

Context of Multiubiquitin Chain Attachment Influences the Rate of Sic1 Degradation

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Summary

The ubiquitin-dependent targeting of proteins to the proteasome is an essential mechanism for regulating eukaryotic protein stability. Here we define the minimal signal for the degradation of the S phase CDK inhibitor Sic1. Of 20 lysines scattered throughout Sic1, 6 N-terminal lysines serve as major ubiquitination sites. Sic1 lacking these lysines (K0N) is stable in vivo, but readdition of any one restores turnover. Nevertheless, ubiquitin chains attached at different N-terminal lysines specify degradation in vitro at markedly different rates. Moreover, although K0N can be ubiquitinated by SCF^{Cdc4}/Cdc34 in vitro in the absence (but not in the presence) of S-CDK, it is degraded slowly. Our results reveal that a single multiubiquitin chain can sustain a physiological turnover rate, but that chain position plays an unexpectedly significant role in the rate of proteasomal proteolysis.

Introduction

The ubiquitin-proteasome pathway has emerged as the central mechanism for regulating protein stability in eukaryotic cells (Hershko and Ciechanover, 1998). Through a series of steps, ubiquitin is shuttled from a ubiquitin-activating enzyme (E1) to a ubiquitin-conjugating enzyme (E2) and then covalently attached onto a lysine of a targeted protein, usually in conjunction with a ubiquitin ligase (E3). The assembly of a lysine 48 linked tetraubiquitin chain upon the substrate results in the protein being recognized and degraded by the 26S proteasome (Thrower et al., 2000).

E3s confer substrate specificity and play a direct role in the transfer of ubiquitin from E2 to substrate. Two major classes of E3s have been identified based on the presence of either a HECT (homologous to E6-AP carboxyl terminus) domain or a RING motif (Pickart, 2001). HECT domain E3s form an obligate thiolester intermediate with ubiquitin. In contrast, RING-based (and the related U box or PHD domain) E3s appear to promote direct transfer of ubiquitin from E2 to substrate.

RING domain proteins comprise the largest group of E3s. In general, these contain a zinc binding RING motif that recruits E2 and a substrate binding site. While single subunit E3s such as Cbl directly contact both substrate and E2, multiprotein E3s such as CBC^{VHL} (Cul2-elongin BC-VHL), APC (anaphase-promoting complex), and SCF (Skp1, cullin, and F box) contain a conserved RING sub-

unit that interacts with E2 and a variable substrate recognition subunit (Tyers and Jorgensen, 2000).

In the instance of SCF, the RING protein Hrt1 (also known as Rbx1 and Roc1) binds directly to E2, while the F box protein confers substrate specificity (Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999). Given the large array of F box proteins, many distinct SCF ligases can assemble to target numerous substrates for ubiquitination (Bai et al., 1996; Patton et al., 1998). Indeed, SCF complexes have been implicated in diverse processes including the cell cycle, inflammation, signal transduction, and transcription (Deshaies, 1999).

In budding yeast, the S-CDK inhibitor Sic1 is phosphorylated during G1 of the cell cycle by G1-CDK (Schneider et al., 1996; Verma et al., 1997a). This results in its association with the F box protein Cdc4 of SCF^{Cdc4}, which promotes its ubiquitination by the E2 Cdc34 (Feldman et al., 1997; Skowyra et al., 1997). Multiubiquitinated Sic1 is degraded by the 26S proteasome, switching on S-CDK activity and allowing cell cycle progression (Verma et al., 2001). Recent work has determined that multiple phosphorylations of Sic1 by G1-CDK are required for its association with SCF^{Cdc4} (Nash et al., 2001). By establishing a minimum threshold for G1-CDK activity, multisite phosphorylation-induced turnover of Sic1 may promote genomic stability by guarding against precocious S phase entry (Lengronne and Schwob, 2002).

Because of the extensive information that exists on its turnover, Sic1 has become an important model for understanding the mechanism and regulation of ubiquitin-dependent proteolysis. Nevertheless, many questions remain regarding the degradation of Sic1 and other 26S proteasome substrates. For example, (1) is a single multiubiquitin chain sufficient to sustain a physiological rate of degradation? (2) Does the position of the multiubiquitin chain within a substrate influence degradation? (3) What effect do tightly bound partners have on a substrate's ubiquitination and degradation? The development of an in vitro system that recapitulates both the ubiquitination and degradation of Sic1 with purified components allows these and other questions to be addressed with a physiological substrate (Verma et al., 2001).

Results

Lysines Are Required for Sic1 Turnover

Previous studies determined that Sic1 turnover requires multiple phosphorylations (Nash et al., 2001; Verma et al., 1997a), but it is unclear what other features of Sic1 may be important for its destabilization. As several reports have suggested that proteins may be targeted to the proteasome either without ubiquitination (Verma and Deshaies, 2000) or by N-terminal ubiquitination (Ciechanover et al., 1999), we sought to determine if lysines are indeed required for Sic1 turnover.

We mutated all 20 lysines to arginine of Sic1 fused to green fluorescent protein (K0-GFP) and assessed the

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behavior of this protein in vivo. Similar to Sic1- Δ 3P which contains mutations in four CDK sites (Verma et al., 1997a), constitutive expression of K0-GFP was toxic (Figure 1A) and induced an elongated bud morphology characteristic of G1 arrest (Figure 1C).

To measure the stability of these proteins, we transiently induced their expression in cells arrested in G1. After release from G1 arrest and promoter shutoff, samples were taken and analyzed. Whereas WT-GFP showed a major decrease after 40 min, K0-GFP and GFP were detectable up to 180 min after release (Figure 1B).

In cells expressing WT-GFP or GFP, endogenous Sic1 disappeared at 20 min and reaccumulated by 120 min after release. K0-GFP expression delayed Sic1 turnover up to 120 min. In contrast, expression of Sic1- Δ 3P blocks cell cycle progression but does not prevent Sic1 turnover (Verma et al., 1997a).

Sic1 is both nuclear and cytoplasmic, while Cdc4 is nuclear, suggesting that Sic1 is shuttled to the nucleus prior to the onset of S phase of the cell cycle (Henchoz et al., 1997). Therefore, we evaluated the localization of K0-GFP to determine if its stability was a result of nuclear exclusion. Whereas GFP was diffusely localized throughout the cell, WT-GFP and K0-GFP were both moderately concentrated in the nucleus (Figure 1C).

K0-Sic1 Inhibits S-CDK Activity and Binds to SCF^{Cdc4}, but Cannot Be Ubiquitinated

We sought to determine if lysine-less Sic1 mimics the known properties of Sic1. Sic1 inhibits S-CDK activity through its stoichiometric association (Nugroho and Mendenhall, 1994; Schwob et al., 1994). Similarly, K0-Sic1 bound to (Figure 2B) and inhibited the histone H1 kinase activity of (Figure 2C) S-CDK. Competition binding experiments revealed that Sic1 and K0-Sic1 bound S-CDK with similar affinities (shown in Supplemental Figure S1 at <http://www.molecule.org/cgi/content/full/11/6/1435/DC1>).

Sic1 is phosphorylated in late G1 by G1-CDK. Once phosphorylated, Sic1 binds to SCF^{Cdc4}, which catalyzes its ubiquitination (Feldman et al., 1997; Skowrya et al., 1997). Incubation of K0-Sic1 with G1-CDK resulted in an ATP-dependent phosphorylation shift (Figure 2D), and this substrate bound SCF^{Cdc4} (Figure 2E) with an affinity similar to WT (Supplemental Figure S2 on *Molecular Cell* website). In spite of this, K0-Sic1 was not ubiquitinated (Figure 2F).

Lysine Residues Near CDK Sites Are Necessary for Sic1 Turnover

Previous work defined CDK sites that when mutated increased Sic1 stability and delayed cell cycle progression (Nash et al., 2001). Of the nine sites, individual mutation of five near the N terminus confers a 3- to 6-fold increase in Sic1 stability. These five CDK sites are juxtaposed to six lysines, clustered in three pairs (Figure 3A). To test if these lysines are important for Sic1 instability, we generated yeast strains that expressed Sic1-GFP fusions: K0C-GFP contained only the 6 N-terminal lysines and K0N-GFP contained only the remaining 14 (Figure 3A). While K0C-GFP turnover was similar to that of WT-GFP, K0N-GFP was stable up to 180 min after release (Figure 3B). Both K0N-GFP and K0C-GFP dis-

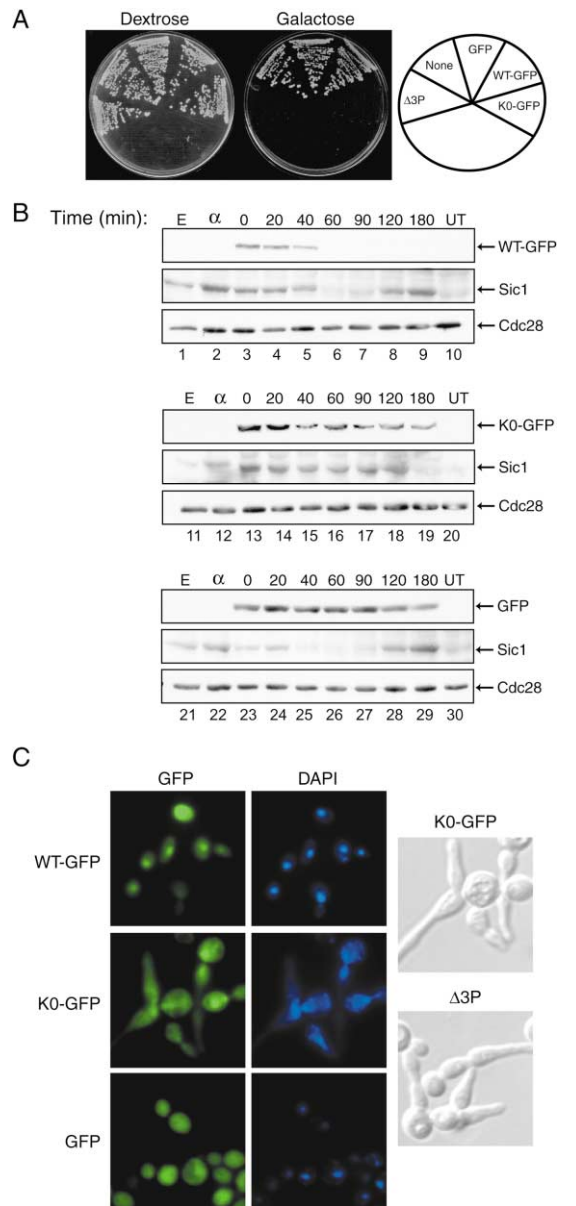


Figure 1. Lysine Residues Are Required for Sic1 Turnover
(A) Constitutive expression of K0-Sic1-GFP is toxic. Δ 3P (RJD1026), none (RJD730), GFP (RJD2456), WT-GFP (RJD2457), K0-GFP (RJD2458) cells were grown on YP dextrose (left) or YP galactose (right) at 30°C for 3 days.
(B) K0-Sic1-GFP is stable in vivo. Yeast strains were arrested in G1 for 2.5 hr, induced with galactose for 2 hr, and washed into glucose-containing media. Samples were prepared and analyzed by Western blotting with antisera against GFP, HA (to detect Sic1-HA expressed from the endogenous *SIC1* locus), and Cdc28 (to confirm equivalent loading). WT-GFP: wild-type Sic1 fused to GFP; K0-GFP: K0-Sic1 fused to GFP; GFP: unfused GFP; Sic1: Sic1^{HA} expressed from the endogenous *SIC1* locus. E: exponentially growing cells, α : α factor-arrested cells, UT: untagged Sic1 control strain.
(C) K0-Sic1-GFP localizes to the nucleus and induces cell morphology characteristic of late G1 phase arrest. Asynchronously growing yeast strains were induced with galactose for 2 hr. Cells were fixed, stained with DAPI for DNA, and mounted onto polylysine-treated slides for microscopy.

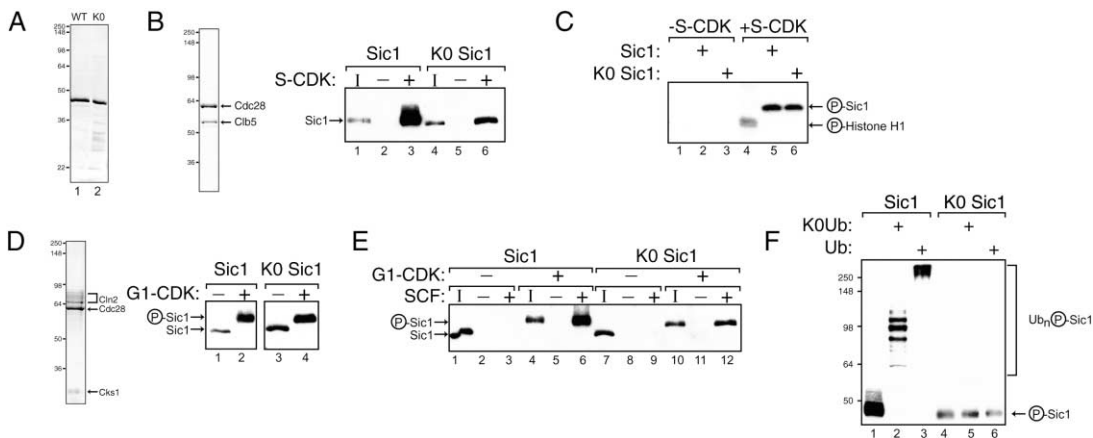


Figure 2. Phosphorylated K0-Sic1 Binds SCF^{Cdc4} but Is Not Ubiquitinated by Cdc34

(A) One microgram of Sic1 (lane 1) and K0-Sic1 (lane 2) purified from *E. coli* was analyzed by SDS-PAGE and Coomassie staining. (B) K0-Sic1 binds S-CDK. Recombinant S-CDK (Coomassie-stained gel, left; approximately 1 μ g) was immobilized on glutathione sepharose and incubated with purified Sic1 or K0-Sic1. After washing, the precipitates were fractionated by SDS-PAGE and analyzed by Western blotting for the T7 tag on Sic1 (right). Inputs (I) represent 10% of the total Sic1 added and 25% of bound material was loaded. (C) K0-Sic1 inhibits S-CDK kinase activity. Glutathione sepharose bound S-CDK complexes were mixed with either Sic1 or K0-Sic1 in the presence of Histone H1 and [γ -³²P]-ATP. After 30 min at room temperature, the reactions were analyzed by SDS-PAGE and autoradiography. (D) K0-Sic1 is phosphorylated by G1-CDK. Recombinant G1-CDK purified on anti-Myc resin (Coomassie-stained gel, left) was incubated with Sic1 or K0-Sic1 in the presence of ATP. After 1 hr at room temperature, the reactions were evaluated as described in (B). (E) Phosphorylated K0-Sic1 binds recombinant SCF^{Cdc4}. Phosphorylated and unphosphorylated Sic1 and K0-Sic1 premixed with S-CDK were incubated with anti-Py resin bound SCF^{Cdc4} complexes purified from baculovirus-infected insect cells. After washing, bead bound material was analyzed as described in (B). (F) K0-Sic1 is not ubiquitinated by SCF^{Cdc4}. Phosphorylated Sic1 or K0-Sic1 was added to ubiquitination reactions containing either K0 ubiquitin or ubiquitin in the presence of S-CDK. Reactions were incubated for 20 min and evaluated as described in (B).

played nuclear partitioning equivalent to WT-GFP in cells arrested in G1 (Figure 3C, left). Similar to that of K0-GFP, KON-GFP expression resulted in stabilization of endogenous Sic1 (data not shown) and an elongated bud phenotype (Figure 3C, right).

Sic1 Containing a Single Lysine within the N-Terminal 90 Amino Acids Is Efficiently Ubiquitinated and Degraded

To further define the minimal degradation signal of Sic1, four of the six N-terminal lysines (K32, K36, K49, and K84) were individually added back to K0-Sic1 (Figure 4A). These proteins were purified, phosphorylated with G1-CDK, mixed with purified S-CDK (Figure 4B), and utilized in ubiquitination assays with either K0 ubiquitin (K0 Ub) or ubiquitin (Ub) (Figure 4C). K0 Ub does not support multiubiquitination and thus allowed us to estimate the number of ubiquitin-accepting lysines in Sic1 (Hofmann and Pickart, 1999). By employing equal amounts of Ub and K0 Ub, it was possible to resolve a ladder of Sic1-Ub adducts from 1 to 10 attachments (an example is shown in Supplemental Figure S3 on *Molecular Cell* website), which we used as a calibration marker for these experiments.

Only a single attachment of K0 Ub was observed for all single lysine substrates. In contrast, Sic1 containing the normal complement of lysines had a molecular weight shift of greater than 50 kDa, indicative of 6 to 7 K0 Ub attachments (Figure 4, and below). Conjugation of wild-type Ub onto Sic1 consistently resulted in a molecular weight shift of greater than 200 kDa, whereas the mass of ubiquitinated single lysine Sic1 was consistently

bracketed between 90 kDa up to greater than 150 kDa, consistent with attachment of 6 to 20 Ub molecules. Given the rigidity of SCF's structure (Zheng et al., 2002), it will be interesting to deduce how it sustains the assembly of ubiquitin chains of such great length.

To determine if individual N-terminal lysines can sustain turnover of Sic1 *in vivo*, we restored lysine residues in the context of KON-GFP. Whereas slight differences in the turnover rates of the various Sic1-GFP fusion proteins were observed (Figure 4D), all were efficiently destabilized in comparison to KON-GFP.

Sic1 Substrates Containing a Single Multiubiquitin Chain Are Degraded at Different Rates by the 26S Proteasome

Because individual N-terminal lysines were ubiquitinated efficiently *in vitro*, we next sought to determine if they supported equivalent rates of Sic1 degradation by the 26S proteasome. Substrates were phosphorylated by G1-CDK in the presence of [γ -³²P]-ATP, ubiquitinated with SCF^{Cdc4}/Cdc34, and premixed with K0-Sic1 (an internal control for ubiquitin-independent degradation) prior to addition of proteasome. Surprisingly, clear differences in the half-lives ($t_{1/2}$) of the different substrates were reproducibly obtained, such that WT ($t_{1/2} \sim 1'$) = K36 ($t_{1/2} \sim 1'$) > K32 ($t_{1/2} \sim 1.5'$) > K49 ($t_{1/2} \sim 1.75'$) > K84 ($t_{1/2} \sim 5'$) > K0 ($t_{1/2} > 10'$) (Figure 4F).

The Association of S-CDK with Sic1 Restricts the Accessible Ubiquitination Sites of Sic1 *In Vitro*

As the presence of an N-terminal lysine was necessary for Sic1 turnover, we next sought to explore the mecha-

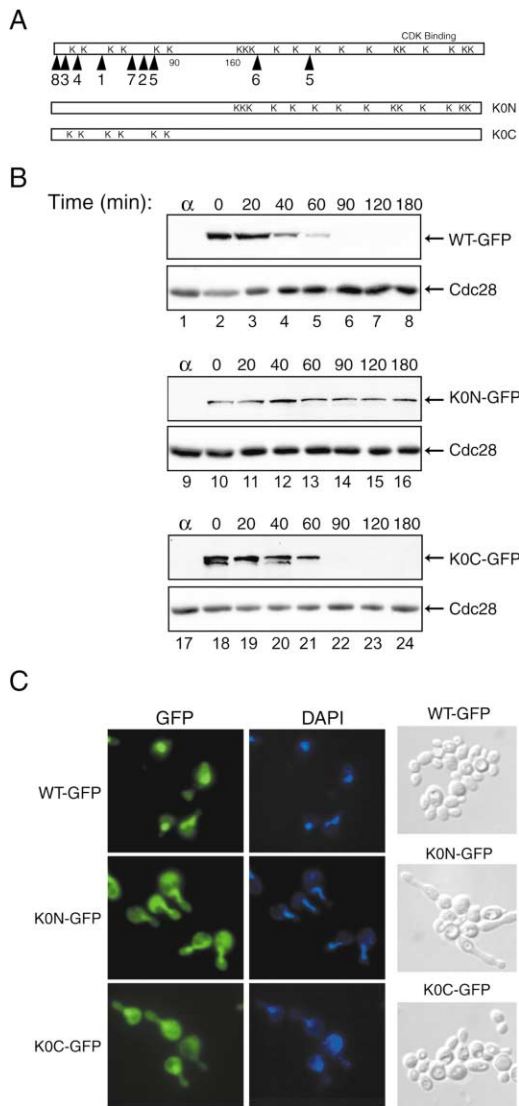


Figure 3. Sic1 Turnover Requires Lysine Residues within the N-Terminal 90 Amino Acids

(A) shows a schematic of Sic1. The positions of lysine residues (K) relative to consensus CDK phosphorylation sites (▲) are shown. Rankings attributed to phosphorylation sites are based on their relative contributions to increased stability of transiently induced Sic1 expression in vivo when individually mutated (Nash et al., 2001). The relative position of lysine residues of K0N (lacking the six N-terminal lysines) and K0C (lacking 14 C-terminal lysines) is shown. (B) N-terminal, but not C-terminal lysines, are required for Sic1 turnover. Yeast strains were arrested in G1 for 2.5 hr, and expression of the Sic1-GFP fusions was induced for 2 hr. After release, protein samples were prepared and analyzed by immunoblotting with anti-GFP and anti-Cdc28 antibodies. (C) shows localization and cell morphology of K0N-GFP, K0C-GFP, and WT-GFP. Cells that had been arrested with α factor (left) were galactose induced, fixed, and analyzed for GFP fluorescence and DNA (DAPI). Asynchronous cells grown in the presence of galactose were examined for cell morphology by DIC (right).

nistic basis for this requirement. The N-terminal CDK sites may position Sic1 on Cdc4 such that only the nearby six N-terminal lysines can be ubiquitinated by Cdc34. This hypothesis implies that lysines outside of

these are incompetent for SCF^{Cdc4}/Cdc34-dependent ubiquitination. Alternatively, C-terminal lysines may serve as ubiquitination sites, but the binding of S-CDK shields them such that only the N-terminal ones remain accessible.

To distinguish between these models, we performed ubiquitination assays with K0 Ub. In the absence of S-CDK, the conjugation of K0 Ub onto Sic1 resulted in a mobility shift of up to 100 kDa, indicative of more than 10 K0 Ub attachments (Figure 5A). However, upon titrating in S-CDK, the mobility of Sic1 decreased to 98 kDa (corresponding to 47 kDa recombinant Sic1 plus 6 to 7 attachments of 8 kDa K0 Ub).

To explore the location of attachment sites, lysines residues were mutated starting with the first six (determined to be required for Sic1-GFP destabilization, Figure 3B) and continuing toward the C-terminal S-CDK binding site. In the absence of S-CDK, all substrates except K0-Sic1 were ubiquitinated (Figure 5B). In contrast, addition of S-CDK suppressed utilization of C-terminal lysines. While some conjugation was observed with Sic1 containing 14 and 11 C-terminal lysine residues, the contribution of these residues was minor because only a relatively small fraction of molecules were converted to modified forms. Consistent with the interpretation that the N-terminal six lysines are the major ubiquitination sites of Sic1, the number of K0 Ub adducts on WT and K0C substrates bound to S-CDK was similar, while little attachment to S-CDK bound K0N was observed (Figure 5D).

The Association of S-CDK with Sic1 Restricts Ubiquitination to Lysines that Support Efficient Degradation by the 26S Proteasome

Although K0N could be extensively conjugated with Ub on multiple lysines in the absence of S-CDK, it was extremely stable in vivo. We reasoned that K0N-GFP stability could reflect either the relative rate of its ubiquitination, the ability of Ub chains attached to C-terminal lysine residues to sustain turnover of Sic1 by the proteasome, or both.

To address this question, in vitro ubiquitination and degradation experiments were performed. Timecourse experiments revealed that K0N was ubiquitinated more slowly than K0C or WT Sic1 (Supplemental Figure S4 on *Molecular Cell* website), suggesting that even in the absence of S-CDK, the C-terminal lysines of Sic1 were less accessible to SCF. To address whether the K0N-ubiquitin conjugates that do form were properly processed by the proteasome, we performed degradation assays. Both ubiquitinated WT and K0C were efficiently degraded by the proteasome, with $t_{1/2}$ of about 1 min whether they were bound to S-CDK (Figure 6A) or not (Figure 6B). Even the most heavily ubiquitinated species of K0N (generated in the absence of S-CDK) were degraded slowly ($t_{1/2}$ of ~3–5 min in multiple experiments).

To determine if the delayed degradation of K0N was due to its inability to be targeted to the proteasome, we performed binding experiments in which proteasome was immobilized on anti-Myc sepharose and mixed with WT or K0N Sic1 in the absence of S-CDK. Ubiquitinated WT efficiently bound to the proteasome (approximately

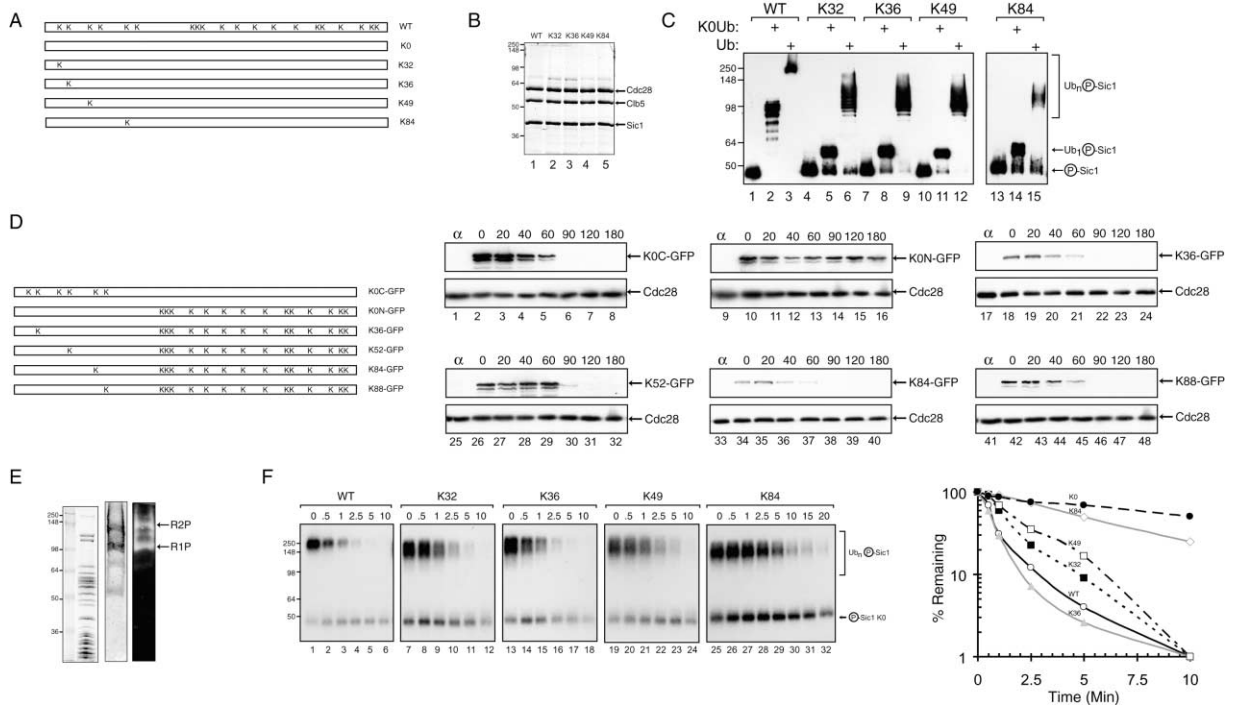


Figure 4. A Single Lysine within the N-Terminal 90 Amino Acids Is Sufficient to Sustain Ubiquitination and Degradation of Sic1

(A) Schematic of the position of lysine residues of Sic1 substrates is shown.
 (B) Substrates (1 μ g) containing either all 20 (WT) or a single lysine (K32, K36, K49, K84) were phosphorylated by G1-CDK and assembled with S-CDK prior to SDS-PAGE and Coomassie staining.
 (C) Ubiquitination of single lysine Sic1 substrates. Substrates shown in (A) were added to ubiquitination reactions containing either K0 Ub or Ub.
 (D) A single N-terminal lysine residue is sufficient for Sic1-GFP turnover. Yeast strains containing the various Sic1-GFP fusions under the *GAL1* promoter were arrested in G1 and transiently induced with galactose. After release and suppression of Sic1-GFP expression, protein samples were prepared. Only a subset of the single lysine substrates is shown. All others showed no significant differences in stability (data not shown). A schematic of the relative position of lysine residues is shown.
 (E) Purified 26S proteasomes are active. 26S proteasome was retrieved from RJD2467 lysate by anti-Myc immunoprecipitation, eluted with TEV protease, and subjected to SDS-PAGE (left) or native gel electrophoresis (right). Native gels were incubated with Suc-LLVY-AMC to assess peptidase activity (far right). Singly (R_1P) and doubly (R_2P) capped proteasomes are indicated.
 (F) Ubiquitination of single lysine substrates are degraded *in vitro*. Substrates that were labeled with [γ - ^{32}P]-ATP by G1-CDK and assembled with S-CDK were ubiquitinated as in (B), supplemented with K0-Sic1/S-CDK, and added to purified 26S proteasome. At the indicated times, samples were taken and analyzed by SDS-PAGE and phosphorimager analysis. The average amount of substrate (WT \circ , K32 \blacksquare , K36 \blacktriangle , K49 \square , K84 \diamond , K0 \bullet) remaining as a function of time was determined from three independent experiments.

25% of input recovered). In contrast, the bulk population of ubiquitinated K0N bound with 1/5th the efficiency (5% of input recovered), and only the highest molecular weight Ub conjugates of K0N were recovered (approximately 10%). Thus, whereas the five to six most C-terminal lysines of Sic1 appear to sustain ubiquitin chain assembly (Figure 5B), only a small fraction of these molecules are competent to serve as proteasome substrates.

Discussion

The experiments shown here allow two significant conclusions about the degradation of ubiquitinated Sic1 by the proteasome, which we suggest generally apply to the degradation of other proteins by this pathway. First, a single ubiquitin chain was necessary and sufficient to sustain turnover of Sic1 at physiological rates. Second, ubiquitin chains attached at different sites in Sic1 were not equally competent to sustain its rapid degradation by the proteasome. Two additional conclusions that draw from our work are: (1) binding of Sic1 to S-CDK

influenced the repertoire of lysines in Sic1 that can be ubiquitinated by SCF^{Cdc4}, effectively shielding those that are unable to sustain proteasome-dependent degradation and (2) SCF was able to catalyze the polymerization of long (>10 ubiquitins) substrate-linked chains on any one of multiple N-terminal lysines. Each of these points is discussed in more detail in the following paragraphs.

How Many Multiubiquitin Chains Are Needed to Specify Substrate Turnover by the Proteasome?

Although some proteins may be degraded either in the absence of ubiquitination (Sheaff et al., 2000; Verma and Deshaies, 2000) or upon ubiquitination of the N terminus (Breitschopf et al., 1998), the former result remains controversial and N-terminal ubiquitination has yet to be directly demonstrated. Our results indicate that Sic1 must contain at least one lysine to be ubiquitinated by SCF^{Cdc4} and degraded by the proteasome.

In addition to a single lysine being *necessary* for Sic1 degradation, our results indicate that a single lysine was also *sufficient*. Prior work on this issue has been less

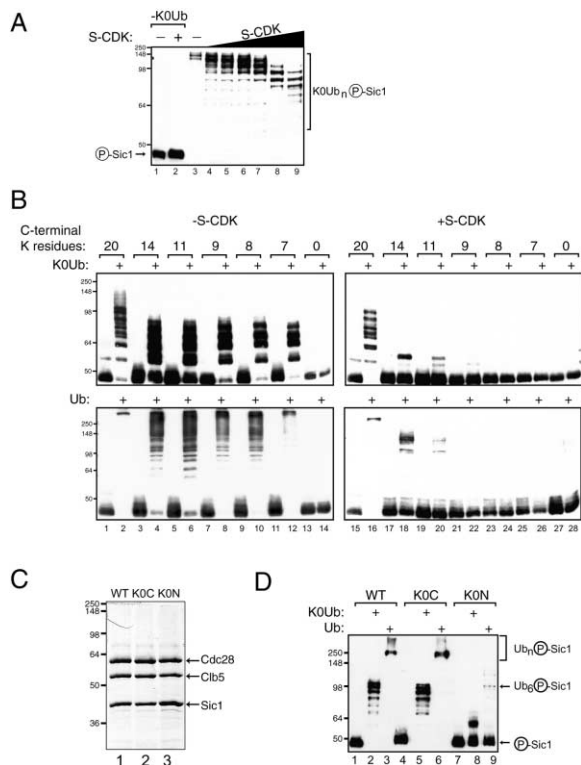


Figure 5. S-CDK Influences the Sites of Sic1 Ubiquitination

(A) The binding of S-CDK restricts the number of lysines in Sic1 that can be ubiquitinated. Sic1 (1 pmol) phosphorylated with G1-CDK was incubated with increasing amounts (0.1 to 10 pmol) of purified S-CDK and added to ubiquitination reactions containing K0 Ub. Reactions were incubated 15 min at room temperature and then fractionated by SDS-PAGE followed by immunoblotting with antibody against the T7 epitope tag on Sic1.

(B) S-CDK blocks ubiquitination of C-terminal lysine residues of Sic1. Substrates containing the indicated number of C-terminal lysine residues were added to ubiquitination reactions containing either K0 Ub or Ub in the absence or presence of S-CDK.

(C) Sic1 containing all 20 lysine residues (WT), 6 N-terminal lysine residues (K0C), or 14 C-terminal lysine residues (K0N) were phosphorylated and assembled with S-CDK prior to analysis by SDS-PAGE and Coomassie staining.

(D) The six N-terminal lysine residues of Sic1 are necessary for efficient ubiquitination in the presence of S-CDK. The Sic1 substrates shown in (C) were utilized in ubiquitination reactions containing either K0 Ub or Ub.

definitive than is perhaps commonly perceived. It has been shown for both I κ B (Scherer et al., 1995) and β -galactosidase (Bachmair and Varshavsky, 1989) that one or two lysines near the N terminus are *necessary* for degradation, but it was not established that they are *sufficient*. Interestingly, addition of more lysines to the N terminus of β -galactosidase dramatically accelerates its rate of turnover (Suzuki and Varshavsky, 1999), which is consistent with the notion that attachment of multiple ubiquitin chains may be required for rapid turnover of this protein (see below).

The most authoritative experiments to address this issue have been the studies on Ub5-dihydrofolate reductase (Ub5-DHFR) and Ub5- β -galactosidase (Ub5- β -gal) reported by Pickart and colleagues (Thrower et al., 2000). We propose that this work can be alternatively

interpreted to suggest that a single ubiquitin chain is not sufficient for the degradation of some substrates. Although Ub5-DHFR can be degraded by purified proteasome in vitro, it is degraded very slowly compared to a more physiological substrate (k_{cat} 0.05 min⁻¹ versus 0.46 min⁻¹ for cyclin-protein A ubiquitin conjugates [Glotzer et al., 1991]). Moreover, Ub5- β -gal is not degraded at all. It was suggested that the stability of Ub5- β -gal in these experiments might arise from a requirement for extraproteasomal chaperones to help unfold the Ub5- β -gal (Thrower et al., 2000). We suggest instead that rapid and processive degradation of tightly folded or multimeric substrates requires more than a single substrate-linked ubiquitin chain—particularly for non-physiological substrates like β -gal and DHFR that have not evolved to be susceptible to the proteasome's intrinsic unfoldase activity (Verma et al., 2001). Given that the proteasome has multiple intrinsic (Rpn10, Rpt5) and extrinsic (Rad23) polyubiquitin receptors (Chen and Madura, 2002; Lam et al., 2002; Wilkinson et al., 2000), the presence of multiple ubiquitin chains on a substrate like β -gal slow its dissociation via an avidity effect enough to provide the proteasome with sufficient time to unfold, translocate, and degrade the β -gal before it could spontaneously dissociate.

If, as we suggest, multiple ubiquitin chains can indeed enhance turnover of model substrates, is the same true for physiological substrates? Our data clearly show that a single ubiquitin chain was sufficient to sustain rapid and efficient turnover of Sic1—even when it was tightly assembled into a complex with S-CDK. Restoration of individual lysines in K0-Sic1 revealed that any one of four lysines in the N-terminal region (K32, K36, K49, or K84) was sufficient to nucleate a multiubiquitin chain of ~6 to 20 residues, which in turn supported degradation by the proteasome. Interestingly, in every case that we are aware of, physiological substrates of the ubiquitin pathway can be conjugated with ubiquitin on multiple lysines in vivo. For example, Sic1 can be ubiquitinated on up to three lysine residues (Skowrya et al., 1997), Gcn4 contains a large number of potential ubiquitination sites spread throughout the polypeptide (Kornitzer et al., 1994), and p53 possesses an intermediate number (four to five) sites clustered near the C terminus (Nakamura et al., 2000). Given our observations on Sic1, we suggest that this does not reflect an absolute requirement for multiple ubiquitin chain attachments, but rather may facilitate efficient ubiquitination and degradation.

Are All Ubiquitin Chains Equivalent?

It is conventionally assumed that the ubiquitin chain is a cis-dominant determinant of protein turnover, and hence a ubiquitin chain placed anywhere in a polypeptide should serve as a sufficient signal for degradation. Our data challenge this view. First of all, in the absence of S-CDK, Sic1 that lacks its N-terminal lysines (K0N) was heavily ubiquitinated in vitro, but the ubiquitin conjugates were degraded very slowly by the proteasome. Admittedly, technical limitations prevent us from knowing the length of any one ubiquitin chain on these K0N molecules. However, even the fraction of the ubiquitinated K0N molecules that exhibited a mobility shift of at least 200 kDa, implying attachment of ~25 or more

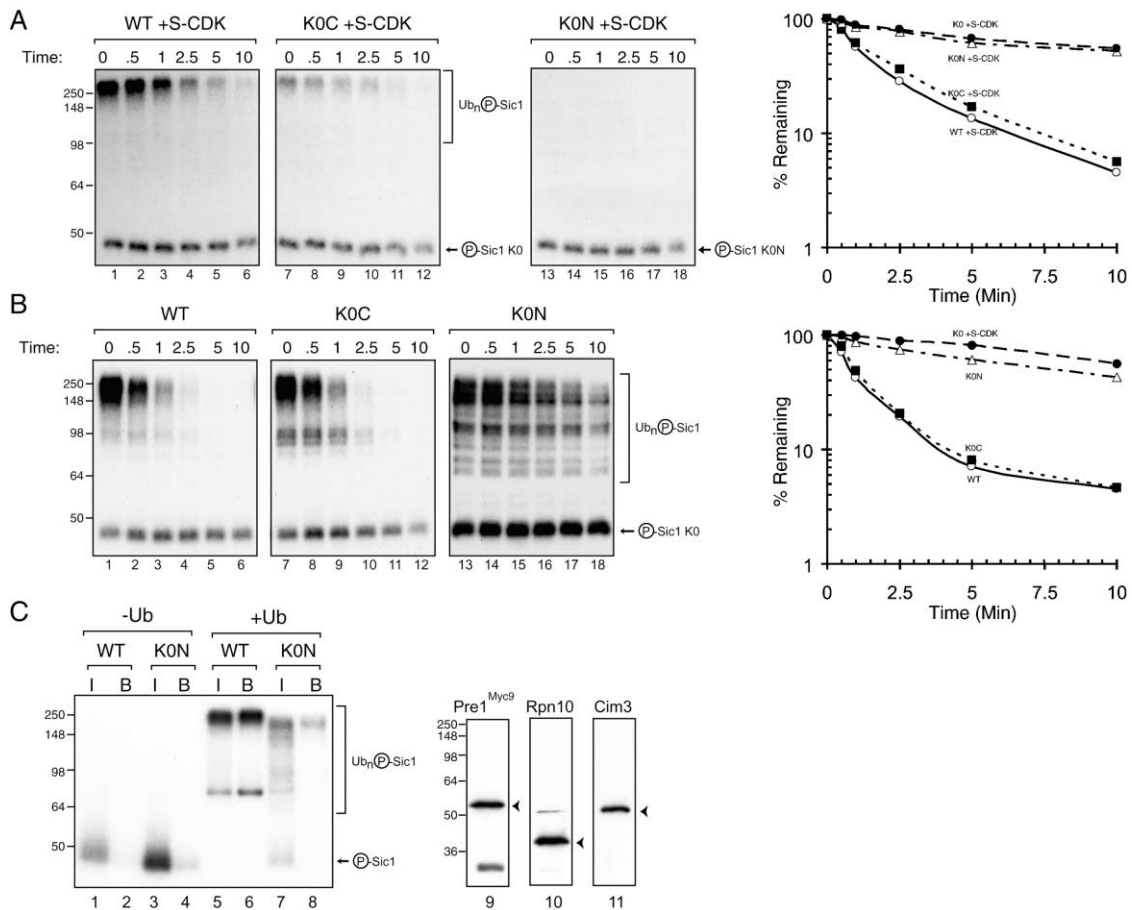


Figure 6. The Association of S-CDK Suppresses Inefficient Sites of Sic1 Ubiquitination that Do Not Confer Recognition by the 26S Proteasome
(A) Ubiquitin-dependent degradation of Sic1 requires exposed lysine residues. Substrates that had been phosphorylated by G1-CDK and [γ - 32 P]-ATP were incubated with S-CDK prior to ubiquitination. After ubiquitination, substrates were mixed with labeled K0-Sic1/S-CDK and added to proteasome preparations. K0-Sic1 was not added to reactions containing K0N in the presence of S-CDK as K0N in these conditions was not efficiently ubiquitinated (Figure 5D). The amount of substrate remaining (WT + S-CDK \circ , K0C + S-CDK \blacksquare , K0N + S-CDK \triangle , K0 + S-CDK \bullet) was determined by phosphorimager analysis from three independent experiments and plotted as a function of time.
(B) The ubiquitination of K0N in the absence of S-CDK does not promote its ubiquitin-dependent degradation. Substrates were ubiquitinated as in (A) except in the absence of S-CDK, premixed with K0-Sic1/S-CDK, and added to purified 26S proteasome. The amount of substrate remaining (WT \circ , K0C \blacksquare , K0N \triangle , K0 + S-CDK \bullet) was determined by phosphorimager analysis from three independent experiments.
(C) K0N is unable to efficiently associate with the 26S proteasome. Proteasome was immobilized on 9E10 beads, treated with 1,10-phenanthroline for 30 min prior to addition of 32 P-labeled substrates. After 30 min at 4°C, bead bound material was analyzed by SDS-PAGE and autoradiography or by immunoblotting to confirm the presence of proteasome subunits (anti-Myc for Pre1, anti-Cim3, and anti-Rpn10). Input amounts of substrate (I) represent 5% of the total ubiquitination reaction and amount bound (B) represents 20% of the recovered material.

ubiquitins, were degraded slowly. Studies with K0 Ub indicate that 5–6 lysines of K0N were conjugated with ubiquitin; even if these were spread equally among the lysines, it is evident that there should be enough ubiquitin present to assemble a tetraubiquitin degradation signal on at least one lysine.

Our evidence that context plays an important role in the targeting function of a ubiquitin chain is not limited to K0N. We also demonstrate that chains of \sim 6 to 20 ubiquitins can be assembled on Sic1 containing unique lysines at positions 32, 36, 49, or 84. Although these ubiquitinated substrates appeared equivalent by SDS-PAGE, they were degraded at markedly different rates. For example, the $t_{1/2}$ of K36 was 1 min, similar to wild-type which contains at least six multiubiquitin chains (Figure 4B), while that of K84 was about 5 min. Restora-

tion of any single N-terminal lysine to K0N-GFP also supported rapid Sic1 turnover in vivo, although the turnover rate varied only modestly. We do not know why turnover rate is more sensitive to chain position in vitro, but it is possible that other processes (nuclear translocation, phosphorylation, ubiquitination) are rate limiting for degradation in vivo and thus mask the effects of ubiquitin chain position, or that the longer time scale of the in vivo experiments makes it difficult to resolve the kinetic differences observed in vitro. Finally, we have also observed that autoubiquitinated Cdc34 (which is present in the same reactions as ubiquitinated Sic1) was competent to bind the 26S proteasome in a ubiquitin-dependent manner (Elsasser et al., 2002; R. Verma, personal communication) but was not degraded whatsoever in a 10' incubation even when >200 kDa of ubiquitin was

conjugated on up to two lysine residues (Verma et al., 2002). Cdc34 is stable in vivo, so we suggest that Cdc34 becomes conjugated with ubiquitin on a portion of the molecule that cannot be readily unraveled by the proteasomal ATPases.

A possible explanation for why the position of the ubiquitin chain might influence the rate of substrate turnover emerges from the elegant work of Matouschek and colleagues (Lee et al., 2001). These workers demonstrated that the position of a peptide degradation signal relative to a protein's termini greatly influences the rate of its degradation by the *E. coli* ClpP protease. These authors argued that a degradation signal must be present in a structural context from which the ClpA and ClpX ATPases can unravel the substrate. As we have pointed out previously (Verma and Deshaies, 2000), degradation of ubiquitinated proteins by the 26S proteasome may be facilitated by loosely structured peptide segments that enable rapid unfolding by the proteasomal ATPases. The results reported here suggest that the relative juxtaposition of such signals in relation to the ubiquitin chain is important.

It is worth noting that in most published studies, the degradation competence of ubiquitinated proteins typically is not directly assessed. Our data indicate that it is important to show that a protein ubiquitinated in vitro with a purified ubiquitin ligase can be degraded by the proteasome in a ubiquitin-dependent manner before concluding that the observed ubiquitination is functionally relevant or physiologically significant.

Implications of Our Work for SCF-Dependent Ubiquitination

Our observation that any one of the six N-terminal lysines of Sic1 could nucleate the assembly of a degradation-competent multiubiquitin chain has three implications for SCF^{Cdc4} function. First, SCF^{Cdc4}/Cdc34 or Sic1 must be sufficiently flexible such that each one of Sic1's N-terminal lysines can be juxtaposed in the vicinity of Cdc34's active site. Alternatively, the ability of SCF^{Cdc4}/Cdc34 to ubiquitinate Sic1 on multiple sites may arise from different phosphate groups on Sic1 sampling the phosphopeptide pocket on Cdc4 (Orlicky et al., 2003), due to repeated rounds of Sic1 dissociation from Cdc4 and reassociation during the timecourse of our reactions. Second, all of the Sic1 single lysine mutants sustained formation of ubiquitin chains of fairly uniform length (6 to 20 molecules). Thus, SCF must be able to catalyze the formation of chains containing 6 to 20 ubiquitin molecules. This raises an interesting conundrum. The gap between Cdc34 and Sic1 in an intact SCF complex is thought to be ~50 Å (Orlicky et al., 2003). However, a chain of ten ubiquitins should span ~130 Å (Cook et al., 1994). Thus, not only is it difficult to envision how the first transfer of ubiquitin bridges the 50 Å gap, but it is also difficult to envision how ubiquitin protomers continue to be added once the chain achieves a length greater than three or four ubiquitins. Any description of the mechanism of action of SCF will need to explain how it can catalyze the polymerization of substrate bound ubiquitin chains that are so long.

Experimental Procedures

Yeast Strains

Sic1-GFP Strains

Sic1 and green fluorescent protein open reading frames (ORFs) were amplified by polymerase chain reaction (PCR) and cloned into pRS306 with the *GAL1* promoter at the 5' end and actin terminator at the 3' end (Sikorski and Hieter, 1989). Site-directed mutagenesis was performed using sequential rounds of oligonucleotide-directed mutagenesis (Quickchange, Stratagene). Plasmids were linearized with EcoRV to target integration at the *URA3* locus and transformed into yeast strain RJD979 (W303 *sic1::SIC1HAHis6::TRP1*) (Verma et al., 1997a).

Pre1-TEV-Myc9 Strain

Epitope tagging of *PRE1* with sequences encoding the Myc9 epitope was performed as described previously (Seol et al., 2001). A PCR product was generated using a 5' oligonucleotide corresponding to the last 14 codons of *PRE1* and a 3' oligonucleotide complementary to the 45 bases downstream of the stop codon. This PCR product was transformed into RJD416 (W303) to yield RJD2467. Descriptions of the plasmids generated and the various yeast strains utilized can be found in Supplemental Tables S1 and S2 at <http://www.molecule.org/cgi/content/full/11/6/1435/DC1>.

Stability of Sic1-GFP and Sic1

Yeast strains were grown to 1.0 OD₆₀₀ in synthetic medium containing 0.67% yeast nitrogen base minus amino acids, 2% raffinose, lacking uracil. Cells were arrested with 2 μg/ml α factor for 2.5 hr, induced with galactose for 2 hr, and washed into synthetic medium containing 2% dextrose. Samples were taken at the indicated times post-release. Lysates were analyzed by Western blotting with anti-GFP antiserum (Clontech) for Sic1-GFP fusions and anti-HA ascites for Sic1-HA expressed from its own promoter (Verma et al., 1997a).

GFP Localization

Yeast strains were grown as in stability experiments, washed with PBS, and fixed for 5 min in 4% formaldehyde, 3.4% sucrose in PBS. Cells were washed with SP (1.2 M sorbitol pH 7.0, 100 mM KPO₄) and mounted onto polylysine-treated slides. For visualization of nuclei, the slides were incubated with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) and washed three times with water prior to microscopy. Images were captured through a 100× objective on a Zeiss Axioskop microscope with a CCD camera.

Recombinant Protein Expression and Purification

Sic1

The *SIC1* ORF was amplified by PCR and cloned into pET11b for *E. coli* expression. The 5' and 3' oligonucleotides used for PCR encoded a T7 tag (Novagen) and a hexahistidine tag, respectively. Successive rounds of mutagenesis were performed using oligonucleotide-directed mutagenesis (Quickchange, Stratagene). Description of the various *E. coli* expression constructs can be found in Supplemental Table S1 on the *Molecular Cell* website.

The Sic1 constructs were transformed into BL21 CodonPlus(DE3) RIL cells (Stratagene), grown to an OD₆₀₀ of 0.7 at 30°C in 2XYT medium containing ampicillin and chloramphenicol, and induced with 0.8 mM IPTG for 2.5 hr. Cells were resuspended in 20 mM HEPES (pH 8.0), 500 mM NaCl, 0.2% Triton X-100, 1 mM EGTA (pH 8.0), 10 mM imidazole, and 10% glycerol, frozen in liquid nitrogen, thawed, and sonicated. Sic1 was purified on NiNTA-agarose in lysis buffer, eluted with 250 mM imidazole, and purified by gel filtration on a PD10 column equilibrated with 20 mM HEPES (pH 8.0), 200 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT (Kinase Buffer).

Sic1 was phosphorylated by immobilized G1-CDK in kinase buffer containing 15% glycerol in the presence of either 2 mM ATP or 10 μCi [γ-³²P]-ATP and 100 μM ATP and incubated for 1 hr at room temperature.

S-CDK

Hi5 cells were infected with GST-Cdc28HA and Clb5 baculoviruses at a ratio of 1:2 with a multiplicity of infection (MOI) of 5. After 36 to 48 hr, lysates were prepared (2 ml lysate per 10 cm² plate) in Sf9 lysis buffer (25 mM HEPES [pH 7.4], 250 mM NaCl, 0.2% Triton X-100, 1 mM DTT, 2 mM EDTA, 5 mM MgCl₂, and 10% glycerol).

S-CDK was purified on glutathione sepharose as described (Verma et al., 2001). Eluted material was dialyzed into 30 mM Tris-HCl (pH 7.6), 200 mM NaCl, 5 mM MgCl₂, 2 mM DTT, and 15% glycerol.

G1-CDK

Hi5 cells were infected with baculoviruses encoding GST-Cdc28HA, Cln2MycHis6 or Cln2, Cks1His6, and Cak1 at a ratio of 2:4:1:1, at a MOI of 5. Lysates were prepared as for S-CDK, and G1-CDK was purified on either glutathione sepharose or protein A coupled with 9E10 monoclonal antibody.

SCF^{Cdc4}

Hi5 insect cells were infected with Py2HA3Cdc4, Cdc53, Skp1HA, and Hrt1 baculoviruses in a ratio of 2:2:1:1 at a MOI of 5 (Seol et al., 1999). After 48 hr, lysates were prepared in Sf9 lysis buffer and incubated with protein A beads coupled with anti-Py monoclonal antibody. After 3 hr at 4°C, the bound material was washed four times with Py wash buffer (20 mM HEPES [pH 7.4], 100 mM NaCl, 0.5% Igepal CA-630, 1 mM EDTA, 1 mM DTT, 10% glycerol). SCF^{Cdc4} was eluted for 2 hr at 4°C with 100 µg/ml Py peptide in Py wash buffer containing 300 mM NaCl, 0.1% n-octylglucoside, and 20 µg/ml arg-insulin. Eluted material was dialyzed into 30 mM Tris-HCl (pH 7.6), 200 mM NaCl, 5 mM MgCl₂, 2 mM DTT, and 15% glycerol and frozen in liquid nitrogen.

Affinity Purification of 26S Proteasome

Yeast strain RJD2467 grown to an OD₆₀₀ of 2.0 in synthetic media lacking histidine. Cells were harvested, washed with water, and resuspended in one pellet volume of 25 mM HEPES (pH 7.4), 250 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 5 mM ATP. This material was passed through a Constant Systems Cell Disrupter at 25 kPsi and centrifuged at 50,000 rpm for 30 min. The lysate was added to protein A sepharose covalently coupled with 9E10 monoclonal antibody (15 ml lysates/ml beads) and incubated for 90 min at 4°C in the presence of 1× ATP regenerating system (ARS) (Verma et al., 1997b). Sepharose bound material was washed four times with 10 volumes of 26S wash buffer (25 mM HEPES [pH 7.4], 250 mM NaCl, 0.2% Triton X-100, 5 mM MgCl₂, 2 mM ATP, 10% glycerol) and two times with 10 volumes of the same buffer without Triton X-100. After resuspension in 1 volume of the final wash buffer, recombinant TEV (tobacco etch virus) protease was added to 100 µg/ml and incubated for 3 hr at room temperature prior to recovery.

To confirm the presence of intact proteasomes, native gels were run and either stained with Coomassie or incubated with N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin to detect peptidase activity (Glickman et al., 1998) (Figure 4E).

In Vitro Binding Assays

Sic1/S-CDK Binding Assay

Lysates from Hi5 cells infected with GST-Cdc28HA and Clb5 baculoviruses were prepared and incubated with glutathione sepharose for 30 min at 4°C (100 µl lysate with 10 µl glutathione sepharose). After washing with Sf9 lysis buffer, 2 pmol of Sic1 was added. The resulting reactions were incubated for 1 hr at 4°C and washed four times with Sf9 lysis buffer prior to analysis by immunoblotting with anti-T7 tag antisera (Novagen).

Sic1/SCF^{Cdc4} Binding Assay

The components of SCF^{Cdc4} were expressed together in baculovirus-infected Hi5 cells and retrieved on anti-Py-coupled protein A beads (25 µl lysate per 10 µl anti-Py sepharose). Approximately 2 pmol of either unphosphorylated Sic1 or Sic1 phosphorylated with G1-CDK was added. After 1 hr at 4°C, the beads were washed four times with Sf9 lysis buffer prior to analysis.

Sic1/26S Proteasome Association Assay

Yeast lysates from RJD2467 were incubated with 9E10 beads for 1 hr at 4°C. The beads were washed three times with 26S wash buffer and twice with the same buffer containing 1 mM EDTA, but lacking Triton and NaCl (26S IP Buffer). After supplementing the bead bound material with ARS to 1×, 1,10-phenanthroline was added to 1 mM and incubated for 30 min at 30°C. After cooling on ice, ³²P-labeled substrates prepared in either the presence or absence of ubiquitin were added and incubated at 4°C for 30 min. The bead bound material was washed four times with 26S IP buffer prior to an analysis.

Histone H1 Kinase Assays

Lysates (100 µl) from Hi5 cells infected with GST-Cdc28HA and Clb5 baculoviruses were incubated for 30 min at 4°C with 10 µl glutathione sepharose. After washing, Sic1 (10 pmol) was added and incubated for an additional 30 min. Histone H1 (2.5 µg) and 10 µCi [³²P]-ATP were added after washing with kinase buffer. After 30 min at room temperature, the reactions were analyzed by SDS-PAGE and autoradiography.

Ubiquitination Assays

Reaction components for the ubiquitination of Sic1 were prepared essentially as described (Seol et al., 1999), except that SCF was eluted from the anti-Py resin. These reactions contained 1 pmol Uba1, 5 pmol Cdc34, 100 pmol Ub or 20 pmol K0 Ub (provided by R. Verma), 5 to 100 pmol of SCF^{Cdc4}, and 1 to 5 pmol of phosphorylated Sic1 in 30 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM MgCl₂, 2 mM DTT, 2 mM ATP in 10 µl total volume. In experiments where S-CDK was added, Sic1 was premixed on ice with S-CDK for 15 min. Reactions were incubated at room temperature for 20 min prior to analysis.

Degradation Assays

Sic1 (1 pmol) labeled by G1-CDK with [³²P]-ATP and ubiquitinated was premixed on ice with approximately equimolar labeled K0-Sic1. Purified 26S proteasome (approximately 10 pmol) was added in a buffer containing 20 mM HEPES (pH 7.4), 5 mM MgCl₂, 2 mM ATP, 1× ARS, 1 mM DTT, 10% glycerol in 50 µl. Reactions were initiated at 30°C and terminated by adding SDS-containing sample buffer. Samples were evaluated by electrophoresis and autoradiography/phosphorimager analysis.

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