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Cell Cycle-dependent Degradation of the *Saccharomyces cerevisiae* Spindle Motor Cin8p Requires APC^{Cdh1} and a Bipartite Destruction Sequence

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Saccharomyces cerevisiae Cin8p belongs to the BimC family of kinesin-related motor proteins that are essential for spindle assembly. Cin8p levels were found to oscillate in the cell cycle due in part to a high rate of degradation imposed from the end of mitosis through the G1 phase. Cin8p degradation required the anaphase-promoting complex ubiquitin ligase and its late mitosis regulator Cdh1p but not the early mitosis regulator Cdc20p. Cin8p lacks a functional destruction box sequence that is found in the majority of anaphase-promoting complex substrates. We carried out an extensive mutagenesis study to define the *cis*-acting sequence required for Cin8p degradation in vivo. The C terminus of Cin8p contains two elements required for its degradation: 1) a bipartite destruction sequence composed of a KEN-box plus essential residues within the downstream 22 amino acids and 2) a nuclear localization signal. The bipartite destruction sequence appears in other BimC kinesins as well. Expression of nondegradable Cin8p showed very mild phenotypic effects, with an increase in the fraction of mitotic cells with broken spindles.

INTRODUCTION

Eukaryotic chromosome segregation is accomplished by the microtubule-based mitotic spindle, a dynamic structure. During mitosis, spindle microtubules undergo dramatic changes in structural organization. Microtubule-based motor proteins orchestrate many of these changes. A major question in the mitosis field concerns the mechanisms by which the activities of the numerous spindle motors are regulated.

The actions of five kinesin-related proteins and a dynein contribute to spindle structure and function in the budding yeast *Saccharomyces cerevisiae* (Hildebrandt and Hoyt, 2000). Two of the kinesins, Cin8p and Kip1p, belong to the conserved BimC subfamily and perform an overlapping and essential function (Hoyt *et al.*, 1992; Roof *et al.*, 1992; Saunders and Hoyt, 1992). Studies of numerous eukaryotic species have demonstrated that the BimC motors are required for spindle assembly (Enos and Morris, 1990; Hagan and Yanagida, 1990; Hoyt *et al.*, 1992; Roof *et al.*, 1992; Sawin *et al.*, 1992; Heck *et al.*, 1993; Blangy *et al.*, 1995). They accomplish this by forming bipolar tetramers with two motor domains at opposite ends of an extended coiled-coil rod (Cole *et al.*, 1994; Gordon and Roof, 1999). The ability of BimC motors to cross-link microtubules from each half spindle presumably is utilized to construct bipolar spindles (Kashina *et al.*, 1996; Sharp *et al.*, 1999). In addition to their role in spindle assembly, Cin8p and Kip1p produce the bulk of spindle elongating activity during anaphase (Saunders *et al.*, 1995). This is probably accomplished by the plus end-directed sliding of cross-linked antiparallel microtubules. BimC motors in other species are believed to perform a similar spindle elongation function (Sharp *et al.*, 2000). Phenotypic analysis has revealed that Cin8p is the most crucial of the *S. cerevisiae* mitotic motors. Indeed, under the appropriate conditions, Cin8p can uniquely support the viability of cells deficient for most of the activities of the five other spindle motors (Cottingham *et al.*, 1999).

The conserved and essential nature of the spindle role of BimC motors has spurred interest in mechanisms that regulate their activity. Previous studies have implicated both transcriptional and posttranslational regulatory mechanisms operating on BimC motors. Phosphorylation of Eg5, the vertebrate BimC family member, by cdc2 (also known as cyclin-dependent kinase 1) and Aurora kinases has been observed (Blangy *et al.*, 1995; Sawin and Mitchison, 1995; Giet *et al.*, 1999). cdc2 phosphorylation is believed to alter Eg5 localization in a cell cycle-specific manner. Some BimC

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Abbreviations used: APC, anaphase-promoting complex; D-box, destruction box; DMSO, dimethyl sulfoxide; HA, hemagglutinin; NLS, nuclear localization signal; ORF, open reading frame; P*GAL1, GAL1* promoter; PCR, polymerase chain reaction.

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motor mRNA and protein levels have been reported to fluctuate in a cell cycle-dependent manner (Hagan and Yanagida, 1992; Giet *et al.*, 1999; Uzbekov *et al.*, 1999). The transcript levels of *CIN8* and *KIP1* fluctuate, peaking in S phase (Cho *et al.*, 1998; Spellman *et al.*, 1998). Our finding that elevated levels of Cin8p interfere with spindle assembly and function suggested the requirement for tight regulation of Cin8p activity (Saunders *et al.*, 1997; Hildebrandt and Hoyt, unpublished observation). In this paper, we report that Cin8p protein levels are also regulated by a cell cycle-specific degradative mechanism.

Targeted degradation is a precise and rapid mechanism for promoting cell cycle transitions. Many mitotic proteins are eliminated by the covalent attachment of ubiquitin and subsequent degradation by the proteosome (King *et al.*, 1996a; Zachariae and Nasmyth, 1999). The mitotic targets for proteolysis are selected by a specific ubiquitin ligase, the anaphase-promoting complex or cyclosome (here referred to as APC). Directed by its association with two homologous regulators, the APC acts at two temporally distinct steps in the cell cycle. In association with Cdc20p, the APC targets the degradation of securin proteins (i.e., *S. cerevisiae* Pds1p), whose elimination is required to initiate anaphase (Cohen-Fix *et al.*, 1996). Exit from mitosis and passage into G1 of the next cycle is facilitated by the APC in association with Cdh1p (also known as Hct1p), a Cdc20p homologue. Known APC^{Cdh1} targets include B-type mitotic cyclins and the spindle midzone protein Ase1p (Juang *et al.*, 1997; Schwab *et al.*, 1997; Visintin *et al.*, 1997). Interestingly, the chromatin-associated kinesin Xkid is an APC substrate, and its destruction is critical for proper chromosome segregation in anaphase (Antonio *et al.*, 2000; Funabiki and Murray, 2000). This suggests that ubiquitin-mediated proteolysis may be an important mechanism for regulating mitotic kinesins.

Substrates recognized by the APC contain a destruction signal sequence, frequently but not exclusively located near the N termini of target proteins. Two types of these destruction sequences have been found: the destruction box (D-box), with a consensus of RxxLxxxxN (where "x" is any amino acid, Glotzer *et al.*, 1991; King *et al.*, 1996b), and the KEN-box with a consensus of KENxxxN/D (Pfleger and Kirschner, 2000). D-box–containing substrates typically appear to be recognized by either APC^{Cdc20} or APC^{Cdh1}. In the case of certain B-type cyclins and *S. cerevisiae* Hsl1p, it has been reported that the D-box is recognized by both APC forms (Schwab *et al.*, 1997; Baumer *et al.*, 2000a; Burton and Solomon, 2000; Pfleger and Kirschner, 2000; Yeong *et al.*, 2000). The KEN-box is believed to be recognized exclusively by APC^{Cdh1}. The KEN-box was recently defined by in vitro ubiquitination and proteolysis studies (Pfleger and Kirschner, 2000). Its role in vivo has been demonstrated for human Cdc6, securin, and *Drosophila* cyclin A, where it partially contributes to the instability of these proteins (Petersen *et al.*, 2000; Zur and Brandeis, 2001; Jacobs *et al.*, 2001).

In this study, we demonstrate that Cin8p protein levels are regulated by the APC and its regulator Cdh1p. No functional D-box was found in Cin8p, but its degradation was dependent on two C-terminal sequence elements: a nuclear localization signal (NLS) and an APC-targeting sequence that contains a KEN-box plus essential amino acids located up to 22 positions downstream. Based on the phenotype of cells expressing a stable form of Cin8p, we propose that Cin8p destruction in late mitosis might be needed to prevent defects in spindle formation in the next cell cycle.

MATERIALS AND METHODS

Yeast Strains and Media

Rich (YPD) and synthetic (S) media were as described by Sherman *et al.* (1983). α-Factor (US Biologicals, Swampscott, MA) was added to 4 μg/ml in pH 4.0 medium except for *bar1*Δ strains for which 0.4 μg/ml α-factor was added and the medium pH was not adjusted. *bar1*Δ strains are hypersensitive to the actions of α-factor (Chan and Otte, 1982). Hydroxyurea (Sigma, St. Louis, MO) was added to 0.1 M in pH 5.8 medium. Nocodazole (Sigma) in dimethyl sulfoxide (DMSO) was added to 10 μg/ml. Strains and plasmids used in this study are listed in Table 1.

Standard techniques were utilized for DNA manipulations (Sambrook *et al.*, 1989). *CLB2* and *CDH1* disruptions were made by one-step gene replacement (Rothstein, 1983) and deletions were confirmed by polymerase chain reaction (PCR). The *clb2::URA3* allele consists of a *URA3*-containing restriction fragment replacing nucleotides 361-1161 of the *CLB2* open reading frame (ORF). The *cdh1::kan*^{R} disruption consists of *a kan*^{R}-containing restriction fragment replacing nucleotides −80 in the *CDH1* promoter to 1326 in the *CDH1* ORF. The *dyn1-Δ3::HIS3* disruption consists of a restriction fragment containing *HIS3* replacing nucleotides 912 to the end of the *DYN1* ORF. The *cin8::URA3*, *cin8::HIS3*, and *kip1::HIS3* disruptions were described previously (Hoyt *et al.*, 1992; Roof *et al.*, 1992; Saunders *et al.*, 1995).

CIN8–3HA encodes three tandem copies of the hemagglutinin (HA) tag fused in-frame to Cin8p at the C terminus. *CIN8–3HA* was able to fully complement a number of *cin8*Δ mutant phenotypes. *cin8::CIN8–3HA* was created by replacing the genomic copy of *CIN8* with *CIN8–3HA* by transplacement (Scherer and Davis, 1979). *6myc-CIN8* contains six tandem copies of the myc epitope tag inserted after the third *CIN8* codon. *6myc-SV40NLS* constructs had the *SV40NLS* inserted in-frame directly after the *6myc* tag at amino acids 96–113.

The following *CIN8* C-terminal truncation alleles were constructed by restriction digests with the indicated sites within *CIN8* (and often a downstream site), followed by fill-in reactions and religation: *CIN8–1031*, *Xho*I(3090); *CIN8–1013*, *Sph*I(3039); *cin8–955*, *Eco*RV(2863); *cin8–871*, *Eco*RI(2611). Other *CIN8* alleles were generated with the use of mutagenic oligonucleotides and either the USE mutagenesis (Nicolas *et al.*, 1997) or megaprimer mutagenesis (Sarkar and Sommer, 1990) protocols. Most mutagenic primers were engineered with diagnostic restriction site changes. All mutations were confirmed by sequencing. Δ*N70-CIN8* was constructed by deleting between the *Msc*I site and a *Cla*I site engineered at nucleotide 215 in *CIN8*. The resulting gene product is missing amino acids 4–73.

The *CIN8* random and site-directed mutant alleles were subcloned into the base set of *CIN8* plasmids as follows (also see Table 1): pEH113 was used for testing Cin8p function in vivo; pEH70 and pEH170 were used for pulse-chase degradation assays on C-terminal mutants; pEH287 was used for moderate overexpression studies.

GAL1 promoter (P_{GAL1})-driven *GAL4BD-CIN8* fusions were generated in two steps. First, a 227-bp *Eco*RV to *Xho*I fragment from *CIN8* was cloned into *Sma*I/*Sal*I-cut pGBD-C1 (James *et al.*, 1996). The resulting plasmid was cut with *Hin*dIII, and the fragment with *GAL4BD-CIN8* was cloned into *Hin*dIII of p415-GAL1 (Mumberg *et al.*, 1994).

Mutagenic PCR and Screen for Stable CIN8 *Mutants*

A 3′ region of the *CIN8* ORF, between *Eco*RV and *Sph*I (176 bp), was PCR amplified under mutagenic conditions (Cadwell and Joyce, 1995). The products were used to replace the wild-type *Eco*RV to

Table 1. Strains and plasmids used in this study

Genotype[a]	
Strains	
K1534*	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 ssd1 bar1::hisG* (Irniger and Nasmyth, 1997)
K4438*	K1534 *cdc16-123* (Irniger and Nasmyth, 1997)
MAY2063	*MATa ade2-101 lys2-801 his3-Δ200 leu2-3, 112 ura3-52 cin8::URA3*
MAY2422	*MATa lys2-801 his3-Δ200 leu2-3, 112 ura3-52 cyh2^R*
MAY4751	*MATa ade2-101 his3-Δ200 leu2-3, 112 ura3-52 cin8::CIN8-3HA cdc15-2*
MAY4822	*MATa lys2-801 his3-Δ200 leu2-3, 112 ura3-52 cin8::CIN8-3HA*
MAY4823	*MATa ade2-101 lys2-801 his3-Δ200 leu2-3, 112 ura3-52 cin8::CIN8-3HA*
MAY5017	*MATa lys2-801 his3-Δ200 leu2-3, 112 ura3-52 cdc20-1 cin8::CIN8-3HA*
MAY5353*	K1534 *cdh1::KAN^R*
MAY5402*	*MATa ade2-1 trp1-1 leu2-3, 112 his3 ura3 bar1::hisG cdc20-1*
MAY5466	MAY4822 *cdh1::KAN^R*
MAY5590	*MATa ade2-101 lys2-801 his3-Δ200 leu2-3, 112 ura3-52 cyh2 cin8::HIS3 kip1::HIS3* pTK97
MAY6752	*MATα lys2-801 his3-Δ200 leu2-3, 112 ura3-52 cyh2 cin8::HIS3 dyn1-Δ3::HIS3* pEH24
MAY6810*	K1534 *clb2::URA3*
MAY6812*	K1534 *clb2::URA3 cdh1::KAN^R*
Plasmids	
pEH24	*CIN8 URA3 CYH2 CEN*
pEH55	P_{GAL1} > *CIN8-3HA LEU2 CEN*
pEH70	P_{GAL1} > *6myc-CIN8 LEU2 CEN*
pEH87	P_{GAL1} > *ΔN70-CIN8-3HA LEU2 CEN*
pEH113	*CIN8 LEU2 CEN*
pEH117	*6myc-CIN8-1031 LEU2 2μ*
pEH136	P_{GALS} > *CIN8 LEU2 CEN*
pEH157	*6myc-SV40NLS-CIN8-1031 LEU2 2μ*
pEH170	P_{GAL1} > *6myc-SV40NLS-CIN8 LEU2 CEN*
pEH287	P_{GALS} > *CIN8 HIS3 CEN*
pEH289	P_{GAL1} > *GAL4BD-cin8 LEU2 CEN*
pEH290	P_{GAL1} > *GAL4BD-cin8-Δ996-1004 LEU2 CEN*
pEH291	P_{GAL1} > *GAL4BD-cin8-alaKEN LEU2 CEN*
pEH292	P_{GAL1} > *GAL4BD LEU2 CEN*
pTK97	*KIP1 URA3 CEN*
pTK138	*6myc-CIN8 LEU2 2μ*

[a] Strains marked with an asterisk are derivatives of W303. All other strains are derivatives of S288C.

*Sph*I fragment of pEH287. The ligation reaction was transformed into *Escherichia coli*, 1150 colonies were pooled, and DNA was isolated. Yeast strain MAY2422 was transformed with the mutant DNA pool, and colonies were selected on SD-histidine medium. A total of 6300 individual colonies were replica plated to 3% glycerol/2% lactate-containing minimal medium, grown for 2 d, and then replica plated to 2% galactose-containing minimal medium. The 216 colonies that failed to grow on the galactose medium were identified; 40 of these mutants were retested after plasmid isolation and retransformation. These 40 mutants were sequenced.

In Vivo Studies

To collect small G1 cells from asynchronous cultures, we used Ficoll gradients (Sloat and Pringle, 1978; Sullivan and Huffaker, 1992). Briefly, cultures were grown in YPD medium to log phase. Cells were harvested, resuspended in 2% Ficoll, and layered onto the top of a gradient with steps of 4, 6, 8, and 10% Ficoll. Gradients were centrifuged for 25 min at 120 × *g*. Fractions were collected from the top of the gradient, and fractions with >90% unbudded cells as judged by microscopy were pooled.

Cin8p expression-pulse assay: To examine the stability of Cin8p in arrested G1 cells, cultures were grown to mid-log phase in S-medium with 2% raffinose at 30°C. α-Factor was added for 3 h followed by the addition of galactose to induce Cin8p expression. Induction times depended on the epitope tag and our ability to detect it by Western blot (i.e., 3HA- and 6myc-tagged Cin8p were induced for 30 min; 6myc-SV40NLS-Cin8p derivatives were induced for 90 min; Gal4BD-Cin8p fusions were induced for 360 min). Cycloheximide (1 mg/ml) and 2% glucose were then added (at t = 0 min.) to terminate gene expression, and samples were collected at times thereafter.

To test the in vivo function of *CIN8* alleles, a plasmid derived from pEH113 but harboring the *CIN8* mutant of interest was transformed into both *cin8Δ kip1Δ* and *cin8Δ dyn1Δ* strains (MAY5590 and MAY6752, respectively). These normally inviable genotypes are supported by a plasmid-borne copy of *CIN8*. The plasmid also carries *URA3*. Therefore, these strains can survive only on 5-fluoroorotic acid-containing media if transformed with another active allele of *CIN8*. *CIN8* mutant allele transformants were tested on 5-fluoroorotic acid at 26 and 37°C. Alleles that supported viability at both temperatures were considered fully proficient for the essential functions of *CIN8*.

Yeast Extracts and Protein Analysis

Yeast protein extracts were prepared one of two ways. Most extracts of cells were made by mechanical lysis with glass beads as previously described (Farr and Hoyt, 1998) in 50 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. For Gal4BD-Cin8p fusions it was necessary to use alkaline-mediated hydrolysis and trichloroacetic acid precipita-

tion (Fujimura-Kamada *et al.*, 1997) to see the Gal4BD epitope by Western blot. Protein samples were separated by SDS-PAGE, and the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) with the use of standard techniques (Sambrook *et al.*, 1989). Equal protein loading was confirmed by India ink staining of the membrane. The HA epitope was detected with 12CA-5 (Boehringer Mannheim, Indianapolis, IN), the myc epitope was detected with 9E10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Gal4BD-Cin8p fusions were detected with anti-Gal4 (Zymed, S. San Francisco, CA). Anti-Clb2 was a generous gift of D. Kellogg. Alkaline phosphatase-conjugated secondary antibodies were from Jackson ImmunoResearch Labs (West Grove, PA). Reagents for chemiluminescence were obtained from Tropix (Bedford, MA).

Microscopy

Indirect immunofluorescence microscopy of 6myc-tagged Cin8p was performed in MAY5590 transformed with the appropriate plasmid. Cells were arrested with preanaphase spindles by growth in minimal glucose medium with 0.1 M hydroxyurea. Cells were fixed for 7 min at 42°C and prepared for immunofluorescence as described before (Pringle *et al.*, 1991; Cottingham and Hoyt, 1997). Myc was stained with 9E10 antibody and a Cy2-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Labs). Tubulin was stained with YOL 1/34 (Harlan Bioproducts for Science, Indianapolis, IN) and rhodamine-conjugated goat anti-rat (Jackson ImmunoResearch Labs) or Alexa Fluor 488-conjugated goat anti-rat (Molecular Probes, Eugene, OR).

RESULTS

Cin8p Levels Fluctuate in the Cell Cycle in Part due to G1-specific Instability

Because Cin8p has a major role in governing mitotic spindle dynamics, we first examined whether its levels fluctuate in the cell cycle. To assay Cin8p levels, we used a 3HA epitope-tagged version of *CIN8* integrated at its normal genomic locus. Cell cycle inhibitors were used to synchronize cells at various cell cycle stages. Cin8p appeared equally abundant in cells arrested in S- and M-phase and by hydroxyurea and nocodazole treatment, respectively, but it was nearly undetectable in G1 cells arrested with α-factor (Figure 1A). To ensure that the disappearance of Cin8p in G1 cells was not a specific effect of α-factor treatment (i.e., specific for cells preparing to mate), we collected small unbudded G1 cells from an untreated asynchronous culture with the use of a Ficoll gradient (Sloat and Pringle, 1978; Sullivan and Huffaker, 1992). Whereas Cin8p was abundant in the total asynchronous culture, it was absent in the G1 cell fraction.

We also examined the cell cycle profile of Cin8p expression in a synchronized culture, beginning with release from α-factor arrest in G1 (Figure 1B). Cin8p levels peaked during spindle assembly. The level stayed high as cells entered anaphase and then declined as bipolar spindles disappeared. Cin8p reaccumulated in the next round of spindle assembly and anaphase. The cycling profile of Cin8p levels closely paralleled the profile for the B-type mitotic cyclin Clb2p.

Two types of regulation could be contributing to Cin8p's cell cycle oscillation: transcription and degradation. It has been demonstrated that *CIN8* transcription is cell cycle regulated and peaks in S-phase (Cho *et al.*, 1998; Spellman *et al.*, 1998). We investigated whether Cin8p stability varies in a cell cycle-dependent manner by examining the fate of Cin8p heterologously expressed in cells arrested in various cell cycle stages (Figure 1C). A pulse of Cin8p was induced from the regulatable, high-level P_{GAL1} for 30 min in the presence of galactose. Expression was then terminated by the addition of glucose and cycloheximide (t = 0 min) to prevent further transcription and translation. Samples were collected at times thereafter, and Cin8p levels were determined by Western blot analysis. Cin8p was unstable in G1-arrested cells, disappearing within 15 min of the pulse. In contrast, Cin8p was stable in S-phase– and M-phase–arrested cells. We also found with the use of the temperature-sensitive *cdc15-2* mutant that Cin8p was stable in cells arrested in late anaphase. However, we found Cin8p was very unstable in the same *cdc15-2* cells if they were first arrested in G1 with α-factor and then shifted to 37°C (Figure 1D). This finding indicates that Cdc15p activity is not directly required for Cin8p degradation. The stabilization of Cin8p observed in *cdc15-2*–arrested cells, therefore, is due to the late mitosis block imposed by this mutant. We conclude that Cin8p becomes unstable during the period between late anaphase and S-phase of the next cell cycle.

Cin8p Instability in G1 Requires APC^{Cdh1}

The instability of Cin8p in G1 cells is typical of proteins degraded via the APC. The requirement for the APC in Cin8p degradation was examined with the use of the *cdc16-123* mutant that is temperature sensitive for function of an essential APC subunit (King *et al.*, 1995; Tugendreich *et al.*, 1995). Cells were arrested in G1 with α-factor and shifted to the nonpermissive temperature, and then the galactose-induced pulse of Cin8p was examined as described above. Cin8p was stable in the *cdc16-123* strain at 37°C in contrast to the isogenic wild-type strain (Figure 2A) or a *cdc16-123* strain carrying a *CDC16* plasmid (Hildebrandt and Hoyt, unpublished results). In a similar type of experiment, we found that Cin8p is stable in α-factor–arrested *cdh1*Δ cells (Figure 2B). These findings indicate that Cin8p degradation requires the Cdc16p subunit of the APC and the APC regulator Cdh1p.

It was reported previously that *cdh1*Δ cells cannot maintain an α-factor–mediated G1 arrest because of the accumulation of B-type cyclins that promote DNA synthesis (Amon *et al.*, 1994; Irniger and Nasmyth, 1997; Schwab *et al.*, 1997). In our experiments >90% of *cdh1*Δ cells treated with α-factor arrested with the unbudded morphology characteristic of G1 cells, but ~50% of the cells accumulated a 2*n* DNA content as judged by flow cytometric analysis (Hildebrandt and Hoyt, unpublished results). Therefore, it was possible that the stability of Cin8p observed in *cdh1*Δ cells is due to their leakage past the α-factor arrest into S-phase. However, two observations demonstrate that Cin8p degradation is indeed Cdh1p dependent. First, small unbudded G1 cells collected from asynchronous *cdh1*Δ cultures showed very high levels of Cin8p and Clb2p compared with wild-type G1 cells, in which Cin8p is barely detectable (Figure 2C). Second, as previously reported, we found that the deletion of *CLB2* from *cdh1*Δ cells reduced B-type cyclin levels sufficiently to allow α-factor to maintain a G1 arrest (Schwab *et al.*, 1997; and confirmed by Hildebrandt and Hoyt, unpublished results). As observed for the *cdh1*Δ single mutant, Cin8p was stable in the α-factor–arrested *cdh1*Δ *clb2*Δ double

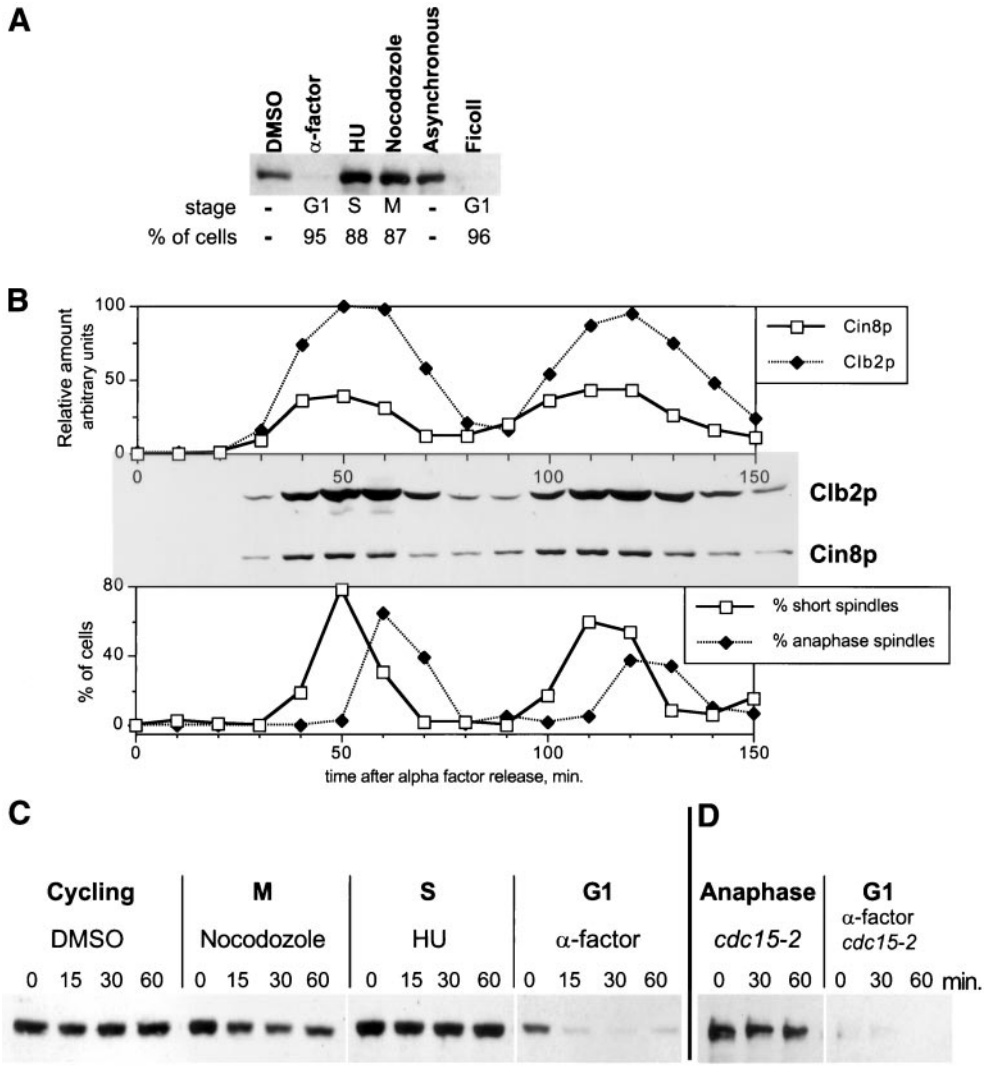


Figure 1. Cin8p oscillates and is unstable in G1. (A) Cin8p is absent from G1 cells. Yeast cells (MAY4823) expressing genomic *CIN8-3HA* were treated with α-factor, hydroxyurea (HU), nocodazole, and DMSO only for 3.7 h. Asynchronous cells were applied to a Ficoll gradient for collection of small G1 cells. The percentage of cells with the morphology characteristic of the respective cell cycle arrest is shown below the blot. (B) Cin8p levels fluctuate in the cell cycle. Yeast cells expressing genomic *CIN8-3HA* (MAY4822) were synchronized with α-factor in G1. Cells were washed and released into medium without α-factor. Samples were collected every 10 min and analyzed for Cin8p and Clb2p levels by Western blot (middle) and for the presence of bipolar spindles by tubulin immunofluorescence (bottom). The top panel shows quantification of Cin8p and Clb2p levels. (C) Cin8p is unstable in G1. Strain K1534 carrying a P_{GAL1} > *CIN8-3HA* plasmid was grown in medium with 2% raffinose. Cells were treated with α-factor, hydroxyurea, nocodazole, or DMSO for 3 h. Cin8p was then induced by incubation with 2% galactose, followed by addition of cycloheximide and glucose (t = 0 minutes). Samples were collected at the indicated times (minutes), and protein extracts were prepared. Equal amounts of protein were separated by gel electrophoresis and analyzed for Cin8p levels by Western blotting. (D) Cin8p is stable in late anaphase. A *cdc15-2* (MAY4751) strain carrying a P_{GAL1} > *6myc-SV40NLS-CIN8* plasmid was grown in synthetic medium plus raffinose at 22°C. Part of the culture was shifted to 37°C for 4.5 h to arrest in late anaphase. Another portion was treated with α-factor at 22°C for 4 h and then shifted to 37°C for 30 min to inactivate Cdc15-2p. Cin8p was induced with galactose for 90 min, followed by addition of cycloheximide and glucose (t = 0). In the α-factor–treated sample nearly all of the Cin8p was degraded by the time the first time sample was processed.

mutant (Figure 2B). The deletion of *CLB2* alone had no effect on Cin8p degradation.

We next investigated whether the APC regulator Cdc20p also affects Cin8p stability. Clb2p has been found to be partially degraded immediately after anaphase initiation via APC^{Cdc20}, whereas the remainder of its degradation occurs late in anaphase via APC^{Cdh1} (Baumer *et al.*, 2000a; Yeong *et al.*, 2000). The Cin8p degradation assay was performed in a *cdc20-1* temperature-sensitive strain arrested in α-factor. Cin8p was observed to be as unstable in the *cdc20-1* strain as in the wild-type control (Figure 2A), suggesting that Cdc20p does not affect Cin8p stability.

Cdc20p is also subject to APC^{Cdh1} degradation in G1 cells (Prinz *et al.*, 1998; Shirayama *et al.*, 1998; Goh *et al.*, 2000; Pfleger and Kirschner, 2000). We were concerned that in our degradation assay with the use of G1 cells Cdc20p was not present to activate the APC. Therefore, this assay may not be a fair test for Cdc20p's possible role in Cin8p degradation. To further address this possibility we utilized a second in vivo assay that does not depend on G1 arrest. Cin8p overexpression (i.e., from P_{GAL1}) is toxic and kills cells (Saunders *et al.*, 1997). However, the amount expressed from a moderate promoter, such as P_{GALS} or P_{MET25} (Mumberg *et al.*, 1994), does not kill (Figure 2D). We speculated that moderate overexpression of Cin8p may kill cells if it cannot be degraded. Indeed, we found that expression of Cin8p from P_{GALS} kills *cdh1*Δ cells when plated on galactose-containing medium on which the promoter is turned on but not on glucose medium on which the promoter is repressed (Figure 2D). In contrast, wild-type and *cdc20-1* cells were able to

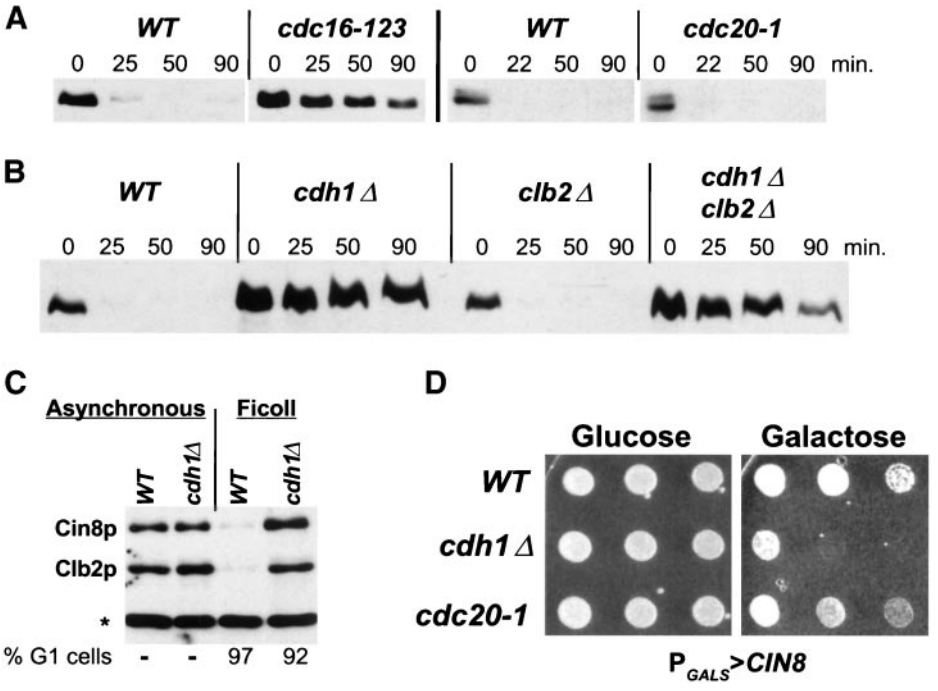


Figure 2. Cin8p degradation requires the APC subunit Cdc16p and the regulator Cdh1p but not Cdc20p. (A) Cin8p's instability in G1 requires *CDC16*, not *CDC20*. Wild-type (K1534, WT), *cdc16-123* (K4438), and *cdc20-1* (MAY5402) strains with P_{GAL1} > *CIN8-3HA* were examined by the G1 expression-pulse protocol described in Figure 1C except that the temperature was increased to 37°C beginning 30 min before the Cin8p pulse. (B) Cin8p is stabilized in G1-arrested *cdh1*Δ cells. Wild-type (K1534), *cdh1*Δ (MAY5353), *clb2*Δ (MAY6810), and *cdh1*Δ *clb2*Δ (MAY6812) strains with P_{GAL1} > *CIN8-3HA* were examined by the G1 expression-pulse protocol described in Figure 1C. (C) Cin8p is absent from G1 cells isolated from an asynchronous culture. Unbudded wild-type (MAY4822) and *cdh1*Δ (MAY5466) cells with genomic *CIN8-3HA* were isolated from a Ficoll gradient and analyzed for Cin8p and Clb2p levels. The asterisk indicates a background band recognized by the Clb2 antibody. (D) Moderate overexpression of *CIN8* kills *cdh1*Δ but not *cdc20-1* cells. Wild-type (MAY4822), *cdh1*Δ (MAY5466), and *cdc20-1* (MAY5017) with P_{GALS} > *CIN8* were spotted in serial dilution (left to right) onto solid synthetic medium containing either glucose (*CIN8* expression off) or galactose (*CIN8* expression on) and grown at 26°C.

tolerate moderate overexpression of Cin8p. Similar effects were found with the use of P_{MET25}-expressed Cin8p (Hildebrandt and Hoyt, unpublished results). Therefore, both assays indicate that it is unlikely that Cin8p degradation is dependent on the APC^{Cdc20} pathway (see DISCUSSION).

Cin8p Degradation Does Not Require a Canonical D-Box but Does Require Nuclear Localization and C-Terminal Sequences

The APC targets the degradation of substrates containing at least one of two destruction signal sequences, the D-box and the KEN-box (see INTRODUCTION). Cin8p has sequences that resemble both elements: a potential D-box (RICLMPAEN), starting at position 35 in its N terminus, and a potential KEN-box (KENATKD), starting at position 970 in the C terminus (Figure 3A). The N-terminal 70 amino acids of Cin8p, upstream of the conserved motor domain, are not required for Cin8p function in vivo (Hildebrandt and Hoyt, unpublished observation). We examined the stability of Cin8p missing the first 70 amino acids (ΔN70-Cin8p). This truncated Cin8p was still degraded with kinetics similar to wild-type (Figure 3B). Although the sequence beginning at amino acid 35 is the only perfect match to the D-box consensus, related sequences can be found beginning at amino acids 196, 292, 621, 751, and 1032 (matching the most highly conserved part of the D-box, RxxL/F; King *et al.*, 1996b). We altered each of these sites by either point mutation or deletion (example given below). In addition, a number of these were combined, creating double and triple mutants. None of these mutant Cin8p forms displayed altered stability (Hildebrandt and Hoyt, unpublished results). In contrast, single amino acid changes in a non–D-box sequence were able to greatly protect Cin8p from degradation (see below). Therefore, we conclude that the D-box-like sequences in Cin8p are not targeting it for degradation.

To assess the role of the C-terminal KEN-box (amino acids 970–976) we used an N-terminal 6myc-tagged Cin8p and made a series of C-terminal truncations. We found that deletion of as few as the seven C-terminal amino acids resulted in dramatic stabilization of Cin8p (Cin8-1031p, Figure 3C). In this case, the missing sequence (RKMLKIE) partially resembles the D-box consensus. However, mutation of the critical D-box residues, R1032 and L1035 to alanine, had no effect on degradation (Hildebrandt and Hoyt, unpublished results). *CIN8-1031* was able to complement *cin8*Δ. Although its protein product partially localized to spindle microtubules, most of it was cytoplasmic (Figure 3D). This is in contrast to the primarily nuclear microtubule localization of the wild type. We could restore localization to the nucleus and mitotic spindle by fusing an SV40NLS to the N terminus of Cin8-1031p, immediately after the 6myc tag (SV40NLS-Cin8-1031p, Figure 3D). Therefore, we have identified a sequence in Cin8p that partially contributes to nuclear localization. This sequence was not identified as an NLS by motif-searching computer programs (Nakai and Horton, 1999). The addition of the 6myc-SV40NLS tag to Cin8p missing its own NLS also restored Cin8p degradation (Figure 3C). We conclude that Cin8p must be properly localized to the nucleus to be targeted for degradation by the APC.

To examine the role of the KEN-box without disturbing Cin8p localization, we made a series of C-terminal trun-

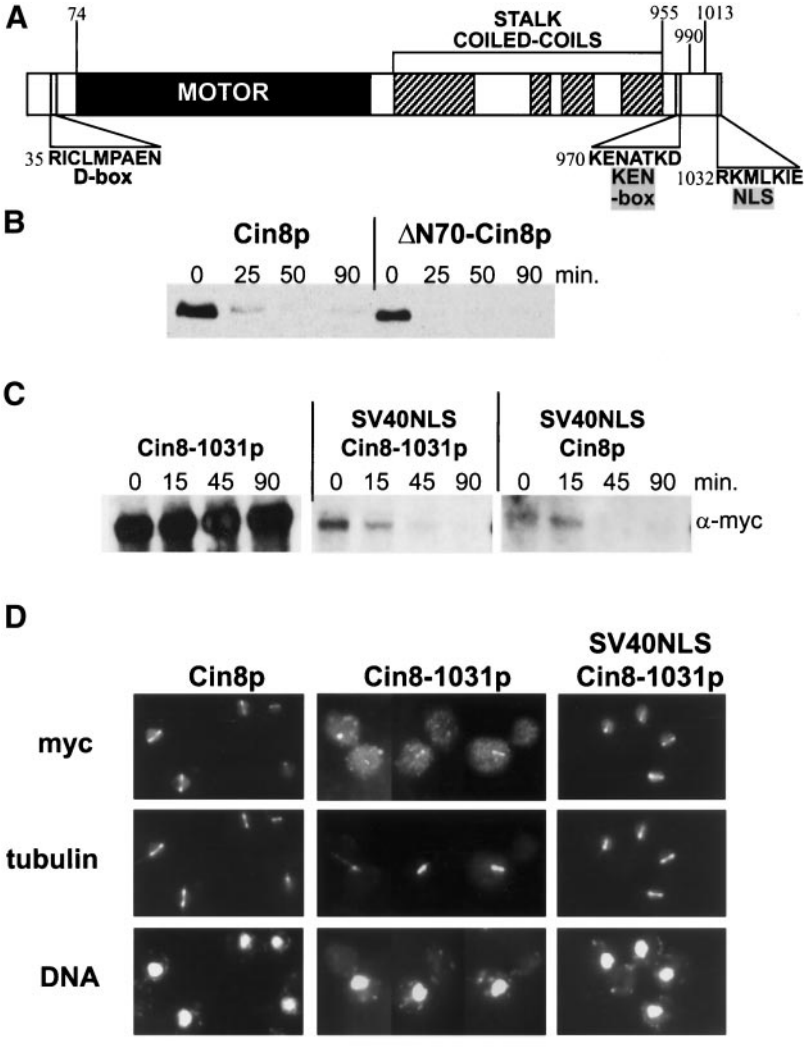


Figure 3. Cin8p degradation is D-box independent but requires localization to the nucleus. (A) Schematic of Cin8p domains drawn to scale. The potential D-box and KEN-box sequences are indicated as well as the region required for Cin8p nuclear localization. (B) Deletion of the D-box does not stabilize Cin8p. Wild-type (K1534) cells with plasmids expressing full-length Cin8p (P_{GAL1} > *CIN8-3HA*) or Cin8p with the D-box deleted (P_{GAL1} > Δ*N70-CIN8-3HA*) were subjected to the G1 expression-pulse protocol described in Figure 1C. (C) Cin8p degradation requires a functional NLS. A wild-type strain (K1534) carrying plasmids expressing Cin8p derivatives with Cin8p's C-terminal NLS and/or an N-terminal SV40NLS (P_{GAL1} > *6myc-CIN8-1031*; P_{GAL1} > *6myc-SV40NLS-CIN8-1031*; P_{GAL1} > *6myc-SV40NLS-CIN8,* from left to right) were subjected to the G1 expression-pulse protocol described in Figure 1C. (D) Demonstration that the SV40NLS restores nuclear localization to *CIN8-1031*. A *cin8*Δ *kip1*Δ strain (MAY5590) carrying 6myc-tagged versions of *CIN8* on high-copy plasmids (*6myc-CIN8; 6myc-CIN8-1031; 6myc-SV40NLS-CIN8-1031,* from left to right) were arrested at the short spindle stage with hydroxyurea and stained for Cin8p (myc), microtubules (tubulin), and DNA. Although most of Cin8-1031p is cytoplasmic, a portion still binds spindles.

cations in a version of Cin8p expressed from P_{GAL1} and containing the 6myc-SV40NLS tag at the N terminus. Surprisingly, a truncation to amino acid 990, which leaves the KEN-box (970–976) intact, was highly stabilized. In marked contrast, a longer form of Cin8p, ending at amino acid 1013, was still degraded with wild-type kinetics (Figure 4A). More extensively truncated forms that removed the KEN-box (955, 871) were stabilized to a similar extent as Cin8-990p. Therefore, if the KEN-box is involved in Cin8p degradation, it is not sufficient to act as the targeting signal. Additional information required for degradation must lie between amino acids 990 and 1013. In a further dissection of this region, we found a truncation to amino acid 998 did not affect Cin8p degradation (Figures 4B and 6C). Therefore, there are no residues essential for Cin8p degradation after amino acid 998. In contrast, removal of three additional residues, to amino acid 995, completely stabilized Cin8p. This indicates that critical residues for Cin8p's degradation lie between amino acids 995 and 998.

The Degradation Signal in Cin8p Is Bipartite and Includes a KEN-Box Plus Downstream Amino Acids

To define amino acids required to specify degradation, we generated random and site-directed mutants in the C terminus of Cin8p. Both types of mutants were subjected to two assays. The first, a colony growth assay, was based on the assumption that a form of Cin8p that is stable because it is missing a *cis*-acting degradation signal will be toxic when moderately overexpressed (from P_{GALS}), just as moderate overexpression of wild-type Cin8p was toxic to *cdh1*Δ cells (Figure 2D). The second test was the G1 expression-pulse analysis described above, utilizing mutant forms of 6myc-SV40NLS-Cin8p expressed from P_{GAL1} ("Degradation assay"). Examples are shown in Figure 5, and all findings are summarized in Figure 6. For the majority of mutants, the two assays resulted in similar conclusions.

To generate random mutants we applied mutagenic PCR to the DNA encoding amino acids 956-1015 of Cin8p. A library was created that expressed the Cin8p random mu-

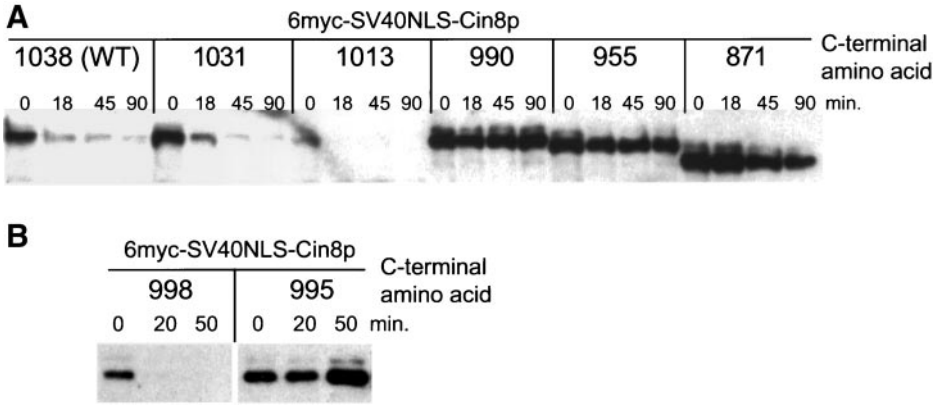


Figure 4. Cin8p's degradation signal is in the C terminus. Wild-type (K1534, WT) cells expressing versions of P_{GAL1} > *6myc-SV40NLS-CIN8,* truncated at the indicated amino acid, were subjected to the G1 expression-pulse protocol described in Figure 1C.

tants from the moderate-level *GALS* promoter. Expressed from P_{GALS}, wild-type Cin8p does not kill wild-type cells. The library was screened for Cin8p mutants that killed on galactose-containing media. Twenty-eight unique mutants that passed this test were identified. Several mutants were isolated two or more times and most of the mutants contained more than one amino acid change. Nineteen mutants had amino acid changes in the potential KEN-box. The majority of these had changes in the first conserved asparagine (KE*N*xxxN/D) including two mutants where this was the only amino acid changed (043 and KED/065). The KEN-box mutants were very toxic on galactose plates and stable in the degradation assay (e.g., KED/065, Figure 5A).

Interestingly, we recovered nine mutants that had an intact KEN-box (Figure 6B). Seven of these had changes that cluster in the region between amino acids 991 and 998, contained within the region that we had identified earlier as important for the degradation of Cin8p. We are designating this second region "element 2" (borders are defined in Figure 8A). The two mutants with changes in only element 2 (090 and 145, Figure 5A) were very toxic on galactose plates and produced stable proteins in the degradation assay. Three of the non–KEN-box mutants had mutations in the residue just before the KEN-box 084, 122, and 182). Mutant 182, with only one amino acid change, exhibited only weakly stabilized Cin8p. A number of KEN-box and element 2 mutants had other changes located upstream, downstream, or between these elements. It is likely that many of these are silent changes caused by the mutagenesis protocol, but we cannot rule out that some affect Cin8p stability. Taken together, the results from this screen demonstrate two important points: first, the KEN-box is required for Cin8p's degradation, and second, an element downstream from the KEN-box is also required.

To further define the sequence requirements for Cin8p degradation, we generated site-directed mutants in the C terminus of Cin8p and tested their stability (Figures 5B and 6C). Combined mutation of all four critical residues in the KEN-box (KENxxxN/D) to alanine (alaKEN) resulted in a stable Cin8p. However, the extent of toxicity in the P_{GALS} assay was not as strong as other stable mutants, including single KEN-box changes (e.g., KED), perhaps because Cin8p activity was affected. The effects of the random and site-directed KEN-box mutants clearly indicate that it is an essential element targeting Cin8p for degradation.

We made various deletion and alanine mutations downstream of the KEN-box (Figures 5B and 6C). Alanine-scanning mutagenesis identified a patch of residues, MPLR (amino acids 993–996), as essential for degradation. Within this patch, changes to alanine of leucine 995 (L995A) and to a lesser extent proline 994 (P994A) had the greatest stabilizing effect. L995 was also identified in the random mutagenesis screen (145). For both mutants the changes at L995 were conservative, suggesting that very specific recognition of this residue is required for degradation.

One other patch of alanine mutations had a stabilizing effect. Changing a series of six serines and asparagines to alanine between amino acids 998 and1004 (SN-NSNS) partially stabilized Cin8p. A deletion encompassing this region was also stabilized (Δ996-1004). Neither scrambling the amino acids in this region (scr996) nor making changes in one or a few of these residues had much effect (LSNI; S998,1002A; ala996; SNSN). In fact, no individual serine alteration, including a potential CDK1 phosphorylation site (S1010), seemed to affect Cin8p degradation, ruling out the likelihood of a critical phosphorylation event. Therefore, it appears that residues after amino acid 998 contribute to Cin8p's degradation signal, in the context of the full-length protein, even though they are not essential.

We investigated the possibility that the region downstream from the KEN-box contains the ubiquitination target residues. There are two pairs of lysines, which when mutated to alanines in pairs (KK991 and KK1012), had only a mild effect on Cin8p stability. When all four lysines were mutated together, Cin8p was partially stabilized (KK991,KK1012, Figure 5B). Providing four C-terminal lysines alone to an otherwise stable Cin8p truncation (992 plus KK) did not rescue degradation. Therefore, it is possible that one (or more) of these downstream lysines serves as a target for the covalent addition of ubiquitin by the APC, although none appeared to be absolutely required for Cin8p degradation. We could not examine the site of ubiquitin addition, unfortunately, because we were unable to detect ubiquitinated forms of Cin8p with the use of established methods (Willems *et al.*, 1996; Zachariae and Nasmyth, 1996). Because the KEN-box has been demonstrated to be a target signal for ubiquitin addition by APC^{Cdh1}, it is likely that a ubiquitinated form of Cin8p is the target for degradation (Pfleger and Kirschner, 2000).

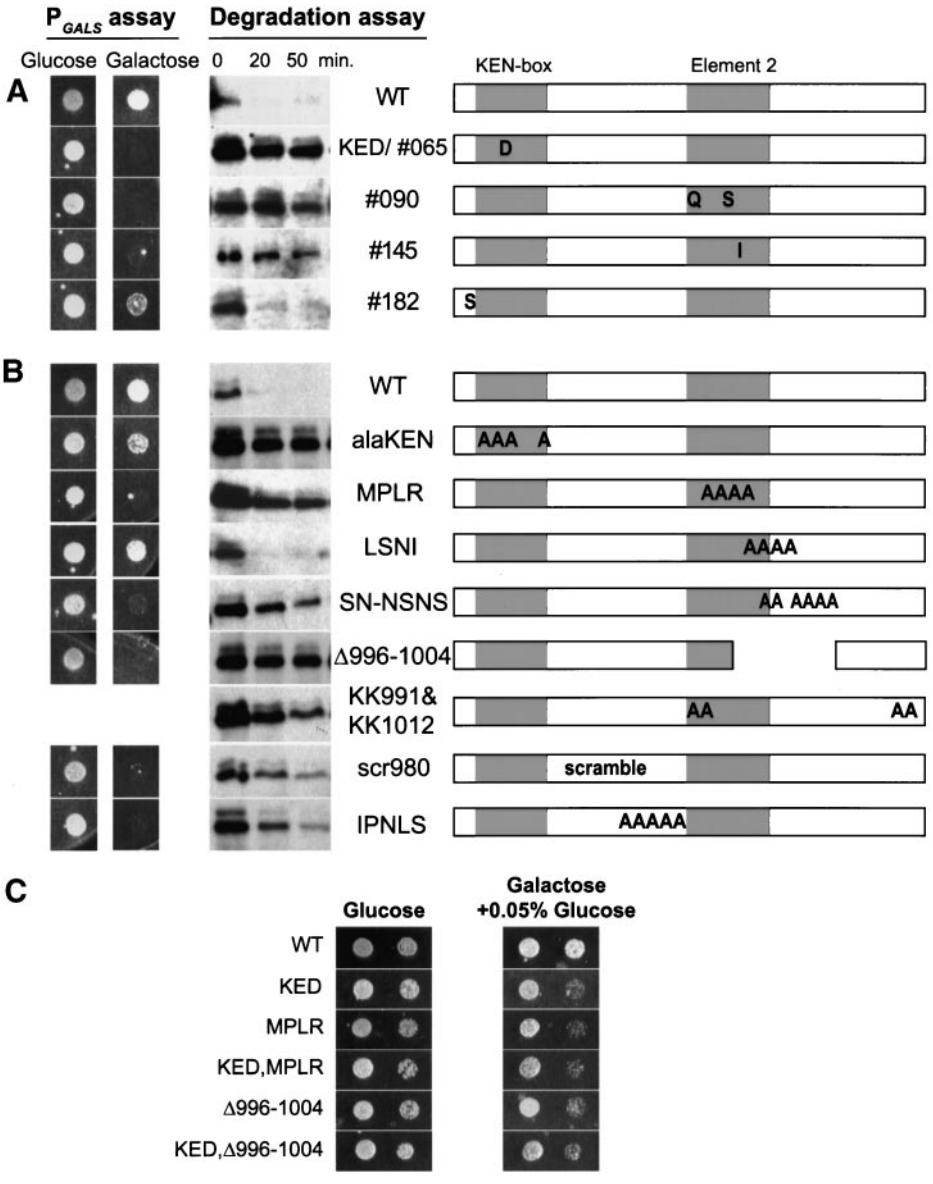


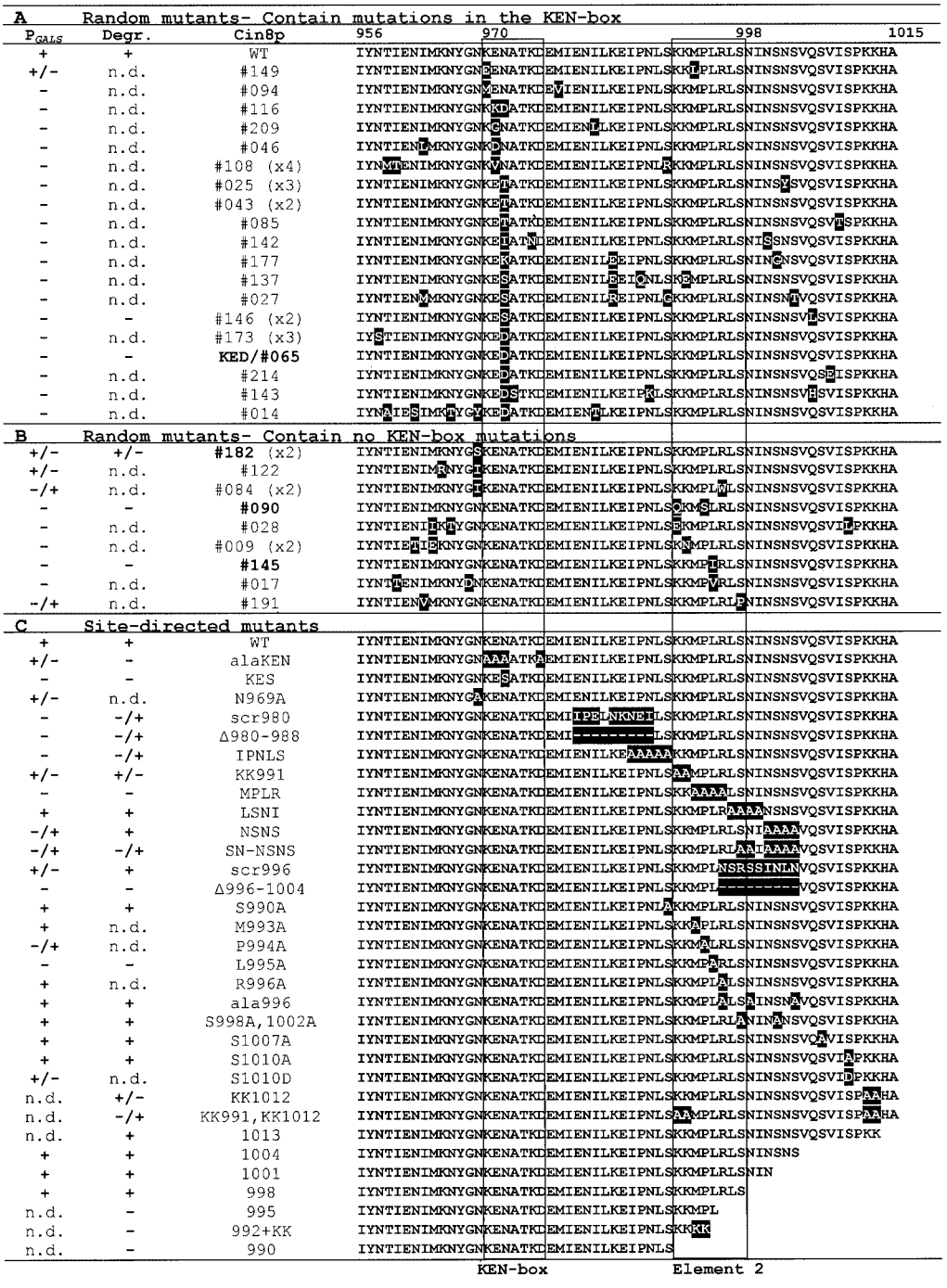
Figure 5. Moderate overexpression and stability of random and site-directed Cin8p mutants. Select random (A) and site-directed (B) Cin8p mutants were tested for toxicity when moderately overexpressed from P_{GALS} (P_{GALS} assay) and for Cin8p stability in vivo (degradation assay). P_{GALS} assay: Wild-type (MAY2422, WT) cells carrying mutant derivatives of P_{GALS} > *CIN8* were spotted onto solid synthetic medium with either glucose or galactose (as in Figure 2D except a single dilution spot is shown). Degradation assay: P_{GAL1} > *6myc-SV40NLS-CIN8* derivatives of the mutants were tested for stability in strain K1534 by the G1 expression-pulse protocol described in Figure 1C. The figures on the right depict the positions of mutant sites in the amino acid 956-1015 region of Cin8p. The boundaries of element 2 are defined in Figure 8A. Precise sequence changes are described in Figure 6. (C) Wild-type (MAY2422) cells carrying mutant derivatives of P_{GALS} > *CIN8* were spotted (as in Figure 2D except two dilution spots are shown) onto solid synthetic medium with either glucose or galactose plus 0.05% glucose as carbon sources.

Mutation and deletion of the region between the KEN-box and element 2 also partially stabilized Cin8p (e.g., scr980 and IPNLS, Figure 5B). We have not identified precise residue requirements in this region. It is possible that this region acts as a spacer between the KEN-box and element 2.

Finally, to address the possibility that the KEN-box and element 2 were acting independently and additively, we created double mutants and compared their effects with single mutants. This was difficult, however, because the single mutants exhibited maximum effects in our two assays. Single mutants often appeared fully stable in degradation assays (i.e., compare the −990 single mutant to the −955 double mutant in Figure 4A) and were lethal in the P_{GALS} assay. Therefore, we compared single and double mutants in the P_{GALS} assay in which the level of transcription was reduced by addition of 0.05% glucose to the galactose medium (Figure 5C). Single mutants in the KEN-box (KED) and element 2 (MPLR and Δ996-1004) were slow-growing compared with wild type but not lethal on galactose/0.05% dextrose medium. Double mutants (KED,MPLR and KED,Δ996–1004) were affected to the same extent as the single mutants.

The C Terminus of Cin8p Is Necessary and Sufficient to Target Degradation

One hallmark of destruction signal sequences is that they can confer instability to a heterologous, normally stable polypeptide. We investigated whether the C-terminal region of Cin8p possessed this property by fusing it to the nuclear-localized DNA-binding domain of Gal4p (Silver *et al.*, 1984). Amino acids 956-1031 of Cin8p, encompassing



P_{GALS}	Degr.	Cin8p	956 970 998 1015
A	Random mutants- Contain mutations in the KEN-box		
+	+	WT	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
+/-	n.d.	#149	IYNTIENIMKNYGNDENATKDEMIENILKEIPNLSKKIPLRLSNINSNSVQSVISPKKHA
-	n.d.	#094	IYNTIENIMKNYGNYENATKDEVIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	n.d.	#116	IYNTIENIMKNYGNKKDATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	n.d.	#209	IYNTIENIMKNYGNKGNATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	n.d.	#046	IYNTIENLMKNYGNKDNATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	n.d.	#108 (x4)	IYNMTENIMKNYGNKVNATKDEMIENILKEIPNLRKKMPLRLSNINSNSVQSVISPKKHA
-	n.d.	#025 (x3)	IYNTIENIMKNYGNKETATKDEMIENILKEIPNLSKKMPLRLSNINSYSVQSVISPKKHA
-	n.d.	#043 (x2)	IYNTIENIMKNYGNKETATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	n.d.	#085	IYNTIENIMKNYGNKETATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVHSPKKHA
-	n.d.	#142	IYNTIENIMKNYGNKEIATNDEMIENILKEIPNLSKKMPLRLSNISSNSVQSVISPKKHA
-	n.d.	#177	IYNTIENIMKNYGNKEKATKDEMIENILEEIPNLSKKMPLRLSNINGNSVQSVISPKKHA
-	n.d.	#137	IYNTIENIMKNYGNKESATKDEMIENILEEIQNLSKEMPLRLSNINSNSVQSVISPKKHA
-	n.d.	#027	IYNTIENYMKNYGNKESATKDEMIENILREIPNLGKKMPLRLSNINSNTVQSVISPKKHA
-	-	#146 (x2)	IYNTIENIMKNYGNKESATKDEMIENILKEIPNLSKKMPLRLSNINSNSVHSVISPKKHA
-	n.d.	#173 (x3)	IYSTIENIMKNYGNKEDATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	-	**KED/#065**	IYNTIENIMKNYGNKEDATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	n.d.	#214	IYNTIENIMKNYGNKEDATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSEISPKKHA
-	n.d.	#143	IYNTIENIMKNYGNKEDSTKDEMIENILKEIPKLSKKMPLRLSNINSNSVHSVISPKKHA
-	n.d.	#014	IYNAIESIMKTYGYKEDATKDEMIENTLKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
B	Random mutants- Contain no KEN-box mutations		
+/-	+/-	**#182** (x2)	IYNTIENIMKNYGSKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
+/-	n.d.	#122	IYNTIENIMRNYGIKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-/+	n.d.	#084 (x2)	IYNTIENIMKNYGIKENATKDEMIENILKEIPNLSKKMPLWLSNINSNSVQSVISPKKHA
-	-	**#090**	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSQKMSLRLSNINSNSVQSVISPKKHA
-	n.d.	#028	IYNTIENIIKTYGNKENATKDEMIENILKEIPNLSEKMPLRLSNINSNSVQSVILPKKHA
-	n.d.	#009 (x2)	IYNTIETIEKNYGNKENATKDEMIENILKEIPNLSKNMPLRLSNINSNSVQSVISPKKHA
-	-	**#145**	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPIRLSNINSNSVQSVISPKKHA
-	n.d.	#017	IYNTHENIMKNYDNKENATKDEMIENILKEIPNLSKKMPVRLSNINSNSVQSVISPKKHA
-/+	n.d.	#191	IYNTIENVMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLPNINSNSVQSVISPKKHA
C	Site-directed mutants		
+	+	WT	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
+/-	-	alaKEN	IYNTIENIMKNYGNAAAATKAEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	-	KES	IYNTIENIMKNYGNKESATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
+/-	n.d.	N969A	IYNTIENIMKNYGAKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKKHA
-	-/+	scr980	IYNTIENIMKNYGNKENATKDEMIIPELNKNELLSKKMPLRLSNINSNSVQSVISPKKHA
-	-/+	Δ980-988	IYNTIENIMKNYGNKENATKDEMI---------LSKKMPLRLSNINSNSVQSVISPKKHA
-	-/+	IPNLS	IYNTIENIMKNYGNKENATKDEMIENILKEAAAAAKKMPLRLSNINSNSVQSVISPKKHA
+/-	+/-	KK991	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSAAMPLRLSNINSNSVQSVISPKKHA
-	-	MPLR	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKAAAALSNINSNSVQSVISPKKHA
+	+	LSNI	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRAAAANSNSVQSVISPKKHA
-/+	+	NSNS	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNIAAAAVQSVISPKKHA
-/+	-/+	SN-NSNS	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLAAIAAAAVQSVISPKKHA
+/-	+	scr996	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLNSRSSINLNVQSVISPKKHA
-	-	Δ996-1004	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPL---------VQSVISPKKHA
+	+	S990A	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLAKKMPLRLSNINSNSVQSVISPKKHA
+	n.d.	M993A	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKAPLRLSNINSNSVQSVISPKKHA
-/+	n.d.	P994A	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMALRLSNINSNSVQSVISPKKHA
-	-	L995A	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPARLSNINSNSVQSVISPKKHA
+	n.d.	R996A	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLALSNINSNSVQSVISPKKHA
+	+	ala996	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLALSAINSNAVQSVISPKKHA
+	+	S998A,1002A	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLANINANSVQSVISPKKHA
+	+	S1007A	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQAVISPKKHA
+	+	S1010A	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVIAPKKHA
+/-	n.d.	S1010D	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVIDPKKHA
n.d.	+/-	KK1012	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPAAHA
n.d.	-/+	KK991,KK1012	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSAAMPLRLSNINSNSVQSVISPAAHA
n.d.	+	1013	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNINSNSVQSVISPKK
+	+	1004	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNINSNS
+	+	1001	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLSNIN
+	+	998	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPLRLS
n.d.	-	995	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKMPL
n.d.	-	992+KK	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLSKKKK
n.d.	-	990	IYNTIENIMKNYGNKENATKDEMIENILKEIPNLS
			KEN-box Element 2

Figure 6. Summary of mutants in the region of KEN-box and element 2. Summary of findings from the two assays (P_{GALS} and degradation assay) to test the stability of random and site-directed Cin8p mutants. A "+" under P_{GALS} indicates wild-type levels of growth on galactose-containing media, and a "−" indicates death on galactose. A "+" in the degradation column (Degr.) indicates a wild-type (WT) level of instability, and a "−" indicates that the mutant was stable. "n.d." means the mutant was not tested. Numbers given in parentheses (e.g., x2) in the Cin8p column indicate the number of times a particular random mutant was isolated. Amino acid positions are indicated at the top of the right column. The KEN-box and element 2 are boxed.

the region after the fourth coiled-coil domain and up to but not including Cin8p's NLS, were fused to Gal4BD (Figure 7A).

The Gal4BD 19-kDa polypeptide was stable ($t_{1/2} \sim 40$ min) when transiently expressed from the *GAL1* promoter in the expression-pulse assay (Figure 7B). In contrast, the

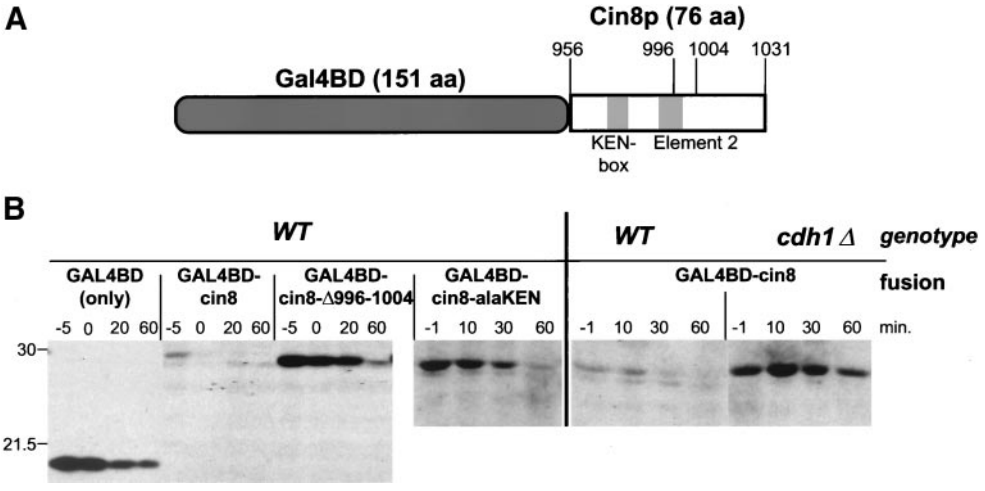


Figure 7. The C terminus of Cin8p destabilizes a heterologous protein. (A) Schematic of Gal4BD-Cin8p fusion (to scale). (B) The C terminus of Cin8p is necessary and sufficient for degradation. Wild-type (K1534, WT) or *cdh1*Δ (MAY5353) strains carrying plasmids expressing Gal4BD (P_{GAL1} > *GAL4BD*) or Gal4BD fused to a C-terminal portion of wild-type Cin8p (P_{GAL1} > *GAL4BD-cin8*), Cin8-Δ996-1004p (P_{GAL1} > *GAL4BD-cin8-Δ996-1004*), or Cin8-alaKENp (P_{GAL1} > *GAL4BD-cin8-alaKEN*) were subjected to the to the G1 expression-pulse protocol described in Figure 1C. Samples were collected 1–5 min before the end of the galactose pulse (−1 or −5 min) and at times indicated after the pulse. The wild-type fusion was nearly completely degraded by the time the first time sample was processed. The positions of molecular weight standards are shown on the left. The Western blot was probed with anti-Gal4BD antibody.

Gal4BD-Cin8p fusion (26.5 kDa) was unstable ($t_{1/2}$ < 5 min) in wild-type cells but stabilized in a *cdh1*Δ strain. Therefore, this 76-amino acid region of Cin8p causes the Gal4BD fusion to be degraded in an APC^{Cdh1}-dependent manner.

We next deleted nine residues in the Cin8p portion of the fusion protein, within element 2. Pulse-chase analysis revealed this fusion was stable ($t_{1/2}$ ~ 30 min, Figure 7B, Gal4BD-cin8-Δ996-1004). Mutation of the KEN-box also stabilized the fusion protein ($t_{1/2}$ ~ 20 min, Gal4BD-cin8-alaKEN). These findings demonstrate that amino acids 956-1031 from the C terminus of Cin8p are sufficient to confer APC-dependent degradation of a heterologous protein and that both the KEN-box and element 2 are important for this degradation signal.

Expression of Stable Cin8p Causes Mild Spindle Defects but Degradation Is Not Essential for Normal Cell Cycle Progression

We predicted that degradation of Cin8p, expressed from its endogenous promoter, would not be an essential process because the regulator Cdh1p is not essential. However, moderate overexpression of stable mutants from P_{GALS} caused lethality, in contrast to wild-type Cin8p (Figure 5). We examined the phenotype of these cells (P_{GALS}-*cin8-Δ996-1004* or P_{GALS}-*cin8-KED* expressed for 3–8 h) and observed that ~50% of cells accumulated at the large-budded stage (compared with 20% for the P_{GALS} control) with mostly unsegregated nuclei. Approximately 60% of budded cells had no spindle (10% for the control). These observations are consistent with an arrest in mitosis with a failure to build or

Table 2. Cells expressing stable Cin8p accumulate mitotic cells with no spindle

	Large-budded (mitotic) cells with[a]			
	No spindle	Short spindle	Anaphase spindle	
Relevant genotype[b]	(%)	(%)	(%)	(n)
CIN8	21	47	32	383
cin8-KED	42	38	20	309
cin8-Δ996-1004	36	42	22	480

[a] The indicated strains were grown to midlog phase, fixed, and stained for microtubules and DNA. Large-budded cells were selected microscopically, and their microtubules were observed by indirect immunofluorescence. Short spindles were defined as a bright bar of microtubule staining approximately half the diameter of the mother cell body (approximately 2–3 μm). Anaphase spindles were defined as a line of microtubule staining extending into both mother and bud and from half to two cell body diameters in length. "No spindle" cells contained no clearly observable bipolar structure but possessed clear arrays of microtubules in either one or both cell bodies.

[b] *cin8*Δ strain (MAY2063) carrying a low-copy *CEN* plasmid with *CIN8*, *cin8-KED*, or *cin8-Δ996-1004*.

maintain bipolar spindles. Moderate overexpression of wild-type Cin8p caused a similar but less severe phenotype.

We assessed the phenotypic consequence of stable forms of Cin8p expressed from its endogenous promoter. Stable Cin8p mutants (e.g., *cin8-alaKEN; cin8-KED; cin8-Δ996-1004; cin8-MPLR; cin8-KED,Δ996-1004*) were fully capable of complementing the *cin8Δ* defect (see MATERIALS AND METHODS). No differences were observed between wild type and stable forms for their ability to support cell growth over a wide range of temperatures. Furthermore, stable Cin8p forms caused no obvious delay to the cell cycle as assessed by flow cytometric analysis of DNA content and cell morphology (Hildebrandt and Hoyt, unpublished results). There was a subtle difference in spindle morphology, however, when we observed the population of mitotic cells in asynchronous cultures. There were 15–21% fewer large-budded cells with obvious bipolar spindle structures in strains expressing stable mutants of Cin8p (*cin8-KED* and *cin8-Δ996-1004*) compared with wild-type Cin8p (Table 2). This reduction in bipolar spindles was attributed to a combined reduction in the fraction of cells with preanaphase spindles (short spindles) and anaphase spindles.

DISCUSSION

Cin8p's Degradation Signal Has Two Essential Parts: a KEN-Box and a Downstream Element

We have found that Cin8p is degraded in an APC^{Cdh1}-dependent manner in the period from late mitosis through G1 phase. We could find no evidence for a functioning canonical D-box sequence in Cin8p. Instead, targeting for degradation appeared to be accomplished exclusively by a C-terminal region that includes a KEN-box. The KEN-box was originally defined by in vitro ubiquitination and proteolysis studies of human Cdc20 (Pfleger and Kirschner, 2000). It's role in targeting proteins for destruction in vivo has been demonstrated for human Cdc6, securin, and *Drosophila* cyclin A, where it appears to share duties with a closely located D-box (Petersen *et al.*, 2000; Jacobs *et al.*, 2001; Zur and Brandeis, 2001). Our finding that KEN-box–mediated destruction occurs in *S. cerevisiae* demonstrates that it is conserved across distantly related eukaryotic species.

In the in vitro analysis of hCdc20, a polypeptide terminating in the sequence KEN was able to serve as a substrate for APC^{Cdh1}-mediated ubiquitination (Pfleger and Kirschner, 2000). The final N/D residue in the KEN-box consensus (KENxxxN/D) was found to contribute to degradation of only certain experimental substrates. In the cases of human Cdc6, securin, and *Drosophila* cyclin A, the extent of the sequence required for KEN-box function was not determined. With the use of an in vivo approach to dissect Cin8p's degradation signal we also identified the KEN-box as being critical for APC^{Cdh1}–mediated degradation. We found that the KEN-box was necessary but not sufficient to target this protein for destruction in vivo. Additional amino acids located up to 22 residues downstream (element 2, Figure 8A) were required. For example, conservative changes affecting leucine 995 resulted in stabilization of Cin8p to the same extent as KEN-box mutations (Figure 5).

We note that element 2 sequences in Cin8p and other related BimC kinesins (see below and Figure 8B) possess a weak match to part of the D-box consensus (RxxL). Although it remains possible that the KEN-box and element 2 could act additively, as observed for the KEN-box and D-box of human Cdc6, securin, and *Drosophila* cyclin A, our findings suggest that for Cin8p they act as a single unit specifying degradation. Mutations affecting either element caused effects of similar magnitude. In addition, KEN-box/element 2 double mutants were no more severely affected than either single mutant in our assays (Figure 5C; also, compare truncation mutant Cin8-990p, which has the KEN-box but not element 2, to Cin8-955p, which has neither in Figure 4A). This is consistent with both elements contributing to the same degradative mechanism.

Until there is structural data for how the APC recognizes substrates, we cannot distinguish whether element 2 is a novel destruction signal, a cryptic D-box, or an extension of the KEN-box. We can, however, suggest a few molecular explanations for Cin8p's apparent bipartite destruction sequence. First, the APC^{Cdh1} may recognize the entire region, with the identity of the intervening amino acids being less critical than the specific sequences of the KEN-box and element 2. The less stringent requirement for downstream amino acids noted by Phleger and Kirschner (2000), therefore, may be a consequence of different experimental substrates or systems. Second, APC-targeting signals may need to appear within a particular protein structure for recognition. For example, the sequence with a perfect match to the D-box consensus in the N terminus of Cin8p did not specify degradation (this study) and the D-box in *Xenopus* cyclin A requires the presence of a downstream region for it to act as a degradation signal (Stewart *et al.*, 1994; King *et al.*, 1996b). Thus, element 2 may contribute to the proper structural context for KEN-box recognition by APC^{Cdh1}. Third, element 2 could confer a specific Cin8p function or localization causing it to become susceptible to the actions of APC^{Cdh1}. This seems unlikely, however, because element 2 was important for degradation of the Gal4BD-Cin8p fusion, which has no motor activity and presumably is bound to DNA rather than microtubules. Finally, it might confer an additional level of regulation to certain APC substrates. For instance, overexpression of *CDH1* reduced Clb2p levels in asynchronous cultures (Schwab *et al.*, 1997; Visintin *et al.*, 1997), implying that Cdh1p is limiting for Clb2p degradation. However, we were unable to see a similar effect on Cin8p levels upon *CDH1* overexpression (Hildebrandt and Hoyt, unpublished results). Therefore, additional factors may be limiting for Cin8p degradation.

Some, but not all, BimC motors possess a KEN-box–like sequence 24–196 residues beyond the end of the predicted coiled-coil regions in their C termini (a notable exception is Kip1p). All those with a C-terminal KEN-box also possess conserved element 2 sequences downstream (Figure 8B, with the exception of KLP61F, which may not have a complete KEN-box). Therefore, it seems likely that the combined KEN-box/element 2 sequence is utilized by the APC^{Cdh1} for targeting of other BimC motors as well.

APC^{Cdh1}- Versus APC^{Cdc20}-mediated Destruction of Cin8p

Some APC substrates are recognized by both APC^{Ccd20} and APC^{Cdh1}. Hsl1p has a D-box, which is responsible for binding interactions with Cdc20p and Cdh1p (Burton and Solomon, 2000). Additionally, the APC substrate Clb2p is degraded in a biphasic manner, first by APC^{Cdc20} in early anaphase and then by APC^{Cdh1} during telophase and G1 (Baumer *et al.*, 2000a; Yeong *et al.*, 2000). Despite the similar cell cycle profiles that we observed for Cin8p and Clb2p, Cin8p appeared to be degraded exclusively via the APC^{Cdh1}. First, *cdc20-1* cells were not sensitive to moderate overexpression of Cin8p, but *cdh1Δ* cells were. Second, Cin8p was stable in late anaphase (Figure 1D), a stage when Cdc20p is presumably active. Third, Cin8p levels were unaffected by

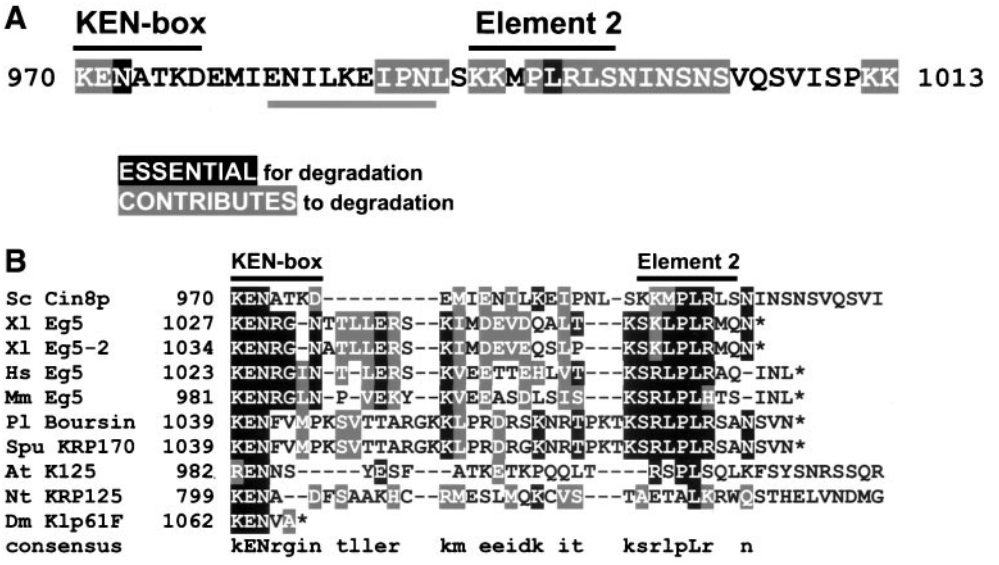


Figure 8. The destruction sequence in Cin8p and possibly other BimC motors is bipartite. (A) Summary of specific amino acid contributions to Cin8p's degradation signal. The C-terminal side of element 2, amino acid 998, is defined by the shortest truncated form that still supports degradation. The N-terminal side is defined by the positions of the random non–KEN-box mutants (Figure 6B). Note that the borders of element 2 are arbitrary because surrounding amino acids are also important. "Essential" amino acids are those with a single point mutant-stabilized Cin8p. "Contributes" to degradation means the highlighted amino acid had a partial effect when individually mutated or Cin8p was completely stabilized when the residue was mutated in combination with other amino acids. Underlined residues show a region that partially affects Cin8p degradation when deleted or scrambled. (B) Alignment of BimC motors that have a KEN-box–like sequence in the C-terminal domain, after the last coiled-coil. The alignment was performed on sequences starting at the KEN-box (amino acid positions indicated) with the use of the PIMA 1.4 (Smith and Smith, 1992) and BoxShade 3.21 alignment programs and final manual adjustment. Asterisks represent the C terminus of the protein. In the consensus line, capital letters represent identical residues, and lower case letters represent conserved residues. Greater than 40% of sequences must agree for shading. Sc, *S. cerevisiae*; Xl, *Xenopus laevis*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Pl, *Paracentrotus lividus*; Spu, *Strongylocentrotus purpuratus*; At, *Arabidopsis thaliana*; Nt, *Nicotiana tabacum*; Dm, *Drosophila melanogaster*.

Cdc20p overexpression (Hildebrandt and Hoyt, unpublished results). Fourth, Cin8p does not have a functioning canonical D-box, the only known targeting signal for APC^{Cdc20}. Finally, because the Cin8p motor makes the greatest contribution to anaphase spindle elongation (Saunders *et al.*, 1995), it seems unlikely that it should be targeted for destruction as cells initiate anaphase. Nevertheless, our studies cannot rule out the possibility that a small fraction of Cin8p degradation is mediated by the APC^{Cdc20} pathway.

Cin8p Must be Nuclear for Degradation

Cin8p's NLS and destruction signal were found in its C-terminal tail domain. Another sequence element conserved in the C-terminal domain of most BimC motors (but not Cin8p and Kip1p) is the "BimC-box." This region contains a recognition sequence for the cdc2 kinase that has been demonstrated to perform an important role in cell cycle-regulated motor localization (Blangy *et al.*, 1995; Sawin and Mitchison, 1995; Sharp *et al.*, 1999). Thus, a conserved property of this motor family may be that determinants of regulation and localization are found in the C-terminal tail.

APC subunits have been found to localize to nuclei and are often associated with spindles, centrosomes, and kinetochores (Tugendreich *et al.*, 1995; Lim *et al.*, 1998; Huang and Raff, 1999; Kurasawa and Todokoro, 1999). In *S. cerevisiae*, mutations disrupting nucleocytoplasmic transport also disrupt APC-mediated degradation (Loeb *et al.*, 1995; Baumer *et al.*, 2000b). In *Schizosaccharomyces pombe*, the failure to localize the proteosome to the nucleus was found to inhibit the destruction of securin and a mitotic cyclin (Tatebe and Yanagida, 2000). Therefore, it is expected that APC substrates may be required to enter the nucleus to become destabilized, especially in fungal cells that do not exhibit mitotic nuclear envelope breakdown. Our findings verify this prediction. C-terminal mutations that reduced the ability of Cin8p to enter the nucleus greatly stabilized the protein. The addition of a heterologous NLS to the N terminus restored its susceptibility to the degradative machinery (Figure 3).

Why Does Cin8p Need to Be Degraded?

Unlike APC^{Cdc20}, the APC^{Cdh1} degradation pathway is not essential for *S. cerevisiae* viability. However, *cdh1*Δ cells display clear growth defects (Schwab *et al.*, 1997; Visintin *et al.*, 1997). Likewise, *S. cerevisiae* cells are quite sensitive to the level of Cin8p activity. When Cin8p was overexpressed in cells arrested with preanaphase spindles, the spindles overelongated as if in anaphase (Saunders *et al.*, 1997). Moderate overexpression of a nondegradable form of Cin8p was toxic to cells (Figure 5). Expression of stable Cin8p mutants from the endogenous promoter caused no obvious growth defects, but we did observe an increased number of preanaphase and late anaphase cells with no bipolar spindles (Table 2).

Bipolar spindle formation can fail when the balance of spindle motor forces is perturbed. This has been observed both for loss of BimC activity (Enos and Morris, 1990; Hagan and Yanagida, 1990; Hoyt *et al.*, 1992; Roof *et al.*, 1992; Sawin *et al.*, 1992; Heck *et al.*, 1993; Blangy *et al.*, 1995) and when Cin8p is overexpressed, leading to spindle overelongation and breakage (Saunders *et al.*, 1997; and this study). We speculate that one of the main functions of Cin8p degradation is to reset motor activity to a low, premitotic state important for the early stage of spindle assembly. Because the degradation pathway for Cin8p is not essential and expression of stable Cin8p is only slightly detrimental to cell division, there may exist an additional mechanism for regulating Cin8p motor activity in vivo.

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