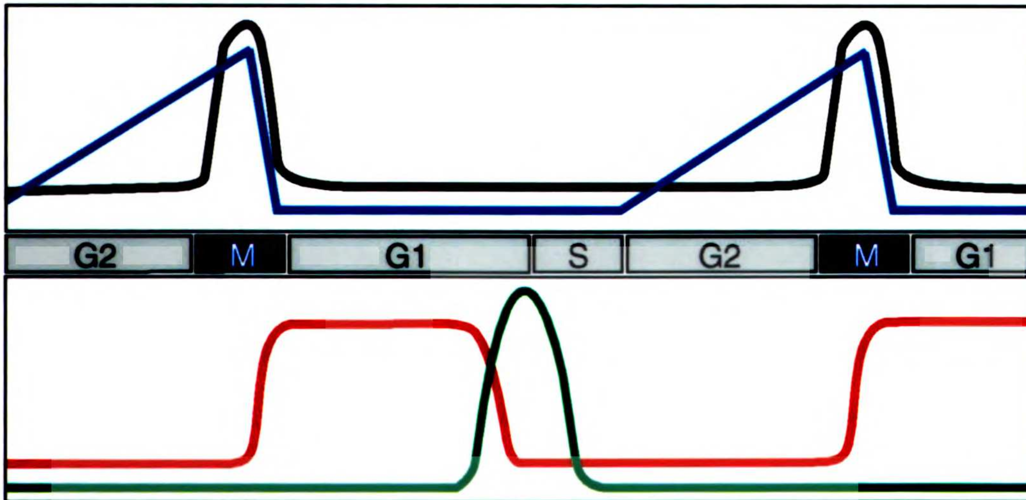
A. In early embryos, the cell cycle is characterized by rapid transitions between interphase and mitosis. The constitutive provision of G1 cyclin activity may result in immediate inhibition of the cyclin B degradation system following inactivation of MPF. This produces very brief periods of cyclin instability.

B. In somatic cells, the appearance of G1 cyclin activity is regulated. Therefore, cyclin proteolysis may not be inactivated immediately following inactivation of MPF, but may remain active throughout most of the G1 period. The induction of G1 cyclins plays a dual role in promoting entry into S phase and stabilizing cyclin B, committing the cell to another round of mitosis after DNA replication.

A. Embryo



B. Somatic Cells



CYCLIN B LEVELS
MPF ACTIVITY
G1 CYCLIN ACTIVITY
CYCLIN B INSTABILITY

Figure 1-3

phase. Mutation of the destruction box of this protein stabilizes it during exit from mitosis and in G1. This suggests that the period of mitotic cyclin instability in mammalian cells, as in yeast, is not confined to mitosis, but rather extends into G1.

What then limits the period of cyclin instability during rapid embryonic cycles? Knoblich et al. (1994) have analyzed the expression pattern of *Drosophila* cyclin E, a G1 cyclin, during early embryogenesis. Cyclin E mRNA is constitutively present during the first 15 cycles, when cells immediately enter S phase after completion of mitosis. However, cyclin E expression is down-regulated during mitosis 16, the final major embryonic mitosis, and cells then arrest in the subsequent G1 phase. These G1-arrested cells contain transcripts for cyclins A and B, but do not accumulate the proteins, suggesting that they may be unstable during this period. Interestingly, expression of cyclin E from a heat-shock promoter at this stage not only triggers S phase but also the entire mitotic cycle, suggesting that the presence of G1 cyclins can lead to the stabilization of G2 cyclins.

While these experiments do not tell us how mitotic cyclins become restabilized, they suggest that a requirement for G1 cyclins to stabilize G2 cyclins may be common to both embryonic systems and somatic cells (Figure 1-3). In early embryos, G1 cyclin activity may be constitutive, promoting immediate entry into S phase and immediate restabilization of mitotic cyclins after exit from mitosis. The ability of MPF to activate cyclin degradation would have to be dominant over the constitutive G1 cyclin activity that turns off degradation. When MPF is inactivated, the activity of G1 cyclins would inactivate the degradation system and initiate the accumulation of cyclin B for the next cell cycle. The dominant ability of MPF to turn on cyclin degradation is supported by the fact that expression of G1 cyclins during mitosis in budding yeast does not inhibit cyclin B degra-

dation (Amon et al., 1994). In somatic cells, where G1 cyclins are not constitutively provided, the cyclin degradation system may continue to be active during early G1. In somatic cells, the mitotic cycle driven by cyclin B alone is not a closed cycle; G1 cyclins stimulated by exogenous growth signals are required to turn off cyclin degradation and restart the mitotic cycle.

The cell cycle through the eyes of cyclin B

The mitotic cell cycle is characterized by three important transitions that drive the cell from one physiological state to another. The first, the interphase-prophase transition, is characterized by the abrupt activation of MPF and the extensive phosphorylation of many cellular components. The second, the metaphase-anaphase transition, induces the irreversible separation of sister chromatids, and initiates the inactivation of MPF and the reversal of mitotic phosphorylation. The third, the START transition, commits the cell to DNA replication and another round of mitosis. These transitions can be viewed through the eyes of cyclin B: cyclin B accumulation, cyclin B degradation, and cyclin B restabilization. The transitions illustrate several important regulatory features. The first transition is characterized by a threshold for cyclin B accumulation. The height of the threshold is a sum of cellular inputs that together are a measure of the cell's preparedness to enter mitosis. The key feature of the second transition is a lag following MPF activation that leads to the activation of the proteolytic system that degrades cyclin and initiates sister chromatid separation. This lag of unknown mechanism must be constructed to accommodate the variability in the time that may be required to assemble the spindle and align the chromosomes properly at the metaphase plate. The third transition, unlike the first two, is dependent on inputs external to the mitotic control circuitry. In embryonic cells, the constitutive provision of G1 cyclins may fulfill this requirement. In somatic cells,

these inputs may be contingent on extracellular signals, often mediated by transcriptional mechanisms. By making G1 cyclin synthesis contingent on external signals the somatic cell inserts a trigger in the otherwise self-sufficient mitotic oscillator.

Chapter 2

Sequence Elements Required for the Ubiquitination of Mitotic Cyclins

Abstract

Mitotic cyclins are rapidly degraded by ubiquitin-dependent proteolysis during mitosis. The destruction box, a conserved 9 or 10- amino acid motif in the cyclin N-terminus, is essential for ubiquitination and proteolysis. In this study, we have defined the sequence elements in the cyclin B N-terminus that are essential for its ubiquitination and subsequent degradation in mitotic *Xenopus* extracts. We find that although the N-terminus of cyclin B is sufficient for rapid degradation, the N-terminus of cyclin A is not. Mutagenic studies suggest that the cyclin A destruction box itself is responsible for this difference. Each of the highly conserved residues of the cyclin B destruction box is essential for rapid destruction. In contrast, non-conserved positions are not essential, but do contribute to the ability of the protein to be degraded. We demonstrate that polyubiquitination is essential for the degradation of cyclin-protein A fusion proteins. We have directly mapped the sites of ubiquitination on this protein and find that cyclin is ubiquitinated throughout the N-terminus, although the predominant site of ubiquitination lies within a cluster of lysines downstream of the destruction box. Mutational analysis indicates that any of the 10 lysines contained in the N-terminal 110 residues of *Arbacia* cyclin B can act as a functional ubiquitin acceptor site. Therefore, in contrast to the strict requirement for a functional destruction box, the secondary requirement for a specific ubiquitination site is relaxed.

Introduction

Mitotic cyclins were first discovered in marine embryos as proteins whose abundance fluctuates in phase with the cell cycle, accumulating during interphase and being degraded late in mitosis (Evans et al., 1983). The unusual dynamics of cyclin stability suggested that the protein might be an important regulator of cell division. This hypothesis was confirmed when cyclin B was identified, in partnership with Cdc2 kinase, as a component of Maturation-Promoting Factor (MPF), a protein kinase that regulates the transition from G2 to mitosis (see Murray and Kirschner (1989b) and Nurse (1990) for reviews). Subsequent work has identified a family of cyclin-dependent kinases (CDKs) and their corresponding cyclin activating subunits that regulate progression through G1 or S phase. However, only A-and B-type cyclins are degraded rapidly during mitosis. Cell-free extracts that undergo multiple mitotic cycles in vitro have provided a crucial experimental system for studying the mechanism of cyclin proteolysis (Murray and Kirschner, 1989; Luca and Ruderman, 1989). Using *Xenopus* extracts, Murray et al. (1989) demonstrated that the cyclin B N-terminus contains an essential determinant for its destruction, as deletion of the N-terminal 90 amino acids stabilized the protein during mitosis. Significantly, this protein dominantly blocked cell cycle progression with elevated levels of cdc2 kinase activity, indicating that cyclin proteolysis is necessary for the inactivation of MPF and exit from mitosis. Subsequent work demonstrated that the N-terminus of cyclin A is also necessary for its destruction (Luca et al., 1991). The destruction of mitotic cyclins appears to be a universal feature of the eukaryotic cell cycle, as non-degradable cyclins arrest cell cycle progression in all organisms so far examined (Murray et al., 1989; Ghiara et al., 1991; Gallant and Nigg, 1992; Surana et al., 1993; Luo et al., 1994; Rimmington et al., 1994).

The N-terminus of Sea Urchin cyclin B contains a signal that is sufficient

to confer M-phase specific destruction to a heterologous protein (Glotzer et al, 1991). Although there is little overall sequence conservation among the N-termini of mitotic cyclins, a 9- or 10- amino acid segment is conserved among A- and B-type cyclins. Mutation of a conserved arginine in this sequence, called the "destruction box," inhibits the degradation of a fusion protein containing the cyclin N-terminus (Glotzer et al., 1991). However, the effects of individual mutations at other positions in this sequence have not been tested in *Xenopus* extracts.

Sequences outside the destruction box also influence the ability of cyclin to be degraded during mitosis. Despite the fact that both cyclin B1 and cyclin B2 contain a destruction box, only the cyclin B1 N-terminus has been reported to be unstable when expressed independently (Holloway et al., 1993; van der Velden and Lohka, 1993). Furthermore, mutations of *Xenopus* cyclins A and B2 that interfere with binding to cdc2 also inhibit their ability to be degraded; such mutations have no effect on the degradation of *Xenopus* cyclin B1 (Stewart et al., 1994; van der Velden and Lohka, 1994). These studies indicate that the signals that target cyclin for proteolysis during mitosis may be complex.

The proteolysis of cyclin B is mediated by the mitosis-specific attachment of ubiquitin (Glotzer et al., 1991). The cyclin destruction box is required for this process, as mutations that interfere with proteolysis also block cyclin ubiquitination. Experiments in clam embryo extracts have demonstrated that inhibitors of polyubiquitin chain formation inhibit the degradation of both A- and B-type cyclins (Hershko et al., 1991). The attachment of a polyubiquitin chain to a substrate is believed to target the substrate for degradation by the 26S proteasome complex. The conjugation of ubiquitin to cyclin B in *Xenopus* and clam embryonic extracts is cell-cycle regulated, and components of the cyclin ubiquitin-conjugation systems have recently been identified (Hershko et al., 1994; Sudakin et al., 1995; King et al., 1995). However, it remains unclear how cyclin is

recognized by this ubiquitin conjugation system. Although the destruction box is essential for ubiquitination, the finding that the N-termini of cyclin A and cyclin B2 are not sufficient substrates suggests that other regions of cyclin, or even cdc2, may also be recognized by the destruction system. Recently, several non-cyclin proteins that are degraded either constitutively or during mitosis have been reported to contain sequences similar to destruction boxes (Brown et al., 1994; Galan et al., 1994). However, since this motif is brief and relatively degenerate, it is difficult to identify a functional destruction box by sequence similarity alone. It is therefore important to define other sequences in cyclin that are necessary for its rapid degradation.

The determinants of substrate specificty for ubiquitin conjugation have been explored in detail for only a few substrates. In general, the ubiquitin-dependent degradation of a protein requires two types of sequences: a sequence element that allows the protein to be recognized by the ubiquitin conjugation machinery, and a lysine residue that can act as a ubiquitin acceptor site. Perhaps the best-studied example is the N-end rule, which relates the half-life of a protein to the identity of its amino-terminal residue (Varshavsky, 1992). In budding yeast, the N-end rule is mediated by the binding of UBR1, a ubiquitin-protein ligase, to the amino terminus of the substrate (Bartel et al., 1990). UBR1 also binds a ubiquitin-conjugating enzyme, UBC2 or RAD6, that is necessary for ubiquitination of the substrate. This complex then catalyzes the attachment of a polyubiquitin chain to a lysine residue located 15-17 amino acids from the N-terminus of the model substrate (Bachmair and Varshavsky, 1989). The c-jun protein is also degraded by a ubiquitin-dependent pathway that requires the presence of the c-jun delta domain for ubiquitination (Treier et al, 1994). In contrast to the N-end rule substrate described above, c-jun appears to be ubiquitinated at multiple lysine residues throughout the protein. However, the sites of ubiquitin conjugation

have not been directly mapped for any natural substrates of the ubiquitin-dependent proteolytic system.

In this study, we have used a series of model substrates to define the sequence elements in the cyclin B N-terminus that are essential for its ubiquitination and subsequent degradation in mitotic *Xenopus* extracts. We find that each of the highly conserved residues of the destruction box is essential for rapid destruction. In contrast, non-conserved positions are not essential, but do contribute to the ability of the protein to be degraded. We demonstrate directly that polyubiquitination is essential for the degradation of cyclin-protein A fusion proteins. We have directly mapped the sites of ubiquitination on this protein and find that cyclin is ubiquitinated throughout the N-terminus, although the predominant site of ubiquitination lies within a cluster of lysines downstream of the destruction box. Mutational analysis indicates that any of the 10 lysines contained in the N-terminal 110 residues can act as a functional ubiquitin acceptor site. Therefore, in contrast to the strict requirement for a functional destruction box, the secondary requirement for a specific ubiquitination site is relaxed.

Materials and Methods

Construction of cyclin derivatives

All constructs for the expression of cyclin fragments and fusion proteins were created in vectors that contained a T7 RNA polymerase promoter. This enabled expression in vitro using T7 RNA polymerase, or in a suitable *E. coli* host that expresses T7 RNA polymerase, such as BL21 (DE3) (Studier and Moffatt, 1986). Plasmids expressing the *Arbacia* cyclin B derivatives 13-91 protein A, 13-66 protein A, and 13-54protein A are previously described (Glotzer et al., 1991). Construct 40-66 protein A was created by PCR-amplifying the region encoding residues 40-66 of *Arbacia* cyclin B. The product was digested with NsiI

and BssHII and used to replace the same fragment from 13-66prA. The resulting construct places methionine and histidine residues before residue 40 of *Arbacia* cyclin. The destruction box of 13-66prA sequence (RAALGNISN) was deleted in frame by PCR mutagenesis. A segment corresponding to residues 13-41 followed by valine and the BstB1 restriction site was amplified. The resulting product was digested with NsiI and BstB1 and used to replace the corresponding fragment from 13-66prA. To make derivatives of 13-66prA containing the destruction boxes of *Xenopus* cyclin B1, *Xenopus* cyclin A1, or *S. cerevisiae* Clb2, mutagenic oligonucleotides were used to amplify the region encoding residues 13-53 of *Arbacia* cyclin. The resulting fragments were used to replace the NsiI/BstBI fragment of 13-66prA. A similar strategy was used to make individual point mutations in the destruction box of 13-66prA (constructs N47V, R42A, L45A, and N50A).

To generate the CS1 derivative of 13-66prA that contained a Factor Xa cleavage site at the cyclin-protein A junction, a PCR reaction was performed using primers that amplified residues 13-66 of cyclin and inserted the residues IEGR following residue 66 of cyclin. The PCR product was digested with NsiI and BssHII and used to replace the corresponding fragment from 13-66 Protein A. To generate the CS2 derivative of 13-66prA that contained a Factor Xa cleavage site downstream of the destruction box, the sequence TAQAG spanning residues 54-58 of cyclin was mutated to IEGRI. Using the construct 13-66prA as a template, a PCR reaction was performed using an upstream mutagenic oligonucleotide and a second oligonucleotide that hybridized downstream of Protein A. This product was cut with BstBI and XbaI, and was used to replace the corresponding sequence from 13-66prA. A similar process was used to convert alanine 57 to methionine for cyanogen bromide cleavage.

To mutate the three N-terminal lysines of 13-66prA to arginine (13-66 prA-

R3), two complementary oligonucleotides that mutated each lysine to arginine were synthesized. These oligonucleotides were hybridized, digested with NsiI and AvaI, and used to replace the corresponding segment from 13-66prA. To mutate the four C-terminal lysines of 13-66prA to arginine (13-66prA-R4), a mutagenic oligonucleotide that converted the sequence KKVVKKD of cyclin (positions 60-66) to RRVVRRD was used in a PCR reaction that amplified residues 13-66 of cyclin. The product was digested with NsiI and BssHII and used to replace the corresponding fragment from 13-66prA. To generate 13-66prA-R7, the AvaI-XbaI fragment of 13-66prA-R4 was used to replace the corresponding fragment of 13-66prA-R3.

Lysine mutants of the *Arbacia* fragment 13-110 were generated in two steps. First, a DNA segment encoding residues 67-110 of *Arbacia* cyclin B was amplified by PCR. The upstream oligonucleotide introduced a silent SpeI mutation at position 66, preceded by an NdeI site. The downstream oligonucleotide introduced tyrosine and proline residues following residue 110, followed by a stop codon and a Bam HI restriction site. The PCR product was digested with NdeI and BamHI and ligated into the T7 expression vector pET11c (Novagen) cut with the same enzymes. The three lysine residues encoded by this fragment were mutated by amplifying the DNA segment encoding residues 67-110 with a mutagenic oligonucleotide. The fragment was digested with SpeI and BamHI and used to replace the corresponding fragment from 67-110. To generate the full length clones, derivatives of 13-66prA (wild-type, R3, R4 or R7) were amplified with oligonucleotides that introduced a SpeI site C-terminal to residue 66. The resulting products were then subcloned into the NdeI/SpeI site of pET11c derivative containing residues 67-110 of cyclin. It should be noted that the 13-110 fragment generated by this method differs from that published previously (Holloway et al., 1993), as it does not contain a pET leader peptide sequence at

its N-terminus.

N-terminal fusions of *Xenopus* cyclin B1 or *Xenopus* cyclin A1 to protein A were constructed by PCR-amplifying the appropriate region from pGEM vectors carrying the cDNAs for the corresponding cyclins (gift of J. Minshull, UCSF). These fragments were cut with NsiI and BssHII and used to replace the corresponding fragment from 13-66 Protein A. 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 between the NdeI and BamH1 sites. All constructs were verified by automated sequencing prior to expression.

Expression of substrates

For expression in reticulocyte lysate, supercoiled plasmid DNA was used to program TNT reticulocyte lysate (Promega) in the presence of ^{35}S-methionine (NEN) according to the manufacturer's instructions. This is a coupled *in vitro* transcription/translation system that contains T7 RNA polymerase and therefore does not require addition of purified mRNA. We have found this system to express proteins at greater and more reproducible levels than traditional reticulocyte expression programmed by addition of mRNA. Proteins were translated for 2 hours at 30 °C. Cycloheximide was added to 100 µg/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 (Glotzer 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) at a concentration of 1 μg/ml. Eggs were crushed by centrifugation 50 minutes after activation. Cycloheximide was added to 100 μg/ml, and extracts were frozen in the presence of 200 mM sucrose. To activate cyclin degradation, bacterially expressed *Arbacia* cyclin B lacking its N-terminal 90 amino acids (Glotzer et al., 1991) was added to thawed interphase extracts at a final concentration of 60 μg/ml. Extracts were incubated at 23 °C for 40 minutes 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. Cyclin degradation remains dependent on addition of Δ90 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 two-fold.

For degradation assays, we mixed 2 μl of TNT reticulocyte lysate with 18 μl of interphase or mitotic *Xenopus* extract containing 0.5 mg/ml bovine ubiquitin. Aliquots of 2 μl were quenched with 18 μl of SDS-sample buffer (63 mM Tris, pH 6.8, 2% sodium dodecyl-sulfate, 10% glycerol, and 100 mM dithiothreitol). Samples were boiled and separated on 12% SDS polyacrylamide gels. Gels were dried and phosphorimaged overnight using a Fuji phosphorimager. 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 which demonstrated the fastest half-lives. We found that the degradation rates using 35S-labeled substrates were comparable to those using the bacterially-expressed and iodinated counterparts.

Preparation of methylated ubiquitin

Bovine ubiquitin (Sigma) was reductively methylated as previously described (Hershko and Heller, 1985) and was dialyzed against XB buffer (10 mM HEPES, pH 7.7, 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM sucrose). The protein was concentrated to 15 mg/ml using a Centricon 3 concentrator, and stored at -70 °C.

Purification of his-tagged conjugates and cleavage with Factor Xa protease

Hexahistidine-tagged ubiquitin was the gift of J. Callis (University of California, Davis). To generate conjugates for cleavage, 60 ng (5 µl) of iodinated substrate (approximately 100 µCi/µg) was added to 25 µl of mitotic *Xenopus* extract in the presence of 20 µl his-tagged ubiquitin (8 mg/ml). Reactions were incubated at room temperature for 20 minutes and quenched by addition of 1 ml quench buffer (50 mM N-ethylmaleimide in 50 mM Tris, pH 8, and 100 mM NaCl). Nickel-agarose beads were added (100 µl) and the mixture incubated for 15 minutes at room temperature with rotation. The beads were pelleted in a microcentrifuge, and washed three times by addition of 1 ml of quench buffer, and twice in quench buffer containing 500 mM NaCl but lacking N-ethyl maleimide. Conjugates were eluted by addition of 200 µl elution buffer (50 mM Tris pH 8.0, 100 mM NaCl, 50 mM EDTA) and incubating for 15 minutes at room temperature. The beads were pelleted and the supernatant retained. Approximately 70% of the counts eluted from the beads under these conditions. Acetylated BSA was added to the eluted conjugates, and the proteins dialyzed into cleavage buffer (20 mM Tris, pH 8, 100 mM NaCl, 2 mM $CaCl_2$). Cleavage reactions were performed in a total of 40 µl and contained 0, 100 ng, 500 ng or 1.5 µg of protease and approximately 7,200 cpm of conjugates. Reactions were incubated for 3 hours 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 was mutated to methionine. The single methionine present in protein A was also mutated to alanine, so that the only internal methionine was provided by the newly engineered methionine. To generate conjugates, the iodinated protein (0.1 µg, approximately 100 µCi/µg) was incubated with 225 µl concentrated high speed supernatant derived from a mitotic extract in the presence of 1 mg/ml bovine ubiquitin. The reaction was incubated 20 minutes at room temperature, and the reaction quenched with 3.2 ml SDS sample buffer. The sample was boiled, and 880µl was loaded on each of four 11x14 cm 5-15% polyacrylamide gels in a single gel-width lane. Following electrophoresis, the sample was transferred to nitrocellulose (Schleicher and Scheull) using a semi-dry blotter (BRL). After drying, the filters were exposed to X-ray film for four hours at room temperature. Using the autoradiograph as a guide, slices of filter were obtained that corresponded to non-ubiquitinated, mono-ubiquitinated, di-ubiquitinated, and higher molecular mass cyclin-ubiquitin conjugates. To elute conjugates, filter strips were incubated for 24 hours at 37 °C with 1 ml of 1.8 M DTT in the presence of 1% Triton in a 1.5 ml tube. This procedure elutes approximately 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 (50µg) was added and the sample dialyzed against H_2O . The sample was then concentrated to 100 µl using a Centricon 10 (Amicon) ultrafiltration unit. The sample was resuspended in 2 ml H_2O and reconcentrated to 100 µl. Fifty microliters of this solution

was 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 H_2O, and evaporated again. The second evaporation step was essential 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

Conserved sequence elements in the N-termini of mitotic cyclins

Our analysis of the sequence elements required for cyclin destruction was guided by the evolutionary relationships evident from an alignment of the N-termini of mitotic cyclins. It was of interest to compare the amino termini of both A-and B-type cyclins, as these proteins are degraded at different periods during mitosis. Cyclin A is degraded during metaphase, while cyclin B degradation does not ensue until the beginning of anaphase. Furthermore, cyclin B is stabilized by agents that disrupt the integrity of the mitotic spindle; cyclin A is not. Figure 2-1 presents a schematic illustration of the N-terminal 120 amino acids of a selected set of A- and B-type cyclins that span a wide evolutionary distance. Among B-type cyclins, the destruction box is the most highly conserved sequence element within the N-terminus. A-type cyclins also contain a conserved destruction box, although the degree of conservation is not as great (see below). A-type cyclins also contain an additional conserved motif, FX(V/I)(F/Y/H)XD, located at position 94-99 of *Xenopus* cyclin A, that is not found in B-type cyclins. Figure 2-1 also indicates the distribution of lysine residues in the N-termini of mitotic cyclins. The N-termini of B-type cyclins appear to be enriched in lysine residues compared to the N-termini of A-type cyclins. We explore potential consequences of this difference below. The cyclin N-terminus is not required for binding to cdc2 kinase;

Figure 2-1. Schematic representation of the N-termini of mitotic cyclins

The N-terminal 120 amino acids of a selected set of mitotic cyclins are shown. The black box indicates the position of the destruction box, the most highly conserved sequence element in the N-terminus. The white box indicates an additional conserved motif, FX(V/I)(F/Y/H)XD that is found only in the N-termini of A-type cyclins. Black circles represent lysine residues, which are enriched in B-type cyclins compared to A-type cyclins.

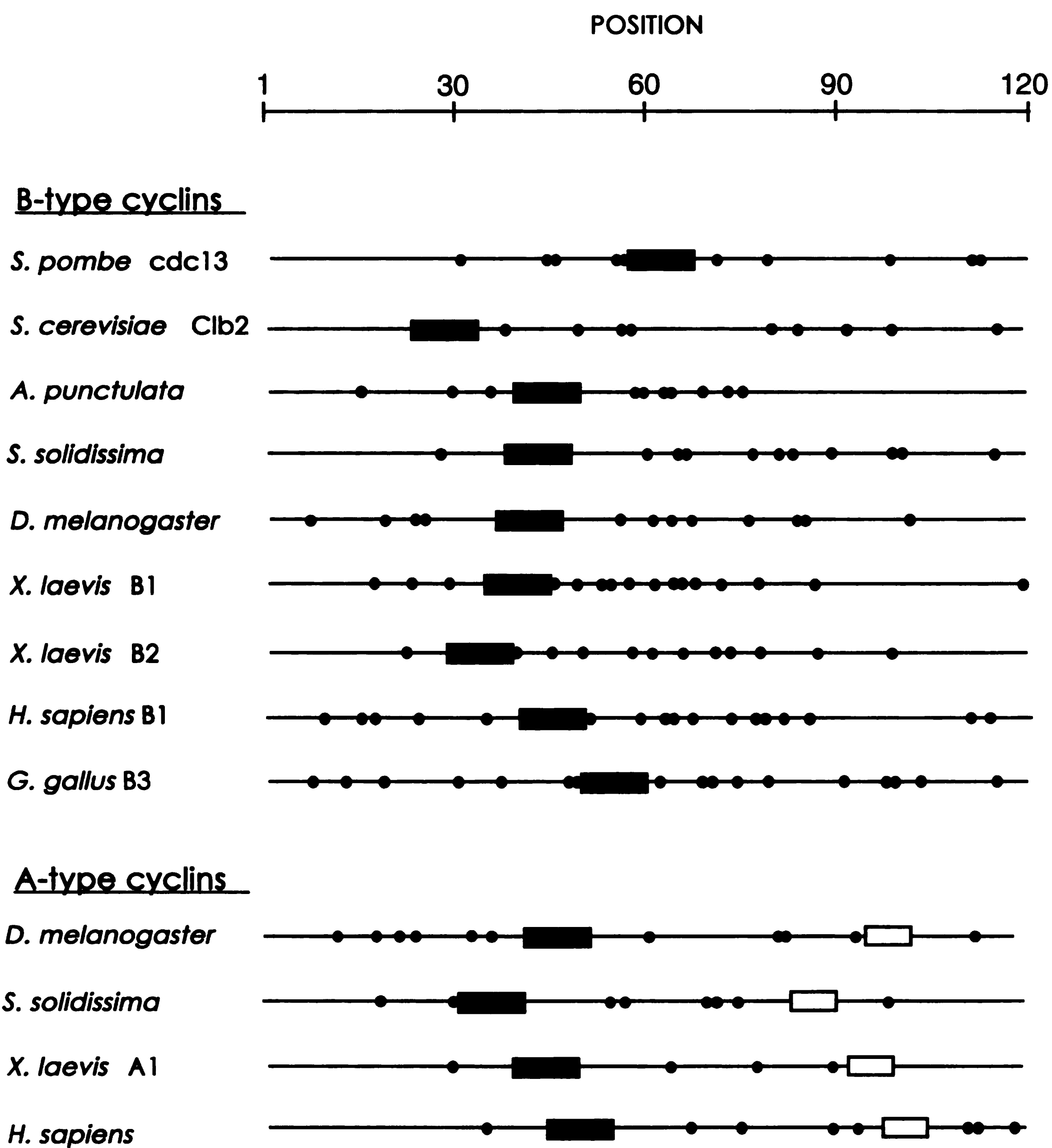


Figure 2-1

rather, binding is mediated by the cyclin box, a conserved sequence located further downstream in the protein. In addition to mediating degradation, the N-terminus of B-type cyclins contains a signal that retains it in the cytoplasm (Pines and Hunter, 1994). In human cyclin B1, this region is found between residues 109 and 154.

Figure 2-2 presents an alignment of sequences containing the destruction box motif found in A- and B-type cyclins. The two most highly conserved residues are found at positions one and four of this sequence, consisting of arginine and leucine, respectively. These residues appear to be conserved to an equivalent extent in both A- and B-type cyclins. Interestingly, a recently-described mitotic cyclin, chicken cyclin B3, contains phenylalanine rather than leucine at position 4. This difference appears to be conserved in *Xenopus* cyclin B3 as well (T. McGarry and M. Kirschner, personal communication). Positions 2 and 3 are not as highly conserved, but consist largely of small aliphatic residues, especially alanine. The second half of the destruction box exhibits interesting differences between A- and B-type cyclins. In general this portion of the destruction box is not as highly conserved among A-type cyclins as it is among B-type cyclins. A- and B-type cyclins differ consistently at positions 6 and 9: in B-type cyclins position 6 is predominantly asparagine, aspartic acid or glutamic acid; in A-type cyclins, valine and threonine predominate. Among B-type cyclins, the asparagine at position 9 is highly conserved; however, in A-type cyclins this position shows considerable variability. Interestingly, those A-type cyclins that do not contain asparagine at position 9 often, but not always, contain asparagine at position 10 or 11. Thus while B-type destruction boxes appear to consist solely of 9 residues, the A-type destruction box may extend to 10 or more residues.

Figure 2-2. Alignment of the destruction box sequences of mitotic cyclins

The N-terminal region of the mitotic cyclins that contains the destruction box is shown. B-type destruction boxes show the highest degree of conservation at positions 1, 4, and 9. A-type destruction boxes show strong conservation only at positions 1 and 4. A- and B-type cyclins differ consistently at position 6: asparagine, aspartate, and glutamate predominate in B-type cyclins, while this residue is valine or threonine in A-type cyclins.

B-type cyclins

		Destruction Box	
		1 2 3 4 5 6 7 8 9	
S. pombe cdc13	M E A T T K	R H A L D D V S N	F H N K E G
S. cerevisiae Clb1	Q K R N V P	R T I L G N V T N	N A N I L Q
S. cerevisiae Clb2	F L R N V Q	R L A L N N V T N	T T F Q K S
A. nidulans	N G T Q R K	R A A L G D V S N	V G K A D N
A. punctulata	V Q K P A Q	R A A L G N I S N	V V R T A Q
S. solidissima	T S H A S Q	R N T L G D I D N	Q V S A I T
X. laevis B1	T N V P K K	R A A L G D L Q N	R G I S R P
X. laevis B2	K P G L R P	R T A L G D I G N	K A E V K V
D. melanogaster	V Q M N S R	R A A L G E I G N	K V T V R G
M. musculus B1	K P G L R P	R T A L G D I G N	K V S E E L
H. sapiens B1	P G L R P N	R T A L G D I G N	K V S E Q L
G. gallus B3	Q G G P K K	R S A F G D I T N	A H K N Q V
A. thaliana	V A K G R N	R Q V L G D I G N	V V R G N Y

A-type cyclins

A. punctulata	G P Q A C K	R A A L G T I T N	V S S T R V
S. solidissima	G L S G P K	R A A L G V I T N	Q V N Q Q V
P. vulgata	N V A V A K	R S A L G T I T N	Q N I R V G
D. melanogaster	S P M S V D	R S I L G V I Q S	S D I S V G
X. laevis A1	Q P N L P Q	R T V L G V I G D	N E Q R R R
X. laevis A2	L V P V G G	R T V L G V L Q E	N H R G P K
G. gallus	A D A P G L	R A A L G T V G E	R R P L A P
B. taurus	A Q Q P R T	R A G L A V L R A	G N S R G P
H. sapiens	V Q Q P R T	R A A L A V L K S	G N P R G L

Figure 2-2

The N-terminus of cyclin B contains sequences sufficient to signal mitosis-specific destruction while the N-terminus of cyclin A does not

Previous studies have examined the ability of the N-terminus of *Arbacia punctulata* (sea urchin) cyclin B to confer mitosis-specific destruction to a heterologous protein, Staphylococcal protein A (Glotzer et al., 1991). It has also been demonstrated that the N-terminus of *Arbacia* cyclin B, when expressed independently, is degraded and ubiquitinated in a mitosis-specific and destruction-box dependent manner in *Xenopus* egg extracts (Holloway et al., 1993; King et al., 1995). These experiments were performed using bacterially-expressed and purified proteins that were subsequently radiolabeled by oxidative iodination. To eliminate the possibility that oxidation of the substrate is essential for its degradation, we expressed these proteins in reticulocyte lysate in the presence of ^{35}S-methionine, and then measured degradation when mixed with a 9-fold volume excess of interphase or mitotic *Xenopus* extract. Stable mitotic extracts were generated from interphase extracts by addition of the non-degradable Δ90 form of *Arbacia* cyclin B. This protein activates cdc2 kinase and the cyclin degradation system in a constitutive fashion (Glotzer et al., 1991). Figure 2-3A indicates that both the fusion protein containing the N-terminal 13-91 amino acids of *Arbacia* cyclin B and the independently expressed N-terminus are stable in interphase extracts, but are rapidly degraded when added to mitotic extracts. Therefore, substrate oxidation does not contribute to instability. Furthermore, reticulocyte lysate added to 10% final volume does not have a significant impact upon the kinetics of degradation. Because of the variability associated with radioiodination, subsequent experiments were performed using proteins expressed in reticulocyte lysate.

We next tested whether N-terminal fragments of *Xenopus* cyclin B1 or *Xenopus* cyclin A1 were degraded in a similar fashion when expressed indepen-

Figure 2-3. The N-terminus of cyclin B is sufficient for degradation, while 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 ^{35}S-methionine. Lysates were then mixed with a nine-fold volume excess of interphase or mitotic Xenopus extract. Aliquots were taken at 0, 5, 10, 20 and 40 minutes following addition, and analyzed by gel electrophoresis and phorphorimaging.

(A) *Arbacia puntulata* cyclin B derivatives.

(B) *Xenopus laevis* cyclin B1 derivatives.

(C) *Xenopus laevis* cyclin A1 derivatives.

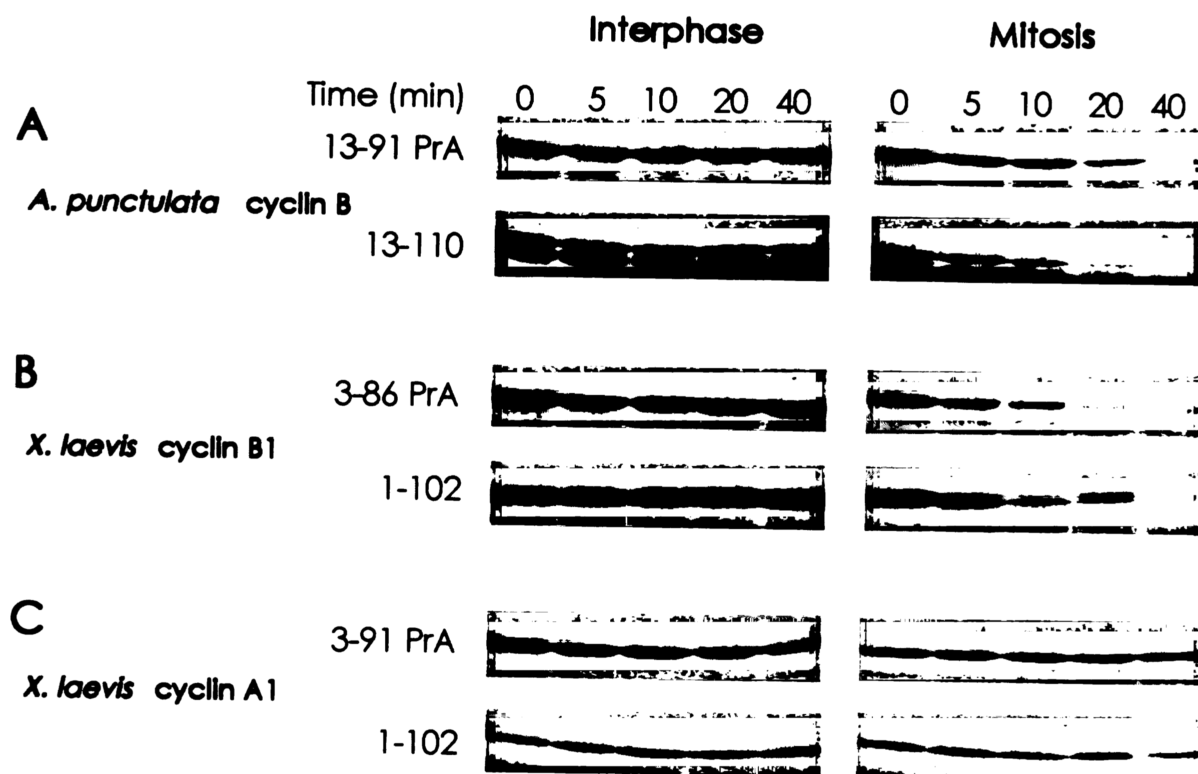


Figure 2-3

dently or as fusion proteins. Figure 2-3B shows that residues 3-86 of *Xenopus* cyclin B1 could efficiently direct the destruction of protein A in a mitosis-specific fashion. A fragment consisting of the N-terminal 102 residues of *Xenopus* cyclin B1 was similarly degraded in mitosis but stable in interphase. However, when residues 3-91 of *Xenopus* cyclin A were fused to protein A, the resulting protein was stable in both mitotic and interphase extracts (Figure 2-3C). The same result was obtained when a fragment consisting of the N-terminal 102 residues of cyclin A was expressed independently. These data suggest either that the cyclin A degradation machinery is not functional in these extracts, or that the cyclin A N-terminus does not contain all of the information necessary to direct mitosis-specific degradation.

Identification of a minimal destruction element

To determine the minimal sequence element in cyclin sufficient to target a heterologous protein for destruction in mitosis, we fused different portions of the cyclin B N-terminus to protein A. These proteins were expressed in reticulocyte lysate and the degradation rates measured in the presence of a 9-fold excess of mitotic extract. Half-lives were determined by quantitative analysis using a phosphorimager. Because we have found that extracts can vary substantially in their ability to degrade cyclin, all of the measurements described below were made using the same preparation of frozen extract.

Figure 2-4A shows that a protein A fusion containing the N-terminal 13-91 amino acids of *Arbacia* cyclin B was rapidly degraded in mitotic extracts with a half-life of 4 minutes. We first analyzed the effect of deleting sequences C-terminal to the destruction box. A fusion containing residues 13-66 was also rapidly degraded, but the half-life was increased to 9 minutes. A construct containing residues 13-54 showed a greater defect, with a half-life extended to 26 minutes.

Figure 2-4. 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 black line represents the cyclin portion, while the gray rectangle represents protein A. Only the cyclin portion is drawn at proper scale; protein A is in fact 4-fold larger than the cyclin sequence. The destruction box is indicated by a black rectangle, and lysine residues are indicated by black circles. Proteins were expressed in reticulocyte lysate and mixed with a 9-fold excess of mitotic extract. Samples were taken at 0, 10, 20, 40, and 60 minutes, and analyzed by gel electrophoresis and phosphorimaging. Half-lives were determined by quantitative phosphorimager analysis.

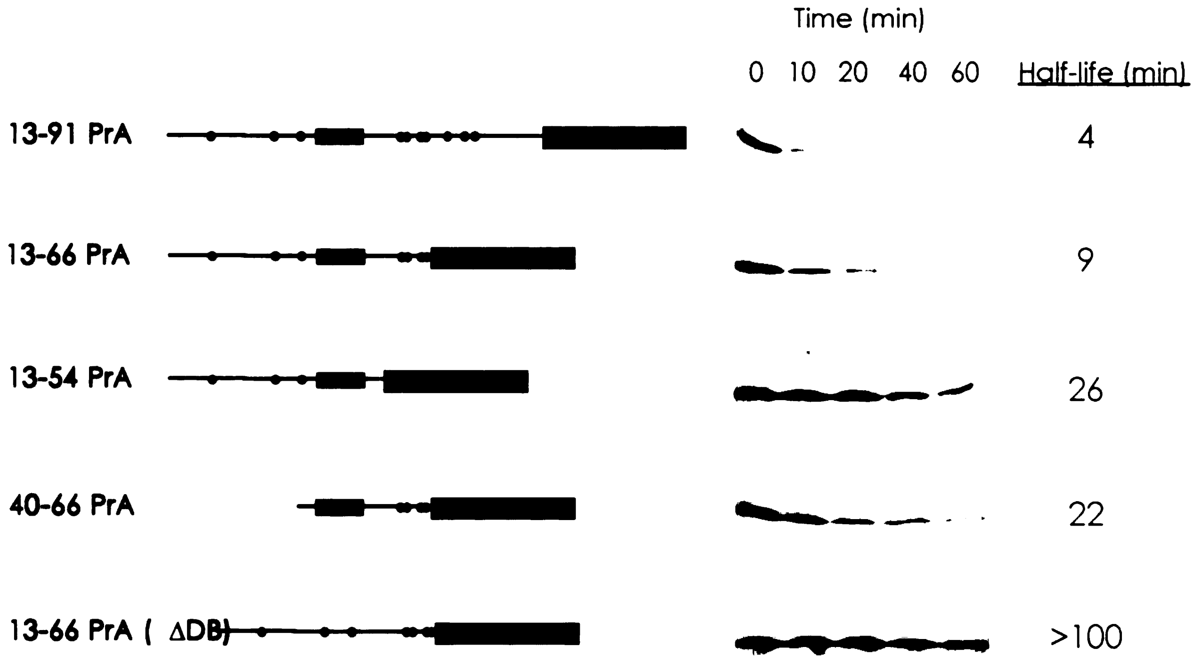


Figure 2-4

We next tested the effect of removing sequences N-terminal to the destruction box. Deleting residues 13-39 from 13-66pr A, yielding 40-66prA, increased half-life from 10 to 20 minutes. Deletion of the destruction box from 13-66prA, however, completely stabilized the protein, indicating that these sequences are essential for rapid proteolysis. In contrast, sequences N-terminal and C-terminal to the destruction box do not appear to be essential for degradation, but can contribute substantially to the overall rate of proteolysis. In other preparations of extract that did not that did not degrade cyclin as rapidly, the half lives of each substrate appeared to increase proportionately. For example, in extracts where 13-66prA exhibited a half-life of 20 minutes, 40-66prA was degraded with a half-life of approximately 40 minutes. Such extracts exhibited a similar defect in degrading 13-54prA.

We performed a similar analysis using the N-terminus of *Xenopus* cyclin B1 fused to protein A (Figure 2-4B). Fusion of cyclin B1 residues 3-86 to protein A yielded a protein that was degraded with a half life of approximately 2 minutes in mitotic extracts. A fusion protein containing residues 31-67 was degraded with a half life of 10 minutes, suggesting that, as for *Arbacia* cyclin B, sequences upstream of the destruction box are not absolutely essential for degradation. We found, however, that residues 31-55 of *Xenopus* cyclin B1 were not sufficient to direct the degradation of protein A (data not shown). Therefore, sequences between residues 55 and 67 of *Xenopus* cyclin B1 are necessary for targeting Protein A for destruction.

Analysis of the destruction box of mitotic cyclins

Mutation of the conserved arginine at the first position of the cyclin B destruction box stabilizes cyclin-protein A fusions in mitotic *Xenopus* extracts (Glotzer et al., 1991). However, the contributions of other positions have not been

tested in this system. In *S. cerevisiae*, mutation of the conserved arginine or leucine in the destruction box of Clb2 results in a partial stabilization that is not as significant as that observed for a deletion of the destruction box (Amon et al., 1994). We explored the effect of mutation of conserved and non-conserved residues in the destruction box on the rate of proteolysis in mitotic *Xenopus* extracts. We chose to use the *Arbacia* fusion protein 13-66prA as a model substrate in these studies. To ensure that the results we obtained would not be an artifactual result of testing *Arbacia* derivatives in a *Xenopus* extract, we first replaced the destruction box of this construct with the corresponding sequence from *Xenopus* cyclin B1. Figure 2-5 indicates that this construct was degraded rapidly, with a half-life of 11 minutes. This experiment demonstrates that the cyclin destruction machinery can recognize the *Xenopus* B1 destruction box in the context of the *Arbacia* N-terminus. This replacement is comparable to a triple mutation of alanine to threonine at position 2, asparagine to aspartate at position 5, and serine to glycine at position 8, indicating that such alterations are well-tolerated.

To further explore the evolutionary conservation of the mitotic destruction machinery, we replaced the *Arbacia* destruction box in 13-66prA with the corresponding sequence from the *S. cerevisiae* mitotic cyclin Clb2 . This is equivalent to the quadruple mutation of alanine to leucine at position 2, glycine to asparagine at position 5, isoleucine to valine at position 7, and serine to threonine at position 8. Figure 2-5 demonstrates that this protein was degraded as rapidly as the *Arbacia* protein in mitotic extracts. This result was somewhat surprising as a fusion of the N-terminal 60 amino acids of Clb2 to protein A was not degraded in mitotic *Xenopus* extracts (data not shown). Thus, although glycine is the predominant amino acid at position 5 of the destruction box, asparagine is also tolerated. This data suggests that the components that recognize the cyclin B

Figure 2-5. Mutagenic analysis of the cyclin destruction box

A series of derivatives containing residues 13-66 of *Arbacia* cyclin B fused to protein A were constructed. The residues that differ from the sequence of wild type *Arbacia* cyclin are indicated in bold. Substrates XI B1 DB, Clb2 DB, and XIA1 DB contain precise replacements of the destruction box of 13-66prA with the corresponding sequences from *Xenopus* cyclin B1, *S. cerevisiae* Clb2, 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.

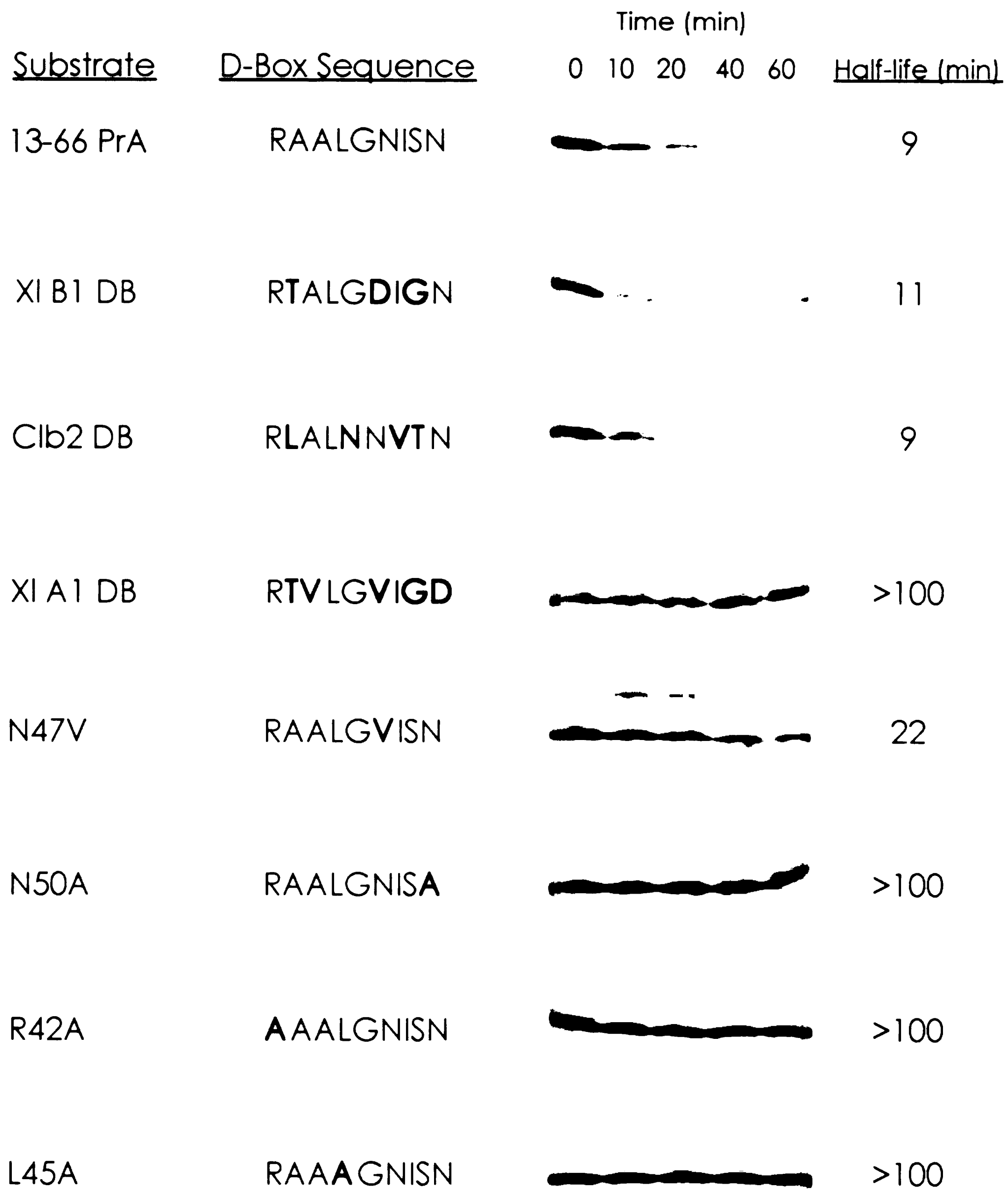


Figure 2-5

destruction box are conserved between *Xenopus* and yeast.

This experimental approach allows us to determine whether the inability of an N-terminal cyclin fragment to be degraded stems from a defect within the destruction box or from sequences outside the destruction box. As described above, the N-terminal 92 residues of *Xenopus* cyclin A1 did not direct the degradation of protein A in mitotic extracts. We therefore replaced the *Arbacia* destruction box with the corresponding 9-amino acid sequence from *Xenopus* cyclin A1. Figure 2-5 shows that the *Xenopus* A1 destruction box did not efficiently support degradation in mitotic extracts. There are three amino acid differences between the *Xenopus* B1 destruction box, which works efficiently in this context, and the *Xenopus* A destruction box, which does not. Position 3 is alanine in cyclin B1, but valine in cyclin A. This is unlikely to cause the defect, as this is a conservative mutation and valine is found at this position in other B-type cyclins, such as chicken cyclin B2. Position 6 of the destruction box, however, differs consistently between A and B-type cyclins. We therefore mutated the asparagine at position 6 of the *Arbacia* destruction box to valine, which is found in *Xenopus* cyclin A1. Figure 2-5 indicates that this point mutation increased the half life of the protein from 11 to 22 minutes. However, the mutation was not completely stabilizing, as we found for the protein containing the complete cyclin A1 destruction box. The final position of the destruction box is a highly conserved asparagine in B-type cyclins. However, in A-type cyclins this position is not highly conserved. Figure 2-5 demonstrates that mutation of asparagine to alanine is alone sufficient to stabilize a protein containing an otherwise wild-type cyclin B destruction box. These studies suggest that changes at positions 6 and 9, but especially the latter, explain why the cyclin A destruction box is not functional in the context of a cyclin B N-terminus.

We also tested the effect of single point mutations at the other highly

conserved positions of the destruction box. Conversion of arginine at position one of the destruction box to alanine completely inhibited degradation, as expected (Figure 2-5). Mutation of leucine at position 4 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 very hydrophobic residues are tolerated at this position.

Polyubiquitination correlates with degradation and is essential for proteolysis

Previous work has demonstrated that cyclin-protein A fusions are degraded by the ubiquitin pathway in mitotic *Xenopus* extracts (Glotzer et al., 1991). Mutation of the conserved arginine to cysteine at position one of the destruction box inhibits ubiquitination in mitotic extracts (Glotzer et al., 1991). To establish that other sequences in the destruction box are involved in targeting the protein for ubiquitination, we tested the mutant proteins described above for their ability to be ubiquitinated in mitotic *Xenopus* extracts. These proteins were expressed in *E. coli*, purified using IgG sepharose affinity chromatography, and radiolabeled with ^{125}I. This method of labeling produces substrates of much higher specific activity and therefore allows the detection ubiquitinated intermediates. Figure 2-6 shows that mutation of any of the conserved positions of the destruction box resulted in an inability to be efficiently ubiquitinated. However, we did notice low levels of ubiquitination of the R42A mutant, while the L45A and N50A mutations completely abolished ubiquitination. We have obtained similar results when the R42A and N50A mutants are tested in the context of the independently-expressed 13-110 N-terminal fragment of Arbacia cyclin B (King et al., 1995; data not shown). Mutation of other positions such asparagine to valine at

Figure 2-6. Ubiquitination of cyclin derivatives containing a mutated destruction box

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 minutes prior to 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 prep to prep.

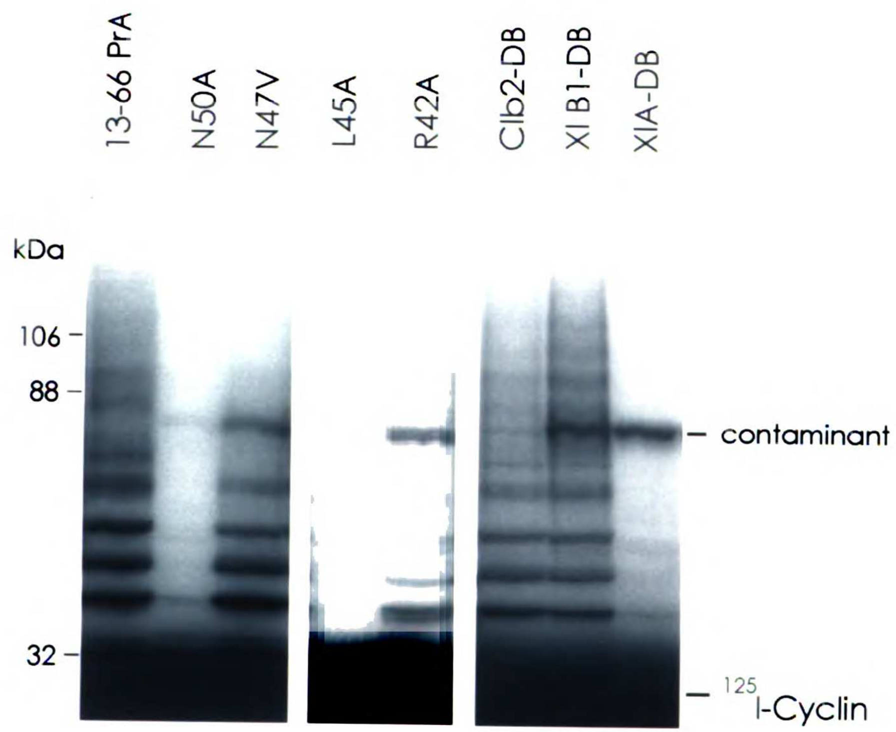


Figure 2-6

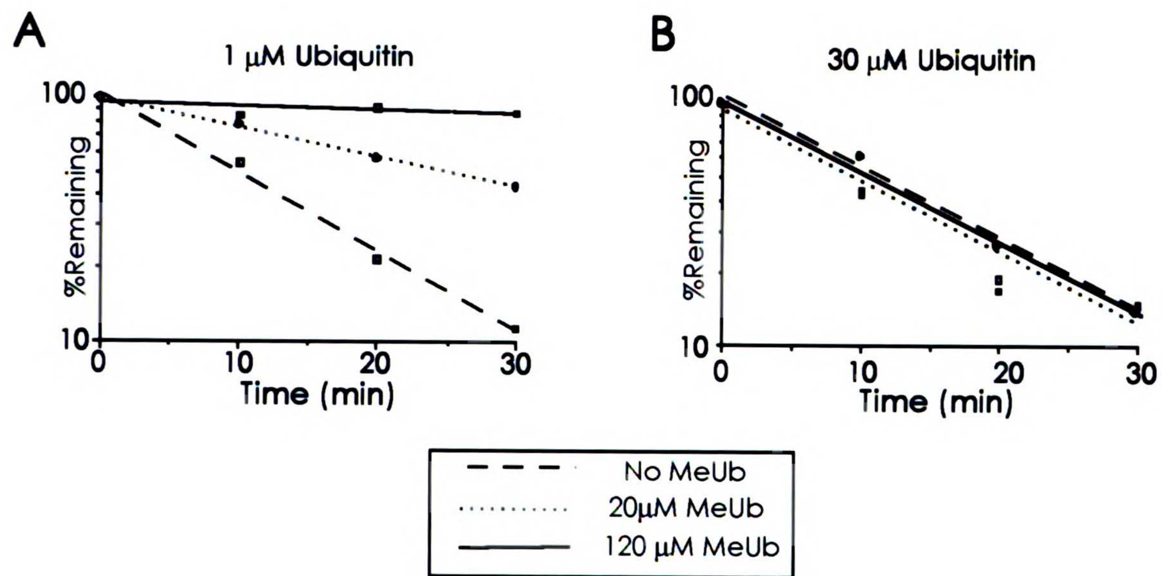
position six resulted in a partial defect in ubiquitination, consistent with the lower rate of proteolysis described above. Proteins containing the *Xenopus* B1 or Clb2 destruction box were efficiently ubiquitinated, while a protein containing the *Xenopus* A-type destruction box was not. In all cases examined, the relative rate of proteolysis correlated with the extent of polyubiquitination. This data provides strong evidence for the hypothesis that the primary function of the destruction box is to target the protein for ubiquitination.

Hershko and colleagues (1991) 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. However, due to the labeling techniques employed, ubiquitinated intermediates could not be observed. We therefore tested whether methylated ubiquitin could inhibit the proteolysis of iodinated *Arbacia* 13-66prA in *Xenopus* extracts, where ubiquitinated intermediates can be observed. We found that methylated ubiquitin strongly inhibited proteolysis in a dose-dependent fashion when added to crude extracts (Figure 2-7A). This inhibition was specific because it could be overcome by addition of unmodified ubiquitin (Figure 2-7B). Because methylated ubiquitin is not incorporated into conjugates as efficiently as unmodified ubiquitin, this reversal of inhibition could be accomplished at a four-fold lower dose of unmodified ubiquitin. In a separate experiment using the iodinated N-terminal *Arbacia* cyclin fragment 13-110, we analyzed the pattern of cyclin-ubiquitin conjugates that accumulated in the presence of 120 μM methylated ubiquitin. In the absence of methylated ubiquitin, high-molecular mass cyclin-ubiquitin conjugates were transiently observed during the course of degradation (Figure 2-7C). In the presence of methylated ubiquitin, the substrate instead accumulated as mono-, di-, and tri-ubiquitinated species; higher molecular mass forms were not observed. Because methylated ubiquitin is incapable of forming polyubiquitin chains, this

Figure 2-7. Polyubiquitination is essential for cyclin degradation

(A, B) Bacterially-expressed and iodinated 13-66 Protein A was added to a mitotic *Xenopus* extract that contained either 1μM unmodified ubiquitin (A) or 30 μM unmodified ubiquitin (B). Increasing amounts of methylated ubiquitin were added to each of these extracts. Samples were taken at 0, 10, 20, and 30 minutes, and analyzed by gel electrophoresis and phosphorimaging.

(C) 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 1, 10, 20, and 30 minutes following substrate addition, and analyzed by gel electrophoresis and autoradiography.



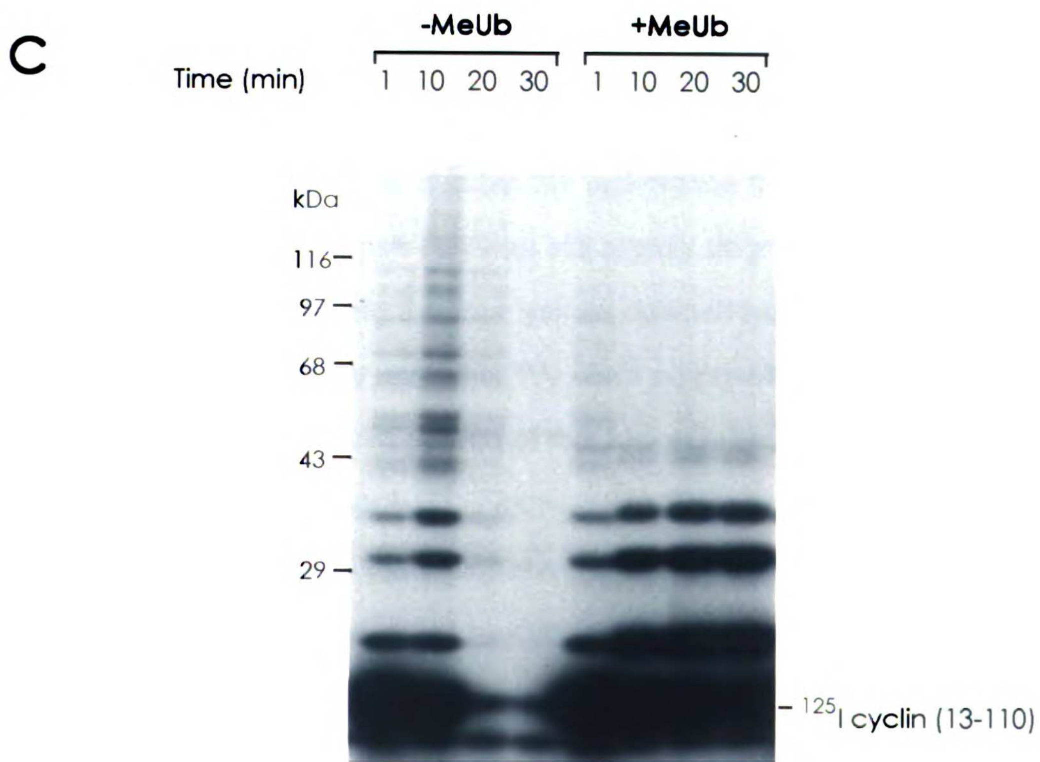


Figure 2-7

data suggests that the cyclin N-terminus can be monoubiquitinated at multiple sites.

Identification of ubiquitination sites in the cyclin N-terminus

The ubiquitination of a substrate requires the presence of at least one lysine residue that can serve as a ubiquitin acceptor site. We expected that sequences neighboring the destruction box in the cyclin N-terminus could function as potential ubiquitin acceptor sites, especially since B-type cyclin N-termini are relatively lysine-rich (Figure 2-1). Deletion of sequences N-terminal or C-terminal to the destruction box of Sea Urchin cyclin B-Protein A fusions resulted in partial stabilization (Figure 2-3). 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 non-ubiquitinable arginine residues. We tested the ability of these mutant proteins, expressed in reticulocyte lysate, to be degraded in mitotic *Xenopus* extract. Figure 2-8 shows that a substrate lacking the three lysines N-terminal to the destruction box (13-66prA-R3) was still rapidly degraded in mitotic extracts. Similarly, a substrate lacking the four lysines downstream of the destruction box (13-66prA-R4) was equally unstable. We were surprised to find that elimination of all seven lysines in the cyclin portion of this fusion protein resulted in only a doubling of half life (13-66prA-R7; Figure 2-8A). These experiments suggest that multiple lysine residues in this substrate, including those in protein A, which contains 18 lysine residues, must be capable as functioning as ubiquitin acceptor sites.

To directly determine the sites of ubiquitin conjugation to 13-66prA, we constructed two mutant proteins that contained a Factor Xa protease cleavage

Figure 2-8. Lysine residues in the cyclin N-terminus are not essential for degradation when the N-terminus is fused to protein A

Three different mutant forms of the *Arbacia* substrate 13-66prA are shown. 13-66 prA-R3 contains the three lysine to arginine substitutions upstream of the destruction box (black rectangle). 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 in the legend to Figure 2-5.

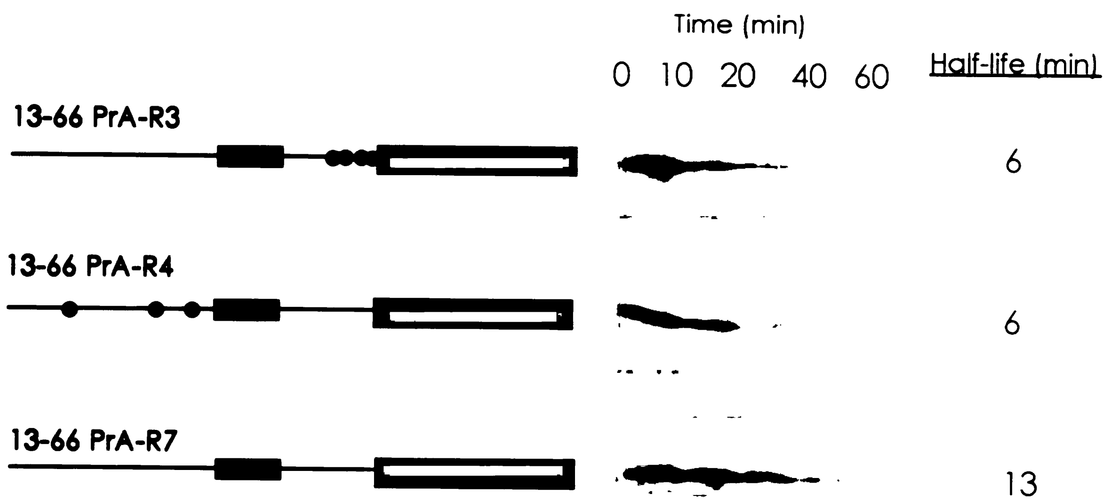


Figure 2-8

site either at the cyclin-protein A junction (Cleavage Substrate 1 [CS1]) or just downstream of the destruction box (Cleavage Substrate 2 [CS2]; Figure 2-9A). These mutations had no effect on the ability of these proteins to be degraded or ubiquitinated in mitotic extracts (data not shown). These fusion proteins were expressed in *E. coli* and purified using IgG- sepharose affinity chromatography. Figure 2-9B shows that cleavage of CS1 produced a C-terminal fragment of the expected size of 20 kDa, while cleavage of CS2 produced a slightly larger product, indicating that cleavage occurred at the introduced site. Because protein A is the only portion of these fusion proteins that contains tyrosine, ^{125}I is incorporated exclusively into this portion of the protein. Therefore, only the C-terminal fragment of CS1 and CS2 remains labeled following cleavage.

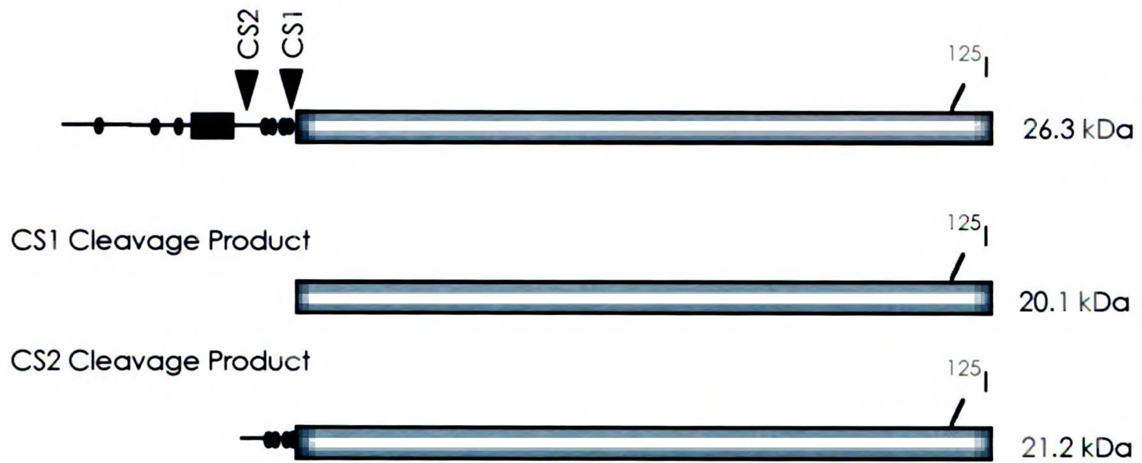
To isolate cyclin-ubiquitin conjugates, we added hexahistidine-tagged ubiquitin (Beers and Callis, 1993) to mitotic extracts in the presence of these iodinated 13-66prA derivatives. Ubiquitinated proteins were purified using nickel affinity chromatography. This purification procedure allowed the isolation of ubiquitinated fusion protein free of non-ubiquitinated substrate (Figure 2-10). The purified conjugates were then cleaved 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 following treatment with protease. This is illustrated in Figure 2-10, which demonstrates that 13-66prA-ubiquitin conjugates that lacked a Factor Xa cleavage site were not cleaved by the protease, although a slight amount of non-specific cleavage was observed at the highest protease concentration. 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 predominant bands that correspond by molecular mass to unmodified protein A and monoubiquitinated protein A. While a slight amount of polyubiquitinated protein A

Figure 2-9. Structure and cleavage products of substrates containing a Factor Xa cleavage site

(A) Schematic representation of cleavage derivatives of the *Arbacia* substrate 13-66prA. Each of these substrates is drawn to scale; protein A is represented by the gray rectangle. The sites of cleavage in substrates CS1 and CS2 are indicated, along with the predicted size of the C-terminal cleavage fragment.

(B) Substrates CS-1 and CS-2 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 hours. Samples were then separated by SDS-PAGE and the gels 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.

A



B

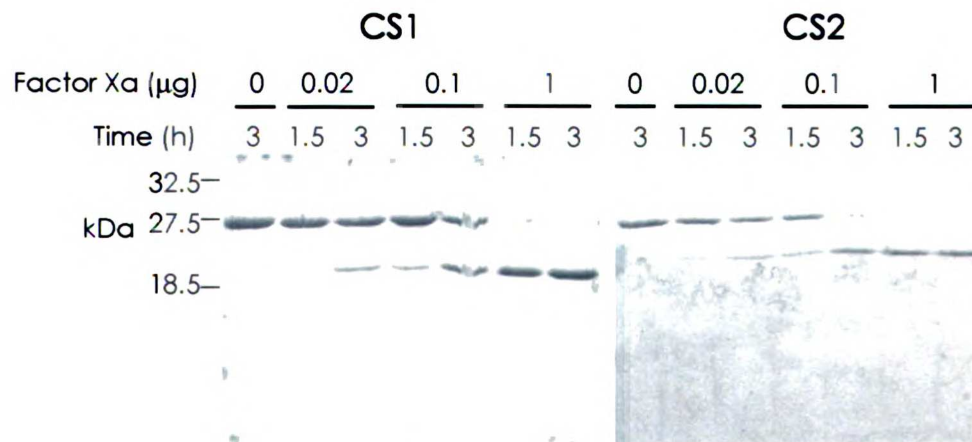


Figure 2-9

Figure 2-10. Identification of sites of ubiquitination using Factor Xa cleavage

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 minute incubation, the reactions were quenched by addition of quench buffer containing N-ethylmaleimide that inactivates ubiquitin-conjugating enzymes and deubiquitinating enzymes. Conjugates were purified using nickel-agarose beads, eluted, and cleaved for 3 hours with increasing amounts of Factor Xa protease. The C-terminal fragments produced by cleavage of substrate CS1 are indicated as CTF(CS1), while those of substrate CS2 are indicated as CTF(CS2).

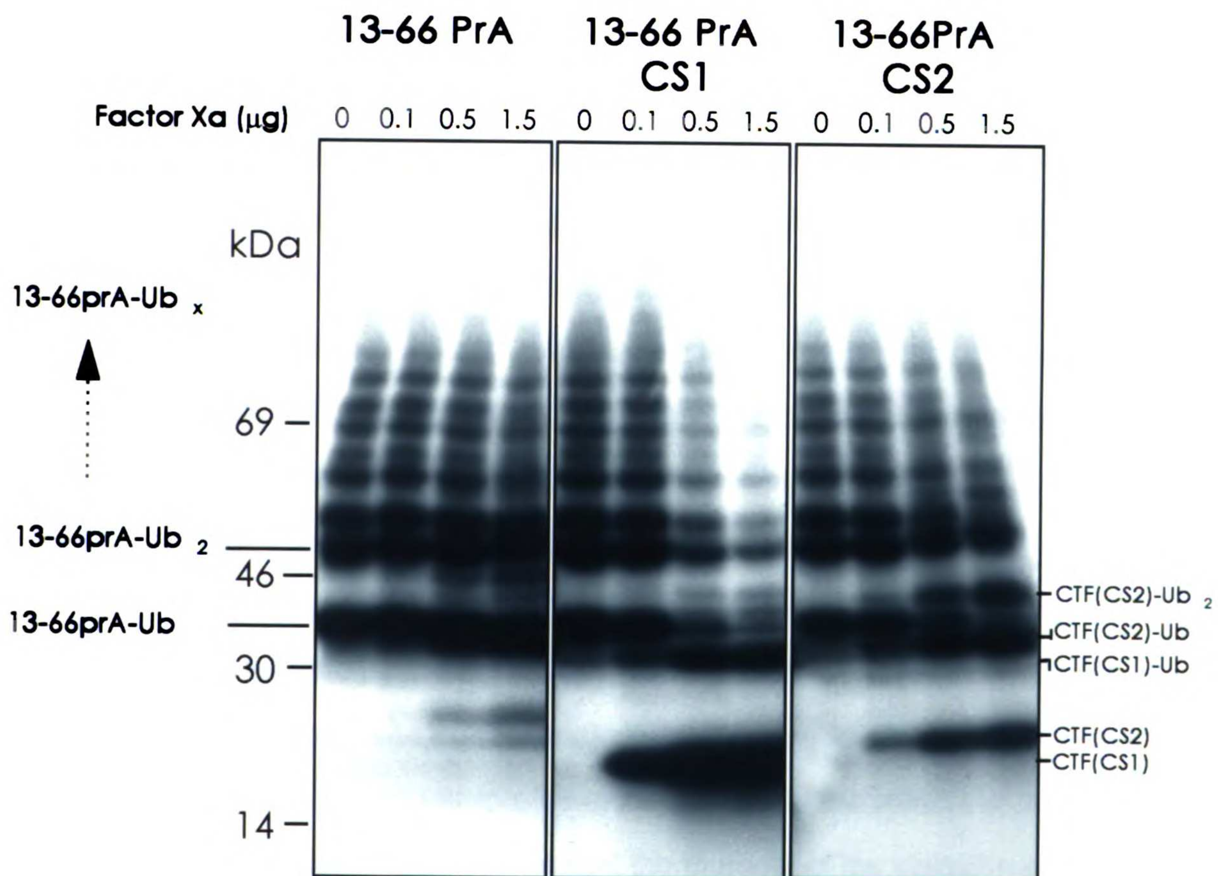


Figure 2-10

is observed following 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. 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 persists following cleavage. Furthermore, the persistence of a small amount of high molecular mass conjugates indicates 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 2-10). In this case, the majority of cleaved conjugates persisted as high molecular mass forms. The mass of the cleaved conjugates was shifted downward by an amount equivalent to the mass of the N-terminal fragment removed (approximately 5 kDa). Cleavage was efficient, as demonstrated by the almost complete shift of the monoubiquitinated species. This data indicates that the majority of the ubiquitin is conjugated to the C-terminal fragment. When interpreted in the context of the results with substrate CS1, these experiments demonstrate that the majority of the ubiquitin must be linked to one of the four lysines that lies between the destruction box and protein A. It should also be noted, however, that cleavage of CS2 also yielded a product equivalent in size to the C-terminal fragment containing no ubiquitin. Because only ubiquitinated conjugates were used as a substrate for cleavage, a subset of the conjugates must therefore contain ubiquitin exclusively N-terminal to the site of cleavage. Therefore, at least one of the three lysine residues N-terminal to the destruction box also functions as a ubiquitin acceptor site.

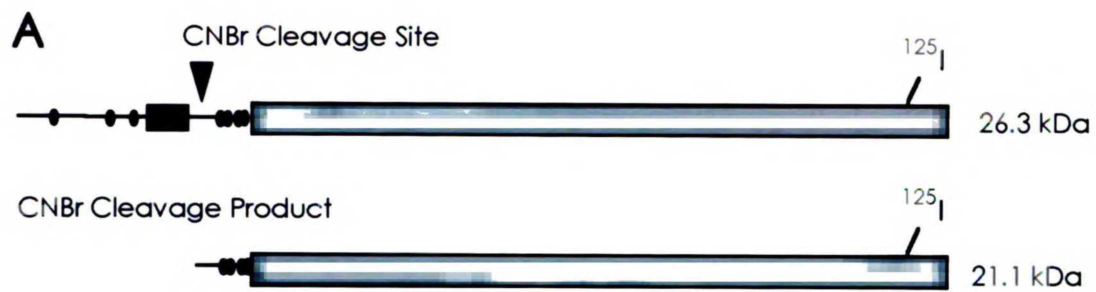
To confirm these results, we used an alternate method of purifying and

cleaving conjugates. 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 downstream of the destruction box and upstream of the cluster of four lysine residues in cyclin (Figure 2-11A). Ubiquitin itself contains only a single amino-terminal methionine and thus is not susceptible to cleavage by cyanogen bromide. The mutant substrate was ubiquitinated and degraded with normal kinetics in mitotic extracts (data not shown). Cyclin-ubiquitin conjugates were generated by adding the iodinated substrate to mitotic extract. The reaction mixture was separated by SDS polyacrylamide gel electrophoresis, and the proteins transferred to a nitrocellulose filter. An autoradiograph of the filter was made and used as a guide to excise conjugates of a particular molecular mass from the filter. Proteins were then eluted from filter slices and cleaved with cyanogen bromide. Untreated and treated samples were then analyzed by SDS-page and autoradiography. Figure 2-11B demonstrates that cleavage of the unconjugated substrate was approximately 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 non-ubiquitinated C-terminal fragment and a mono-ubiquitinated fragment. This demonstrates that ubiquitin must be present both N- and C-terminal to the site of cleavage. Cleavage of a di-ubiquitinated conjugate produced non-ubiquitinated, mono-ubiquitinated, and di-ubiquitinated C-terminal fragments, again indicating that ubiquitin is dispersed to either side of the cleavage site. Cleavage of conjugates containing 6-7 ubiquitin molecules produced a similar pattern, although di-, tri- and tetra-ubiquitinated products predominated. This data suggests a slight bias for ubiquitination occurring C-terminal to the site of cleave, but indicates that a fraction of the material is

Figure 2-11. Identification of ubiquitination sites using cyanogen bromide cleavage

(A) Schematic diagram of a mutant version of the *Arbacia* derivative 13-66 protein A that contains a single internal methionine residue at position 57 of the cyclin sequence. The predicted cleavage product is also diagrammed.

(B) This protein 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 dithiothreitol to reduce methionine sulfoxides generated during iodination or electrophoresis. The conjugates were dialyzed and the treated either with 70% formic acid (uncut lanes, U) or formic acid plus cyanogen bromide (cut lanes, C) and the products analyzed by electrophoresis and autoradiography. The filter fragments excised corresponded to non-ubiquitinated cyclin (Cyc), mono-ubiquitinated cyclin (Cyc-Ub), di-ubiquitinated cyclin (Cyc-Ub_2), tetra-ubiquitinated cyclin (Cyc-Ub_4) and a mixture of higher mass forms (Cyc$Ub_{6\text{-}7}$). The unmodified and ubiquitinated C-terminal cleavage fragments are indicated on the right (CTF).



B

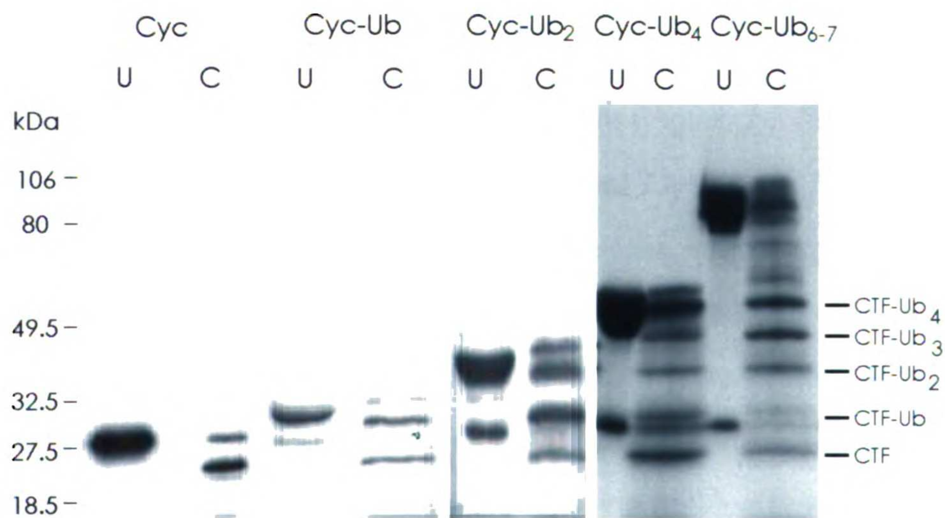


Figure 2-11

polyubiquitinated N-terminal to the site of cleavage.

These experiments indicate that ubiquitin conjugation can occur at multiple sites throughout 13-66prA, including protein A itself. This finding explains why elimination of the lysine residues in the cyclin portion of 13-66prA is not sufficient to stabilize the protein. We therefore analyzed the lysine requirements for degradation in a substrate that does not contain protein A. We measured the degradation rates of series of lysine to arginine mutants constructed in the *Arbacia* cyclin N-terminal fragment 13-110. This substrate contains ten lysine residues (Figure 2-12A) that were converted to arginine in various combinations. Figure 2-12B demonstrates that the ^{35}S-labeled wild-type substrate was rapidly degraded in mitotic extracts with a half-life of 4 minutes. A mutation of asparagine to alanine at position 9 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 following the destruction box (R4), or the three C-terminal lysines (R3C) had no effect on half-life (Figure 2-12B). Similarly, eliminating the N-terminal 7 lysines (R7), the C-terminal 7 lysines (R7C), or the six N- and C-terminal lysines (R6) had no effect on half life, indicating that any single cluster of lysine residues was sufficient to support degradation (Figure 2-12B). Stabilization was achieved only when all ten lysines were simultaneously converted to arginine (R10; Figure 2-12B). 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 lysines throughout the protein.

Discussion

In this study we have examined the sequences in the N-termini of mitotic cyclins that are required to target cyclin for ubiquitination and degradation during

Figure 2-12. Multiple lysine residues in the cyclin N-terminus can serve as ubiquitin acceptor sites sufficient for rapid proteolysis

(A) Sequence of residues 13-80 of *Arbacia* cyclin B. The destruction box and lysine residues are indicated in bold type.

(B) Schematic diagram and degradation analysis of derivatives of the *Arbacia* N-terminal fragment 13-110. The N-terminal fragment is drawn to scale with respect to the location of the destruction box (black rectangle) and lysine residues (circles). Proteins were expressed in reticulocyte lysate, and degradation assays performed as indicated for Figure 2-5.

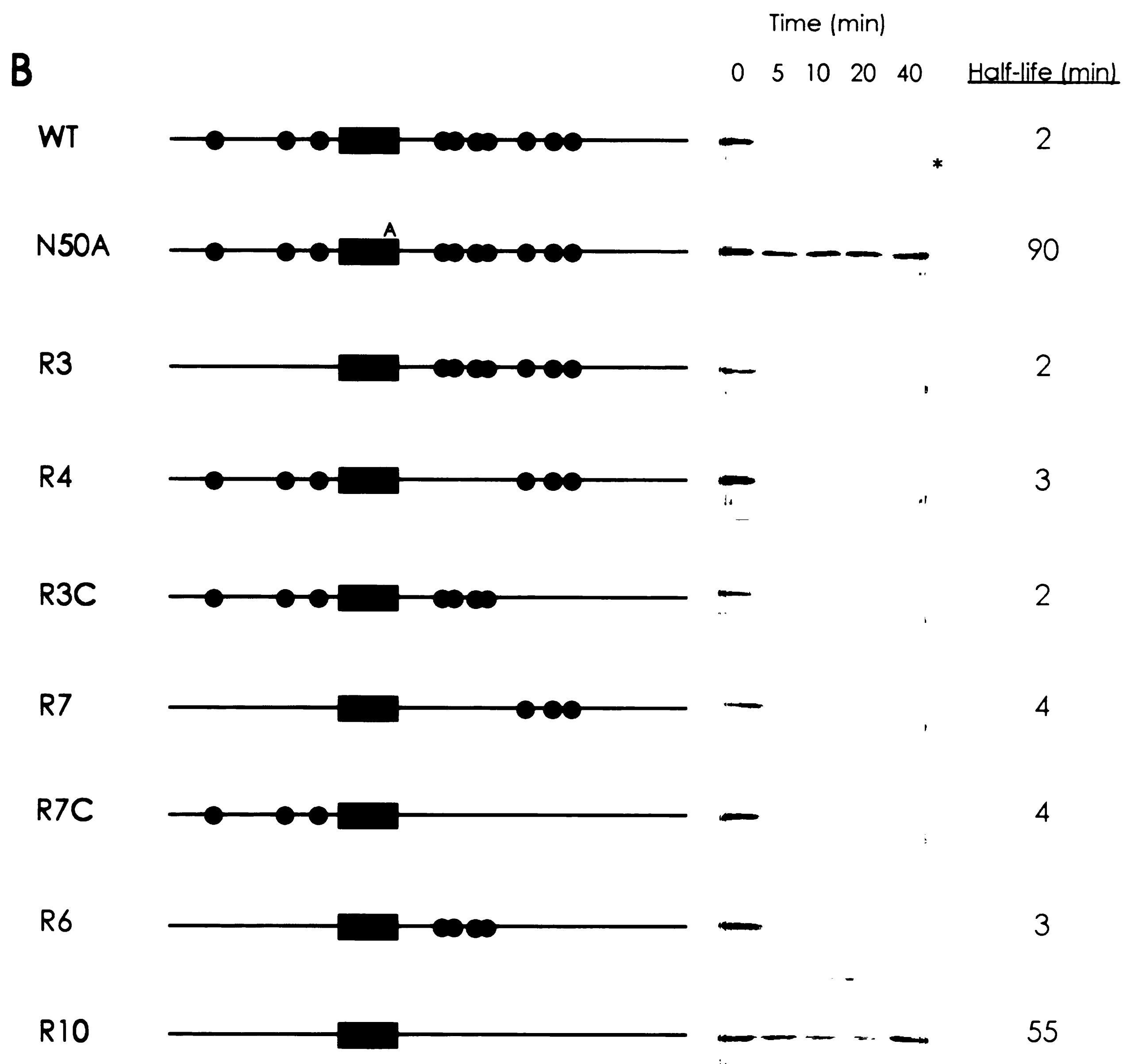


Figure 2-12

mitosis. Our results indicate that although the cyclin B N-terminus contains signals sufficient for mitotic destruction, the cyclin A N-terminus does not. Our data further indicate that the minimal destruction box sequence of A-type cyclins is not functional when placed in the context of an otherwise functional cyclin B N-terminus. Mutagenic analysis suggests that this defect may stem from the lack of conservation of sequences in the C-terminal half of the destruction box. Positions one, four and nine of the destruction box of B-type cyclins are highly conserved, and our mutagenic data indicates that such conservation is essential for ubiquitination and degradation. However, only positions one and four are conserved among A-type cyclins. The lack of conservation at the final position is perplexing, given that the conserved positions of the cyclin A destruction box are essential for rapid degradation of the full length protein (Kobayashi et al., 1992). Furthermore, position 6 of the destruction box differs consistently between A and B-type cyclins. We show that a cyclin B destruction box that contains a cyclin A-type residue at position 6 is a poorer substrate for proteolysis and ubiquitination. These data suggest that there are important differences between the way that cyclin A and cyclin B are recognized by the cyclin destruction machinery, differences that probably stem from variations in the structure of destruction box.

These findings are perhaps not surprising when considered in the context of the physiology of cyclin A and B destruction. Cyclin A is degraded during metaphase, while cyclin B proteolysis does not commence until the metaphase-anaphase transition (Whitfield et al., 1990; Minshull et al., 1990; Hunt et al., 1992; Pines and Hunter, 1991). Furthermore, agents that disrupt spindle integrity stabilize cyclin B, but not cyclin A (Whitfield et al., 1990; Hunt et al., 1992; Minshull et al., 1994). These data suggest that the cyclin A proteolytic system is activated in advance of that for cyclin B. We tested the degradability of substrates in extracts that had been activated to enter mitosis with a non-degradable, re-

combinant cyclin B protein. One possibility is that these extracts do not degrade the cyclin A N-terminus because they have passed the point at which the cyclin A degradation system is active. However, we found that full length *Xenopus* cyclin A was degraded in these extracts, although at slower rates than for *Xenopus* cyclin B1 (data not shown), indicating that the cyclin A proteolytic machinery was active. Clam extracts activated with non-degradable cyclin B also maintain the cyclin A destruction system in an active state (Luca et al., 1991).

If cyclin A and cyclin B are recognized by the same ubiquitin conjugation system, there must be structural features of cyclin A or an associated protein that allow it to be efficiently recognized. As shown in Figure 1, the cyclin A N-terminus contains a short conserved sequence element, FXXVDE that is present in all cyclin A N-termini but is not found in B-type cyclins. However, this motif is not essential for the degradation of full length cyclin A (Stewart et al., 1994). A more attractive hypothesis is that the substandard destruction box of cyclin A is compensated by association with another protein that is also recognized by the cyclin degradation system. This idea was originally suggested when it was discovered that mutations in cyclin A that inhibit binding to cdc2 also stabilize the protein (Stewart et al., 1994). However, cdc2 itself may not contribute to the recognition process. Instead, another component of the cyclin A-cdc2 complex may provide a recognition site. It has been shown that cyclin A-cdk2 complexes contain additional proteins that are essential for cell cycle progression (Zhang et al., 1995). Such proteins could play an important role in regulating cyclin A destruction if they were necessary for recognition of cyclin A by the destruction machinery. Such a mechanism may be responsible in part for the difference in timing of cyclin A and cyclin B degradation during the cell cycle. In this context, it would also be interesting to test whether the binding of cyclin A to cdc2 vs. cdk2 influences its ability to be degraded.

Our finding that the destruction box of Clb2 from budding yeast can substitute efficiently for the *Xenopus* sequence indicates that the components that recognize the destruction box are highly conserved. However, it is interesting to note that the N-terminus of Clb2 could not direct protein A degradation. This is unlikely to be a consequence of a lack of a ubiquitin acceptor site, since other experiments demonstrate that protein A can fulfill this function. Therefore, sequences outside the destruction box, which are not highly conserved, may nonetheless be important for recognition. This hypothesis is supported by the finding that the *Arbacia* constructs 13-66prA, 40-66prA, and 13-54prA were degraded more slowly than 13-91prA. The same result was obtained for the deletion derivatives of the *Xenopus* cyclin B1-prA fusions; in fact the sequence 31-55 of Xenopus B1 did not support destruction at all, even though it contained an intact destruction box. We consider it unlikely that the increase in half life was the result of loss of ubiquitin acceptor sites, since the effect of deletion was more severe than mutation of the lysine residues contained in the deleted region. Instead, these sequences may also be important for recognition by the ubiquitin conjugation system.

It is worth pointing out that there is a region of quasi-low sequence complexity downstream of the destruction box, stretching from residues 55-67 of *Xenopus* cyclin B1 and residues 59-71 of *Arbacia* cyclin B. This sequence is highly basic, and contains the most highly conserved phosphorylatable residue in the cyclin B N-terminus. However, as this residue is located at position 67 of *Arbacia* cyclin, it is unlikely to be required for proteolysis, since a fragment of 13-66 of *Arbacia* cyclin can target protein A for destruction efficiently. Furthermore, *Xenopus* cyclin B1 contains alanine at this position, but is nevertheless efficiently degraded. The finding that the Clb2 and *Xenopus* B1 destruction boxes can replace that of *Arbacia* indicate that no specific phosphorylatable residue in the

destruction box that is essential for cyclin degradation. Nonetheless, we mutated each of the four phosphorylatable residue in the 13-66 portion of 13-66prA to nonphosphorylatable residues. Only mutation of serine 49 of *Arbacia* cyclin to alanine had an effect, increasing half-life two to four fold, but this effect was quite variable (unpublished results). Two independent studies have mapped the sites of cyclin phosphorylation during mitosis and meiosis, and have found that phosphorylation of cyclin is not essential for its degradation (Izumi and Maller, 1991; Li et al., 1995). However, these experiments do not rule out the possibility that phosphorylation of cyclin may protect it from proteolysis when checkpoint signals become activated (Minshull et al., 1994).

A sequence comparison indicated that the N-termini of B-type cyclins are enriched in lysine residues, especially in the region downstream of the destruction box. This suggested that this region might function as the primary ubiquitin acceptor site in cyclin B. We were therefore surprised to find that elimination of all seven lysine residues in the cyclin portion of 13-66prA failed to stabilize the protein. This finding indicated that multiple lysine residues could potentially act as ubiquitin acceptor sites. Furthermore, the substrate became multi-ubiquitinated even in the presence of methylated ubiquitin, which cannot form polyubiquitin chains, indicating that multiple lysine residues must serve as ubiquitin attachment sites. To demonstrate this directly, we mapped the sites of ubiquitination using independent chemical and protease cleavage techniques. Both approaches indicated that ubiquitin is distributed both N- and C-terminal to the destruction box in the substrate 13-66prA. In fact, cleavage of individual conjugates with cyanogen bromide indicates that both sections of the protein undergo multiubiquitination. Cleavage of purified ubiquitin conjugates of 13-66prA using Factor Xa protease demonstrated that the majority of ubiquitin is conjugated to one of the four lysine residues located between the destruction box and protein

A. Importantly, this experiment also demonstrated that some amount of ubiquitin becomes conjugated to protein A itself. Because protein A could serve as a functional ubiquitin acceptor site, we also analyzed the requirements for lysine residues in the independently-expressed cyclin N-terminus. Stabilization of the substrate was achieved only when all 10 lysine residues were simultaneously mutated to arginine. Together, these experiments suggest that although the lysine cluster closest to the destruction box is the primary ubiquitin acceptor site, any lysine in the cyclin N-terminus can serve this purpose.

What significance does the finding that the cyclin N-terminus can be ubiquitinated at multiple sites have for our understanding of cyclin degradation? There is little overall sequence conservation among the N-termini of mitotic cyclins, suggesting this region of the protein may not be highly structured. This is borne out by NMR analysis of the *Arbacia* N-terminal fragment 13-110, which does not have significant structure in solution (H. Yu and M.W.K., unpublished). Such conformational flexibility could explain how many different lysine residues could function as ubiquitin acceptor sites. However, the mobility of the N-terminus may normally be restricted as part of the full length molecule, and this flexibility may be modulated by the binding of cyclin to cdc2 or other proteins. Therefore, it will be interesting to determine whether there are more stringent requirements for a particular lysine residue for the destruction of the full length protein. However, some degree of conformational flexibility may be essential for recognition by the cyclin ubiquitination system. For example, fusion of heterologous proteins such as glutathione-S-transferase to the N-terminus of full length cyclin renders the protein indestructible.

Our data indicates that the cyclin-ubiquitin conjugation system has the capability to ubiquitinate multiple lysine residues both N- and C-terminal to the destruction box. Studies of beta-galactosidase degradation mediated by the N-

end rule pathway have demonstrated that a destruction signal on one subunit of the tetrameric protein can direct the ubiquitination of another subunit, even if that subunit does not contain a degradation signal (Johnson et al., 1990). Although there are no known physiological substrates degraded by such "trans-ubiquitination," the flexibility with which the cyclin degradation system can ubiquitinate its substrates suggests that proteins associated with cyclin might also be ubiquitinated during mitosis. This does not appear to be the case for cdc2, however, which is stable throughout the cell cycle. It will be interesting to see if any cyclin-associated proteins that lack destruction boxes are degraded in a mitosis-specific fashion.

Cyclin B is the first physiologically important substrate of the ubiquitin proteolytic pathway for which the sites of ubiquitination 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 located 15 or 17 amino acids downstream from the N-terminal amino acid (Bachmair and Varshavsky, 1989; Chau et al., 1989). Histone H2A , which is stably ubiquitinated, also appears to be polyubiquitinated at a single position (Nickel and Davie, 1989). Mapping of ubiquitin conjugates of yeast cytochrome c generated in reticulocyte lysate 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; Sokolik and Cohen, 1992). The degradation of c-jun, in contrast, is mediated by multiple lysine residues, since elimination of individual lysines does not appreciably alter degradation rate or the pattern of ubiquitin conjugates observed (Treier et al., 1994). The ubiquitination of a substrate at multiple positions may thus be a general feature of the ubiquitin-dependent degradation of cellular regulators.

Because the consensus sequence of the cyclin destruction box is short and degenerate at several positions, it is not difficult to find proteins with close matches to the consensus. For example, mutation of an arginine residue in a destruction box-like sequence of yeast uracil permease stabilizes the protein against degradation under starvation conditions (Galan et al., 1994). However, the destruction of this protein is not cell-cycle regulated. What then distinguishes a true destruction box from an impostor? Our experiments reveal few hard and fast rules beyond what evolution has already told us. However, our data do suggest that non-conserved regions surrounding the destruction box may contribute to instability. In this vein, it is interesting that in *S. cerevisiae*, deletion of the destruction box only increases the half life of Clb2 from one minute to ten minutes *in vivo*, suggesting that sequences outside the destruction contribute strongly to proteolysis (Amon et al., 1994). This may provide a useful system for defining other signals in cyclin B that contribute to its rapid proteolysis during mitosis.

Chapter 3

Ubiquitin Conjugating Enzymes Involved in Cyclin B Proteolysis

Abstract

Mitotic cyclins are degraded at the end of mitosis by a ubiquitin-dependent proteolytic system that recognizes a short sequence in the N-terminus of cyclin called the destruction box. We have fractionated mitotic *Xenopus* egg extracts into a mitotically-regulated fraction and an unregulated fraction that are together required for mitosis- and destruction-box-dependent ubiquitination of cyclin B. The unregulated fraction contains at least two distinct ubiquitin conjugating enzymes that is each sufficient to support cyclin B ubiquitination. One of these, UBC4, is a ubiquitous enzyme involved in the turnover of regulatory and misfolded proteins. The second enzyme, UBCX, is a novel ubiquitin conjugating enzyme most closely related to UBC2. In contrast, we find that a *Xenopus* homolog of UBC9, an enzyme implicated in cyclin degradation in yeast, is not required for ubiquitination of cyclin B *in vitro*.

Introduction

Mitotic cyclins bind to cdc2 kinase to promote entry into mitosis in all eukaryotic cells. While stable in interphase, mitotic cyclins share the unusual property of becoming unstable at a point late in mitosis that correlates with the onset of anaphase. Degradation of cyclin B is necessary for the inactivation of cdc2 kinase and exit from mitosis (Gallant and Nigg, 1992; Ghiara et al., 1991; Murray et al., 1989; Surana et al., 1993), and is dependent upon a conserved 9 amino acid sequence in the cyclin N-terminus termed the destruction box (Glotzer et al., 1991). The degradation of mitotic cyclins is mediated by the ubiquitin pathway (Glotzer et al., 1991; Hershko et al., 1991; Hershko et al., 1994), in which isopeptide bond formation between the C-terminus of ubiquitin and a lysine residue of the target protein is catalyzed by a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin-protein ligase (E3). Multiple ubiquitin polypeptides can then be conjugated to one another to form a chain that is presumed to target the substrate for degradation by the 26S proteasome complex.

Substrate specificity in the ubiquitin pathway is thought to be determined by the E2 and E3 proteins. There are currently at least twelve distinct ubiquitin conjugating enzymes that have been identified in *S. cerevisiae* that are involved in the degradation of a wide number of substrates (Jentsch, 1992). Two of these ubiquitin conjugating enzymes have been implicated in control of the cell cycle. The CDC34 gene of *S. cerevisiae* encodes a ubiquitin conjugating essential for passage for passage from G1 into S phase (Goebl et al., 1988). Several substrates of the CDC34-dependent degradation system have been identified and include transcription factors such as GCN4 (Kornitzer et al., 1994), and the G1 cyclin Cln2 (Deshaies et al., 1995). The G1 cell cycle arrest of *cdc34* mutants is, however, a consequence of a failure to degrade the CDC28 kinase inhibitor

p40^{sic1} (Schwob et al., 1994). Deletion of p40 allows CDC34 mutants to enter S phase; however, the cells arrest again in G2/M, suggesting that CDC34 might be important for other cell cycle functions as well.

The second ubiquitin conjugating enzyme implicated in cell cycle control is *S. cerevisiae* UBC9, mutants of which arrest in the G2/M phase of the cell cycle (Seufert et al., 1995). Mutations in this ubiquitin conjugating enzyme can stabilize both Clb5, one of a pair of B-type cyclins involved in initiation of S-phase (Schwob and Nasmyth, 1993), and Clb2, a B-type cyclin involved in mitosis. However, it is not clear whether UBC9 is required directly for the destruction-box dependent ubiquitination of cyclins or whether its function lies upstream. A gene closely related to UBC9, called *hus5+*, was identified in *S. pombe* in a screen for genes involved in the checkpoint control that restrains mitosis in the presence of unreplicated or damaged DNA. *hus5* deletion mutants are impaired for growth and undergo abnormal mitoses, suggesting this gene is required for normal cell cycle progression.

Biochemical fractionation of the cyclin ubiquitination system in clam extracts has identified an activity, called E2-C, that can participate in cyclin ubiquitination *in vitro* (Hershko et al., 1994). The activity of this component, which has the hallmarks of a ubiquitin-conjugating enzyme, is not regulated during the cell cycle. Instead, another factor, that in clam extracts is associated with particulate material, is responsible for the mitotic specificity of cyclin ubiquitination (Hershko et al., 1994).

We have fractionated mitotic *Xenopus* egg extracts into a mitotically-regulated fraction and an unregulated fraction that are together required for mitosis- and destruction-box-dependent ubiquitination of cyclin B. The unregulated fraction contains at least two different ubiquitin conjugating enzymes that is each sufficient to support cyclin B ubiquitination. One of these, UBC4, is a ubiqui-

tous enzyme involved in the turnover of regulatory and misfolded proteins. The second enzyme, UBCX, is a novel ubiquitin conjugating enzyme most closely related to UBC2. In contrast, we find that a *Xenopus* homolog of UBC9, an enzyme implicated in cyclin degradation in yeast, is not required for ubiquitination of cyclin B *in vitro*.

Materials and Methods

Preparation of extracts

Concentrated cytoplasmic extracts were prepared from *Xenopus laevis* eggs as described (Murray, 1991), except that eggs were activated with the calcium ionophore A23187. Cycloheximide was added to arrest the extracts in interphase; extracts were subsequently induced to enter mitosis by addition of bacterially-expressed non-degradable Δ90 fragment of sea urchin cyclin B, which keeps the cyclin degradation system constitutively activated (Glotzer et al., 1991). Extracts were diluted 10-fold in buffer Q-A (10 mM Tris-HCl, pH 7.7, 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol [DTT]) containing an energy-regenerating system (7.5 mM creatine phosphate, 1 mM adenosine triphosphate (ATP), 0.1 mM EGTA, 1mM $MgCl_2$, pH 7.7) and okadaic acid (1 μM), and were centrifuged for 1 h at 115,000 x g at 4 °C. The supernatant was reconcentrated to 50% of the original extract volume in Centriprep-10 concentrators (Amicon). Pellet and membrane fractions were separately resuspended in the dilution buffer, centrifuged again, and resuspended in 50% of the original volume.

Ubiquitination assays

An N-terminal fragment of sea urchin cyclin B, consisting of residues 13-110, was expressed in *E. coli*, and purified as described (Holloway et al, 1991).

Proteins containing a wild type or mutated (R42A, A44R) destruction box were labeled to a specific activity of ~100 µCi/µg protein using the chloramine T procedure (Parker, 1990). Ubiquitination assays were performed in a total volume of 10 µl and contained energy-regenerating system (Murray, 1991), 1.25 mg/ml bovine ubiquitin (Sigma) and 12.5 ng (100 nM) labeled substrate. Reactions were incubated at room temperature for 15 min. and quenched by addition of sodium-dodecyl-sulfate (SDS)-sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Dried gels were then analyzed by autoradiography or by phosphorimaging using a Molecular Dynamics phosphorimager.

Thioester assays

For thioester assays, protein fractions were incubated with 0.3 µg radiolabeled ubiquitin (10 µCi/µg) in a total volume of 10 µl in the presence of 5 mM Tris-HCl (pH 7.7), 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM DTT, and 1 U inorganic pyrophosphatase (Sigma). After 5 min. at room temperature, reactions were stopped by addition of 10 µl urea sample buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 4 M urea, 20% glycerol), and reaction products analyzed by non-reducing SDS-PAGE and autoradiography. Thioester assays with recombinant E2s employed 1 µg of purified recombinant wheat E1 with 50 µg/ml of recombinant E2 in a final volume of 10 µl.

Ion-exchange chromatography of high-speed supernatants

Typically, 20-40 ml of diluted S100 was applied to a 6 ml Resource Q column on an FPLC system (Pharmacia) equilibrated with buffer Q-A. Flow-through fractions were combined (Q1), the column washed with 5 column volumes, and bound proteins eluted with 0.5 M KCl in Q-A. Eluted fractions were combined (Q2), desalted on PD10 columns (Pharmacia), and Q1 and Q2 recon-

centrated to 50% of the original extract volume. Fractionation was carried out at 4°C; concentrated fractions were stored at -70°C.

S chromatography of fraction Q1

Fraction Q1 derived from 50 ml of diluted S100 was precipitated with 80% ammonium sulfate, redissolved in buffer S-A (10 mM PIPES-KOH, pH 6.5, 30 mM KCl, 1 mM DTT) and gel filtered into the same buffer. The fraction was applied to a 6 ml Resource S column on an FPLC system equilibrated with buffer S-A. After collection of the flow-through fraction (Q1A), the column was washed with 2 column volumes of buffer S-A, and bound proteins eluted with 0.5 M KCl in S-A (Q1B). Q1A and Q1B were gel filtered into buffer Q-A and reconcentrated as described for fractions Q1and Q2.

MPM-2 antibody immunodepletion

Purified monoclonal MPM-2 antibodies (Davis et al., 1983) were the kind gift of Dr. J. Kuang (University of Texas, Houston). 0.25 volumes of MPM-2 antibodies (10mg/ml) or control antibodies (purified total mouse IgG [Sigma], 10 mg/ml) were incubated with fraction Q1 or Q2 for 20 minutes on ice in a final volume of 13.5 µl. Protein A-Sepharose beads (5 µl; Sigma) were added, incubated on ice for 40 minutes and subsequently removed by centrifugation. The supernatants were tested for ubiquitination activity.

Gel filtration of fraction Q1

Typically, 1-2 ml samples of concentrated Q1A or Q1B were separated on a Superdex 75 column (1.6 x 60 cm) equilibrated with buffer Q-A on an FPLC system (Pharmacia) at a flow rate of 0.75 ml/min. 5 ml fractions were collected and concentrated 10- to 25-fold. In some cases, ubiquitin or bovine serum albu-

min (BSA) was added to the fractions to a final concentration of 0.2 mg/ml before reconcentration.

Cloning of *Xenopus* UBC9

A *Xenopus* homolog of UBC9 was identified by polymerase chain reaction (PCR) using degenerate primers based on *S. cerevisiae* UBC9. The upstream primer was derived from amino acids 14-24 of S.c. UBC9 (KKWRKDHPFGF); the downstream primer was based on amino acids 144-153 (YDKKVLLQAK). The primer sequences were as follows (I indicates inosine; degenerate positions are indicated by parentheses): Upstream primer: 5'-[CCC TCT AGA GGA TCC AA(A/G) AA(A/G)TGG AG(A/G) AA(A/G) GAI CA(C/T) CCI TT(C/T) GGI TT]; Downstream primer: 5'-[GGG AAG CTT GAA TTC TTI GC(C/T) TGI AG(C/T) AAI AC(C/T) TT(T/C) TTI TC(G/A) TA]. PCR reactions contained 1 μM each primer, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.25 U Taq polymerase (Cetus), 200 μM of each deoxyribonucleoside triphosphate, and oligo dT-primed cDNA derived from total *Xenopus laevis* egg RNA. The PCR cycle began with a 5 minute step at 97°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 37°C, and 1 minute at 72°C. The reaction yielded a product of approximately 440 base pairs, which was cloned into the pBluescript Vector SK^+ using the pCR-Script cloning system (Stratagene). The cloned PCR fragment was then used to screen a *Xenopus laevis* oocyte lambda gt10 library. DNA from positive plaques was isolated, digested with EcoRI, and cloned into Bluescript SK^+. Both strands were sequenced using an automated DNA sequencing system. This clone was introduced into pTS210 (T. Stearns, Stanford University), a yeast centromere vector with the GAL1 promoter and the ACT1 transcriptional terminator and tested for its ability to complement the strain ywo102α which carries a temperature sensitive mutation in UBC9 (Seufert et al., 1995).

Generation of *Xenopus* UBC9 antisera

A peptide derived from the C-terminal 14 amino acids of UBC9 was used to generate polyclonal rabbit antisera. This antiserum recognized a predominant band of 18 kDa in crude *Xenopus* extracts that comigrated with bacterially-expressed UBC9 in immunoblot experiments; this reactivity could be blocked by preincubation of the serum with an excess of UBC9 C-terminal peptide. Antibodies were purified by affinity chromatography using UBC9 C-terminal peptide affinity beads. Purified antibodies were gel filtered into phosphate-buffered saline, concentrated to 2 mg/ml, and stored at 4°C. For immunodepletion, 50 μl of purified UBC9 antibody or 50 μl of crude non-specific rabbit antiserum was incubated with 20 μl Protein A-Sepharose beads for 90 minutes with frequent mixing at 4°C. Beads were then washed 5 times with buffer Q-A. The gel filtration fraction of peak activity derived from Q1B (elution volume 81-86 ml) was depleted by diluting 20 μl of the concentrated fraction with 100 μl buffer Q-A containing 0.5 mg/ml BSA, adding 20 μl of the washed antibody beads, and incubating for 90 minutes at 4°C with constant rotation. Beads were removed by centrifugation, and the supernatant reconcentrated to a final volume of 12 μl. A portion of the reconcentrated material (2.1 μl) was then tested for ubiquitination activity by mixing with an equal volume of fraction Q2. Residual UBC9 content was measured by immunoblotting the depleted fractions, using affinity-purified UBC9 antibodies at a final concentration of 2 μg/ml.

UBC4 antisera

Rabbit antibodies raised against human UBC4 (Rolfe et al., 1995) recognized a major band of 15 kDa in crude *Xenopus* egg extract and in fraction Q1 that was absent from fraction Q2. Crude antisera were used for all immunoblotting experiments at a final dilution of 1:300. As the antibodies were

raised against denatured protein, they did not immunoprecipitate native UBC4.

Cloning of a *Xenopus* homolog of UBC4

A *Xenopus* homolog of UBC4 was identified by polymerase chain reaction (PCR) using degenerate primers based on sequences conserved among ubiquitin conjugating enzymes. The upstream primer was based on the sequence EYPSKPPKV and the downstream primer based on the sequence PNPNSPA . The primer sequences were as follows (I indicates inosine; degenerate positions are indicated by parentheses): Upstream primer: 5'-CCC TCT AGA GGA TCC GAI TA(C/T) CCI TCI AA(A/G) CCI CCI AA(A/G) GT; Downstream primer: 5'-GGG AAG CTT GAA TTC (C/T)TG IGC IGG I(G/C)(A/T) (G/A)TT IGG (G/A)TT IGG. PCR reactions contained 1 μM each primer, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.25 U Taq polymerase (Cetus), 200 μM of each deoxyribonucleoside triphosphate, and oligo dT-primed cDNA derived from total *Xenopus laevis* egg RNA. The PCR cycle began with a 5 minute step at 97°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 37°C, and 1 minute at 72°C. The reaction yielded a product of approximately 221 base pairs, which was cloned into the pBluescript Vector SK^+ (Stratagene). DNA sequencing of this fragment revealed it to encode a gene highly related to yeast and human UBC4. A full length cloned was obtained by generating specific primers based on the sequence of the cloned fragment. These primers were then used to screen a Xenopus neurula library (Lustig and Kirschner, 1995) that had been subdivided into pools of approximately 300 clones each. The positive pools were subdivided in a matrix and reassayed by PCR to identify a single positive clone.

Preparation of recombinant ubiquitin conjugation enzymes

Recombinant *Xenopus* UBC9 protein was prepared by constructing an in-

frame fusion with the C-terminus of the glutathione S-transferase (GST) gene using the vector pGEX2T (Pharmacia). This vector contains a thrombin protease recognition sequence immediately following the GST moiety. Fusion protein was expressed, purified, and cleaved with thrombin directly from the GST matrix according to manufacturer's instructions (GST Gene Fusion System, Pharmacia). Gel filtration analysis indicated that the cleaved proteins chromatographed as monomers. Human UBC2 and UBC4 proteins were expressed in *E. coli* and purified by conventional chromatographic methods (Rolfe et al., 1995). Recombinant purified human CDC34 protein was the kind gift of P.R. Yew (Harvard Medical School).

Recombinant *Xenopus* UBC4 was expressed in *E. coli* and purified by S column chromatography. A 100 ml culture of *E. Coli* strain BL21(DE3) harboring a Xenopus UBC4 expression plasmid under the control of the T7 promoter was induced for 3 hours with 1 mM IPTG. The cells were pelleted and resuspended in 10 ml of column buffer (20 mM Pipes, pH 6.5, 1 mM EDTA, 1mM DTT and 10 μg/ml each pepstatin, leupeptin, and chymostatin). Lysozyme was added to 0.1 mg/ml and the cells incubated on ice for one hour. The lysed cells were sonicated on ice, and the debris pelleted by centrifugation for 15 min. at 10,000*g*. The supernatant was filtered through a 0.45 μm filter and applied to a 5 ml HiTrap SP column (Pharmacia). The column was washed with column buffer, and eluted with a linear 0-250 mM KCl gradient. The UBC4 that eluted at approximately 225 mM KCl was 80% pure as judged by SDS-PAGE and coomassie blue staining.

Purification and cloning of UBCX

The Q1 fraction derived from approximately 150 ml of interphase extract (7.5 g of extract protein) was precipitated in 80% ammonium sulfate. The pellet was then resuspendeD in buffer P-A (20 mM Tris, pH 7.7, 1.2 M ammonium

sulfate, 1 mM DTT), the sample was centrifuged again, and the supernatant was applied, in several runs, to a 16/10 HiLoad phenyl sepharose column (Pharmacia). The column was eluted with a linear gradient of buffer P-B (20 mM Tris, pH 7.7, 1 mM DTT). Fractions eluting between 0.05 and 0 M ammonium sulfate were pooled and gel filtered into buffer Q-A (20 mM Tris pH 9.0, 1 mM DTT), and applied to a 5 ml HiTrap Q column. Activity eluted from the column between 100-150 mM KCl. This material was then fractionated on a 1.6x60cm Superdex 75 column (Pharmacia). Activity eluting from this column copurified with a single thioester of 29 kDa and a single band on silver-stained gels of approximately 21 kDa. Fractions were blotted to PVDF for microsequence analysis. Peptide sequence obtained from the 21 kDa band identified a human expressed sequence tag of high similarity in the expressed sequence tag database (dbEST). This EST was used to isolate a clone from a human testis cDNA library using the Gene Trapper system (Gibco).

Results

Fractionation of mitotic extracts into regulated and non-regulated fractions

As a substrate for the cyclin ubiquitination reaction, we used an iodinated N-terminal fragment of *Arbacia punctulata* (sea urchin) cyclin B, consisting of residues 13-110. We monitored the formation of radiolabeled cyclin-ubiquitin conjugates by SDS-PAGE and autoradiography. The ubiquitination and degradation of this protein is dependent upon the cell-cycle state of crude extracts and requires an intact D-box (Holloway et al., 1993; see Chapter 1). As a source of factors required for cyclin ubiquitination, we prepared concentrated interphase extracts from *Xenopus* eggs. Stable mitotic extracts that constitutively degrade cyclin B were then obtained by adding a non-degradable mutant of cyclin B lacking its N-terminal 90 residues (Glotzer et al., 1991).

As a first step in isolating components required for cyclin B ubiquitination, we prepared pellet, membrane, and supernatant (S100) fractions by high-speed centrifugation of diluted, crude mitotic extracts. Okadaic acid (1μM) and an energy-regenerating system were included to maintain the mitotic state during dilution. Cyclin ubiquitination activity was recovered in the S100 fraction after reconcentration to the original volume, as indicated by the ladder-like appearance of higher-molecular-weight species (Figure 3-1A). Cyclin-ubiquitin conjugates were observed within one to two minutes of incubation in the mitotic supernatant, and reached a steady state within five minutes (data not shown). The substrate was degraded in this fraction with a half-life of five minutes, similar to that observed in crude extracts (see Chapter 1). No ubiquitination activity was detectable in the washed pellet or membrane fractions (Figure 3-1A).

We fractionated mitotic S100 by anion exchange chromatography, using Resource Q as a resin, yielding a flow through fraction (Q1) and a 0.6 M KCl eluate (Q2). While neither fraction alone catalyzed cyclin ubiquitination, mixing the fractions fully reconstituted activity (Figure 3-1B). A substrate containing a mutated D-box (R42A, A44R) produced only low-molecular-mass conjugates. To determine whether both of these fractions were mitotically regulated, we prepared Q1 and Q2 from interphase S100 (designated $Q1^i$ and $Q2^i$). Cyclin ubiquitination was observed when Q1 was replaced by $Q1^i$ but not when Q2 was replaced by $Q2^i$ (Fig. 3-1C), indicating that only Q2 is mitotically regulated.

We had observed that addition of the monoclonal antibody MPM-2, which recognizes a phosphorylated epitope shared by a discrete number of mitotic phosphoproteins (Davis et al., 1983), could inhibit cyclin ubiquitination and degradation when added to crude mitotic extracts (J. Kuang, J. Penkala, M. Glotzer, R.W.K., M.W.K, unpublished data). We found that immunodepletion of fraction Q2 with the MPM-2 antibody strongly inhibited cyclin ubiquitination, while deple-

Figure 3-1. Fractionation of cyclin ubiquitination activity in mitotic *Xenopus* egg extracts into a regulated and an unregulated fraction

(A) Pellet (P), membrane (M) and supernatant (S100) fractions obtained from mitotic extracts were assayed for their ability to convert a radiolabeled N-terminal fragment of cyclin B ([^{125}I]cyc) into ubiquitin conjugates.

(B) Flow-through (Q1) and eluate (Q2) fractions were obtained from mitotic S100 by Resource Q chromatography and assayed individually or after mixing equal volumes. Destruction box dependence was tested by addition of a radiolabeled fragment containing two destruction box point mutations [Q1 + Q2 (*)].

(C) Flow through ($Q1^{i}$) and eluate ($Q2^{i}$) fractions were prepared as in (B) using interphase S100 and tested for their ability to replace Q1 and Q2 derived from mitotic S100. (D) Fraction Q1 and Q2 were immunodepleted with either purified total mouse IgG control antibody ($Q1^{c}$, $Q2^{c}$) or with purified MPM-2 monoclonal antibody ($Q1^{m}$, $Q2^{m}$) and assayed as in (A).

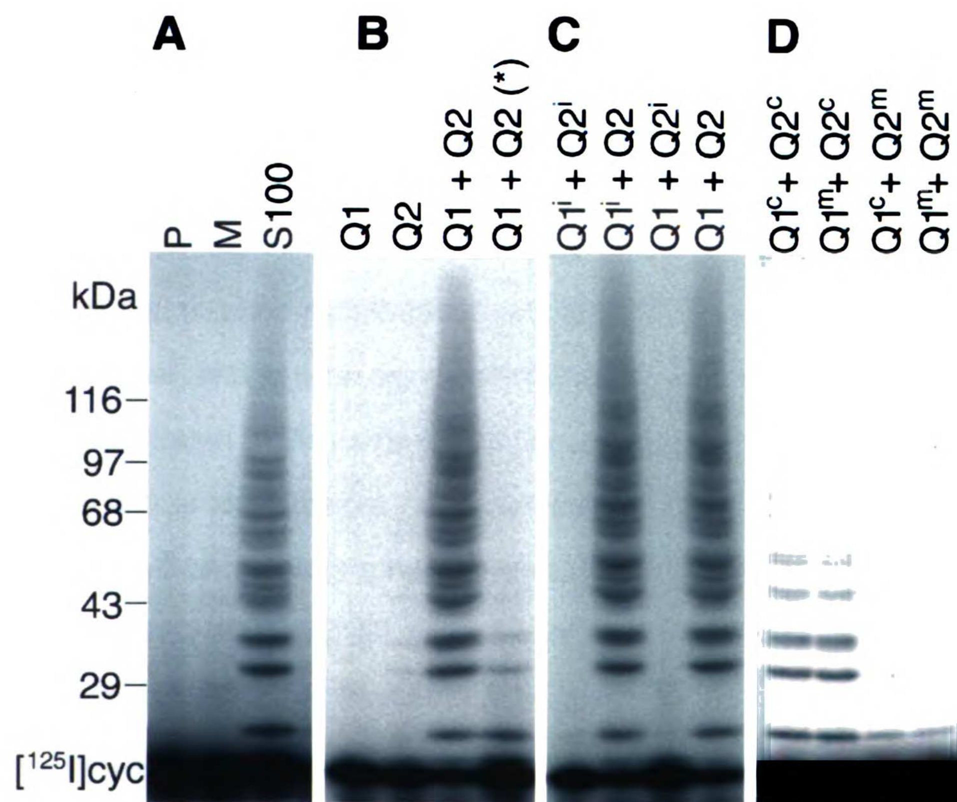


Figure 3-1

tion of fraction Q1 had no effect (Fig. 3-1E). Control depletions using total mouse IgG or an unrelated monoclonal antibody were not inhibitory. This result further suggests that fraction Q2 is subject to mitotic regulation.

Fraction Q1 contains two distinct E2s that each support cyclin ubiquitination

To identify components in the unregulated fraction required for cyclin ubiquitination, we further fractionated interphase Q1 by gel filtration and tested each fraction for its ability to complement Q2. We found complementing activity in a broad molecular weight range corresponding to 15-40 kDa, suggesting that multiple components in Q1 might each be sufficient to complement Q2 (Figure 3-2). Complementation was highly dependent on reconcentration of these fractions. To ensure that this complementation was physiological, we fractionated Q2 in a similar manner, and saw no significant complementation activity when fractions were concentrated to the same extent (data not shown).

To further characterize these unregulated components, we fractionated Q1 by Resource S chromatography, and generated a flow-through fraction (Q1A) and a 0.6 M KCl eluate (Q1B). Each fraction was sufficient to complement Q2, and was therefore further fractionated by gel filtration over a Superdex 75 column. The peak of activity in fraction Q1A eluted at 70 ml, corresponding to a molecular weight of ~30 kDa; activity in fraction Q1B peaked at 81 ml, corresponding to ~20 kDa (Fig. 3-3A). The pattern of ubiquitin conjugates generated by Q1A and Q1B was different: Q1A favored the production of low-molecular-mass conjugates, whereas Q1B generated higher-molecular-mass conjugates, suggesting that these two activities are distinct. Ubiquitination mediated by both Q1A and Q1B required an intact D-box (data not shown).

Because ubiquitin-conjugating enzymes (E2s) typically range in size from

Figure 3-2. Gel filtration analysis of fraction Q1

Interphase fraction Q1 was applied to a Superdex-75 column and fractions analyzed for the ability to complement mitotic fraction Q2. The positions of elution of BSA (67 kDa) and RNase A (14 kDa) are indicated.

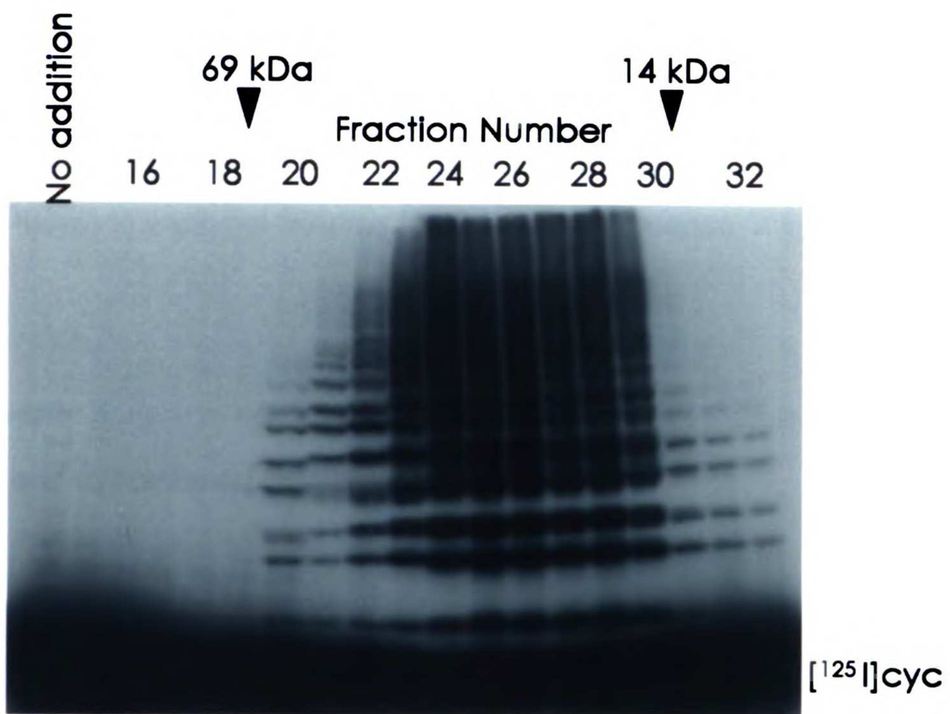


Figure 3-2

Figure 3-3. Fraction Q1 contains two distinct E2s that is each sufficient to support cyclin ubiquitination

Fraction Q1 was subjected to Resource S chromatography, and the flow through (Q1A) and eluate (Q1B) fractions analyzed by gel filtration. Elution volume (ml) of each fraction is indicated at the top of the figure.

(A) Cyclin ubiquitination activity measured by addition of labeled cyclin N-terminus ([^{125}I]cyc) to each gel filtration fraction in the presence of an equal volume of Q2.

(B) Thioester activity in each fraction was measured by addition of E1 in the presence of radiolabeled ubiquitin ([^{125}I]ub).

(C, D) Immunoblot detection of UBC4 and UBC9 in each fraction.

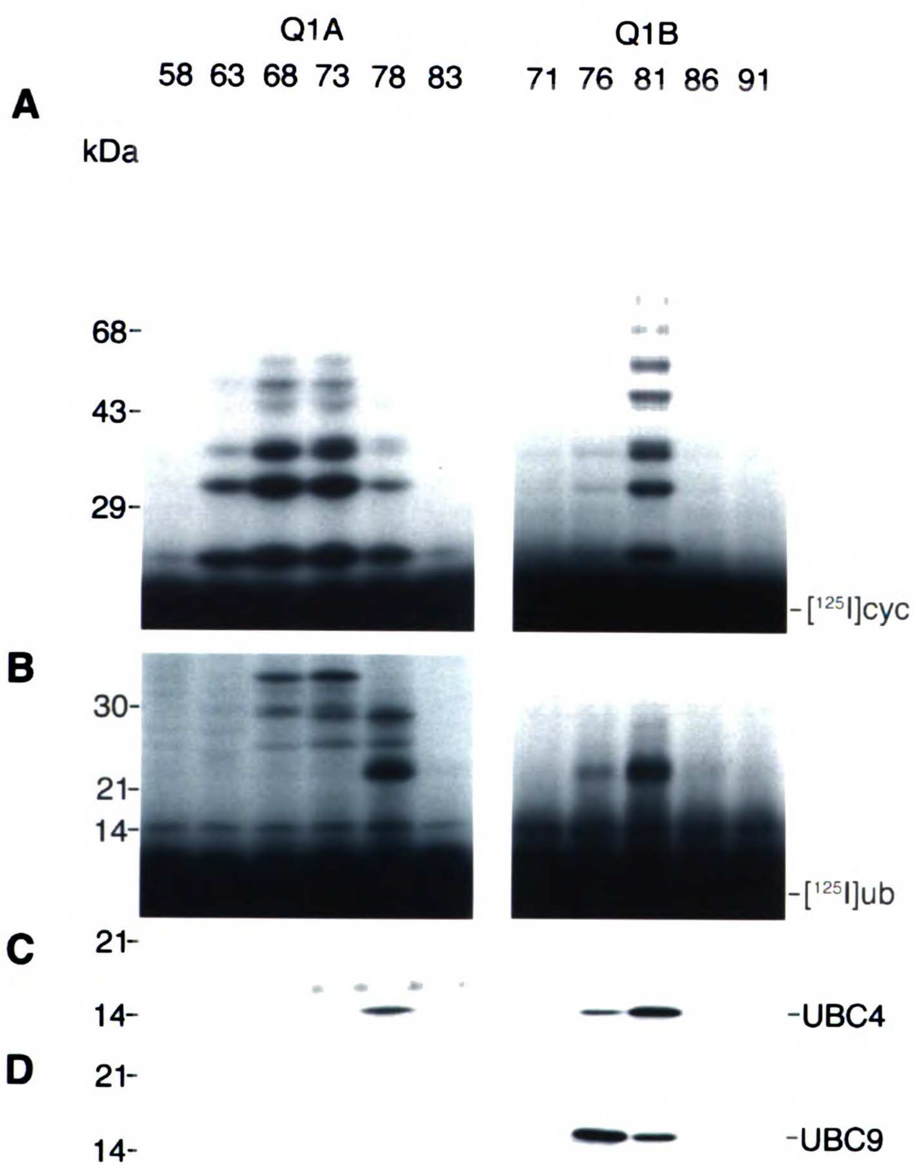


Figure 3-3

14 to 34 kDa, we tested gel filtration fractions derived from Q1A and Q1B for the presence of E2s by asking whether ubiquitin thioesters were formed in the presence of radiolabeled ubiquitin and purified E1. Fraction Q1A contained multiple ubiquitin thioesters of 24 - 34 kDa that copurified with cyclin-ubiquitin conjugating activity (Fig. 3-3B). In contrast, the activity in fraction Q1B copurified with a single predominant thioester of 22 kDa, suggesting the presence of a 15-16 kDa E2 (Fig. 3-3B). As this thioester did not cofractionate with the peak of activity in fraction Q1A, there appear to be at least two different E2s in fraction Q1 sufficient to complement fraction Q2.

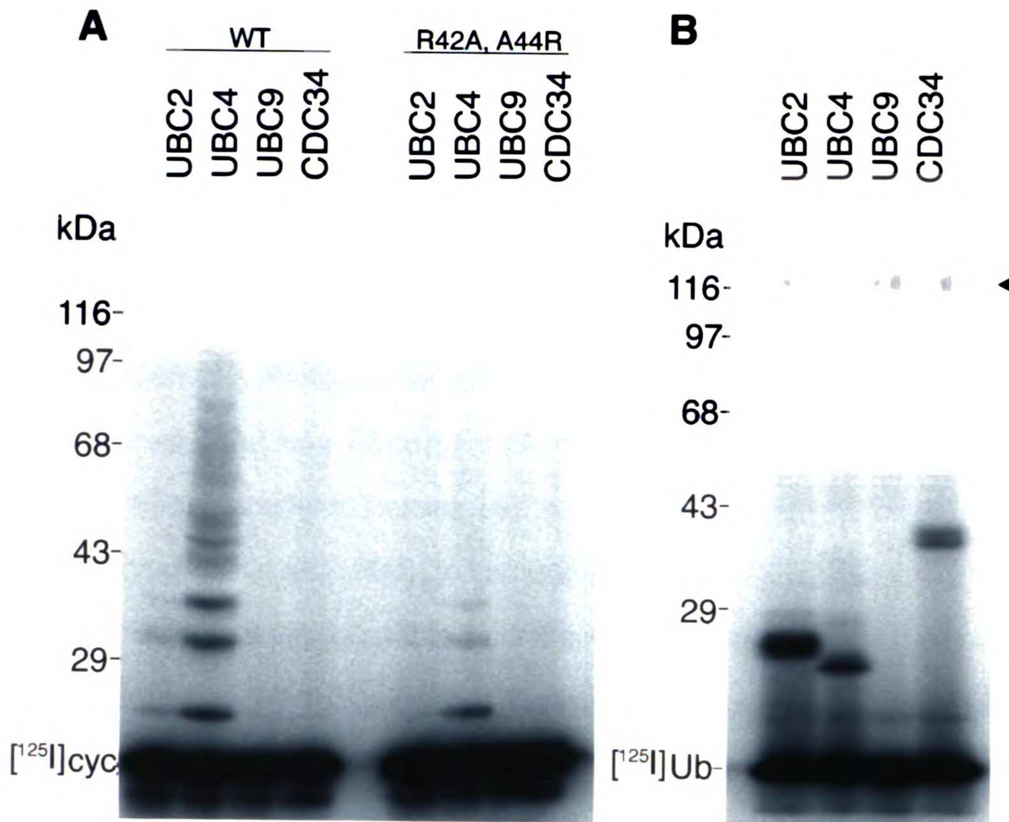
UBC4 is present in fraction Q1B, and recombinant UBC4 can complement fraction Q2

Few E2s characterized to date have been reported to bind cation exchange resins like the activity in fraction Q1B. One of these, a *Xenopus* homolog of UBC9, did not cofractionate with activity (Fig 3-3D, see below). Another is the UBC4/5 class of E2s in budding yeast, homologs of which have been demonstrated to bind cation exchange resins (Girod and Vierstra, 1993; Scheffner et al., 1993). Fraction Q1B showed strong UBC4 immunoreactivity that cofractionated with cyclin ubiquitination activity during gel filtration (Figure 3-3C). Fraction Q1A contained only a small amount of UBC4 that did not copurify with the majority of activity (Figure 3-3C). Therefore, we tested whether bacterially-expressed and purified human UBC4 was sufficient to complement fraction Q2. UBC4 supported cyclin ubiquitination in a dose-dependent fashion (Figs. 3-4A,C). Strong complementation was observed at 6 μg/ml and was maximal at 25 μg/ml (Fig. 3-4C). Quantitative immunoblotting demonstrated that UBC4 is present in fraction Q1 at a concentration of 4 μg/ml (data not shown). A D-box mutant yielded only low-molecular weight conjugates in the presence of recombinant UBC4 (Fig. 3-4A),

Figure 3-4. Recombinant UBC4 complements fraction Q2 in destruction-box-dependent cyclin ubiquitination

(A) Recombinant purified human UBC2, human UBC4, *Xenopus* UBC9 and human CDC34 proteins (25 µg/ml each) were tested for their ability to complement fraction Q2 in ubiquitination assays in which an N-terminal fragment of cyclin B, containing a wild type (WT) or mutant (R42A,A44R) destruction box, was used as a substrate ([^{125}I]cyc).

(B) This panel of E2s (50 µg/ml each) was also tested for the ability to form thioesters with iodinated ubiquitin ([^{125}I]ub) in the presence of E1. The position of the E1-ubiquitin thioester is indicated by an arrowhead.



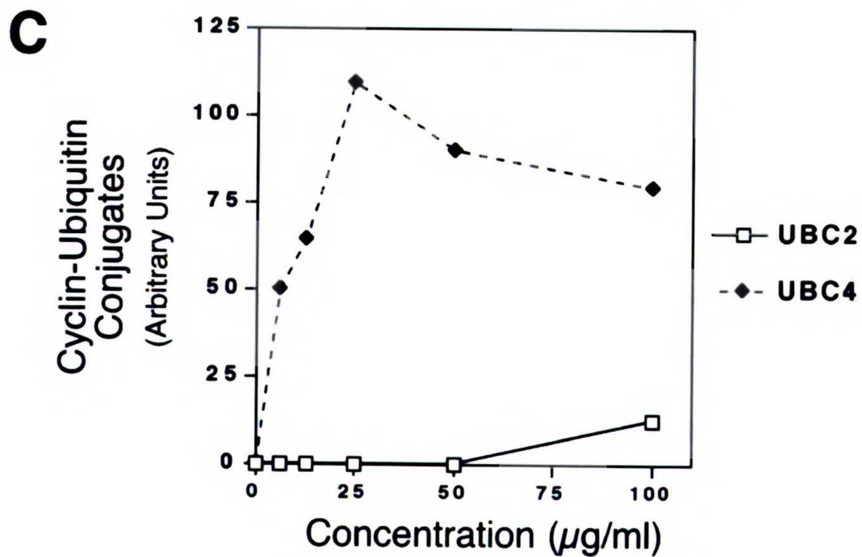


Figure 3-4

suggesting that substrate specificity is maintained in this reconstituted system. Other purified E2s such as human CDC34 did not complement fraction Q2, even at high dose; human UBC2 only weakly supported cyclin ubiquitination (Figs. 3-4A, C). This was not due to a difference in the ability of these E2s to accept ubiquitin from E1, because UBC2, UBC4 and CDC34 formed similar amounts of ubiquitin thioesters (Fig. 4B).

To verify that this complementation was not an artifact of using human UBC4, we cloned a *Xenopus* homolog of UBC4 (Figure 3-5). As shown in Figure 3-6, the cloned gene was 79 percent identical at the amino acid level to yeast UBC4 and 99% identical to human UBC4. Another human UBC4-related protein, UBCH5 (Scheffner et al., 1994), is somewhat less related (89% identical). *Xenopus* UBC4 was expressed in *E. coli* and tested for ability to complement fraction Q2. Figure 3-7 indicates that *Xenopus* UBC4 stimulated ubiquitination at concentrations between 4-8 µg/ml, similar to what was observed for human UBC4. Notably, recombinant *Xenopus* UBC4 supported the formation of high-molecular-mass cyclin-ubiquitin conjugates.

A *Xenopus* homolog of UBC9 is not required for cyclin ubiquitination *in vitro*

Recent work in *S. cerevisiae* has identified a novel ubiquitin-conjugating enzyme, UBC9, that is involved in the degradation of the S-phase cyclin Clb5 and the mitotic cyclin Clb2 (Seufert et al., 1995). To test whether UBC9 participates directly in cyclin ubiquitination in *Xenopus* extracts, we isolated a full-length *Xenopus* homolog of *S. cerevisiae* UBC9 (Figure 3-8). This gene is highly similar to both *S. cerevisiae* UBC9 and to a recently-identified UBC9 relative in *S. pombe*, *hus5*$^+$ (Al-Khodairy et al., 1995). The *Xenopus* gene encodes a 158 amino acid protein with a predicted molecular mass of 18 kDa and a predicted

Figure 3-5. Sequence of a *Xenopus* homolog of *S. cerevisiae* UBC4

A *Xenopus* homolog of *S. cerevisiae* UBC4 was obtained by degenerate PCR. The sequence was used to identify a full-length UBC4 clone from a *Xenopus* neurula-stage embryo library.

TTAGAACGCATCTGCTGGG
ACGTCGGAGCAAGCTTGATTTAGGTGACACTATAGAATACAAGCTACTTGTTCTTTTTCAGGATCCCATCGATTCGGAA
TTCGGCTCGAGCTTCCTCGCCTCCGCCCAGCTCAGCCCGAAGCACCCGCCCGGCAGCAGTAGCGACAGCATT

ATG GCG CTG AAA CGG ATC CAC AAG GAA CTC AAT GAT TTG GCT CGT GAT
M A L K R I H K E L N D L A R D
CCT CCA GCT CAG TGT TCC GCC GGC CCA GTT GGA GAT GAT ATG TTT CAC
P P A Q C S A G P V G D D M F H
TGG CAA GCA ACA ATA ATG GGA CCT AAT GAC AGC CCA TAT CAA GGT GGT
W Q A T I M G P N D S P Y Q G G
GTG TTT TTC TTG ACG ATT CAT TTT CCA ACA GAC TAT CCC TTT AAA CCT
V F F L T I H F P T D Y P F K P
CCT AAA GTT GCG TTT ACA ACA AGA ATC TAC CAT CCA AAT ATT AAC AGC
P K V A F T T R I Y H P N I N S
AAT GGC AGC ATT TGT CTT GAT ATT CTC AGA TCA CAG TGG TCC CCA GCT
N G S I C L D I L R S Q W S P A
TTA ACT ATT TCT AAA GTT CTT TTG TCA ATT TGT TCA CTG TTG TGT GAC
L T I S K V L L S I C S L L C D
CCA AAC CCA GAT GAC CCT CTA GTG CCA GAG ATT GCA CGC ATC TAC AAA
P N P D D P L V P E I A R I Y K
ACA GAT AGG GAA AAG TAC AAC AGA ATA GCC CGG GAA TGG ACT CAG AAG
T D R E K Y N R I A R E W T Q K
TAT GCT ATG TGA TGCTACCTTCAAGTCTGAACTAACCTGCATTATAGCTGGAATAAACTTTAAATTACTGTTTTCC
Y A M

CTCTCCCTTTCAGACCTCATCTACTTGCCCTTTCCCCACTTCTTTTTTTTTT

Figure 3-5

Figure 3-6. Sequence comparison of UBC4 homologs from *Xenopus* and human with those of yeast

The sequence of *Xenopus* UBC4 is aligned with sequences of similar proteins from yeast and human. Residues identical to *Xenopus* UBC4 are boxed. *Xenopus* UBC4 is 99% identical (one amino acid change) to human UBC4, and is 88% identical to UBCH5. *Xenopus* UBC4 is 79% identical to UBC4 and UBC5 from *S. cerevisiae*.

X. laevis UBC4	M A L - K R I H K E L N D L A R D P P A	19
H. sapiens UBC4	M A L - K R I H K E L N D L A R D P P A	19
H. sapiens UBCH5	M A L - K R I Q K E L S D L Q R D P P A	19
S. cerevisiae UBC4	M S S S K R I A K E L S D L E R D P P T	20
S. cerevisiae UBC5	M S S S K R I A K E L S D L G R D P P A	20

X. laevis UBC4	Q C S A G P V G D D M F H W Q A T I M G	39
H. sapiens UBC4	Q C S A G P V G D D M F H W Q A T I M G	39
H. sapiens UBCH5	H C S A G P V G D D L F H W Q A T I M G	39
S. cerevisiae UBC4	S C S A G P V G D D L Y H W Q A S I M G	40
S. cerevisiae UBC5	S C S A G P V G D D L Y H W Q A S I M G	40

X. laevis UBC4	P N D S P Y Q G G V F F L T I H F P T D	59
H. sapiens UBC4	P N D S P Y Q G G V F F L T I H F P T D	59
H. sapiens UBCH5	P P D S A Y Q G G V F F L T V H F P T D	59
S. cerevisiae UBC4	P A D S P Y A G G V F F L S I H F P T D	60
S. cerevisiae UBC5	P S D S P Y A G G V F F L S I H F P T D	60

X. laevis UBC4	Y P F K P P K V A F T T R I Y H P N I N	79
H. sapiens UBC4	Y P F K P P K V A F T T R I Y H P N I N	79
H. sapiens UBCH5	Y P F K P P K I A F T T K I Y H P N I N	79
S. cerevisiae UBC4	Y P F K P P K I S F T T K I Y H P N I N	80
S. cerevisiae UBC5	Y P F K P P K V N F T T K I Y H P N I N	80

X. laevis UBC4	S N G S I C L D I L R S Q W S P A L T I	99
H. sapiens UBC4	S N G S I C L D I L R S Q W S P A L T I	99
H. sapiens UBCH5	S N G S I C L D I L R S Q W S P A L T V	99
S. cerevisiae UBC4	A N G N I C L D I L K D Q W S P A L T L	100
S. cerevisiae UBC5	S S G N I C L D I L K D Q W S P A L T L	100

X. laevis UBC4	S K V L L S I C S L L C D P N P D D P L	119
H. sapiens UBC4	S K V L L S I C S L L C D P N P D D P L	119
H. sapiens UBCH5	S K V L L S I C S L L C D P N P D D P L	119
S. cerevisiae UBC4	S K V L L S I C S L L T D A N P D D P L	120
S. cerevisiae UBC5	S K V L L S I C S L L T D A N P D D P L	120

X. laevis UBC4	V P E I A R I Y K T D R E K Y N R I A R	139
H. sapiens UBC4	V P E I A R I Y Q T D R E K Y N R I A R	139
H. sapiens UBCH5	V P D I A Q I Y K S D K E K Y N R H A R	139
S. cerevisiae UBC4	V P E I A H I Y K T D R P K Y E A T A R	140
S. cerevisiae UBC5	V P E I A Q I Y K T D K A K Y E A T A K	140

X. laevis UBC4	E W T Q K Y A M	147
H. sapiens UBC4	E W T Q K Y A M	147
H. sapiens UBCH5	E W T Q K Y A M	147
S. cerevisiae UBC4	E W T K K Y A V	148
S. cerevisiae UBC5	E W T K K Y A V	148

Figure 3-6

Figure 3-7. Complementation of fraction Q2 with *Xenopus* UBC4

Varying concentrations of purified recombinant *Xenopus* UBC4 were mixed with total fraction Q2 in the presence of the 13-110 N-terminal fragment of cyclin. Reactions were incubated for 15 minutes and then analyzed by SDS-PAGE and phosphorimaging.

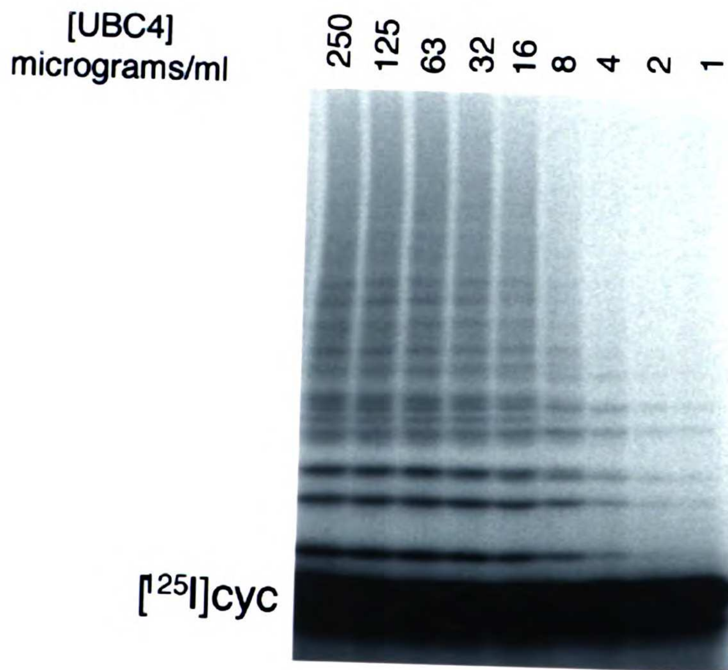


Figure 3-7

Figure 3-8. Sequence of a *Xenopus* homolog of *S. cerevisiae* UBC9

Reverse-transcribed *Xenopus* egg RNA was used in a PCR reaction with degenerate oligonucleotide primers to identify a fragment encoding a *Xenopus* homolog of yeast UBC9. This fragment was used to screen an oocyte library. Two independent clones were obtained and sequenced and found to be identical.

GAATTCCGCCCTGAA

ATG	TCT	GGC	ATA	GCC	CTG	AGC	AGA	CTT	GCA	CAG	GAG	AGA
M	S	G	I	A	L	S	R	L	A	Q	E	R
AAA	GCT	TGG	AGA	AAA	GAC	CAT	CCT	TTT	GGT	TTT	GTG	GCA
K	A	W	R	K	D	H	P	F	G	F	V	A
GTA	CCA	ACG	AAA	AAT	CCA	GAT	GGC	ACA	ATG	AAT	TTG	ATG
V	P	T	K	N	P	D	G	T	M	N	L	M
AAC	TGG	GAA	TGT	GCT	ATT	CCA	GGC	AAG	AAA	GGG	ACC	CCC
N	W	E	C	A	I	P	G	K	K	G	T	P
TGG	GAA	GGT	GGC	TTA	TTT	AAA	TTA	CGG	ATG	CTT	TTT	AAG
W	E	G	G	L	F	K	L	R	M	L	F	K
GAT	GAT	TAT	CCC	TCG	TCA	CCT	CCT	AAA	TGT	AAA	TTT	GAG
D	D	Y	P	S	S	P	P	K	C	K	F	E
CCA	CCC	CTA	TTT	CAT	CCG	AAT	GTC	TAT	CCT	TCA	GGC	ACA
P	P	L	F	H	P	N	V	Y	P	S	G	T
GTG	TGT	CTG	TCT	ATC	TTA	GAA	GAA	GAT	AAG	GAT	TGG	AGG
V	C	L	S	I	L	E	E	D	K	D	W	R
CCA	GCA	ATC	ACA	ATT	AAA	CAG	ATC	TTG	TTA	GGA	ATA	CAA
P	A	I	T	I	K	Q	I	L	L	G	I	Q
GAA	CTT	CTA	AAT	GAA	CCA	AAT	ATA	CAA	GAT	CCA	GCT	CAA
E	L	L	N	E	P	N	I	Q	D	P	A	Q
GCA	GAG	GCA	TAC	ACA	ATT	TAC	TGC	CAA	AAC	AGA	GTT	GAA
A	E	A	Y	T	I	Y	C	Q	N	R	V	E
TAT	GAA	AAA	AGA	GTC	AGA	GCA	CAA	GCC	AAG	AAG	TTT	GCG
Y	E	K	R	V	R	A	Q	A	K	K	F	A
CCA	TCA	TAA ACTTCAACCTTGCAGGATCTAAAAAAAAAAA										
P	S											

Figure 3-8

isoelectric point of 8.7. *Xenopus* UBC9 is 56% identical to *S. cerevisiae* UBC9 and 66% identical to *S. pombe hus5$^+$* (Figure 3-9). To verify that the gene we had cloned was indeed a homolog of yeast UBC9, we tested whether *Xenopus* UBC9 would complement a temperature-sensitive mutation of *S. cerevisiae* UBC9 (Seufert et al., 1995). A low copy number plasmid expressing *Xenopus* UBC9 under the control of the gal promoter supported growth of the temperature-sensitive strain at 37 °C when plated on galactose-containing medium; no complementation was observed when this strain was plated on medium containing glucose (Figure 3-10A). Mutation of the putative active site cysteine and a neighboring leucine (cysteine 93, leucine 96) to serine residues abolished complementation activity (Figure 3-10B). No inhibition of growth was observed when this mutant was expressed in a wild-type background, indicating that it did not act in a dominant-negative fashion (Figure 3-10C).

Xenopus UBC9 is a basic protein, and thus would be expected to bind cation exchange columns. To test whether UBC9 was present in our fractions, we prepared affinity-purified polyclonal antibodies raised against a C-terminal peptide derived from *Xenopus* UBC9. This antiserum recognized a single band of 18 kDa in crude *Xenopus* extracts (Figure 3-11A). Quantitative immunoblotting of crude extracts demonstrated that UBC9 is present at a concentration of approximately 6 µg/ml (data not shown). The majority of UBC9 was found in fraction Q1, as expected; however, a small amount was also present in fraction Q2 (Figure 3-10B). The UBC9 protein present in fraction Q1 bound the Resource S cation-exchange column quantitatively, as UBC9 was found in fraction Q1B but not Q1A (Figure 3-3D). The UBC9 protein contained in fraction Q1B chromatographed over gel filtration with a molecular mass of approximately 20 kDa, indicating that this protein is monomeric under the conditions tested (Figure 3-3D). Although UBC9 did not precisely cofractionate with cyclin-ubiquitination activity in this

Figure 3-9. Sequence comparison of *Xenopus* UBC9 with *S. cerevisiae* UBC9 and *S. pombe hus5⁺*

The sequence of *Xenopus* UBC9 is aligned with homologous sequences from *S. cerevisiae* and *S. pombe*. Residues identical to the *Xenopus* sequence are boxed. The *Xenopus* gene is 56% identical to *S. crevasse* UBC9 and 66% identical to *S. pombe hus5+*. *S. cerevisiae* UBC9 and *S. pombe hus 5+* are 60% identical.

X. laevis UBC9	M S G I A L S R L A Q E R K A W R K D H	20
S. cerevisiae UBC9	M S S L C L Q R L Q E E R K K W R K D H	20
S. pombe hus5+	M S S L C K T R L Q E E R K Q W R R D H	20
X. laevis UBC9	P F G F V A V P T K N P D G T M N L M N	40
S. cerevisiae UBC9	P F G F Y A K P V K K A D G S M D L Q K	40
S. pombe hus5+	P F G F Y A K P C K S S D G G L D L M N	40
X. laevis UBC9	W E C A I P G K K G T P W E G G L F K L	60
S. cerevisiae UBC9	W E A G I P G K E G T N W A G G V Y P I	60
S. pombe hus5+	W K V G I P G K P K T S W E G G L Y K L	60
X. laevis UBC9	R M L F K D D Y P S S P P K C K F E P P	80
S. cerevisiae UBC9	T V E Y P N E Y P S K P P K V K F P A G	80
S. pombe hus5+	T M A F P E E Y P T R P P K C R F T P P	80
X. laevis UBC9	L F H P N V Y P S G T V C L S I L E E D	100
S. cerevisiae UBC9	F Y H P N V Y P S G T I C L S I L N E D	100
S. pombe hus5+	L F H P N V Y P S G T V C L S I L N E E	100
X. laevis UBC9	K D W R P A I T I K Q I L L G I Q E L L	120
S. cerevisiae UBC9	Q D W R P A I T L K Q I V L G V Q D L L	120
S. pombe hus5+	E G W K P A I T I K Q I L L G I Q D L L	120
X. laevis UBC9	N E P N I Q D P A Q A E A Y T I Y C Q N	140
S. cerevisiae UBC9	D S P N P N S P A Q E P A W R S F S R N	140
S. pombe hus5+	D D P N I A S P A Q T E A Y T M F K K D	140
X. laevis UBC9	R V E Y E K R V R A Q A K K F A P S	159
S. cerevisiae UBC9	K A E Y D K K V L L Q A K Q Y S K	157
S. pombe hus5+	K V E Y E K R V R A Q A R E N A P	157

Figure 3-9

Figure 3-10. *Xenopus* UBC9 complements a temperature-sensitive mutation of *S. cerevisiae* UBC9

(A) The *Xenopus* UBC9 gene was placed on a low copy vector under the control of the gal promoter, and tested for its ability to complement a strain carrying a temperature-sensitive mutation in *ubc9* (Seufert et al., 1995). Two independent transformants expressing *Xenopus* UBC9 were streaked.

(B) A mutant version of *Xenopus* UBC9 (designated C93S in the figure) carrying two point mutations, one at the putative active site cysteine, cysteine 93 to serine, and one at a neighboring leucine, leucine 96 to serine, was tested for its ability to rescue the *ubc9* mutant strain. Two independent transformants expressing the mutant plasmid were streaked.

(C) The mutant *Xenopus* UBC9 construct was tested for its ability to inhibit growth in a wild type background. Two independent transformants were streaked. Although growth on glucose was lower than on galactose, no differences between strains harboring the mutant vs. the wild-type gene could be observed.

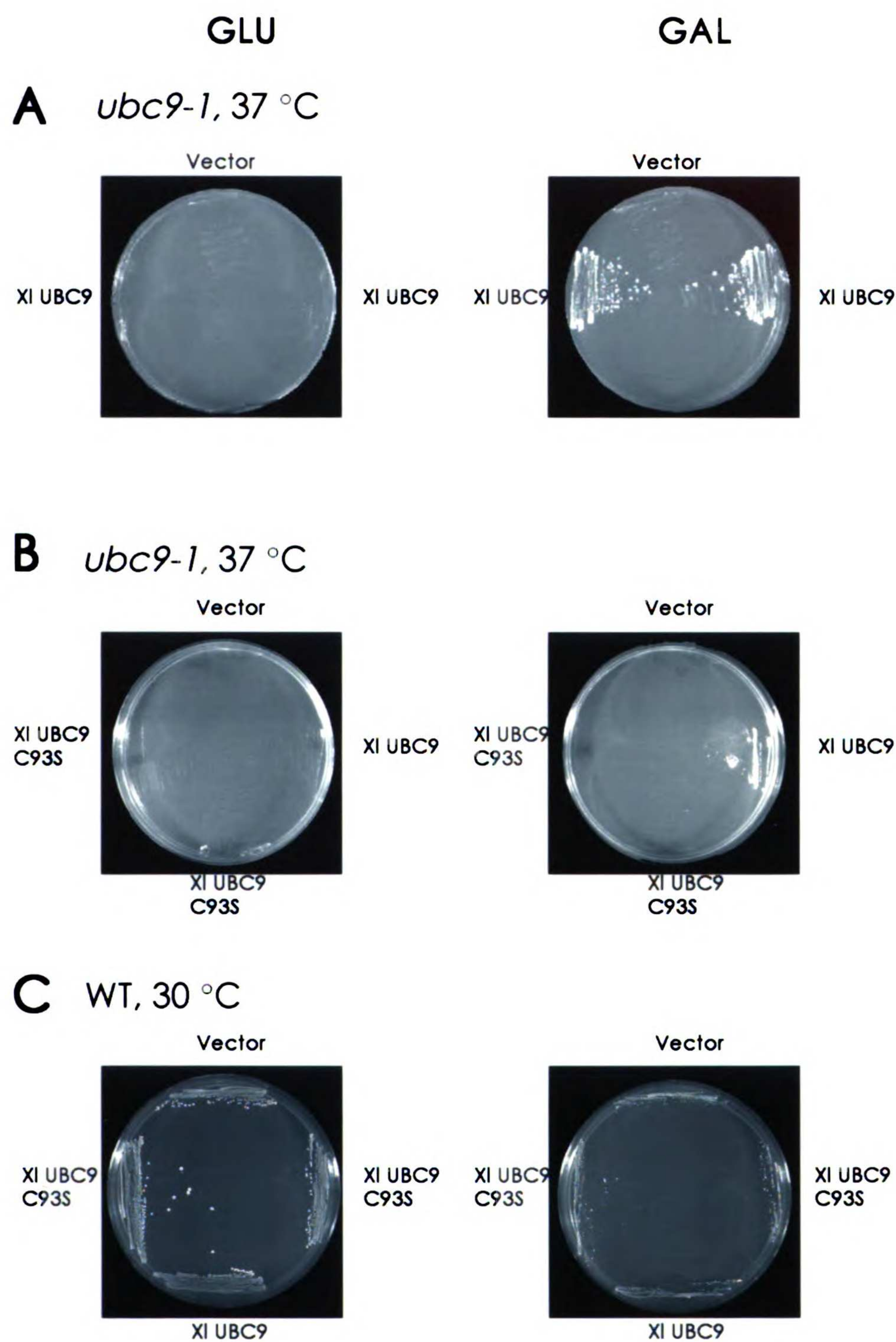


Figure 3-10

Figure 3-11. *Xenopus* UBC9 is present in fraction Q1 in monomeric form and in fraction Q2 as a large complex

(A) Affinity-purified antibodies generated against a C-terminal *Xenopus* UBC9 peptide were used to immunoblot crude interphase (I) or mitotic (M) extracts.

(B) Immunoblot of mitotic fractions Q1 and Q2 with anti-UBC9 antibodies.

(C) Mitotic S100 was applied to a Resource Q column and eluted with a 100-600 mM KCl gradient. Fractions were desalted, reconcentrated, and assayed for the presence of UBC9 by immunoblotting.

(D) Mitotic fraction Q2 was fractionated on a Superdex 200 gel filtration column. Fractions were tested for the presence of UBC9 by immunoblotting.

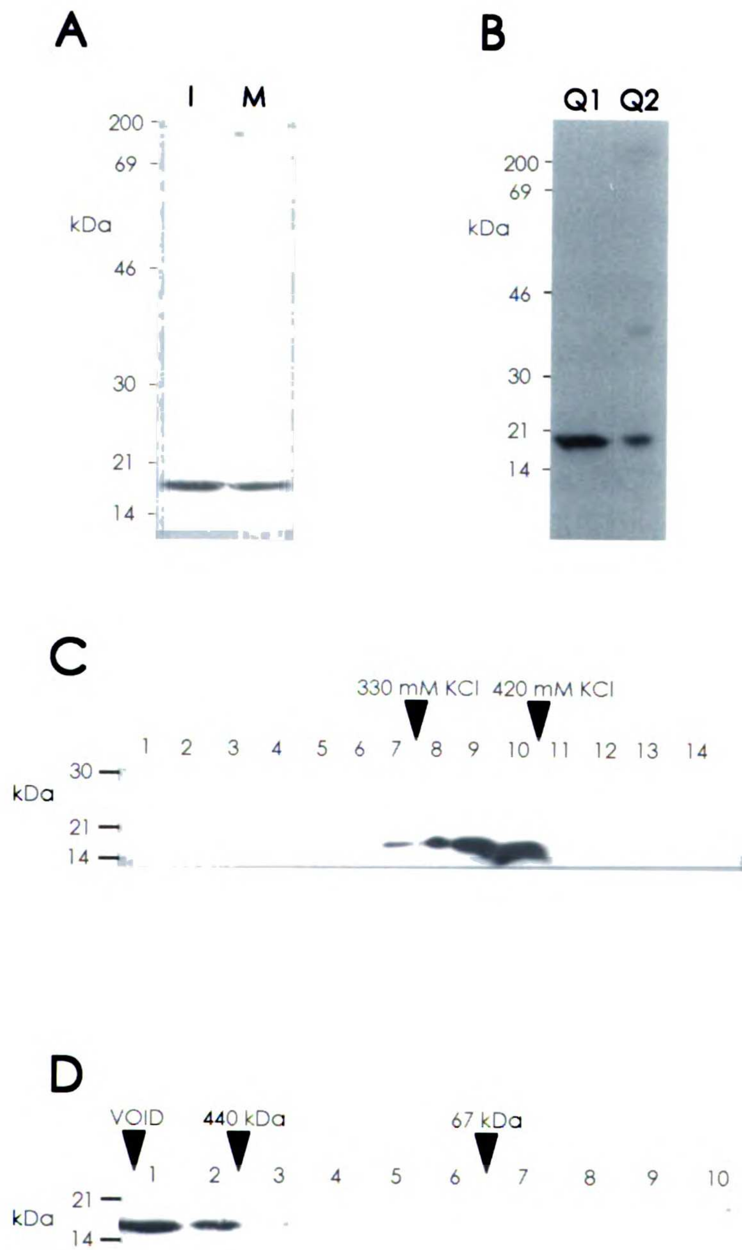


Figure 3-11

experiment (Figure 3-3A), UBC9 protein was clearly present in the complementing fraction. To test whether UBC9 was required for this activity, we immunodepleted the protein from this fraction (81 ml in Figure 3-3). Figure 3-12A shows that immunodepletion of UBC9 had little effect on the ability of this fraction to complement fraction Q2. This result strongly suggests that monomeric UBC9 is not required for cyclin ubiquitination in vitro.

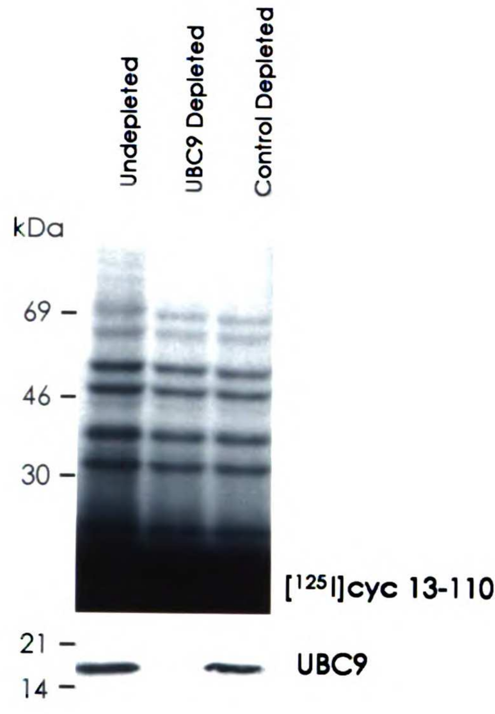
Surprisingly, the UBC9 protein contained in these fractions did not appear to form a ubiquitin thioester in the presence of recombinant E1 and radiolabeled ubiquitin. Figure 3-3B shows that the peak fraction of complementing activity (81ml) contains both UBC4 and UBC9, but only the 22 kDa UBC4-ubiquitin thioester is observed. This suggested that the UBC9 protein present in these fractions may be inactive. We therefore tested whether a bacterially-expressed form of UBC9 could complement fraction Q2 or form a ubiquitin thioester. Figures 3-4A and 3-4B indicate that bacterially-expressed UBC9 was similarly incapable of complementing fraction Q2 or forming a ubiquitin thioester in the presence of recombinant E1. This is unlikely to be due to simple inactivation of UBC9, because other ubiquitin-conjugating enzymes, such as UBC2, UBC4, or CDC34, prepared in a similar manner, were capable of accepting ubiquitin from E1 (Figure 3-4B).

We were surprised to find that a fraction of *Xenopus* UBC9 bound to the Q column, given its predicted isolectric point of 8.7. To test whether this form of UBC9 is required for cyclin ubiquitination, we immunodepleted either total fraction Q1 or fraction Q2 and tested the fractions for activity (Figure 3-12B). We found that depletion of UBC9 from both fractions had little effect on the ability to ubiquitinate cyclin. The small decrease in formation of higher molecular mass conjugates was the result of slight dilution of the fractions during immunodepletion. However, we should point out that it was difficult to completely

Figure 3-12. *Xenopus* UBC9 is not required for cyclin ubiquitination *in vitro*

(A) The Q1B gel filtration fraction of peak activity (81 ml in Fig. 3-3) was immunodepleted with either affinity-purified UBC9 antibodies or with control antibodies and tested for its ability to complement fraction Q2. Below is shown the immunoblot detection of UBC9 in undepleted and depleted fractions.

(B) Total fraction Q1 or Q2 was immunodepleted of UBC9 and tested for activity. The substrate in this experiment was a fusion protein containing an N-terminal fragment of cyclin B fused to protein A. Below is shown the immunoblot detection of UBC9 in undepleted and depleted fractions.



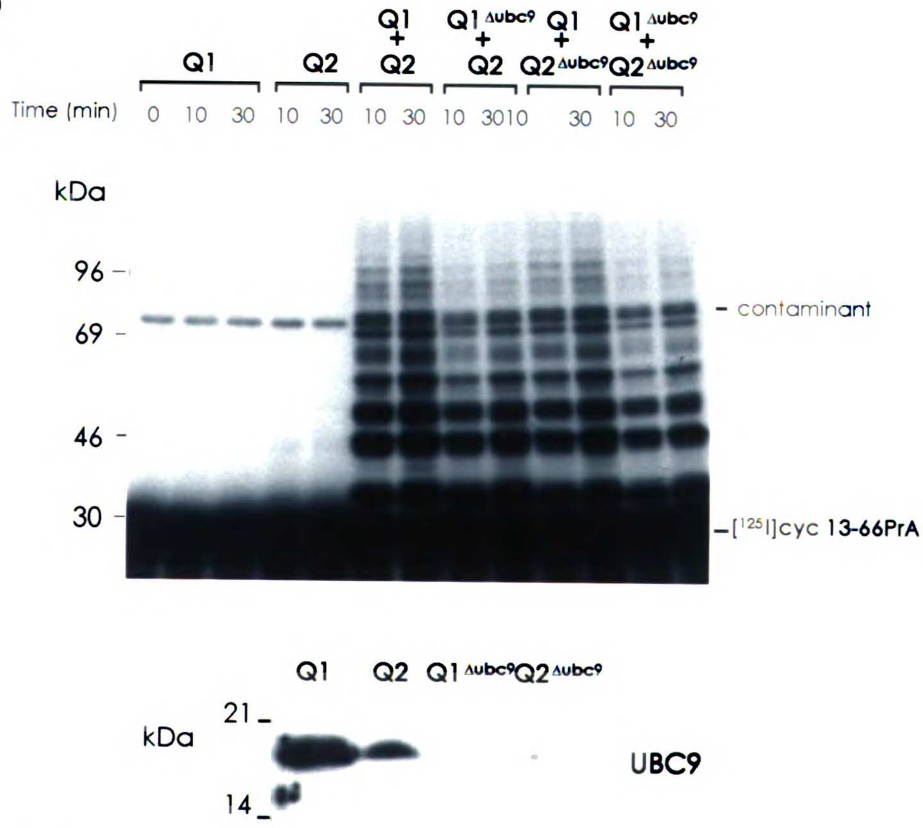


Figure 3-12

immunodeplete UBC9 from fraction Q2, suggesting that the protein in this fraction might not be as accessible to the antibody. To further characterize this form of UBC9, we fractionated mitotic high-speed supernatants over Resource Q and eluted the column with a 0.1-0.6M KCl gradient. UBC9 eluted as a single peak at a salt concentration of approximately 350 mM (Figure 3-11C). To determine whether UBC9 in fraction Q2 was part of a complex, we applied this fraction to a Superdex 200 column (fractionation range 10 kDa to 600 kDa). Surprisingly, UBC9 contained in this fraction did not elute as a monomer of 18 kDa, as was found for UBC9 contained in fraction Q1, but rather as a large complex in the void volume of the column (Figure 3-11D).

Purification of the ubiquitin-conjugating enzyme in Fraction Q1A

To identify the activity present in fraction Q1A, we developed the following purification strategy (Figure 3-13): total fraction Q1 derived from approximately 150 ml of interphase *Xenopus* extract was precipitated in 80% ammonium sulfate, and the pellet resuspended in 1.2 M ammonium sulfate. This material was applied to a 22-ml phenyl sepharose column and eluted with a decreasing ammonium sulfate gradient. Two peaks of Q2-complementing activity were observed. The first peak of activity, eluting between 0.4 and 0.3 M ammonium sulfate, cofractionated with UBC4. The second, eluting at 0.05-0 M ammonium sulfate, did not contain detectable UBC4 as measured by immunoblotting. This step yielded a very high degree of purification as very few proteins bound this tightly to the column. Fractions from the second peak of activity were pooled, desalted, and the pH raised to 9.0. This material was then applied to an anion-exchange column, and the protein eluted with a 0-0.6 M KCl gradient. Complementing activity eluted as a discrete peak between 100 and 150 mM KCl. This activity was then fractionated by gel filtration chromatography using a Superdex

Figure 3-13. Purification of UBCX

(A) Fractionation scheme.

(B) Column fractions from the Superdex 75 column were tested for their ability to complement mitotic fraction Q2.

(C) Thioester assay of column fractions in the presence of recombinant E1 and radiolabeled ubiquitin.

(D) Silver-stained gel of the column fractions.

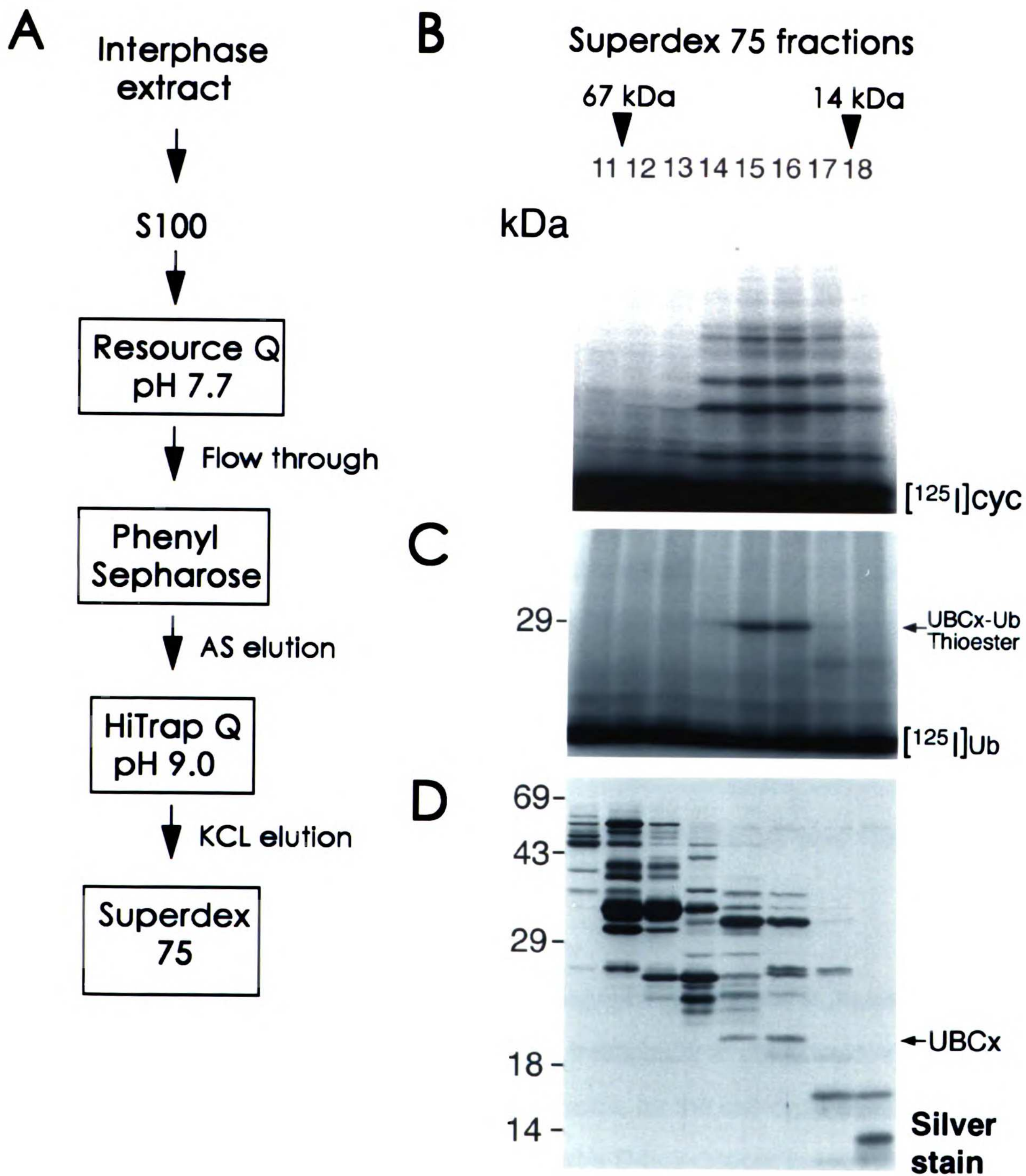


Figure 3-13

75 column. Figure 3-13 shows that complementing activity eluted at molecular mass corresponding to approximately 28 kDa. Addition of recombinant E1 and radiolabeled ubiquitin to these fractions indicated the presence of a single predominant 29 kDa thioester that cofractionated with complementing activity. Silver-stained polyacrylamide gels of these fractions indicated a single cofractionating 21 kDa protein, a size consistent with the observed 29 kDa thioester with ubiquitin (8.5 kDa).

These proteins were transferred to PVDF, and the 21 kDa band excised and submitted for proteolytic digestion and peptide sequencing. Two peptides of good quality were obtained and used to search the database. Both peptides identified a human expressed-sequence tag that had homology to ubiquitin conjugating enzymes. We used the sequence of this EST to obtain a full length clone from a human testis cDNA library. The open reading frame predicts a protein of 21 kDa, and translation of this clone *in vitro* yields a protein of the expected size. Sequence comparison of this clone with known ubiquitin conjugating enzymes reveals that it is a novel ubiquitin-conjugating enzyme. It is most similar to human UBC2, with which it shares 46% identity over 128 amino acids.

Discussion

We find at least two ubiquitin-conjugating enzymes in *Xenopus* extracts that are each sufficient to complement fraction Q2 to ubiquitinate cyclin B. Neither of these E2s appears to be responsible for the cell-cycle specificity of cyclin ubiquitination. Both E2 activities support a D-box-dependent reaction, suggesting that they act through a common recognition mechanism. Our data strongly suggest that one of the two E2 activities is a *Xenopus* homolog of UBC4. This protein copurifies with ubiquitination activity over ion exchange and gel filtration chromatography. Furthermore, both human and *Xenopus* UBC4 can complement

fraction Q2 for D-box-dependent cyclin ubiquitination at concentrations similar to those found in our fractions. We have recently purified and cloned the second E2 activity present in our fractions. UBCX is distinct from UBC4 and UBC9, and may correspond to the recently described activity in clam eggs called E-2C, for which sequence information has not yet been reported (Hershko et al., 1994). The *Xenopus* homolog of yeast UBC9, an enzyme implicated in cyclin degradation (Seufert et al., 1995), does not cofractionate with either of our two E2 activities.

UBC4 has been implicated in the degradation of many different proteins. In yeast it is required in conjunction with a closely related enzyme, UBC5, for the turnover of abnormal proteins (Seufert and Jentsch, 1990), and for the degradation of the MATα2 repressor (Chen et al., 1993). A UBC4 homolog present in wheat germ extract appears capable of supporting ubiquitin conjugation to many proteins *in vitro* (Girod and Vierstra, 1993) Two mammalian homologs of UBC4 are capable of mediating the ubiquitination of p53 catalyzed by the E6AP/E6 complex (Rolfe et al., 1995; Scheffner et al., 1994). The involvement of UBC4 in such varied ubiquitination reactions makes it an unlikely determinant of substrate specificity. Taken together with our data, these findings suggest that UBC4 may act in conjunction with multiple E3s.

Mutation of UBC4 family members in yeast has not been reported to cause a clearly-defined cell cycle arrest (Seufert and Jentsch, 1990). This could be due to either the pleiotropy of the mutation or to functional redundancy among E2s involved in cyclin ubiquitination. However, deletion of UBC4 is synthetically lethal in yeast strains carrying a temperature-sensitive mutation in CDC23 (Irniger et al., 1995), a component of APC (see Chapter 4), suggesting that these proteins function in the same biochemical pathway. Furthermore, *ubc4* mutants are hypersensitive to Clb2 overexpression (A. Amon, personal communication).

UBCX is a novel ubiquitin-conjugating enzyme involved in cyclin ubiquitination. The gene we have identified is most similar to human UBC2, perhaps explaining why we observed some complementation of fraction Q2 with high doses of human UBC2 (Figure 3-4). We have noted consistent differences in the pattern of ubiquitin conjugates produced by addition of UBC4 vs. UBCX to fraction Q2. UBC4 promotes formation of high molecular mass forms; UBCX favors the production of mono-, di, and tri-ubiquitinated species. Since it is believed that the proteasome recognizes longer ubiquitin chains, the activity of UBCX may not be sufficient to target cyclin for degradation. One hypothesis is that both UBC4 and UBCX may be necessary for rapid degradation *in vivo*. Unfortunately, we have not been able to immunodeplete UBC4 from crude extracts to test whether this protein is essential for degradation. We have also not detected any synergy between the function of semi-purified UBC4 and UBCX in vitro. We are in the process of expressing recombinant UBCX in *E. coli*. Dose response experiments with the purified recombinant protein should enlighten us as to the relative roles of these E2s in cyclin degradation.

Searches of the sequence databases with the human UBCX sequence have not identified a yeast gene of high similarity. It is perhaps surprising that previous screens for genes required for cell division have not identified this E2. However, if multiple ubiquitin conjugating enzymes can support cyclin proteolysis, as our data suggest, this finding would not be unexpected. Another possibility is that different ubiquitin conjugating enzymes participate in cyclin proteolysis in different organisms. Although this might explain our inability to observe a role for UBC9 in cyclin proteolysis in *Xenopus*, we consider this possibility unlikely, as the E3 complex involved in cyclin degradation is conserved from yeast to man (see Chapter 4). Our identification of UBCX should enable the yeast homolog to be identified, if it exists. Knockouts of this gene in combination with UBC4 mutations

should yield insight into the physiological roles of these E2s in mediating cyclin destruction.

Xenopus UBC9 is not essential for cyclin ubiquitination *in vitro*

Our analysis of *Xenopus* UBC9 has yielded several surprises. We were disappointed to find that *Xenopus* UBC9 did not appear to cofractionate with our activities; nor is recombinant UBC9 sufficient to complement the ubiquitination reaction. It remains formally possible that UBC9 is required at some other step in promoting proteolysis. For example, it could be required for a post-conjugative degradation step affecting the degradation of many proteins. It is important to point out that the yeast *ubc9-1* mutant was not tested for defects in degrading substrates other than cyclins (Seufert et al., 1995). It is therefore possible that it, like UBC4, participates in the degradation of a wide variety of proteins, and is not specifically involved in cyclin degradation. Another possibility is that UBC9 is involved in the activation of cyclin proteolysis or its regulation. We have immunodepleted UBC9 from crude interphase extracts and have not detected any defect in activating cyclin ubiquitination with recombinant cyclins. Experiments in *S. pombe* have revealed that deletion of *hus 5* causes a high number of abortive mitoses with abnormal mitotic spindles formed. Such conditions might activate the checkpoint pathway that stabilizes cyclin in response to misattached chromosomes. It should be possible to test this hypothesis by determining whether mutations in this checkpoint pathway (Hoyt et al., 1991; Li and Murray, 1991) are necessary for stabilization in *ubc9* mutants.

The biochemistry of UBC9 activation also seems unique. We have been unable to detect a thioester with either endogenous monomeric UBC9 or with bacterially-expressed protein. We feel it is unlikely that the protein is simply inactivated, since other E2s form readily-detectable thioesters under the same

conditions. Instead, this may indicate that UBC9 must be activated in some manner to accept ubiquitin from E1, that an enzyme other than E1 serves as the ubiquitin donor, or that other proteins must be present to transfer ubiquitin from E1 to UBC9. The latter possibility is especially interesting given that a fraction of UBC9 in *Xenopus* extracts forms a very high molecular mass complex. We are currently testing whether this form of UBC9 can form a thioester in vitro, and we are attempting to purify this complex by affinity methods using UBC9 antibodies. We know that the UBC9 complex is neither the proteasome nor the Anaphase-Promoting Complex involved in cyclin ubiquitination (Chapter 4) although it does demonstrate similar chromatographic properties. UBC9 is unique among E2s from a biochemical perspective, and it is clearly important for some aspect of mitosis, and perhaps in recovery from DNA-damage induced checkpoint arrest (Al-Khodairy et al., 1995). Our data suggest that it is not involved directly in ubiquitinating cyclin. It will be interesting to learn what its true substrates are.

Chapter 4

A 20S Complex containing CDC16, CDC23, CDC27, and BimE Catalyzes the Mitosis-Specific Conjugation of Ubiquitin to Cyclin B

Abstract

Cyclin B is degraded at the onset of anaphase by a ubiquitin-dependent proteolytic system that involves the ubiquitin conjugating enzymes UBC4 and UBCX. The mitotic specificity of cyclin ubiquitination is determined by a 20S complex that contains homologs of *S. cerevisiae* CDC16, CDC23 and CDC27 and *A. nidulans* BimE. Because these proteins are required for anaphase in yeast and mammalian cells, we refer to this complex as the Anaphase-Promoting Complex (APC). CDC27 antibodies deplete APC activity, while immunopurified CDC27 complexes are sufficient to complement either interphase extracts or a mixture of recombinant UBC4 and the ubiquitin-activating enzyme E1. These results suggest that APC functions as a regulated ubiquitin-protein ligase that targets cyclin B for destruction in mitosis. Purification of the interphase and mitotic forms of APC indicates that both forms of the complex contain eight subunits, four of which appear to be novel proteins. Several components of APC become phosphorylated in mitosis. Fractionation of mitotic extracts has revealed that cdc2 kinase can phosphorylate at least two subunits of the complex. However, this phosphorylation is not sufficient for activating the interphase form of APC, suggesting the importance of other regulators.

Introduction

Entrance into mitosis is governed by the protein kinase cdc2, whose positive regulatory subunits, the mitotic cyclins, accumulate throughout interphase. Exit from mitosis requires the inactivation of cdc2, initiated by rapid cyclin B proteolysis that commences at anaphase. Restabilization in the subsequent interphase enables cyclin B to accumulate again, initiating a new mitotic cycle. In the embryonic cell cycle, the regulated activation and inactivation of mitotic cyclin destruction transforms continuous cyclin synthesis into alternating periods of interphase and mitosis (King et al., 1994; Nasmyth, 1993).

Mitotic cyclins contain a short N-terminal sequence, called the destruction box (D-box), that is required for their rapid degradation (Glotzer et al., 1991). Ectopic expression of non-degradable cyclins arrests the cell cycle with elevated levels of cdc2 kinase activity (Gallant and Nigg, 1994; Ghiara et al., 1991; Luca et al., 1991; Murray et al., 1989). This arrest occurs in telophase, suggesting that cyclin degradation is required to exit mitosis (Holloway et al., 1993; Surana et al., 1993). Treatments that interfere with the proteolysis of endogenous cyclin B, however, arrest cell division earlier, at anaphase (Holloway et al., 1993). This discrepancy can be explained by hypothesizing that chromosome segregation and cyclin proteolysis depend upon common components. Support for this idea has emerged recently from studies in budding yeast, where CDC16 and CDC23, genes required for progression through anaphase, have been shown to be required for the proteolysis of B-type cyclins (Imiger et al., 1995). The proteins encoded by these genes form a complex with the CDC27 protein (Lamb et al., 1994), a homolog of which is also required for anaphase progression in mammalian cells (Tugendreich et al., 1995).

Biochemical evidence suggests that cyclin proteolysis is mediated by the ubiquitin pathway: cyclin B-ubiquitin conjugates can be observed in mitotic, but

not interphase *Xenopus* extracts (Glotzer et al., 1991); mutations in the D-box that block degradation also interfere with ubiquitination; and methylated ubiquitin, an inhibitor of polyubiquitin chain formation, interferes with the proteolysis of A- and B-type cyclins in extracts of clam eggs (Hershko et al., 1991).

A complex, multistep pathway is required for the covalent attachment of polyubiquitin chains to substrate proteins (reviewed by Ciechanover (1994)). The polypeptide ubiquitin is first activated at its C-terminus via thioester formation with E1, the ubiquitin-activating enzyme. E1 subsequently transfers ubiquitin to a family of ubiquitin-conjugating enzymes (E2s), again forming thioester intermediates. Although certain E2s can transfer ubiquitin directly to substrates *in vitro*, the physiologic reaction often requires a third component, termed a ubiquitin-protein ligase, or E3. This component can directly mediate substrate specificity and may also be required to synthesize the polyubiquitin chain that is presumed to target the substrate for degradation by the 26S proteasome complex (reviewed by Peters (1994)). In certain cases, substrate specificity is mediated by an additional component, such as the human papillomavirus E6 protein, which interacts with a cellular E3 to ubiquitinate p53 (Scheffner et al., 1993).

The cyclin ubiquitination reaction is unusual in that it exhibits specificity at two levels: substrate recognition which is reflected in the requirement for an intact D-box, and temporal control in the limitation of its activity to a specific phase of the cell cycle, late mitosis and early G1 (Hunt et al, 1992; Amon et al., 1994). Little is known regarding the components involved in cyclin B ubiquitination. Studies in budding yeast have implicated a ubiquitin conjugating enzyme, UBC9, in the degradation of both S- and M-phase cyclins (Seufert et al., 1995); however, it remains unclear whether this enzyme is required for D-box-dependent ubiquitination. Fractionation of clam egg extracts has separated two activities distinct from E1 that are required for cyclin ubiquitination (Hershko et

al., 1994). While one activity is found only in mitotic extracts, the other is active throughout the cell cycle, and appears to be an E2. To date, no components required for D-box-dependent cyclin ubiquitination have been molecularly identified.

We have fractionated mitotic *Xenopus* egg extracts to identify components required for cyclin B ubiquitination. We find that the mitotic specificity of cyclin ubiquitination is determined by a 20S complex that contains homologs of the *S. cerevisiae* tetratricopeptide-repeat proteins CDC27, CDC23, and CDC16. Additionally, this complex contains at least five additional stoichiometric subunits, one of which we have identified as a Xenopus homolog of *A. nidulans* gene BimE. This complex, activated in mitosis, can act in conjunction with recombinant UBC4 and E1 to ubiquitinate cyclin B, suggesting that it functions as a temporally-regulated cyclin-ubiquitin ligase.

Materials and Methods

Preparation of extracts and high-speed supernatants

Interphase extracts were prepared as described (Murray, 1991), except that eggs were activated with the calcium ionophore A23187 (CalBiochem) at a concentration of 1 μg/ml. Cycloheximide was added to arrest the extracts in interphase. To generate mitotic Δ90 extracts, a bacterially-expressed non-degradable Δ90 fragment of sea urchin cyclin B was added to interphase extracts at a concentration of 60 μg/ml. A different mitotic extract was prepared from non-activated metaphase II-arrested eggs in the presence of 1 μM okadaic acid (CalBiochem) according to the protocol of Kuang et al. (1991). For the generation of high-speed supernatants, extracts were diluted 10-fold in buffer Q-A (20 mM Tris-HCl, pH 7.7, 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM DTT) containing an energy-regenerating system (Murray, 1991) and okadaic acid (1 μM,

mitotic extracts only), and were centrifuged for 1 h at 115,000 x g. The superna-tant was reconcentrated to 50% of the original extract volume in Centriprep-10 concentrators (Amicon). The fractions were stored at -70 °C. For preparation of fraction QE2, extracts from metaphase II-arrested eggs were thiophosphorylated using the protocol described by Kuang et al. (1991) with modifications (J. Kuang, C. Ashorn, M. Nelman-Gonzalez, and T. Stukenberg, in preparation).

Protein fractionation

Column chromatography was done with an FPLC system (Pharmacia) at 4 °C. For fractionation of Q2, 40 ml of diluted mitotic S100 was applied to the Resource Q column. Bound proteins were eluted with 6 column volumes of a linear salt gradient (0-500 mM KCl in Q-A). Fractions (2.5 ml) were collected, desalted and reconcentrated to 0.5 ml. Similar amounts of S100 prepared from metaphase II extracts or thiophosphorylated QE2 fractions were fractionated using the same protocol.

To determine the S-value of APC, fraction QE2 was rechromatographed over Resource Q, and the fraction containing APC analyzed by sucrose gradient centrifugation (15-40%) for 13.5 h at 37,500 rpm in an SW40 rotor (Beckman). Fractions (0.85 ml) were diluted 1:4 with buffer Q-A and concentrated to 150 µl. The positions of the 15S p97-ATPase (Peters et al., 1990) and a 10S complex of elongation factors (J.-M.P., unpublished) separated in the same gradient were used as references.

To determine the apparent molecular weight of APC, 0.2 ml of a reconcen-trated Q column fraction was applied to a 24 ml Superose 6 column at a flow rate of 0.5 ml/min. Fractions of 1 ml were reconcentrated to 0.1 ml and assayed in the presence of interphase S100.

Ubiquitination assays

An N-terminal fragment of sea urchin cyclin B, consisting of residues 13-110, and a corresponding fragment containing a mutated D-box (R42A, A44R; Holloway et al., 1993) were labeled to a specific activity of ~100 µCi/µg using the chloramine T procedure. Ubiquitination assays were performed in a total volume of 5 µl. They contained energy-regenerating system, 1.25 mg/ml bovine ubiquitin (Sigma), 12.5 ng (100 nM) labeled substrate, 2-3 µl of column fraction and in some cases either 1.25 µl of interphase S100 or 130 µg/ml recombinant human E1 and 25 µg/ml recombinant human UBC4 (Rolfe et al., 1995). Reactions were incubated at 22°C for 10 min., quenched by addition of SDS-sample buffer, and analyzed by SDS-PAGE followed by autoradiography or phosphorimaging.

Immunodepletion and reconstitution experiments

To immunodeplete CDC27 from fraction QE2, 4 volumes of anti-CDC27 or preimmune serum were incubated with one volume of protein A-affiprep beads (Biorad) for 2 hours at 4°C. Beads were washed 5 times in buffer Q-A, incubated in 2 volumes of fraction QE2 for 2 hours at 4°C and removed by centrifugation.

For reconstitution experiments, 0.5 volumes of CDC27 antiserum or preimmune antiserum was bound to one volume protein A beads as described above. The beads were incubated with 5 volumes of fraction QE2 for 2 hours at 4 °C and subsequently washed 5 times in buffer Q-A containing 500 mM KCl, and three times in Q-A. For immunoblot analysis, the beads were eluted with SDS-sample buffer. For ubiquitination assays, 5 µl of washed beads was incubated with 6 µl of reaction mix containing energy-regenerating system, ubiquitin, and labeled substrate and either interphase S100, or a mixture of recombinant E1 (260 µg/ml) and UBC4 (50 µg/ml).

To analyze the protein composition of the CDC27 immunoprecipitate,

antibodies were bound to protein A beads as above and covalently coupled using the dimethylpimelimidate method (Harlow and Lane, 1988). Fifty µl of the immune and preimmune beads were each incubated with 750 µl of fraction QE2 for 2 hours at 4°C. Subsequently, the beads were washed 5 times in Q-A containing 500 mM KCl and 0.5% NP-40, once in Q-A, and once in 10 mM KPO_4 (pH6.8). Bound proteins were eluted with 400 µl of 100 mM glycine-HCl, pH 2.1. The eluate was neutralized, concentrated 10-fold and analyzed by SDS-PAGE.

Large scale purification of APC

To obtain amounts of APC that could be used for microsequence analysis, we prepared interphase extract from approximately 3L of laid eggs. These eggs were activated with ionophore, and interphase extracts were prepared as described above, except that the packing spin was omitted. This procedure yielded approximately 500 ml of extract with a protein concentration of 30 mg/ml. The extract was frozen directly in this form to allow subsequent processing in batches. The frozen extract was then rapidly thawed, diluted 5-fold in buffer Q-A and a high speed supernatant was generated by centrifugation in an SW28 rotor for 75 minutes at 28,000 rpm. This material was applied to a 60 ml resource Q column (1.5 g protein capacity), washed with buffer Q-A, and eluted with a 100-600 mM salt gradient. Fractions were collected, desalted into Q-A, reconcentrated, and frozen. Fractions were analyzed by immunoblot analysis for the presence of CDC27, and the peak fractions, eluting at approximately 450 mM KCl, were used in the immunoaffinity step. After all of the column chromatography was completed, the peak fractions were thawed pooled, and concentrated to a volume of approximately 5 ml. A small fraction of this material was saved for use in activation experiments. The remainder was incubated for 2 hours at 4°C with 2.5 ml of covalently-coupled CDC27 antibody beads, prepared as described

above. The beads were then washed extensively with buffer Q-A containing 500 mM KCl and 0.5% NP-40. Protein was eluted from the beads with 2x2.5 ml 100 mM glycine-HCl, pH 2.1. The eluate was neutralized, concentrated, and used for subsequent analysis.

For analysis by coomassie blue staining, 5% of this prep was loaded on a single lane of a 5-15% gradient gel. A similar purification procedure, on a 10-fold smaller scale, was used to purify the mitotic form of APC. In this case, the starting material was prepared by homogenizing eggs in the presence of 2 volumes of Q-A buffer containing 1 µM okadaic acid and energy-regenerating system. This mixture was spun at 100,000x*g* for one hour, and the supernatant processed as described above. These conditions maintain CDC27 in its phosphorylated state.

To obtain peptide sequence, 80% of the interphase immunoprecipitate was loaded onto two wide lanes of a 5-15% gradient gel, electrophoresed, and blotted onto PVDF sequencing membrane (Biorad). The filters were stained with amido black, and individual bands were excised and submitted for proteolytic digestion and peptide sequencing. This preparation yielded 50-100 pmoles of each subunit as determined by amino acid analysis.

Results

Characterization of a mitotically-regulated factor essential for cyclin ubiquitination

We had previously demonstrated that the components required for cyclin ubiquitination could be isolated in soluble form following high speed centrifugation of diluted *Xenopus* extracts (Chapter 3). Importantly, the cell-cycle dependent regulation of cyclin ubiquitination was preserved in the soluble system. Fractionation of the system indicated that the regulated component(s) bound to anion exchange columns (fraction Q2), while the non-regulated components, the

ubiquitin-conjugating enzymes, did not (fraction Q1). Immunoblotting experiments demonstrated the presence of E1, the ubiquitin activating enzyme, in fraction Q2 only. However, its activity did not appear cell-cycle regulated, as Q2 fractions derived from mitotic and interphase extracts both supported the formation of a prominent 120 kDa E1-ubiquitin thioester (data not shown). Neither purified *Xenopus* E1 nor recombinant E1 was sufficient to complement fraction Q1, suggesting the fraction Q2 contained additional components required for cyclin ubiquitination.

We used an interphase S100-complementation assay to identify the regulated factor(s) present in fraction Q2. To ensure that such mitotic factors would not be rapidly inactivated upon mixing with interphase S100, we prepared a thiophosphorylated fraction that had been stably phosphorylated with ATP-γ-S (Kuang et al., 1991). This treatment renders phosphoproteins less sensitive to the actions of protein phosphatases. Thiophosphorylated S100 was applied to a Resource Q column and eluted with a 100-600 mM linear salt gradient. Activity in the presence of interphase S100 eluted at approximately 450 mM KCl (Figure 4-1A). To test whether this component was directly involved in cyclin ubiquitination rather then acting as an activator of this process, we tested each fraction for the ability to complement a mixture of recombinant UBC4 and E1. Figure 4-1B indicates that activity in the presence of E1 and UBC4 cofractionated with activity in the S100, suggesting the complementation was the result of providing a component that is directly involved in cyclin ubiquitination. E1 eluted much earlier and did not complement either reaction, as expected (Figure 4-1C).

To determine whether thiophosphorylation was essential for the stability of this activity, we fractionated a mitotic extract in precisely the same manner, but did not thiophosphorylate the fraction prior to Q column chromatography. Figure 4-2A indicates that S100 complementing activity eluted at approximately the

Figure 4-1. Identification of a mitotically-regulated activity in thiophosphorylated fraction Q2 that can complement interphase S100 or a mixture of E1 and UBC4

(A) A thiophosphorylated Q fraction (see methods) was applied to a 6 ml resource Q column and eluted with a 100-600 mM KCl gradient. Column fractions were tested for their ability to complement interphase S100 to ubiquitinate the N-terminal cyclin fragment 13-110. The peak of activity eluted at approximately 450 mM KCl.

(B) The same column fractions were tested for their ability to complement a mixture of recombinant human UBC4 and E1.

(C) Radiolabeled ubiquitin was added to each fraction, and a non-reducing gel was run to detect the E1-ubiquitin thioester.

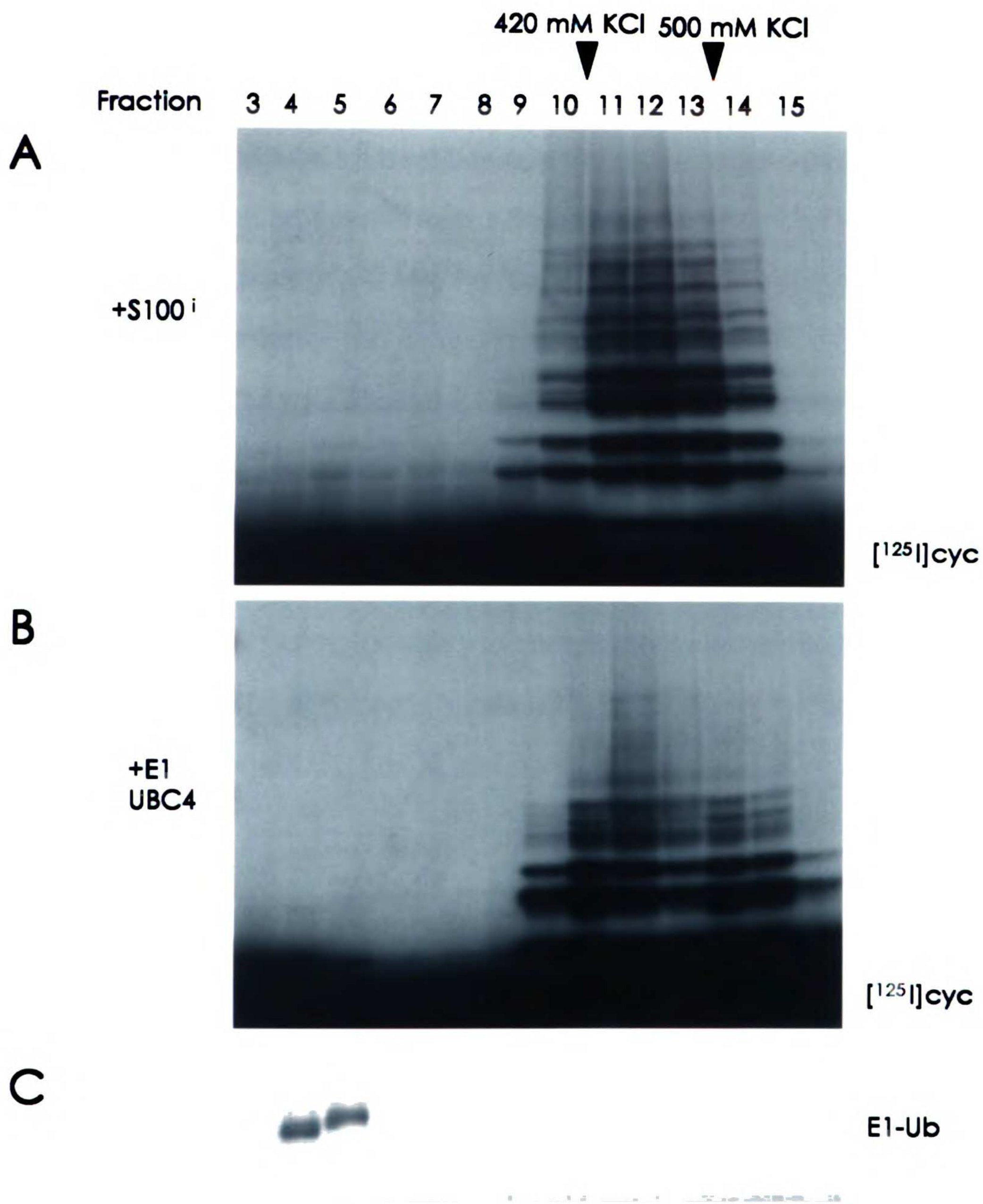


Figure 4-1

Figure 4-2. Thiophosphorylation of mitotic extracts is not necessary for complementation of interphase S100

(A) Mitotic S100 derived from metaphase II-arrested eggs was applied to a Resource Q column and eluted with a linear gradient from 100-600 mM KCl (corresponding to fractions 1-14). Fractions were desalted and tested for ability to complement interphase S100 for cyclin ubiquitination activity (upper panel), or were assayed for the presence of E1 by addition of radiolabeled ubiquitin, followed by non-reducing SDS-PAGE (lower panel; the E1-ubiquitin thioester is marked by an arrowhead).

(B) Fraction 11 was tested for ability to complement interphase S100 in the presence of wild-type or mutant (*) substrate, or for ability to complement a mixture of recombinant E1, UBC4 and purified ubiquitin.

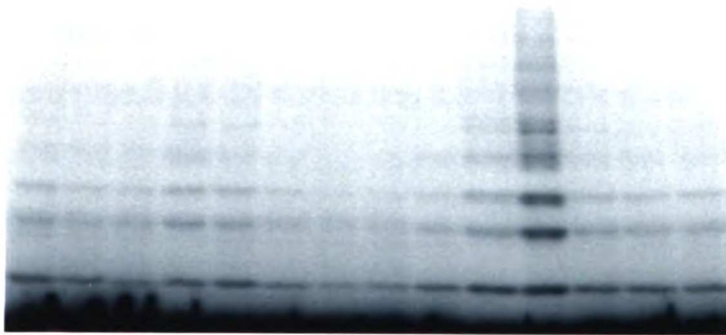


Figure 4-2

same salt concentration as in the previous experiment. Although the activity was not as robust, this experiment indicates that thiophosphorylation is not essential for complementation. Formation of high-molecular-weight conjugates was substantially reduced when the D-box mutant was assayed (Figure 4-2B). Significantly, fraction 11 was also sufficient to complement a mixture of recombinant E1, UBC4 and ubiquitin (Fig. 4-2B), although the formation of high-molecular-weight conjugates was slightly reduced compared to the reaction using complete interphase S100. These results suggest that the mitotic factor functions as a mitosis-specific cyclin-ubiquitin ligase, rather than indirectly activating ubiquitination in the interphase S100 by converting it to a mitotic state.

The mitotically-regulated factor is a 20S complex containing homologs of CDC27 and CDC16

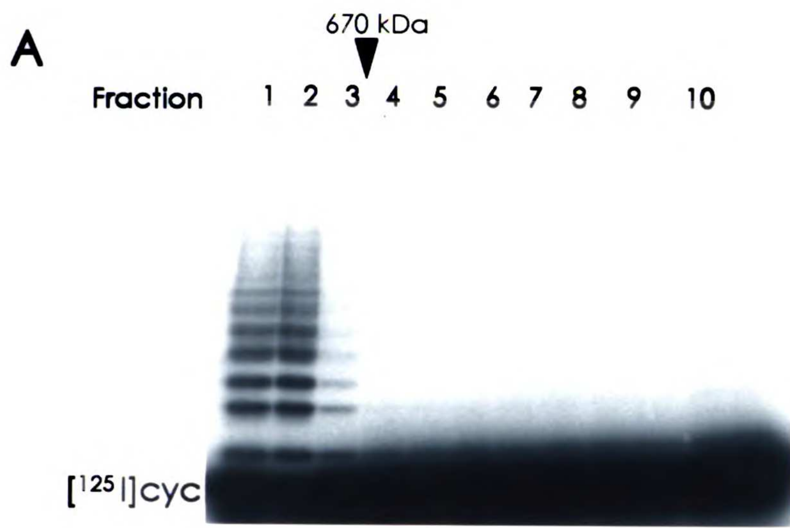
To estimate the size of the activity present in Fraction Q2, the thiophosphorylated S100 was fractionated as described for Figure 4-1, and the peak of activity (called QE2) applied to a Sephacryl 300 column. Fractions were reconcentrated and tested for the ability to complement interphase S100. Figure 4-3A indicates that the activity eluted in the void volume of the column, indicating an apparent molecular weight greater than 1 million Daltons. Analysis using a Superose 6 column indicated an apparent molecular mass between 1000 and 1500 kDa (data not shown). The peak of activity from the Resource Q column was also analyzed by sucrose gradient centrifugation. Figure 4-3B (top panel) indicates that interphase S100-complementing activity sedimented as a discrete complex of 20-22S. Peak fractions could also complement a mixture of recombinant E1 and UBC4 (data not shown).

Recent work in yeast has shown that CDC16 and CDC23, genes required for anaphase, are also necessary for the proteolysis of B-type cyclins *in vivo*

Figure 4-3. The mitotically-regulated factor is a 20S complex containing *Xenopus* homologs of yeast CDC27 and CDC16

(A) Fraction QE2 was refractionated by Resource Q chromatography, and the peak of activity was applied to a 1.6x60 Sephacryl 300 column. Fractions were reconcentrated and assayed for activity in the presence of interphase S100. The position of elution of thyroglobulin (670 kDa) is shown.

(B) Fraction QE2 was refractionated by Resource Q chromatography, and the peak of activity (designated "L") was analyzed by sucrose gradient centrifugation and assayed for ubiquitination activity in the presence of interphase S100 (upper panel). Arrows indicate the positions of proteins sedimenting at 10S and 15S, from left to right. Corresponding fractions were tested for the presence of CDC27 and CDC16 by immunoblotting (lower panels).



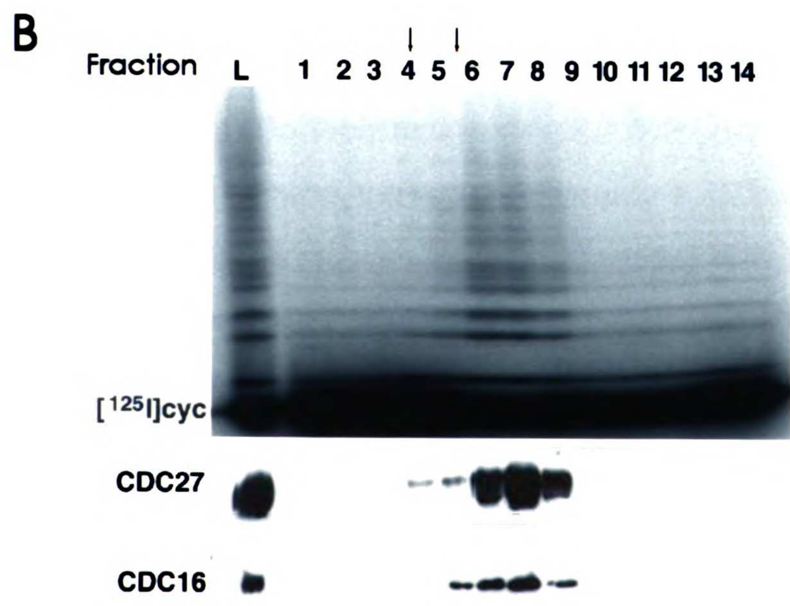


Figure 4-3

(Irniger et al., 1995). Their protein products have been demonstrated to physically interact with the CDC27 protein (Lamb et al, 1994). In human cultured cells, CDC16 and CDC27 are components of a 20S complex (S.T. and P.H., unpublished observations). Therefore, we immunoblotted the sucrose gradient fractions with antibodies generated against human homologs of CDC16 and CDC27. Figure 4-3B (lower panels) indicates that CDC27 and CDC16 both cofractionated with activity. The corresponding preimmune sera showed no reactivity (data not shown). Additional immunoblotting experiments revealed that both proteins also cofractionated with ubiquitination activity during Resource Q and gel filtration chromatography (data not shown).

To determine whether CDC27 is required for ubiquitination activity, we immunodepleted the active Q column fraction with CDC27 antibodies and assayed the supernatant for the ability to complement interphase S100. Figure 4-4A shows that CDC27 antibodies depleted activity, while preimmune sera had no effect. This treatment effectively removed both CDC27 and CDC16, suggesting that, as in yeast and humans, the two proteins form a complex. However, complete depletion of CDC16 required higher antibody concentrations than were necessary to deplete CDC27 or ubiquitination activity. CDC16 antibodies did not deplete CDC16, CDC27, or ubiquitination activity (data not shown).

We next tested whether the CDC27 complex was the only mitotic component required for cyclin ubiquitination. Figure 4-4B demonstrates that stringently-washed CDC27 immunoprecipitates derived from fraction QE2 were sufficient to complement interphase S100, while control immunoprecipitates were not. This activity required the presence of an intact D-box for generation of high-molecular weight conjugates (Figure 4-4B).

To determine whether the CDC27 immunoprecipitate contained cyclin-ubiquitin ligase activity, we assayed the mitotic immunoprecipitate against a

Figure 4-4. A complex containing CDC27 is required for ubiquitination activity, and is sufficient to complement UBC4 and E1

(A). Fraction QE2 was depleted with CDC27 antibodies (anti-CDC27) or control antibodies (preimmune), and tested for ability to complement interphase S100 (upper panel). CDC27 and CDC16 protein remaining after depletion was assayed by immunoblotting (lower panel).

(B,C) CDC27 immunoprecipitates (CDC27-IP) or control immunoprecipitates (pre-IP) derived from fraction QE2 were assayed for the ability to complement either interphase S100 (B) or recombinant E1 and UBC4 (C), using either wild-type or mutant (*) cyclin proteins.

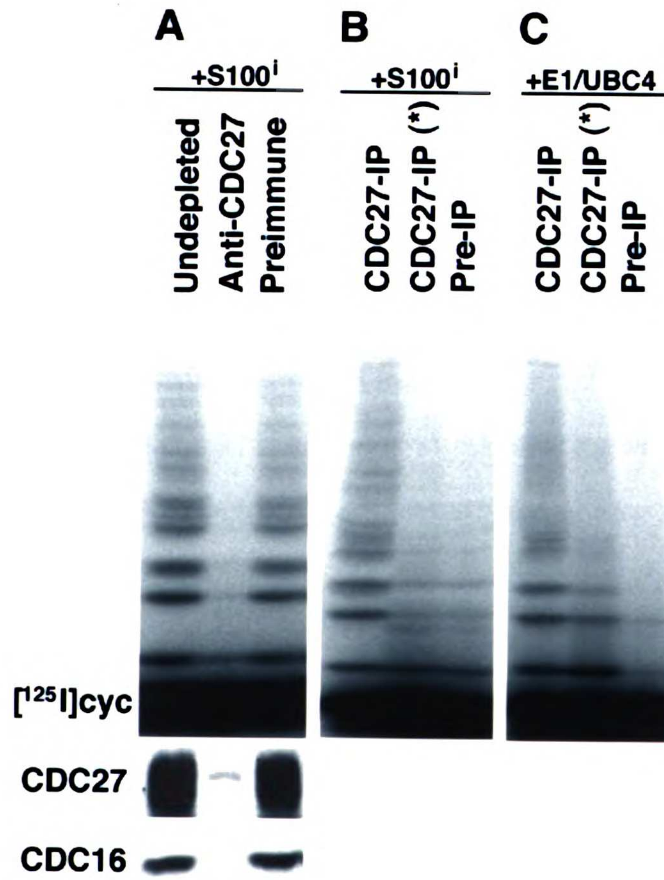


Figure 4-4

defined system composed of recombinant E1, UBC4, and purified ubiquitin. Figure 4-4C shows that a CDC27 immunoprecipitate complemented these components, while the control immunoprecipitate did not. Although the immunopurified CDC27 complex retained some degree of D-box dependence in this defined system (Figure 4-4C), the discrimination between the wild-type and mutant substrate was not as strong as when the immunoprecipitate was tested against interphase S100. The reduced specificity in the defined reaction is unlikely to be related to the use of purified UBC4, because the D-box dependence of the reaction is fully maintained when UBC4 is used to complement total fraction Q2 (Chapter 2). Instead, this result may indicate that components required for full D-box dependence may be partially lost during immunoprecipitation.

These experiments suggested that the CDC27-containing complex is the only component of the cyclin ubiquitination system that is subject to mitotic regulation. To demonstrate this directly, we immunoprecipitated CDC27 from crude interphase or mitotic extracts, and assayed the immunoprecipitates for activity in the presence of either interphase S100 or a mixture of E1 and partially purified fractions containing UBC4 or UBCX. Figure 4-5 indicates that reactions containing the mitotic immunoprecipitate were significantly more active than those containing the interphase immunoprecipitate. Importantly, the immunoprecipitated complex could support ubiquitination in the presence of either UBC4 or UBCX. However, ubiquitination did not appear as processive in the presence of these UBCs compared to the activity in the presence of S100. These experiments provide strong evidence for the hypothesis that a component of the CDC27 complex is directly regulated during the cell cycle.

Our sucrose gradient fractionation data and immunodepletion experiments suggested that CDC27 and CDC16 might be associated with one another in *Xenopus* extracts, as they are in yeast (Lamb et al., 1994). To test this possibility

Figure 4-5. The activity of the immunoprecipitated CDC27 complex is mitotically regulated

CDC27 immunoprecipitations were performed from crude interphase or mitotic extracts. The immunoprecipitates were washed three times in Q-A buffer containing 500 mM KCl and three times in buffer Q-A. The immunoprecipitate was then assayed for ubiquitination activity in the presence of Interphase S100 ($S100^{i}$), or a mixture of recombinant E1 plus UBC4 or UBCX that had been partially purified from fraction Q1.

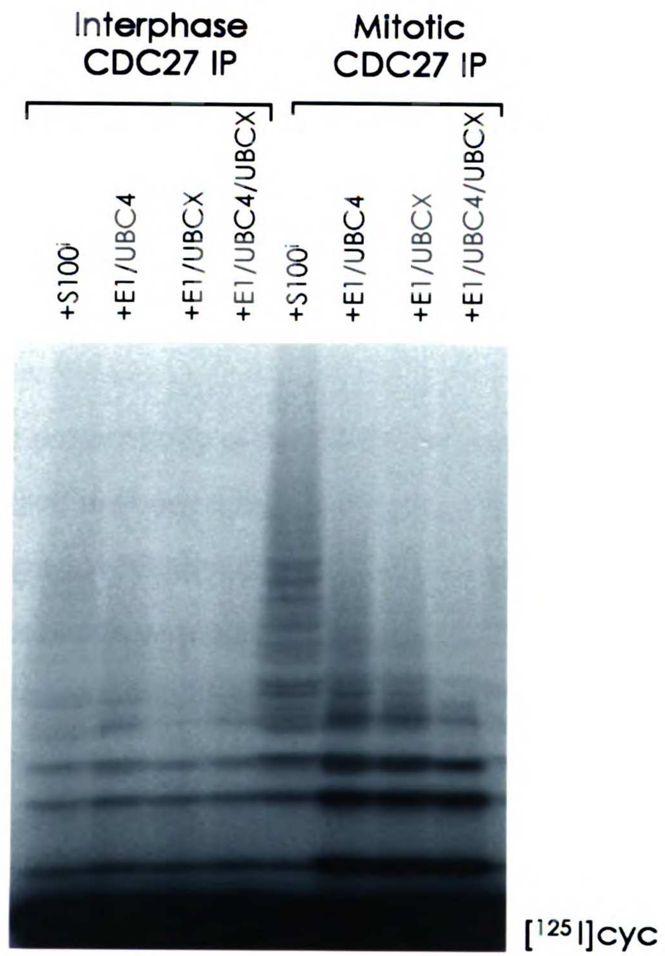


Figure 4-5

directly, we analyzed CDC27 immunoprecipitates derived from either interphase S100 or fraction mitotic fraction QE2 for the presence of CDC27 and CDC16. Figure 4-6A indicates that CDC27 immunoprecipitated from interphase S100 migrated as a 100 kDa protein on denaturing polyacrylamide gels. However, when derived from mitotic fraction QE2, CDC27 migrated as a 130-140 kDa protein, suggesting that it becomes modified in mitosis. Immunoblot analysis revealed that CDC16 coimmunoprecipitated with CDC27 from both the interphase and mitotic fractions (Figure 4-6A). CDC16 derived from interphase S100 migrated as a 72 kDa protein, while CDC16 derived from fraction QE2 was shifted to 74 kDa, indicating that it may also be modified in mitosis. These data suggest that at least a fraction of CDC16 is complexed with CDC27 during both interphase and mitosis.

To determine the complexity of the CDC27 immunoprecipitate, we incubated covalently-coupled antibody beads in fraction QE-2 and eluted bound proteins at low pH. Figure 4-6B shows that total fraction QE2 contained a large number of polypeptide bands as judged by coomassie blue staining. CDC27 immunoprecipitates derived from this fraction, however, yielded a discrete pattern of polypeptide bands that were not present in the control immunoprecipitate. Immunoblot analysis indicated that two of these bands comigrated with CDC27 and CDC16 (Figure 4-6B). The specific coimmunoprecipitation of these proteins with CDC27 in roughly stoichiometric amounts suggests that they represent additional components of the CDC27 complex. Because this complex contains homologs of genes essential for anaphase progression in yeast and mammalian cells, we refer to it as the Anaphase-Promoting Complex (APC).

Identification of the major subunits of the CDC27 complex

To identify the remaining subunits of the CDC27 complex, and to deter-

Figure 4-6. CDC 27 and CDC16 are associated in both interphase and mitosis

(A) Preimmune (Pre-IP) or CDC27 (CDC27-IP) immunoprecipitates derived from either interphase S100 (S100^{i}) or mitotic fraction QE2 were assayed for the presence of either CDC27 or CDC16 by immunoblotting. The strongly-reacting band at 55 kDa is IgG heavy chain derived from the immunoprecipitate.

(B) Proteins in total fraction QE2 or proteins immunoprecipitated from this fraction were separated by SDS-PAGE and the gels either stained with Coomassie blue or immunoblotted for the presence of CDC27 and CDC16. The positions of CDC27 and CDC16 in the immunoblots are marked by a bracket and an arrowhead, respectively. Coomassie blue-stained polypeptide bands that comigrated with CDC27 and CDC16 have been indicated by the same symbols. The identity of the faster-migrating polypeptide band that cross-reacts with CDC27 antibodies (middle panel) is probably a *Xenopus* homolog of CDC23 as determined by subsequent peptide sequence analysis.

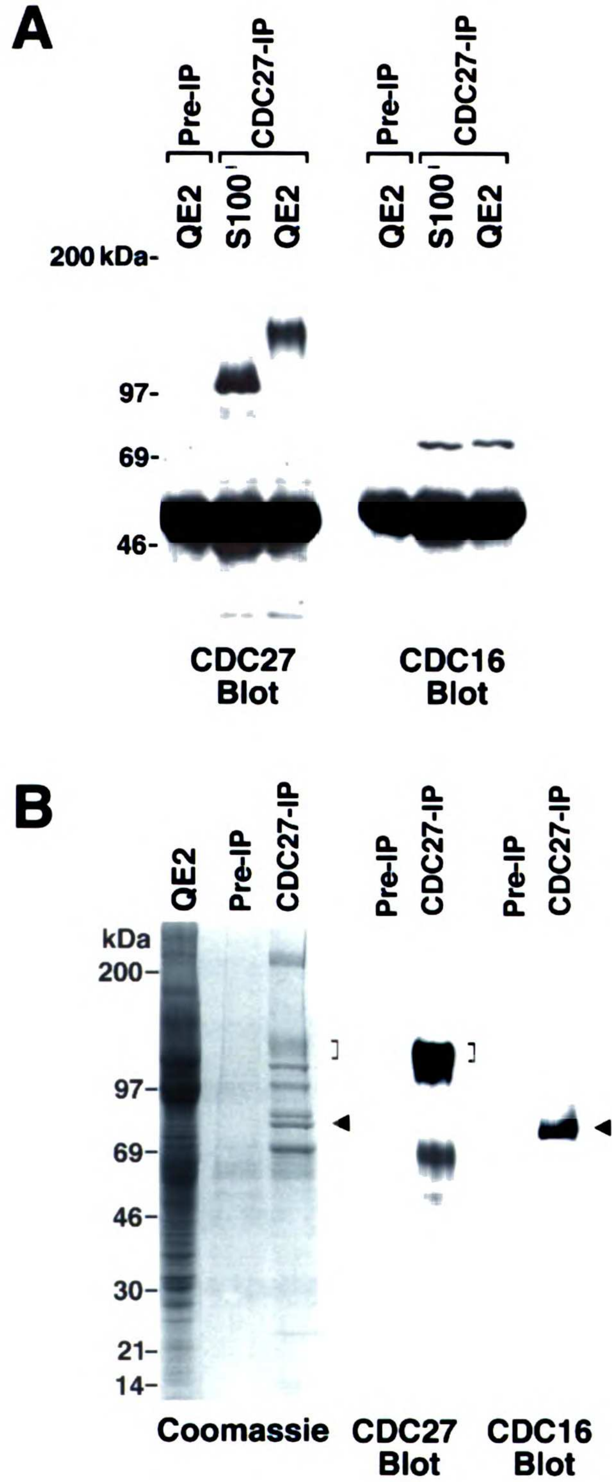


Figure 4-6

mine whether the subunit composition of the complex varies during the cell cycle, we performed a large-scale purification of APC from interphase and mitotic extracts (see methods for details). Figure 4-7 shows a coomassie blue-stained SDS-gel of the composition of the complex as purified from interphase or mitotic extracts. In addition to the eight subunits present in roughly stoichiometric quantities, a number of lower molecular mass proteins are present at substoichiometric levels (data not shown). Immunoblot analysis suggests that APC-γ corresponds to CDC27, and that APC-ζ corresponds to CDC16. The basic subunit composition of the complex appears similar in interphase and mitosis; however, as for CDC27, a number of the subunits exhibit different migration in interphase and mitotic extracts. This is most clearly evident for APC-α, which migrates at 200 kDa in interphase extracts and 220 kDa in mitotic extracts; APC-θ also changes mobility, from 60 kDa in interphase to 70 kDa in mitosis.

We purified the interphase form of the complex from approximately 500 ml (15 g total protein) of *Xenopus* extract (see methods for details). The immunoprecipitate was separated by SDS-gel electrophoresis and the proteins transferred to PVDF. Membrane slices corresponding to each subunit were obtained, and each appeared to be a well-resolved band with the exception of the gamma and delta subunits, which comigrate when isolated from interphase extracts. Each of these subunits was subjected to proteolytic cleavage using Lys C endopeptidase and the peptides separated by HPLC. Peptides of high mass and reasonable purity as determined by mass spectroscopy were subjected to Edman degradation.

Computer-based searches of GenBank and the expressed-sequence tag databases (dbEST) using these peptide sequences revealed that APC-α, APC-γ, APC-ζ, and APC-η are similar to known proteins (Figure 4-8). Peptide sequences from the other four subunits have not indicated any similarity with known pro-

Figure 4-7. Composition of the interphase and mitotic forms of the Anaphase-Promoting Complex

Interphase or mitotic S100 was fractionated by Q column chromatography, and the peak CDC27-containing fractions pooled and immunoprecipitated using covalently-coupled antibody beads. Beads were washed in buffer containing 0.5 M KCl and 0.5% NP-40, and eluted at low pH. The eluate was neutralized, concentrated and analyzed by electrophoresis on 5-15% polyacrylamide gels subsequently stained with coomassie blue. The eight approximately stoichiometric subunits have been designated APCα- APCθ.

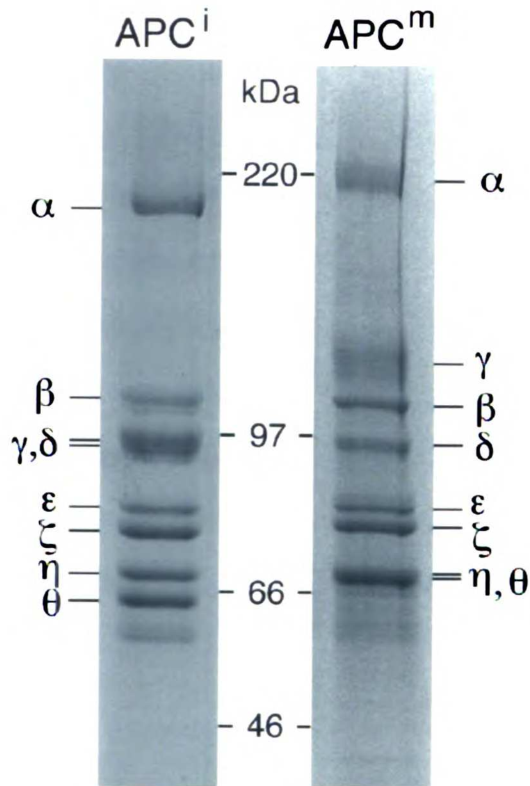


Figure 4-7

Figure 4-8. Peptide sequences of known components of APC

Peptide sequence was obtained from each subunit of APC. The peptide sequences for those subunits which matched known proteins are indicated above. Two peptides derived from APC-α matched a published sequence in the database, tsg24. Three peptides obtained from APC-γ matched showed a high degree of similarity to human CDC27. Similarly, three peptides from APC-ζ showed good agreement with human CDC16. The identity of APC-γ and APC-ζ have also been confirmed by immunoblot with CDC27 and CDC16 antibodies, respectively. APCη peptide sequence matched a human expressed sequence tag (H59410) perfectly over 18 amino acids. Search of the data base with this EST reveled the highest degree of similarity with *Drosophila* CDC27, followed closely by *S. cerevisiae* CDC23. However, this EST is more highly related to *S. cerevisiae* CDC23 than to human CDC27, suggesting that it is a human homolog of CDC23.

APCα

APCα: PK105 DYI APLPF QVAN VXP
tsg24:168-182 DYI APLPF QVAN NWA

APCα: PK182 E E Q NAVL NL - D QL GT P QH GMT T SSLTA X LR
tsg24:277-306 E E E NAVL K F P E QA GT L QN A T T S SSLTA H LR

APCγ

APCγ: PT114 LL HL PAAL GPL NP Q FGIL
CDC27Hs: 268-285 LL GG PAAL S PL T P S FGIL

APCγ: PT60 V SILFANEK
CDC27Hs: 709-717 A SILFANEK

APCγ: PT85 S ALQELEELK
CDC27Hs: 720-729 L ALQELEELK

APCζ

APCζ: PT49 2° G L X LTAQY
CDC16Hs: 45-52 C L Y LTAQY

APCζ: PT49 1° CYDFDVHTMK
CDC16Hs: 543-552 CYDFDVHTMK

APCζ: PT47 EF DF E R
CDC16Hs: 566-571 EF EV E K

APCη

APCη: PK153 EFFLAHIYTELQLIEEAL
EST H59410 EFFLAHIYTELQLIEEAL

Figure 4-8

teins. However, peptides derived from APC-β, APC-ε, and APC-θ show a high degree of similarity with unknown human proteins found in the expressed-sequence tag database. We are currently in the process of obtaining full-length clones of these proteins. Although we have obtained high-quality peptide sequence from APC-δ, we have not successfully identified homologous EST sequences for this protein.

Figure 4-8 presents an alignment of the peptide sequences derived from APC-α, APC-γ, APC-ζ, and APC-η and the proteins with which they show the most similarity. Not surprisingly, APC-γ and APC-ζ show a high degree of similarity with human CDC27 and human CDC16, respectively. This result was expected based on our immunoblot results. Peptide sequence of APC-η showed 100% similarity across 18 amino acids with a human protein in the expressed-sequence tag database. When the protein sequence of this clone was used to search the database, *Drosophila* CDC27 and *S. cerevisiae* CDC23 showed the highest degrees of similarity. However, this sequence is more similar to *S. cerevisiae* CDC23 than to human CDC27, indicating that it is likely to be the human homolog of CDC23. This finding was expected based on the fact that CDC16, CDC23, and CDC27 are known to form a complex in yeast (Lamb et al, 1994), and that CDC23 is essential for cyclin proteolysis in yeast (Imiger et al., 1995).

The most interesting finding came from analysis of the peptides generated from APC-α, the largest subunit of the complex. Database search revealed homology between two peptides of APC-α and a 216 kDa protein expressed in mouse testis, termed tsg-24 (Starborg et al., 1994). The mouse protein is 30% identical to the BimE protein of *Aspergillus nidulans*, a protein essential for passage through the metaphase-anaphase transition, and that has been proposed to be involved in a checkpoint control that negatively regulates entrance into mitosis

(Engle et al., 1990; Osmani et al., 1991; Osmani et al., 1988). To confirm APC-α as a homolog of mouse tsg-24, we used tsg-24 specific antibodies to immunoblot preparations of APC immunopurified from enriched fractions or crude extracts. Figure 4-9A indicates that tsg-24 antibodies specifically recognized the APC-α subunit of purified APC isolated from interphase or mitotic fractions. The mitotic form of APC-α undergoes an approximately 20 kDa upward mobility shift in its mitotic form, and this shift was observed in the tsg-24 immunoblot. Figure 4-9B indicates that CDC27 immunoprecipitates derived from crude interphase extracts specifically immunoprecipitate tsg-24-reactive protein as well, and can substantially deplete tsg24, suggesting that most tsg24 is contained in the CDC27 complex.

Modification of CDC27

Dramatic mobility shifts have been observed for a number of proteins that become phosphorylated in mitosis, such as Cdc25 (Kumagai and Dunphy, 1992). We have found that treatment of mitotic CDC27 immunoprecipitates with phosphatase can reverse the mitotic mobility shift (unpublished data), suggesting this modification is due to phosphorylation. To determine the timing of CDC27 phosphorylation during the cell cycle, we prepared cycling *Xenopus* extracts and assessed them for CDC27 status and nuclear morphology. Figure 4-10 demonstrates that CDC27 becomes shifted only during a brief 10 minute period each cell cycle that correlates with mitosis as observed by morphological examination of nuclei (Figure 4-10B). This experiment indicates that CDC27 modification occurs during normal mitotic cycles, and that the shifts we observe are not simply due to prolonged time in the mitotic state.

Because the onset of ubiquitination correlates with CDC27 modification as extracts pass from interphase to mitosis (data not shown), we developed a

Figure 4-9. APC contains a *Xenopus* homolog of a BimE-related protein

(A) Immunoblot of purified interphase (I) or mitotic (M) forms of APC, as prepared in Figure 4-7, with antibodies against mouse tsg-24.

(B) Crude interphase *Xenopus* extracts were immunodepleted using either preimmune or CDC27 antisera. The beads were washed extensively, eluted with sample buffer, and the eluate analyzed by immunoblotting with anti-tsg24 antibodies. The arrowhead indicates tsg24. The lower panel shows tsg-24 immunoreactivity following control or CDC27 immunodepletion.

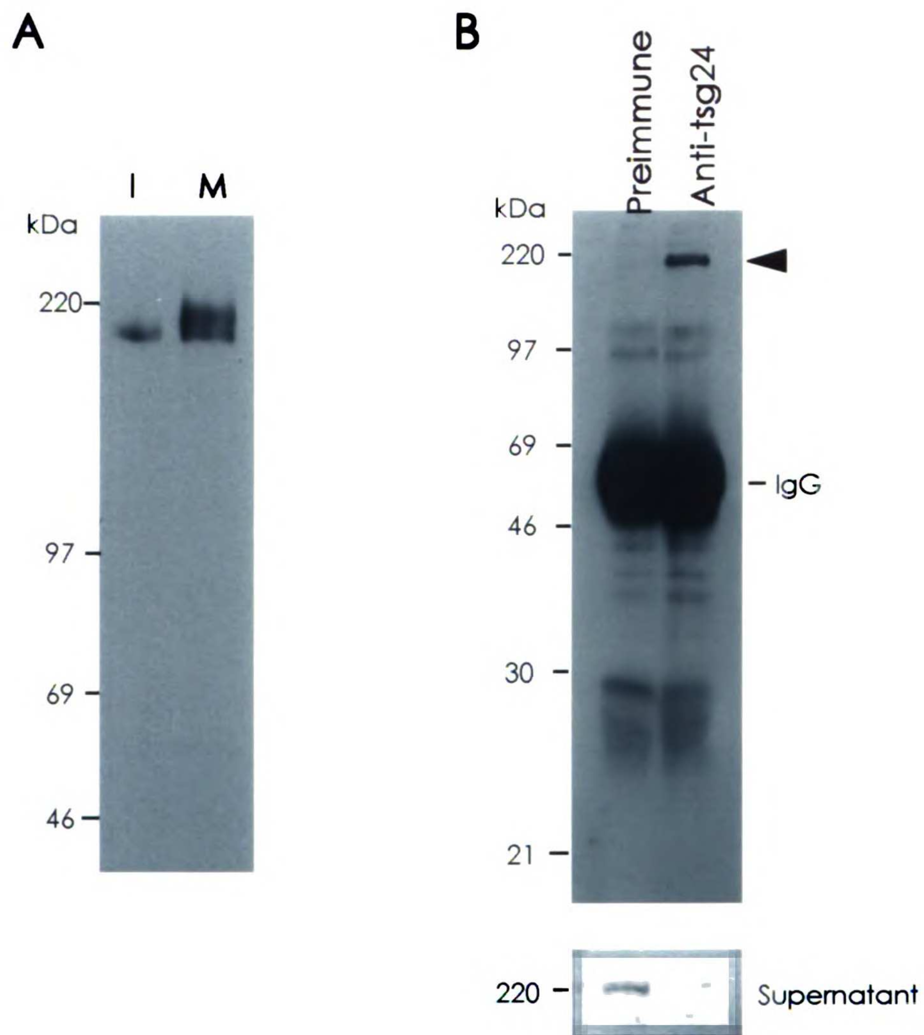
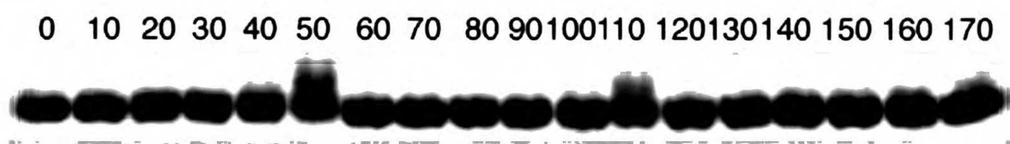


Figure 4-9

Figure 4-10. Analysis of CDC27 modification in cycling *Xenopus* extracts

Aliquots from a cycling *Xenopus* extract containing sperm nuclei were taken every 10 minutes and analyzed for (A) CDC27 by immunoblot, or (B) for nuclear morphology by Hoechst staining.

A



B

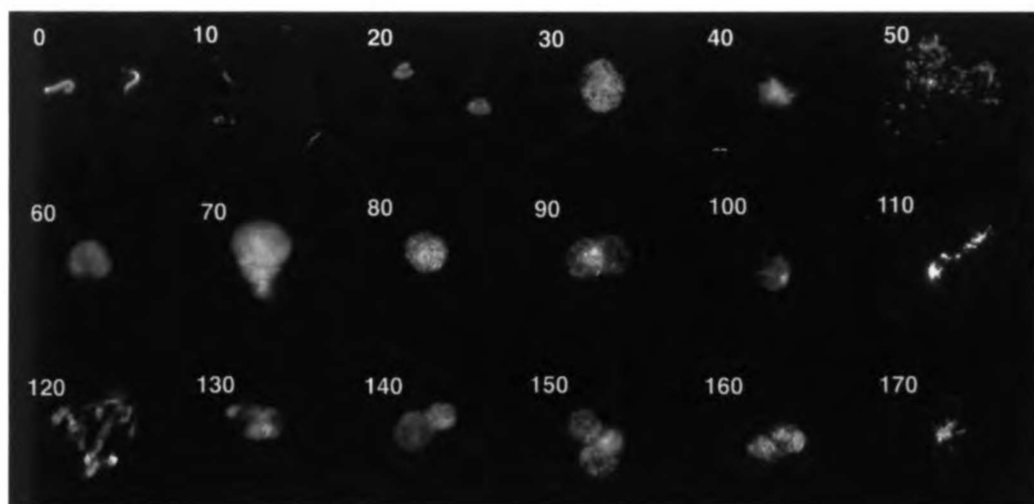


Figure 4-10

CDC27 modification assay that could potentially identify activators of APC. We tested mitotic fraction Q1 and Q2 for the ability to phosphorylate and upshift exogenous partially-purified interphase APC. Figure 4-11A demonstrates that mitotic fraction Q1 did not produce a CDC27 upshift, while fraction Q2 induced CDC27 upshift in the presence or absence of okadaic acid. Interestingly, fraction Q1 seemed to inhibit the ability of fraction Q2 to modify CDC27.

We next applied S100 derived from a cyclin Δ90 activated mitotic supernatant, and eluted the column with a linear salt gradient. Fractions were desalted, concentrated, and tested for their ability to modify CDC27 in the presence of okadaic acid. Figure 4-11B indicates that APC-phosphorylating activity eluted early from the column. Interestingly, this kinase was also capable of modifying the Xenopus homolog of tsg24 (Xl BimE).

Immunoblot analysis and H1 kinase assays of the column fractions indicated co-fractionation of cdc2 and H1 kinase activity. Cofractionation was maintained when this activity was analyzed by gel filtration (data not shown). Therefore, we tested whether the peak of activity from the Q column could be depleted with $p13^{suc1}$ beads. Figure 4-11C indicates that $p13^{suc1}$ beads completely removed CDC27 phosphorylating activity, while control beads had no effect. These data strongly suggest that the major APC-kinase, which can phosphorylate both CDC27 and tsg24, is cdc2/cyclin B.

We next tested whether the kinase-containing fractions could activate the ubiquitination activity of interphase APC. Unfortunately, we did not observe activation under any conditions tested. This data suggests that other activators may be necessary to activate the interphase form of APC. For this reason, we turned back to crude extracts to determine if our partially purified interphase APC could complement a crude mitotic extract depleted of APC. Figure 4-12A indicates that interphase APC could indeed complement a CDC27-depleted mitotic extract,

Figure 4-11. APC-modification activity in *Xenopus* extracts cofractionates with cdc2 kinase and can be depleted with p13-suc1 beads

(A) Mitotic fractions Q1 and Q2 were incubated for 60 minutes with interphase APC in the presence or absence of okadaic acid. Samples were then analyzed by CDC27 immunoblot.

(B) Mitotic S100 derived from a cyclin Δ90-activated mitotic extract was applied to a Resource Q column and eluted with a 100 mM (fraction 4) to 600 mM (fraction 15) linear KCl gradient. Column fractions were desalted, reconcentrated and incubated with partially-purified interphase APC in the presence of 1 μM okadaic acid for 60 minutes. Samples were then analyzed by immunoblotting for *Xenopus* BimE and CDC27 (upper panel). The column fractions were also immunoblotted for Cdc2 or assayed for H1 kinase activity (lower panels). The slower-migrating forms in lanes 10 and 11 represent endogenous phosphorylated APC present in the mitotic extract.

(C) APC-kinase can be depleted by suc1 beads. The Q column fraction of peak activity (Fraction 6) was incubated with suc1 beads or control beads for 1 hour at 4 °C. The beads were pelleted, and the supernatant mixed with interphase APC in the presence of okadaic acid. After 60 minutes, the reaction was stopped and analyzed by CDC27 immunoblotting.

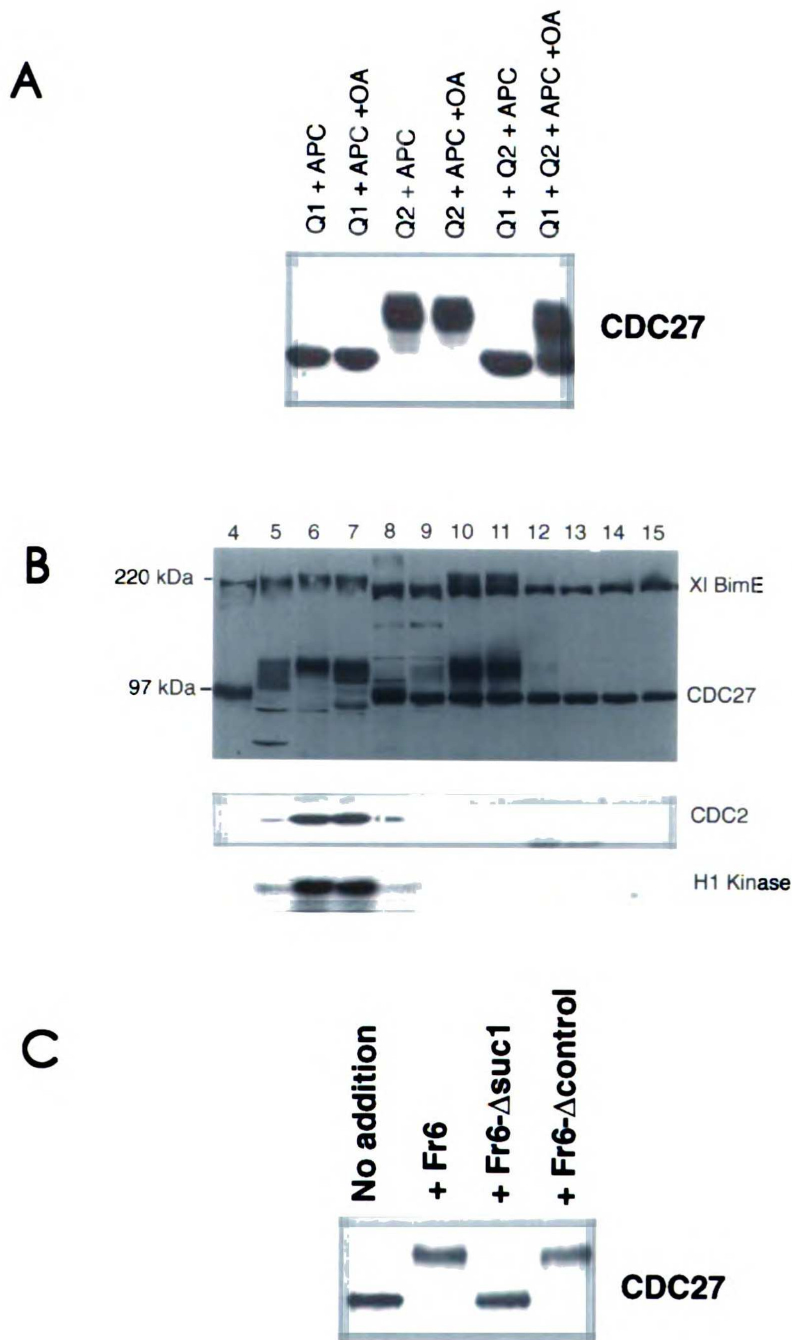


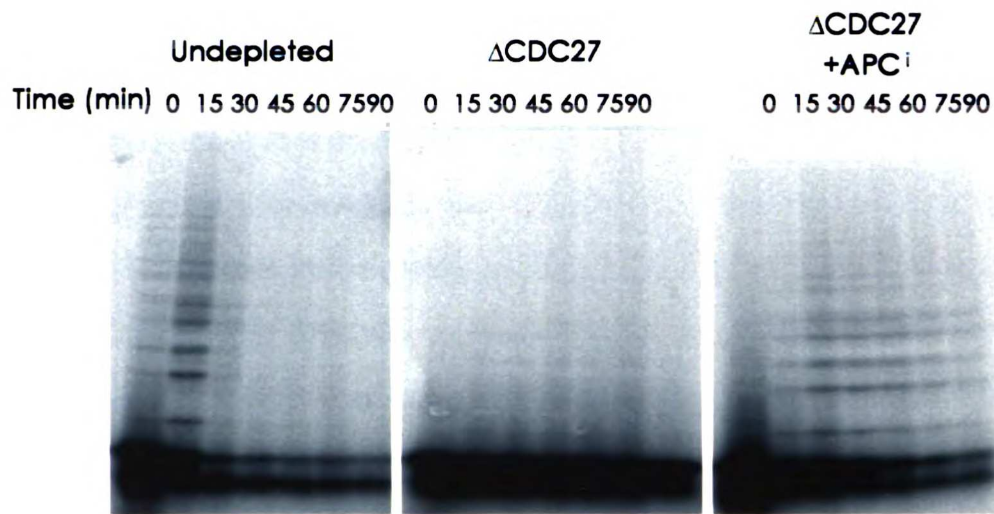
Figure 4-11

4-12. Okadaic acid is inhibitory for the activation of cyclin degradation in complete extracts and in a reconstituted system

(A) Interphase APC can be activated by CDC27-depleted mitotic extracts. Degradation assays were performed by addition of the N-terminal cyclin fragment to crude mitotic extracts (left panel), CDC27-depleted mitotic extracts (middle panel), or CDC27-depleted mitotic extracts to which partially purified interphase APC was added following depletion.

(B) The experiment was performed as in (A), except that 1 μM okadaic acid (OA) was added at the time of APC addition to one reaction.

A



B

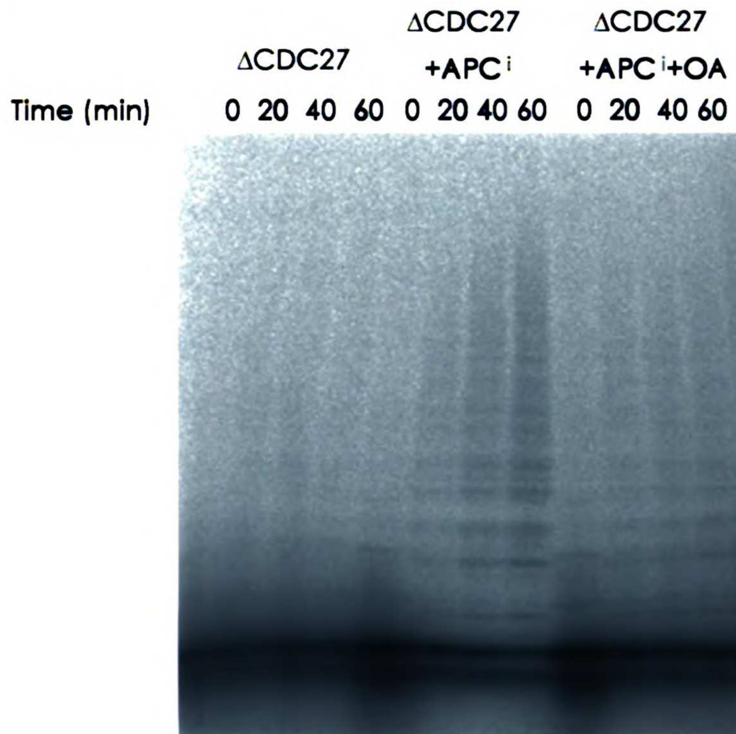


Figure 4-12

supporting both ubiquitination and degradation of the substrate. Activation of the interphase form occurred with a lag of about 15 minutes.

We had found that addition of okadaic acid to crude interphase extracts could inhibit the activation of cyclin degradation by recombinant cyclins (unpublished data). We therefore tested whether addition of 1 μM okadaic acid inhibited the ability of interphase APC to complement CDC27-depleted mitotic extracts. Figure 4-12B shows that okadaic acid was partially inhibitory for the activation of cyclin ubiquitination. This may indicate that a phosphatase is required for activation of cyclin ubiquitination, explaining why treatment of interphase APC with cdc2 kinase is insufficient to activate ubiquitination.

Discussion

The proteolysis of mitotic cyclins is a key event in the cell cycle that irreversibly initiates exit from mitosis. Through the activation of cdc2 kinase a process is initiated that targets cyclins for destruction by the ubiquitin system. To determine the basis of the cell cycle specificity of cyclin ubiquitination, we have fractionated mitotic extracts to identify components required for this process. In addition to the universally-required components E1 and ubiquitin, we have found three distinct activities that can reconstitute mitotic cyclin ubiquitination. The first two activities are a set of E2s that include UBC4; the third activity is a 20S complex that meets the functional criteria for a ubiquitin-protein ligase and appears to be the major determinant of cell cycle specificity in the cyclin ubiquitination system. Because this complex contains homologs of CDC16, CDC23, CDC27, and BimE, proteins essential for the onset of anaphase, we refer to it as the Anaphase-Promoting Complex (APC)

APC contains a cell-cycle regulated cyclin-ubiquitin ligase activity

Our data suggest that the cell-cycle specificity of cyclin ubiquitination is mediated by APC, as this complex is the only mitotic component required to reconstitute cyclin ubiquitination in interphase fractions. The ability of APC to complement recombinant E1 and UBC4 indicates that it functions as a ubiquitin-protein ligase. We have identified four components of APC, CDC16, CDC23, CDC27, and BimE. Our analysis of immunopurified APC indicates that it contains four additional stoichiometric subunits, consistent with its sedimentation coefficient of 20S. The findings that these proteins are required for cyclin degradation in yeast (Irniger et al., 1995) and for anaphase progression in fungi and mammalian cells (Hirano et al., 1988; O'Donnell et al., 1991; Osmani et al., 1988; Tugendreich et al., 1995), suggest that APC plays an essential role in cyclin ubiquitination and anaphase progression in all eukaryotes.

We have not yet determined which of the subunits of APC is responsible for its ubiquitin-protein ligase activity. To date, only two distinct types of E3s have been cloned. Yeast UBR1 mediates N-end rule degradation (Bartel et al., 1990); mammalian E6AP participates in p53 ubiquitination (Scheffner et al., 1993) and shares C-terminal homology with a family of other proteins that may also function as E3s (Scheffner et al., 1995). UBR1 and E6AP do not share homology with each other or with CDC16, CDC23, CDC27, or BimE. Recently it has been demonstrated that E6AP can accept ubiquitin from UBC4, forming a thioester at a conserved cysteine residue essential for ligase activity (Scheffner et al, 1995). This E3-ubiquitin thioester may be the ultimate ubiquitin donor for the conjugation reaction. We have not yet been able to observe the formation of a similar ubiquitin thioester with any of the APC subunits, but we have found that APC activity is sensitive to the sulfhydryl-reactive reagent N-ethyl maleimide (unpublished results). Formation of such thioesters may require high concentrations of

the components or the E2 enzymes. Our recent cloning of UBCX (Chapter 3) should facilitate the identification of an E3 thioester, if one exists.

It is also unclear how this complex recognizes cyclin, and which component of the complex is responsible for recognizing the destruction box. The fact that some destruction-box specificity is lost during immunoprecipitation suggests that the recognition factor might only be weakly associated with APC. Repeated attempts using the cyclin N-terminus as an affinity reagent to identify factors involved in destruction box recognition have not been successful. Similarly, crosslinking experiments with fractions enriched in APC or immunopurified complexes have not yielded clearly interpretable result. A clear remaining challenge is to identify which subunit(s) of this complex recognizes cyclin, and how the destruction box participates in this process. Our data also do not address whether this complex recognizes both A- and B-type cyclins. Recently, Hershko, Ruderman and colleagues have characterized a high molecular weight complex in clam extracts, called the cyclosome, that can complement a partially purified ubiquitin conjugating enzyme to support cyclin ubiquitination. Although the chromatographic behavior of this particle differs from Xenopus APC (Sudakin et al., 1995), functional data suggest that it is the clam cognate of our activity. This complex was able to weakly ubiquitinate a cyclin A N-terminal fragment *in vitro*. However, this does not rule out the possibility that different factors might be involved in recognizing A- or B-type cyclins.

BimE is a component of APC

Our results demonstrate that *Xenopus* APC contains a homolog of mouse tsg24(Starborg et al., 1994), a gene related to *Aspergillus* BimE (Osmani et al., 1988). Although the ability of mouse tsg 24 to complement a BimE deficient strain has not been tested, we feel that our functional data make it very likely that these

proteins are functional cognates. BimE mutants arrest in mitosis with condensed chromosomes and a short spindle (Osmani et al., 1988), consistent with a functional role at the metaphase-anaphase transition, like other components of APC. What is somewhat more difficult to explain, but also more exciting, is the potential role that has been ascribed to BimE as a negative regulator of entrance into mitosis (Osmani et al., 1991). Mutations in BimE allow cells that are arrested in G2 or S phase to enter mitosis, suggesting that one function of this protein is to restrain entrance into mitosis. This finding may suggest that APC has functional roles at other points in the cell cycle, perhaps restraining entry into M phase as well as regulating exit from M-phase by catalyzing ubiquitination. However, if such a function were a general function of APC, one might have expected to see similar phenotypes for mutations in other subunits of the complex; such phenotypes have not been reported. Clearly it will take much additional work to determine whether APC function is important in regulating other transitions of the cell cycle. It is interesting to point out that BimE contains several short hydrophobic segments in its C-terminal third that have been proposed to be potential membrane-spanning domains (Engle et al., 1990). This region of the protein shares the greatest degree of similarity with tsg24. Since APC, and hence BimE, is largely soluble, we find it unlikely that BimE is a membrane protein. Instead, we speculate that these hydrophobic stretches may be important for interaction with other components of APC, such as the TPR motifs of CDC16, CDC23, and CDC27.

Regulation of APC

The activation of cyclin B-cdc2 kinase is required for the activation of cyclin degradation (Murray et al., 1989; Luca et al., 1991). Purified cdc2 kinase triggers cyclin degradation in interphase extracts (Felix et al., 1990b); however

there is a 15-minute lag period preceding activation, suggesting that the activation of APC by cdc2 kinase may not be direct. Furthermore, the MPM-2 monoclonal antibody, which recognizes a discrete set of mitotic phosphoproteins, can deplete APC activity from crude fractions (this paper), and can immunoprecipitate CDC27 and CDC16 (unpublished results). Phosphorylation of this epitope appears to be mediated, at least in part, by kinases distinct from cdc2 (Kuang et al., 1991). These kinases provide interesting candidates for regulators that function downstream of cdc2 kinase to contribute to the activation of APC in mitosis.

Immunoblotting experiments revealed that CDC27 undergoes a dramatic upward electrophoretic mobility shift during the course of mitotic activation. Preliminary experiments indicate that treatment of CDC27 immunoprecipitates with phosphatase can completely reverse this mobility shift (S.T., P.H., unpublished results), suggesting that altered mobility is a consequence of phosphorylation. Such large mobility shifts have been reported for other MPM-2 epitopes, such as Cdc25 (Kumagai and Dunphy, 1992; Kuang et al., 1994). During the course of mitotic activation, the CDC27 upshift precedes the activation of cyclin ubiquitination, suggesting either a requirement for a threshold level of modification or the existence of additional controls (unpublished results). We have fractionated mitotic extracts and found that a very active CDC27-modifying activity is present. This activity cofractionates with cdc2 kinase, and can be depleted with suc1 beads. This finding may explain, in part, how cdc2 can activate cyclin degradation in interphase extracts. However, we were not able to successfully activate interphase APC with this mitotic kinase alone. Further evidence suggests that a dephosphorylation step may be required for activating the complex. A good candidate for such an activity is protein phosphatase 1, whose activity is necessary for the metaphase-anaphase transition in yeast and flies (Axton et al., 1990; Ohkura et al., 1989). We postulate that activation of APC may require both posi-

tive activation of the complex, perhaps by cdc2-mediated phosphorylation, and also removal of inhibitory phosphorylation, perhaps catalyzed by phosphatase 1. Such inhibitory phosphorylation would provide an elegant means of restraining APC activity during mitosis; dephosphorylation of the complex would then be the trigger initiating cyclin degradation.

A common set of components for cyclin destruction and anaphase: A common mechanism?

Biochemical and genetic evidence have now converged to suggest that cyclin degradation and sister chromatid segregation are mediated by a common set of components including CDC16, CDC23, CDC27, and BimE. This complex may have functions in addition to cyclin ubiquitination, as certain mutant alleles of CDC16 and CDC27 block anaphase progression but do not interfere with cyclin degradation (Imiger et al., 1995). One such function of this complex might be the ubiquitination of substrates other than mitotic cyclins whose degradation is required for the onset of anaphase. The existence of such proteins is suggested by the finding that an N-terminal fragment of cyclin B can inhibit the onset of anaphase in extracts that contain only a non-degradable form of cyclin (Holloway et al., 1993). Furthermore, methylated ubiquitin interferes with sister chromatid separation, suggesting the involvement of ubiquitin-mediated proteolysis in this process. APC may therefore ubiquitinate several proteins whose degradation is required for anaphase, explaining why some but not all CDC16 alleles interfere with cyclin proteolysis in yeast. Alternatively, APC may have functions that are independent of its ubiquitin-protein ligase activity.

Why the cyclin-ubiquitin ligase is contained in such a large complex is presently a mystery. Some insight into this problem may be found in the observations in fungi and mammalian cells that homologs of CDC27 and CDC16 localize

to the mitotic spindle and centrosomes (Mirabito and Morris, 1993; Tugendreich et al., 1995), structures that are also associated with MPM-2 antigens and cyclin B (Debec and Montmory, 1992; Engle et al., 1988; Maldonado-Codina and Glover, 1992; Vandre et al., 1986). The association of APC with spindle components may be important for the proper regulation of anaphase, perhaps by restraining APC activity until the chromosomes are aligned on the metaphase plate. Only then would the spindle signal the activation of APC, initiating both cyclin proteolysis and the segregation of sister chromatids.

Chapter 5

Conclusions and Future Directions

The identification of cyclin B as a substrate of the ubiquitin-dependent proteolytic system demonstrated that ubiquitination plays a central role in regulating the metaphase-anaphase transition and exit from mitosis. Ubiquitin-mediated proteolysis appears to be a general mechanism for gating passage through the cell cycle, as recent work in yeast suggests that ubiquitination regulates the G1/S transition as well (Schwob et al., 1994). Perhaps the most interesting aspect of cyclin proteolysis is its regulation during the cell cycle. Our work has indicated that the ubiquitin-protein ligase component of this system is regulated; cyclin is currently the only substrate whose ubiquitination is known to be regulated in this manner. Our goal has been to understand how cyclin is recognized by this ubiquitination system, to identify the components responsible for ubiquitinating cyclin, and to understand how they are regulated during the cell cycle.

Our work began with an analysis of the signals within cyclin that target the protein for ubiquitination. We found that each of the conserved residues within the destruction box of cyclin B is essential for ubiquitination and degradation. Individual residues outside this region appeared to have little influence on degradation rate; however, deletion of large segments of the N-terminus neighboring the destruction box partially stabilize the protein, suggesting these regions might be important for recognition by the ubiquitination machinery. In contrast to the strict requirement for a destruction box, there appears to be no requirement for a particular lysine residue to serve as a ubiquitin acceptor site. Direct mapping experiments demonstrated that the ubiquitination of cyclin occurs at multiple sites, perhaps because the cyclin N-terminus is conformationally mobile. This mobility may be essential for the rapid destruction of the protein.

Although the cyclin B N-terminus appears to contain all the information necessary to be rapidly degraded during mitosis, the cyclin A N-terminus does not. Our experiments demonstrate that this defect stems in part from the destruc-

tion box sequence of cyclin A, which is not efficiently recognized by the cyclin B ubiquitination system in crude extracts. The clam cyclosome, the regulated cyclin-ubiquitin ligase in clam extracts, can monoubiquitinate an N-terminal fragment of clam cyclin A; this reaction appears less efficient than for the cyclin B N-terminal fragment (Sudakin et al., 1995). We have not yet tested whether cyclin A is a substrate for purified Xenopus APC in vitro. It is possible that additional or distinct components may be required for cyclin A to be recognized and ubiquitinated; alternatively, binding of cyclin A to cdc2 or to some other protein may be essential for the protein to be recognized by APC. Differences in the destruction box may be responsible for the different timing of cyclin A and cyclin B destruction. This could be tested by swapping the destruction boxes of the full length proteins and determining whether the timing of destruction changes. The localization of cyclin A and cyclin B is distinct, and this difference may also influence the timing of destruction. The sequences responsible for cyclin localization have recently been identified (Pines and Hunter, 1994), so it should be possible to swap localization elements and determine the effect on degradation rate. Recent experiments in *Drosophila* suggest that the difference in timing of cyclin A and cyclin B destruction may be functionally important, as N-terminal deletion mutants of cyclin A delay passage through metaphase, while N-terminal deletion mutants of cyclin B arrest cells in telophase (Sigrist et al., 1995).

Figure 5-1 summarizes our current understanding of the components involved in cyclin ubiquitination. The E2 function can be fulfilled by either of two E2 enzymes, UBC4 or UBCX. Each of these enzymes can act together with APC to ubiquitinate cyclin B in a mitosis-specific and destruction-box dependent manner. Because UBC4 is involved in the degradation of many cellular proteins, we believe that substrate specificity in this system is determined by a component of

Figure 5-1. Components of the cyclin ubiquitination system

Schematic representation of components of the cyclin ubiquitination system. The E2 enzyme requirement can be fulfilled by one of two distinct E2s, UBC4 or UBCX. The Anaphase-Promoting Complex (APC) functions as a cell-cycle regulated E3, and is composed of at least eight distinct subunits.

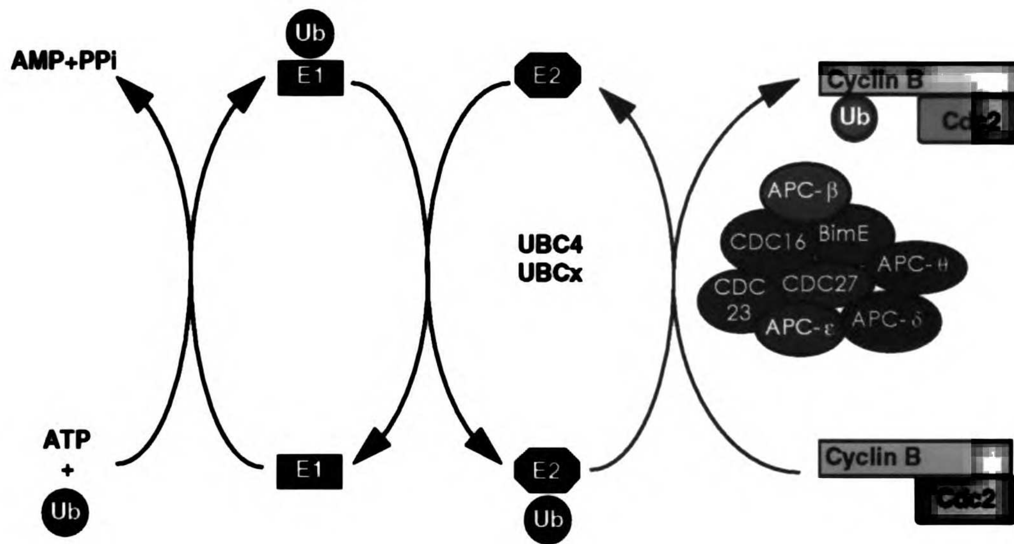


Figure 5-1

APC. We have recently obtained a recombinant form of UBCX, which will allow us to test in more detail the relative roles of these two E2s in promoting cyclin ubiquitination. Dose-response experiments with UBC4 have demonstrated that the recombinant protein is active at concentrations similar to what is found in crude extracts; performing a similar experiment with UBCX should be informative. We are developing antibodies against each of these proteins to perform immunodepletion experiments in crude extracts. These experiments should demonstrate whether each of these E2s is required for rapid cyclin degradation. We are also generating inactive UBC4 and UBCX mutants by changing the active site cysteine to serine. We will test whether mutant UBCX can inhibit the ability of wild-type UBC4 to promote cyclin ubiquitination, and vice versa. These experiments will demonstrate whether UBC4 and UBCX function through binding to the same site of APC.

Our experiments demonstrate that UBC4 and UBCX generate a different pattern of cyclin-ubiquitin conjugates. UBC4 promotes the formation of a relatively balanced distribution of conjugates, whereas UBCX favors formation of low-molecular mass conjugates. In the presence of methylated ubiquitin, the pattern of UBC4-generated conjugates is altered to look like the UBCX pattern, suggesting that UBCX may act primarily to monoubiquitinate the substrate at up to three distinct sites. Using a mutant version of the cyclin N-terminus that contains only a single lysine residue, we should be able to demonstrate directly that UBCX tends to monoubiquitinate the substrate, while UBC4 favors the formation of polyubiquitin chains. These findings have important implications for cyclin proteolysis, because polyubiquitin chains are thought to be the signal for degradation by the proteasome. We will therefore test whether cyclin-ubiquitin conjugates generated by UBC4 or UBCX are substrates for the proteasome in vitro.

The cell-cycle specificity of cyclin ubiquitination resides with APC, the

Anaphase-Promoting Complex. Figure 5-1 presents a schematic representation of this complex which consists of at least eight distinct polypeptides, four of which we have identified as *Xenopus* homologues of known proteins. The combined molecular mass of these eight proteins totals 700 kDa, approximately half the size suggested by gel filtration chromatography. However, the 20 S complex as measured by sucrose gradient sedimentation corresponds, for a symmetric complex, to a molecular mass of 800-900 kDa. One explanation for the discrepancy in size as measured by gel filtration and sucrose gradient sedimentation is that the complex may be asymmetric. However, we have noted that there are several substoichiometric subunits present in our immunopurified preparations of the interphase form of APC. These may be weakly associated subunits that are partially lost during the stringent washing conditions of the immunoprecipitation. It will therefore be important to perform a conventional purification of the complex. We have found that the interphase form of APC binds to cation exchange resins, while the mitotic form of APC does not. This step may therefore be useful to conventionally purify the interphase form of the complex. A conventionally-purified complex should also be suitable for analysis by electron microscopy; such a study would yield important information as to the structural features of the complex, and could explain the discrepancy in the size estimates obtained by gel filtration and sucrose gradient sedimentation.

The four known components of APC, CDC16, CDC23, CDC27, and BimE, are each required for passage through anaphase in the fungi in which they were first identified. This complex appears to be highly conserved across all eukaryotes (Figure 5-2). However, much work remains to determine whether homologues of all of the components of Xenopus APC can be found in fungi. For example, a homolog of BimE has not been reported yet in *S. cerevisiae* or *S. pombe*. Furthermore, the remaining four subunits of APC appear to be novel

Figure 5-2. Evolutionary conservation of APC components

This figure shows a comparison of the *Xenopus* APC components and their orthologs in organisms in which components have been identified.

Evolutionary Conservation of APC

Xenopus	Human	Mouse	S. cerevisiae	S. pombe	A. nidulans
APC- α	-	tsg24	-	-	BimE
APC- β	-	-	-	-	-
APC- γ	CDC27Hs	-	CDC27	nuc2^{+}	BimA
APC- δ	-	-	-	-	-
APC- ε	-	-	-	-	-
APC- ζ	CDC16Hs	-	CDC16	cut9^{+}	-
APC- η	-	-	CDC23	-	-
APC- θ	-	-	-	-	-

Figure 5-2

proteins without significant homology to known fungal proteins. If such homologues can be identified, it should be possible to demonstrate their involvement in cyclin proteolysis, as has been done for CDC16 and CDC23 in *S. cerevisiae* (Irniger et al., 1995). Identification of BimE homologs in *S. cerevisiae* and *S. pombe* will be especially useful, as this protein has been proposed to have a role in restraining entrance into mitosis in *Aspergillus*. This hypothesis is based on the finding that mutations in BimE cause premature entrance into mitosis in cells arrested in S phase with hydroxyurea, or in G2 phase by a mutation in the protein kinase nimA (Osmani et al., 1988). We have attempted to demonstrate a similar role for APC in *Xenopus* extracts by immunodepleting the complex from cycling extracts arrested in interphase by addition of high concentrations of nuclei and DNA synthesis inhibitors. However, such experiments are technically difficult, and it is not clear that depletion of the complex will result in the same phenotype as mutation of a single subunit. Therefore, genetic experiments will be crucial in confirming the role of BimE in regulating entrance into mitosis in other eukaryotes. Although mutations in other components of *S. cerevisiae* APC, such as CDC16, CDC23, and CDC27 have not been reported to have a defect in checkpoint control, it is interesting that mutation of BimA, the *Aspergillus* CDC27 homolog, can also bypass the nimA requirement (James et al., 1995). This is perhaps the best data to suggest that negative regulation of entrance into mitosis is a general function of APC rather than a specific function of BimE. Although BimE is a major component of APC, it is possible that a non-APC associated form of BimE exists in cells, and that this form of BimE could be involved in regulating entrance into mitosis. We are currently in the process of determining whether the majority of BimE in *Xenopus* eggs is found within APC or whether it is present in non-complexed forms as well.

We hypothesize that the inability of CDC16, CDC23, CDC27, and BimE

mutants to execute the metaphase-anaphase transition stems from a defect in the ability of these mutants to ubiquitinate and degrade a protein that inhibits the segregation of sister chromatids. Experiments in *Xenopus* extracts suggest that a destruction-box-containing protein must be degraded in order for sister chromatids to separate (Holloway et al., 1993). Such a protein could act as a cohesion component, physically linking sister chromatids; alternatively, the protein could be a regulator of sister chromatid segregation. One possibility for such a component would be an inhibitor of topoisomerase II, whose activity is essential for sister chromatids to separate (Shamu and Murray, 1992). However, bulk topoisomerase II activity does not increase at the metaphse/anaphase transition, suggesting that if this mechanism regulates chromatid separation, regulation must occur on a local level. Another candidate inhibitor of sister chromatid segregation is an inhibitor of type I phosphatases (PP1). PP1 activity varies through the cell cycle, peaking during interphase and again during mitosis (Walker et al., 1992). PP1 is essential for anaphase in several organisms, including *Aspergillus* (Doonan and Morris, 1989), *Drosophila* (Axton et al., 1990), and *S. pombe* (Kinoshita et al., 1990). However, PP1 mutants may be incapable of executing the metaphase-anaphase transition because they indirectly activate the metaphase checkpoint; alternatively, PP1 may be involved in the activation of APC rather than being a specific regulator of sister chromatid segregation. A complete understanding of how anaphase is regulated awaits the identification of novel substrates of APC.

Biochemistry and genetics have converged to provide a useful working model that suggests that both cyclin destruction and sister chromatid segregation are mediated by ubiquitin-proteolysis catalyzed by components of APC. However, many important questions remain. First, how are proteins that contain a destruction box recognized by APC? Second, how does APC function enzymatically to act as a ubiquitin-protein ligase? Third, how is the activity of APC regulated

between interphase and mitosis? Finally, what are the mechanisms that stabilize cyclin during the metaphase arrest characteristic of mature oocytes and cells that have not properly aligned chromosomes at the metaphase plate?

Although the destruction boxes of cyclin A and cyclin B are essential for their ubiquitination and proteolysis, we still do not understand how this sequence is recognized by the cyclin ubiquitination system. Structural studies of the cyclin N-terminus have been uninformative, as this region of the protein does not appear to adopt a clearly defined conformation. We also do not yet know which component of the cyclin ubiquitination system is responsible for recognizing cyclin. Because both UBCX and UBC4 can support a destruction-box dependent reaction, we believe that recognition is likely to be mediated by a component of APC. However, we have observed that reactions using immunopurified APC and recombinant UBC4 lose some degree of destruction box dependence compared to reactions in crude extracts or with partially purified factors. This may indicate that the component responsible for specificity is only loosely associated with the complex. Such a specificity factor has been identified in virally-mediated p53 ubiquitination (Scheffner et al., 1993). This factor, encoded by the E6 gene of the human papilloma virus, recognizes p53, and then binds the E3, E6-AP, which catalyzes ubiquitination. The destruction box of cyclin could be recognized by a factor similar to E6 that has only a relatively weak affinity for the E3 component of APC. It will therefore be important to refractionate extracts to identify factors in fraction Q2 that enhance the specificity of the reaction.

However, the only specificity control that we have tested is a substrate in which the first and third positions of the destruction box have been scrambled (RAAL to AARL). In crude extracts, this substrate is weakly ubiquitinated. We will therefore test whether substrates containing stronger mutations, such as L45A, N50A, or a destruction box deletion mutant, reveal a greater degree of specificity

in the reconstituted reaction.

We have taken two approaches to identify components of the cyclin ubiquitination system that interact with cyclin. First, we have attempted affinity chromatography using an N-terminal cyclin fragment as an affinity ligand. We have attempted to fractionate either crude extracts or fraction Q2, but have not been able to deplete activity using this type of column. This may indicate that the interaction between the cyclin-binding component and the destruction box is quite weak. We have also attempted crosslinking experiments in which the cyclin N-terminal fragment is labeled with an iodinated photoactivatable cross-linking reagent. We have added this substrate to fraction Q2, initiated cross-linking, and then immunoprecipitated with CDC27 antibodies. We have not been able to detect a specific cross-linked band under a variety of washing conditions. However, it is possible that interaction with the complex may also require the presence of the proper E2. Using recombinant enzymes, we can now test whether a cross-link can be formed in the presence of UBC4 or UBCX. If the interaction between APC and cyclin is weak, the use of higher concentrations of conventionally-purified APC may allow the interaction to be detected. Furthermore, the availability of recombinant forms of the APC subunits may enable the detection of even a very weak interaction, especially if the subunit does not have to be complexed with APC to recognize cyclin B. Initial experiments would include co-immunoprecipitation of proteins expressed in vitro, or demonstration of an interaction using the yeast 2-hybrid system. However, the recognition of cyclin B by APC may be the step at which cyclin ubiquitination is regulated; if so, recombinant APC subunits may be inactive unless modified appropriately.

We also have yet to determine how APC functions as a ubiquitin-protein ligase for cyclin B. None of the four known components of APC show similarity to the two classes of E3 enzymes, represented by UBR1 and E6-AP. We are in the

process of obtaining cDNA clones for the remaining subunits of the complex, which may be simlar to known E3s. However, because APC is the first example of an E3 activity contained in a large complex, it may function through a novel mechanism. Previous characterization of ubiquitin-protein ligases suggest that they may have some affinity for either the substrate or the E2. Thus in addition to defining the component of APC that recognizes cyclin, it will be important to determine whether any individual subunit can interact with UBC4 or UBCX. Once again, the availability of recombinant forms of each subunit will facilitate this analysis. E3 enzymes of the E6-AP variety can accept ubiquitin from an E2 enzyme to form a thioester (Scheffner et al., 1995). We have not observed a thioester between an APC subunit and ubiquitin in the presence of recombinant E1 and UBC4. However, these experiments used immunoprecipitated APC, which may not be present at concentrations sufficient to see a thioester. Studies with E6-AP required the use of recombinant protein. Furthermore, we have not tested whether UBCX can promote thioester formation on a subunit of APC. The availability of recombinant UBCX will enable us to perform this experiment.

Cloning of the subunits of APC will also enable us to examine how this complex is assembled, and may eventually allow cyclin ubiquitination to be reconstituted from recombinant components. The protein products of the CDC16, CDC23, and CDC27 genes all contain tetratricopeptide repeat (TPR) motifs. These domains probably play an important role in mediating protein-protein interactions within APC. Interestingly, the BimE protein contains a series of hydrophobic repeats that are conserved between *Aspergillus* and mouse (Starborg et al., 1994). These domains were originally proposed to be potential membrane-spanning regions (Engle et al., 1990). We believe these regions of BimE may mediate interaction with proteins in APC, perhaps defining a new motif that interacts with TPR proteins. This hypothesis can be tested by expressing mutant

versions of BimE and determining whether they are incorporated into APC. It will also be interesting to determine how the remaining novel components of APC are assembled into the complex, and to define the sequence elements within those proteins that mediate their interaction with other components of APC.

Perhaps the most interesting question relating to APC function is its regulation during the cell cycle. Several lines of evidence indicate that MPF activity is required to activate cyclin ubiquitination; however, this activation must occur after a lag period sufficient to allow time for the mitotic spindle to assemble. It is unlikely, therefore, that cdc2 kinase is the direct trigger for cyclin degradation. One possible mechanism for activating APC in mitosis would be association of an activating subunit, or dissociation of an inhibitory subunit. Our data indicate that the basic composition of APC remains unchanged during the cell cycle. However, this conclusion is based on the composition of stringently-washed immunoprecipitates. It will therefore be important to compare the composition of interphase and mitotic forms of the complex purified using less stringent conditions. We have found that mitotic regulation of APC is preserved in stringenly washed complexes, suggesting that direct modification of APC may be responsible for modulating its activity. An obvious candidate for regulatory modification is phosphorylation, as several subunits of the complex are phosphorylated during mitosis. It has been recently reported that the mitotic form of the clam cyclosome can be inactivated by treatment with phosphatase, suggesting that some form of phosphorylation is essential for activity (Lahav-Baratz et al., 1995). However, our data suggest that phosphorylation itself may not be sufficient to activate the interphase form of APC. We have partially purified a kinase from mitotic extracts, which is likely to be cyclin B/cdc2, that can phosphorylate both CDC27 and BimE *in vitro*; however, phosphorylation with this kinase is not sufficient to activate the complex. This may indicate that a distinct type of phosphorylation, cata-

lyzed by a different kinase, is essential for activation. Alternatively, our data suggest that dephosphorylation of interphase APC may also be essential for activation, because okadaic acid can inhibit the activation of interphase APC when the complex is added to CDC27-depleted mitotic extracts. Experiments in budding yeast have demonstrated that the activity of G1 cyclins in concert with CDC28 is essential to inactivate cyclin proteolysis, suggesting that inhibitory phosphorylation may play a role in regulating APC activity (Amon et al., 1994).

This hypothesis can be tested in several ways. First, protein phosphatase Type 1 (PP1) is a good candidate for a potential activator of APC, because mutation of PP1 produces a metaphase arrest in several organisms (Axton et al., 1990; Doonan and Morris, 1989; Kinoshita et al., 1990). Specific inhibitors of PP1 are available and can be used to determine whether PP1 activity is essential for the activation of cyclin degradation in crude extracts. A more direct approach would be to demonstrate dephosphorylation of a subunit of APC in mitosis. This could be accomplished by adding calcium to CSF-arrested extracts in the presence of labeled orthophosphate. If inhibitory phosphorylation occurs during interphase, labeled phosphate will be incorporated into APC. The pattern of labeled protein could then be compared to a similar extract which has been activated to enter mitosis by addition of non-degradable cyclin. Comparison of the labeling pattern of APC components immunoprecipitated from interphase or mitotic extracts should reveal the changes in phosphorylation state that occur between interphase and mitosis. However, it may be essential to perform phosphopeptide mapping for each subunit, because activating phosphorylation could mask activating dephosphorylation.

We have found that interphase APC can be activated in either a crude mitotic extract or high-speed supernatant depleted of CDC27. This demonstrates that components essential for activation of APC are not tightly associated with

the complex. Fractionation of this supernatant should lead to the identification of components necessary for activation of APC. Our data suggests that several components may be required to activate the interphase form of APC; furthermore, these components may have to be added in a particular order to achieve activation of the complex. It is interesting to note that regulation of APC activity may parallel closely the regulation of MPF activity. Activation of MPF requires both activating phosphorylation by cdc2-activating kinase (CAK), and removal of inhibitory phosphorylation. This dual form of regulation allows MPF activity to be tightly regulated in response to checkpoints, such as incompletely replicated DNA, and may enhance the cooperative activation of the kinase during the transition from interphase to mitosis (see Chapter 1). APC activity may be regulated in a similar manner. Activating phosphorylation, catalyzed by MPF or perhaps other kinases activated during mitosis, would occur early in mitosis, during prophase. However, APC would remain inactive due to inhibitory phosphorylation catalyzed during the previous interphase period. As is the case for MPF activation, the trigger for APC activation would be removal of inhibitory phosphorylation, catalyzed by a phosphatase that becomes active at the time of the metaphase-anaphase transition. This mechanism would allow for a lag period between the activation of MPF and the activation of the cyclin destruction system.

This model predicts that there are activities present in interphase extracts that should be capable of inactivating the mitotic form of APC. We have found that the mitotic form of the complex remains active for about 20 minutes when added to interphase extracts. After this period, loss of activity is observed, and this correlates with dephosphorylation of CDC27. It should be possible to fractionate interphase extracts to identify activities capable of inactivating mitotic APC. By analogy with the requirement for G1 cyclins in turning cyclin destruction off in yeast, we have tested whether vertebrate G1 cyclin-dependent kinases,

such as cyclin E/cdk2, are involved in the regulation of cyclin B proteolysis in *Xenopus* extracts. To test this idea, we have added the cyclin E/cdk2 inhibitor protein p21 to CSF-arrested extracts, released the extracts with calcium, and determined the timing of activation and inactivation of cyclin ubiquitination. We have found that cyclin ubiquitination is rapidly activated following addition of calcium to CSF extracts; the system is inactivated approximately 20 minutes following calcium addition. Addition of p21 did not have any ability to prolong the activity of the cyclin degradation system. This may indicate that there are several pathways of inactivating APC in *Xenopus* extracts. We have not yet tested whether purified cyclin E/cdk2 can inhibit the activity of purified mitotic APC.

The lag between activation of MPF and the activation of cyclin ubiquitination suggests that there may be intermediate regulators involved in the activation of cyclin proteolysis. An interesting candidate for such a regulator is the CDC20 gene of *S. cerevisiae* (Hartwell et al., 1973) Strains containing the *cdc20-1* mutation arrest at the restrictive temperature as large budded cells with a single nucleus positioned in the neck (Pringle and Hartwell, 1981). Ultrastructural analysis indicates that mutant cells arrest with a short spindle, suggesting CDC20 function is essential for anaphase (Byers and Goetsch, 1974). However, CDC20 mutants exhibit general defects in microtubule organization and microtubule-dependent processes such as karyogamy (Sethi et al., 1991), suggesting that metaphase arrest could be a consequence of activating the spindle assembly checkpoint. Molecular cloning of CDC20 indicates that it contains WD-40 repeats (Sethi et al., 1991), a sequence motif originally found in β-transducin. Recently, a gene with similarity to CDC20, called *fizzy*, has been identified in *Drosophila*. Mutations in *fizzy* cause a metaphase arrest in *Drosophila* (Dawson et al., 1993), and stabilize both A- and B-type cyclins (Dawson et al., 1995; Sigrist et al., 1995).This finding suggests that *fzy* function may be directly re-

quired for cyclin proteolysis, because agents that stabilize cyclin through activation of the spindle assembly checkpoint will stabilize only cyclin B in *Drosophila* (Whitfield et al., 1990). However, the mitotic spindles of *fizzy* mutants demonstrate an excess of microtubules, indicating that *fizzy* function is also required for normal microtubule organization (Dawson et al., 1995). It is possible that the WD-40 motif of CDC20/*fizzy* interacts directly with TPR-containing components of APC such as CDC16, CDC23 or CDC27. For example, the TPR-protein Ssn6 interacts with TUP1, a WD-40 protein, to function as a general transcriptional repressor (Keleher et al., 1992). Other WD-40-containing proteins may regulate exit from mitosis, as a *Xenopus* clone containing WD-40 repeats can suppress a *cdc15* mutation in *S. cerevisiae* (Spevak et al., 1993). CDC15 has sequence similarity to protein kinases and probably functions during a late step in mitosis (Schweitzer and Philippsen, 1991).

Cyclin degradation may be regulated through additional mechanisms distinct from the direct activation of APC. For example, it is possible that isopeptidases that remove ubiquitin from cyclin may be inactivated during mitosis. This may potentiate the formation of long polyubiquitin chains on cyclin during mitosis. This hypothesis could be tested by incubating purified cyclin-ubiquitin conjugates with fractions derived from interphase or mitotic extracts. The relative localization of APC may also be important for the proper regulation of cyclin degradation. In mammalian tissue culture cells, APC is localized predominantly to the centrosome during interphase, and to the centrosome and mitotic spindle during mitosis (Tugendreich et al., 1995). This may provide a mechanism to localize the destruction of cyclin B. For example, during the syncytial mitoses of early *Drosophila* embryos, it is impossible to detect fluctuations in the level of cyclins or MPF activity (Edgar et al., 1994). A similar situation appears to hold in physarum, another organism with syncytial mitoses (Cho and

Sauer, 1994). In these organisms, cyclin may be degraded locally on the spindle, driving the nuclear cycles. Degradation of cytoplasmic cyclin B may therefore only be essential only for cytokinesis. In fact, fluctuations in H1 kinase levels and cyclin B levels become evident in *Drosophila* around the time of cellularization. It would be interesting to determine whether APC is localized solely to the spindle in these organisms, or whether it is found throughout the syncytium. We believe the latter to be more likely, because a maternal stockpile of APC is probably recruited to the spindle from the cytoplasm as nuclei proliferate. This may indicate the presence of mechanisms that ensure that only the APC localized to the spindle is activated during each cell cycle. This is in dramatic contrast to the situation in *Xenopus* embryos, in which cytokinesis, a process incompatible with high MPF levels, requires that APC be activated globally to destroy cytoplasmic cyclin. However, one interesting possibility is that even in *Xenopus* embryos, localization of APC to the spindle may be required for proper orientation of the plane of cleavage. It would be interesting to test whether the cleavage plane could be redirected by injection of the mitotic form of APC into the equatorial region of the embryo.

Cyclin stability is also regulated by additional mechanisms in certain circumstances. For example, most cells possess a checkpoint that monitors the attachment of kinetochores to the mitotic spindle (see Murray (1992) for review). If kinetochores are not properly attached to spindle microtubules, the cell cycle is arrested in metaphase and cyclin B is stabilized until a proper attachment can be made. Once a proper attachment is made, anaphase proceeds and cyclin B is degraded. Although the molecular event that signals arrest has not been identified, tension on the kinetochore may be one aspect of spindle dynamics that is monitored by this system (Li and Nicklas, 1995). Genetic analysis of this checkpoint has identified several components that participate in this signaling system

(Hoyt et al., 1991; Li and Murray, 1991). As both cyclin B destruction and anaphase progression are blocked when the checkpoint is activated, APC may be the final downstream target of the checkpoint system. Alternatively, it is possible that substrates such as cyclin B and the hypothetical inhibitor of sister chromatid segregation may be modified directly by components of the checkpoint system to prevent their degradation. The latter possibility is suggested by the finding that although cyclin B is stabilized by the checkpoint, cyclin A is not (Hunt et al., 1992; Minshull et al., 1994; Whitfield et al., 1990). It will therefore first be important to determine whether APC or the substrates are the targets of the checkpoint system. This could be tested by purifying APC from nocodazole-arrested cells and determining whether the complex has activity. Alternatively, tagged cyclin could be modified in an extract derived from checkpoint arrested cells, and then incubated with active APC to determine whether its ability to function as a substrate is impaired.

A similar situation exists in metaphase-II arrested *Xenopus* eggs which contain high levels of MPF activity but stable cyclin B. This arrest is mediated by an activity called cytostatic factor (CSF). Although all of the components of CSF have not been identified, at least two different types of components are essential to establish a CSF arrest. The first is mos, a protein kinase whose synthesis is required for oocyte maturation. Injection of mos mRNA is sufficient to induce a cleavage arrest in dividing eggs, whereas mos antibodies can deplete CSF activity from extracts (Sagata et al., 1989). The second component is another protein kinase, cdk2, whose activity is essential to produce a metaphase II arrest (Gabrielli et al., 1993). The latter finding is especially interesting in light of work form yeast that suggests that G1 cyclins are necessary to inactivate the cyclin degradation system as cells cross START (Amon et al., 1994). There are several parallels between the cell cycle arrest induced by CSF and the checkpoint arrest

that results from incomplete spindle assembly. First, both produce arrest in metaphase. Second, both stabilize cyclin B. Third, the activity of MAP kinase appears to be important for both arrests (Haccard et al., 1993b; Minshull et al., 1994). It is therefore tempting to speculate that similar mechanisms operate to stabilize cyclin in each of these arrests. It will therefore be important to determine whether APC or cyclin B is the ultimate downstream target of CSF activity. Preliminary experiments indicate that purified MAP kinase does not inactivate the mitotic form of APC in vitro, suggesting the target of MAP kinase may lie further upstream.

It will be interesting to determine whether mechanisms that establish the checkpoint arrest are also utilized during normal cell cycles to regulate cyclin proteolysis. For example, components of the checkpoint pathway may be important for establishing the kinetic lag between MPF activation and the onset of cyclin degradation. It is also conceivable that the machinery that inactivates cyclin proteolysis in telophase (embryos) or in G1 (yeast) may also share components with the checkpoint system. For example, there are several myelin-basic protein kinases that become active for a brief period at the end of each mitotic cycle in early *Xenopus* embryos (Minshull et al., 1994). These kinases are also activated during the metaphase checkpoint in embryo extracts. It would be interesting to test whether inactivation of these kinases using the MAP kinase-specific phosphatase MKP-1 would inhibit the inactivation of cyclin proteolysis during normal embryonic cell cycles. Perhaps the combined activities of MAP-kinase and cdk2 are essential for inactivation of cyclin proteolysis, as both activities are necessary for the establishment of the metaphase arrest induced by c-mos or CSF.

The study of cyclin degradation has yielded new insights into the importance of proteolysis in regulating the transitions of the cell cycle. Understanding

how these proteolytic systems are regulated, and identifying new substrates of these systems remain two major challenges that lie ahead. The discovery of cyclin ubiquitination has brought the field of ubiquitin-dependent protein degradation to the forefront of cell cycle research. The cyclin ubiquitination system is surprisingly complex, for it requires the participation of at least 11 distinct components. Unraveling how each of these components participate in the enzymology of cyclin ubiquitination will undoubtedly prove fascinating.

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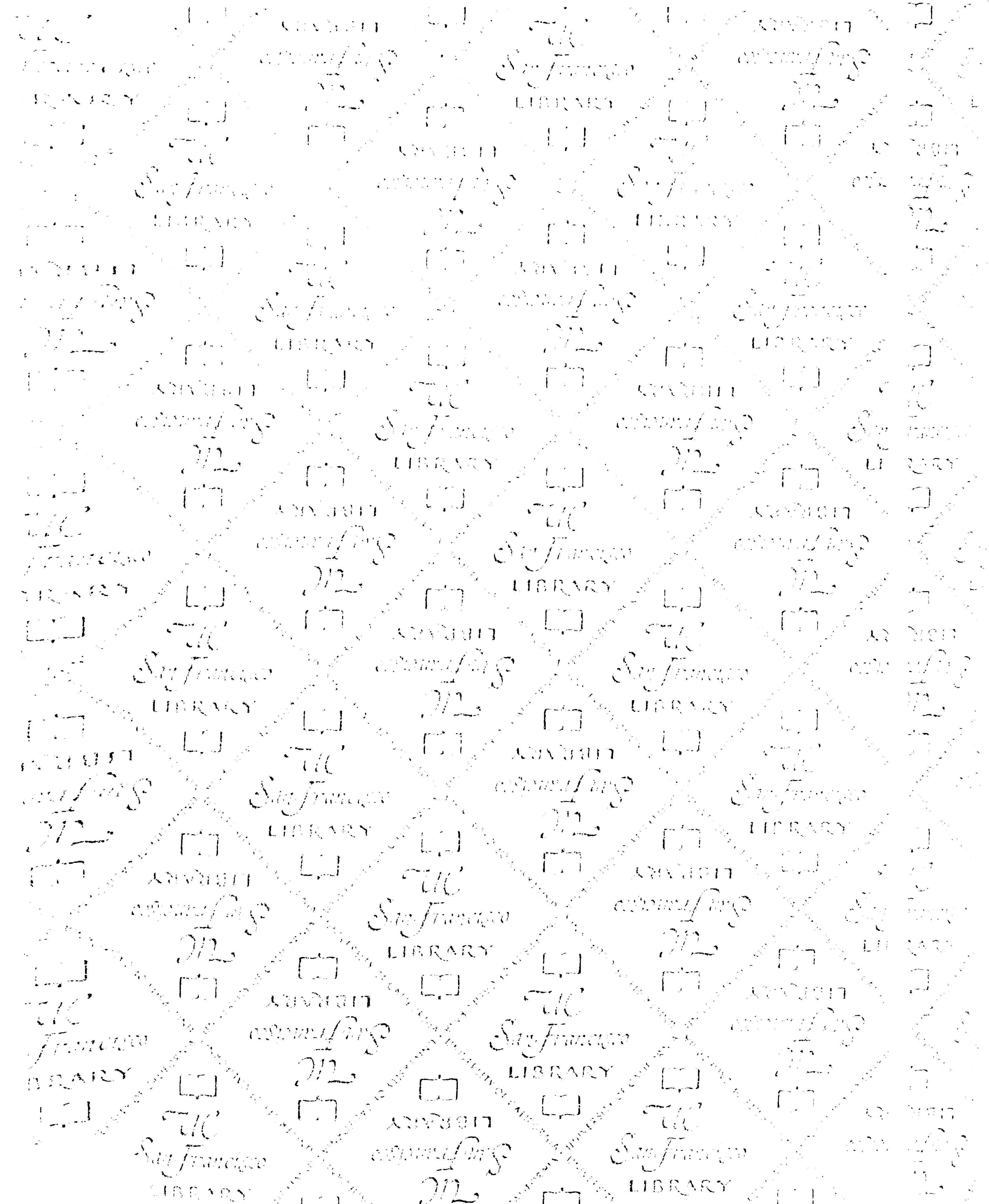
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