End of the line: proteolytic degradation of cyclin-dependent kinase inhibitors

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Cyclin-dependent kinase inhibitors (CKIs) are crucial regulators of cell-cycle progression. The CKI Sic1 controls the timing of DNA replication by inhibiting Clb–Cdc28 kinase. Phosphorylation of Sic1 by Cln–Cdc28 kinase alleviates this inhibition by targeting Sic1 for degradation through the ubiquitin-mediated proteolytic pathway.

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Cell proliferation requires duplication of all cellular components, followed by their segregation into identical daughter cells. The mechanisms controlling this process are described by the cell-cycle paradigm, in which the two major events of proliferation — DNA replication and chromosome segregation/cytokinesis (cellular division) — are temporally ordered by gap phases that separate them [1]. Because loss of cell-cycle control, leading to unregulated proliferation, is a hallmark of tumorigenesis, much work in the last 20 years has focused on identifying the molecular components that facilitate or inhibit cell-cycle transitions.

The ultimate targets of proliferative signals are the cyclin-dependent kinases (CDKs), which catalyze positive transitions through the cell cycle by transferring the gamma phosphate from ATP to protein substrates [1]. The simplicity of this reaction is in contrast to the complexity of the regulatory systems through which it is controlled. As the name implies, CDKs are positively activated by their association with a member of the cyclin family of proteins, and by other posttranslational modifications. The temporal order of cell-cycle progression is maintained in part by the sequential expression and activation of specific cyclin–CDK complexes at each transitional period.

Recent attention has focused on a novel mechanism for negatively regulating cell-cycle transitions by inhibiting the activity of these CDKs [2]. The CDK inhibitors, or CKIs, are ubiquitous proteins found in cells from diverse species (from yeast to humans); they function by directly binding to and inactivating the cyclin–CDK complex (Fig. 1). Given the variety of cyclin–CDK complexes, it is not surprising that different classes of CKIs are used to regulate their activity. They are thought to have critical roles in such diverse processes as withdrawal from the cell cycle, maintenance of cell-cycle timing, checkpoint controls (mechanisms preventing inappropriate cell-cycle progression during processes such as DNA replication or repair), and differentiation [2].

The cell uses three basic mechanisms to establish and maintain the exquisitely complex interactions amongst the proteins comprising the cell-cycle machinery: modulation of protein synthesis rates, biological activity, or half life. Although the control of protein expression and activity has long been recognized as integral to control of cell-cycle progression, the significance of selective proteolysis has only recently been elevated to equal status. The ubiquitin-mediated degradation of proteins provides the targeting specificity and irreversibility necessary in a temporally ordered regulatory system such as the cell cycle. Here, we summarize the emerging evidence indicating a critical role for ubiquitin-mediated proteolysis of CKIs in cell-cycle regulation.

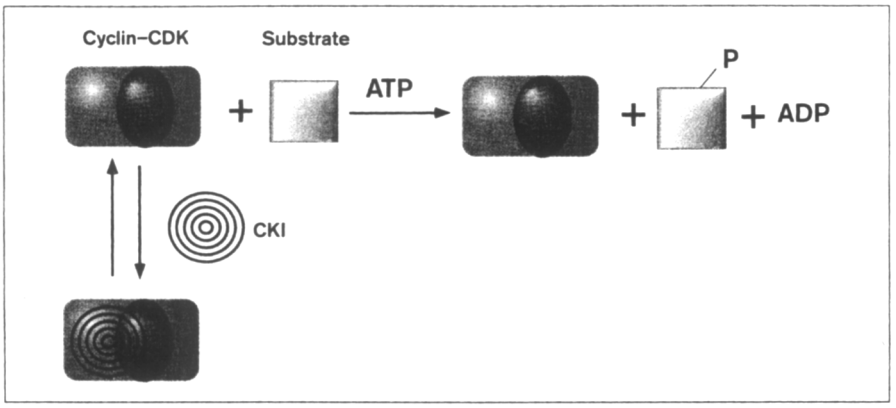
Sic1 degradation is required for S-phase entry in yeast

The clearest example of ubiquitin-mediated destruction of a CKI comes from the yeast *Saccharomyces cerevisiae*. The fact that these yeast double every 90 minutes under optimal conditions, combined with the ease of genetic analysis, makes them an attractive organism in which to study the cell cycle. The CDK homolog in yeast is Cdc28, which is positively activated by association with the Cln or Clb family of cyclins.

The sequence of events required for progression from G1 through S phase is depicted in Figure 2. Midway through G1, the cell evaluates its capacity to complete replication of chromosomal DNA and cellular division. A commitment to proliferation, called Start, is dependent upon activation of the cyclin-dependent kinase Cdc28 by association with the Cln (G1 type) cyclins [3]. Progression through Start simultaneously initiates parallel pathways culminating in DNA replication, duplication of the spindle pole body (the yeast microtubule-organizing center), and bud emergence. Cln3–Cdc28 launches this program by activating transcription of Clns 1 and 2 and Clbs 5 and 6 in G1 [4]. Although both Clns and Clbs associate with Cdc28 after synthesis, only the Cln–Cdc28 complexes are immediately activated. The Clb–Cdc28 complexes are maintained in an inactive state through association with Sic1, whereas Cln–Cdc28 kinase activity is unaffected by this inhibitor [5].

Deletion of the genes for Clns 1–3 causes cell-cycle arrest in G1 without bud emergence, spindle-pole duplication,

Figure 1



Inhibition of cyclin–CDK kinase activity by CKIs. The phosphorylation of a protein substrate by a cyclin–CDK complex is shown. CKIs inhibit this reaction by binding to the enzyme.

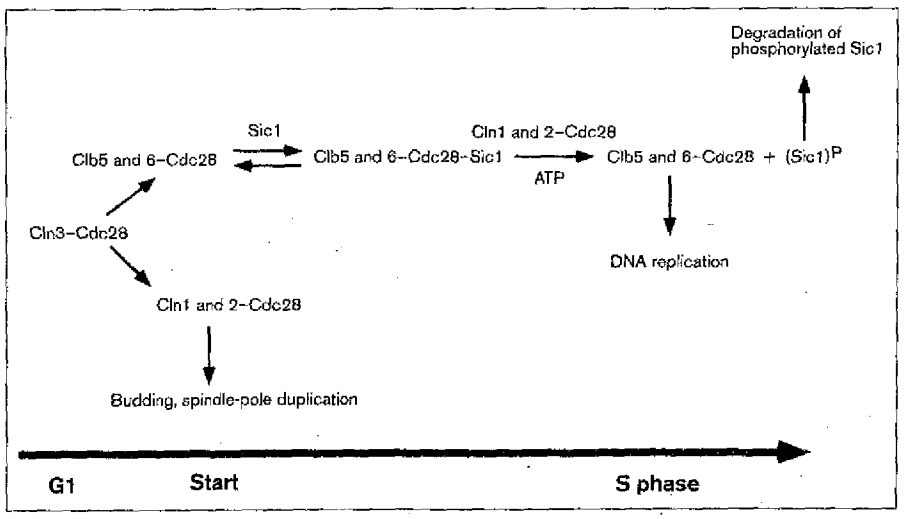
or DNA replication [6]. In contrast, overexpression of Clns 1–3 results in G1 acceleration, indicating that they are rate limiting for progression through Start [7]. Clns 1 and 2 are not sufficient for initiating DNA replication on schedule, however, as deletion of Clbs 5 and 6 delays S-phase entry by 30 minutes [8]. Nevertheless, bud emergence and spindle-pole duplication are still initiated on time, indicating that Clb5 and 6 are dispensable for these mitotic events. The dependence of S-phase entry on Clb5 and 6–Cdc28 kinase activity requires that Sic1 be inactivated. Thus, Sic1 coordinates the timing of S-phase initiation with other cell-cycle events by preventing the pre-mature activation of the Clb5 and 6–Cdc28 kinases [9].

It has been known for some time from genetic experiments that exit from G1 into S phase also requires the gene products of *CDC34*, *CDC4*, and *CDC53* [10]. Mutants in *CDC34* duplicate spindle-pole bodies and bud repeatedly, but fail to enter S phase, similar to the *clb5* and *clb6* double-deletion mutants [9]. Despite high levels of the G1 Cln–Cdc28 kinase activity in these cells, the cells fail to progress through G1. The failure to enter S phase is a consequence of the dearth of Clb5 and 6–Cdc28 kinase activity in the *cdc34* mutants, coincident with an accumulation of Sic1. The discovery that *CDC34* encodes an E2 enzyme of the ubiquitin conjugating system immediately suggested a mechanism in which degradation of Sic1 restores the Clb–Cdc28 kinase activity required for initiating DNA replication [9].

Targeting Sic1 for degradation

Cdc34 substrates share two features: they are phosphorylated, and they contain internal PEST sequences (i.e., proline, glutamic acid, serine, and threonine) [11]. Phosphorylation is a common means of marking a protein for ubiquitination, and probably facilitates recognition by ubiquitin-conjugating enzymes. Although the exact function of the PEST sequence is unclear, it is a common

Figure 2

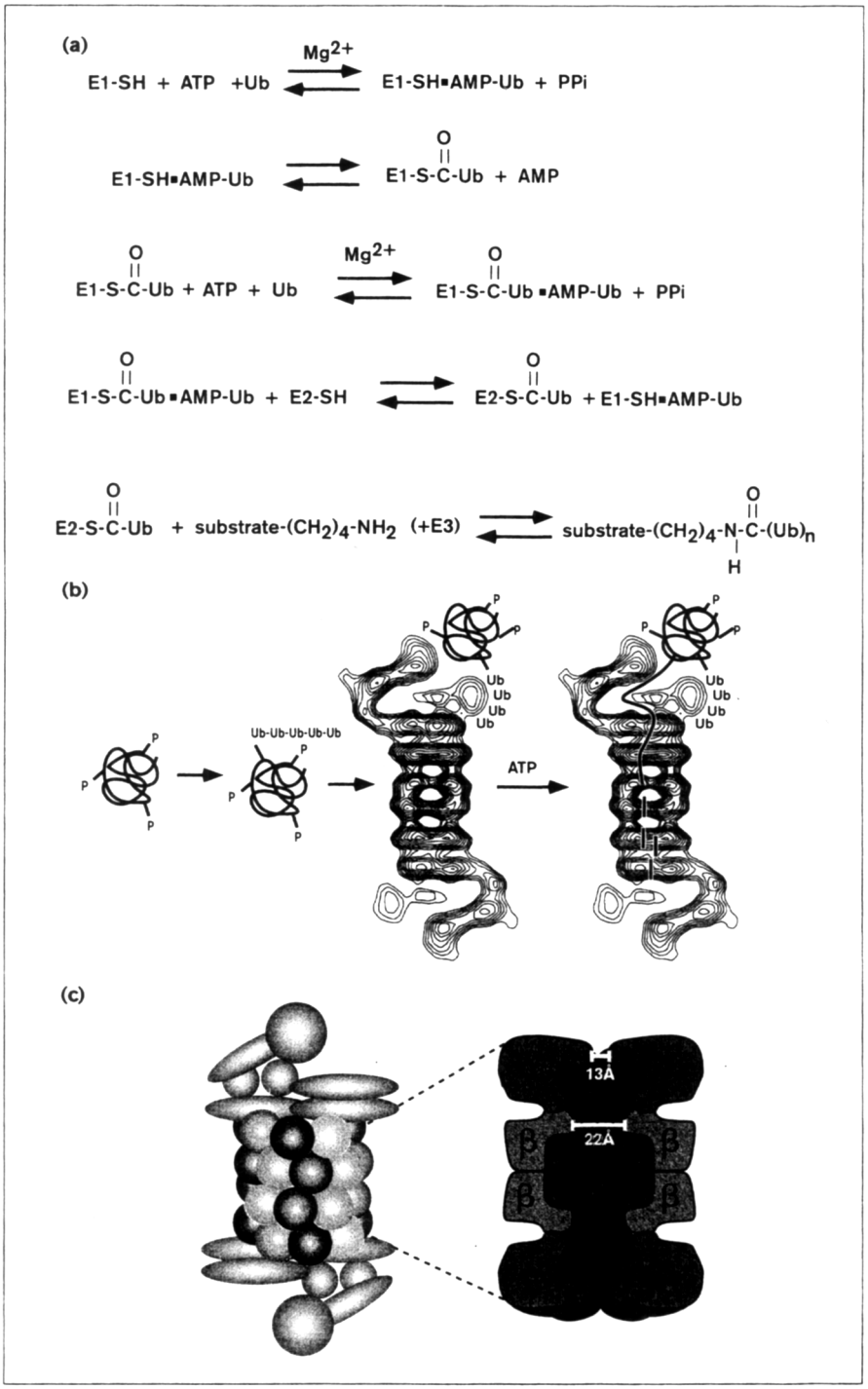


G1/S-phase progression in yeast. Cln3–Cdc28 kinase activity in G1 phase activates transcription of the genes encoding Clns1 and 2 and Clbs 5 and 6. While Cln–Cdc28 complexes are immediately activated, Clb–Cdc28 kinase activity is inhibited by Sic1. Cln–Cdc28 phosphorylates Sic1, leading to its degradation and subsequent activation of Clb–Cdc28 kinase, which is required for entry into S phase.

motif found in unstable proteins and is required for maximal rates of degradation. It may direct ubiquitination at another site in the protein, facilitate Cdc34 recognition, or perhaps prevent de-ubiqutination.

Although purified Sic1 specifically inhibits Clb–Cdc28 kinase activity by direct interaction, Cln–Cdc28 kinases are refractory to Sic1 inhibition. In fact, Sic1 is a substrate for Cln–Cdc28 [12]. Sic1 contains a PEST sequence, is highly phosphorylated in the cell, and its phosphorylation state correlates with the appearance of Cln–Cdc28 kinase activity [12]. Cln–Cdc28 phosphorylates Sic1, targeting it for degradation through the ubiquitin pathway and alleviating Clb–Cdc28 inhibition (Fig. 2).

Figure 3



The ubiquitin-mediated proteolytic pathway. **(a)** The enzymatic reactions of the ubiquitin pathway. Covalent bonds are depicted by dashes, noncovalent binding by squares. Action of the E1, E2 and E3 enzymes results in attachment of multiple ubiquitin groups to lysine residues in the target protein. **(b)** The substrate targeted for degradation is anchored to the proteasome by its ubiquitin chain. The substrate is then simultaneously unwound and threaded into the catalytic center of the proteasome for proteolytic degradation. **(c)** Schematic representation of the 26S proteasome (left) and cross section (right) of the central 20S portion of the particle. The 20S proteasome is composed of two central β-subunit rings, with two outer α-subunit rings, each with seven members. The space between the α-subunit and β-subunit rings defines the small outer chambers, while the space between the two β-subunit rings defines the large central chamber. Proteolysis takes place within the large central chamber. The target protein must enter the chamber through a 13 Å pore in the center of the α-subunit ring.

Genetic experiments lend support to this model. Deletion of the gene encoding Sic1, while not lethal, results in premature DNA replication and genetic instability (broken or lost chromosomes) [13]. Furthermore, deleting Sic1 suppresses the phenotype of the *cdc34* mutation, indicating that Sic1 is the only essential target of this enzyme that must be degraded to transverse the G1/S phase boundary [9]. It should be noted that the *cdc34|sic1* double mutant still experiences growth slowdown, since Cdc34 targets other substrates for degradation. Finally, the *sic1* deletion rescues the cln triple deletion, suggesting that phosphorylation of Sic1 is an essential function of Cln–Cdc28 kinases [12,14]. There are obviously other important Cln substrates, however; although the *sic1|cln* mutant cells progress through G1 and enter S phase, they are very sick.

Mechanism of ubiquitin-mediated degradation

The first event in targeting a protein for destruction is the conjugation of ubiquitin to the target protein by a cascade of enzymatic reactions (Fig. 3a) [15]. Ubiquitin is an abundant 76 amino acid protein, which is highly conserved from yeast to humans. The ubiquitin-activating enzyme E1 first binds to ubiquitin and ATP; magnesium-dependent hydrolysis of ATP at the α-phosphate results in the formation of ubiquitin adenylate (Ub-AMP) tightly bound to E1. The ubiquitin adenylate intermediate acts as a donor of ubiquitin to an active-site sulfhydryl group of E1 in a magnesium-independent fashion through a high-energy thioester bond. Interestingly, the E1-ubiquitin thioester undergoes another round of magnesium-dependent Ub-AMP formation, resulting in the ternary complex of E1-ubiquitin noncovalently bound to Ub-AMP.

The activated ubiquitin in the ternary complex is tranfered by transacylation to a thiol group of the E2 family of ubiquitin-carrier proteins. E2-ubiquitin thiol esters can donate the ubiquitin directly to the substrate by isopeptide bond formation with the ε amino groups of substrate lysine residues. Alternatively, transfer of ubiquitin from E2 may be assisted by an E3 family member protein, which functions as a ubiquitin ligase (Fig. 3a). Additional ubiquitins are added to the first by the same mechanisms, forming one or more chains of ubiquitin, which are thought to serve as signals for transportation to the proteasome. Most cells have only a single E1, whereas there are at least 12 genes encoding E2 enzymes in yeast [15]. Thus, the targeting specificity associated with the ubiquitin pathway arises through the E2 and E3 enzymes.

Upon arrival at the 26S proteasome, the ubiquitin chains of the substrate are used for docking (Fig. 3b) [16]. The substrate is then unfolded in an ATP-dependent manner, followed by cleavage and recycling of the ubiquitin chain. The central, 20S portion of the proteasome is a multiple subunit, barrel-shaped particle, with four stacked rings of 7 polypeptides each (Fig. 3c). The crystal structure shows a central channel with three chambers — two small 13 Å openings at the ends of the barrel, and a larger chamber in the middle composed of β subunits [17]. The narrow openings are only wide enough to permit passage of unfolded proteins to the two proteolytic active sites sequestered in the middle chamber, ensuring that only ubiquitinated proteins are degraded.

A unique mechanism of catalysis uses the hydroxyl group of the amino-terminal threonine of the β subunit as the nucleophile. The crystal structure of the proteasome in a complex with an inhibitor suggests that the transition state is similar to that of the serine proteases, although the familiar catalytic triad is lacking. The α amino group of the amino-terminal threonine is thought to serve as the basic amino acid, which accepts the proton from the threonine hydroxyl in the transition state. Spatial orientation of the two active sites is such that they are spanned by a polypeptide chain of 7–8 amino acids, the fragment size generated by proteolysis [17].

Proteolytic control of the G1/S phase transition – a twice told tale?

Although our understanding of CKI regulation by proteolytic degradation is still limited, the available evidence provides important insights into the control of cell-cycle progression. In yeast, the Cln1 and 2–Cdc28 kinase phosphorylates Sic1, targeting it for degradation. The dependence of Clb–Cdc28 activation on Cln–Cdc28 activity nicely illustrates the temporal nature of cell-cycle progression achieved through a system of checks and balances.

It should be pointed out, however, that involvement of the ubiquitin-mediated pathway in Sic1 regulation has not yet been directly demonstrated. Changes in the Sic1 half-life at S phase have not been determined, and ubiquitinated Sic1 intermediates have not been observed. Even less is known about proteolytic regulation of CKIs in mammalian cells, although ubiquitinated intermediates have been isolated [18]. Nevertheless, the realization that CKIs, at least in yeast, are both inhibitors and substrates of cyclin–CDK complexes is particularly intriguing. This duality has currently attracted little attention from researchers in the mammalian cell-cycle field, but if past examples from yeast are any indication, it should be seriously considered.

Acknowledgements

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