Sgt1p and Skp1p Modulate the Assembly and Turnover of CBF3 Complexes Required for Proper Kinetochore Function

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INTRODUCTION

Kinetochore proteins are essential for the proper segregation of chromosomes. Kinetochore complexes are composed of many proteins that are required for the attachment and movement of chromosomes along the mitotic spindle. The kinetochore is also critical for the spindle checkpoint, a surveillance system that ensures proper kinetochore–microtubule attachments are formed before the onset of anaphase. The recent identification of a large number of kinetochore proteins suggests that the processes of microtubule attachment, chromosome movement, and coordination with the cell cycle are complex and require the organization of a large number of proteins at the centromere (Cheeseman et al., 2002; McAinsh et al., 2003).

Remarkably, in the budding yeast Saccharomyces cerevisiae, this large array of proteins is organized on a compact centromere of 125 base pairs that is divided into three regions: CDEI, CDEII, and CDEIII (Clarke and Carbon, 1985). Although each region plays an important role in kinetochore function, mutational analyses suggest that only the CDEII region is essential for kinetochore function (Hegemann et al., 1988). Biochemical purification of proteins that bind CDEII and subsequent genetic analyses identified the CBF3 complex, which is comprised of three proteins: Ctf13p (p58), Cep3p (p64), and Ndc10p (p110) (Lechner and Carbon, 1991; Doheny et al., 1993; Jiang et al., 1993; Lechner, 1994; Strunnikov et al., 1995). Additional genes critical for the proper function of CBF3 were identified in dosage suppression studies; SKP1 was found as a dosage suppressor of a mutation in CTF13 (ctf13-30); similarly, SGT1 was found as a dosage suppressor of a mutation in SKP1 (skp1-4) (Connelly and Hieter, 1996; Kitagawa et al., 1999). Mutations in SKP1 and SGT1 abolish CBF3 and in vitro studies suggest that Skp1p and Sgt1p are required for the biochemical activity of Ctf13p (Kaplan et al., 1997; Kitagawa et al., 1999). Although CBF3 is required for kinetochore function, it does not directly contact microtubules. Instead, it acts to nucleate and organize a large number of outer kinetochore complexes (Basu et al., 1999; Ortiz et al., 1999; He et al., 2001; Jones et al., 2001; Janke et al., 2002; Li et al., 2002; Measday et al., 2002). Although its interaction with microtubules is indirect, it has been recently shown that Skp1p directly interacts with the spindle checkpoint protein Bub1p, suggesting that CBF3 may be intimately connected to the regulatory machinery that ensures proper kinetochore–microtubule attachment has occurred before the onset of anaphase (Kitagawa et al., 2003).

Studies of CBF3 organization suggest that multiple DNA contacts made by all three subunits are required for CBF3 to stably bind CEN DNA and that Ctf13p is the core subunit of the complex, making contacts with both Cep3p and Ndc10p (Espelin et al., 1997; Russell et al., 1999). The importance of Ctf13p is underscored by its central position in the complex and by the fact that mutations in the SKP1 and SGT1 genes inactivate Ctf13p, leaving it unable to form a functional CBF3 complex (Kaplan et al., 1997; Kitagawa et al., 1999). Skp1p was shown to interact directly with a conserved amino terminal F-box motif in Ctf13p and mutations in the F-box disrupt Skp1p association with Ctf13p, inactivating Ctf13p and abolishing CBF3 function (Russell et al., 1999).
Similarly, a mutation in SGT1 that blocks its interaction with Skp1p inhibits the formation of CBF3 (Kitagawa et al., 1999). Biochemical and genetic studies suggest that Skp1p and Sgt1p modify Ctf13p, possibly through protein phosphorylation or the action of HSP90 type chaperones (Kaplan et al., 1997; Stemmann et al., 2002). However, the precise biochemical changes required for Ctf13p activation and how activation contributes to CBF3 function remain unclear. Given the central role of CBF3 in nucleating kinetochore formation and its involvement in the spindle checkpoint, it is critical to precisely understand how Skp1p and Sgt1p contribute to CBF3 function.

Here, we examine how Skp1p and Sgt1p contribute to the function of the CBF3 complex and how this process relates to the normal function of the kinetochore. We report that Skp1p and Sgt1p are required for the assembly of CBF3, which occurs in a stepwise manner. Mutations in Skp1p and Sgt1p are required for the assembly of CBF3, the normal function of the kinetochore. We report that function of the CBF3 complex and how this process relates to Skp1p and Sgt1p contribute to CBF3 function.

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**Materials and Methods**

**Plasmids, Yeast Strain Construction and Antibodies**

The coding sequence of SGT1 was polymerase chain reaction (PCR)-amplified from yeast genomic DNA. Primers (sequences provided upon request) were designed with appropriate restriction sites to insert SGT1 DNA into the pFastBac (Invitrogen, Carlsbad, CA) and pFBNhis10HA baculovirus vectors. The sequence was confirmed, recombinant viruses were generated, and High-five cells were used to produce recombinant proteins as described previously (Kaplan et al., 1997; Russell et al., 1999). The CTF13 gene used to generate recombinant protein was synthetically constructed using short oligonucleotides to optimize codon usage (kindly provided by X. He and P. Sorger, Massachusetts Institute of Technology, Cambridge, MA). Yeast strains were constructed in the indicated backgrounds (Table 1) by homologous recombination by using standard PCR-based targeting techniques (Longtine et al., 1998). The integrity of both 5′ and 3′ recombination sites was confirmed using PCR, and the fusion verified by immunoblotting.

Viral infections of insect cells were carried out and lysates were prepared as described previously (Russell et al., 1999). Recombinant MBCs-10×his-HA-Sgt1p was purified using standard metal chelate chromatography conditions. Polyclonal antibodies against Sgt1p were produced in rabbits after injection of purified recombinant MBCs-10×his-HA-Sgt1p by using standard procedures (Harlow, 1988). Antiserum against Ctf13p, Cep3p, and Skp1p were made as described previously (Kaplan et al., 1997; Russell et al., 1999). The monoclonal antibody to the hemagglutinin tag (12C5) was purchased from Roche Diagnostics (Indianapolis, IN), and the rat tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

**Yeast Growth and Extract Preparation**

Yeast strains were grown at 30°C unless otherwise indicated in standard YEP media supplemented with 2% dextrose or 2% rafinose and 2% galactose as indicated previously (Guthrie and Fink, 1991). For chromosome loss assays, yeast were grown to log phase in minimal media lacking histidine and containing 2% galactose and rafinose. Cells were transferred to YEP containing the indicated carbon source for 1 h. Cells were plated on YEP-agar plates containing 2% galactose and rafinose. The number of red colonies (more than half red) and sectored colonies (more than half red and sectored colonies (more than half red and sectored colonies (more than half red) were determined, corrected for the number of cell divisions and the fold increase of each colony type calculated by comparing cells with a wild-type

**Table 1. Strains used in this study**

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mM KCl, 5 mM EGTA, 5 mM EDTA, 100 mM β-glycerophosphate, 10% glycerol, 14 mM 2-mercaptoethanol, 10 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, and 0.4 mM phenylmethylsulfonyl fluoride) and then resuspended in a volume of extract buffer supplemented with a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM N-tosyl-l-phenylalanine chloromethyl ketone, and 10 µl/mg leupeptin, pepstatin, and chymostatin) equal to the size of the cell pellet. The resulting cell slurry was immediately frozen in liquid N₂ and processed as described previously (Kaplan and Sørgør, 1997).

Results

Ctf13p, Skp1p, and Sgt1p Form a Single Complex Involved in the Activation of Ctf13p

Skp1p and Sgt1p are required to activate Ctf13p, a critical event for the formation of CBF3 complexes that can bind stably to centromeric (CEN) DNA. To understand how Skp1p and Sgt1p contribute to CBF3 formation, we analyzed the interactions of recombinant CBF3 proteins by using wild-type or mutant versions of SKP1. To validate the relevance of this in vitro system, we first characterized the behavior Sgt1p when coexpressed with recombinant CBF3 subunits in insect cells. Although Sgt1p is not required to activate Ctf13p in insect cells (Kaplan et al., 1997), presumably because the insect Sgt1p can substitute, we observed a 13-fold increase in the specific activity of Ctf13p when coexpressed with yeast Sgt1p (Figure 1A). Furthermore, the increase in specific activity of Ctf13p remained dependent on Skp1p, because Ctf13p coexpressed with Sgt1p failed to be activated in the absence of Skp1p or in the presence of Skp1-4p (Figure 1B).

We note that the small amount of active Ctf13p observed in Skp1-4p lysates may reflect the partial suppression of the skp-4 mutation due to overexpression of Sgt1p, consistent with previously reported genetic interactions (Kitagawa et al., 1999). The contribution of Sgt1p to Ctf13p activation and its dependence on Skp1p allowed us to further characterize the role of Skp1p and Sgt1p in CBF3 formation.

Mutations in SGT1 that block the interaction between Sgt1p and Skp1p prevent the formation of CBF3 complexes. To test whether this is the case for SKP1 mutations, we coexpressed Sgt1p with either wild-type Skp1p or Skp1-4p in insect cells, Sgt1p complexes were isolated using a polyclonal serum against Sgt1p and, as expected, Skp1p specifically coprecipitated with Sgt1p, consistent with their demonstrated interaction in yeast extract (Kitagawa et al., 1999). In contrast, Skp1-4p failed to coprecipitate with Sgt1p and the complementary immunoprecipitation confirmed the reduction in affinity between Skp1-4p and Sgt1p (Figure 1, C and D). The small amount of Sgt1p that coprecipitates with Skp1-4p is consistent with the partial suppression we observed in the bandshift assay (Figure 1B). Thus, a SKP1 mutation that blocks Ctf13p activation also prevents Skp1p and Sgt1p interaction.

Although previous studies suggest that the interaction between Skp1p and Sgt1p is critical for CBF3 function, it is not clear to what extent Sgt1p contributes to the formation of CBF3 complexes (Kitagawa et al., 1999). To address whether Skp1p, Sgt1p, and Ctf13p form a single complex, we coexpressed a GST-Ctf13p fusion with Sgt1p and either Skp1p or Skp1-4p. After the isolation of GST-Ctf13p, we observed the copurification of Skp1p and low but specific levels of Sgt1p copurifying (Figure 2A). The higher affinity of Sgt1p for Ctf13p in the absence of Skp1p or in the presence of Skp1-4p argues that Sgt1p binds directly to Ctf13p and that this interaction is destabilized by Skp1p association (Figure 2A, compare lane 2 with lanes 3 and 4). To assess whether Skp1p, Sgt1p, and Ctf13p can form a single complex, we analyzed the migration of Skp1p on glycerol gradients after fractionating extracts containing Skp1p and Sgt1p, Skp1p and Ctf13p, or all three proteins. Significantly, a subtraction of Skp1p (16%) migrates in more dense fractions when coexpressed with Sgt1p and Ctf13p, consistent with the formation of a single complex containing all three proteins (Figure 2B, fractions 9–12). The low percentage of triple complex is consistent with the relatively weak inter-
conclude that the Skp1p-Sgt1p interaction observed between Sgt1p and the Ctf13p-Skp1p heterodimer in the pull down assays (Lingelbach and Kaplan; unpublished data). Our results indicate that both Skp1p and Sgt1p contact Ctf13p, raising the possibility that interactions between all three proteins contribute to the activation of Ctf13p.

Activation of Ctf13p Is Required for Its Association with Ndc10p

Skp1p and Sgt1p may be important for assembly of CBF3 complexes, or they may facilitate the binding of the already assembled complex to CEN DNA. To distinguish between these possibilities, we asked whether there are Skp1p-dependent interactions between Ctf13p and the other CBF3 subunits. We expressed GST-Ctf13p alone or in the presence of Skp1p or Skp1-4p. We confirmed that Skp1p is required for GST-Ctf13p activity by demonstrating that it can be complemented with separately expressed Cep3p and Ndc10p in a bandshift reaction (Figure 3A). As observed in our binding experiments, Sgt1p partially suppressed the Skp1-4p extracts, resulting in a low level of active GST-Ctf13p. We used advantage of this material to evaluate the interaction of Cep3p and Ndc10p with active, partially active or inactive Ctf13p. Active GST-Ctf13p produced in the presence of wild-type Skp1p bound specifically to both Ndc10p and Cep3p (Figure 3B). In contrast, partially active GST-Ctf13p, produced in the presence of Skp1-4p, bound similar levels of Cep3p but more than twofold less Ndc10p. Inactive GST-Ctf13p produced in the absence of Skp1p bound similar levels of Cep3p, but bound only background levels of Ndc10p (Figure 3B and C). Although unable to bind Ndc10p, inactive GST-Ctf13p does not seem misfolded because it could bind both Cep3p and Skp1p or Skp1-4p. We conclude that the Skp1p-Sgt1p-mediated activation of Ctf13p is required for the assembly of the CBF3 complex and specifically for the binding of Ndc10p to Ctf13p.

The Binding of Ndc10p to Ctf13p Is a Rate-limiting Step for CBF3 Assembly In Vivo

The short half-life of Ctf13p in yeast and the stability of both Cep3p and Ndc10p suggest that production of active Ctf13p may limit the assembly of CBF3 complexes (Kaplan et al., 1997). To characterize the steps that lead to CBF3 formation, we synchronized the assembly of CBF3 complexes in vivo by placing the chromosomal copy of CTF13 under control of the GAL1 promoter and fusing CTF13 to a TAP tag. This strain grows with wild-type characteristics when cultured in the presence of galactose with no obvious cell cycle delays or loss of viability (our unpublished data). When switched to media containing dextrose, the levels of TAP-Ctf13p quickly decrease in agreement with previous measurements of Ctf13p half-life (Figure 4A; Kaplan et al., 1997). The rapid loss of Ctf13p in dextrose-containing media and the stability of other CBF3 components (our unpublished data) makes this a unique system for asking questions about how Ctf13p activation is related to CBF3 assembly. When cells are shifted to media containing galactose, significant levels of TAP-Ctf13p were induced after only 10 min and a peak of Ctf13p was observed after 40 min (Figure 4A). Quantitation of TAP-Ctf13p and CBF3 levels during induction demonstrates that the appearance of Ctf13p precedes CBF3 formation by 15–20 min, suggesting the presence of a rate-limiting step in CBF3 assembly (Figure 4B).

To identify the rate-limiting step in CBF3 assembly, we harvested yeast in 10-min intervals after the induction of TAP-Ctf13p and purified these complexes using a two-step affinity chromatography protocol (Rigaut et al., 1999). Isolated complexes were analyzed by immunoblot or silver...
staining of gels after SDS-PAGE. A small amount of Ctf13p was detected in cells grown in dextrose, suggesting that not all of the TAP-Ctf13p was degraded or that there is leaky repression of the GAL1 promoter under these conditions (Figure 4C, arrow 3). A 64-kDa band copurified specifically with TAP-Ctf13p at all times after induction and was confirmed by immunoblot to be Cep3p (Figure 4C, arrow 2; our unpublished data). Skp1p was also observed to copurify with TAP-Ctf13p at all time points by immunoblot and was particularly enriched in later time points (Figure 4C, arrow 4; our unpublished data). Although not detectable by silver staining, Sgt1p was observed by immunoblot to be weakly associated with these complexes (our unpublished data). Notably, the band corresponding to Ndc10p (110 kDa) occurred only at later time points, beginning to peak at 40 min (Figure 4C, arrow 1). Quantification of protein levels confirms that Ctf13p and Cep3p association precedes CBF3 formation and that the kinetics of Ndc10p binding exactly match those of CBF3 formation (Figure 4D). These data argue that the association of Ctf13p with Ndc10p represents the rate-limiting step in CBF3 assembly.

What prevents the interaction between Ctf13p and Ndc10p at early stages of the induction time course? We entertained two possibilities: Ndc10p in extracts may be inactive or, as our in vitro binding studies would suggest, Ctf13p may only become active at later time points (i.e., >20 min). To distinguish between these possibilities, we added recombinant, active Ctf13p or Ndc10p to extracts derived from cells starved for Ctf13p, and from cells after Ctf13p induction for 15 and 45 min; in this experiment, a strain containing a 3×HA-fusion to Ctf13p under control of the GAL1 promoter was used to facilitate analyses. Similar levels of 3×HA-Ctf13p were observed at 15 and 45 min after induction (Figure 4E), although CBF3 complexes were not observed until 45 min (Figure 4F, buffer controls). Addition of recombinant Ndc10p resulted in only a minor increase in CBF3 formed in extracts, whereas the addition of Ctf13p completely complemented extracts to wild-type levels (Figure 4F), arguing that both Ndc10p and Cep3p are functional in these extracts. These results indicate that the lag in CBF3 assembly is due to a lack of active Ctf13p. Thus, activation of Ctf13p is required for Ndc10p binding and is rate limiting in the assembly of CBF3.

To address the assembly of CBF3 complexes in living cells, we analyzed the behavior of a Cep3-GFP fusion during the synchronized assembly of CBF3. Because the binding of Cep3p to CEN DNA depends on intact CBF3, we reasoned that Cep3p would be a good marker to monitor CBF3 assembly in live cells. Consistent with the distribution of other kinetochore proteins in yeast, Cep3-GFP was observed to

Figure 2. Sgt1p contributes transiently to the activation of Ctf13p. (A) Insect cell lysates containing equal levels of GST-Ctf13p and the indicated recombinant proteins were bound to glutathione Sepharose. Associated proteins were detected by immunoblot using antibodies against the indicated proteins. Lysates containing a 10×His-Ctf13p instead of GST-Ctf13p were used to determine the level of background binding to glutathione beads. Input lanes represent the levels of protein before purification. (B) Insect cell lysates containing the indicated recombinant proteins were equalized for the levels of Ctf13p and Skp1p and separated by glycerol gradient sedimentation. Fractions were collected from the top of the gradient to the bottom (fractions 1–14), and the position of Skp1p was determined by immunoblotting with polyclonal antibodies against Skp1p. The fractions containing the triple complex of Ctf13p, Skp1p, and Sgt1p are indicated with a bracket. (C) Quantification of Skp1p levels are plotted for each glycerol gradient as a fraction of the total protein detected on the gradient.
cluster in cells grown in the presence of galactose (Figure 4G, arrow, gal/raf). After cells were grown in dextrose for 300 min, Cep3p-GFP was dispersed throughout cells (Figure 4G, Dex, 300/H11032). After the return of cells to galactose containing media for 15 min, Cep3-GFP was observed in puncta (Figure 4G, arrows in gal/raf 15/H11032; our unpublished data) and by 45 min, Cep3-GFP returned to its clustered position in the majority of cells, consistent with its binding to centromeres (Figure 4G, arrow in 45/H11032). In separate experiments, we observed Cep3p localization by indirect immunofluorescence to reflect the behavior of the GFP fusion and, after the assembly of CBF3, to cluster near spindle poles overlapping with chromosomes (Figure 1, S-). We note that this pattern is distinct from the localization of outer kinetochore proteins under these same conditions (Figure 7), suggesting that Cep3-GFP uniquely transits through multiple cellular compartments during CBF3 assembly. However, our main conclusion that CBF3 assembly as measured in extracts correlates with kinetochore formation in vivo is supported by these data.

The Assembly and Turnover of CBF3 Complexes Are Affected by Mutations in SKP1

To examine how SKP1 mutations affect CBF3 complexes in vivo, we compared the assembly of CBF3 in wild-type, skp1-4, and skp1-3 strains containing a 3 × HA-fusion to Ctf13p under control of the GAL1 promoter. Whereas the skp1-4 mutation has been shown to block Ctf13p activation, the skp1-3 mutation has been reported to affect the SCF ubiquitin ligase and arrests cells in G1 with wild-type CBF3 levels (Connelly and Hieter, 1996; Kaplan et al., 1997). Cells were grown in the presence of raffinose to inhibit 3 × HA-Ctf13p expression and induced with galactose at 37°C for 90 min, sufficient time for CBF3 to assemble in wild-type strains. When grown in the presence of raffinose, 3 × HA-Ctf13p decreased to similar levels in all strains, regardless of the temperature cultured (Figure 5A). CBF3 assembly was monitored after the induction of 3 × HA-Ctf13p in each of the strains. After 90 min of induction, CBF3 was observed at similar levels in wild-type and skp1-3 cells at both permissive and nonpermissive temperatures (Figure 5B). As expected, CBF3 levels were reduced in skp1-4 cells at permissive temperature and entirely absent in cells grown at nonpermissive temperature (Figure 5B). We confirmed that skp1-4 blocked 3 × HA-Ctf13p activation by showing biochemical complementation of CBF3 with activated recombinant Ctf13p but not with Ndc10p or Cep3p (Figure 5C, 37°C).

A prediction based on our in vitro binding studies is that the skp1-4 mutation blocks Ctf13p activation by preventing the interaction between Skp1p and Sgt1p. To examine this interaction during the synchronized assembly of CBF3, we immunoprecipitated Sgt1p from extracts and examined the levels of coprecipitated Skp1p. In wild-type cells, barely detectable levels of Skp1p were observed to coprecipitate with Sgt1p before the induction of 3 × HA-Ctf13p even though both proteins were present at wild-type levels (Figure 5D, Skp1p visible on longer film exposures; our unpublished data). Interestingly, significantly higher levels of Skp1-3p coprecipitated with Sgt1p under all conditions. These results suggest that the Skp1-3p has a higher affinity for Sgt1p. In contrast, Skp1-4p failed to coprecipitate with Sgt1p under any conditions, consistent with our in vitro
Figure 4. Ndc10p binding to TAP-Ctf13p is the final step in the synchronous assembly of CBF3 in yeast. (A) Cells containing the GAL1 promoter upstream of TAP-CTF13 were cultured in galactose (0 min, top) and then transferred to media containing dextrose for 15, 30, 60, and 120 min; extracts were prepared and immunoblotted to detect the TAP-fusion. After 5 h of growth in dextrose (0 min, middle), cells were transferred to media containing galactose for 5, 10, 20, 40, and 60 min, and extracts were prepared and immunoblotted as described above.
observations that the skp1-4 mutation disrupts the interaction with Sgf1p (Figure 5D).

To test the prediction that inactive Ctf13p results in the partial assembly of CBF3, we performed immunoprecipitations of 3 × HA-Ctf13p followed by immunoblot detection of Cep3p. We observed similar levels of Cep3p coprecipitation with 3 × HA-Ctf13p from wild-type and mutant strains, grown at permissive or nonpermissive temperature (Figure 5D). The small variations in the levels of Cep3p that coprecipitate with 3 × HA-Ctf13p at 90 min reflects differences in the amount of 3 × HA-Ctf13p induced after addition of galactose (Figure 5A). Together, these results argue that CBF3 assembles in at least two steps; the first step involves the interaction between Ctf13p, Cep3p, Skp1p, and Sgf1p; the second step only occurs when Skp1p and Sgf1p interact directly to activate Ctf13p, allowing Ndc10p binding and the formation of CBF3 complexes.

In addition to demonstrating that Skp1p and Sgf1p are required for the assembly of CBF3, these experiments also highlight the transient nature of the CBF3 complex. Depletion of 3 × HA-Ctf13p causes the rapid disappearance of CBF3 from extracts with a half-life of 40 min (our unpublished data). Interestingly, CBF3 levels are not reduced in strains carrying the skp1-3 mutation when grown in the presence of raffinose (Figure 5B, 0-min time point). Because 3 × HA-Ctf13p levels are equally reduced in all strains cultured in raffinose, this stabilization is not due to a general failure to degrade 3 × HA-Ctf13p (Figure 5A, compare 0-min time points). Therefore, we speculate that skp1-3 may prevent the turnover of CBF3 complexes and argues that Skp1p is involved in both the assembly and the turnover of CBF3 complexes.

The Machinery Required to Assemble CBF3 Is Active throughout the Cell Cycle

The assembly of CBF3 and its binding to CEN DNA is required for the association of all other kinetochore complexes with CEN DNA, and thus it can be considered the first step in de novo kinetochore formation. De novo formation of kinetochores may be restricted to discrete points in the cell cycle. For example, assembly of CBF3 may be limited to G1 or S when newly replicated centromeres are assembling a kinetochore. To determine whether pathways that control the assembly of CBF3 are influenced by cell cycle progression, we monitored CBF3 assembly in cells containing GAL1-3-factor (Figure 6). Expression of CBF3 at specific stages of the cell cycle in the presence of raffinose and α-factor cells are arrested in G1 with depleted 3 × HA-Ctf13p. The addition of galactose induced the production of 3 × HA-Ctf13p and examination of bud morphology, fluorescence-activated cell sorting profiles, and tubulin staining suggest that cells remain largely arrested in G1 (Figure 6, A–C; our unpublished data). Normal kinetics and levels of CBF3 were observed under these conditions (compare Figure 6A with Figure 5). In separate experiments, we also observed the movement of Cep3-GFP to kinetochores in G1 cells with similar kinetics (Figure 4G; our unpublished data). These results argue that CBF3 can assemble during the G1 stage of the cell cycle, possibly to enable de novo kinetochore formation at newly replicated centromeres.

To examine whether CBF3 assembly is restricted to G1, we analyzed assembly in cells that had been arrested in G2/M with the microtubule poison nocodazole. Cells arrested with large-bud morphology as expected; Clb2 levels accumulated and remained high throughout the experiment, consistent with cells being arrested in mitosis. Surprisingly, CBF3 assembly occurred with normal kinetics, whereas cells were arrested in mitosis (Figure 6, D–F). To rule out that microtubule depolymerization artificially induced CBF3 assembly, we analyzed cells enriched in G2 after release from α-factor arrest. Cells were arrested with α-factor, depleted for 3 × HA-Ctf13p, and released from the arrest in raffinose-containing media. After the appearance of medium budded cells, galactose was added to the media and samples were collected at the indicated time points (Figure 6G). The appearance of 2N DNA levels and the increase in Cib2p levels suggest that these cells have progressed into G2 (Figure 6, G–I). Induction of 3 × HA-Chl3p resulted in the formation of CBF3 complexes with similar kinetics as observed in cells arrested with nocodazole (Figure 5D). Together, these results suggest that the machinery required to assemble CBF3 is active throughout the cell cycle and this could allow for the continual assembly of kinetochores.

The Balance of CBF3 Assembly and Turnover Is Required for Proper Kinetochore Function

To understand how CBF3 assembly contributes to kinetochore function, we tested the effect of altering CBF3 levels on chromosome segregation. To this end, we first examined the segregation of a chromosome III fragment carrying SLIP11, a suppressor of the ade2-101 mutation, in a strain where CTF13 is under control of the GAL1 promoter. Cells with compromised kinetochore function lose the chromosome fragment at high rates, resulting in the failure to suppress the ade2-101 mutation and the accumulation of red or red-sectored colonies (Spencer et al., 1990). When cells were grown in the presence of galactose to induce 3 × HA-CTF13, we observed a significant increase of red sectored-colonies (colonies with more than 2 sectors) compared with the background chromosome loss observed in the wild-type strain with CTF13 under normal transcriptional control (10.8-fold), although only a small increase in red colonies (1.7-fold), suggesting that elevated levels of CBF3 cause a modest negative effect in chromosome segregation (Figure 7A). In contrast, we observed a significant increase in the percentage of red colonies when cells were grown for short periods (as little as 1 h) in the presence of raffinose or dextrose to inhibit CBF3 assembly before plating on media containing galactose (4.8- and 8.9-fold, respectively), suggesting that limiting CBF3 assembly also interferes with normal chromosome segregation (Figure 7A). We conclude that maintaining the proper balance of CBF3 complexes is critical for kinetochore function and the most dramatic effect on chromosome segregation is observed when CBF3 assembly is inhibited.

To address in more detail how inhibition of CBF3 assembly compromises kinetochore function, we examined the ability of kinetochores to form on CEN DNA. After the
inhibition of CBF3 assembly, we were unable to detect CBF3 complexes in extracts by bandshift analysis (Figures 4 and 5); however, chromatin immunoprecipitation revealed levels of CEN DNA coprecipitating with Cep3p antibodies comparable with wild-type cells (Figure 7B). We also examined the association of CBF3 with CEN DNA in a skp1-4 strain and observed that similar levels of CEN DNA precipitated with antibodies to Cep3p at both permissive and nonpermissive temperatures (Figure 7C). In contrast, the ndc10-1 allele dramatically reduced the ability of CBF3 to associate with CEN DNA, consistent with published reports that this allele prevents kinetochore nucleation (Figure 7D). To determine whether inhibiting CBF3 assembly affected the recruitment of other kinetochore proteins to CEN DNA, we performed chromatin immunoprecipitation on strains with Spc24-myc, Okp1-myc, and Dam1-myc. No significant changes in the levels of coprecipitating CEN DNA were observed when CBF3 assembly was inhibited (Figure 7E). We conclude that mutations that block CBF3 assembly (i.e., skp1-4 and sgt1-3; our unpublished data) do not release CBF3 or outer kinetochore proteins from CEN DNA; however, mutations in the core DNA binding subunits (i.e., ndc10-1 and ctf13-30 (our unpublished data) cause CBF3 to dissociate from DNA.

Although kinetochores may form on CEN DNA, it is possible that they are not properly organized. One characteristic of properly organized kinetochore proteins in yeast is their juxtaposition to spindle poles throughout the majority of the cell cycle (Jin et al., 2000). To examine
the affect of inhibiting CBF3 assembly on the positioning of kinetochore proteins near spindle poles, we monitored the localization of the outer kinetochore protein Dam1p after fusion to GFP in a strain also containing Spc42-CFP to mark spindle poles. When cells were grown in galactose, Dam1-GFP clustered adjacent to spindle poles in G1 or late mitotic cells or between replicated spindle poles in early mitotic cells as expected (Figure 7F, arrows indicate early mitotic cells and arrowheads indicate late mitotic cells). Inhibition of CBF3 assembly after growth of cells in dextrose had no detectable effect on Dam1-GFP localization (Figure 7F). Similarly, GFP fusions to Spc24p and Okp1p also maintained their normal localization after inhibition of CBF3 assembly (our unpublished data). In contrast, the mutation ndc10-1 at nonpermissive temperature caused a dramatic loss of Dam1-GFP from their juxtapolar position, presumably because the CBF3 complex itself was released from CEN DNA (Figure 7H). We conclude from these data that interfering with normal CBF3 assembly by overexpressing Ctf13p, or more dramatically, by inhibiting CBF3 assembly compromises chromosome segregation independent of kinetochore formation on CEN DNA. We cannot rule out that depleting CBF3 results in a subtle perturbation of kinetochores that we cannot detect by these assays. However, it is interesting to speculate that CBF3 may have a noncentromeric role required for proper chromosome segregation.

**DISCUSSION**

The large number of kinetochore proteins and their organization into multiple discrete complexes highlights the importance of understanding their assembly into a functional kinetochore. Our studies of Skp1p and Sgt1p implicate these well conserved proteins in the assembly of CBF3, raising the possibility that they may perform a general role in the assembly of protein complexes. Specifically, we have shown that Skp1p and Sgt1p are required for the final and rate-limiting step in CBF3 assembly, a step that is essential for its ability to associate with CEN DNA. Finally, our work suggests that the de novo assembly of CBF3 occurs throughout the cell cycle and is required for proper kinetochore function, independent of its role in recruiting proteins to CEN DNA. We propose that a balance between CBF3 assembly and turnover may help to modulate kinetochore function.

**Mechanism of Skp1p-Sgt1 Action**

Skp1p and Sgt1p are well conserved and implicated in the function of a number of protein complexes important for the
regulation of cell growth, cell cycle advance, and chromosome segregation (Kitagawa et al., 1999; Dubacq et al., 2002). Although a biochemical function for these proteins has not yet been determined, CBF3 offers a useful model complex to dissect their actions. Our results indicate that Skp1p and Sgt1p form important contacts with each other and with the F-box protein Ctf13p that allow Ctf13p to associate with Ndc10p and ultimately bind CEN DNA. Two models have been proposed to explain the “activation” of Ctf13p: 1) posttranslational modification in the form of phosphorylation (Kaplan et al., 1997), and 2) the recruitment of HSP90 chaperones (Stemmann et al., 2002). Our efforts to distinguish between these two possibilities suggest that Ctf13p activation may be controlled by multiple pathways. We have observed a mobility shift for Ctf13p, Skp1p, and Sgt1p during the synchronous assembly of CBF3 that is reversed by phosphatase treatment, but mutation of modified sites in Ctf13p and Sgt1p has not revealed an essential function (our unpublished data). Also, our results suggest that the role of HSP90 chaperones in Ctf13p activation is not straightforward; inhibition of HSP90 function in insect cells expressing recombinant Ctf13p reduces the levels of protein produced but not its specific activity (our unpublished data). Given the complicated relationship between CBF3 assembly and turnover, it is not unreasonable to speculate that multiple pathways work in parallel to maintain proper CBF3 levels. Although this work does not resolve the mechanism by which Skp1p and Sgt1p activate Ctf13p, it provides important details concerning the role of these proteins in...
maintaining CBF3 complexes and thus provides a critical framework from which to more carefully dissect the molecular mechanisms underlying Skp1p-Sgt1p function.

Skp1p and Sgt1p also have been reported to play critical roles in the function of the SCF ubiquitin ligase. In this light, it is interesting to note certain similarities between CBF3 and the SCF. Both complexes are composed of an F-box protein, Skp1p, and Sgt1p. The F-box protein in both complexes provides a protein interaction function; as a scaffold in CBF3 to bind other subunits and as a ligand receptor in the context of SCF. Perhaps a more intriguing similarity is that both complexes exhibit a transient quality. In both cases, F-box proteins are degraded after ubiquitin modification, a process proposed to be important for the formation of new SCF complexes (Zhou and Howley, 1998). The turnover of Ctf13p may also contribute to the ability to create new CBF3 complexes, a process that may be linked to maintenance of the spindle checkpoint (Kitagawa et al., 2003). Interestingly, it has been reported that Skp1p is required for the turnover of Rcy1p, a protein involved in the recycling of SNARE complexes, independent of its role in SCF (Galan et al., 2001); this result and our observations concerning the stability of CBF3 in skp1-3 cells raises the possibility that Skp1p contributes to the turnover of proteins independent of ubiquitin-mediated degradation. Whether degradation of Ctf13p itself serves as the lynchpin for CBF3 turnover, or some other process involving chaperones is involved, will require a more detailed examination of how CBF3, and perhaps SCF, complexes are turned over.

Assembly of CBF3 Is Important for Chromosome Segregation but Not for Kinetochore Formation

The relatively constant levels of CTF13 message (Cho et al., 1998) and our results that CBF3 can assemble throughout the cell cycle raise the possibility that the continual assembly of CBF3 is important for proper kinetochore function. Indeed, both overexpression and inhibition of CBF3 assembly dramatically increases chromosome loss even though kinetochore complexes are properly recruited to CEN DNA. There are several possible explanations for this observation: 1) inhibition of CBF3 results in a small number of defectively formed kinetochores, leading to a low rate of chromosome loss without detectable dissociation of kinetochore proteins from CEN DNA; 2) inhibition of CBF3 assembly preferentially blocks the recruitment of factors to CEN DNA required to maintain the fidelity of chromosome segregation; and 3) CBF3 plays some extracentromeric role that is required for accurate chromosome segregation. Although it is possible that only a few kinetochores are defective in every cell, the high rate of chromosome loss and the drop in cell viability observed after inhibition of CBF3 assembly suggests a massive failure of kinetochore function. More comprehensive analyses of the composition of kinetochores and the movement of chromosomes after inhibition of CBF3 assembly will help to distinguish between these possibilities.

In summary, our results document the biochemical details that underlie the formation of kinetochores in the budding yeast S. cerevisiae. The job of assembling such a large protein structure is likely to be carefully orchestrated. The data we present suggest that maintaining careful control over CBF3 assembly and turnover is important for proper chromosome segregation.

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