CHAPTER 12

Cell Cycle Control by Ubiquitin-Dependent Proteolysis

Jan-Michael Peters, Randall W. King, and Raymond J. Deshaies

1. INTRODUCTION

The prime directive of the dividing cell is to pass on two identical copies of its genetic blueprint. This requires accurate replication of DNA and accurate segregation of replicated chromosomes. The cell cycle control machinery regulates progression from one phase of the chromosome cycle to the next, ensuring that DNA replication (S phase) alternates with chromosome segregation (mitosis) to maintain a constant ploidy. In most cells, growth is coordinated with division to maintain a constant average cell size; the length of the gap phases that occur before (G1) and after (G2) S phase must therefore be regulated. In contrast, early embryonic division occurs in the absence of cell growth, so that the cell cycle consists solely of rapidly alternating S and M phases. Despite these distinct physiologies, a common biochemical machinery regulates cell division in all eukaryotic cells.
Transitions from one stage of the cell cycle to the next are often associated with the activation or inactivation of protein kinases known as the cyclin-dependent kinases (CDKs; reviewed in Morgan, 1995; King et al., 1994; Nasmyth, 1993). Although the cell cycle of fungi is controlled by only a single CDK (called Cdc28p in budding yeast and Cdc2p in fission yeast), a family of divergent cyclins, which are essential activators of the kinase, affords functional diversification. In budding yeast, the cyclins Cln1p, Cln2p, and Cln3p are expressed early in the cell cycle and promote events that are unique to G1 phase. Clb5p and Clb6p are expressed later and promote S phase, whereas the mitotic cyclins Clb1p through Clb4p drive entry into mitosis. Although the family of CDKs has expanded in higher eukaryotes, they appear to regulate the same events as their fungal relatives. The CDKs of higher eukaryotes form complexes with functional homologues of the budding yeast cyclins, with cyclins D and E functioning during G1, cyclins E and A during S phase, and cyclins A and B during mitosis.

This chapter reviews the important role that ubiquitin-mediated proteolysis plays in regulating cellular division (for related reviews see King et al., 1996a; Deshaies, 1995a). The proteolysis of cell cycle regulators is intimately coupled to protein phosphorylation catalyzed by CDKs: Phosphorylation plays an important role in regulating ubiquitin-mediated proteolysis during the cell cycle; conversely, degradation of cyclins and CDK inhibitors is essential for the proper regulation of CDKs. Although many of the components involved in regulating cell cycle progression have been identified, we have only begun to catch a glimpse of how the complex interplay of protein phosphorylation and protein degradation produces orderly progression through the cell cycle.

In the first half of this chapter, we discuss a ubiquitin-dependent proteolytic pathway, the CDC34 pathway, that regulates the transition from G1 to S phase. In the second half of the review, we describe a second ubiquitin-dependent proteolytic system, the APC pathway, that triggers anaphase and the exit from mitosis by initiating the destruction of proteins that regulate sister chromatid cohesion and mitotic cyclins, respectively (Fig. 1).

2. THE ROLE OF UBIQUITIN-DEPENDENT PROTEOLYSIS IN THE G1/S TRANSITION

In most eukaryotic cells, division is made contingent on cell growth that occurs during G1. Cells become committed to a round of cell division only after they have reached a point in G1 called START in budding yeast or the Restriction Point in animal cells. A yeast or animal cell that is deprived of essential nutrients or growth factors while it is in early G1 phase will not divide and instead will enter a period of quiescence (G0). However, once the cell has passed START, it will
Figure 1. Schematic representation of the classic phases of the cell division cycle (G1, S, G2, and M) and the chromosome cycle (bottom panel), indicating when the Cdc34p- and APC-dependent ubiquitination pathways control important transitions during the cell cycle. The relative stability of Sic1p, a key substrate of the Cdc34p pathway, and of mitotic cyclins, the most prominent substrates of the APC pathway, are also indicated. Sic1p is a protein that has so far only been found in budding yeast, but functionally related proteins are thought to play a similar role in the G1/S transition in other eukaryotes. Although the stability of Sic1p and mitotic cyclins appears complementary, their proteolysis seems to be regulated by principally different mechanisms (for details see text and Figs. 2 and 5). It is also important to note that the restabilization of mitotic cyclins at the G1–S transition does not depend on the CDC34 pathway and can occur, although reversibly, in cdc4mut cells (Schwob et al., 1996; Amon et al., 1994). Note that the metaphase–anaphase transition, the “point of no return” in the process of chromosome segregation, does not coincide with any of the classical transitions from one cell cycle phase to the next but instead divides mitosis into two separate phases.

divide regardless of the concentration of nutrients and growth factors in the growth medium.

Whereas both passage through START and the subsequent transition from G1 to S phase require the activity of CDKs complexed with G1-specific cyclins, these two events are temporally separable and are regulated distinctly. In budding yeast, different genes are required for START (e.g., CDC28, G1 cyclins, CDC37) and the G1/S transition (CDC4, CDC34, CDC53 and SKP1). At START, budding yeast cells assemble active G1 cyclin/CDK complexes, accumulate DNA replication proteins, and inactive S phase-inducing cyclin/CDK complexes, and extinguish pathways characteristic of the pre-START G1 phase (e.g., cyclin B proteolysis). Later, at the G1/S transition, a further series of changes occurs: Latent S phase-inducing cyclin/CDK complexes are activated, a G1-specific pattern of
gene expression yields to a pattern characteristic of S phase, and pathways restricted to post-START G1 phase, such as the initiation of bud growth, are shut off. Recent evidence indicates that the conversion from a post-START G1 state to an S phase state at the G1/S transition is regulated by the phosphorylation of the CDK inhibitor Sic1p, which renders it susceptible to ubiquitination by the CDC34 pathway and subsequent destruction.

2.1. Physiology and Biochemistry of the CDC34 Pathway

2.1.1. Background

An important role for ubiquitin-mediated proteolysis in cell cycle control was suggested by the observation that the CDC34 gene of Saccharomyces cerevisiae encodes a ubiquitin-conjugating enzyme (Goebel et al., 1988). On incubation at the restrictive temperature, strains harboring cdc34ts mutations accumulate high levels of G1 cyclins (Deshaias et al., 1995; Yaglom et al., 1995), produce new daughter buds, and complete spindle pole body duplication. Nevertheless, cdc34ts cells fail to initiate DNA replication and remain arrested in a G1-like state (Goebel et al., 1988). This constellation of phenotypes indicates that CDC34 function is dispensable for the execution of START, but is required for cells to transit from G1 to S phase. Besides CDC34, genetic analyses have revealed that CDC4, CDC53 (Mathias et al., 1996), SKP1 (Bai et al., 1996), and G1 cyclins (CLNI–3) (Schneider et al., 1996) are also required for cells to pass from G1 into S phase (Table 1). Genetic and biochemical studies summarized below indicate that the products of

<table>
<thead>
<tr>
<th>Gene</th>
<th>Implicated in proteolysis in vivo (protein)</th>
<th>Implicated in ubiquitination in vitro (protein)</th>
<th>Gene essential?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC4</td>
<td>Yes (Sic1p, Cdc6p)</td>
<td>Yes (Sic1p)</td>
<td>Yes</td>
<td>Piatti et al. (1996), Schwob et al. (1994)</td>
</tr>
<tr>
<td>CDC34</td>
<td>Yes (Cln3p)</td>
<td>Yes (Sic1p)</td>
<td>Yes</td>
<td>Deshaies et al. (1995), Yaglom et al. (1995), Schwob et al. (1994)</td>
</tr>
<tr>
<td>CDC53</td>
<td>Yes (Cln2p, Sic1p)</td>
<td>Yes (Sic1p)</td>
<td>Yes</td>
<td>Willems et al. (1996), Mathias et al. (1996)</td>
</tr>
<tr>
<td>SKP1</td>
<td>Yes (Cln2p, Sic1p)</td>
<td>Yes (Sic1p)</td>
<td>Yes</td>
<td>Bai et al. (1996)</td>
</tr>
<tr>
<td>GRR1</td>
<td>Yes (Cln2p)</td>
<td>Not tested</td>
<td>No</td>
<td>Barral et al. (1995)</td>
</tr>
<tr>
<td>SCM4</td>
<td>Not tested</td>
<td>Not tested</td>
<td>No</td>
<td>Smith et al. (1992)</td>
</tr>
<tr>
<td>UBS1</td>
<td>Not tested</td>
<td>Not tested</td>
<td>No</td>
<td>Prendergast et al. (1996)</td>
</tr>
<tr>
<td>CLNI–3</td>
<td>Yes (Sic1p)</td>
<td>Yes (Sic1p)</td>
<td>Yes</td>
<td>Schneider et al. (1996)</td>
</tr>
</tbody>
</table>
these genes act in the same pathway and that Cdc34p, Cdc4p, Cdc53p, and Skp1p are part of a multisubunit ubiquitin-conjugating complex. Thus, for simplicity, we will hereafter refer to CDC34, CDC4, CDC53, SKP1, and their products collectively as the CDC34 pathway.

2.1.2. Physiological Role of the CDC34 Pathway in G1 Cells

How do CDC4, CDC34, CDC53, and SKP1 promote the transition from G1 to S phase? An elegant study by Schwob et al. (1994) indicated that the CDC34 pathway is required for the activation of a set of cyclin B/Cdc28p complexes that trigger the initiation of DNA synthesis. A budding yeast strain deleted for all six known cyclin B genes (CLB1–CLB6) and sustained by expression of CLB5 from the regulatable GAL1 promoter arrests cell division at the same point as CDC34 pathway ts mutants when expression of CLB5 is extinguished. This observation suggested that the CDC34 pathway might be required for the generation of active Clb/Cdc28p complexes. Indeed, CDC34 pathway ts mutants accumulate inactive Clb5p/Cdc28p complexes, and extracts from these mutants contain high levels of a Clb5p/Cdc28p inhibitor. A candidate for this inhibitor was Sic1p/Sdb25p (for simplicity, we will use "Sic1p" throughout the remainder of this review), which was previously identified as both a tightly bound inhibitor of the Cdc28p protein kinase (Nugroho and Mendenhall, 1994; Mendenhall, 1993), and a multicopy suppressor of the lethal anaphase arrest of dbf2ts mutants (Donovan et al., 1994). Remarkably, cdc34ts sic1Δ, cdc4ts sic1Δ, cdc53ts sic1Δ, and skp1ts sic1Δ double mutants do not accumulate Clb5p/Cdc28p inhibitory activity, and are able to replicate DNA at the nonpermissive temperature (Bai et al., 1996; Schwob et al., 1994). Furthermore, whereas Sic1p disappears as wild-type cells transit from G1 to S phase, Sic1p stably accumulates in CDC34 pathway ts mutants. These observations led to the hypothesis that the CDC34 pathway specifies the ubiquitin-independent destruction of Sic1p, which otherwise restraints Clb5p/Cdc28p complexes from triggering the initiation of DNA synthesis (Schwob et al., 1994). This hypothesis has recently been confirmed by the observation that expression of a nondegradable form of Sic1p blocks cell cycle progression at the G1/S boundary (Verma et al., 1997a).

Although its role in promoting the destruction of Sic1p accounts for the cell cycle arrest of CDC34 pathway mutants, the CDC34 pathway may also contribute to the regulation of G1/S by limiting the accumulation of G1 cyclins. The G1 cyclins Cln2p and Cln3p are substrates of the CDC34 pathway (Willems et al., 1996; Deshaies et al., 1995; Yaglom et al., 1995), and cis-acting mutations that stabilize Cln2p and Cln3p shorten G1 and perturb the regulation of START (Lanker et al., 1996; Yaglom et al., 1995; Cross and Blake, 1993; Tyers et al., 1992).
2.1.3. Biochemical Functions of CDC34 Pathway Components

How does the CDC34 pathway promote the destruction of Sic1p at the G1/S transition? Because CDC34 encodes a ubiquitin-conjugating enzyme, the most parsimonious model is that Cdc34p ubiquitinates Sic1p, thereby targeting it for degradation by the 26 S proteasome. Although the molecular function of Cdc34p can be inferred from its sequence, the sequences of Cdc4p, Cdc53p, and Skp1p are less informative. CDC4 encodes an 86-kDa protein that contains a recently described Skp1p interaction motif named the "F box," 7-8 copies of the WD-40 repeat, and unique N- and C-terminal domains (Bai et al., 1996; Peterson et al., 1984). Characterization of cdc4 mutant alleles suggests that all four domains of CDC4 are essential (Bai et al., 1996; Mathias et al., 1996; Johnson, 1991). CDC53 encodes a 94-kDa protein that is related to at least five genes of unknown function in a variety of organisms including humans and nematodes (Kipreos et al., 1996; Mathias et al., 1996). SKPL encodes a 23-kDa protein (Bai et al., 1996) that is 48% identical to a human protein that was originally identified via its association with SKP2/cyclin A/CDK2 complexes (Zhang et al., 1995). Human SKPL can complement a yeast skpl" mutant (Bai et al., 1996).

Although the functions of Cdc4p, Cdc53p, and Skp1p are unclear, genetic and biochemical data suggest that they collaborate with Cdc34p to effect multiubiquitination of unstable proteins. All double combinations of cdc4"s, cdc34"s, and cdc53"s mutations are inviable at the normally permissive temperature, and overexpression of CDC53 or SKPL suppresses cdc4"s, and overexpression of CDC4 suppresses skpl"s and cdc53"s mutants (Bai et al., 1996; Mathias et al., 1996). As is often the case, such genetic interactions are indicative of physical interactions between these polypeptides: Cdc4p and Cdc53p are associated with Cdc34p in yeast lysates (Mathias et al., 1996), and Cdc53p–Cdc34p, Cdc53p–Skp1p, and Skp1p–Cdc4p binary interactions can be demonstrated with recombinant proteins (Feldman et al., 1997; Skowrya et al., 1997; Bai et al., 1996; Willems et al., 1996).

Besides interacting with Cdc4p and Cdc34p, Cdc53p has also been identified as a component of affinity-purified Cln2p/Cdc28p complexes (Willems et al., 1996). CDC53 is required for rapid Cln2p destruction, and Cdc53p associates with phosphorylated, but not unphosphorylated Cln2p complexed with Cdc28p, suggesting that Cdc53p targets phosphorylated Cln2p for Cdc34p-dependent ubiquitination and destruction. No direct reconstitution of Cdc53p interaction with phosphorylated Cln2p was reported, however, leaving open the possibility that Cdc53p and phospho-Cln2p interact through the agency of an unidentified protein.

Recently, reconstitution of Sic1p ubiquitination in vitro has provided direct evidence that Cdc34p, Cdc4p, Cdc53p, and Skp1p define a novel ubiquitination pathway. Sic1p is extensively multiubiquitinated on incubation in wild-type, but not cdc4"s or Cdc34p-depleted extracts, and addition of recombinant Cdc34p or Cdc4p
rescues the defect of the corresponding mutant extract (Verma et al., 1997b). Furthermore, efficient ubiquitination of Sic1p can be reconstituted by mixing together purified El enzyme, ubiquitin, Cln2p/Cdc28p (see Note Added in Proof), Cdc34p, Cdc4p, Cdc53p, and Skp1p. These data indicate that the genetically identified members of the Cdc34p pathway define a set of components that is both necessary and sufficient to catalyze Sic1p ubiquitination. This in vitro system should now permit a systematic dissection of the functions of the enigmatic Cdc4p, Cdc53p, and Skp1p proteins.

Because Cdc4p, Cdc53p, and Skp1p assist Cdc34p in the ubiquitination of substrates, these proteins appear to fulfill the operational definition of an E3 enzyme, a component that is required for substrate ubiquitination in the presence of a ubiquitin-charged E2 (Hershko et al., 1983). Intriguingly, there is no apparent homology between Cdc4p/Cdc53p/Skp1p and Ubr1p (see Chapter 8) or the E6-AP family (see Chapters 3 and 11) of E3s. Thus, Sic1p ubiquitination may proceed by a mechanism different from that described for the E6-AP-mediated ubiquitination of p53, and Cdc4p, Cdc53p, Skp1p may be charter members of a novel class of E3 enzymes.

At least five lines of evidence suggest that Cdc4p, Cdc53p, and Skp1p do not always function in lockstep to mediate the ubiquitination of Cdc34p pathway substrates. First, besides its role in Sic1p destruction, Skp1p also serves as an essential subunit of the centromere-binding CBF3 complex (Connelly and Hieter, 1996; Stemmmand and Lechner, 1996). Second, whereas CDC4, CDC53, and CDC34 function are required for the destruction of Sic1p, CDC4 is not required for the CDC53- and CDC53-dependent destruction of Cln2p (R. J. Deshaies, unpublished data). Third, although Cln2p proteolysis is severely reduced in grr1Δ cells (Barral et al., 1995), Grr1p is unlikely to be directly involved in Sic1p proteolysis because grr1Δ cells are viable and exhibit a shortened G1 phase. Fourth, Cdc4p apparently does not associate with the pool of Cdc53p that binds to phosphorylated Cln2p (Willems et al., 1996). Fifth, mutant alleles of SKP1 have reciprocal effects on proteolysis; skp1-11s stabilizes Sic1p but not Cln2p, whereas skp1-12s stabilizes Cln2p but not Sic1p (Bai et al., 1996). These observations can be reconciled by assuming that the individual subunits of the putative Cdc4p/Cdc53p/Skp1p complex are in dynamic equilibrium in living cells. For example, by substituting Grr1p for Cdc4p, the substrate specificity of the complex could readily be changed from Sic1p to Cln2p. Such rearrangements could be mediated by the Skp1p-binding F box domains of Cdc4p and Grr1p (Bai et al., 1996). Likewise, Skp1p might tether Cdc53p and its partners to the kinetochore, thereby accounting for the Cdc34p-dependent ubiquitination of the kinetochore protein Ndc10p (Yoon and Carbon, 1995). The shorthand used so far in this review, “CDC34 pathway,” is thus probably an oversimplification of the boundaries of Cdc34p, Cdc4p, Cdc53p, and Skp1p function.
2.2. Regulation of CDC34-Dependent Ubiquitination

2.2.1. CDK-Dependent Phosphorylation Triggers Destruction of Sic1p and G1 Cyclins

Accumulating evidence indicates that substrate phosphorylation triggers Cdc34p-dependent ubiquitination (Fig. 2). A causal link between substrate phosphorylation and ubiquitin-dependent destruction via the Cdc34p pathway was originally suggested by the observations that (1) Cln2p and Cln3p are partially stabilized by cdc28ts mutations; (2) destruction of β-galactosidase-Cln3p chimeras requires Cdc28p-dependent phosphorylation of a CDK consensus site in the destabilization domain of Cln3p; and (3) Cdc34p-dependent ubiquitination of Cln2p in vitro requires Cdc28p activity (Deshaies et al., 1995; Yaglom et al., 1995). Lanker et al. (1996) strengthened this hypothesis by demonstrating that a mutated version of Cln2p lacking seven consensus Cdc28p phosphorylation sites (Cln2p-4T3S) is dramatically stabilized in vivo. Surprisingly, Cln2p-4T3S is

![Diagram](image)

**Figure 2.** A model of the G1/S transition in budding yeast. This figure summarizes data presented in this review on the mechanism and regulation of Sic1p destruction. In G1 cells Sic1p binds to and inhibits Cdc28p-Cib kinase complexes and thus prevents the initiation of DNA replication. This inhibition is released once Sic1p becomes phosphorylated by Cdc28p-Cln2p kinase, which allows the ubiquitination and subsequent destruction of Sic1p by the CDC34 pathway. It is unknown if Cdc4p, Cdc53p, Skp1p, and Cdc34p exist as a stable particle. Although the putative Cdc4p/Cdc53p/Skp1p complex is shown to be interacting directly with phosphate groups on Sic1p, it is not yet known how phosphorylation targets Sic1p for ubiquitination (see Section 2.3.2 and Fig. 3).
substantially more stable than either a deletion mutant of Cln2p (Cln2p-Δxs) that is unable to bind Cdc28p or wild-type Cln2p expressed in cdc28ts cells (Lanker et al., 1996; Deshaies et al., 1995). In a double mutant analysis, the Δxs mutation is epistatic to the 4T3S allele, implying that there exist two pathways for Cln2p destruction: The first targets Cln2p that is bound to and phosphorylated by Cdc28p, whereas the second preferentially degrades free Cln2p, and does not depend on Cdc28p activity. It is important to note that proteolysis of phosphorylated Cln2p persists in cdc34ts cells (albeit at a rate about fourfold slower than that observed in wild type) (Willems et al., 1996; Deshaies et al., 1995). This residual destruction of Cln2p may be related to either the leakiness of the cdc34ts mutant, the activity of the phosphorylation-independent Cln2p destruction pathway mentioned above, or the presence of yet a third pathway for Cln2p proteolysis. Regardless of the underlying cause, the complex interplay of destruction pathways may confound analyses of ubiquitin-dependent proteolysis (Salama et al., 1994).

Recently, Blondel and Mann (1996) published a study of Cln1p and Cln2p degradation in various mutant strains. The major findings in this paper are as follows. Both Clb-deficient and ubc9 mutants accumulate stable Cln1/2p. Furthermore, the stabilization of Cln2p in cdc34 mutants (Bai et al., 1996; Willems et al., 1996, Deshaies et al., 1995) is suggested to be an artifact of cell cycle arrest at the G1/S boundary, as Cln1/2p are unstable in cdc34 siclΔ mutants. These data suggest that Clbs antagonize Cln accumulation both by inhibiting CLN transcription (Amon et al., 1993) and by stimulating Cln proteolysis, although it is unclear if Clb-dependent proteolysis of Cln1/2p is important for proper cell cycle regulation. Whereas the data in this paper appear to be at odds with the suggestion that Cln2p is a Cdc34p substrate (Bai et al., 1996; Willems et al., 1996; Deshaies et al., 1995), several points deserve comment. First, it is unclear if the polyclonal antibody used in this report detects primarily Cln1p or Cln2p; little has been published regarding the degradation of Cln1p, and it is unclear if it behaves as a Cdc34p substrate. Second, it is difficult to exclude the possibility that deletion of SIC1 derepresses a normally cryptic Cln1/2p degradation pathway. Third, it will be interesting to see if stabilization of Cln2p in cdc53 and skp1 mutants is SIC1-dependent, as these genes have been reported to be required for Cln2p proteolysis at cell cycle positions other than the G1/S boundary (Bai et al., 1996; Willems et al., 1996).

Several lines of evidence suggest that Cdc34p-dependent destruction of Sic1p is triggered by the Cdc28p-dependent phosphorylation of Sic1p at the G1/S transition. In vivo analyses have revealed that Sic1p destruction is prevented in cells arrested at the cdc28ts block (Schwob et al., 1994), is substantially delayed in a ctn1Δcln2Δ mutant (E. Schwob, personal communication), and requires continuous expression of G1 cyclins on reversal of the cdc34ts block (Schneider et al., 1996). In fact, switching on Sic1p destruction probably is, along with the polarization of membrane assembly, a key function of G1 cyclins since deletion of
SIC1 advances the timing of S phase entry in a cln1Δcln2Δ mutant (Dirick et al., 1995) and suppresses the lethal cell division arrest of a cln1Δcln2Δcln3Δ mutant (Schneider et al., 1996; Tyers, 1996). What exactly are the G1 cyclins doing to activate Sic1p destruction? By analogy to Cln2p, one might imagine that G1 cyclin/CDK complexes phosphorylate Sic1p, rendering it competent for ubiquitination by the CDC34 pathway. Alternatively (or in addition), Cln2p/Cdc28p might activate components of the CDC34 pathway or quench inhibitors that oppose the CDC34 pathway. Evaluation of Sic1p ubiquitination reactions reconstituted in vitro revealed that G1 cyclin activity is required for the assembly of multiubiquitin chains on Sic1p, and Sic1p is an excellent substrate for Cln2p/Cdc28p protein kinase complexes (Verma et al., 1997b). Moreover, purified phospho-Sic1p is ubiquitinated by the CDC34 pathway in the absence of Cln2p/Cdc28p activity, indicating that direct phosphorylation of Sic1p by Cln2p/Cdc28p is both necessary and sufficient to trigger its ubiquitination (Verma et al., 1997a). The hypothesis that phosphorylation triggers Sic1p destruction is further supported by the analysis of nonphosphorylatable mutants of Sic1p (Verma et al., 1997a). Mutation of CDK phosphorylation sites in Sic1p both reduces Sic1p ubiquitination in vitro and stabilizes Sic1p in vivo. Furthermore, expression of nonphosphorylatable Sic1p in vivo inhibits progression into S phase, strongly suggesting that phosphorylation-dependent destruction of Sic1p is a prerequisite for the initiation of DNA replication.

2.2.2. Are Cdc34p, Cdc4p, Cdc53p, and Skp1p Regulated?

Little is known about the regulation of the activity, abundance, localization, or posttranslational modification state of CDC34 pathway components in vivo. Cdc34p is phosphorylated by an unknown kinase on serine residues, but the role of this modification and its cell cycle timing have not been reported (Goebel et al., 1994). Although the phosphorylation status of Cdc4p, Cdc53p, and Skp1p have not been directly evaluated, Cdc53p does not serve as a substrate for the tightly bound Cln2p/Cdc28p protein kinase (Willems et al., 1996). Both Cdc34p and Cdc53p form thiol-insensitive conjugates with ubiquitin in vivo, but the significance of these modifications is unclear (Willems et al., 1996; Goebel et al., 1994).

The experiments summarized in the previous section are consistent with the notion that destruction of CDC34 pathway substrates is controlled not by regulating the activity of the CDC34 pathway enzymes, but by modulating the phosphorylation of the substrate. According to this view, substrates submit themselves, on their phosphorylation by regulated protein kinases, to be disposed of via the action of a constitutively active CDC34 pathway. The CDC34 pathway is active at least throughout the period of CDK activity in vivo: CDC34 pathway (and CDK) substrates Cln2p (Willems et al., 1996), Sic1p (Bai et al., 1996), and Cdc6p (Piatti et al., 1996) are unstable in cells arrested during S phase or mitosis. An important
caveat is that it is not yet clear whether phosphorylation of \textit{CDC34} pathway substrates by CDK complexes is sufficient to trigger their ubiquitination \textit{in vivo}; certain forms of regulation (i.e., compartmental localization) may not be preserved in the \textit{in vitro} ubiquitination system.

\subsection{2.3. Recognition of Substrates by the \textit{CDC34} Pathway}

\subsubsection{2.3.1. Other Substrates of Cdc34p}

Besides Sic1p, Cln2p, and Cln3p, several other candidate substrates of the Cdc34p pathway have been identified (Table II). The transcription factor Gcn4p (Kornitzer \textit{et al.}, 1994) and the G1 cyclin/Cdc28p inhibitor Far1p (Henchoz \textit{et al.}, 1997; McKinney \textit{et al.}, 1993) accumulate in \textit{cdc34} cells, and the mult ubiquitination of both proteins in yeast extract is stimulated by Cdc4p and Cdc34p (Y. Chi and R. J. Deshaies, unpublished data). Likewise, the normally unstable Cdc6p is dramatically stabilized in nocodazole-arrested \textit{cdc4} cells (Piatti \textit{et al.}, 1996), and serves as a substrate for Cdc4p/Cdc34p-stimulated ubiquitination \textit{in vitro} (Y. Chi and R. J. Deshaies, unpublished data). The Cbf2p subunit of the CBF3 kinetochore-binding complex may also be a Cdc34p substrate; ubiquitinated forms of Cbf2p are detected in wild-type but not \textit{cdc34} cells at the nonpermissive temperature. Cbf2p is not extensively mult ubiquitinated, and the formation of short ubiquitin chains on Cbf2p does not require \textit{CDC4} (Yoon and Carbon, 1995). Perhaps ubiquitination of Cbf2p influences some aspect of its function other than

\begin{table}[h]
\centering
\caption{Known and Suspected Substrates of the \textit{CDC34} Ubiquitination Pathway}
\begin{tabular}{|l|c|c|c|}
\hline
Protein & \textit{CDC34}-dependent instability \textit{in vivo}? & Cdc34p-dependent ubiquitination \textit{in vitro}? & References \\
\hline
Sic1p & Yes & Yes & Schwob \textit{et al.} (1994) \\
Cln1p & Not tested & Yes & Y. Chi and R. J. Deshaies (unpublished data) \\
Cln2p & Yes & Yes & Deshaies \textit{et al.} (1995) \\
Cln3p & Yes & Not tested & Yaglom \textit{et al.} (1995) \\
Far1p & Not tested & Yes & McKinney and Cross (1995), Y. Chi and R. J. Deshaies (unpublished data) \\
Gcn4p & Yes & Yes & Kornitzer \textit{et al.} (1994), Y. Chi and R. J. Deshaies (unpublished data) \\
Cdc6p & Yes & Yes & Piatti \textit{et al.} (1996), Y. Chi and R. J. Deshaies (unpublished data) \\
Cbf2p & No & Yes & Yoon and Carbon (1995) \\
\hline
\end{tabular}
\end{table}
its stability. It will be interesting to determine if Cbf2p is targeted for ubiquitination by its CBF3 partner Skp1p (Connelly and Hieter, 1996; Stemmann and Lechner, 1996). Lastly, whereas cdc34ts sic1Δ double mutants fail to arrest at the G1/S transition, they remain temperature-sensitive for growth and arrest in G2/M phase, suggesting that the accumulation of a Cdc34p substrate other than Sic1p can block cell division (Schwob et al., 1994).

2.3.2. Signals Involved in Substrate Recognition

Sequence comparisons have failed to identify conserved destruction signals in known CDC34 pathway substrates, but most Cdc34p substrates contain multiple segments rich in proline, glutamate/aspartate, serine, and threonine (PEST sequences), which are commonly found in unstable proteins (Rogers et al., 1986). It is important to note that PEST elements are related by composition, not by primary sequence similarity. Mutational analyses have shown that PEST segments of Cln2p, Cln3p, and Gcn4p can be important for maximal rates of proteolysis, but individual elements are neither essential nor sufficient for degradation (Yaglom et al., 1995; Kornitzer et al., 1994; Salama et al., 1994). Surprisingly, ~40% of the proteins predicted from the yeast genome sequence contain high-scoring PEST regions (D. Mathog and R. J. Deshaies, unpublished data). Taken together with the mutational data, this observation suggests that the PEST algorithm is an imperfect prognosticator of a bona fide destruction-targeting sequence. An accurate description of the true destruction signal embedded within Cdc34p substrates that contain active PEST regions will probably require detailed analyses of the interaction between these substrates and components of the CDC34 pathway. It is interesting to speculate that the CDC34 pathway may be the physiological route of destruction for rapidly degraded proteins that contain destabilizing PEST domains.

Besides PEST sequences, a second feature shared by all CDC34 pathway substrates is that phosphorylation appears to play an important role in their ubiquitin-mediated destruction. All candidate Cdc34p substrates except for Gcn4p are phosphorylated in a CDC28-dependent manner prior to either their ubiquitination or degradation, and mutation of Cdc28p consensus phosphorylation sites or inactivation of Cdc28p itself stabilizes Cln2p (Lanker et al., 1996; Deshaies et al., 1995), Cln3p (Yaglom et al., 1995), Sic1p (see Section 2.2.1), and Far1p (Henchoz et al., 1997). In this instance, Gcn4p may be the exception that proves the rule: in vitro studies suggest that Cdc34p/Cdc4p-dependent ubiquitination of Gcn4p requires a Gcn4p kinase that is distinct from Cdc28p (Y. Chi and R. J. Deshaies, unpublished data). An important question is how phosphorylation serves to target substrates to the CDC34 pathway. Two possible mechanisms are presented in Fig. 3. Phosphorylation may create an epitope that is directly recognized by the
ubiquitination machinery, much like tyrosine phosphorylation creates an epitope recognized by SH2 domains. Alternatively, phosphorylation may perturb the conformation of protosubstrates, revealing a cryptic peptide-based ubiquitination signal.

As PEST elements are often enriched in the S/T P dipeptide that serves as a minimal Cdc28p phosphorylation site, the essence of PEST-mediated instability may be the propensity of PEST sequences to serve as a prominent target for phosphorylation (Yaglom et al., 1995). The actual destruction signal encrypted within PEST elements is probably more complex, however, because many proteins are phosphorylated in eukaryotic cells, and it is improbable that all CDK substrates are rendered unstable by phosphorylation. Rather, it seems likely that either a protein must be phosphorylated on multiple residues, or a phosphorylated residue must be juxtaposed with a specific peptide “cosignal” to allow for effective substrate recognition by the CDC34 pathway. Similar context effects have been observed for the recognition of phosphorylated tyrosine residues by SH2 domains.

### 2.4. Conservation of CDC34 Pathway Function

#### 2.4.1. Vertebrate CDC34

A vertebrate CDC34 homologue was isolated in a screen for human genes that can rescue a budding yeast checkpoint mutant that fails to arrest cell division.
in the presence of unreplicated DNA (Plon et al., 1993). Human CDC34 suppresses the thermosensitive growth of cdc34<sup>ts</sup> strains, implying that yeast and human Cdc34p perform similar functions. One of the targets of human Cdc34p may be the CDK inhibitor p27, which participates in the regulation of the Restriction Point (Coats et al., 1996). p27 is stable in quiescent cells but is unstable in proliferating cells (Pagano et al., 1995). Furthermore, the destruction of p27 in both reticulocyte and fibroblast cell extracts is inhibited by dominant-negative human Cdc34p (Pagano et al., 1995). Like p27, the myogenic regulator MyoD is also stabilized by dominant-negative Cdc34p (Song et al., 1996). A Cdc34p-like protein has also been identified in Xenopus eggs, and Xenopus egg extracts immunodepleted of Cdc34p and affiliated polypeptides fail to sustain cyclin E/Cdk2-dependent replication of double-stranded sperm DNA (Yew and Kirschner, 1997). The picture emerging so far is consistent with the notion that vertebrate Cdc34p, like budding yeast Cdc34p, counteracts the activity of an inhibitor(s) of DNA replication by targeting it for ubiquitin-mediated destruction.

2.4.2. The cullin Family

CDC53 is also conserved among eukaryotes. The lin-19 gene of C. elegans (renamed cul-1, for cullin) encodes a protein 30% identical to CDC53 (Kipreos et al., 1996). Comparison of cul-1 and CDC53 sequences with those present in expressed sequence tag databases revealed that there are at least six cullins in humans, five in C. elegans, three in budding yeast, and one in fission yeast (Kipreos et al., 1996). All five of the C. elegans cul genes encode proteins of similar length (~740–800 amino acids), and all pairwise combinations with CUL1 exhibit 24–28% sequence identity. Cdc53p and CUL1 probably possess similar biochemical activities, as human CUL1 complements the thermosensitive phenotype of a cdc53<sup>ts</sup> mutant (S. Lyapina and R. J. Deshaies, unpublished data). In contrast to the cell division arrest of cdc53<sup>ts</sup> mutants in yeast, cul-1 mutants exhibit hyperplasia of all larval tissues, suggesting that wild-type CUL1 normally restrains cell division (Kipreos et al., 1996). A possible explanation for this discrepancy is that CUL-1 may be responsible only for the degradation of positive cell cycle regulators, such as G1 cyclins, whereas other cullin family members may be important for the degradation of a Sic1p-like protein. It will be interesting to see whether any members of the cullin family behave as tumor suppressor genes in humans. Cullins may also play a role in mitotic degradation events, as a subunit of the anaphase-promoting complex has recently been found to be a novel member of this protein family (see Section 3.1.2b; J.-M. Peters, H. Yu, R. W. King, and M. Kirschner, unpublished data; W. Zachariae and K. Nasmith, personal communication).
2.5. Links between Ubiquitin-Dependent Proteolysis and the Control of G1 Phase in Vertebrate Cells

The classical experiments of Pardee and co-workers led to the hypothesis that the accumulation of an unstable polypeptide(s) positively regulated the commitment to cell division during G1 phase (Pardee, 1987). Subsequent molecular analyses have revealed that both positive (cyclins D and E, c-Myc, E2F) and negative (p27, p53) regulators of G1s progression are unstable (Clurman et al., 1996; Hateboer et al., 1996; Hofmann et al., 1996; Won and Reed, 1996; Pagano et al., 1995; Matsushima et al., 1991; Ramsay et al., 1984; Reich and Levine, 1984). With the exceptions of p27 and the virally targeted destruction of p53 in cells infected by human papillomavirus (for reviews, see chapter 11 and Deshaies, 1995b), the identity of the pathways by which these proteins are degraded in normal cells remains unknown. Nevertheless, cyclin E (Clurman et al., 1996; Won and Reed, 1996) shares an important characteristic of Cdc34p substrates inasmuch as it is stabilized by mutation of a CDK consensus phosphorylation site. The same may be true for p53 (Lin and Desiderio, 1993).

Progression through G1 phase may also be influenced by proteins that disassemble multiubiquitin chains, because two genes that encode deubiquitinating enzymes—tre-2 and DUB1—have been linked to cell proliferation (Matoskova et al., 1996a,b; Zhu et al., 1996; Papa and Hochstrasser, 1993; Nakamura et al., 1992; see also Chapter 4). However, detailed insight into the substrates and physiological roles of both DUB-1 and tre-2 awaits future investigations.

3. UBQUITIN-DEPENDENT PROTEOLYSIS IN MITOSIS

Mitosis is the process by which the cell divides its duplicated DNA and other cellular contents. Whereas the early events of mitosis such as chromosome condensation and spindle assembly are thought to be initiated by the activation of CDC2 and other mitosis-specific protein kinases, later events such as the separation of sister chromatids require the activation of a ubiquitin-dependent proteolytic system. The first substrates of this system that were identified are the mitotic cyclins, whose destruction inactivates CDC2 and allows exit from mitosis. Recent studies have revealed that the degradation of other substrates regulates the metaphase–anaphase transition and the disassembly of the mitotic spindle (Fig. 4). An important question is how the CDK-dependent entry into mitosis is coordinated with the exit from mitosis, which is driven by proteolysis. The cell cycle machinery must allow sufficient time for the chromosomes to condense and align on the spindle before the proteolytic system that initiates sister chromatid
Figure 4. Mitotic functions of the APC pathway. Schematic representation of the morphological phase of mitosis, indicating how APC-mediated ubiquitination regulates progression through mitosis. Degradation of anaphase inhibitors such as budding yeast Pds1p and fission yeast Cut2 is required for the initiation of chromosome segregation, whereas the proteolysis of mitotic cyclins such as cyclin B is essential for exit from mitosis (for references see text).

separation is activated. Although the central players of the mitosis-specific proteolytic pathway have recently been identified, little is known about how these components are switched on and off during the cell cycle.

3.1. Mitotic Cyclin Degradation

In rapidly dividing marine embryos, mitotic cyclins accumulate steadily throughout interphase and are rapidly degraded at anaphase (Evans et al., 1983). This intriguing observation provided an early clue that proteolysis may regulate cell division. This hypothesis was confirmed by two important findings: first, that maturation-promoting factor (MPF), a kinase activity capable of inducing entry into mitosis, is composed of a heterodimer of cyclin B and a homologue of fission yeast Cdc2p (Labbé et al., 1989; Arion et al., 1988; Dunphy et al., 1988); and second, that degradation of cyclin B is necessary for inactivation of MPF and exit from mitosis (Murray et al., 1989). The latter finding has subsequently been confirmed in many organisms, emphasizing the universal importance of cyclin degradation in regulating exit from mitosis (Yamano et al., 1996; Luo et al., 1994; Rimmington et al., 1994; Surana et al., 1993; Gallant and Nigg, 1992; Ghiara et al., 1991).
Higher eukaryotes contain two subclasses of mitotic cyclins, called A and B, which share the capacity to activate CDC2; cyclin A is also able to activate the related kinase CDK2, and plays a role in regulating S phase. Although the mitotic destruction of cyclins A and B in clam embryos appears to be mediated by the same pathway (Sudakin et al., 1995), their proteolysis has been reported to differ in two respects: First, cyclin A is degraded in advance of cyclin B; second, B-type cyclins are preferentially stabilized by an arrest mechanism, called the spindle assembly checkpoint, that prevents the separation of sister chromatids if they are not properly attached to the mitotic spindle (Edgar et al., 1994; Minshull et al., 1994; Hunt et al., 1992; Whitfield et al., 1990). Why the destruction of cyclins A and B differs in these respects and whether these differences are important for the orderly progression through mitosis are presently unknown.

Although degradation of mitotic cyclins can be observed in most dividing somatic cells and early embryos, the syncytial nuclear divisions of the early Drosophila embryo provide an interesting exception. In this system, bulk fluctuations in cyclin levels are only observed once cellularization occurs (Edgar et al., 1994). A similar situation has been reported for the syncytial slime mold Physarum polycephalum (Cho and Sauer, 1994). This suggests that widespread cyclin degradation and kinase inactivation may be necessary only for cell cycles that involve cytokinesis. In syncytial cycles, however, local degradation of cyclin may suffice to allow nuclear division.

Several features of cyclin proteolysis are worth noting. First, cyclin degradation appears relatively specific, as the majority of labeled proteins in marine embryos remains stable through mitosis (Evans et al., 1983). Second, in early clam embryos, cyclin was found to be unstable during only a brief period at the end of mitosis (Hunt et al., 1992), suggesting that the activity of the cyclin degradation system is regulated during the cell cycle. Subsequent studies have indicated that the cyclin degradation system remains active from late mitosis through G1 in cells whose cell cycle contains G1 and G2 phases such as budding yeast (Amon et al., 1994) and somatic mammalian cells (Brandeis and Hunt, 1996).

The identification of nondegradable mutants of cyclin B was crucial to the biochemical analysis of the cyclin degradation system, whose fleeting activity made it difficult to study. Deletion of the N-terminal 90 amino acids of cyclin B results in a protein (cyclin B Δ90) that is stable in mitosis, but retains the capacity to activate CDC2 (Murray et al., 1989). Xenopus egg extracts containing cyclin B Δ90 remain arrested in a mitotic state with a constitutively activated cyclin degradation system. Using these mitotically arrested extracts, Glotzer et al. (1991) found that the N-terminus of sea urchin cyclin B is sufficient to target a heterologous protein (protein A) for mitosis-specific degradation. Labeling of cyclin B–protein A chimeras to high specific radioactivity revealed the presence of ubiquitinated intermediates during the course of degradation, providing the first evidence that cyclin B is degraded by the ubiquitin pathway. Ubiquitinated intermediates
are 10-fold more abundant in mitotic extracts than in interphase extracts, where cyclins are stable, suggesting that regulated ubiquitination can account for the cell cycle dependence of cyclin proteolysis. Subsequent experiments in clam extracts demonstrated that methylated ubiquitin, an inhibitor of polyubiquitin chain formation, inhibits cyclin degradation, confirming that mitotic cyclin proteolysis is ubiquitin-dependent (Hershko et al., 1991).

3.1.1. Signals that Target Mitotic Cyclins for Destruction

The N-termini of mitotic cyclins share little overall sequence similarity, with the exception of a 9-amino-acid motif that has been called the destruction box (D box; Glotzer et al., 1991; Table III). Mutation of conserved positions of the D box is sufficient to prevent both ubiquitination and proteolysis of cyclins A and B, suggesting that it serves as a ubiquitination signal (King et al., 1996b; Sudakin et al., 1995; Kobayashi et al., 1992; Lorca et al., 1992a; Glotzer et al., 1991). Although a cyclin B N-terminal fragment as short as 27 amino acids can target a heterologous protein for degradation, physiological rates of degradation (half-life less than 5 min) appear to require the entire N-terminus (King et al., 1996b). This may indicate that the N-terminus is necessary for the proper presentation and recognition of the D box, which is presumed to be recognized by a component of the anaphase-promoting complex (see below). There does not appear to be a strict requirement for a particular lysine residue to serve as a ubiquitin acceptor site, as cyclin B can be ubiquitinlated at multiple lysine residues in a manner sufficient to support degradation at physiological rates (King et al., 1996b).

Although the N-terminal fragments of Arbacia cyclin B and Xenopus cyclin B1 are rapidly degraded in mitotic Xenopus extracts, the N-terminal fragments of other cyclins, such as Xenopus cyclin A1 or cyclin B2, are not (Stewart et al., 1994; van der Velden and Lohka, 1993). This may reflect the fact that these cyclins need to be bound to CDC2 to be degraded (Stewart et al., 1994; van der Velden and Lohka, 1994). Surprisingly, the D box of cyclin A is not sufficient to substitute for that of cyclin B (King et al., 1996b; Klotzbücher et al., 1996), although the cyclin B D box can target A-type cyclins for destruction (Klotzbücher et al., 1996). Although A- and B-type cyclins are thought to be ubiquitinated by the same components (Sudakin et al., 1995), structural differences between A- and B-type D boxes may contribute to the distinct physiological properties of cyclin A and cyclin B degradation discussed earlier.

Recently, D boxes have also been found to be important for the mitosis-specific ubiquitination and degradation of several noncyclin proteins (see below). A comparison of D boxes in cyclins and noncyclins shows that only residues 1 (arginine), 4 (isoleucine, leucine, or phenylalanine), and 9 (usually asparagine) are conserved.
Table III  
Destruction Box Sequences\(^a\)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-type cyclins</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pombe</em> Cdc13p</td>
<td>59</td>
<td>RHALDDVSN</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Clb2p</td>
<td>25</td>
<td>RLALNNVTN</td>
</tr>
<tr>
<td><em>A. nidulans</em> NIME</td>
<td>52</td>
<td>RAALGDVSN</td>
</tr>
<tr>
<td><em>A. thaliana</em> Cyc1</td>
<td>38</td>
<td>RQVLGDIGN</td>
</tr>
<tr>
<td><em>D. melanogaster</em> Cyc B</td>
<td>37</td>
<td>RAALGDQGN</td>
</tr>
<tr>
<td><em>A. punctulata</em> Cyc B</td>
<td>42</td>
<td>RAALGNISN</td>
</tr>
<tr>
<td><em>S. solidissima</em> Cyc B</td>
<td>40</td>
<td>RNTLGDIQN</td>
</tr>
<tr>
<td><em>X. laevis</em> Cyc B1</td>
<td>36</td>
<td>RTALGDIQN</td>
</tr>
<tr>
<td><em>X. laevis</em> Cyc B2</td>
<td>30</td>
<td>RAALGEIGN</td>
</tr>
<tr>
<td><em>G. gallus</em> Cyc B2</td>
<td>32</td>
<td>RAVLEEIGN</td>
</tr>
<tr>
<td><em>G. gallus</em> Cyc B3</td>
<td>51</td>
<td>RSAFGDITN</td>
</tr>
<tr>
<td><em>H. sapiens</em> Cyc B1</td>
<td>42</td>
<td>RTALGDIQN</td>
</tr>
<tr>
<td>A-type cyclins</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. melanogaster</em> Cyc A</td>
<td>160</td>
<td>RSILGVIQS</td>
</tr>
<tr>
<td><em>S. solidissima</em> Cyc A</td>
<td>33</td>
<td>RAALGVTN</td>
</tr>
<tr>
<td><em>X. laevis</em> Cyc A1</td>
<td>41</td>
<td>RTVLGVIGDN</td>
</tr>
<tr>
<td><em>X. laevis</em> Cyc A2</td>
<td>26</td>
<td>RTVLGVLEN</td>
</tr>
<tr>
<td><em>G. gallus</em> Cyc A</td>
<td>93</td>
<td>RAALGTVGE</td>
</tr>
<tr>
<td><em>M. musculus</em> Cyc A1</td>
<td>37</td>
<td>RTVLGVLEN</td>
</tr>
<tr>
<td><em>H. sapiens</em> Cyc A</td>
<td>47</td>
<td>RAALAVLSGN</td>
</tr>
<tr>
<td>Other APC substrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Pds1p</td>
<td>85</td>
<td>RLPLAAKDN</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Asclp</td>
<td>760</td>
<td>RQLFPIPLN</td>
</tr>
<tr>
<td><em>X. laevis</em> Geminin</td>
<td>33</td>
<td>RRTLKVQIP</td>
</tr>
</tbody>
</table>

\(^a\)Amino acid sequences are given in one-letter code. The position of the first shown amino acid residue is indicated. For a detailed discussion see King *et al.* (1996b).

### 3.1.2. Components Involved in Mitotic Cyclin Degradation

Both biochemical and genetic approaches have played an important role in the identification of components involved in mitotic cyclin degradation. The clam system was the first to be successfully fractionated into several distinct activities required for ubiquitination of cyclin B N-terminal fusion proteins *in vitro* (Hershko *et al.*, 1994). Cyclin ubiquitination in clam extracts requires two fractions in addition to ubiquitin and E1: an E2 activity, called E2-C, and a putative E3 activity that associates with particulate material and is active only when derived from mitotic extracts. On solubilization, this E3-containing component, called the cyclosome, sediments as a large particle on glycerol gradients (Sudakin *et al.*, 1995).
Fractionation of *Xenopus* extracts yielded similar, although not identical results (King et al., 1995). In this system, all components are soluble, and two distinct E2 activities can sustain D box-dependent cyclin ubiquitination *in vitro*. As in the clam system, both E2 activities are constitutively active through the cell cycle, and a large protein complex, containing the putative E3 activity, is active only when purified from mitotic extracts. The purified complex contains eight distinct proteins (Peters et al., 1996; King et al., 1995), several of which are homologous to proteins required for cyclin proteolysis in budding yeast (Zachariae et al., 1996; Irniger et al., 1995). Earlier genetic studies in different fungi had shown that these conserved proteins are essential for the transition from metaphase to anaphase (see below). The E3 complex purified from *Xenopus* and characterized in yeast was therefore called the anaphase-promoting complex (APC).

### 3.1.2a. Ubiquitin-Conjugating Enzymes

One of the E2s involved in cyclin ubiquitination in *Xenopus* extracts is a homologue of the budding yeast Ubc4/5p subfamily of E2 enzymes, which has been implicated in the ubiquitination of many different proteins (King et al., 1995). Mutation of Ubc4p family members in yeast has not been reported to cause a cell cycle arrest (Seufert and Jentsch, 1990), but deletion of *UBC4* is synthetically lethal in yeast strains carrying a mutated form of the APC subunit Cdc23p (Irniger et al., 1995), suggesting that Ubc4p and APC function in the same biochemical pathway.

Purification and cloning of the second E2 activity from *Xenopus* and clam extracts (called UBCx and E2-C, respectively) has revealed that they are orthologues (Aristarkhov et al., 1996; Yu et al., 1996). Dominant-negative versions of the human relative, designated UBC-H10, arrest cells at metaphase, consistent with a role of this E2 in the APC pathway (Townsley et al., 1997). This E2 appears to be a novel enzyme, most closely related to human UBC2. In *Xenopus*, either UBC4 or UBCx is sufficient to support D box-dependent cyclin ubiquitination in the presence of E1 and purified APC. UBCx is active at slightly lower concentrations than UBC4, and at saturating concentration can convert twice as much substrate into ubiquitin conjugates. However, the conjugates formed are of lower average molecular mass than those formed in the presence of UBC4, suggesting that the reaction may be less processive (Yu et al., 1996).

UBCx and E2-C appear to have homologues in budding and fission yeast, although the role of the yeast enzymes in cyclin degradation has not been directly tested. Mutation of the *S. pombe* gene UbcP4 leads to a metaphase arrest that can be rescued by overexpression of the APC subunit Cut9p, suggesting that this enzyme functions in the APC pathway (Osaka et al., 1997).

In budding yeast, mutation of yet another E2-related enzyme, Ubc9p, stabilizes the B-type cyclins Clb2p and Clb5p (Seufert et al., 1995). However, unlike Clb2p, whose ubiquitination and degradation is APC-dependent (see below),
Clb5p stability is not cell cycle regulated (Seufert et al., 1995), does not require a D box, and is not affected in cdc16 and cdc23 mutants (Irninger et al., 1995), suggesting that its degradation is not mediated by APC. In *Xenopus* extracts a homologue of Ubc9p is not required for APC-dependent cyclin B ubiquitination (King et al., 1995), and mutation of *UBC9* does not interfere with Clb2p ubiquitination in yeast extracts *in vitro* (Zachariae and Nasmyth, 1996). Recently, vertebrate UBC9 has been found to assemble with two other proteins, RanGAP1 and RanBP2, into a complex that is involved in regulating nuclear transport of karyophilic proteins (Mahajan et al., 1997; Saitoh et al., 1997). The stabilization of Clb2p in *ubc9* mutants could therefore be related to a defect in the nuclear transport machinery, which would prevent the nuclear uptake of proteins that are required for progression into mitosis or for mitotic cyclin proteolysis in the yeast nucleus. Interestingly, the targeting of the UBC9–RanGAP1–RanBP2 complex to the nuclear pore complex requires modification with SUMO-1, a ubiquitin related protein (Mahajan et al., 1997; see Chapter 2 for more details). UBC9 can form a thioester with SUMO-1 (M. Scheffner, personal communication), explaining the previous observation that *Xenopus* UBC9 is unable to form thioesters with conventional ubiquitin (R. W. King, J.-M. Peters, and M. W. Kirschner, unpublished results).

### 3.1.2b. The APC/Cyclosome.

Several genes have been identified in yeasts and filamentous fungi that are required for progression through the metaphase–anaphase transition. A subset of these proteins contain the tetra-tricopeptide repeat (TPR) motif, a repeated 34-amino-acid sequence that is thought to mediate protein–protein interactions (Hirano et al., 1990). Consistent with this hypothesis, the TPR-containing proteins Cdc27p, Cdc16p, and Cdc23p are part of a macromolecular complex in budding yeast (Lamb et al., 1994). Mutations in homologous proteins in fission yeast (Cut9 and Nuc2; Samejima and Yanagida, 1994; Hirano et al., 1988) and *Aspergillus* (BIMA; O’Donnell et al., 1991) also cause defects in anaphase, emphasizing the universal role of these proteins in regulating progression through mitosis. Furthermore, injection of antibodies raised against a human homologue of CDC27 into mammalian tissue culture cells results in a metaphase arrest (Tugendreich et al., 1995).

A genetic screen in budding yeast identified three genes required for mitotic cyclin proteolysis: *CDC16*, *CDC23*, and *CSE1* (Irninger et al., 1995). However, it was unclear whether the corresponding proteins regulate or directly catalyze mitotic cyclin proteolysis. Utilizing antibodies raised against human CDC27 and CDC16, it was found that the large E3 complex in *Xenopus* extracts cofractionates with both proteins, and that immunopurified CDC27 complexes are sufficient to support cyclin B ubiquitination in the presence of recombinant UBC4 and E1 (King et al., 1995). Purification and peptide sequencing of the components of the E3 complex has revealed that it consists of at least eight tightly associated subunits.
(Table IV), including *Xenopus* homologues of budding yeast Cdc27p, Cdc16p, and Cdc23p (Peters et al., 1996). Four of the remaining proteins have previously not been identified, whereas the final subunit is a homologue of the *Aspergillus* protein BIME (Peters et al., 1996), which is required for anaphase progression (Osmani et al., 1988). BIME was originally proposed to be a transmembrane protein (Engle et al., 1990), but in *Xenopus* extracts BIME is only detectable in association with APC, which is soluble (Peters et al., 1996). Extension of the screen for genes required for cyclin proteolysis in *S. cerevisiae* has demonstrated that *CDC26*, and a yeast homologue of BIME, called *APC1*, are also required for Clb2p ubiquitination (Zachariae et al., 1996). A BIME-related protein in fission yeast is also required for mitotic cyclin ubiquitination and is a component of APC in that organism (Yamashita et al., 1996). The budding yeast APC has also been affinity-purified and shown to contain several subunits in addition to Cdc16p, Cdc23p, Cdc27p, and the homologue of BIME. Three of these are homologous to *Xenopus* APC2, APC4, and APC5 (W. Zachariae, M. Mann, and K. Nasmyth, personal communication).

Neither Cse1p, a protein required for accurate chromosome segregation in budding yeast (Xiao et al., 1993), nor its vertebrate relative CAS (Schertf et al., 1996; Brinkmann et al., 1995) are tightly associated with APC in yeast or *Xenopus* (Zachariae et al., 1996; Peters et al., 1996), and the role of this protein in cyclin degradation is presently unclear. Whereas yeast extracts from *cse1* mutants are deficient in Clb2p ubiquitination (Zachariae and Nasmyth, 1996), cyclin B ubiquitination can be reconstituted from purified *Xenopus* enzymes in the absence of *Xenopus* CAS (Peters et al., 1996). Another protein that may play a role in mitotic cyclin ubiquitination is Suc1, a small CDK-binding protein in fission yeast that has homologues in budding yeast (Cks1p), mammals (CKS), and *Xenopus* (p9). In the absence of Suc1, fission yeast cells arrest in mitosis with high levels of Cdc2 kinase activity (Basi and Draetta, 1995; Moreno et al., 1989) and *Xenopus* extracts immunodepleted with p9 antibodies are unable to degrade cyclin B (Patra and Dunphy, 1996). The observation that *Xenopus* p9 is required for cyclin B degradation in calcium-treated cytosolic factor (CSF) extracts, in which APC has already been activated (see Section 3.4.2), suggests that p9 may play a direct role in cyclin ubiquitination, perhaps by facilitating recognition of cyclin B–CDC2 complexes by APC. This view is supported by the recent observation that the mitotic form of the clam cyclosome can bind to fission yeast Suc1 in vitro (Sudakin et al., 1997).

Given the large number of components involved in cyclin ubiquitination, it is perhaps not surprising that the reaction mechanism remains obscure. Two possible models of how APC may ubiquitinate substrates are shown in Fig. 5. Although APC meets the functional criteria for a ubiquitin-protein ligase or E3, as it is sufficient for ubiquitination in the presence of E1 and E2, none of the APC subunits identified to date bear sequence resemblance to either one of the two well-defined E3 enzymes Ubr1p and E6-AP (see Chapters 8 and 11). However,
### Table IV

**Subunits of Xenopus APC Purified from Interphase (i) and Mitotic (m) Extract and Their Fungal Homologues**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>$M_r$ (x 10$^3$)</th>
<th>Homologues</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i</td>
<td>m</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>APC1</td>
<td>210</td>
<td>220</td>
<td>Apc1p</td>
</tr>
<tr>
<td>APC3</td>
<td>100</td>
<td>130</td>
<td>Cdc27p</td>
</tr>
<tr>
<td>APC4</td>
<td>100</td>
<td>100</td>
<td>Apc4p</td>
</tr>
<tr>
<td>APC5</td>
<td>82</td>
<td>82</td>
<td>Apc5p</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Cdc26p</td>
</tr>
</tbody>
</table>
Fig 5. Two models of how APC could mediate cyclin ubiquitination. For a detailed discussion see Yu et al. (1996).

both human APC2 and its budding yeast homologue show limited but significant sequence similarity with members of the cullin family which includes Cdc53p, a component of the CDC34 pathway (J.-M. Peters, H. Yu, R. W. King and M. W. Kirschner, unpublished data; W. Zachariae and K. Nasmyth, personal communication). It is therefore conceivable that the APC and the CDC34 pathways utilize similar substrate recognition or ubiquitination mechanisms.

The formation of ubiquitin thioester intermediates, characteristic for members of the E6-AP family of E3s and also observed with Ubrlp (see Chapters 3 and 8), has not been detected with APC (King et al., 1995). This suggests that APC may not serve as the final ubiquitin donor for the reaction, but rather that APC may serve to bind both the E2 and the substrate. Substrate specificity is presumed to be mediated by a component of the APC rather than by the E2, although it has not yet been determined whether the substrate binds the APC or the E2 directly.

3.1.2c. The 26 S Proteasome. Several genetic experiments have suggested that the 26 S proteasome plays an important role in mitotic cyclin proteolysis and exit from mitosis. Mutation of proteasome subunits in both budding and fission yeast results in a G2/M arrest that is similar to the arrest point of APC mutants (Gordon et al., 1996, 1993; Ghislain et al., 1993), and budding yeast proteasome mutants accumulate Clb2p (Ghislain et al., 1993). It has also been reported that budding yeast proteasome mutants can be rescued by deletion of the CLB2 gene (Friedman and Snyder, 1994) and are growth-arrested by overexpression of Clb2p (Richter-Ruoff and Wolf, 1993) or Clb5p (Seufert et al., 1995). Although there is no direct biochemical experiment demonstrating that ubiquitinated mitotic cyclins are degraded by the 26 S proteasome, cyclin B proteolysis is inhibited in cells (Sherwood et al., 1993) or extracts (J.-M. Peters and M. W.
Kirschner, unpublished data) treated with proteasome inhibitors. The 26 S proteasome itself does not appear to be regulated during mitosis, because model substrates such as ubiquitinated lysozyme (Mahaffey et al., 1993) and purified cyclin–ubiquitin conjugates (J.-M. Peters, M. Glotzer and M. W. Kirschner, unpublished results) are rapidly degraded in both interphase and mitotic Xenopus extracts.

3.2. Chromosome Segregation

In most cell types, cyclin B is degraded at about the time that sister chromatids separate, suggesting that its proteolysis might be functionally related to anaphase. Furthermore, cyclin B is stabilized in two physiological circumstances in which sister chromatid segregation is also prevented: the spindle assembly checkpoint, and the natural arrest of vertebrate eggs at metaphase of the second meiotic division (see below). The finding that nondegradable mutants of cyclin B block exit from mitosis in Xenopus extracts (Murray et al., 1989) and budding yeast (Ghiara et al., 1991) suggested that inactivation of CDC2-cyclin B kinase might trigger chromosome segregation. However, it was found that both frog egg extracts and yeast cells containing nondegradable cyclin B arrest in telophase rather than metaphase (Holloway et al., 1993; Surana et al., 1993). The reconstitution of sister chromatid segregation in Xenopus egg extracts (Shamu and Murray, 1992) revealed that even though nondegradable cyclin B does not interfere with anaphase, chromosome segregation is inhibited by high concentrations of a D box-containing fragment of cyclin B, which presumably acts as a competitive inhibitor of APC-mediated ubiquitination reactions (Holloway et al., 1993). The same result was obtained in extracts supplemented with methylated ubiquitin and in extracts lacking endogenous full-length cyclins, suggesting that D box-containing proteins other than cyclin B must be degraded by the APC pathway to initiate anaphase. The recent finding that components necessary for cyclin degradation are also required for anaphase in yeast and mammalian cells further suggests that the two processes are mediated by the same APC-dependent proteolytic pathway (Irniger et al., 1995; Tugendreich et al., 1995).

The work of Holloway et al. (1993) predicted the existence of an anaphase inhibitor that would be degraded through D box-dependent, and hence APC-mediated, proteolysis. Recently, two such candidates have been identified in budding and fission yeast. In budding yeast, the PDS1 gene was identified in a screen for mutants that undergo precocious dissociation of sister chromatids in the presence of microtubule inhibitors (Yamamoto et al., 1996a,b). Although the null mutant is viable, pds1Δ mutants have a high rate of chromosome loss, suggesting that Pds1p is important for anaphase fidelity. Although it is not clear how Pds1p regulates anaphase, the protein has all of the hallmarks of a substrate of the APC pathway: Pds1p is degraded during anaphase, and is unstable when expressed during G1, as is the case for mitotic cyclins; Pds1p contains a sequence element
similar to the cyclin D box that is essential for its degradation in vivo, and Pds1p is stabilized in either cdc16 or cdc23 mutants (Cohen-Fix et al., 1996). Biochemical studies support the contention that Pds1p is a substrate of APC: yeast Pds1p is rapidly degraded in a mitosis-specific and D box-dependent fashion in Xenopus egg extracts, and Pds1p is ubiquitinated in vitro by purified Xenopus APC (Cohen-Fix et al., 1996). Intriguingly, whereas cdc23 or cdc16 mutants arrest prior to anaphase, about 50% of pds1 cdc23 or pds1 cdc16 double mutants progress through anaphase, suggesting that failure to degrade Pds1p partially accounts for the metaphase arrest of cdc23 and cdc16 mutants (Yamamoto et al., 1996b). This hypothesis is strongly supported by the observation that transient expression of a Pds1p mutant lacking the D box results in a metaphase arrest (Cohen-Fix et al., 1996).

Another putative anaphase inhibitor has been identified in fission yeast (Funabiki et al., 1996). Degradation of Cut2, which contains two D box-related sequences, appears essential for anaphase, as overexpression of a nondegradable deletion mutant of Cut2 blocks sister chromatid separation (this does not demonstrate, however, that destruction of physiological levels of Cut2 is required for anaphase). The levels of wild-type Cut2 drop only about twofold during anaphase, suggesting that only a subpopulation of Cut2 may have to be degraded for the initiation of sister chromatid separation. Like other cut (cells untimely torn) mutants, cut2 mutants initiate septum formation despite being unable to separate chromosomes. Cut2 accumulates in cells containing a mutated version of Cut9, a homologue of budding yeast Cdc16p, suggesting that Cut2 is a substrate of APC.

How Pds1p and Cut2 regulate anaphase is unknown. Holloway et al. (1993) proposed that a chromosomal protein might shield sister chromatids from the activity of topoisomerase II until the proteolytic machinery is activated at the metaphase–anaphase transition. Alternatively, the key substrate could be a soluble protein that regulates sister chromatid segregation. Pds1p and Cut2 may have distinct functions, as they share little sequence similarity except for the presence of an N-terminal D box. Moreover, their intracellular distribution differs, with Cut2 localized on the mitotic spindle, and Pds1p distributed diffusely throughout the nucleus. Lastly, whereas Cut2 is essential, cells deleted for PDS1 are viable but sick. Null mutants of cut2 arrest at metaphase, indicating that Cut2 may independently promote and inhibit anaphase.

Additional proteins that may regulate the metaphase–anaphase transition have been identified through the analysis of the Drosophila mutants pimples (pim; Stratmann and Lehner, 1996) and three rows (thr; D’Andrea et al., 1993; Philp et al., 1993). Homozygous pim and thr mutants fail to separate sister chromatids once the maternal store of wild-type protein has been exhausted after embryonic cell cycle 14. As DNA replication continues in these embryos, chromosomes accumulate with up to four pairs of unseparated sister chromatids held together at the centromeric region. PIM and THR cannot be detected by immunofluorescence
microscopy in cells that have undergone anaphase, suggesting that both proteins are either redistributed or degraded at the metaphase–anaphase transition. This resembles the loss of cyclin staining observed during anaphase of Drosophila embryos and therefore suggests that PIM and THR may be targets of APC. It has been proposed that PIM and THR function as activators of anaphase and that their mitotic degradation may be required to prevent premature chromosome segregation in the subsequent cell cycle (Stratmann and Lehner, 1996). The phenotype of null cut2 mutants suggests, however, that proteins required for anaphase may also function as anaphase inhibitors (Funabiki et al., 1996).

3.3. Other Proteins Degraded in Mitosis

We presently know of two APC functions in mitosis: the initiation of anaphase, regulated by the ubiquitination of proteins such as Pds1p, and the exit from mitosis, mediated by the ubiquitination of mitotic cyclins (Fig. 4). However, there are several other proteins that are degraded specifically in mitosis, and at least some of these may be substrates of APC (see Table V). This raises the interesting possibility that the execution of other mitotic events may depend on the proteolysis of specific proteins. For example, the budding yeast protein kinase Cdc5p, a homologue of polo in Drosophila and Plk-1 in vertebrate cells, is degraded in an APC-dependent manner (M. Shirayama and K. Nasmyth, personal communication). Several microtubule-binding proteins (MAPs) are degraded at the end of mitosis, including CENP-E in mammalian cells (Brown et al., 1994) and Ase1p in budding yeast (Pellman et al., 1995). Degradation of Ase1p requires both CDC23 function and a sequence element in the Ase1p C-terminus that resembles the cyclin B3 D box, indicating that it is a substrate of the APC pathway (Juang et al., 1997). Overexpression of a nondegradable Ase1p mutant in telophase delays exit from mitosis, whereas overexpression in G1 arrests cells at G2/M. This arrest is dependent on MAD2, a gene essential for the spindle assembly checkpoint, suggesting that expression of Ase1p in G1 leads to formation of an abnormal mitotic spindle. Degradation of Ase1p and perhaps other MAPs may therefore prevent premature binding of MAPs to microtubules during G1 and also facilitate spindle disassembly at the end of mitosis.

Yet another APC substrate, called geminin, has been identified in Xenopus based on its instability in mitotic egg extracts (T. McGarry and M. Kirschner, personal communication). Geminin contains a D box that is required for its mitosis-specific ubiquitination and degradation. Xenopus embryos microinjected with a D box mutant develop into cellularized embryos that lack nuclei, suggesting that the degradation of geminin may be involved in some aspect of nuclear division, such as DNA replication or chromosome segregation.

To date, all known substrates of APC contain a D box and are degraded in mitosis and G1. However, given the multisubunit nature of APC, it is conceivable
Table V
Substrates of the APC Ubiquitination Pathway

<table>
<thead>
<tr>
<th>Protein</th>
<th>APC-dependent degradation <em>in vivo</em>?</th>
<th>APC-dependent ubiquitination <em>in vitro</em>?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-type cyclins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. punctulata</em> B</td>
<td>—&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Clb2p</td>
<td>Yes</td>
<td>Yes</td>
<td>Zachariae and Nasmyth (1996), Irniger <em>et al.</em> (1995)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Clb3p</td>
<td>Yes</td>
<td>Not tested</td>
<td>S. Irniger and K. Nasmyth (personal communication)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Clb5p</td>
<td>Yes</td>
<td>Not tested</td>
<td>S. Irniger and K. Nasmyth (personal communication)</td>
</tr>
<tr>
<td>A-type cyclins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. solidissima</em> A</td>
<td>—</td>
<td>Yes</td>
<td>Sudakin <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>Other APC substrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>X. laevis</em> Geminin</td>
<td>Not tested</td>
<td>Yes</td>
<td>T. McGarry, J.-M. Peters, and M. Kirschner (unpublished results)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Pds1p</td>
<td>Yes</td>
<td>Yes</td>
<td>Cohen-Fix <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>S. pombe</em> Cut2</td>
<td>Yes</td>
<td>Not tested</td>
<td>Funabiki <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Ase1p</td>
<td>Yes</td>
<td>Yes</td>
<td>Juang <em>et al.</em> (1997), J.-M. Peters and D. Pellman (unpublished results)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Cdc5p</td>
<td>Yes</td>
<td>Not tested</td>
<td>M. Shirayama and K. Nasmyth (personal communication)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Degradation of A- and B-type cyclins *in vivo* has been observed in higher eukaryotes (e.g., Evans *et al.*, 1983; Hunt *et al.*, 1992), but APC dependence *in vivo* has not been demonstrated.
that the complex has additional substrates that could be recognized through different substrate binding sites, perhaps allowing ubiquitination at other points in the cell cycle. This speculation arises from three reports in the literature that cannot be easily explained by the known mitotic function of APC. First, *cdc16* and *cdc27* mutants of budding yeast have been reported to overreplicate their DNA, suggesting that APC function may be required to restrict DNA replication to once per cell cycle (Heichman and Roberts, 1996). In another study it has been reported, however, that most of the DNA synthesis that occurs in APC mutants is the result of replication of mitochondrial DNA (Pichler *et al.*, 1997). Second, *Aspergillus bimE* mutants can override the interphase arrest induced by certain drugs and mutations (*Ye et al.*, 1996; *James et al.*, 1995; *Osmani et al.*, 1991), suggesting that in these cells APC may normally restrain entry into mitosis. Third, fission yeast cells that contain a mutated version of the APC subunit Cut4 are hypersensitive to heavy metals (*Yamashita et al.*, 1996). This suggests that APC may be required for the ubiquitin-dependent degradation of misfolded proteins that are induced by heavy metal treatment. However, this phenotype is not shared by other fission yeast APC mutants such as *cut9* or *nuc2*. It is important to note that in all three cases it remains unclear whether the observed phenotypes can be attributed to novel interphase functions of APC, or whether they result indirectly from interference with the mitotic ubiquitination activity of the complex.

### 3.4. Regulation of APC-Dependent Ubiquitination

#### 3.4.1. Mitotic Regulation of the APC Pathway

Mitotic cyclin stability could in principle be regulated at the substrate level, as is the case for substrates of the *CDC34* pathway, or through the periodic activation of components of the APC pathway. Unlike the G1 cyclins or Sic1p, there is no evidence that phosphorylation of mitotic cyclins targets these proteins for degradation during mitosis (*Li et al.*, 1995; *Izumi and Maller*, 1991). Instead, the activity of the mitotic cyclin ubiquitination system is regulated. Regulation of this pathway is apparently achieved by modulating the intrinsic activity of cyclosome/APC. Whereas UBC4 and UBCx/E2-C are equally active during interphase and mitosis, the cyclosome/APC is at least fivefold more active during mitosis (*King et al.*, 1995; *Sudakin et al.*, 1995). Curiously, purified interphase APC consistently exhibits a low level of cyclin ubiquitination activity, whereas little or no mitotic cyclin ubiquitination occurs in crude interphase extracts (*Peters et al.*, 1996). Suppression of this basal activity to the low levels seen in crude interphase extracts is related to the presence of inhibitors that are removed during purification of APC (J.-M. Peters and M. W. Kirschner, unpublished results). Posttranslational modification of APC during mitosis could render the complex
insensitive to such an inhibitor, enabling the complex to be fully active in mitotic extracts.

Biochemical experiments in clam and *Xenopus* suggest that phosphorylation of the cyclosome/APC plays an important role in its activation during mitosis. At least four subunits of *Xenopus* APC become phosphorylated in mitosis (Peters *et al.*, 1996), and partially purified clam cyclosome and affinity-purified APC are inhibited by incubation with protein phosphatases (Peters *et al.*, 1996; Lahav-Baratz *et al.*, 1995). Although the kinase(s) that phosphorylate APC in mitosis are unknown, purified CDC2-cyclin B kinase, which can activate cyclin degradation when added to crude interphase extracts (Félix *et al.*, 1990), can partially activate interphase (Sudakin *et al.*, 1995) or phosphatase-treated forms of the clam cyclosome (Lahav-Baratz *et al.*, 1995) and interphase *Xenopus* APC (J.-M. Peters, P. T. Stukenberg, and M. W. Kirschner, unpublished data). However, CDC2 kinase is activated early in mitosis, well before B-type cyclins become unstable. *In vitro* experiments indicate that CDC2 kinase activates cyclosome fractions with a lag period that may account for the delayed activation of cyclin B destruction observed *in vivo* (Sudakin *et al.*, 1995). Alternatively, additional kinases that regulate APC activity may become activated later in mitosis. One candidate is protein kinase A, which is activated following CDC2 kinase, and whose activity is essential for cyclin degradation in *Xenopus* extracts (Grieco *et al.*, 1996). Mitotic APC is recognized by the MPM-2 monoclonal antibody (King *et al.*, 1995), which reacts with a subset of mitotic phosphoproteins (Davis *et al.*, 1983), indicating that APC may be phosphorylated by the recently identified MPM-2 epitope kinase PLX-1 (Kumagai and Dunphy, 1996; Kuang and Ashorn, 1993).

Although CDC2-cyclin B kinase is a well-established activator of the cyclin degradation system, the ability of cyclin A to activate the degradation machinery is somewhat controversial. Initial reports demonstrated that cyclin A is incapable of activating the degradation machinery, despite the fact that it could activate histone-H1 kinase activity when added to interphase extracts (Grieco *et al.*, 1996; Lorca *et al.*, 1992b; Luca *et al.*, 1991). However, a recent study demonstrated that cyclin A, when added at higher concentrations sufficient to activate nuclear envelope breakdown, could also initiate both cyclin A and cyclin B degradation (Jones and Smythe, 1996). Whether cyclin A plays a physiological role in activating the destruction system remains to be determined.

Dephosphorylation has also been discussed as a possible mechanism for activating mitotic cyclin proteolysis (Ishii *et al.*, 1996). Protein phosphatase-1 (PP1) activity is essential for the metaphase–anaphase transition in fungi (Hishimoto *et al.*, 1994; Doonan and Morris, 1989; Ohkura *et al.*, 1989), *Drosophila* (Axton *et al.*, 1990), and mammalian cells (Ghosh and Paezetz, 1992). A multicopy suppressor of fission yeast PP1 mutants, *sds22+*, is also required for sister chromatid segregation (Ohkura and Yanagida, 1991), whereas another suppressor, *sds23+*, can also suppress mutations in APC subunits (Ishii *et al.*, 1996). However, it is not known whether PP1 directly regulates APC, or whether mutation of
PPI stabilizes mitotic cyclins and inhibits anaphase indirectly through activation of the spindle assembly checkpoint.

Another potential regulator of cyclin proteolysis is the budding yeast gene *CDC20* (Hartwell et al., 1973). Although Cdc20p activity is not essential for cyclin ubiquitination in extracts of G1-arrested cells (Zachariae and Nasmyth, 1996), *cdc20* mutants arrest prior to anaphase (Sethi et al., 1991). Mutation of a related gene in *Drosophila*, *fizzy*, produces a similar arrest and stabilizes both A- and B-type cyclins (Dawson et al., 1995, 1993; Sigrist et al., 1995). Recent data suggest that *CDC20/fizzy* are substrate specific activators of the APC pathway (see Note Added in Proof).

Genetic studies in budding yeast suggest that additional mechanisms may regulate mitotic cyclin stability. In this organism the proteins Cdc5p, Cdc15p, Cdc14p, Lte1p, Dbf2p/Dbf20p, Ras1p/Ras2p/Rsr1p, Spo12p, and Tem1p are required for exit from mitosis and the complete degradation of mitotic cyclins but not for the initiation of anaphase (Shirayama et al., 1996, 1994a,b; Morishita et al., 1995; Toyn and Johnston et al., 1994, 1993; Kitada et al., 1993; Surana et al., 1993; Johnston et al., 1990). The molecular functions of these proteins can be inferred from their sequences that show similarities with either protein kinases (Cdc5p, Cdc15p, Dbf2p/Dbf20p), protein phosphatases (Cdc14p), GTPases (Ras1p/Ras2p/Rsr1p, Tem1p), or guanine nucleotide release factors (Lte1p), suggesting that these proteins may be components of a signaling pathway that somehow regulates Clb2p stability.

The findings that these proteins are required for Clb2p proteolysis but not for chromosome segregation suggest that the degradation of various APC substrates may be differentially regulated, perhaps at the level of substrate recognition. Further support for this hypothesis comes from studies of the CDK inhibitor Sic1p (Mendenhall, 1993), which, when overexpressed, is sufficient to induce Clb2p proteolysis in *cdc15* mutants or in cells arrested in nocodazole (Amon, 1997). Sic1p is expressed late in mitosis, when B-type cyclins become unstable, and its expression is dependent on Dbf2p and Cdc14p (Donovan et al., 1994). Sic1p may serve as a targeting factor to direct cyclin B for ubiquitination by APC; alternatively, it has been proposed that CDC2-cyclin B activity negatively regulates APC in metaphase, and that Sic1p induces cyclin proteolysis by inactivating the kinase (Amon, 1997). However, if this is a universal mechanism for regulating cyclin stability, it would not explain why the cyclin B degradation system remains activated in *Xenopus* extracts containing nondegradable cyclin B and consequently high levels of CDC2 kinase activity (Glotzer et al., 1991). Furthermore, although cells deleted for *SIC1* show a delay in exiting mitosis, they are nevertheless viable (Donovan et al., 1994), suggesting that Sic1p-independent mechanisms for turning on cyclin proteolysis must exist.

Following exit from mitosis, the cyclin degradation machinery must be inactivated to allow the accumulation of new cyclins for the next mitosis. In the budding yeast cell cycle, the mitotic cyclin degradation system remains active
through early G1 until G1 cyclin/Cdc28p kinase activity appears (Amon et al., 1994). This suggests a simple model for maintaining the orderly progression of cell cycle phases in yeast: Mitotic cyclins cannot accumulate and thus mitosis cannot occur until the G1-Cdc28p kinase switches off the mitotic cyclin degradation system. It has been observed that the ability of G1 kinases to inhibit mitotic cyclin degradation remains reversible until the CDC4-dependent step (Schwob et al., 1996; Amon et al., 1994), suggesting that inactivation of APC may occur in two distinct steps. Recent experiments with extracts prepared from synchronized mammalian cells indicate that mitotic cyclins are also degraded during the G1 phase of higher eukaryotic somatic cells (Brandeis and Hunt, 1996). It will be interesting to determine whether CDKs that are active at the end of G1 are required for the inactivation of the cyclin degradation in metazoans. Experiments in Drosophila suggest that this may be the case because expression of cyclin E is necessary for the accumulation of mitotic cyclins (Knoblich et al., 1994). Although G1-cyclin-dependent kinases may play a universal role in extinguishing cyclin proteolysis, the mechanisms through which these kinases inactivate cyclin degradation remain obscure.

3.4.2. Additional Mechanisms that Regulate APC-Dependent Ubiquitination

The spindle assembly checkpoint is a control mechanism that delays anaphase until every chromosome has become properly attached to the spindle, thus ensuring accurate segregation of sister chromatids (for a review, see Rudner and Murray, 1996). This mechanism is also activated by microtubule-depolymerizing or stabilizing agents such as nocodazole and taxol. Elegant micromanipulation experiments in grasshopper spermatocytes suggest that unequal tension across the meiotic spindle can generate an anaphase-inhibiting signal (Li and Nicklas, 1995). This signal may originate from the kinetochore of misattached sister chromatids where phosphopeptidases have been observed that disappear as soon as chromosomes become properly linked to the spindle (Nicklas et al., 1995). Genetic studies in budding yeast have identified several proteins that are required for the spindle assembly checkpoint (Hoyt et al., 1991; Li and Murray, 1991). Reconstitution of spindle assembly checkpoint conditions in Xenopus extracts has shown that the checkpoint-induced inhibition of mitotic cyclin proteolysis depends on the activation of MAP kinases (Minshull et al., 1994). The next crucial step is to learn how the components of the spindle assembly checkpoint stabilize substrates of the APC-dependent ubiquitination system, such as Cut2, Pds1p, and the mitotic cyclins. Whether stabilization is achieved through inhibition of APC or through protection of its substrates is unknown.

Anaphase is also inhibited physiologically during the meiotic maturation of vertebrate eggs (for a review, see Sagata, 1996). Cell cycle arrest occurs at
metaphase of meiosis II, and is overcome by the calcium influx into the ooplasm induced by fertilization. Metaphase-arrested eggs contain high levels of CDC2 kinase activity and mitotic cyclins, but do not enter anaphase because of the activity of cytostatic factor (CSF), a component of which is the protein kinase c-mos (Sagata et al., 1989). CSF must somehow inhibit APC-dependent proteolysis. The CSF-induced arrest may depend on mechanisms similar to those activated by the spindle assembly checkpoint because activated MAP kinase is required in both systems (reviewed in Sagata, 1996). Interestingly, APC is active when purified from metaphase-II arrested extracts (R. W. King, J.-M. Peters, and M. W. Kirschner, unpublished data), suggesting that additional meiotic factors either block active APC, or protect its substrates from ubiquitination.

4. PERSPECTIVES

The recent dissection of the CDC34 and APC pathways has changed our understanding of how cell cycle progression is controlled. Although protein phosphorylation was initially thought to be the major mechanism by which cell division was regulated, it is now clear that proteolysis is an integral part of the cell cycle control machinery (King et al., 1996a, 1994; Nasmyth, 1996a,b; Deshaies, 1995a).

Although the existence of the CDC34 and the APC pathways and their physiological importance is becoming widely recognized, many questions remain unanswered. For example, our present inventory of known CDC34 pathway components may not be complete, and our understanding of how this pathway functions in higher eukaryotes is rudimentary. There may also be yet unidentified components in the APC pathway. Even though the minimal set of proteins that is required for the reconstitution of APC-dependent ubiquitination reactions is known, little is known about the factors that turn APC on and off during the cell cycle. In both pathways, the mechanisms of substrate recognition and ubiquitination remain poorly understood and may be different from the mechanisms proposed for other ubiquitination reactions, such as the E6-AP-dependent ubiquitination of p53.

For about a decade mitotic cyclins were the only cell cycle regulators that were known to be controlled by proteolysis. Within only a few years this has changed and we now know of several CDK inhibitors, kinases, anaphase inhibitors, and MAPs that are subject to cell cycle-regulated proteolytic control. Presently there is no reason to believe that the list of APC and Cdc34p substrates is near completion. Thus, an important goal for the future is to identify new physiological substrates for these pathways, and to assess how their degradation contributes to cell cycle progression.
Note Added in Proof

Since this article was written a number of discoveries have been made in the field of cell cycle-regulated proteolysis. We apologize to those authors whose recent papers we cannot mention in this added note due to space constraints.

Two recent papers (Feldman et al., 1997; Skowyra et al., 1997) have established that Cdc53p, Cdc4p, and Skp1p assemble into a ubiquitin ligase complex (dubbed SCF^{Cdc4} for Skp1p-Cdc53p or Cullin-F box receptor subunit) that interacts with Cdc34p to catalyze ubiquitination of phosphorylated Sic1p. Phospho-Sic1p binds selectively to Cdc4p/Skp1p, which in turn bind to Cdc53p and Cdc34p. Intriguingly, the Cdc4p subunit of SCF^{Cdc4} can be replaced by the F-box protein Grrlp. SCF^{Grr1} complexes bind tightly to phospho-Cln2p, but not phospho-Sic1p, suggesting that the substrate specificity of SCF complexes is dictated by the identity of the F-box subunit. Similar ubiquitin ligase complexes appear to be present in human cells, since human SKP1, CUL1, and SKP2 proteins assemble into complexes in vivo and in vitro, and human CUL1 associates with ubiquitin-conjugating activity in HeLa cell extracts (Lisztwan et al., 1998, EMBO J. 17:368–383; Lyapina et al., submitted).

The cloning of the human APC subunits APC2, APC4, APC5, APC7 and APC8 (CDC23) has been completed (Yu et al., 1998, Science, in press). Homologs of APC2, APC4 and APC5 and three additional subunits of low molecular mass (Apc9p, Apc10p/Doc1p, Apc11p) have been identified in budding yeast (Zachariae et al., 1998, Science, in press; Kramer et al., 1998, EMBO J. 17:498–450; Hwang and Murray, 1997, Mol. Biol. Cell 8:1877–1887).

Genetic experiments in budding yeast and flies have recently identified two related WD40 repeat proteins as essential components of the APC pathway. In yeast Cdc20p is required for the degradation of Pds1p and Clb5p, whereas Hct1p/Cdh1p is essential for Clb2p and Aselp proteolysis (Schwab et al., 1997, Cell 90:683–693; Visintin et al., Science 278:460–463, 1997). In flies fizzy and fizzy-related may perform similar functions (Sigrist and Lehner, 1997, Cell 90:671–681). Overexpression studies indicate that these WD40 proteins may be substrate specific activators of APC.

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