Degradation of the kinesin Kip1p at anaphase onset is mediated by the anaphase-promoting complex and Cdc20p

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Edited by Joan V. Ruderman, Harvard Medical School, Boston, MA, and approved September 6, 2001 (received for review April 30, 2001)

Kip1p of *Saccharomyces cerevisiae* is a bipolar kinesin in the conserved bimC kinesin subfamily that mediates mitotic spindle–pole separation. Here, we show that Kip1p is regulated immediately after anaphase initiation by its rapid degradation. Degradation required the ubiquitin protein ligase called the anaphase-promoting complex, the anaphase-promoting complex activating protein Cdc20, and a unique 43-aa sequence in Kip1p. Degradation also required import of Kip1p into the nucleus, but occurred independently of spindle association. A mutation that stabilized Kip1p impaired anaphase progression. The timing of degradation suggests that Kip1p functions primarily during spindle assembly and metaphase, and that Kip1p degradation facilitates structural changes in the mitotic spindle as anaphase progresses.

Assembly of the mitotic spindle and segregation of chromosomes during mitosis is accomplished by a series of microtubule-based movements generated in part by motor proteins. Progress in elucidating the function of mitotic motor proteins has made it clear that distinct motor proteins are specialized to perform particular movements. For instance, one class of motor proteins bundles microtubules and catalyzes microtubule–microtubule sliding; several different types of motor proteins located at the kinetochores mediate microtubule capture and chromosome positioning (1, 2). Motor protein specialization implies that the activities of different motor proteins must be regulated so that the events of mitosis occur in the correct order and at the right time. Thus, one key to understanding mitosis is to elucidate how motor protein activity is turned on and off.

A major class of kinesins that cross-link and slide adjacent microtubules is the bimC subfamily, whose members share particularly high sequence conservation in their motor domains and, where examined, have similar functions (3). Inhibition of bimC kinesin function in many organisms results in the formation of monopolar spindles (4–12). Although the nonmotor (tail) domains of bimC kinesins show little sequence conservation, hydrodynamic analysis and rotary shadow electron microscopy of KLP61F from *Drosophila* and of Kip1p from *Saccharomyces cerevisiae* have revealed that subunit composition and domain arrangement are remarkably conserved (13, 14). Both KLP61F and Kip1p are homotetramers with pairs of motor domains at opposite ends of a long stalk, a bipolar arrangement that would facilitate cross-linking of adjacent microtubules. Microtubule cross-linking in the spindle midzone has been demonstrated directly for KLP61F, which could slide apart interpolar microtubules to promote pole separation (15). In addition, bimC kinesins are localized near the spindle poles, where they may organize microtubules in parallel orientation during spindle assembly (3).

In *S. cerevisiae*, Kip1p and another kinesin (Cin8p) from the bimC subfamily interchangeably function to separate the spindle poles during spindle assembly, metaphase, and possibly anaphase (8–10, 16). Kip1p and Cin8p localization have been detected on unassembled spindles and on preanaphase spindles, but not on anaphase spindles (8, 9). Mutants singly defective in *KIP1* or *CIN8* are viable, but conditional double mutants arrest with duplicated but unseparated spindle poles, suggesting a role in mitotic spindle assembly (8, 9). The loss of *KIP1* and *CIN8* function after spindle assembly but before anaphase results in the collapse of the spindle, indicating a role in the maintenance of pole separation (10). After anaphase initiation, the loss of function results in slowed anaphase progression, suggesting that these kinesins play a nonessential role in pole separation during anaphase (16, 17).

One mechanism that controls mitotic transitions is regulated proteolysis mediated by a multisubunit ubiquitin-protein ligase known as the anaphase-promoting complex (APC) or cyclosome (18). The APC, in conjunction with other ubiquitination enzymes, adds a polyubiquitin chain to substrate proteins, which then are recognized by the 26S proteosome and degraded (19). Ubiquitination of known substrates frequently requires a conserved 9-aa motif in the substrate protein termed the cyclin destruction box (20). In the first APC-mediated cell-cycle transition, degradation of the anaphase-inhibitor protein securin, called Pds1p in *S. cerevisiae*, elicits sister chromatid separation and, thereby, initiates the metaphase-to-anaphase transition (21, 22). The APC subsequently initiates the exit from mitosis by inactivating mitotic cyclin-dependent kinases (23). The substrate and temporal specificities of the APC that underlie this sequence of events requires additional evolutionarily conserved proteins, called APC-activating proteins, which bind to and activate the APC at distinct times. In *S. cerevisiae*, proteolysis of the anaphase-inhibitor Pds1p at the onset of anaphase requires the APC activator Cdc20p, whereas complete proteolysis of the mitotic cyclin Clb2p at mitotic exit requires the APC activator Cdh1p (also called Hct1p) (24–26). The currently known substrates of the APC are regulatory proteins (27), with the exceptions of the spindle protein Ase1p and the chromokinesin Xkid. Proteolysis of Ase1p at the end of anaphase is mediated by Cdh1p-activated APC (APC^{cdh1}; ref. 25), and when degradation of overexpressed Ase1p is inhibited by mutations in its destruction box, mitotic spindle disassembly is prolonged (28). APC-targeted degradation of Xkid in *Xenopus* egg extracts during anaphase is required for the poleward movement of chromosomes (29, 30).

Here, we show that Kip1p is regulated by degradation. Kip1p increased in abundance during S, G_2, and metaphase, then abruptly disappeared during the initial stage of anaphase spindle elongation. Degradation of Kip1p required the APC, the APC-activating protein Cdc20, and a unique 43-aa segment of Kip1p. Degradation also required a nuclear localization sequence (NLS), but degradation occurred independently of Kip1p spin-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: APC, anaphase-promoting complex; NLS, nuclear localization sequence; GST, glutathione *S*-transferase.

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dle association. We suggest that Kip1p degradation facilitates structural rearrangement of the mitotic spindle.

Materials and Methods

Growth Conditions, Strains, and Plasmids. Media and genetic manipulations were as described (31). For synchronization by cell-cycle arrest and release, strain DS138 was grown at 30°C in yeast extract/peptone/dextrose medium to early log phase, α-factor (Research Genetics, Huntsville, AL) was added to 5 μg/ml, and incubation was continued until microscopy showed that >95% of the cells were arrested in G_1. The cells were collected by filtration, washed in water, resuspended in fresh prewarmed medium lacking α-factor, and aliquots were removed at 15-min intervals. Strain DS138 is a *ura3–52 leu2–3,112 trp1*Δ*1 his3*Δ*200* and is a derivative of S288c.

To measure the half-life of Kip1p, the genomic *KIP1* promoter was replaced with the inducible *GAL1–10* promoter by homologous recombination with the kanMX4′-promGAL1 module (32). The parent strains were W303 derivatives DY327 (*MAT*a *trp1–1 ade2–1 his3–11,115 leu2–3,112 ura3–1 can1–100*) and DY331 (*MAT*a *trp1–1 ade2–1 his3–11,115 leu2–3,112 ura3–1 ade3*Δ *cdc23–1*), obtained from D. Pellman (Dana-Farber Cancer Institute, Boston, MA). Cells were grown to early log phase at 23°C in yeast extract/peptone medium containing 2% (wt/vol) raffinose, and the cells were arrested in G_1 by adding α-factor to 5 μg/ml or were arrested in S by adding hydroxyurea to 0.1 M. When >95% of the cells were arrested, galactose was added to 2% to induce *KIP1* transcription. After 1.5 h, glucose (2%; wt/vol) and cycloheximide (1 mg/ml) were added to repress transcription and translation, and cells were collected at 5–10 min intervals. Maintenance of the G_1- or S-phase arrest was confirmed at all time points by microscopy.

For measurement of the steady-state level of Kip1p and glutathione *S*-transferase (GST)-Kip1p fusion proteins, cells were arrested in G_1 by adding α-factor to 5 μg/ml and were harvested when >95% of the cells were arrested. To express *KIP1* from the *ADH* promoter, the *ADH1* promoter was PCR amplified and cloned into plasmid pRS416 (*URA3 CEN ARS*) (33), creating plasmid pDR731. A *Bam*HI-*Not*I fragment containing *KIP1* (13) was cloned into pDR731 to create pDR716. To fuse *KIP1* to the GST gene (*GST*), a 700-bp fragment encoding *GST* was cloned downstream of the *ADH1* promoter, creating pDR782. Various *KIP1* fragments were generated by PCR so that, upon insertion into pDR782, they formed in-frame fusions with the upstream *GST* gene. To construct the NLS-GST plasmids, a PCR-generated DNA fragment encoding the sequence MAPKKKRKVGTIM, which includes the SV40 NLS (underlined) and the first methionine of GST (underlined), was inserted at the GST N terminus. The correct sequence of all PCR-amplified regions was confirmed by DNA sequencing.

To construct the *kip1*Δ*43* mutation, the region between the *Bsp*HI and *Bst*1107I sites in *KIP1* was deleted, which removed the codons for 43 amino acids (EHTRPEASVIKALPLLDYPK QFQIYRDAENKSKDDTSNSRTCI). The *kip1*Δ*43* mutation was synthetic lethal with a *cin8*-deletion mutation but was found to be recessive in complementation tests with *KIP1 cin8*Δ by using plasmid shuffle tests described (9). Point mutations in *KIP1* were made by site-directed mutagenesis with the Transformer kit (CLONTECH). Mutations in potential destruction box sequences were as follows: *kip1–160* is R3A, L6A; *kip1–161* is R438A, L441A; *kip1–162* is R647A, L650A; *kip1–163* is R755A, F758A. The nuclear localization signal mutation is *kip1–139*, which is K1083A, R1084A, R1085A. All *kip1* mutations were confirmed by sequencing and were introduced into genomic *KIP1* by homologous recombination with an insertion of *HIS3* downstream of *KIP1* as a selectable marker.

For immunofluorescence microscopy of Kip1p abundance, *CDH1* was disrupted with the HIS3MX6 module (32) to create strain DS1243; correct deletion of restriction sites in *CDH1* was confirmed by PCR and the GST-Kip1 (766–1111) plasmid pDR874 was introduced. The *cdc20* deletion mutant (34) was strain 8352 (*MAT*a *cdc20*::*LEU2 pds1*::*URA3 clb5*::*HIS3* YIp*GALLCDC20*::*ADE2*, obtained from K. Nasmyth, Research Institute of Molecular Pathology, Vienna, Austria), into which pDR874 was introduced.

Western and Northern Blots. Yeast-cell extracts were prepared by vortexing cells with glass beads in buffer [20 mM Hepes, pH 7.4/350 mM NaCl/1 mM $MgCl_2$/1 mM EGTA/1% Triton X-100/1% glycerol and protease inhibitors (1 mM PMSF, 1 mM benzamidine HCl, and 10 μg/ml leupeptin, pepstatin A, and chymostatin)]. After centrifugation at 16,000 × *g* for 15 min at 4°C, protein concentrations were determined by using the Micro BCA Assay (Pierce). A total of 100–150 μg of extract was loaded per lane for SDS/PAGE and Western blot transfer. Kip1p was detected by using the affinity-purified polyclonal anti-Kip1p antibodies described (13); GST-fusion proteins were detected with monoclonal antibody GST-2 (Sigma), and tubulin was detected with the monoclonal antibody BIBE2, a gift of F. Solomon (Massachusetts Institute of Technology, Cambridge, MA). Chemiluminescence detection was performed with the ECL kit (Amersham Pharmacia) or the Supersignal Femto kit (Pierce). For Northern blotting, total RNA was isolated from cells with a hot acidic-phenol protocol (35), and polyA mRNA was isolated from total RNA with the Oligotex mRNA mini kit (Qiagen, Chatsworth, CA). *KIP1*, *ACT1*, and *CLB2* mRNA were detected with ^{32}P-labeled DNA probes.

Microscopy. Immunofluorescence microscopy was performed as described (31). Primary antibodies were anti-GST polyclonal antibodies (Molecular Probes) and anti-tubulin monoclonal antibody BIBE2. Secondary antibodies were goat anti-rabbit antibody conjugated to rhodamine (Accurate Chemicals) and goat anti-mouse antibody conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch). DNA was stained with the dye 4{prime},6-diamindino-2-phenylindole (DAPI, Sigma). To lower the background fluorescence, the anti-GST antibody and both secondary antibodies were incubated for 1 h at 4°C with yeast cells that had been fixed and permeabilized with the immunofluorescence microscopy protocol (31). Some background staining was evident with the anti-GST antibodies even in control strains that contained no GST-fusion protein, but this staining occurred throughout the cell and was easily distinguished from the nuclear-localized GST-fusion protein staining. For the cell-cycle stage-distribution analysis, isogenic strains DS140 (*MAT*α *ura3–52 leu2–3,112 trp1*Δ*1 his3*Δ*200*), DS116 (*MAT*α *ura3–52 leu2–3,112 trp1*Δ*1 his3*Δ*200 kip1*Δ*1*::*HIS3*), and DS541 (*MAT*α *ura3–52 leu2–3,112 trp1*Δ*1 his3*Δ*200 kip1*Δ*43*/*HIS3*) were grown at 30°C in yeast extract/peptone/dextrose and fixed in the culture medium.

Results

Kip1p Abundance Is Cell Cycle-Dependent and Its Degradation Requires the APC. To investigate regulation of Kip1p during the cell cycle, we measured the abundance of Kip1p in a population of cells that was synchronously progressing through the cell cycle. Wild-type Kip1p expressed from its native promoter was undetectable in G_1 cells, and the Kip1p level rose as cells with short spindles accumulated and fell as cells entered anaphase (Fig. 1). Kip1p reached its highest level at the time cells established a preanaphase spindle, coincident with the proposed role of Kip1p in the assembly of a bipolar mitotic spindle (8, 9). The rise and fall of Kip1p abundance was preceded by a corresponding increase and decrease of *KIP1* mRNA (Fig. 1), consistent with the cell cycle-dependent regulation observed in genome-wide transcription studies (36, 37).

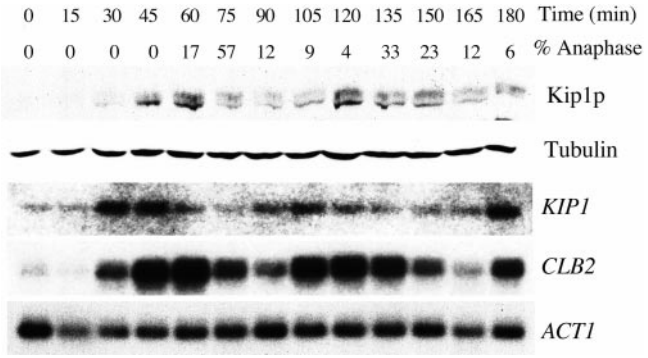

Fig. 1. The abundance of Kip1p and *KIP1* mRNA is cell cycle-dependent. A culture of wild-type strain DS138 was synchronized in G_1 and allowed to progress through two cell cycles. At each time point, samples were removed, and total yeast extracts were analyzed by Western blotting for Kip1p abundance (first panel). Tubulin abundance was measured as a loading control (second panel). mRNA was isolated from the same samples and analyzed by Northern blotting for *KIP1*, *CLB2*, and *ACT1* mRNA abundance (bottom three panels). The percentage of cells in anaphase was scored by tubulin immunofluorescence microscopy.

We noticed that in G_1, the *KIP1* mRNA was detectable, whereas the protein was not detectable, suggesting that Kip1p abundance is subject to posttranscriptional control. To test whether Kip1p stability depended on the stage of the cell cycle, we measured the half-life of Kip1p in G_1 and S with a promoter-shutoff procedure. In wild-type cells, Kip1p was stable in S but

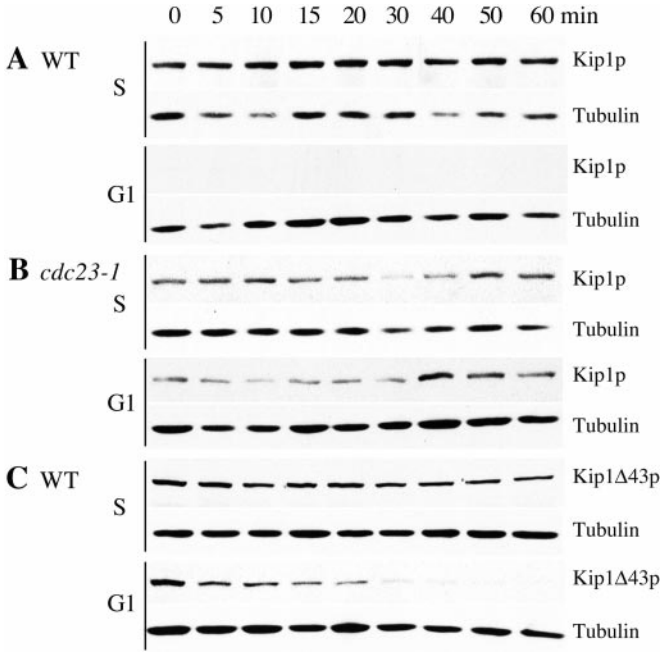

Fig. 2. Kip1p degradation is cell cycle-dependent and requires the APC. To measure the half-life of Kip1p, the *KIP1* gene was expressed from the pGAL promoter, the cells were arrested in G_1 or S, *KIP1* transcription and translation were repressed by the addition of glucose and cycloheximide, and, at the indicated times, samples were harvested for Western blotting using antibodies against Kip1p and tubulin. (*A*) Cell cycle-dependence. Wild-type strain background DY327 was used. Kip1p was undetectable in G_1 cells even after prolonged expression from the *pGAL1* promoter. As a control, the Kip1p half-life was measured in cells arrested in S, where Kip1p was stable. (*B*) APC dependence of Kip1p stability. In the isogenic *cdc23–1* strain DY344, Kip1p was stable in G_1. (*C*) Requirement for a 43-aa region of Kip1p. Kip1Δ43p showed a prolonged half-life in G_1, expressed in wild-type strain DY327.

was undetectable in G_1 (Fig. 2*A*), suggesting that the rate of Kip1p degradation in G_1 exceeded the rate of its synthesis. The APC-substrate Clb2p also failed to accumulate in G_1 when expressed from the *GAL1* promoter (38).

One posttranscriptional mechanism that could regulate Kip1p abundance is protein degradation targeted by the APC. To test whether Kip1p degradation requires the APC, we measured the half-life of Kip1p in a mutant defective in the APC subunit Cdc23p. Degradation of several APC substrates during G_1 is defective in *cdc23* mutants, including Ase1p, Pds1p, and Clb2p (21, 28, 39). In *cdc23–1* cells, Kip1p was stable in G_1 (Fig. 2*B*), confirming that Kip1p degradation requires a functional APC. Northern blots showed that *KIP1* mRNA levels were unaffected by the *cdc23–1* mutation, when *KIP1* was expressed either from its native promoter or from the constitutive *ADH1* promoter (data not shown).

Sequences in Kip1p Necessary for Kip1p Degradation. One signal that could influence Kip1p degradation is the association and potential disassociation of Kip1p with the mitotic spindle. To test whether microtubule binding influences Kip1p degradation, we deleted from *KIP1* the region encoding the 393 NH_2-terminal amino acids, which includes the entire kinesin-related domain. Immunofluorescence microscopy showed that the truncated Kip1p was located throughout G_2/metaphase nuclei without increased concentration on the spindle microtubules (data not shown, also see Fig. 4*A*). The abundance of the truncated Kip1p in G_1 and S exhibited the same pattern of stability as full-length Kip1p (Fig. 3, lines 1 and 2). Thus, cell cycle-dependent degradation of Kip1p is independent of spindle binding.

Many substrates of the APC contain a conserved consensus sequence (RxxLxxxxN) called the cyclin destruction box that is required for APC-dependent proteolysis (20). There are three RxxL sequences and one RxxF sequence in Kip1p, beginning at Kip1p Arg residues 3, 438, 647, and 755, respectively. We tested whether these sequences specified Kip1p degradation by using site-directed mutagenesis to convert the Arg and Leu or Phe residues to Ala. Combinations of mutations at sites 3, 438, and 647 as well as at sites 3, 438, and 755 did not stabilize Kip1p during G_1 (Fig. 3, lines 3 and 4). In addition, we tested a deletion of the first 765 residues of Kip1p that removes all of these sites. *KIP1* codons 766-1111 were fused to the *GST* and expressed from the constitutive *ADH1* promoter. This fusion protein was stable in S but degraded in G_1 phase (line 6), indicating that sequences in the first 765 residues of Kip1p are nonessential for cell cycle-dependent degradation.

A signal that is potentially required for Kip1p degradation is an NLS, because the APC subunits Cdc16p, Cdc23p, Cdc26p, Cdc27p, and Apc1p are confined to the nucleus (40, 41). We tested the abundance of Kip1p that was excluded from the nucleus because of point mutations in the Kip1p NLS. The *kip1-nls*$^-$ mutation (K1083A, R1084A, R1085A) in both the full-length *KIP1* and the *GST-KIP1* (766–1111) genes prevented the accumulation of Kip1p in the nucleus, as observed by immunofluorescence microscopy (data not shown), and stabilized the protein in G_1 (Fig. 3, lines 5 and 7), suggesting nuclear localization is required for degradation. To ensure that the increased Kip1p abundance conferred by the *kip1-nls*$^-$ mutation was caused solely by abrogation of nuclear localization and not by a different effect of the mutation, we restored nuclear localization to the GST-Kip1-nls$^-$ protein by introducing a heterologous (SV40) NLS and tested Kip1p abundance. The SV40 NLS restored degradation in G_1 (Fig. 3, line 8), confirming that the *kip1-nls*$^-$ mutation stabilized Kip1p by abolishing nuclear localization.

To determine whether a short segment of Kip1p could confer degradation to another protein, we fused fragments of *KIP1* to *GST* and tested the abundance of the resultant fusion protein in

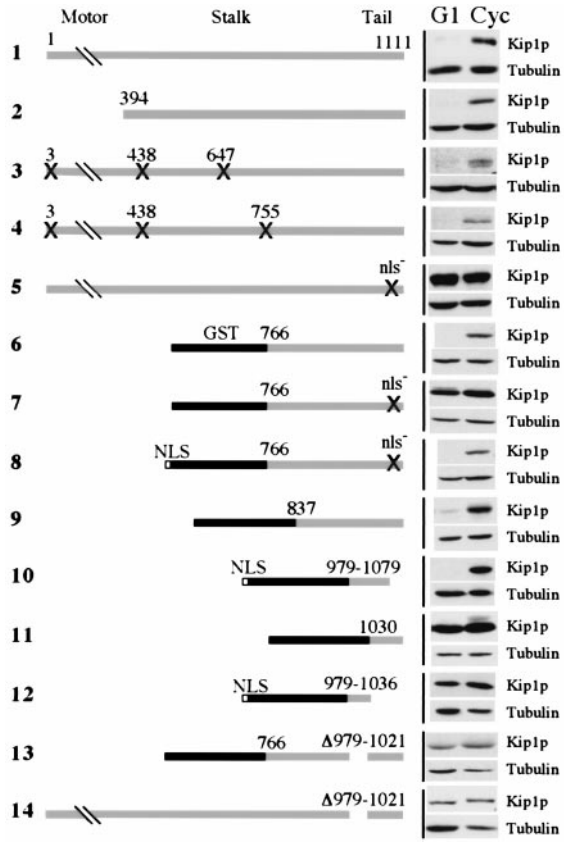

Fig. 3. Domains of Kip1p required for degradation. Schematic of Kip1p- and GST-Kip1p-fusion proteins. The Kip1 polypeptide is represented by a gray box; the 26-kDa GST moiety is shown as a black box; the SV40 nuclear localization signal is an open box; and the location of mutations are shown with an ''x''. The abundance of each Kip1 protein in G_1-arrested cells or in cycling cells was measured by Western blotting (*Right*). All of the Kip1-fusion proteins were expressed from the *ADH1* promoter.

G_1 and in cycling cells. A diagram of the different fusion proteins tested is shown in Fig. 3. The smallest fragment of Kip1p tested that exhibited degradation in G_1 contained 101 residues of Kip1p (residues 979-1079) fused to GST and to the SV40 NLS (Fig. 3, line 10). When this region was subdivided into two overlapping fusion proteins (residues 979-1036 and 1030–1111), neither fusion protein exhibited degradation in G_1 (Fig. 3, lines 11 and 12). Thus, a 101-aa region is sufficient to confer cell cycle-dependent degradation to GST.

To further delineate the sequences necessary for Kip1p degradation, we made an inframe deletion (named *kip1Δ43*) that removed the sequence-encoding amino acids 979-1021 and introduced this deletion into the GST-Kip1 (766–1111) fusion plasmid. Unlike GST-Kip1p (766–1111), GST-Kip1Δ43p accumulated in G_1-arrested cells (Fig. 3, line 13). The 43 amino acids deleted in the *kip1Δ43* mutant include the sequence RTCI (residues 1019–1022), which resembles the RxxL/RxxF destruction box consensus sequence. However, mutations that changed the RTCI codons to ATCA, as well as a deletion of the RTCI codons, did not stabilize the GST-Kip1p (766–1111) fusion protein (data not shown). Thus a 43-aa region of Kip1p is necessary for degradation of the fusion protein, but the RTCI sequence within this interval is not essential. In addition to testing the effect of the *kip1Δ43* mutation in the context of the GST-Kip1p (766–1111) fusion protein, we introduced this mutation into the full-length *KIP1* gene and tested the resulting protein for cell cycle-dependent degradation. When the *kip1Δ43* gene was expressed from the constitutive *ADH1* promoter, the protein was abundant in cells arrested in G_1 and in cycling cells (Fig. 3, line 14). To define the extent of stabilization conferred by the *kip1Δ43* mutation, we examined the kinetics of degradation by using the *GAL1*-promoter shutoff procedure. When the APC is inactive in S phase, both wild type and Kip1Δ43p were stable for >60 min. In contrast to the immeasurably short half-life of wild-type Kip1p in G_1 cells, Kip1Δ43p in G_1 cells accumulated during the induction period and was detectable for 40 min after transcription and translation were repressed (Fig. 2*C*). Thus, the *kip1Δ43* mutation strongly stabilizes Kip1p from APC-mediated degradation. Some degradation occurred, indicating that additional sequences also may target Kip1p degradation. But the residual instability may not be significant in proliferating cells, because results presented below indicate that a Kip1Δ43p fusion is abundant throughout the cell cycle.

There is evidence that the assembly state of a target protein can influence ubiquitin-mediated degradation. For example, degradation of cyclin A and B2 in *Xenopus* requires association of the cyclins with Cdc28p (42, 43). To test whether the *kip1Δ43* mutation interfered with formation of the Kip1p homotetrameric complex, we examined the subunit composition of Kip1Δ43p by sedimentation (13). The sedimentation coefficient of both wild-type Kip1p and Kip1Δ43p was 8.2 S, suggesting that the stabilization of Kip1Δ43p in G_1-arrested cells was not due to abnormal complex assembly.

Kip1p Degradation Occurs Early in Anaphase and Requires the APC-Activating Protein Cdc20p. To define the precise stage of mitosis at which Kip1p is degraded, we used immunofluorescence microscopy to correlate Kip1p abundance with mitotic spindle length in individual cells. The GST-Kip1p (766–1111) fusion protein was used because this protein does not associate with microtubules, and, therefore, the intensity of the immunofluorescence signal was not substantially perturbed by elongation of the spindle during anaphase or by the possibility of Kip1p dissociation from the spindle. We found that Kip1p abruptly disappeared after anaphase initiation (Fig. 4*A*). Whereas all cells in metaphase (spindle length ≈1 μm and undivided DNA) exhibited GST-Kip1p staining, only 35% of the cells in early anaphase (spindle length 2–4 μm and undivided but elongated DNA) exhibited staining. None of the cells in mid-anaphase or telophase (spindle length 4–7 μm and divided DNA) exhibited staining (250 cells were counted). Given a rate of spindle pole separation during the first stage of anaphase of ≈1.3 μm/min (44, 45), we infer that the Kip1p-fusion protein was degraded within 2 to 3 min of anaphase initiation. The degradation of Kip1p early in mitosis suggested that Kip1p is targeted by the activating protein Cdc20p, which is active at anaphase onset. To test this idea, we examined Kip1p abundance in the absence of Cdc20p. The essential function of Cdc20p stems only from the need to degrade Pds1p and Clb5p, and thus *CDC20* can be deleted if *PDS1* and *CLB5* are also deleted (34). In proliferating *cdc20Δ pds1Δ clb5Δ* mutant cells, the GST-Kip1p (766–1111) fusion protein was abundant at all stages of the cell cycle (Fig. 4*B*), indicating that Cdc20p is required for Kip1p proteolysis.

In addition to defining the requirement of Cdc20p for Kip1p degradation early in anaphase, we examined whether Kip1p degradation also could be targeted by the APC-activator Cdh1p. Mutants defective in *CDH1* are viable and exhibit only a small delay in late anaphase (24, 25). We found that in proliferating *cdh1Δ* cells, the constitutively expressed GST-Kip1p fusion was present in all metaphase cells, but was undetectable in all mid- and late-anaphase cells (107 cells were counted; Fig. 4*C*). Thus, APC^{cdh1} is not required for Kip1p degradation during mid and late anaphase. The stabilizing effect of the *kip1Δ43* mutation shown in Fig. 2*C* was characterized in G_1-arrested cells, whereas microscopy indicated that, in proliferating cells, Kip1p is de-

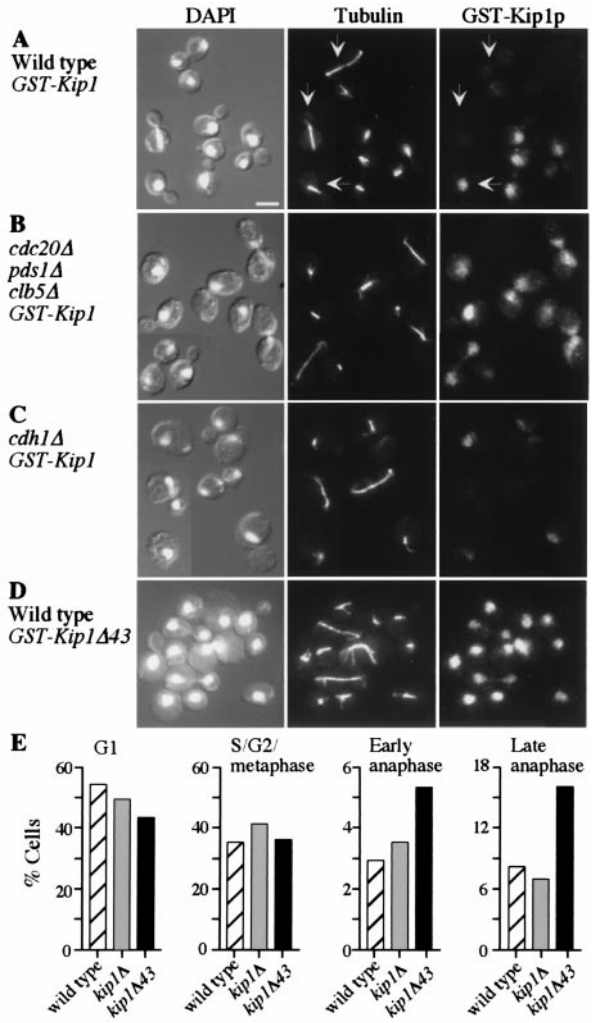

Fig. 4. The timing of Kip1p degradation and the effect of the *kip1Δ43* mutation. (*A*) Abundance of the GST-Kip1 (766–1111) fusion protein in a wild-type strain. The first panel shows DAPI staining that has been superimposed on differential interference contrast microscopy images, the second panel shows anti-tubulin staining, and the third panel shows anti-GST staining. GST-Kip1p is confined to the nucleus, and the faint anti-GST staining present throughout the cells is nonspecific and also was present in strains without a GST-fusion protein. The vertical arrows indicate mid- and late-anaphase cells in which Kip1p has been degraded, and the horizontal arrow indicates a metaphase cell in which Kip1p is abundant. (Bar = 4 μm.) (*B*) Abundance of the GST-Kip1 (766–1111) fusion protein in a *cdc20*-deletion mutant. Mid- and late-anaphase cells in which GST-Kip1p has not been degraded are shown. (*C*) Abundance of GST-Kip1p in a *cdh1*-deletion mutant. Like in wild type, GST-Kip1p is degraded in anaphase. (*D*) Abundance of GST-Kip1Δ43p in a wild-type strain. GST-Kip1Δ43p is present in all cells. (*E*) Cell-type distribution in wild-type, *kip1*Δ, and *kip1Δ43* strains. Proliferating cultures were grown at 30°C and fixed for microscopy. Cells were placed into one of four stages of the cell cycle based on bud morphology, mitotic spindle length, and nuclear DNA localization. For each strain, at least 840 cells from duplicate cultures were scored.

graded in early anaphase. This result raised the possibility that the stabilizing effect of the *kip1Δ43* mutation was specific to G_1, and that a different site in Kip1p mediates degradation during anaphase. We tested this idea by examining GST-Kip1Δ43p abundance by microscopy and found that the protein was stable throughout anaphase (Fig. 4*D*), indicating that the *kip1Δ43* mutation inhibits APC^{cdc20}-mediated degradation.

To address the physiological purpose of Kip1p degradation, we inhibited Kip1p degradation by using the *kip1Δ43* mutation and examined the effect on the duration of anaphase. Proliferating cultures of wild type, *kip1*Δ, and *kip1Δ43* strains were fixed and stained to visualize the mitotic spindle and DNA, and the percentage of cells at different stages of the cell cycle was scored by microscopy (Fig. 4*E*). Cells in mid-anaphase increased from 2.9% in the wild-type strain to 5.3% in the *kip1Δ43* mutant (1.8-fold), and cells with a mitotic spindle that spanned the length of the mother and daughter cells increased from 8.1% in wild type to 16% in the *kip1Δ43* mutant (a 2-fold increase), indicating that Kip1Δ43p prolongs the duration of anaphase. The isogenic *kip1*Δ-null mutant exhibited nearly wild-type numbers of mid- and late-anaphase cells, suggesting that the prolonged anaphase in the *kip1Δ43* mutant is not due to the loss of Kip1p function. We also measured the rate of anaphase directly in live cells by using NUF2-GFP to observe spindle pole position and found that the mean rate in *kip1Δ43* was 1.5-fold slower than in the *kip1*Δ-null mutant (data not shown).

Discussion

Here, we demonstrate that the kinesin Kip1p is regulated by its degradation, which occurs within 2–3 min of anaphase initiation. A key aspect of our approach is that we used microscopy to measure Kip1p abundance during anaphase in proliferating cells. By correlating the abundance of Kip1p with mitotic spindle length, the timing of degradation could be defined more precisely than is possible by assaying Kip1p in extracts of synchronized cell populations. The degradation of Kip1p at anaphase onset suggests that Kip1p predominantly functions during spindle assembly and metaphase, consistent with the essential function inferred from analysis of *kip1 cin8* mutants (8–10) and the timing of wild-type Kip1p localization (9).

We show that Kip1p degradation in anaphase requires APC^{cdc20} but not APC^{cdh1} by using mutants defective in the APC subunit Cdc23p and in the APC-activating proteins Cdc20p and Cdh1p. By using the viable *cdc20 pds1 clb5* triple mutant (34), we were able to assay the effect of a *cdc20*-null mutation on Kip1p during anaphase in proliferating cells. The dependence upon APC^{cdc20} is consistent with the timing of Kip1p degradation at anaphase onset, when APC^{cdc20} is active. Although APC^{cdc20} is both necessary and sufficient for Kip1p degradation during anaphase, APC^{cdh1} also may be capable of targeting Kip1p degradation and potentially helps keep Kip1p levels low during G_1.

A unique sequence targets Kip1p for degradation, unlike the other known substrates of APC^{cdc20}, which contain RxxL destruction boxes. A 101-aa region of Kip1p is necessary and sufficient for degradation and confers degradation to GST. This region contains no destruction boxes or Lys-Glu-Asn sequences (KEN boxes; ref. 46), nor does this region show strong similarity to other proteins. The Kip1p destruction signal apparently spans at least 17 amino acids within the 101-aa region (Fig. 3), suggesting that the signal does not consist only of a short primary amino acid sequence. A unique, dispersed destruction signal in *Drosophila* cyclin A also has been identified (47). The fact that soluble Kip1p in yeast extracts is homotetrameric (13) argues against the possibility that degradation is conferred by means of a destruction box located in a persistently associated heterologous subunit.

Degradation of Kip1p within the first few minutes of anaphase suggests that Kip1p destruction facilitates the extensive remodeling of the spindle that must occur during anaphase. Although a few microtubules increase in length during anaphase to enable the poles to separate, three-dimensional reconstructions of spindles from serial electron micrographs show that the majority of microtubules shorten by about 10-fold (the median microtubule length decreases from ≈300 nm to ≈30 nm; ref. 48). We hypothesize that bipolar Kip1p molecules cross-link and stabilize microtubule arrays during metaphase, and that Kip1p degrada-

tion during anaphase facilitates depolymerization of these microtubules. However, Kip1p degradation by means of the APC^{Cdc20} would not be expected to be essential for anaphase progression, because spindle elongation and cell proliferation proceed without Cdc20p when Pds1p and Clb5p also are deleted (ref. 34, and see Fig. 4). To address whether the absence of Kip1p degradation more subtly affects anaphase, we used the *kip1Δ43* mutation that stabilizes Kip1p. Microscopy showed that the duration of anaphase increased by 1.5- to 1.8-fold in *kip1Δ43* compared with both wild type and a *kip1*-deletion mutant, consistent with an impairment of anaphase progression caused by the abnormal presence of Kip1p. Because the *kip1Δ43* mutation abolishes *KIP1* function, it is possible that a functional defect contributes to the observed anaphase impairment. However, the effect of the mutation would have to be more severe than that caused by the *kip1*-deletion mutation, as the *kip1Δ* mutation did not impair anaphase in our assays.

The APC was originally viewed as a mechanism that controls the activity of regulatory proteins and enzymes (18, 27), but it is becoming clear that the APC has broader utility. For instance, the APC regulates the chromokinesin Xkid, the degradation of which is required in anaphase to allow poleward chromosome movement (29, 30). Two additional mitotic kinesins and a kinetochore protein also are known to be degraded during anaphase (49–51). Finally, degradation seems to facilitate remodeling of large protein structures, as illustrated by Kip1p in early anaphase and by Ase1p, whose destruction facilitates spindle disassembly (28). We suggest that additional proteins that stabilize transient protein assemblies are strong candidates for regulated degradation.

We thank Todd DeZwaan for constructing the *kip1Δ43* and *kip1-nls* mutations, Olga Smirnova for technical assistance, and Frank Luca and Erika Holzbaur for hospitality. We also thank David Pellman for strains and discussions, Orna Cohen-Fix for discussions and a critical reading of the manuscript, and A. Amon, S. Holloway, J. Kahana, K. Nasmyth, W. Seufert, and P. Silver for providing strains or plasmids. This work was supported by National Institutes of Health Grant GM57441.

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