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Requirement of Skp1-Bub1 Interaction for Kinetochore-Mediated Activation of the Spindle Checkpoint

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Summary

The spindle checkpoint transiently prevents cell cycle progression of cells that have incurred errors or failed to complete steps during mitosis, including those involving kinetochore function. The molecular nature of the primary signal transmitted from defective kinetochores and how it is detected by the spindle checkpoint are unknown. We report biochemical evidence that Bub1, a component of the spindle checkpoint, associates with centromere (*CEN*) DNA via Skp1, a core kinetochore component in budding yeast. The Skp1's interaction with Bub1 is required for the mitotic delay induced by kinetochore tension defects, but not for the arrest induced by spindle depolymerization, kinetochore assembly defects, or Mps1 overexpression. We propose that the Skp1-Bub1 interaction is important for transmitting a signal to the spindle checkpoint pathway when insufficient tension is present at kinetochores.

Introduction

Mistakes in cell division result in aneuploidy that is manifested in genetic disorders and cancer. Cell cycle checkpoints are cellular control systems that detect errors in the proper completion of ordered events within the cell cycle and prevent premature progression through the cell cycle when errors occur (for review see Hartwell and Kastan, 1994). The spindle checkpoint is a surveillance system that can delay mitotic progression by transiently inhibiting the anaphase-promoting complex (APC, also called cyclosome) in response to either a defect in spindle organization or a failure of the chromosomes to attach correctly to the spindle (for review see Amon, 1999). Genes involved in the spindle checkpoint were first isolated from the budding yeast *Saccharomyces cerevisiae* and include *MAD1*, *MAD2*, and *MAD3* (*m*itotic *a*rrest *d*eficient); (Li and Murray, 1991) *BUB1*, *BUB2*, and *BUB3* (*b*udding *u*ninhibited by *b*enzimidazole) (Hoyt et al., 1991); and *MPS1* (*m*ono*p*olar spindle) (Weiss and Winey, 1996). When treated with drugs that depolymerize microtubules, *mad* or *bub* mutants fail to undergo arrest in mitosis and enter anaphase without a functional spindle; these events lead to aneuploidy and rapid death. Mammalian orthologs of the spindle checkpoint proteins have been shown by microscopy to localize to kinetochores (for review see Kitagawa and Hieter, 2001). Consistent with a link between aneuploidy and cancer, mutations in human homologs of Bub1 have been found in subtypes of colorectal cancer that exhibit chromosome instability (CIN tumor) (Cahill et al., 1998).

The kinetochore is a complex of proteins bound to centromere (*CEN*) DNA. Kinetochores play an important role in relaying the checkpoint signal to the spindle checkpoint pathway. The core of the kinetochore in budding yeast is a protein complex called CBF3, which is bound to an essential conserved *CEN* DNA element (CDEIII) and consists of four essential proteins: Ndc10, Cep3, Ctf13, and Skp1 (for review see Kitagawa and Hieter, 2001). Recently, Gardner et al. showed that elimination of CBF3 components by the degron system abolishes the spindle checkpoint response, a result that indicates that CBF3 is not only monitored by the spindle checkpoint, but also required for checkpoint signaling (Gardner et al., 2001).

In mammalian cells, CENP-E, a kinesin-like motor protein, interacts with BubR1, a Bub1-related protein (also termed Mad3Hs or Ssk1) (Chan et al., 1998). *Xenopus* egg extracts from which CENP-E is immunodepleted fail to undergo cell cycle arrest in response to spindle damage (Abrieu et al., 2000). In addition, immunodepletion of Bub1 abolishes the binding of CENP-E, Mad1, Mad2, and Bub3 to the kinetochore, suggesting that Bub1 binds closer to the core of the kinetochore than these other proteins (Sharp-Baker and Chen, 2001). Despite its importance, the molecular linkage between kinetochores and the spindle checkpoint has not been well characterized.

Unattached kinetochores (Rieder et al., 1995) or a lack of tension on kinetochores (Li and Nicklas, 1995) is thought to produce a checkpoint signal. Recently, Biggins and Murray have shown that Ipl1 is required for the delay triggered by chromosomes whose kinetochores are not under tension, but is not required for arrest induced by spindle depolymerization, which is suggesting that Ipl1 is required to monitor tension at the kinetochore (Biggins and Murray, 2001).

Here we report biochemical evidence that Bub1 interacts with *CEN* DNA via Skp1 in budding yeast and that Skp1's function to interact with Bub1 is required for the mitotic delay induced by outer kinetochore defects or cohesion defects, but not for the arrest induced by kinetochore assembly defects or spindle depolymerization.

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We propose that the Skp1-Bub1 interaction is required by the spindle checkpoint pathway for detecting lack of sufficient tension at kinetochores.

Results

Bub1 Binds to Skp1

When we performed yeast systematic genome-wide two-hybrid screens using Skp1 and its homologs from other organisms as bait (to be described elsewhere), we found that Skp1 interacted with Bub1; this interaction was not detected in a previous screen (Uetz et al., 2000). We first confirmed the interaction by performing the reciprocal two-hybrid assay (see Supplemental Data at http://www.molecule.org/cgi/content/full/11/5/1201/DC1). Next, to confirm the specificity of the Skp1-Bub1 interaction, we performed in vitro binding experiments in which Bub1 and Skp1 were coexpressed in insect cells and reciprocal coimmunoprecipitations were performed. When 6×His-tagged Bub1 and FLAG-tagged Skp1 were coexpressed, immune complexes precipitated by an anti-FLAG antibody contained FLAG-tagged Skp1 and 6×His-tagged Bub1 (Figure 1A, left panel). Likewise, when 6×His-tagged Bub1 and untagged Skp1 were coexpressed, an anti-6×His antibody precipitated immune complexes containing tagged Bub1 and untagged Skp1 (Figure 1A, right panel).

To assess the biological significance of Bub1's binding to Skp1, we identified the Skp1 binding domain of Bub1. Various deletion proteins (Figures 1B and 1C) were expressed and labeled with ^{35}S-methionine by an in vitro translation system, and the lysates were mixed with extract containing Skp1-HA that was expressed separately. Skp1-HA was immunoprecipitated by using anti-HA Sepharose to test which deletion proteins can bind Skp1 (Figure 1B). We found that the Skp1 binding domain is located between amino acids 181 and 360 (Figure 1C). Interestingly, this region contains the Bub3 binding domain of Bub1 (amino acids 313–356) and a domain that is required for Bub1's localization to the kinetochore (Taylor et al., 1998). Because Bub1 and Bub3 in higher eukaryotes require each other to localize to kinetochores and because Skp1 is a CBF3 component in budding yeast, Bub3 may also localize to the kinetochore via Skp1.

To examine whether Skp1 directly binds to Bub1, we purified GST-fused Skp1 and a 6×His-tagged Bub1 polypeptide (amino acid 141–608) containing the Skp1 binding domain (Figure 1D), both of which had been expressed in *E. coli*. The purified Bub1 polypeptide was incubated with the GST-Skp1 fusion protein or GST protein and immunoprecipitated with anti-6×His antibodies. GST-Skp1, but not GST alone, was coprecipitated with the Bub1 polypeptide (Figure 1E).

Taken together, our results indicate that Skp1, a component of the core kinetochore complex (CBF3), directly binds to Bub1, a component of the spindle checkpoint complex.

Bub1 Associates with *CEN* DNA In Vivo

Bub1 homologs in other organisms were shown by microscopy to localize to kinetochores (Bernard et al., 1998; Jablonski et al., 1998; Taylor and McKeon, 1997). To examine whether Bub1 associates with *CEN* DNA in vivo in budding yeast, we performed chromatin immunoprecipitation (ChIP) assays (Hecht and Grunstein, 1999; Meluh and Koshland, 1997; Tanaka et al., 1997) with Myc-tagged Bub1. Myc-tagged Bub1 strains as well as wild-type strains grew in the presence of benomyl (see Supplemental Data at http://www.molecule.org/cgi/content/full/11/5/1201/DC1), indicating that the myc-tagged Bub1 was functional (Roberts et al., 1994). Bub1-myc was immunoprecipitated from chromatin preparations of log phase cells and the chromatin bound to Bub1 was analyzed by PCR to determine whether *CEN* DNA was present in the immunoprecipitates. The templates from total chromatin and from the immunoprecipitates were titrated to determine the linear range for PCR (data not shown). Regions of *CEN3*, *CEN1*, and *CEN16* were amplified specifically from the Bub1 immunoprecipitate, but not from the immunoprecipitate of the untagged strain (Figure 2A). A non-*CEN* locus, *PGK1*, was not amplified from the immunoprecipitate (Figure 2A). Thus, we obtained biochemical evidence that Bub1, a component of the spindle checkpoint, associates with *CEN* DNA in vivo in budding yeast.

Mammalian homologs of Bub1 localize to kinetochores during mitosis (Jablonski et al., 1998; Taylor and McKeon, 1997). We therefore examined yeast cells arrested in different stages of the cell cycle. Cells were arrested in the G2/M phase by treatment with nocodazole and in G1 phase by treatment with α factor (Figure 2B). The Bub1-*CEN* binding in G2/M cells was significantly increased compared to that in G1 cells (Figure 2B), a result that is consistent with the previous observation that mammalian Bub1 homologs are enriched at kinetochores during mitosis (Jablonski et al., 1998; Taylor and McKeon, 1997).

Bub1-*CEN* Binding Was Abolished in *skp1-4* Cells

Next we tested the effects of several kinetochore mutants (*ctf13-30*, *sgt1-3* [G2 allele; G2/M arrest at nonpermissive temperature; kinetochore deficient], *skp1-3* [G1 allele; G1 arrest at nonpermissive temperature; SCF deficient], *skp1-4* [G2 allele; G2/M arrest at nonpermissive temperature; kinetochore deficient], and *ndc10-1*) on Bub1-*CEN* binding. In *skp1-4* cells grown logarithmically at 25°C, the Bub1-*CEN* binding was abolished, but binding occurred in *ctf13-30*, *sgt1-3*, *skp1-3*, and *ndc10-1* cells under the same conditions. This result indicates that Bub1-*CEN* binding depends on *SKP1* (Figure 2C). The abolishment of Bub1-*CEN* binding was not due to a reduction in the amount of Bub1 protein: Western blot analysis showed that almost equal amounts of Bub1 were expressed in the mutant cells (Figure 2C, bottom panel).

Benomyl-Hypersensitive *skp1* Mutants

Many kinetochore mutants exhibit increased sensitivity to microtubule-depolymerizing drugs, presumably because of the kinetochore deficiency rather than defects in the spindle checkpoint function. The *skp1-4* mutation causes a defect in CBF3/*CEN* DNA assembly (Connelly and Hieter, 1996; Kaplan et al., 1997) as evidenced by DNA mobility shift assays monitoring CBF3/*CEN* DNA binding in vitro (Figure 3F). Consistently, these *skp1-4*

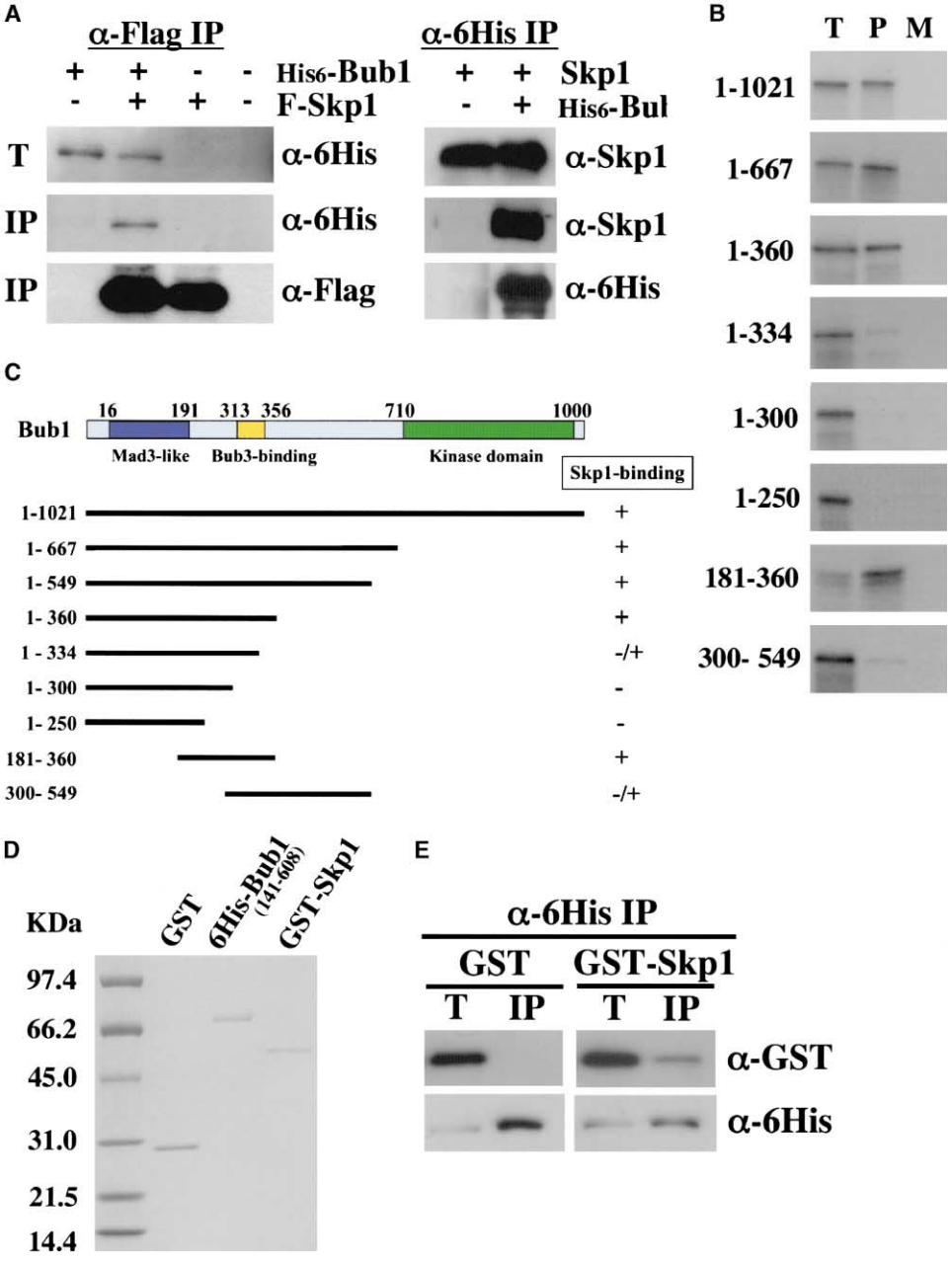

Figure 1. Interaction between Skp1 and Bub1

(A) Left panel: the 6×His-tagged Bub1 (His6-Bub1) and FLAG-tagged Skp1 (F-Skp1) proteins were coexpressed in insect cells, and FLAG-tagged Skp1 was immunoprecipitated from the cell extract by an anti-FLAG antibody (α-Flag). Immunoblot analyses were performed with an anti-6×His (α-6×His) or anti-FLAG antibody. Right panel: 6×His-tagged Bub1 protein and Skp1 were coexpressed in insect cells. In both experiments, 6×His-tagged Bub1 was immunoprecipitated from the cell extract by an anti-6×His antibody. Immunoblot analyses of the precipitates (IP) were performed with anti-Skp1 and anti-6×His antibodies. T, total lysates (2% of the starting material).

(B) The indicated proteins were expressed and labeled with ^{35}S-methionine in the in vitro translation system, and the lysates were mixed and incubated for 1 hr at 30°C with a different extract containing Skp1-HA that was expressed separately in insect cells. Skp1-HA was immunoprecipitated by using anti-HA-Sepharose, the immunoprecipitates were eluted and subjected to SDS-PAGE, and radioactive bands were identified by autoradiography. T, total lysates (5% of the starting material); P, immunoprecipitate; and M, mock (without Skp1-HA extract).

(C) An illustration of the Bub1 deletion proteins used in the Skp1 binding assay and the results are shown.

(D) Approximately 100 ng of each purified protein was loaded, and the gel was stained with Coomassie blue R-250.

(E) Approximately 200 ng of the purified proteins was used. The 6×His-Bub1 polypeptide was incubated with GST only or GST-Skp1 for 1 hr at 30°C and then immunoprecipitated by using anti-6×His antibody bound to protein A-Sepharose. The eluted protein was subjected to immunoblotting by using anti-GST antibody and anti-6×His antibody.

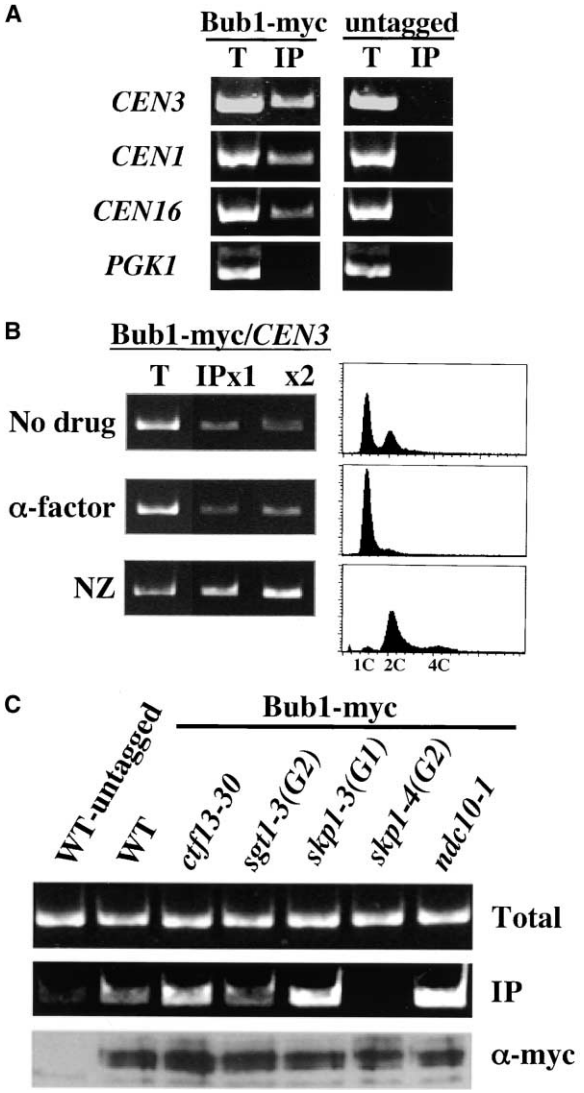

Figure 2. Skp1-Mediated Association between Bub1 and *CEN* DNA

(A) Log phase cells that expressed myc-tagged or untagged Bub1 were analyzed by ChIP assays using an anti-myc antibody. The templates used for PCR were total chromatin (T) or immunoprecipitate (IP).

(B) ChIP assays were performed by using untreated cells (No drug), α factor-treated cells (2 hr treatment), or nocodazole-treated cells (15 μg/ml for 2 hr). Twice the amount of immunoprecipitate was used for IP×2.

(C) ChIP assays were performed by using the indicated strains that were incubated at 25°C (Connelly and Hieter, 1996; Doheny et al., 1993; Goh and Kilmartin, 1993; Kitagawa et al., 1999). All the strains showed similar FACS profiles (data not shown). Immunoblot analyses of the indicated strains were performed by using anti-myc antibodies. Equal amounts of protein extracts from the indicated cells were loaded.

cells exhibit substantial chromosome missegregation, which is the most common phenotype of kinetochore mutants (Connelly and Hieter, 1996) (Figure 3B), and arrest at G2/M at nonpermissive temperature in a spindle checkpoint-dependent manner. Furthermore, at semipermissive temperature, *skp1-4* cells exhibit cell cycle progression delay at G2/M, grow slower than wild-type cells (Figure 3A), and are sensitive to benomyl (Figure 3A).

If the Skp1-Bub1 interaction is specifically important for a checkpoint response, we reasoned that it may be possible to generate a *skp1* mutant that is specifically deficient in the checkpoint response but not in chromosome segregation. Given this hypothesis, the mutant should be hypersensitive to microtubule depolymerizing drugs but not show a phenotype of chromosome missegregation. To test this hypothesis, we performed a PCR-based method (Connelly and Hieter, 1996) to screen for *skp1* mutants that are hypersensitive to benomyl. We isolated two benomyl-sensitive *skp1* mutants, *skp1-s6* and *skp1-s8* (Figure 3A). Next, we evaluated the extent of chromosome missegregation in *skp1-s6* and *skp1-s8*. A colony color assay that measures the stability of a marker chromosome fragment revealed that *skp1-s8,* but not *skp1-s6*, shows increased chromosome missegregation (Figure 3B); however, *skp1-s6* was not as sensitive to benomyl as *skp1-s8* or *skp1-4* (Figure 3A). We found that *skp1-s6* has one missense mutation (E129G) and *skp1-s8* has two missense mutations (E122V, G150D) (Figure 3E). *skp1-s6* appeared to be a weak separation of function allele in which checkpoint function, but not chromosome segregation function, was altered. In order to create a stronger separation of function allele, we created the *skp1-AA* allele in which amino acid residues 129 and 130 were mutated (E129A and M130A) and tested whether these changes enhanced benomyl sensitivity but did not increase the level of chromosome missegregation. Indeed, of all the mutants, the *skp1-AA* cells were more sensitive to benomyl than the other *skp1* mutants (Figure 3A; note that *skp1-4* cells grow slowly in the absence of benomyl), and they did not show a phenotype of chromosome missegregation (i.e., the increase in the loss of the marker chromosome fragment was less than 10-fold) (Figure 3B). Furthermore, the skp1-AA protein did not interact with Bub1 as evidenced by two-hybrid analysis (Figure 3C and Supplemental Data at http://www.molecule.org/cgi/content/full/11/5/1201/DC1), and the Bub1-*CEN* DNA interaction was abolished in *skp1-AA* cells as evidenced by ChIP analysis (Figure 3D and Supplemental Data at http://www.molecule.org/cgi/content/full/11/5/1201/DC1).

To further confirm that the ability of Skp1 to serve as component of the core kinetochore remained intact, we performed bandshift assays that evaluated the kinetochore (CBF3) assembly activity. Compared with extracts of wild-type cells, extracts of *skp1-AA* cells were not deficient in CBF3-forming activity (Figure 3F), although extracts of *skp1-4* cells and *ctf13-30* cells were deficient under the same condition as previously reported (Kaplan et al., 1997; Kitagawa et al., 1999). This finding indicates that the CBF3 assembly activity is intact in *skp1-AA* cells. Furthermore, we utilized the one-hybrid system to test whether other proteins can associate with *CEN* DNA in *skp1-AA* cells. Ctf13 and Mad1, but not Bub1, associated with *CEN* DNA in *skp1-AA* cells (Figure 3G). These results strongly suggest that the kinetochore structure is not disrupted in *skp1-AA* cells.

If the Skp1-Bub1 interaction is required for signaling in the spindle checkpoint pathway, then spindle checkpoint activation induced by Bub1 overexpression might

be able to suppress the benomyl sensitivity of *skp1-AA* cells. We found that overexpression of Bub1 or the constitutively active mutant bub1-5 protein (Farr and Hoyt, 1998) suppressed the benomyl sensitivity of *skp1-AA* substantially but not the benomyl sensitivity of other kinetochore-defective mutants, i.e., *ctf13-30*, *skp1-4*, *dam1-1*, and *cep3-1* (Figure 3H and Supplemental Data at http://www.molecule.org/cgi/content/full/11/5/1201/DC1). This finding suggests that Bub1 functions downstream of Skp1 in the signaling pathway.

Taken together, these results strongly suggest that the Skp1-Bub1 interaction is important in the signaling pathway of the spindle checkpoint.

The Cell Cycle Delay Induced by Outer Kinetochore or Cohesion Defects Is Abolished in the *skp1-AA* Mutant

We tested whether *skp1-AA* cells have the same phenotypes observed in previously characterized checkpoint mutants. Upon treatment with high levels of microtubule-destabilizing drugs, *skp1-AA* cells arrested in G2/M (Figure 4A) with high levels of Pds1 (Figure 4B) and did not exhibit a rebudding phenotype (Hoyt et al., 1991) (data not shown) or chromosome reduplication (Schott and Hoyt, 1998) (Figure 4A). We suspected that *skp1-AA* cells retained the ability to signal defects caused by spindle microtubule depolymerization, but might be unable to signal other specific types of kinetochore defects. To examine this possibility, we determined whether the delayed progression through G2/M that is caused by a mutant kinetochore is abolished in *skp1-AA* mutants. It was previously shown that the G2/M delay of both *ctf13-30* and *ctf19*Δ mutants depends on *BUB1* and *MAD2*, indicating that kinetochore defects require the proteins encoded by *BUB1* and *MAD2* in order to be detected by the spindle checkpoint (Hyland et al., 1999; Pangilinan and Spencer, 1996; Wang and Burke, 1995). To test the effect of the *skp1-AA* mutation on kinetochore defect-induced G2/M delay, we introduced null mutations for the outer kinetochore proteins Ctf19 and Ctf3 (Hyland et al., 1999; Ortiz et al., 1999). *CTF19* and *CTF3* are not essential for viability, but cells lacking either gene experience a high level of chromosome missegregation (Hyland et al., 1999; Measday et al., 2002) and a G2/M delay that is observed as a marked accumulation of cells with G2 DNA content in a flow cytometric profile (Hyland et al., 1999; Measday et al., 2002) (Figure 4C and data not shown). In contrast, a *ctf19*Δ*skp1-AA* double mutant did not accumulate in G2/M (Figure 4C). This lack of accumulation of cells with a G2 DNA content is not due to suppression of the mitotic defects of *ctf19*Δ by the *skp1-AA* mutation because the double mutant still showed a high level of chromosome missegregation (data not shown). Identical results were observed in *ctf3*Δ and *ctf3*Δ *skp1-AA* double mutants (data not shown). Together, these results demonstrate that the G2/M accumulation of *ctf19*Δ or *ctf3*Δ cells is dependent on *SKP1* and strongly suggest that the Skp1-Bub1 interaction is required for transmitting a signal to the spindle checkpoint from compromised kinetochores.

Previous studies have shown that *skp1-4* cells exhibit a G2/M arrest when shifted to the nonpermissive temperature (Connelly and Hieter, 1996), due to activation of the spindle checkpoint (our unpublished data). However, our results suggest that the skp1-4 mutant protein apparently does not interact with Bub1, as evidenced by the Bub1-Skp1 two-hybrid (Figure 3C) and Bub1-*CEN* ChIP assays (Figure 2C). Thus, we hypothesized that the Bub1-Skp1 interaction may be only required for detecting outer kinetochore defects but not CBF3 defects. To test this possibility, we examined whether the G2/M accumulation of *ctf13-30* cells is dependent on *SKP1* because Ctf13 is also a component of CBF3. We found that the *ctf13-30 skp1-AA* mutants still showed accumulation in G2/M (Figure 4C), a result that supports the hypothesis that the Skp1-Bub1 interaction is not required for transmitting a signal induced by defects in CBF3 activity nor by defects caused by microtubule depolymerization.

Our results are consistent with the view that there may be at least two distinct mechanisms to detect kinetochore defects. Two different stimuli, i.e., from unattached kinetochores (Rieder et al., 1995) or via lack of tension on kinetochores (Li and Nicklas, 1995), are thought to activate the spindle checkpoint. Recently, Biggins and Murray have shown that Ipl1 is required for the delay triggered by chromosomes whose kinetochores are not under tension, but is not required for arrest induced by spindle depolymerization, a result that suggests that Ipl1 is required to detect loss of tension at the kinetochore but not unattached kinetochores (Biggins and Murray, 2001). Thus, we considered the possibility that Skp1-Bub1 monitors kinetochore tension but not attachment in budding yeast and tested this hypothesis directly by looking at mutants that destroy tension at the kinetochore.

Ctf8 and Scc1/Mcd1 are required for proper sister chromatid cohesion (Guacci et al., 1997; Mayer et al., 2001; Michaelis et al., 1997). In *ctf8* and *scc1* mutants, microtubules can still attach to kinetochores, but because the linkage between sister chromatids is compromised, the tension at kinetochores is reduced. The *ctf8*Δ and *scc1-73* cells accumulate in G2/M (Figure 5A), and the G2/M arrest caused by *ctf8*Δ has been shown to be dependent on Mad2 (Mayer et al., 2001). We found that the cell cycle progression delays associated with *ctf8*Δ and *scc1-73* were abolished in the *ctf8*Δ*skp1-AA* and *scc1-73skp1-AA* double mutants (Figure 5A). In addition, we arrested *ctf8*Δ, *ctf8*Δ*skp1-AA*, and the wild-type cells in G1 with α factor at 30°C and then released into YPD medium without α factor. We observed a delay in the degradation of Pds1 in *ctf8*Δ cells, indicating that a checkpoint is activated (Figure 5B), which was abolished in the *ctf8*Δ*skp1-AA* double mutant (Figure 5B). These results strongly suggest that the Skp1-Bub1 interaction is required by the spindle checkpoint to delay cells in which tension at kinetochores has been relaxed by a cohesion defect.

Next, we performed an independent set of experiments to confirm the requirement of the Skp1-Bub1 interaction for spindle checkpoint signaling from kinetochore tension defects. In the absence of DNA replication, kinetochore tension cannot be generated because kinetochores lack sisters. DNA replication can be prevented by depleting Cdc6 (which is required for the initiation of replication) without affecting the interac-

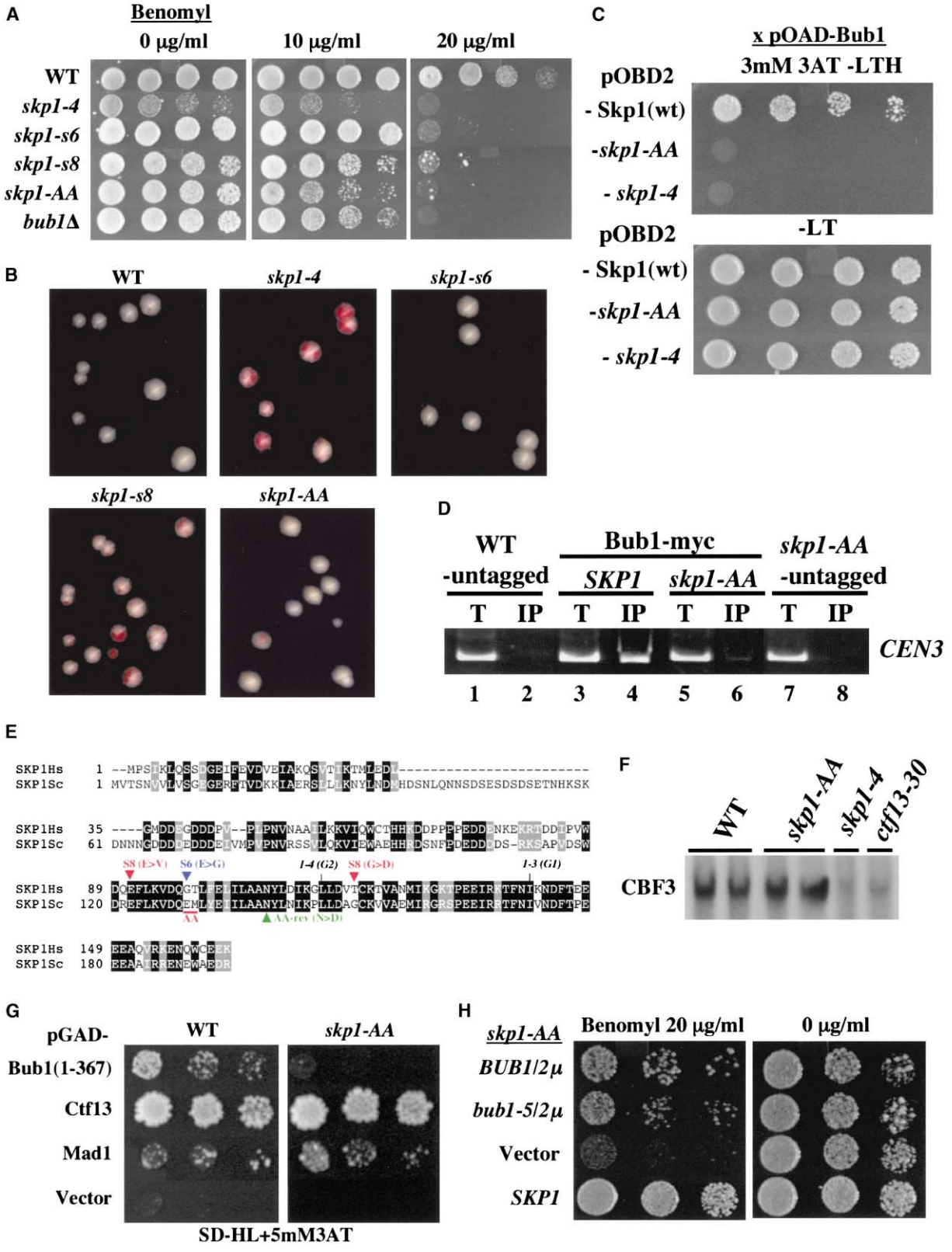

Figure 3. Benomyl-Sensitive *skp1* Mutants

(A) The indicated strains were spotted onto YPD plates containing 0, 10, or 20 μg/ml benomyl, and the plates were then incubated for 3 days at 30°C. The numbers of cells that were spotted onto each plate (left to right in the figure) were 5×10^4, 1×10^4, 2×10^3, and 4×10^2.

(B) Chromosome fragment segregation was analyzed in wild-type (WT) and *skp1* mutants by using a colony color assay (Koshland and Hieter, 1987). Loss of the nonessential chromosome fragments results in a red sector in a white colony.

tion between microtubules and kinetochores (Piatti et al., 1995). In these cells, Pds1 is stabilized in a spindle checkpoint-dependent manner (Stern and Murray, 2001). We therefore examined the effect of the *skp1-AA* mutation in cells that failed to replicate DNA. Cells depleted of Cdc6 protein (*cdc6*Δ; when *GAL-CDC6* is off) were arrested in G1 with α factor and released from the arrest, and Pds1 levels were monitored as cells progressed through the cell cycle. In Cdc6-depleted cells containing unreplicated DNA, Pds1 levels are stabilized for at least 1.5 hr (Figure 5C). The Pds1 stabilization requires the spindle checkpoint because it is abolished in *cdc6*Δ*mad2*Δ cells (Figure 5C). We observed that Pds1 levels are also not stabilized in *cdc6*Δ*skp1-AA* cells (Figure 5C), with degradation kinetics almost identical to that observed in the *cdc6*Δ*mad2*Δ *cells*. Therefore, we concluded that the Skp1-Bub1 interaction is required for the spindle checkpoint response that delays cells whose kinetochores are not under tension.

Mps1 overexpression ectopically activates the spindle checkpoint, causing cell cycle arrest with a bipolar spindle. To test the effect of the *skp1-AA* mutation on this arrest, Mps1 was inducibly overexpressed from the *GAL1* promoter in wild-type cells and *skp1-AA* mutant cells. After 3 hr, both the wild-type cells and the *skp1-AA* mutant cells were arrested in G2/M properly (Figure 5D). These results indicate that overexpression of Mps1 can activate the spindle checkpoint independently of the Skp1-Bub1 interaction.

Restoration of the Bub1 Binding Ability of skp1-AA Protein Restores the Tension Checkpoint of *skp1-AA* Cells

To address whether the lack of function in the tension checkpoint of *skp1-AA* is due to the lack of the interaction between skp1-AA protein and Bub1, we used a PCR-based method (Connelly and Hieter, 1996) to screen for skp1-AA "revertant" proteins that can bind to Bub1 in the two-hybrid assay. We obtained such a revertant (*skp1-AA-rev*) (Figures 6A and 6B and Supplemental Data at http://www.molecule.org/cgi/content/full/11/5/1201/DC1), which contains a third mutation (N139D) in addition to the two original AA mutations (E129A and M130A) (Figure 3E). Expression of either skp1-AA-rev protein or expression of the wild-type Skp1 protein, but not skp1-AA protein, suppressed the benomyl sensitivity of *skp1-AA* cells (Figure 6C). In addition, *skp1*deletion strains carrying pRS315-*skp1-AA*-rev plasmid were not as sensitive to benomyl as the *skp1* deletion strains carrying pRS315-*skp1-AA* plasmid (Figure 6D and Supplemental Data at http://www.molecule.org/cgi/content/full/11/5/1201/DC1). Furthermore, expression of the skp1-AA-rev protein or of wild-type Skp1 protein, but not skp1-AA protein, restored the G2 delay of *skp1-AActf8*Δ cells (Figure 6E). Finally, the association of Bub1 with *CEN* DNA, that is disrupted in *skp1-AA* cells, is restored in *skp1-AA-rev* cells (Figure 6F).

Taken together, these coreversion results strongly suggest that the absence of the tension checkpoint in *skp1-AA* cells is due to the lack of interaction between skp1-AA protein and Bub1.

Discussion

In this study, we demonstrate that Skp1, a protein within the core of the kinetochore, interacts with Bub1, a component of the spindle checkpoint, and that Bub1 associates with *CEN* DNA via Skp1. We also show that mutations in Skp1 that specifically disrupt the Skp1-Bub1 interaction abrogate the cell cycle delay response induced by kinetochore tension defects. Furthermore, restoration of the Bub1 binding ability of the skp1-AA revertant protein restored the tension checkpoint in *skp1-AA* cells. Based on these findings and those of earlier studies, we propose a model for the signaling of kinetochore defects to the spindle checkpoint that includes Skp1-Bub1-dependent (detecting lack of tension) and Skp1-Bub1-independent (detecting failure in attachment) functions (Figure 7).

CBF3 and the Spindle Checkpoint Pathway

Gardner et al. showed that the elimination of CBF3 components by the degron system abolishes the spindle checkpoint response, a result that indicates that CBF3 is required for checkpoint signaling (Gardner et al., 2001). Thus, Bub1-*CEN* binding should depend on the CBF3 complex. However, at the permissive temperature, CBF complexes do exist in the *skp1-4*, *ctf13-30*, *sgt1-3*, and

(C) The ability of skp1-AA protein and skp1-4 protein to bind to Bub1 was compared with that of Skp1 in the two-hybrid assay. The colonies were grown on a Sc-Leu-His-Trp plate containing 3 mM 3-amino-1,2,4-triazole (3AT) (left panel) or on a Sc-Leu-Trp plate (LT) (right panel). The dilution was done in the same way as in (A).

(D) ChIP assays of the indicated strains were performed. The myc antibody was used for immunoprecipitation. Total chromatin (T) and immunoprecipitate (IP) were the templates for amplification of *CEN3*. The controls were wild-type Skp1 (lanes 1 and 2) and skp1-AA that lacked a myc tag (lanes 7 and 8); the combinations of Bub1 and Skp1 that were analyzed were the following: wild-type Skp1 and myc-tagged Bub1 (lanes 3 and 4); skp1-AA and myc-tagged Bub1 (lanes 5 and 6).

(E) An alignment of the amino acid sequences of budding yeast Skp1 (SKP1Sc) and human Skp1 (SKP1Hs) is shown. Identity is indicated by the black boxes; conservative changes are indicated by the shaded boxes. The location and nature of mutations found in the indicated mutants are shown above or below the aligned sequences. The high level of chromosome missegregation in *skp1-s8* could be due to G150D mutation because its location is near the L146S mutation in *skp1-4*.

(F) Protein extracts of the indicated mutant cells were subjected to the bandshift assay to examine the CBF3 assembly activity. A ^{32}P-labeled 88 bp *CDEIII* fragment (Lechner and Carbon, 1991) served as the probe.

(G) The one-hybrid strain (wild-type: YLJ128) and the one-hybrid strain carrying the *skp1-AA* mutation (Y977) were used in the in vivo *CEN* DNA binding assay as described previously (Warren et al., 2002). Each strain harboring pGAD-Bub1 (1-367), pGAD-Ctf13, or vector only was spotted onto an SD-Leu-His plate containing 5 mM 3AT. The plates were then incubated at 30°C for 9 days.

(H) The *skp1-AA* cells containing BUB1/2μ plasmid, bub1-5/2μ plasmid, or vector only were spotted onto YPD plates containing 0 or 20 μg/ml benomyl, and the plates were incubated for 3 days at 30°C. The numbers of transformed cells that were spotted onto the plate were 1×10^4, 2×10^3, and 4×10^2 (from left to right in the figure).

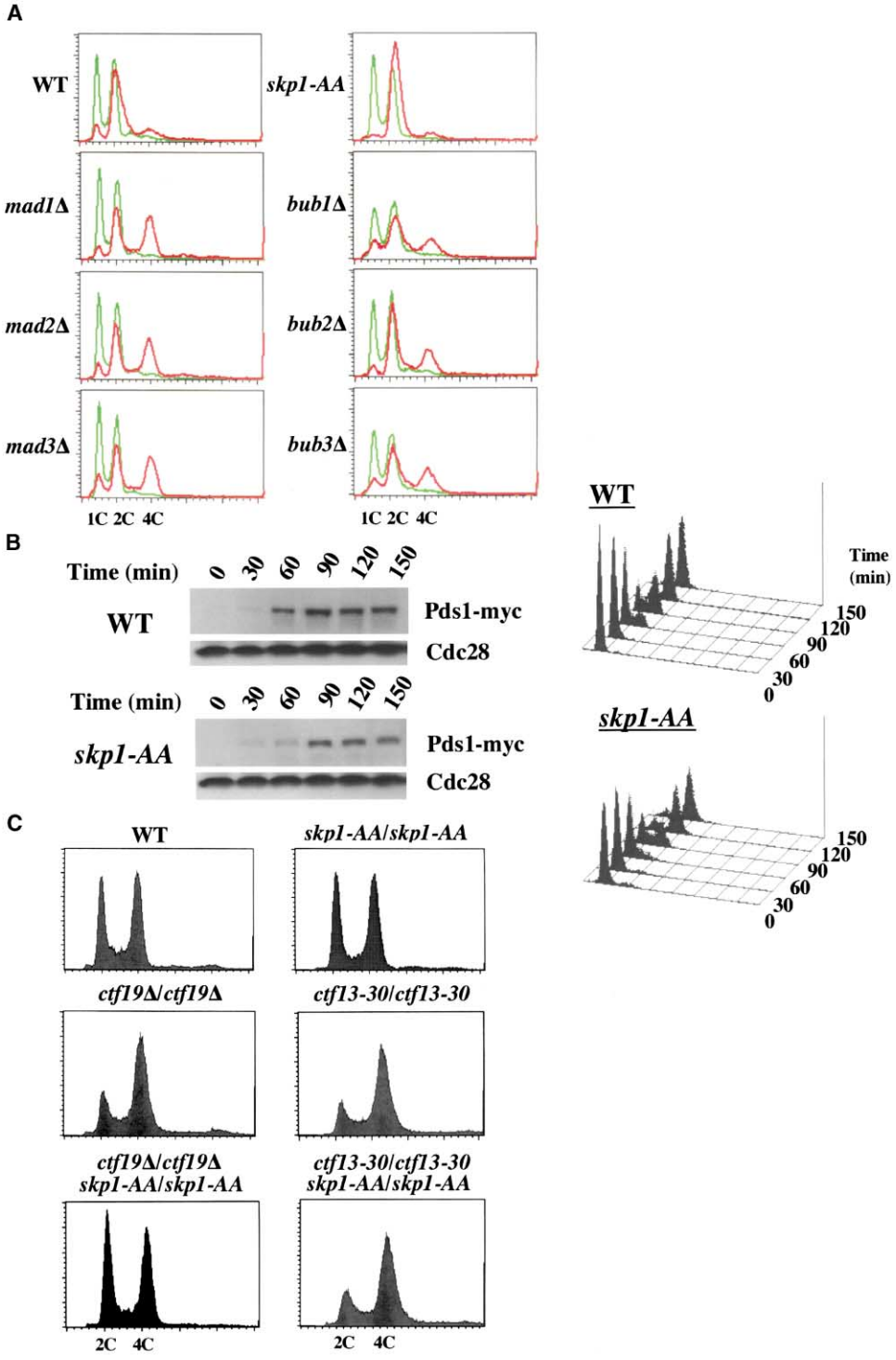

Figure 4. No Requirement for the Skp1-Bub1 Interaction in the Cell Cycle Arrest Induced by Spindle Depolymerization or CBF3 Defects

(A) Fluorescence-activated cell sorting (FACS) profiles of cells in early log phase (green lines) and cells in the same phase of growth but treated with 15 μg/ml nocodazole for 3 hr at 30°C (red lines) before analysis. The abbreviations 1C, 2C, and 4C represent ploidies.

(B) Cells were arrested with α factor for 2 hr and released into YPD media containing 15 μg/ml nocodazole. Samples were taken at the indicated times for FACS analysis (two bottom panels) and for preparing protein extracts. The protein extracts were immunoblotted with anti-myc antibody and anti-Cdc28 antibody, respectively. The same amount of lysate was used for each lane as indicated by the Cdc28 protein level.

(C) FACS profiles of the indicated strains. Before analysis, cells were grown to the early log phase at 30°C.

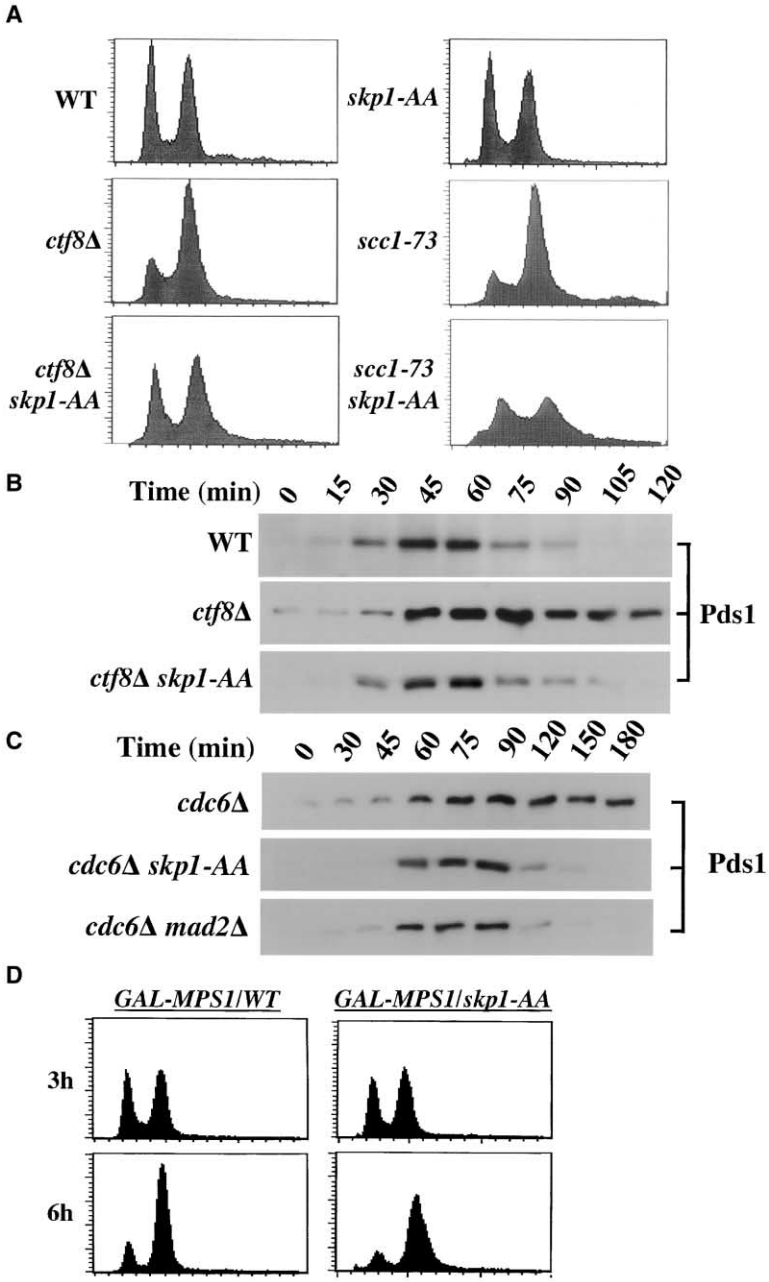

Figure 5. Requirement for the Skp1-Bub1 Interaction in the Cell Cycle Delay Induced by Kinetochore Tension Defects

(A) FACS profiles of the indicated strains. Before analysis, cells were grown to the early log phase at 30°C.

(B) The indicated strains were arrested in G1 with α factor, and were released to YPD media. Pds1-myc levels were monitored by immunoblotting using anti-myc antibody. The same amount of lysate was used for each lane as indicated by the Cdc28 protein level (data not shown).

(C) Cells depleted of the Cdc6 were grown and arrested in G1 with α factor and were released in the presence of glucose (YPD media) to keep *GAL-CDC6* repressed. Pds1 levels were monitored by immunoblotting using anti-myc antibody. Two independent *cdc6Δskp1-AA* strains (Y909 and Y910) were used for this analysis, and identical results were observed.

(D) Y755 *MAT***a** *GAL1-MPS1:URA3* and Y762 *MAT***a** *GAL1-MPS1:URA3 skp1-AA:LEU2* cells were grown on Sc-raffinose plates for a couple of days, and grown in Sc-raffinose media overnight. 1/10th volume of 20% galactose was added to the culture to induce expression of Mps1. Samples were taken for FACS analysis at the indicated times.

ndc10-1 mutant cells; therefore, it is not surprising that we were able to detect Bub1-*CEN* binding in these mutants, except those that had lost their ability to interact with Bub1. Importantly, *skp1-AA* cells did not exhibit substantial chromosome missegregation, and CBF3 activity was intact in the *skp1-AA* cells. Therefore, in *skp1-AA* cells, Bub1 does not associate with functional kinetochore complexes that can segregate chromosomes properly.

Our results indicate that the *skp1-4* mutant protein does not interact with Bub1. However, confirming the finding from an earlier study, *skp1-4* cells exhibited a G2/M delay when shifted to the nonpermissive temperature, an effect that is caused by the activation of the spindle checkpoint (Connelly and Hieter, 1996) (Figure 7A). In addition, the G2/M delay caused by the *ctf13-30* mutation was consistently unaffected by the *skp1-AA* mutation. Together, these results suggest that the defects in these mutant CBF3 components are detected by the spindle checkpoint pathway in a Skp1-Bub1-independent manner (e.g., monitoring of CBF3 assembly or detection of microtubule attachment) (Figure 7B).

Cohesion Defects and the Spindle Checkpoint Pathway

Ctf8 and Scc1 are required for proper sister chromatid cohesion. In *ctf8* and *scc1* mutant cells, kinetochore tension is relaxed and the spindle checkpoint is activated, which results in cell cycle delay at G2/M (Figure 7A). In *skp1-AA* cells, the G2/M delay caused by *ctf8* and

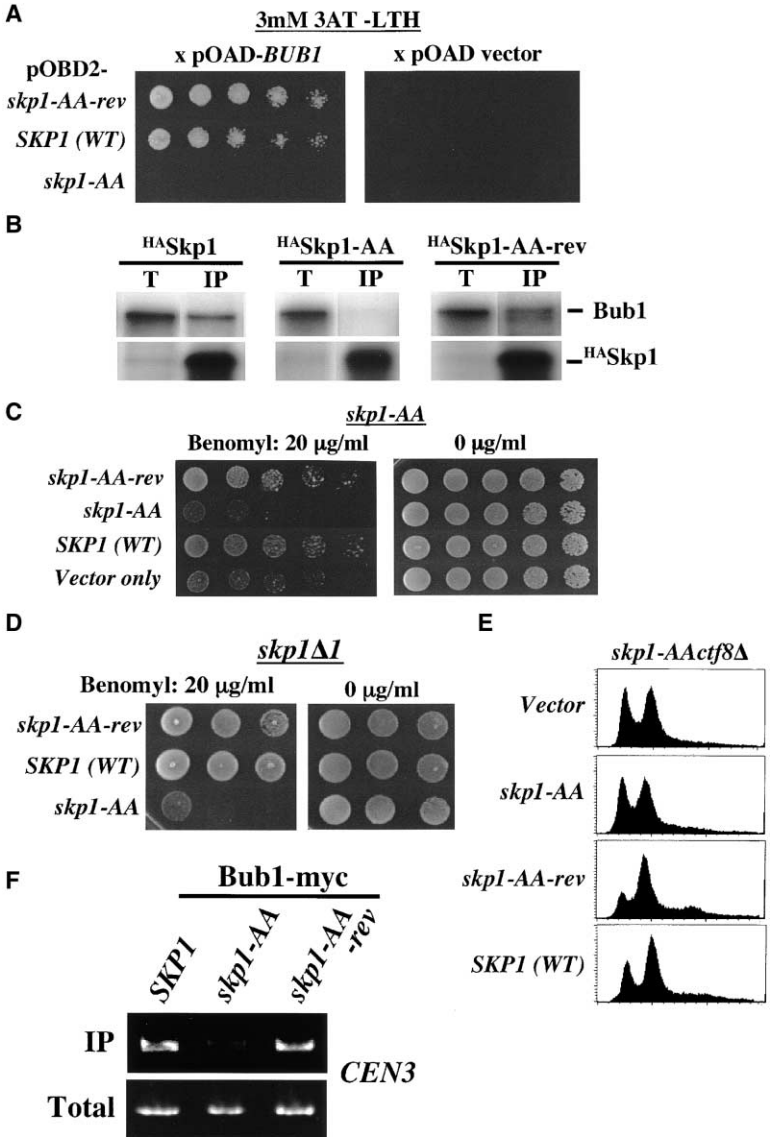

Figure 6. The Absence of the Tension Checkpoint in *skp1-AA* Cells Is Due to the Lack of Interaction between skp1-AA Protein and Bub1

(A) The ability of skp1-AA-rev protein to bind to Bub1 was examined in the two-hybrid assay. Colonies were grown on an Sc-Leu-His-Trp plate containing 3 mM 3-amino-1,2,4-triazole (3AT) (left panel) or on an Sc-Leu-Trp plate (LT) (right panel). The dilution was done in the same way as in Figure 3C.

(B) The indicated Skp1 wild-type and mutant proteins were expressed and labeled with ^{35}S-methionine in the in vitro translation system, and the lysates were mixed and incubated for 1 hr at 30°C with a different extract containing Bub1. Skp1-HA was immunoprecipitated by using anti-HA-Sepharose A, the immunoprecipitates were eluted and subjected to SDS-PAGE, and radioactive bands were identified by autoradiography. T, total lysates (5% of the starting material); IP, immunoprecipitate.

(C) The *skp1-AA* cells containing *skp1-AA-rev/CEN* plasmid, *skp1-AA/CEN* plasmid, *SKP1/CEN* plasmid, or vector only was spotted onto YPD plates containing 0 or 20 μg/ml benomyl, and the plates were incubated for 3 days at 30°C. The numbers of transformed cells that were spotted onto the plate were 1×10^4, 2×10^3, 4×10^2, and 1×10^2 (from left to right in the figure).

(D) The *skp1*Δ cells containing *skp1-AA-rev/CEN* plasmid, *SKP1/CEN* plasmid, or *skp1-AA/CEN* plasmid was spotted onto YPD plates containing 0 or 10 μg/ml benomyl, and the plates were incubated for 3 days at 30°C. The numbers of transformed cells that were spotted onto the plate were 1×10^4, 2×10^3, and 4×10^2 (from left to right in the figure).

(E) FACS profiles of the *skp1-AActf8*Δ strains containing vector only, *skp1-AA/CEN* plasmid, *skp1-AA-rev/CEN* plasmid, or *SKP1/CEN* plasmid. Before analysis, cells were grown to the early log phase at 30°C.

(F) ChIP assays of the indicated strains were performed. The anti-myc antibody was used for immunoprecipitation. Total chromatin (T) and immunoprecipitate (IP) were the templates for amplification of *CEN3*.

scc1 mutations is not observed. These results strongly suggest that the Skp1-Bub1 interaction is required to detect loss of kinetochore tension (Figure 7B). These results also suggest that cohesion defects are detected at kinetochores, which is consistent with the observations in fission yeast that the loss of chromatid cohesion in *mis4-242* and *rad21-K1* mutants leads to activation of the Mad2- and Bub1-dependent checkpoint and that the cohesion mutation greatly increases the duration of kinetochore localization of Bub1 (Toyoda et al., 2002).

The Tension Checkpoint

Stern and Murray demonstrated that monooriented chromatids, in which kinetochores are not under tension, can trigger the spindle checkpoint (Stern and Murray, 2001). Cells lacking Cdc6, which is required for initiating DNA replication, do not activate the DNA replication checkpoint and thus enter mitosis without replication (Piatti et al., 1995). In this situation, chromosomes lack paired sister chromatids, and kinetochores presumably randomly attach to microtubules and the chromosomes move to one pole or the other (Figure 7A). We found that the delay in Pds1 degradation in *cdc6* Δ cells due to spindle checkpoint activation is not seen in *skp1-AA* cells. Therefore, we conclude that the Bub1-Skp1 interaction is required to detect lack of kinetochore tension.

Ctf19 is an outer kinetochore protein that forms a complex with Mcm21 and Okp1 (Hyland et al., 1999; Ortiz et al., 1999). Recently, Measday et al. showed that Ctf3 is an outer kinetochore protein that forms another complex with Mcm22 and Mcm16 and that the Ctf3-Mcm22-Mcm16 complex is located on the outer surface of the Ctf19-Okp1-Mcm21 complex, toward the spindle (Measday et al., 2002). Our results indicate that the spindle checkpoint detects the absence of these outer kinetochore proteins via the Skp1-Bub1 interaction (Figure 7B). Therefore, the simplest interpretation would be that

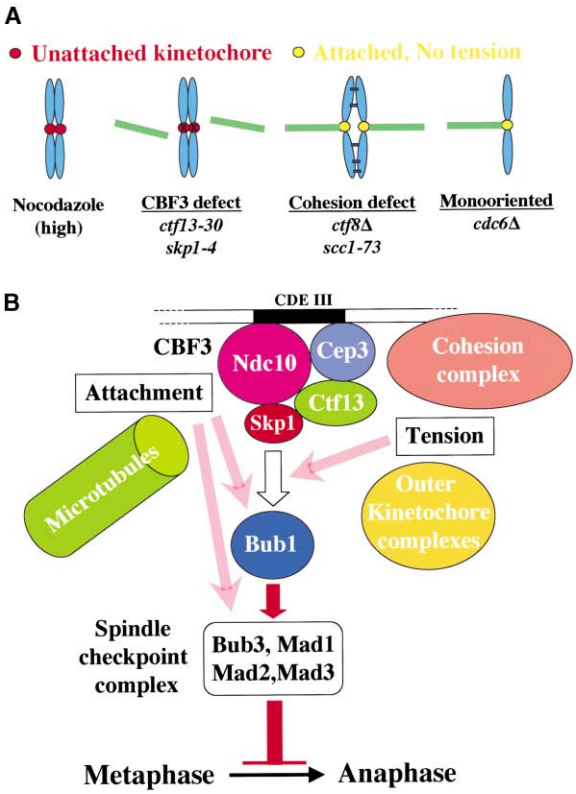

Figure 7. A Model of the Spindle Checkpoint Signaling Pathway

(A) Schematic view of the status of kinetochore-microtubule attachment and kinetochore tension under various conditions. The elongated ovals indicate sister chromatids, and the green rectangles represent microtubules.

(B) We propose that the Skp1-Bub1 interaction is required to detect defects in outer kinetochores and cohesion, presumably in kinetochore tension. A shaded red arrow pointing from the cohesion complex and the outer kinetochore complex indicates a checkpoint signal that detects insufficient tension at kinetochores. The other shaded red arrows indicate checkpoint signals that detect a failure in the attachment of kinetochores to microtubules.

the absence of the outer kinetochore proteins could cause loss of kinetochore tension. Further investigation is necessary to elucidate the potential role of outer kinetochore proteins in promoting kinetochore tension.

Initially, we isolated *skp1* mutants that did not grow in the presence of a low (normally sublethal) concentration of benomyl. However, the benomyl-hypersensitive *skp1* mutants arrested in G2/M in the presence of a high (normally lethal) concentration of nocodazole. Skoufias et al. reported that in mammalian cells the arrest induced by a low concentration of a microtubule inhibitor correlates with a loss of tension at the kinetochore, and that in response, the checkpoint proteins Bub1 and BubR1 are recruited to the kinetochore (Skoufias et al., 2001). Therefore, we think that in *skp1-AA* mutants, in the presence of a low concentration of benomyl, kinetochore tension is relaxed, but the checkpoint signal is ignored, resulting in the lethality of the mutant cells.

The *skp1-AA* mutation did not alter the G2/M arrest induced by overexpression of Mps1, a finding that is consistent with the observation that Mps1 overexpression can activate the G2/M checkpoint in the absence of functional kinetochores (Fraschini et al., 2001). Therefore, the Mps1 pathway may be independent from the Bub1-Skp1 pathway, or the Mps1 pathway can activate multiple spindle checkpoint pathways.

Two Different Checkpoint Signals from the Kinetochore?

In any case, our data may be revealing at least two different checkpoint signals from the kinetochore, one for assembly/attachment (Bub1-Skp1 interaction independent, perhaps assessing some assembly intermediate) and one for later events (Bub1-Skp1 interaction dependent, perhaps assessing tension) (Figure 7B). However, it is possible that the loss of the tension checkpoint may result from the partial loss of activity of the spindle checkpoint (i.e., the spindle checkpoint activity in the *skp1-AA* cells is sufficient to detect a loss of attachment but not a lack of tension). Because Skp1 is essential for viability, it is impossible to test the effects of a complete of loss-of-function mutation. Biggins and Murray recently showed that Ipl1, a member of the Aurora protein kinase family in budding yeast, is required for the G2/M delay triggered by loss of tension but is not required for arrest induced by spindle depolymerization (Biggins and Murray, 2001). Hence, the relationship between the Skp1-Bub1 interaction and the Ipl1 pathway is an interesting area that requires further study.

High Levels of Bub1 at Kinetochores Is Not Necessary for the Mictrotubule-Kinetochore Attachment Checkpoint

Our results also strongly suggest that Bub1's localization to kinetochores (at least at high steady-state level) is not required for signaling a checkpoint signal from unattached kinetochores. This interpretation implies that the other spindle checkpoint proteins may detect failure in attachment. However, because *bub1* deletion strains fail to respond to microtubule depolymerizing drugs, Bub1 itself should be involved in the attachment signaling pathway elsewhere but not at kinetochores. Martin-Lluesma et al. reported very recently that depletion of Hec1 by siRNA from human cells induced spindle checkpoint activity while they could not detect Mad1/Mad2 complexes at kinetochores in the Hec1-depleted cells (Martin-Lluesma et al., 2002). In the Hec1-depleted cells, Bub1 was still localized at kinetochores, although the kinetochore association was reduced by 50% (Martin-Lluesma et al., 2002). Because kinetochores were not stretched in the Hec1-depleted cells, it is plausible that the checkpoint response was caused by a lack of tension (Martin-Lluesma et al., 2002). Therefore, it would be interesting to examine whether Bub1 needs to be at kinetochores to detect tension defects in mammalian cells.

Bona Fide Kinetochore Proteins Could Be Cancer-Related Targets

In most colon cancers, and probably in many other cancer types, a chromosomal instability (CIN) phenotype is observed. Cahill et al. have shown that CIN is consistently associated with the loss of function of the spindle checkpoint and that in two independent CIN cancers,

the human homologs of *BUB1* are mutated (Cahill et al., 1998). Michel et al. reported that *Mad2*$^{+/-}$ mice develop lung tumors at high rates after long latency periods (Michel et al., 2001). These results strongly suggest that proteins required for the spindle checkpoint could be cancer-related targets. However, recent sequencing analyses of many tumor cells suggest that mutations in the human orthologs of known spindle checkpoint components are rare (Cahill et al., 1999; Hernando et al., 2001; Imai et al., 1999; Yamaguchi et al., 1999). Therefore, additional unknown proteins involved in the spindle checkpoint function may exist. The results presented here and elsewhere indicate that mutations in kinetochore proteins can eliminate a spindle checkpoint function. Thus, it would be worthwhile to determine whether tumor cells contain mutated bona fide kinetochore proteins.

Experimental Procedures

Protein Expression and Immunoprecipitation

Baculovirus that express FLAG-tagged Skp1 and untagged Skp1 were previously described (Kaplan et al., 1997; Kitagawa et al., 1999; Skowyra et al., 1997). For expression of 6×His-Bub1 in insect cells, pFastBac-HTa-BUB1 (Invitrogen) was constructed; expression was performed as recommended by the supplier. For protein expression, insect cells (Sf9; Invitrogen) were infected with the indicated virus combinations for 40 hr. Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Nonidet P-40, 10 mM NaF, 10 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin), and debris was removed by centrifugation. Typically, 3 ml of lysis buffer was used to lyse 0.5×10^8 cells. For immunoprecipitation, 0.3–0.6 ml of cell lysate was incubated at 4°C for 16 hr with 4 μg of anti-6×His or anti-FLAG antibody and 15 μl of protein A-conjugated Sepharose. For in vitro expression of protein, PCR products that encoded polypepides of the expected sizes (Figure 1C) were used as templates in the PCR-TNT in vitro transcription/translation system (Promega), and the proteins were labeled with ^{35}S-methionine. Approximately equal amounts of proteins were used for immunoprecipitation with an anti-HA antibody. For bacterial protein expression, BL21(DE3) cells were transformed with pGEX-4T-SKP1, pGEX-4T (Amersham), and pTR124 (Roberts et al., 1994), and expression was induced by the addition of IPTG (1 mM) and incubation for 3 hr at 37°C. The GST-tagged and 6×His-tagged proteins were affinity purified according to the manufacturer's instruction (Amersham and Qiagen). Approximately 200 ng of the purified proteins were mixed in 200 μl of the lysis buffer and incubated at 30°C for 1 hr. The proteins were then subjected to immunoprecipitation with an anti-6×His antibody. Immune complexes were washed five or seven times with 1.5 ml of lysis buffer before they underwent SDS-PAGE and immunoblotting. Total lysates (T) were represented by material in a volume that was equivalent to 2%–5% of that of the starting material.

ChIP Assay

ChIP experiments were performed as described (Hecht and Grunstein, 1999; Meluh and Koshland, 1997; Tanaka et al., 1997), but the following alterations were made. Cells were fixed (cellular proteins and DNA were cross-linked) by incubation in 1% formaldehyde at 4°C overnight. Cell extracts were sonicated six times for 10 s each to shear the chromatin to an average size of 500 bp. Anti-Myc (9E10) affinity matrix (Covance; volume, 20 μl) was used to immunoprecipitate protein-DNA complexes. At least 1.5 mg of lysate was used in each immunoprecipitation. Depending on the liner range of detection of PCR, the amount of template used in PCR ranged from 1/200 to 1/30 of total chromatin and from 1/30 to 1/15 of total immunoprecipitate. The primers for *CEN3*, *CEN1*, and *CEN16* used in PCR analysis were identical to the region from nucleotide 113,925–113,945 of chromosome III, 151,379–151,681 of chromosome I, and 555,845–556,189 of chromosome XVI (Meluh and Koshland, 1997). Primers for *PGK1* were identical to the region from nucleotide 138,557–138,845 of chromosome III (Meluh and Koshland, 1997).

CDC6 Depletion

The *GAL-CDC6* experiment was performed as essentially described previously (Biggins and Murray, 2001; Stern and Murray, 2001). In brief, cells were grown in YPG (2% galactose + 1% raffinose) and arrested in α factor for 2 hr at 30°C. The cells were released into YPG medium for 20 min at 30°C and were then transferred to YPD medium (2% glucose) to repress Cdc6 expression. Protein samples were taken at the indicated times. α factor was added again after 70 min to rearrest cells in the next cell cycle.

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