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Cohesin's Binding to Chromosomes Depends on a Separate Complex Consisting of Scc2 and Scc4 Proteins

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Summary

Cohesion between sister chromatids depends on a multisubunit cohesin complex that binds to chromosomes around DNA replication and dissociates from them at the onset of anaphase. Scc2p, though not a cohesin subunit, is also required for sister chromatid cohesion. We show here that Scc2p forms a complex with a novel protein, Scc4p, which is also necessary for sister cohesion. In *scc2* or *scc4* mutants, cohesin complexes form normally but fail to bind both to centromeres and to chromosome arms. Our data suggest that a major role for the Scc2p/Scc4p complex is to facilitate the loading of cohesin complexes onto chromosomes.

Introduction

The segregation of sister chromatids to opposite poles of the cell during mitosis is crucial for the proliferation of eukaryotic cells. Segregation of sister chromatids depends on the pulling forces exerted by microtubules that usually attach to a single region of chromosomes called the centromere. How cells ensure that sister centromeres attach to microtubules that emanate from, or extend to, opposite poles (known as bipolar attachment) is not fully understood. It is presumed that cohesion between sister chromatids is necessary for the bipolar attachment. Indeed, it is difficult to envisage how sister centromeres would avoid attaching to spindles extending to the same pole, were sister chromatids not closely held together after DNA replication (Miyazaki and Orr-Weaver, 1994).

Sister chromatids remain associated from S phase until the onset of anaphase (Guacci et al., 1994). Cohesion between sisters must be established during DNA replication (Uhlmann and Nasmyth, 1998). In yeast, a multisubunit "cohesin" complex, containing Scc1p (also known as Mcd1p), Scc3p, Smc1p, and Smc3p (Toth et al., 1999), is essential for sister chromatid cohesion. A related complex has also been implicated in holding sisters together in vertebrate cells (Losada et al., 1998). In yeast, cohesin binds to chromosomes shortly before S phase and remains associated with them until metaphase (Michaelis et al., 1997). At the metaphase-to-anaphase transition, a separin protein, Esp1p (Ciosk et al., 1998), causes proteolytic cleavage of Scc1p, which triggers loss of cohesion and thereby segregation of sisters to opposite poles of the mitotic spindle (Uhlmann et al., 1999).

Cohesin binds to both centromeres and specific sites along chromosome arms (Blat and Kleckner, 1999; Megee et al., 1999; Tanaka et al., 1999). However, very little is known about the cohesive structures that are generated after DNA replication. Two of cohesin's subunits, Smc1p and Smc3p, are members of a family of conserved proteins (called the SMC family), which have roles in chromosome segregation in a variety of organisms, including bacteria (Sharpe, 1999). Smc1p and Smc3p are most related to Smc4p and Smc2p (respectively), which are subunits of a condensin complex required for chromosome condensation in many (if not all) eukaryotic organisms (Hirano, 1999). Smc proteins from bacteria are thought to be homodimers whose terminal globular domains are separated by two long stretches of antiparallel coiled coils interrupted by a hinge region (Melby et al., 1998). Condensin and cohesin are presumed to contain Smc2p/Smc4p and Smc1p/Smc3p heterodimers, respectively. Condensin's ability to condense chromosomes has been proposed to stem from its ability to generate (in an ATP-dependent fashion) large supercoil loops in a single molecule of DNA (Kimura et al., 1999). Whether cohesin might have a related activity, but involving two different DNA molecules, is not known.

Building cohesion between sisters seems to be a multistep reaction. In addition to cohesin, at least two other classes of proteins are needed for sister cohesion. Genetic studies in yeasts have shown that Eco1p (also known as Ctf7p) and Scc2p (Mis4p in *S. pombe*) are also required for sister cohesion (Michaelis et al., 1997; Furuya et al., 1998; Skibbens et al., 1999; Toth et al., 1999). Neither of these two proteins is a stable constituent of the cohesin complex. Eco1p is necessary for establishing sister cohesion during S phase but not for maintaining it during G2 or M phases. In *eco1* mutants, cohesin appears to associate with chromatin normally, but despite this, effective physical association between sisters is never formed. These findings, some genetic interactions between *eco1 (ctf7)* and DNA replication mutants (Skibbens et al., 1999), and the observation that cohesin must be present during S phase to form cohesion between sisters suggest that Eco1p is an S phase–specific factor required for formation of cohesive structures between sisters, following cohesin's binding to chromosomes.

Unlike Eco1p, we found that Scc2p and its partner Scc4p (sister chromatid cohesion protein 4) are essential for binding of cohesin to chromosomes (which is thought to be a prerequisite for the Eco1-mediated step during S phase). We show here that in *scc2* and *scc4*

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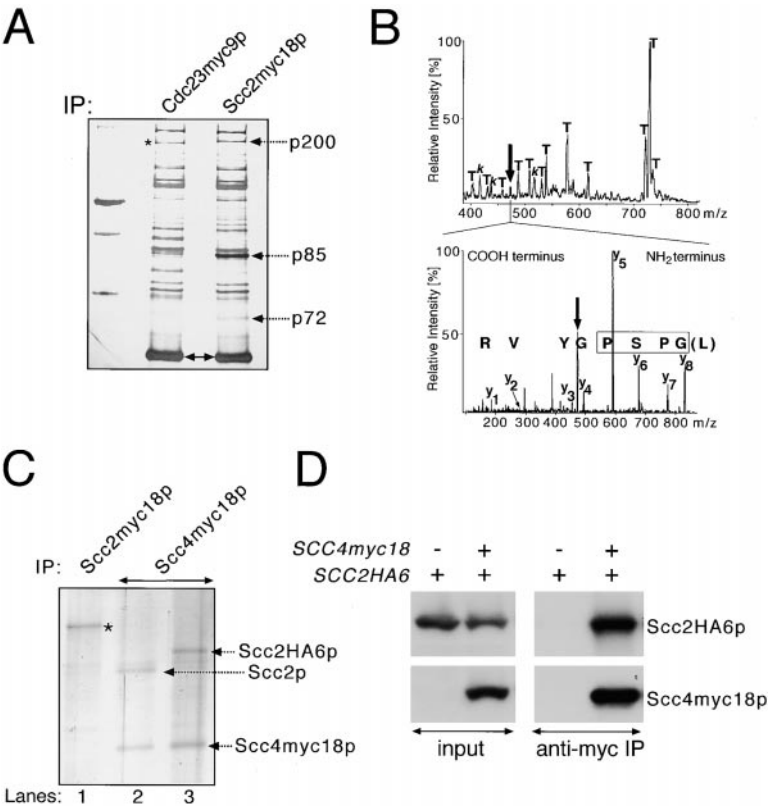

Figure 1. Scc2p Forms a Complex with a 72 kDa Protein

(A) Coimmunoprecipitation of a 72 kDa protein with Scc2myc18p. Protein extracts were prepared from control cells (expressing Cdc23myc9p) and cells expressing a myc18-tagged version of Scc2p. The myc-tagged proteins were immunoprecipitated with the 9E10 mAb specific for the myc epitope. Precipitated proteins were separated on an SDS-polyacrylamide gel and detected by silver staining. Three protein bands, p200, p85, and p72, were specifically present in the Scc2myc18p's precipitate. Asterisk, Apc1. Double arrow indicates antibody heavy chain.

(B) Sequencing of the 72 kDa band by nanoelectrospray tandem mass spectrometry. Only a femtomole amount of the protein was available, and therefore, parent ion scanning for the daughter ions m/z 86 (immonium ions of leucine and isoleucine) was employed to distinguish peptide ions from chemical noise. A part of the mass spectrum of unseparated p72 digest is presented in the upper panel. Autolysis products of trypsin are designated with a T, and peptide ions that originated from keratins (ubiquitous protein contamination) are designated with a k. A single peptide precursor ions having m/z 473.0 (designated with closed arrow) did not belong to trypsin and keratin peptides, and upon its collisional fragmentation allowed identification of the Yer147c protein. The tandem mass spectrum acquired from this peptide precursor ion is presented in the lower panel. A peptide sequence tag (Mann and Wilm, 1994) was assembled by considering the precise mass difference between the adjacent fragment ions containing the C terminus of the peptide (y ions) (Biemann, 1988) (boxed) and used for searching a database. The search produced the peptide sequence LGPSPGYVR from the yeast protein Yer147c as a single hit. The hit was further confirmed by matching the masses of fragment ions calculated from the peptide sequence to the masses of ions observed in the spectrum. This allowed identifying the protein with high certainty, albeit a tandem mass spectrum from the single peptide precursor ion was acquired.

(C) Reverse coimmunoprecipitation of Scc2p with Scc4p (Yer147p). Protein extracts were prepared from cells containing *SCC2myc18* (lane 1), *SCC4myc18* (lane 2), and *SCC4myc18 SCC2HA6* (lane 3). The myc-tagged proteins were immunoprecipitated with 9E10 antibody. Bound proteins were separated on an SDS-polyacrylamide gel and detected by silver staining. Scc2p was confirmed to be present in Scc4myc18p's precipitates based on the decreased electrophoretic mobility of Scc2p upon HA tagging. The asterisk indicates Scc2myc18p. Scc4p is not visible in this precipitate (lane 1), because the untagged protein runs well below the myc18-tagged version and does not stain well with silver.

(D) Coimmunoprecipitation of Scc2HA6p with Scc4myc18p (IP-Western). Protein extracts were prepared from cells of the indicated genotype. The plus sign indicates presence of the epitope-tagged version of *SCC2* or *SCC4*.

mutants, cohesin complexes assemble normally, but the binding of intact (i.e., already assembled) cohesin complexes to specific sites within chromosome arms and to centromeres is abolished. We also show that Scc2p and Scc4p are required for the establishment but not the maintenance of sister chromatid cohesion, suggesting that Scc2p/Scc4p complex is unlikely to be part of cohesive bridges holding sisters together until the onset of anaphase. Our data imply that the Scc2p/Scc4p complex facilitates the loading of cohesin complexes onto chromosomes.

Results

Scc2p Forms a Stable Complex with Scc4p, a 72 kDa Protein Encoded by the *YER147C* ORF

To determine whether Scc2p interacts with other proteins, we employed silver staining to detect proteins immunoprecipitated with Scc2myc18p (Figure 1A). The identities of three proteins (p200, p85, and p72) that were present in Scc2myc18p but not in Cdc23myc9p immunoprecipitates (IPs) were determined by mass spectrometry (Figure 1B). MALDI mass spectrometric analysis showed that p200 was Scc2p, whereas p85 was a major coat protein from the *S. cerevisiae* virus LA (SwissProt entry P32503). The 72 kDa protein, which we named Scc4p, was identified by nanoelectrospray tandem mass spectrometry as a product of an ORF, *YER147C*. The viral coat protein is a frequent contaminant in IPs prepared from strains carrying the virus. Indeed, p85 was not detected in IPs from another strain expressing Scc2myc18p (data not shown). It remains possible that we failed to identify additional components of the Scc2p/Scc4p complex due to high background in the Scc2myc18p immunoprecipitate.

To confirm the specificity of interaction between Scc2p and Scc4p, we precipitated Scc4myc18p from strains expressing either Scc2p or Scc2HA6p (Figure 1C). We found that Scc2p, whose electophoretic mobility is decreased upon HA tagging (Figure 1C, lane 3), seemed to be present in roughly stoichiometric amounts in Scc4myc18p precipitates. The specificity of interaction between Scc2p and Scc4p was further confirmed by IP immunoblot experiments (Figure 1D).

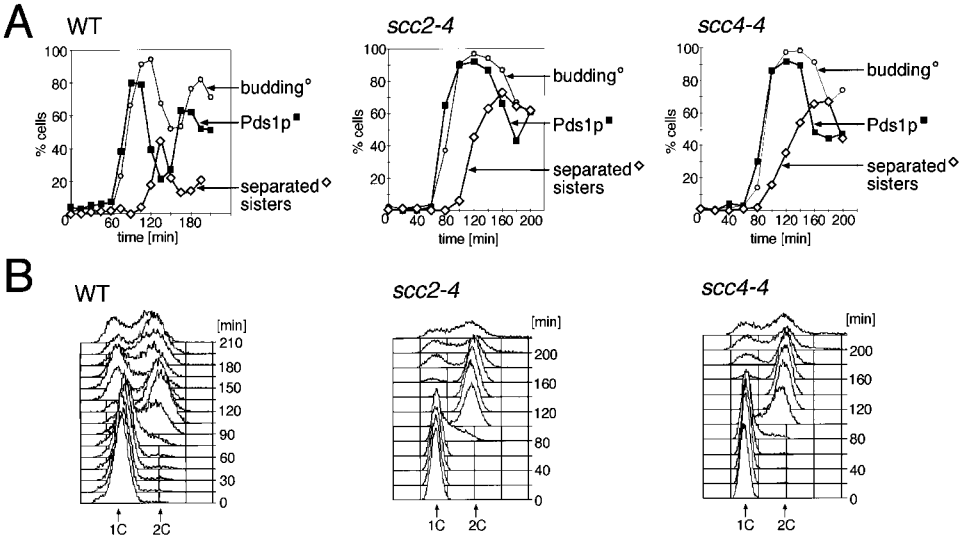

Figure 2. *scc2* and *scc4* Mutants Separate Sister Chromatids Prematurely

(A) Sister chromatids separate prematurely in *scc2-4* and *scc4-4* mutant cells. Elutriated G1 cells of wild-type (K6803), *scc2-4* (K8076), and *scc4-4* (K8349) strains, containing *PDS1myc18* and *CEN5*-proximal sequences marked with GFP, were incubated at 37°C. Pds1myc18 protein was detected by indirect immunofluorescence. Only cells completely lacking Pds1myc18p staining were counted as negative. Sequences in the vicinity of *CEN5* were visualized by the GFP autofluorescence. In wild-type cells (WT), degradation of the anaphase inhibitor Pds1p always precedes sister separation. In both the *scc2* and *scc4* mutants, sisters separate much earlier, when Pds1p is still present. The wild type shown here is derived from Michaelis et al. (1997).

(B) Corresponding DNA contents measured by flow cytometry (FACS).

Scc2p and Scc4p Are Required for Sister Chromatid Cohesion

In wild-type cells (WT), APC/C (anaphase promoting complex/cyclosome)-mediated proteolysis of the securin Pds1p, which inhibits the separin Esp1p, precedes separation of sister chromatids (Figure 2A) (Cohen-Fix et al., 1996; Michaelis et al., 1997; Ciosk et al., 1998). In cohesin mutants, however, sisters separate prematurely, soon after cells form bipolar spindles and long before Pds1p degradation. In fact, Pds1p degradation is delayed, presumably due to activation of the Mad2p-dependent spindle checkpoint (Rudner and Murray, 1996; Alexandru et al., 1999). We have previously shown that inactivation of Scc2p permits sister separation in *cdc16 (apc/c)* mutants, which fail to degrade Pds1p (Michaelis et al., 1997). To determine whether *scc2* single mutants separate sisters prior to Pds1p degradation, we isolated by elutriation small G1 cells of a *scc2-4* strain and incubated them at 37°C. In these cells, sequences adjacent to the centromere of chromosome V *(CEN5)* were visualized as green dots due to binding of Tet repressor–GFP fusion proteins to multiple tet operators integrated at the *URA3* locus (Michaelis et al., 1997). Cells with two separate GFP dots accumulated around 40 min after budding, which is about 10 min earlier than in wild type. Meanwhile, however, Pds1p degradation was substantially delayed, so that 50% or more of the *scc2-4* mutant cells separated sisters in the presence of high levels of Pds1p (Figure 2A). We conclude that Scc2p is essential for preventing precocious sister separation in wild-type cells as well as in cells arrested in metaphase due to an APC/C mutation.

SCC4 was found to be an essential gene; following germination, spores lacking *SCC4* underwent one or two divisions and then died. To determine whether Scc4p is required for sister chromatid cohesion, we generated temperature-sensitive (ts) alleles of *SCC4* (see Experimental Procedures). Three independent alleles arrested with similar phenotypes soon after a shift to 37°C (data not shown). We found that in the ts *scc4-4* mutants, sister chromatids separated prior to Pds1p degradation (Figure 2A). We conclude that both Scc2p and Scc4p are essential for sister chromatid cohesion.

Scc2p Is Essential for the Establishment of Cohesion

To test whether Scc2p is required for the establishment and/or maintenance of sister cohesion, we isolated by elutriation small G1 cells of a *scc2-4* strain and incubated them at either 25°C or 37°C. In the culture incubated at 37°C from the outset, cell viability dropped around the time of DNA replication (Figure 3A; DNA content, sister separation, and Pds1p levels in this culture are shown in Figure 2). In contrast, when an aliquot from the 25°C culture was shifted to 37°C at 100 min (when most of the cells had replicated their DNA), cells maintained high viability through G2 and M phases (most cells completed mitosis by 180 min, as judged by the number of binucleated cells) (Figure 3A). These cells eventually died during S phase of the second cell cycle (after 200 min). Our data suggest that Scc2p function is completed around the time of DNA replication, when sister cohesion is being established.

Neither Scc2p nor Scc4p Is Required for Sister Cohesion during Metaphase

To test more directly whether Scc2p and Scc4p are dispensable for maintaining sister cohesion once it has

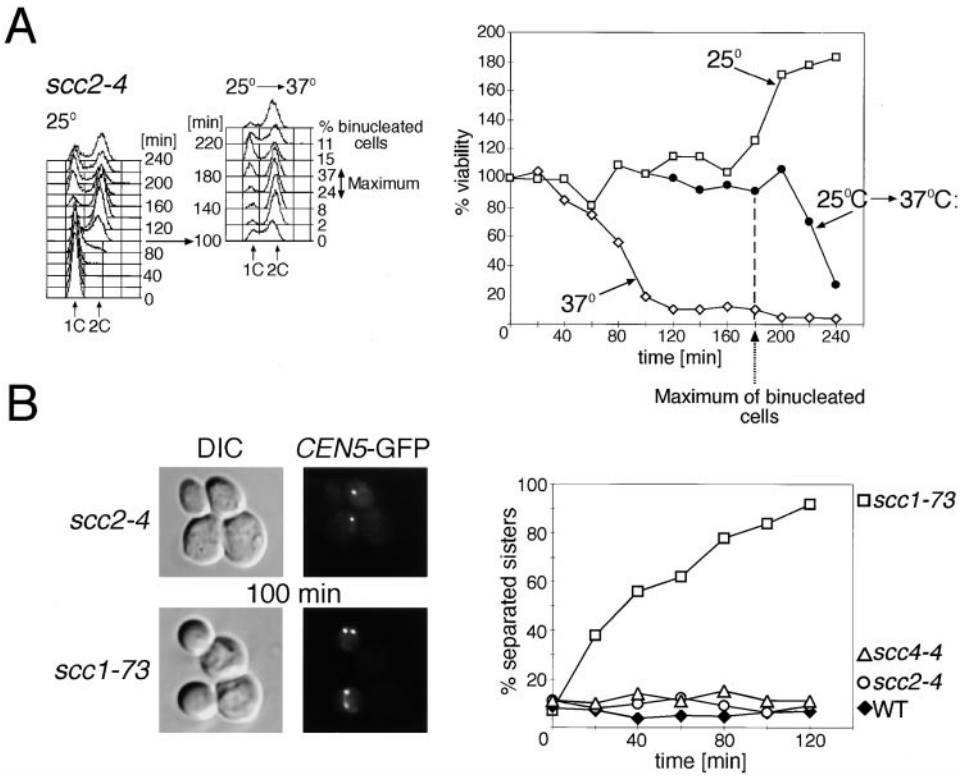

Figure 3. Scc2p and Scc4p Are Required for the Establishment, but Not Maintenance, of Sister Chromatid Cohesion

(A) Scc2p's function is essential around S phase, but not during G2 and M phases. Small G1 cells of the *scc2-4* (K8076) strain were incubated at 25°C and 37°C. DNA contents and percentages of viable cells were determined in each of these cultures every 20 min. An aliquot from the culture grown at 25°C was shifted to 37°C at 100 min (when cells were undergoing DNA replication). FACS of the 37°C culture is shown in Figure 2B. Cells incubated at 37°C lost viability around S phase. However, if cells were allowed to initiate DNA replication at 25°C and were then shifted to 37°C, they maintained high viability until S phase of the second cell cycle.

(B) Scc2p and Scc4p are not required to maintain sister cohesion in metaphase-arrested cells. Strains K8465 (*cdc20::LEU2 GALCDC20*), K8466 (*cdc20::LEU2 GALCDC20 scc2-4*), K8467 (*cdc20::LEU2 GALCDC20 scc4-4*), and K8468 (*cdc20::LEU2 GALCDC20 scc1-73*) containing the *CEN5*-GFP were grown in YEPraf+gal medium at 21°C for more than 12 hr. Expression of the *CDC20* gene was repressed by incubating cells in glucose-containing medium (YPD) at 21°C for 3.5 hr. In order to inactivate Scc2, Scc4, and Scc1 proteins, mutant cells were shifted to restrictive temperature by placing cells in YPD medium that was prewarmed to 35°. Aliquots were taken every 20 min and analyzed for DNA contents (not shown), and separation of the *CEN5*-proximal sequences was marked with GFP. The wild-type cells depleted of Cdc20p arrested in metaphase with 2C DNA content (data not shown) and tightly associated sister chromatids. Inactivation of Scc1p in the Cdc20p-depleted cells was found to result in the dramatic loss of sister cohesion. However, inactivation of Scc2p or Scc4p had no effect on sister cohesion.

been established during S phase, we constructed *scc2-4*, *scc4-4*, and *scc1-73* strains in which Cdc20p was expressed exclusively from the *GAL1-10* promoter. Cdc20p is an unstable protein (Shirayama et al., 1997), which activates the APC/C to mediate ubiquitination (and thereby proteolysis) of Pds1p (Zachariae and Nasmyth, 1999). Removal of galactose caused cells proliferating at 21°C to accumulate in metaphase with unseparated sister chromatids. These metaphase-arrested cells were then shifted to 35°C (restrictive temperature for all the mutants), which caused separation of *CEN5*-GFP in the *scc1-73* mutant cells but not in the wild-type, *scc2-4*, or *scc4-4* mutant cells (Figure 3B). These data suggest that unlike Scc1p, neither Scc2p nor Scc4p is needed for maintaining sister cohesion during metaphase.

Cohesin Complexes Form Normally but Fail to Associate with Chromosomes in *scc2* and *scc4* Mutants

We have previously reported that Scc2p (but not Eco1p) is needed for the stable association of two cohesin subunits with chromosomes (Toth et al., 1999). To distinguish whether Scc2p is required for the assembly of cohesin or for the loading of assembled complexes, we analyzed the integrity and chromatin binding of cohesin complexes in wild-type, *scc2-4*, and *scc4-4* mutant cells progressing through the cell cycle. All three strains (containing *SCC3myc18* and *SMC1HA6*), were released at 33°C from a pheromone-induced G1 arrest. The amounts of Scc1p, Smc1HA6p, and Scc3myc18p in whole extracts (marked WE) were similar in wild type and both mutants from G1 until mitosis. Despite this, at all time points, the amounts of Scc1p, Scc3p, and Smc1p present in chromatin pellets (marked P) were considerably lower in both the *scc2-4* (Figure 4A) and *scc4-4* (Figure 5A) mutants than in the wild type. In wild type, Scc1p and Scc3p appeared in the chromatin pellet following release from G1 arrest, decreased at anaphase (around 60 min, Figure 4A), and increased again when cells reentered the next cell cycle. In the *scc2-4* and *scc4-4* mutants, little or no Scc1p and Scc3p, and only low amounts of Smc1p were found in chromatin pellets

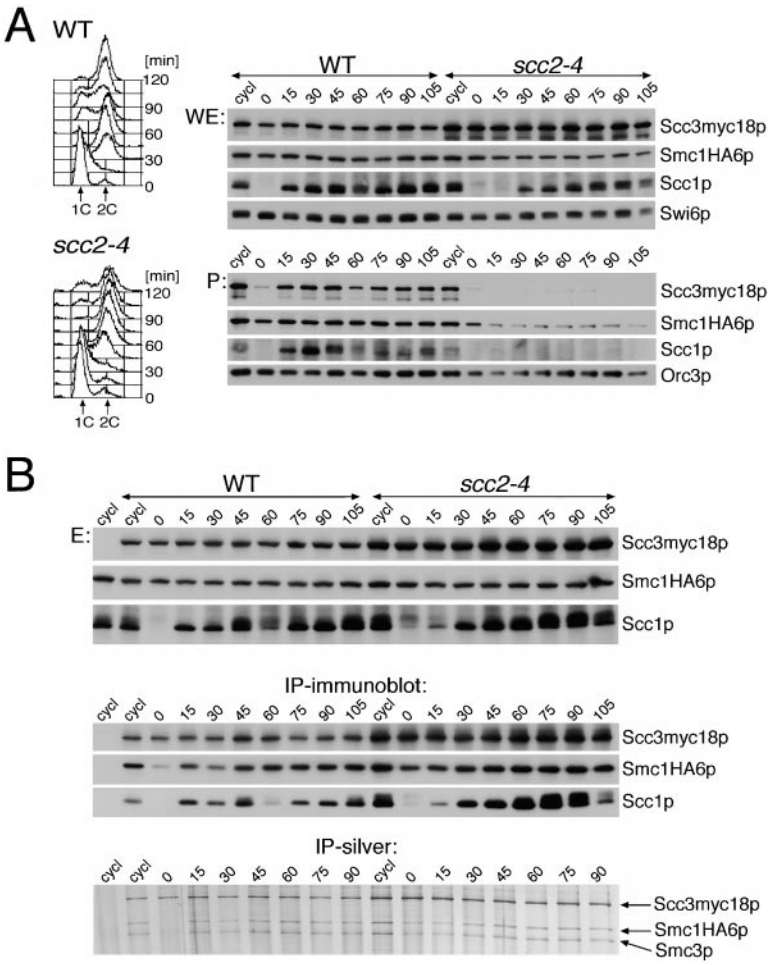

Figure 4. Scc2p Is Required for Cohesin's Association with Chromatin but Not for the Complex Assembly

Wild-type (K7691) or *scc2-4* (K8250) cells containing *SCC3myc18* and *SMC1HA6* were synchronized in G1 with α factor and incubated at 33°C. WT cells expressing both Scc3myc18p and Smc1HA6p grow a bit slower at 37°C, so lower temperature, 33°C, was used instead (at this temperature, cells behaved identically to the untagged strain). Aliquots were taken every 15 min and analyzed for DNA content and cohesin's association with chromatin (A) and integrity of the cohesin complex (B). Aliquots from cultures grown at 23°C were taken for cycling (cycl) samples. The myc-tagged and HA-tagged proteins were detected by immunoblotting using the 9E10 or 12CA5 monoclonal antibodies, respectively.

(A) Cohesin fails to associate with chromatin in *scc2-4* mutants. Chromatin pellets were separated from the crude extract on sucrose cushions. Crude extracts (WE) and chromatin pellets (P) were tested for the presence of the indicated proteins by immunoblotting. Swi6p and Orc3p are the loading controls for the WE and the P fractions, respectively. In the *scc2-4* mutants, cohesin complex (represented by Scc3myc18p, Smc1HA6p, and Scc1p) failed to associate with chromatin at any time in the cell cycle.

(B) The cohesin complex forms normally in *scc2-4* mutants. Precleared extracts (E) and immunoprecipitates (IP) were tested for the presence of the indicated proteins by immunoblotting (two top panels) or by silver staining (bottom panel). The first lane from the left shows a control IP from extract of cells (K7695) containing *SMC1HA6* but no myc tags on Scc3p. In the absence of Scc2p's function, all four known cohesin components were found to associate normally.

throughout this interval of time. In contrast, there was little or no difference in the amount of Orc3p (a subunit of the origin recognition complex) in chromatin pellets from either the wild type or the mutants.

To address whether cohesin's failure to bind chromatin in *scc2/4* mutants might stem from a defect in cohesin complex assembly, we used immunoblotting to measure the amount of Scc1p and Smc1HA6p associated with Scc3myc18p, as wild-type and mutant cells progressed through the cell cycle at the restrictive temperature (Figures 4B and 5B). Similar amounts of Smc1HA6p and Scc1p were coprecipitated with Scc3myc18p in wild type and mutants from S phase until mitosis. We were also able to detect Scc3myc18, Smc1HA6, and Smc3 proteins in the Scc3myc18 IPs by silver staining (Scc1p does not stain well with silver), and this confirmed that the stoichiometry of interaction between Scc3p and Smc1p/Smc3p is not altered in *scc2-4* or *scc4-4* mutants (Figure 4B, bottom panel, and data not shown). We conclude that cohesin complexes form normally in *scc2* or *scc4* mutants but fail to bind to chromatin.

We also noticed that in G1-arrested wild-type cells, less Smc1p and Smc3p was associated with Scc3myc18p, suggesting that Scc1p (absent at this stage of the cell cycle) is necessary for efficient binding of Scc3p to Smc1p and Smc3p. Interestingly, this effect was much less pronounced in *scc2-4* mutants.

The Scc2p/Scc4p Complex Is Specifically Required for Cohesin's Binding to Chromatin

It is possible that *scc2* and *scc4* mutants might be also defective in binding to chromatin of proteins not required for sister cohesion. However, mutations in both *SCC2* and *SCC4* genes (generated independently and by different means) specifically affect sister cohesion but no other cell cycle events. For example, cell growth, DNA replication, or cytokinesis take place normally in *scc2* and *scc4* mutants. We showed that (consistently with unperturbed DNA replication) Orc3p (a DNA-binding protein essential for DNA replication) binds to chromatin normally in *scc2* and *scc4* mutants. Furthermore, we also tested whether chromatin binding of Smc2p, a protein related to cohesin's Smc proteins, might be affected in *scc2* mutants. We found little or no difference in Smc2p's abundance in chromatin pellets between the wild-type and *scc2-4* mutant cells (Figure 5C). Thus, Scc2p is unlikely to be required for the association of yeast condensin with chromatin. Taken together, the

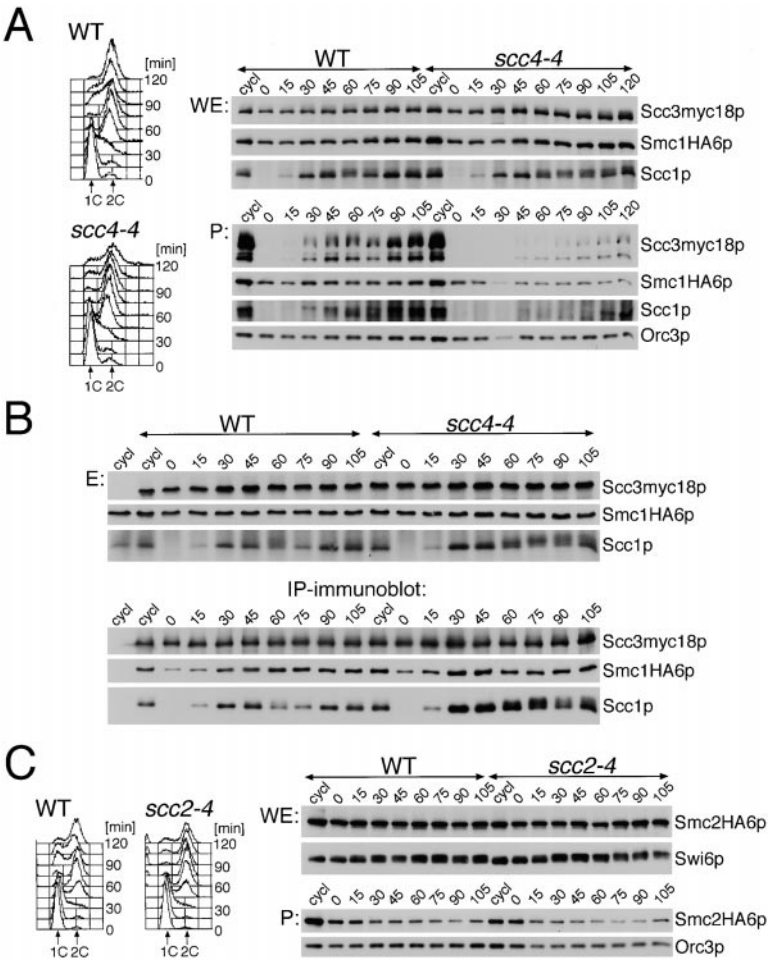

Figure 5. The Scc2p/Scc4p Complex Is Required Specifically for Cohesin's Binding to Chromatin

Wild-type (K7691) or *scc4-4* (K8373) cells containing *SCC3myc18* and *SMC1HA6* (A and B), and wild-type (K8275) or *scc2-4*(K8276) cells containing *SMC2HA6* (C), were synchronized in G1 with α factor and incubated at 33°C or 35°C, respectively. Aliquots were taken every 15 min and analyzed for DNA content and cohesin's association with chromatin (A), integrity of the cohesin complex (B), and for DNA content and Smc2p's association with chromatin (C). Aliquots from cultures grown at 23°C were taken for cycling (cycl) samples.

(A) Cohesin fails to associate with chromatin in *scc4-4* mutants. Chromatin pellets were separated from the crude extract on sucrose cushions. Crude extracts (WE) and chromatin pellets (P) were tested for the presence of the indicated proteins by immunoblotting. In the *scc4-4* mutants, cohesin complex (represented by Scc3myc18p, Smc1HA6p, and Scc1p) failed to associate with chromatin at any time in the cell cycle.

(B) Cohesin complex forms normally in *scc4-4* mutants. Precleared extracts (E) and immunoprecipitates (IP) were tested for the presence of the indicated proteins by immunoblotting. Scc3myc18p was precipitated using the 9E10 mAb. In the absence of Scc4p's function, cohesin components were found to associate normally.

(C) Smc2p's binding to chromatin does not depend on Scc2p. Chromatin pellets derived from wild-type and *scc2-4* cells were separated from the bulk extract on sucrose cushions. Crude extracts (WE) and chromatin pellets (P) were tested for the presence of Smc2HA6 by immunoblotting. Chromatin association of Smc2HA6p was not affected by the loss of Scc2p function.

Scc2p/Scc4p complex is unlikely to be a general factor regulating protein–chromatin interactions or chromatin structure. Instead, our data suggest that the Scc2p/Scc4p complex may be specifically required for cohesin's binding to chromatin.

Scc1p Fails to Associate with Specific Cohesin Association Sites on Chromosome Arms and with Centromeres in *scc2* and *scc4* Mutants

The distribution of cohesin along chromosomes is not random. Cohesin binds to both centromeres and specific sites within chromosome arms (Blat and Kleckner, 1999; Tanaka et al., 1999). To investigate the roles of Scc2p and Scc4p in recruiting cohesin to specific association sites, we used chromatin immunoprecipitation (CHIP), following formaldehyde-induced cross-linking of cells released from pheromone-induced G1 arrest (Figure 6C). We followed the binding of Scc1p to *CEN6* (Figure 6A) and to two sites (549.7 and a site adjacent to 558) on the right arm of chromosome V (Figure 6B). Scc1p's association with all three sites was severely reduced in *scc2-4* and *scc4-4* mutants. Thus, Scc2p and Scc4p are essential for recruiting cohesin both to centromeres and to arm sites.

Scc2p and Scc4p Colocalize on Chromosome Spreads but with Distinct Properties and at Different Loci Than Cohesin

There appear to be at least two pools of cohesin at most stages of the cell cycle; one that is tightly associated with specific sequences along chromosomes and another that is largely soluble. The same is true for Scc2p and Scc4p. A large fraction of both proteins is found in "chromatin" pellets, but some is also found in a soluble form. Most if not all of the soluble Scc2p is stably bound to Scc4p. To test whether what appears to be "chromatin-bound" Scc2p and Scc4p are present at the same loci as cohesin, we compared the distribution of Scc1p, Scc2p, and Scc4p on "super-spread" chromatin (Toth et al., 1999). We found that Scc2HA6p and Scc4myc18p were present in multiple foci. Furthermore, the distribution of the two proteins was very similar if not identical; that is, most foci of Scc2HA6p coincided with Scc4myc18p (Figure 7A). In contrast, Scc1myc18p, which is also distributed in multiple speckles on chromosome spreads, did not (as reported previously in Toth el al. [1999]) colocalize with Scc2HA6p. We also found that the amount of Scc2HA6p associated with chromatin pellets (P) was reduced in *scc4-4* but not in *scc1-73* mutants (Figure 7B). Finally, we failed to detect Scc2p

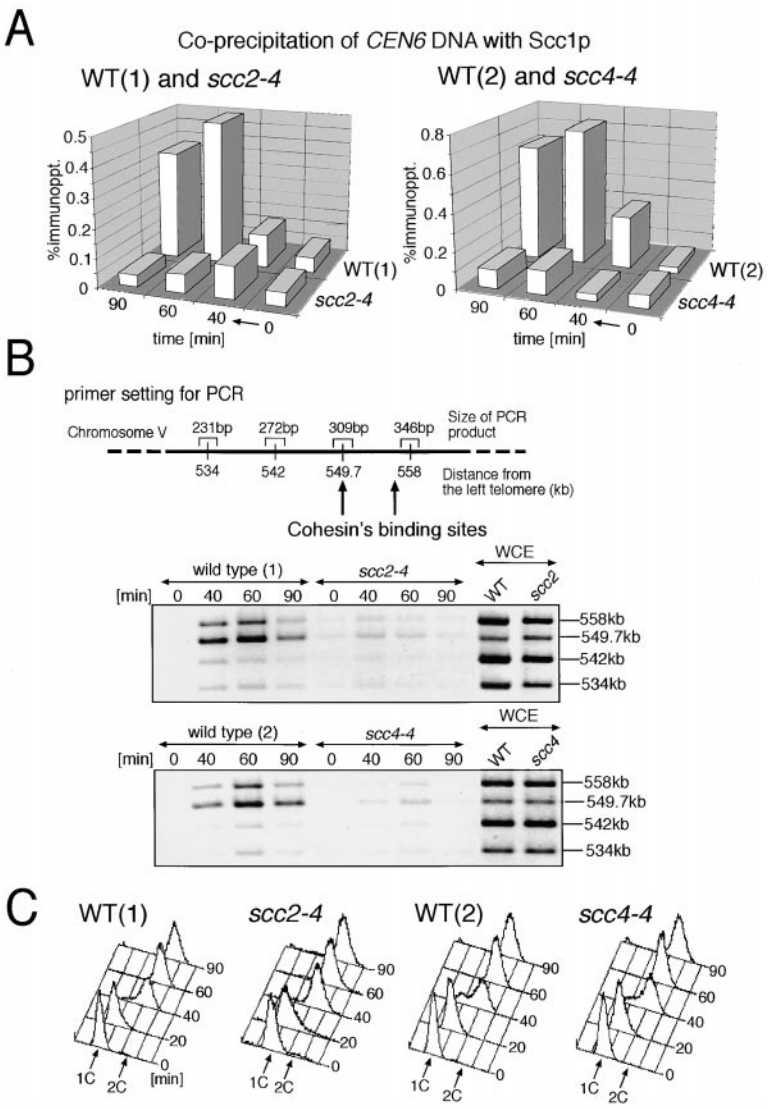

Figure 6. The Scc2p/Scc4p Complex Is Required for Cohesin's Binding at Both Centromeres and along Chromosome Arms

K6565 (*SCC1myc18*), K6575 (*SCC1myc18, scc2-4*), and K8417 (*SCC1myc18, scc4-4*) cells were synchronized in G1 with α factor. Cells were harvested by filtration and incubated (time 0 min) in fresh YPD medium containing nocodazole (15 μg/ml) at 33°C. Chromatin immunoprecipitations (CHIP) are shown in (A) and (B); DNA contents are shown in (C). Aliquots of wild-type (1) and (2) (both K6565) cells were processed together with those from the K6575 and K8417 strains, respectively. Scc1myc18p was immunoprecipitated from these extracts, and the presence of Scc1p-associated centromeric (A) or chromosome arm (B) DNAs were determined by PCR reactions using specific primers.

(A) Scc2p/Scc4p complex is required for cohesin's binding to centromeres. Fractions of *CEN6* DNA present in the Scc1myc18p precipitates were quantified by comparing amounts of PCR products amplified from the serial dilutions of total and immunoprecipitated DNAs (Tanaka et al., 1999). Scc1myc18p's binding to the *CEN6* sequence was found to be reduced in both the *scc2-4* and *scc4-4* mutants.

(B) Cohesins fail to bind along chromosomal arms in *scc2-4* and *scc4-4* mutants. The upper panel shows genomic fragments on the right arm of chromosome V that were amplified simultaneously in the PCR reactions. Lower panels show the reactions. Whole-cell extract DNA (WCE) was obtained from cells at time 0 min. Cohesin's association with the two sites on the left arm of chromosome V was found to depend on both Scc2p and Scc4p.

(C) DNA contents of cells measured by FACS.

bound to cohesin association sites using CHIP (data not shown). More detailed CHIP-based mapping will be required to determine whether Scc2p binds to specific sites on chromosomes that are distinct from cohesin association sites.

The different localization of Scc2p/Scc4p complexes and cohesin inspired us to address whether Scc2p/Scc4p association with chromatin depends on the integrity of DNA. We digested with micrococcal nuclease (MNase) whole chromatin pellets (WPs) prepared from strains expressing Scc1HA6p, Scc2HA6p, and Scc4HA6p. After digestion, pellets were spun through sucrose cushions to separate insoluble material (Pel) from proteins that have been solubilized (Sup). Unlike Orc3p and Scc1HA6p, neither Scc2HA6p nor Scc4HA6p was efficiently released from chromatin pellets by micrococcal nuclease treatment (Figure 7C). Thus, association of Scc2p/Scc4p complex with chromatin is spatially and biochemically different from that of cohesin. It is currently unclear whether Scc2p and Scc4p speckles in chromosome spreads represent complexes bound to DNA. Scc2p and Scc4p might, for example, bind to the proteinaceous axis of chromosomes (chromosome scaffolds), whereas cohesin binds directly to DNA. Our data nevertheless show that both soluble and insoluble (possibly chromatin-bound) Scc2p is tightly associated with Scc4p. They also imply that neither Scc2p nor Scc4p is stably associated with chromosomal loci at which cohesin is bound. It is therefore unlikely that cohesin complexes are recruited to sites on chromatin at which Scc2p/Scc4p complexes had previously bound.

Our finding that the association of cohesin with chromosomes requires a specialized factor composed of Scc2p and Scc4p suggests that the interaction between cohesin and chromosomes may be a complex one. Unlike Orc3p, which is washed off chromatin by 0.4M KCl, the chromatin association of Scc1p is highly salt resistant (Figure 7D). Interestingly, the same was found to be true for Scc2p and Scc4p.

Discussion

A Specialized Factor Required for Cohesin's Association with Chromatin?

Scc2p and its homolog in *S. pombe*, Mis4p, are essential for sister chromatid cohesion. Both premature sister

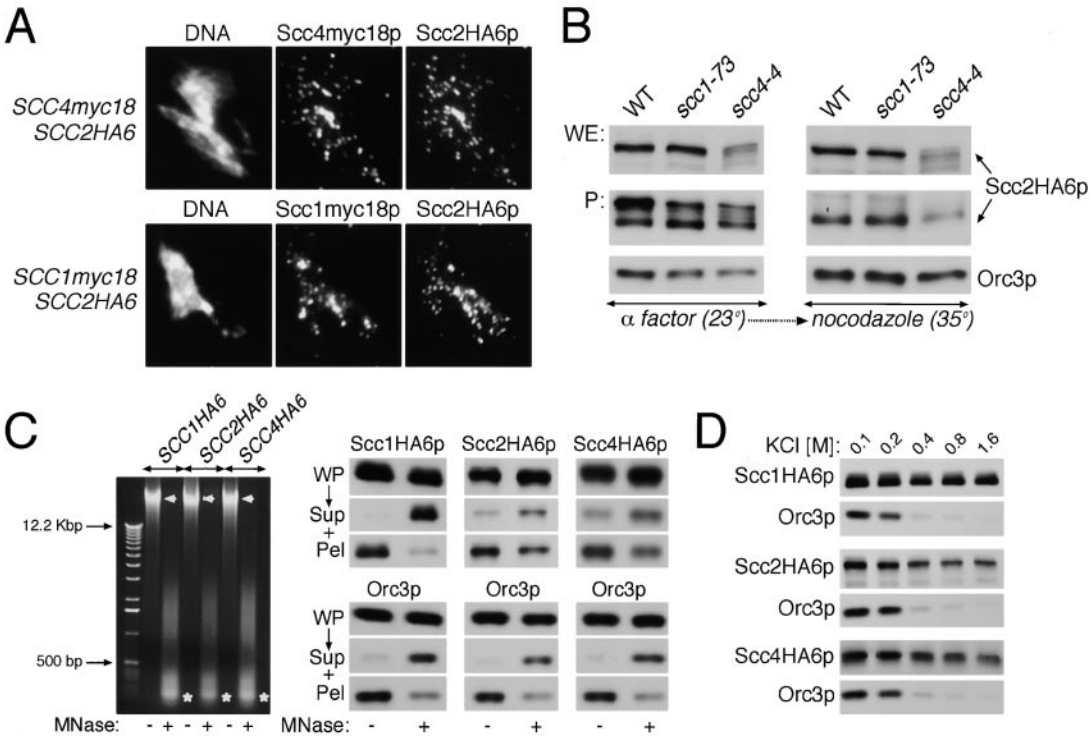

Figure 7. Cohesin and Scc2p/Scc4p Complexes Bind to Chromatin throughout the Cell Cycle, but Their Associations Have Different Characteristics

(A) Scc2p colocalizes with Scc4p but not Scc1p on "super-spread chromatin." Mechanically dispersed chromosome spreads were prepared from strains expressing Scc2HA6p in combination with either Scc4myc18p (K8519) or Scc1myc18p (K7709). DNA was stained with DAPI. Myc epitopes were detected with anti-myc mouse mAb 9E10 and anti-mouse cy3-conjugated goat Ab (Amersham). HA-tagged protein was visualized with anti-HA rat mAb 3F10 (Boehringer) and anti-rat cy2-conjugated goat Ab (Amersham).

(B) Scc2p's association with chromatin depends on Scc4p, but not on Scc1p. Wild-type (K8278), *scc1-73* (K8505), and *scc4-4* (K8504) cells containing *SCC2HA6* were synchronized at 23°C in G1 with α factor and incubated at 35°C in media containing nocodazole. Whole-cell extracts (WE) were fractionated on sucrose cushions, and chromatin pellets (P) were examined for the presence of Scc2HA6p by immunoblotting.

(C) Cohesin's (but not Scc2p/Scc4p's) chromatin association is sensitive to DNA digestion. Whole chromatin pellets (WE) prepared from cells containing *SCC1HA6* (K7694), *SCC2HA6* (K8278), or *SCC4HA6* (K8277) were divided into two parts. One part was incubated with micrococcal nuclease (MNase). Both the untreated (−) and treated (+) WPs were spun through sucrose cushions, resulting in the insoluble pellet (Pel) and soluble supernatant (Sup) fractions. To confirm chromatin digestion upon the MNase treatment, DNAs isolated from the (−) and (+) WPs were subjected to agarose gel electrophoresis (shown on the left). White arrowheads point to genomic DNAs derived from the (−) extracts. White asterisks mark the shortest DNA fragments produced after the MNase digest. Shown on the right are whole pellet (WP), supernatant (Sup), and pellet (Pel) fractions that were tested for presence of the indicated proteins by immunoblotting. Upon the MNase treatment, most of Scc1HA6p (similar to Orc3p) became soluble. However, a large fraction of Scc2HAp and Scc4HA6p remained associated with the insoluble material.

(D) Cohesin's and Scc2p/Scc4p's associations with chromatin are highly resistant to salt extraction. Whole-cell extracts (WE) derived from cells containing *SCC1HA6* (K8502), *SCC2HA6* (K8278), or *SCC4HA6* (K8277) were incubated in buffers containing increasing concentrations of KCl (0.1–1.6 M). After 10 min incubation, WEs were spun through sucrose cushions, and the resulting pellets were examined for the presence of Scc1HA6p, Scc2HA6p, Scc4HA6p, and Orc3p. Orc3p, but not the other proteins, dissociated from chromatin at moderate salt concentrations (0.2–0.4 M). Scc1p (and to a lesser degree, Scc2p/Scc4p) was found to resist up to the 1.6 M salt extraction.

separation and S phase–linked lethality of *mis4* mutants resemble phenotypes of *scc2/4* mutants. However, because *mis4* cells released from G2 or M arrest seem to lose viability during mitosis, Mis4p, unlike Scc2p, has also been proposed to function in M phase. Interestingly, despite loss of viability, chromosome segregation seems to proceed normally in *mis4* mutants released from the G2 arrest. Thus, it is possible that the M phase lethality of *mis4* mutants does not stem from Mis4p function in sister cohesion. It might also be that the *mis4-242* cells, when transferred from restrictive to permissive temperature during M phase, die because they do not reactivate ts Mis4p by the time a new round of DNA replication starts (in fission yeast, G1 phase of the cell cycle is very short so that S phase begins very soon after mitosis). It has also been proposed that two different types of connection might be required for holding sister chromatids together: one based on cohesin and another on Scc2p/Mis4p, which has been called adherin (Furuya et al., 1998).

More recently, we noticed that two different cohesin subunits, Scc1p and Scc3p, fail to associate stably with chromosomes in *scc2* mutants (Toth et al., 1999). We show here that Scc2p forms a stable complex with another protein, Scc4p, which is also necessary for sister chromatid cohesion. We show that Scc2p and Scc4p colocalize in chromosome spreads, that both proteins are essential for cohesin to bind to sequences within

chromosome arms and to centromeres, and that neither protein is needed for the formation of soluble cohesin complexes. We find that Scc2p must function around S phase but is not needed during G2 or M phase. We also show that neither Scc2p nor Scc4p is required for maintenance of cohesion between sisters in metaphase-arrested cells. These data suggest that neither Scc2p nor Scc4p is a constituent of the bridges that physically hold sister chromatids together during G2 and early M phase. We propose instead that the essential function of the Scc2p/Scc4p complex is to promote loading of cohesin complexes onto chromatin.

Mutations in *SCC2* and *SCC4* genes specifically affect sister cohesion but no other cell cycle events. Several aspects of chromatin physiology, including general transcription and DNA replication, seem to take place normally in *scc2* and *scc4* mutants. We also showed that chromatin binding of Smc2p, a condensin component, is not affected in *scc2* mutants, suggesting that Scc2p and Scc4p are dispensable for chromosome condensation. Consistent with this, chromosome condensation was shown to be normal in *mis4* mutant cells. These data suggest that Scc2p/Scc4p may be required specifically for cohesin's binding to chromosomes. It will be interesting to see if Scc2p/Scc4p also functions during meiosis, for example, to ensure association of the meiotic version of cohesin (containing Rec8 instead of Scc1p) with chromosomes.

Is Scc2p/Scc4p Activity Cell Cycle Regulated?

Our data show that in *scc2* or *scc4* mutants, cohesin fails to associate with chromosomes at any point in the cell cycle, with the result that cohesin accumulates in a soluble form from late G1 until the onset of mitosis. Using the *GAL1-10* promoter, it is possible to delay Scc1p expression until after cells have undergone S phase. Under these circumstances, cohesin complexes both form and bind efficiently to chromosomes despite the failure to establish functional connections between sister chromatids (Uhlmann and Nasmyth, 1998). In contrast, ectopic expression of Scc1p during G1 fails to induce the appearance of cohesin on chromosomes. This failure is due to the activity of the separin protein Esp1p, which causes Scc1p's proteolytic cleavage from the onset of anaphase until late G1 in the next cell cycle (Uhlmann et al., 1999). However, the ability of a non-cleavable Scc1p variant to bind chromosomes during early G1 suggests that the presence of stable Scc1p is sufficient for cohesin's assembly and that cohesin is in principle capable of binding yeast chromosomes throughout the yeast cell cycle. Because Scc2p/Scc4p activity is essential for cohesin's binding to chromatin and cohesin can bind to chromosomes during most of the cell cycle, Scc2p/Scc4p activity is unlikely to be cell cycle regulated.

How Does Cohesin Bind to Chromosomes?

In G1 cells (in the absence of Scc1p), Scc3p does not bind to chromosomes. Also, neither Scc1p nor Scc3p can bind to chromosomes in the absence of each other, or in the absence of Smc1p and Smc3p in nocodazole-arrested cells (Toth et al., 1999). These data suggest that all four cohesin subunits bind to chromosomes efficiently only as part of an intact protein complex. Our finding that in G1 cells (in the absence of Scc1p), Scc3p not only does not bind to chromatin but also does not associate efficiently with Smc1p and Smc3p is consistent with such a hypothesis. These data suggest that formation of soluble cohesin may be an important prerequisite for binding of its constituent subunits to chromatin.

The C-terminal domains of Smc proteins, including that of Smc1p, are capable of binding DNA in vitro, in particular to sequences with secondary structures (Akhmedov et al., 1998). Cohesin contains two Smc subunits (Smc1p and Smc3p), suggesting that these proteins might be responsible for cohesin's association with chromatin. It is clear, however, that the binding of cohesin to yeast chromosomes in vivo must be a far more complex process. During Scc1p's absence in early G1, much less Smc1p (and possibly Smc3p) is bound to chromosomes (Figure 5A). In addition, formation of intact complexes is insufficient for cohesin's association with chromosomes. A specialized complex composed of Scc2p and Scc4p is also necessary.

As might be expected for a complex that interacts with DNA, cohesin is efficiently released from chromatin pellets by micrococcal nuclease digestion. However, its association is highly salt resistant, suggesting that cohesin's association with chromosomes might involve the formation of higher order structures analogous to nucleosomes or the large superhelical coils thought to be formed by condensin (Kimura et al., 1999). The structures formed on chromosomes by cohesin must be assembled even in the absence of DNA replication and then yet further elaborated following the passage of replication forks to produce Eco1p-mediated connections between sister chromatids.

A Mechanism of Cohesin's Binding to Chromosomes

The ability of ts *scc2* mutants to undergo mitosis normally when shifted to the restrictive temperature during G2 suggests that the Scc2p/Scc4p complex, while required for the initial binding of cohesin to chromosomes, might not be required to maintain cohesin's association with chromatin. We envision several types of mechanisms by which Scc2p/Scc4p might facilitate cohesin's loading onto chromosomes. Although we did not detect any change in cohesin's composition or appearance in *scc2* or *scc4* mutants, one possibility is that Scc2p/Scc4p directly catalyzes a change in the conformation of cohesin that is essential for its binding to chromatin. In this case, Scc2p/Scc4p's facilitation of cohesin binding would not necessarily depend on Scc2p/Scc4p's own association with chromosomes.

Another possibility is that Scc2p/Scc4p might, by transient binding at cohesin association sites, catalyze a change in chromatin structure at these sites, which would be necessary for cohesin's binding. Alternatively, Scc2p/Scc4p might bind to cohesin association sites and then act as a kind of receptor. A precedent for such a phenomenon might be the recruitment of worm dosage compensation complexes, which also contain two Smc proteins, to X chromosomes by SDC2p (Dawes et al.,

1999). A substantial amount of Scc2p/Scc4p complex does indeed appear to be associated with chromosomes at defined locations. However, the foci of Scc2p/Scc4p complexes in chromosome spreads do not coincide with those of cohesin. We were also unable to detect Scc2p at cohesin association sites using chromatin immunoprecipitation (CHIP). These observations are inconsistent with the notion that Scc2p/Scc4p catalyzes conformational changes at cohesin association sites or that it recruits cohesin to sites at which it is itself bound.

Finally, it is possible that through binding to defined chromosomal sites (for example, at the base of chromosomal loops) Scc2p/Scc4p might generate a domain permissive for the loading of cohesin. In this scenario, Scc2p/Scc4p would promote cohesin's loading indirectly, without any direct, physical association with cohesin. According to this hypothesis, cohesin association sites within a domain generated by Scc2p/Scc4p need not coincide with sites at which Scc2p/Scc4p is itself associated. Interestingly, Nipped-B, a *Drosophila* homolog of Scc2p, has been proposed to facilitate long-range enhancer–promoter interactions, possibly by looping out the interstitial chromatin (Rollins et al., 1999). Why would binding of cohesin require special chromatin domains? Cohesin's binding to chromatin may be inhibited by transcription (Tanaka et al., 1999). Thus, Scc2p/Scc4p-mediated changes in chromatin might "protect" cohesin association sites from phenomena (such as transcription) that could potentially disturb cohesin's binding.

Distinguishing between these different possibilities will very likely require an in vitro system in which the binding of cohesin to DNA or chromatin is Scc2p/Scc4p dependent. Our preliminary experiments aimed at developing such an assay suggest that Scc2p/Scc4p facilitates binding of cohesin complexes to DNA in crude cell extracts, which resembles the process that occurs in vivo (data not shown).

Do Scc2p/Scc4p-like Complexes Exist in All Eukaryotes?

It is possible that similar cohesin-loading complexes exist in most, if not all, eukaryotes. Proteins homologous to Scc2p exist in fission yeast (Mis4p) (Furuya et al., 1998), in *Coprinus* (Rad9p) (Seitz et al., 1996), in *Drosophila* (Nipped-B) (Rollins et al., 1999), in mouse, and in man. Thus far, we have been unable to detect proteins homologous to Scc4p, but this may be due to the low sequence conservation. Mis4p in *S. pombe* has been reported to exist in a large complex distinct from cohesin, which would be consistent with it being complexed with other proteins. Mis4p, like Scc2p, is necessary for sister chromatid cohesion, whereas Rad9p in *Coprinus* is crucial for meiotic chromosome segregation and for recombination. The phenotypes of *mis4* and *rad9* mutants are therefore consistent with a role in loading cohesin onto chromosomes. Meanwhile, Nipped-B mutations in *Drosophila* affect enhancer–promoter interactions, and it has been suggested that the mutations affect long-range chromosome behavior. Whether Nipped-B and other animal Scc2p homologs are also involved in establishing sister chromatid cohesion remains to be tested.

Experimental Procedures

Yeast Strains and Growth Conditions

All strains are derivatives of W303. YEP medium (Rose et al., 1990) was supplemented with either 2% raffinose (YEPraf), 2% raffinose and 2% galactose (YEPraf+gal), or 2% glucose (YEPD). To obtain synchronous cultures, cells were grown in YEPraf medium at 23°C, and small G1 cells were isolated by centrifugal elutriation (Schwob and Nasmyth, 1993). Alternatively, MAT**a** cells grown at 23°C were synchronized in G1 by adding α factor for 2–2.5 hr (typically; 2 μg/ml at 0 min and additional 1.5 μg/ml at 1 hr). To arrest cells in an S phase–like state, hydroxyurea (HU) was added to 100 mM. M phase–like arrest was achieved by adding nocodazole to 15 μg/ml.

Strain Constructions

Disruption or epitope tagging of *SCC4* was achieved by homologous recombination at its chromosomal locus using a PCR-based method (Knop et al., 1999). The heterozygous *SCC4* Δ*scc4* diploid strain was sporulated, and tetrad analysis showed that none of the Δ*scc4* spores were viable. The lethality of the Δ*scc4* spores was rescued by a centromeric plasmid containing the wild-type *SCC4* gene (YCplac33*SCC4*; C3892). Strains expressing epitope-tagged Scc2p or Scc4p proliferated normally at 37°C, demonstrating that the fusion proteins were functional.

To construct the K8468 strain (*cdc20::LEU2 GALCDC20 scc1-73*), the N terminus of the *TRP1* gene in the strain K7246 was replaced by *LEU2* (from *K. lactis*), and the plasmid C2329 (YIp*GALCDC20*::TRP1) was then integrated (after linearization with EcoRV) into the remaining C-terminal part of the *trp1* gene. The copy number of *GALCDC20* was determined by Southern analysis. The resulting strain was crossed to K7428 to obtain K8468.

Construction of Conditional Alleles of *SCC4*

To deplete cells of Scc4p, we initially expressed Scc4p from the galactose-inducible and glucose-repressible *GAL1-10* promoter. Cells were transformed with several independent constructs consisting of the integrative pRS303 plasmid and a 5′ fragment of *SCC4* fused to the *GAL1-10* promoter. Integration into the genome generated a truncated *SCC4* and a full-length *SCC4* expressed from *GAL1-10*. Most transformants were viable on glucose-containing (YEPD) plates, suggesting that either repression of the *GAL* promoter was insufficient or Scc4p is a particularly stable protein. However, one construct did result in lethality on YEPD plates. Sequencing of this particular construct revealed an aberrant junction between *GAL1-10* and 5′*SCC4*, resulting in the expression of a truncated version of Scc4p. Although the depletion was taking too long (several cell divisions) to use this strain for studying the Δ*scc4* phenotype, we used this Scc4$^{(\Delta N)}$p-expressing strain to facilitate isolation of temperature-sensitive alleles of *SCC4*. To achieve this, *SCC4* was PCR mutagenized (Stark, 1998), and the PCR products were cotransformed with the YCplac111 plasmid carrying a gapped *SCC4* gene (most of which had been removed) into the strain expressing Scc4$^{(\Delta N)}$p. Transformants were recovered on −Leu YEPD plates, and about 5000 colonies were then replica plated on −Leu YEPD plates at 25°C and 37°C. Two hundred temperature-sensitive (ts) colonies were streaked for single cells to check for ts sensitivity. Eventually, 17 transformants showing strong ts phenotypes (which were rescued on YEPraf+gal plates) were chosen. Mutated *SCC4* rescued from seven independent transformants were recloned into the integrative YIplac128 plasmid. These plasmids were linearized with ClaI and integrated at the *leu2* genomic locus of a Δ*scc4::HIS3* strain (K8290) kept alive by an *SCC4* gene on a centromeric plasmid, YCplac33 (C3892). 5-FOA selection was used to isolate cells that lost the YCplac33*SCC4* plasmid. Finally, phenotypes of three strains containing a single copy (as determined by Southern analysis) of integrated *scc4* were compared. Since the phenotypes appeared similar, only one strain (K8326) containing the ts *scc4-4* allele (plasmid C3894) was chosen for further studies.

Mass Spectrometric Identification of Scc4p

Scc2p-associated proteins were eluted from beads, separated by one-dimensional gel electrophoresis, and visualized by staining with silver (Shevchenko et al., 1996b). Proteins specifically present in the

immunoprecipitate (but not in the control) were excised from the gel, reduced with dithiothreitol, alkylated with iodoacetamide, and digested in-gel with trypsin (unmodified, sequencing grade, Boehringer Mannheim) at 37°C overnight. Proteins were identified by high mass accuracy MALDI peptide mapping (Jensen et al., 1996) and nanoelectrospray tandem mass spectrometric sequencing (Wilm et al., 1996), combined in a layered approach (Shevchenko et al., 1996a). A 0.5 μL aliquot of the digest was analyzed by MALDI mass spectrometry on a REFLEX time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). If peptide mass mapping failed to identify the protein, the digestion products were extracted from the gel pieces with 5% formic acid and acetonitrile, and the extracts were pooled together and dried down in a vacuum centrifuge. The sample was redissolved in 5% formic acid, desalted, and concentrated on a glass capillary containing 100 nL of POROS R3 reversed phase resin (PerSeptive Biosysytems, Framingham, MA), and the unseparated pool of peptides was sequenced on a API III triple quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a nanoelectrospray ion source (Wilm and Mann, 1996) developed in EMBL.

A comprehensive sequence database was searched using PeptideSearch version 3.0 software developed in EMBL. No limitations were imposed on the molecular weight of proteins and species of origin.

Salt Extraction of Chromatin Pellets

Whole-cell extracts (WEs) and chromatin pellets were prepared as described in Liang and Stillman (1997), with the exception that spheroplasts were lysed in buffer already containing Triton X-100 (EBX). Whole-cell extracts were mixed with equal volumes of EBX buffer containing increasing concentrations of KCl (2×). After 10 min incubation, WEs were spun through sucrose cushions, and the resulting pellets were examined by immunoblotting for the presence of Scc1HA6p, Scc2HA6p, Scc4HA6p, and Orc3p.

MNase Digestion of Chromatin Pellets

Chromatin pellets were prepared as described in Liang and Stillman (1997). These pellets were gently resuspended in the EBX buffer, divided into two tubes, and supplemented with $CaCl_2$ to 1 mM. Micrococcal nuclease (40 U) was added to one tube, and both samples were then incubated at 37°C for 2 min, followed by addition of EGTA to 1 mM to stop Mnase digestion. At this stage, aliquots were taken from these MNase (−) and (+) whole pellets (WP) to show that MNase treatment did not affect protein levels. Next, (−) and (+) WPs were further divided into two tubes, (a) and (b), which were processed further as follows.

To one aliquot (a), SDS was added to 2%, and genomic DNA was isolated by phenol:chloroform extraction and ethanol precipitation. Precipitated genomic DNAs were resuspended in TE buffer supplemented with RNase. DNAs were subjected to electrophoresis in a 0.5% agarose gel. DNAs derived from the (−) WPs run predominantly as single bands of high molecular weight. DNAs derived from the (+) WPs produced DNA fragments of different sizes (which appeared as a smear), with a large fraction of DNA cut to the mononucleosome size.

The other aliquot (b) was spun in an Eppendorf centrifuge for 5 min through a 30% sucrose cushion. This fractionation step was used to separate proteins that were solubilized (Sup) upon the MNase treatment from the proteins that remained associated with insoluble material (Pel). Levels of Scc1HA6p, Scc2HA6p, Scc4HA6p, and Orc3p in the different fractions were determined by immunoblotting.

Other Techniques

A FACSscan (Becton-Dickinson) was used for flow cytometric analysis of cellular DNA content as described (Epstein and Cross, 1992). Visualization of yeast chromosomes using the tetR-GFP/tetO system was performed as described previously (Michaelis et al., 1997). Viability assay, protein extracts, Western blot analysis, and immunoprecipitations were performed as described in Toth et al. (1999). Chromatin immunoprecipitations (CHIP) were performed as described previously (Tanaka et al., 1997).

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