SMC2, a *Saccharomyces cerevisiae* gene essential for chromosome segregation and condensation, defines a subgroup within the SMC family

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We characterized the *SMC2* (structural maintenance of chromosomes) gene that encodes a new *Saccharomyces cerevisiae* member of the growing family of SMC proteins. This family of evolutionary conserved proteins was introduced with identification of *SMC1*, a gene essential for chromosome segregation in budding yeast. The analysis of the putative structure of the Smc2 protein (Smc2p) suggests that it defines a distinct subgroup within the SMC family. This subgroup includes the ScII, XCAPE, and cut14 proteins characterized concurrently. Smc2p is a nuclear, 135-kD protein that is essential for vegetative growth. The temperature-sensitive mutation, *smc2-6*, confers a defect in chromosome segregation and causes partial chromosome decondensation in cells arrested in mitosis. The Smc2p molecules are able to form complexes in vivo both with Smc1p and with themselves, suggesting that they can assemble into a multimeric structure. In this study we present the first evidence that two proteins belonging to two different subgroups within the SMC family carry nonredundant biological functions. Based on genetic, biochemical, and evolutionary data we propose that the SMC family is a group of prokaryotic and eukaryotic chromosomal proteins that are likely to be one of the key components in establishing the ordered structure of chromosomes.

[Key Words: *S. cerevisiae*; SMC family; chromosome segregation; condensation; chromosome structure]

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The structure and protein composition of chromosomes change in the course of the cell cycle and during embryonic development of multicellular organisms (Manuelidis 1990; Singh et al. 1991; Wolffe 1991; Wagner et al. 1993). In particular, the segregation of chromosomes in mitosis requires the ordered structural reorganization of chromosomes, facilitating their successful distribution in anaphase. Morphologically this reorganization appears as chromosome condensation. The chromosomes of eukaryotic cells in their segregation-ready state have been proposed to have at least six levels of structural hierarchy (Filipski et al. 1990; Saitoh and Laemmli 1994). Only the formation of 10- and 30-nm fibers, the first two levels of chromatin structure, is understood relatively well. The interactions between DNA and the core histones are responsible for formation of 10-nm fibers (DNA wrapped around nucleosomes) (Richmond et al. 1984). Formation of 30-nm fibers (solenoid) is mediated by histone H1 and its variants as well as some non-histone proteins (Dimitrov et al. 1993; Zhao et al. 1993; Graziano et al. 1994; Ner and Travers 1994). Solenoid formation involves both protein-DNA contacts, which

cause a bend in the internucleosomal DNA, and protein-protein contacts between the histones and the nonhistone proteins. In general, proteins involved in chromosome structure at levels higher than 30-nm fibers remain unknown. Whereas one protein, topoisomerase II, has been implicated in mediating higher levels of chromosome organization, including the formation of 10- to 50-kb loops (Ohsumi et al. 1993; Saitoh and Laemmli 1994) its exact role as a structural protein remains controversial (Hirano and Mitchison 1993). The requirements for DNA sequence to be recognized by the putative loop-stabilizing proteins are also unknown. However, several models have been proposed that implicate A/T-rich stretches of DNA (Kas et al. 1993) in the organization of loops and in the formation of rosettes and 100-nm fibers. In the absence of conclusive data, extremely diverse hypotheses regarding the generation of higher order chromatin structure have been proposed (Cook 1994; Timsit and Moras 1994). Therefore, it is critical to identify proteins specific for the packaging of mitotic chromosomes.

We have initiated a study of mitotic chromosome structure in the budding yeast *Saccharomyces cerevisiae* using fluorescent in situ hybridization (FISH) (Guacci et al. 1994). With this method we have shown that sister chromatids in mitosis are paired and condensed as they

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are in the cells of higher eukaryotes. Moreover, we estimated that the mitotic chromatin condenses in budding yeast only twofold compared to interphase chromatin, versus five- to sevenfold effect in animal cells (Manuelidis and Chen 1990). Whereas the magnitude of condensation is less in budding yeast, the process is important for mitosis: Decondensed yeast chromosomes would not segregate successfully as their length surpasses the length of the mitotic spindle (Guacci et al. 1994). The difference in the level of condensation between higher eukaryotes and *S. cerevisiae* could reflect a simple difference in the density of condensation initiation sites or a more fundamental difference in the mechanism of condensation. To understand the mechanism of yeast chromosome condensation and its relationship to condensation in higher eukaryotes, it has been necessary to study yeast chromosome condensation at the molecular level. It has been anticipated that FISH combined with the availability of mutants impairing chromosome segregation will allow such comprehensive studies on mitotic chromosomes of budding yeast, despite the small chromosome size (0.2×10^6 to 1.5×10^6 bp).

Studies of a chromosome segregation mutant, *smc1*, led us to the characterization of Smc1p, an essential nuclear protein that is a candidate for a specific structural component of yeast mitotic chromosomes (Strunnikov et al. 1993). The depletion of the *SMC1* (structural maintenance of chromosomes) product from yeast leads to a severe defect in chromosome segregation resulting in a cut phenotype (the undivided nucleus is split by the ongoing cytokinesis) and rapid cell death. It was shown that an essential activity of Smc1p is required in mitosis but not at other stages of cell cycle. It was also apparent that Smc1-like proteins (SMC family) are ubiquitous among cellular organisms, including *Prokaryota*, suggesting a common and very basic biological role for these proteins in all types of cells. All SMC family members contain a specific signature, the evolutionary conserved carboxy-terminal region (DA box). The putative structure of Smc1p and its homologs is reminiscent of force-generating "motor" proteins (Strunnikov et al. 1993), in that both SMC and motor proteins contain a putative nucleotide-binding (NTP-binding) site and two extensive coiled-coil regions. Based on these observations we postulated that Smc1p and its homologs in other organisms might be structural chromosomal proteins that are involved in the formation of mitotic chromosome structure in eukaryotic cells (Guacci et al. 1993; Strunnikov et al. 1993). This hypothesis has been validated with the recent characterization of SMC family proteins in higher eukaryotes. The homologs from chicken (ScII) (Saitoh et al. 1994) and frog (XCAPC and XCAPE) (Hirano and

Mitchison 1994) have been shown to be components of condensed chromosomes.

Concurrently with the above mentioned studies of the SMC proteins from vertebrates, we characterized the second SMC gene from *S. cerevisiae*. This gene was found fortuitously as a partially sequenced open reading frame (ORF) that shared a significant homology with the DA box of Smc1p (Strunnikov et al. 1993). As we proposed that the DA box is a signature for the whole SMC family, the finding of an ORF containing a DA box suggested that the budding yeast have another gene of the SMC family, *SMC2*. In this report we describe the cloning of the full-length *SMC2* gene and show that its product (Smc2p) identifies a new structural subfamily within the SMC family. We characterize *SMC2* and its product genetically, cytologically, and biochemically. Taking into account the profound chromosome-related phenotypes of *smc1* and *smc2* mutants, we suggest that the original SMC acronym (stability of minichromosomes) (Larionov et al. 1985) be redefined as structural maintenance of chromosomes.

Results

SMC2 product is a new member of the SMC family

In a previous study (Strunnikov et al. 1993) we found a sequence homology between the carboxy-terminus of Smc1p (DA-box) and a partially sequenced ORF from the chromosome VI (Shirahige et al. 1993). We cloned a fragment that contained the full-length gene for the potential SMC protein and determined its nucleotide sequence (Fig. 1a,b). The sequence analysis revealed the presence of an ORF capable of encoding a protein with a predicted molecular mass of 134 kD (1170 residues). The conceptual protein showed 27% identity and 51% similarity to Smc1p. The new protein also contained all putative domains found in Smc1p: a nucleotide-binding region, two coiled-coil regions (Fig. 1c), and a DA box. All putative domains were positioned within the polypeptide almost exactly as they are in Smc1p, suggesting that these two proteins are structurally related. Based on that fact and the finding that the newly identified gene (see below), as well as *SMC1*, is involved in maintaining chromosome fidelity (Strunnikov et al. 1993), we considered the new protein functionally related to Smc1p and named the new gene *SMC2*.

We showed previously that the proteins homologous to Smc1p and Smc2p (the SMC family) are ubiquitous among the cellular organisms, including bacteria. With the identification of Smc2p and new SMC proteins from other organisms (Chuang et al. 1994; Hirano and Mitch-

Figure 1. Map position and sequence analysis of *SMC2*. (a) Physical map position of *SMC2* on chromosome VI. An ORF upstream of *SMC2* encodes a putative ribosomal protein. The *smc2-Δ1* and *smc2-Δ2* deletions are shown. (b) Sequence analysis of *SMC2* gene. The flanking restriction sites *Xba*I and *Sal*I are shown. The signature sequences for the NTP-binding site and the DA box are underlined. The sequence data are available from GenBank under accession number U05820. (c) Prediction of the regions with the coiled-coil structure within the Smc2 protein. The Newcoil program (Lupas et al. 1991) was used to calculate the probability of coiled-coil formation within the Smc2p.

Figure 1. (See facing page for legend.)

Figure 2. A comparison of the structure of Smc2p and other SMC family members. (a) The pairwise alignment of the DA box and flanking sequences of different SMC proteins and the acidic cluster from the RecN protein (*E. coli*). The DA box sequence is underlined. The region used for the phylogenetic analysis is bracketed. (b) Dot matrix analysis of ScII/Smc2p and Smc1p/Smc2p similarity. The matrix was built using a 23-residue window with the requirement of 25% identity (6 residues). (c) Phylogenetic analysis of the SMC family and distantly related proteins. A 56-residue region that included the DA box (a) from each SMC protein was used for the molecular phylogenetic analysis of the SMC family. The phylogenetic tree was built using the Fitch–Margoliash algorithm with the evolutionary clock and was validated by resampling (see Materials and methods). The percentages at the forks indicate the probability that all of the species to the right of that fork fall into this group. References for the sequences used are given in the text. (*M. h.*) p115 from mycoplasma; (*Rh. r.*) partial ORF from purple bacteria (see Strunnikov et al. 1993); (*B. s.*) *B. subtilis*; (*E. c.*) *E. coli*.

ison 1994; Saitoh et al. 1994; Saka et al. 1994) it was possible to update our previous comparative structural analysis of this family. First, a comparison of carboxy-terminal regions of SMC proteins showed that Smc2p is much more similar to the ScII protein than to Smc1p (Fig. 2a). The greater similarity between Smc2p and ScIIp extends over the entire length of the proteins (Fig. 2b). Similar pairwise comparison suggests that the XCAPE protein from *Xenopus laevis* (Hirano and Mitchison 1994) and the cut14 protein from *Schizosaccharomyces pombe* (Saka et al. 1994) fall into the Smc2p group. In contrast, the sequences of mouse and human homologs of Smc1p (MSmcA and HSmcA; A.V. Strunnikov, unpubl.) show a higher degree of similarity to Smc1p than to Smc2p (Fig. 2a), suggesting that mammalian SmcA is a member of the Smc1 subgroup. Other members of the Smc1 subgroup include the Dpy-27 protein from *Caenorhabditis elegans* (Chuang et al. 1994), the XCAPC protein from *X. laevis* (Hirano and Mitchison 1994), and the cut3 protein from *S. pombe* (Saka et al. 1994). These data suggest that not only are SMC proteins evolutionary

conserved but the structural (and probably functional) difference between the subfamilies (e.g., Smc1p and Smc2p) is conserved as well, at least from fungi to animals.

To search for other distantly related sequences we masked the coiled-coil regions of Smc2p with a wildcard character (Green et al. 1993) and used this sequence as a query in a BLAST search (Altschul et al. 1990). By this method, several additional proteins distantly related to Smc2p were identified. The most promising similarity was found with the RecN proteins from *Bacillus subtilis* and *Escherichia coli* (Fig. 2a) (Rostas et al. 1987; Van Hoy and Hoch 1990). These proteins also have a putative NTP-binding site as well as an acidic cluster with limited similarity to the DA box, separated by a domain with weak similarity to a coiled-coil (data not shown). Another group distantly related to SMC proteins is the family of transport-related ATPases (McGrath and Varshavsky 1989; Shea and McIntosh 1991; Cole et al. 1992). However, in those proteins, the putative NTP phosphate-binding site and the acidic cluster lie in close

proximity to each other as observed also in helicases (Gorbaleyna et al. 1989).

To assess quantitatively the evolutionary relationships between all known SMC family members and the distantly related proteins, we performed the phylogenetic analysis (see Materials and methods) of the DA box and the encompassing region of SMC proteins using the corresponding "matching" sequences from related proteins as a control. The resulting phylogenetic tree (Fig. 2c) corroborates our previous pairwise analysis, suggesting that Smc1p and Smc2p are in different subfamilies. Furthermore, the phylogenetic analysis places the DA box-containing group (SMC family) well away from other proteins (RecN proteins are shown as an example), supporting our view that their similarity is a result of the convergent evolution (Doolittle 1994). We also noticed that the Smc1p group is less homogeneous than the Smc2p group. This fact combined with the unclear phylogenetic position of the bacterial SMC proteins within the family (Fig. 2c) suggests that new structural subgroups are yet to be discovered.

The SMC2 gene is essential for chromosome condensation and segregation

To assess the function of the *SMC2* product, we disrupted the chromosomal copy of the *SMC2* gene (see Materials and methods). The disruption of the DA box-containing region of *SMC2* (*smc2-Δ1*), as well as the deletion of whole ORF from the yeast genome (*smc2-Δ2*) (Fig. 1a), produced inviable spores, as determined by the tetrad analysis of the diploid strains heterozygous for these deletions. The spores bearing either of the *smc2* deletions germinated normally but stopped growing after one to three generations. The resulting cells displayed gross abnormalities in their appearance and distribution of their chromosomal DNA (data not shown). Thus, the *SMC2* gene is essential for vegetative growth of yeast and its DA box-containing region is critical for the function of Smc2p. The latter conclusion is corroborated by the observation that an in-frame deletion of just the DA box of Smc1p eliminated the ability of Smc1p to function in vivo (data not shown).

The fact that both *SMC2* and *SMC1* are essential genes suggests that they perform distinct biological roles in yeast, despite their structural resemblance. This conclusion is supported by several experimental observations. Not only did the single copy of *SMC1* present in the genome fail to rescue the *smc2-Δ2* strains, but these strains were also inviable in the presence of multiple copies of the *SMC1* gene (see Materials and methods). The reciprocal experiment produced the same outcome: The presence of multiple copies of *SMC2* failed to rescue the *smc1-Δ2* strain. Therefore, *SMC1* and *SMC2* are not functionally redundant. In addition, we constructed strains containing both *smc1-2* and *smc2-6* mutations (see below), and no synthetic lethality was observed at the permissive temperature. This observation further supports the conclusion that Smc1 and Smc2 proteins have at least some nonoverlapping functions.

To better understand the biological function of Smc2p and the relationship between the *SMC2* and *SMC1* genes, 12 independent conditional (temperature-sensitive) *smc2* alleles were isolated by plasmid-shuffling (see Materials and methods). These alleles were designated *smc2-1* to *smc2-12*. While present on a CEN plasmid in the *smc2-Δ2* strain, these alleles were analyzed for temperature-related phenotypes. All alleles showed similar phenotypes at the nonpermissive temperature, so one allele was chosen randomly for further studies. This mutation, *smc2-6*, was introduced into the *smc2* locus on chromosome VI (see Materials and methods). Like the other *smc2* mutants, the *smc2-6* strains showed a cell-cycle arrest after two divisions at 37°C (Fig. 3). Although we already have shown that the *SMC1* gene could not suppress a deletion of *SMC2*, it was possible that the delay of the arrest (two cell divisions) of *smc2-6* strains at 37°C was caused by the ability of the *SMC1* gene to suppress partially the *smc2-6* temperature-sensitive mutation. However, we did not find any indication of such suppression: Multiple copies of *SMC1* (2 μ -based plasmid) failed to complement the temperature-sensitive defect of the *smc2-6* mutation, despite the increase in cellular content of Smc1p (data not shown). At 37°C, 45% of *smc2-6* cells were viable after a 4-hr temperature shift. This relatively high viability prevented us from mapping an essential function of *SMC2* in the cell cycle as has been done for *SMC1* (Strunnikov et al. 1993). Also the *smc2-6* diploid strains did not show a significant increase in chromosome III loss at permissive temperatures.

The morphology of chromosomal DNA and mitotic spindle have been examined in *smc2-6* and *SMC2* cells using the standard cytological methods. The previous FISH analysis of wild-type yeast cells suggests that individual yeast chromosomes reach the peak of their con-

Figure 3. The time course analysis of *smc2-6* temperature sensitivity. Data are from a typical experiment using the 3aAS283 strain (*smc2-6*). Growth curves were generated for two temperatures (23°C and 37°C) by counting the cells in a hemocytometer after sonication. The growth of the *smc2-6* strain arrests after 3 hr at 37°C, which corresponds to three divisions of an *SMC2* strain (YPH499) at this temperature (data not shown).

densation at the time of mitosis [Guacci et al. 1994]. A manifestation of this property is that chromosomes segregate into two distinct masses by the time the spindle has elongated notably (Fig. 4a). In contrast, during the first divisions after the temperature shift (37°C), the *smc2-6* culture had up to 15% of cells with a fully elongated mitotic spindle but with their DNAs failing to separate into two masses (Fig. 4b). This morphology is consistent with a defect in chromosome condensation. Upon the continuous incubation at the nonpermissive temperature, the *smc2-6* cells with the stretched nuclear DNA disappear while unbudded cells and large budded cells with a single nucleus accumulate (Fig. 4b). The video-assisted observations of *smc2-6* cells after the transient exposure to 37°C showed that all unbudded cells are inviable while most of the large budded cells are viable (data not shown). We suggest that the *smc2-6* cells with the stretched DNA resolve by cutting the segregated chromosomes, thereby generating inviable unbudded cells. The cells escaping the mentioned cutting subsequently arrest in the cell cycle prior to chromosome segregation and remain viable (large budded cells with a single nucleus and a short spindle).

Because the transient morphology of *smc2-6* cells was suggestive of a chromosome condensation defect, we directly measured chromosome XVI condensation in *smc2-6* cells using our established FISH condensation assay [Guacci et al. 1994]. For comparison we also examined chromosome condensation in the wild-type and *smc1* strains (Table 1). A logarithmic culture of each strain was treated with nocodazole for 2.5 hr and then subjected to a half-hour temperature shift in the presence of nocodazole. After the cells were prepared for *in situ* hybridization, three probes were used to assess the

condensation state of chromosome XVI: A pericentromeric probe; a probe separated from the centromere by 110 kb, or a probe separated from the centromere by 240 kb [Guacci et al. 1994]. When the longer chromosome distance (240 kb) was examined, an increase in the separation of the fluorescent spots in *smc2-6* was evident (Table 1), though it did not reach the maximal interphase value observed for that interval (Table 1). These results suggest that Smc2p is required to maintain mitotic chromosomes in the condensed state. Furthermore, the FISH experiments revealed another phenotypic difference between the *smc2-6* and *smc1-2* mutants, as no significant effect on the condensation of chromosome XVI was observed in the *smc1* mutant (Table 1). The failure to observe decondensation of the 110-kb interval of chromosome XVI in the same set of experiments (data not shown) may reflect that the methodology is not sensitive enough to detect differences in this range or that the *smc2* mutation affects only highest orders of chromosome structure in yeast.

Smc2p molecules physically associate with themselves and with Smc1p

The putative structure of SMC proteins suggests the formation of two extensive coiled-coil regions. For many other proteins this structure has been demonstrated to mediate dimerization, trimerization, and/or higher order protein-protein interactions. Two preliminary observations indicated that Smc1p and Smc2p possibly interact with each other. First, while Smc1p and Smc2p are both in low abundance in yeast, they are in equivalent amounts when present either in a single copy or when placed on a multicopy plasmid. This was shown by

Figure 4. The temperature-related characteristics of the *smc2-6* cells. (a) The *SMC2* strain (YPH499) was grown for 3 hr at 37°C, fixed, and stained with anti-tubulin antibodies and DAPI to reveal microtubules and chromosomal DNA. The late anaphase spindles and corresponding nuclei are marked with arrowheads. (b) The morphology of *smc2-6* cells (3aAS283) at restrictive temperature. After 3 hr at 37°C, the population accumulates large budded cells with elongated spindles and “stretched” nuclei (marked with arrowheads in left panels). After 6 hr at 37°C the population accumulates large budded cells with an undivided nucleus and a short spindle (marked with arrowheads in the right panel). (c) Quantitative data for the distribution of cell types in *smc2-6* cultures at 37°C. The combination of 3aAS283 and 1cAS287 data gives the standard error. (Left) The distribution of large budded cells; (right) the distribution of the unbudded cells and cells with a small bud.

Table 1. The distance (μm) between two FISH signals on chromosome XVI

Value:	Median			Mean	
Cell-cycle stage:	G_1^b	G_2/M	G_2/M (36.5°C)	G_2/M	G_2/M (36.5°C)
Strain ^a					
Smc ⁺	1.0 ± 0.01	0.70 ± 0.01	0.63 ± 0.06	0.76 ± 0.01	0.68 ± 0.06
<i>smc2-6</i>	1.1 ± 0.1	0.72 ± 0.11	0.90 ± 0.12	0.78 ± 0.18	0.90 ± 0.06
<i>smc1-2</i>	no data	0.65 ± 0.06	0.70 ± 0.01	0.68 ± 0.11	0.76 ± 0.04

^aSmc⁺ was YPH102; *smc2-6* was 2aAS283 and 2bAS283; and *smc1-2* was 3aAS273.

^bThe G_1 value corresponds to the cells released from G_0 (see Materials and methods); G_2/M is a nocodazole-arrested population.

Western blot analysis, comparing the intensity of signals corresponding to Smc2p and Smc1p marked with the c-myc epitope tag (data not shown). Second, we showed that the Smc2 protein is localized to the nucleus (Fig. 5), as was shown previously for Smc1p (Strunnikov et al. 1993).

To test experimentally the physical interaction between the SMC proteins, we asked whether different SMC proteins coimmunoprecipitate. In these experiments we used strains that overexpressed Smc2p and/or Smc1p ~10-fold as a means of overcoming their low abundance (see Materials and methods). However, even after overexpression, Smc1p and Smc2p were present in

relatively low amounts in these cells. Crude protein extracts were prepared from these overexpressing strains and subjected to a high-speed spin to remove all insoluble proteins (see Materials and methods). We chose conditions (0.2 M NaCl, 0.2% Triton X-100, 50 mM Tris-HCl at pH 8.0) under which the bulk of Smc1p and Smc2p was soluble, but antibodies were still able to bind corresponding antigens with high efficiency. At this level of analysis, one cannot differentiate between the formation of the complex containing two SMC molecules or more than two. So, we chose to use the terms homomer and heteromer to designate such complexes, rather than homodimer and heterodimer.

In the first set of immunoprecipitation experiments we tested the ability of Smc2p to form homomers. When extracts from cells co-overexpressing two forms of Smc2p (Smc2p-myc and Smc2p-ha) were analyzed, it was found that these two forms can be reproducibly coprecipitated by the anti-hemagglutinin (anti-HA) antibodies (Fig. 6a). A control antibody, anti-t7, did not mediate any coprecipitation of Smc2p molecules (Fig. 6a). These results demonstrate that Smc2p can form homomers. Because the Smc1 protein was not overexpressed in these cells, it was present in substoichiometric amounts in the extract. Therefore, the formation of Smc2p homomers was probably independent of Smc1p.

Under these experimental conditions we could not determine what fraction of the complexes was formed in the cells (in vivo) versus in the extracts (in vitro). To address this question, Smc2p-ha and Smc2p-myc were expressed in two independent cultures that were pooled, and the extraction and immunoprecipitation steps were performed identically to the previous experiment. In this case, Smc2p-ha and Smc2p-myc reproducibly failed to coprecipitate (Fig. 6b). Therefore, the association of Smc2p molecules, when coexpressed in the same cell, must occur in vivo. This result also provides a control, showing that coprecipitation of Smc2p-ha and Smc2p-myc coexpressed in the same cell cannot be explained by the cross-reaction of the anti-HA antibodies with Smc2p-myc.

To ask whether Smc2p also forms heteromers with the Smc1 protein, we constructed a strain that overexpressed both of the proteins and used the same experimental design as in the previous experiments (Fig. 6c). When the affinity-purified anti-Smc1p antibodies were used for

Figure 5. Immunolocalization of the epitope-tagged Smc2p. The *smc2-Δ2* strain (1-6dAS268) was transformed with the pAS428 plasmid (*SMC2::ha*) and the corresponding plasmid lacking the HA-epitope, pAS405 (*SMC2*). The anti-HA antibodies (see Materials and methods) were used for indirect immunofluorescent staining of Smc2p-ha- and Smc2p-expressing cells. Cells were fixed for a short time (5–10 min) to preserve the antigen. The control isogenic strain expressing Smc2p with no HA epitope gave no staining. The analogous experiments were done with the 1-6dAS268/pAS447 strain expressing Smc2p-myc. The staining pattern revealed by anti-c-myc antibody staining (data not shown) was indistinguishable from that observed for Smc2p-ha.

Figure 6. Analysis of Smc2p–Smc2p and Smc2p–Smc1p interaction by coimmunoprecipitation and Western blot analysis. The schematic representations of Smc2p and Smc1p show the positions of the epitope tags (open boxes) and the putative domains: NTP binding (circle), coiled–coils (thick bar), and the DA box (ellipse). Both the precipitates (*IP*) and the supernatant fractions (*Sup*) are shown for all experiments. *Right* panels show efficiency and specificity of immunoprecipitation; *left* panels show coimmunoprecipitation results. (a) Analysis of coimmunoprecipitation of Smc2p–myc and Smc2p–ha. The extracts were prepared from the 6aAS268 strain expressing both Smc2p–myc (pAS443 plasmid) and Smc2p–ha (pAS428). Smc2p–ha was immunoprecipitated by anti-HA antibodies (HA), and the resulting supernatant and pellet were probed for Smc2p–myc using anti-myc antibodies. The anti-t7-tag antibodies (t7) were used for the mock precipitations. (b) The experiment identical to a except the cells of the 6dAS268/pAS443 and 6aAS268/pAS428 strains were mixed just before preparing the extracts. (c) Coprecipitation of Smc1p–myc and Smc2p–ha. Extracts were prepared from the AS248 strain carrying both pAS428 (Smc2p–ha) and pAS258 (Smc1p–myc) plasmids. The *top* panel shows that Smc2p–ha is coimmunoprecipitated when the Smc1p–myc is precipitated with anti-Smc1p antibodies (Smc1). The rabbit serum [(-)Smc1] depleted from the anti-Smc1p antibodies was used for the mock precipitations. The *bottom* panel shows that Smc1p–myc is coimmunoprecipitated when the Smc2p–ha is precipitated by anti-HA antibodies. Anti-t7-tag (t7) antibodies were used for the mock precipitations.

the immunoprecipitation of Smc1p–myc, a significant amount of Smc2p–ha was detected in the precipitate. Reciprocally, when the anti-HA antibodies were used to

precipitate Smc2p–ha, a substantial fraction of Smc1p–myc was precipitated. The mock antibodies did not precipitate either of SMC proteins (Fig. 6c). These results show that at least a portion of Smc1p and Smc2p is present in the same complex, resistant to 0.2 M salt, detergent, and antibody treatment. Association of Smc2p and Smc1p is not dependent on DNA presence in the extracts (data not shown). So, it is likely that Smc2p and Smc1p form protein–protein complexes both with each other and with themselves. Implications of the immunoprecipitation results for the structural and functional analysis of the SMC proteins are presented in the Discussion.

Discussion

In this report we present the characterization of *SMC2*, a second member of the SMC family from budding yeast. Identification of *SMC2* was based on the hypothesis that the DA box is a necessary and sufficient characteristic signature of the SMC family. This hypothesis was validated by the subsequent finding that the Smc2 protein contains all putative domains characteristic for a typical SMC family member: a nucleotide-binding region, two coiled–coil regions separated by a “hinge,” and the DA box itself. The structural identity of the SMC family is also confirmed by the same type of organization shared by all recently characterized SMC-like proteins.

Our data strongly suggest that Smc2p plays an essential role in chromosome segregation and condensation in yeast. The implication of Smc2p in chromosome condensation is based on several experimental facts. First, mitotic chromosomes in temperature-sensitive *smc2-6* cells show a unique transient morphology consistent with a defect in chromosome condensation. The chromosomes trail along the elongating mitotic spindle producing a “stretched nuclear DNA” appearance. Second, as assayed by FISH, chromosome XVI partially decondensed when mutant Smc2p was inactivated in mitosis. Third, in two other organisms, the SMC proteins were shown to play a significant role in mitotic chromosome condensation. The XCAPE and XCAPC proteins from *X. laevis* are important for the establishment and, probably, for the maintenance of the condensed structure of chromatin in vitro (Hirano and Mitchison 1994); the mutation in the *cut14* gene of *S. pombe* results in a chromosome condensation defect (Saka et al. 1994).

The effect of the *smc2* mutation on chromosome condensation seems to be small, relative to the range of condensation observed in cells of higher eukaryotes. This may reflect in part the generally low level of chromosome condensation in budding yeast. However, the addition of anti-XCAPC antibodies to mitotic chromosomes in vitro also led to only a very limited change in their condensation (Hirano and Mitchison 1994). Thus, the results of our studies in vivo corroborate those obtained using an in vitro system. These experimental data, when coupled with the structural resemblance of the SMC proteins, strongly suggest that the mechanism of chromosome condensation is conserved between

yeast and vertebrates. Moreover, the observed partial decondensation upon inactivation of specific SMC proteins suggests that other molecules participate in condensation, perhaps other SMC proteins, topoisomerase II, or other proteins yet to be identified.

It is noteworthy that the structural role of SMC proteins in other systems may be broader than just the condensation of chromosomes in mitosis. For example, in addition to Smc1 and Smc2 subgroups of the SMC family, a subgroup of bacterial homologs also exists (Strunnikov et al. 1993). The characterization of bacterial SMC proteins could provide a missing link between the prokaryotic and eukaryotic modes of chromosome organization. It has been shown that topoisomerases of the second type (DNA gyrase and topoisomerase IV) play a major role in the formation of the looped tertiary structure of bacterial nucleoid DNA (Adams et al. 1992; Schmid and Sawitzke 1993). Functional analogs of histones also have been identified in bacterial cells (Roubiere-Yaniv and Yaniv 1978; Hulton et al. 1990). However the proteins that stabilize the compact tertiary structure of the bacterial nucleoid and promote even higher levels of compaction, for example, in response to environmental factors and developmental stimuli (Gualerzi and Pon 1985; Drlica and Riley 1990; Kellenberger and Arnold-Schulz 1992), have not yet been identified. The SMC proteins might serve that purpose. Identification of such proteins in a well-studied prokaryote could shed additional light on their functions.

Despite the observed structural similarity between the SMC proteins, in this report we proposed that Smc1p and Smc2p perform distinct functions in yeast cell. This statement is based on several experimental facts. First of all, because we showed that both *SMC1* and *SMC2* genes are essential, their functions must be distinct. This conclusion is affirmed by the failure of either of these genes to substitute for each other in vivo when present in multiple copies per cell. Second, we showed that Smc1p and Smc2p fall into two distinct evolutionary conserved subfamilies within the SMC family. Third, several phenotypic differences were observed between the *smc1* and *smc2* mutants, including different cell morphology, arrest kinetics, and viability. Also, the condensation of a reporter chromosome as assayed by FISH was affected significantly by the *smc2-6* mutation but only marginally by the *smc1-2* mutation. Although we cannot exclude the possibility that these differences reflect the specific properties of particular alleles that we have studied, it seems unlikely, as our data suggest that these mutations represent loss-of-function alleles (this report; Strunnikov et al. 1993). All of these considerations suggest that Smc2p and Smc1p must have distinct biological functions even though their structural similarity implies common primary biochemical activities.

To explain these structural and functional relationships between Smc1p and Smc2p, we can propose two hypotheses. These two proteins can be essential subunits of the same complex; in this case, specificity of their action can be mediated by the ability of different accessory proteins to bind to the regions of Smc1p and

Smc2p that are not conserved. Alternatively, Smc1p and Smc2p can be specialized (functionally or spatially) for performing independent essential activities. Support for the second model comes from the study of Dpy-27, which has already demonstrated the spatial specialization of a SMC protein. This protein is localized only to the X chromosomes in *C. elegans*, where it performs specific modulation of transcription (Chuang et al. 1994). Also, the difference in the effect on chromosome XVI condensation in the *smc1* and *smc2* mutants could be explained if both Smc1p and Smc2p participate in chromosome condensation but have differential distribution along a chromosome arm or some other kind of specialized intranuclear localization.

Insight into the molecular function of Smc1p and Smc2p in condensation may be obtained through the analysis of their putative structure. We noted previously the gross structural similarity between Smc1p and known mechanochemical molecules, or motor proteins (Strunnikov et al. 1993). As chromatin loops move closer together during the condensation, it is reasonable to assume that SMC proteins are condensation motors (Guacci et al. 1993). By immunoprecipitating SMC proteins from yeast extracts, we have shown that Smc2p can form complexes with other Smc2p molecules as well as with Smc1p. This indicates that like the well-studied motor molecules, kinesin and myosin, SMC proteins can oligomerize presumably through the association of their coiled-coil domains. The degree of oligomerization could range from a dimer to multimeric filament (Fig. 7). Based on the analysis of *X. laevis* XCAPC and XCAPE proteins, Hirano and Mitchison (1994) proposed an obligatory heterodimer as well as a filament model. Taking into account the diversity in the modes of assembly of heteromeric filaments (McKeon et al. 1986; Mitchison 1992), other models for filament formation are possible. As an example, we suggest a facultative heterodimer model, that is, one in which both homodimers and heterodimers can be formed. This model provides a potential mechanism to generate SMC complexes with distinct specificity. A virtue of this model is that it can explain the distinct phenotypes of *smc* mutants and the divergent functions of SMC proteins in mitosis and dosage compensation. The identification of the biochemical activities of the SMC proteins and the direct structural studies (e.g., filament assembly in vitro) will be required to discriminate between the structural models presented in this report and those suggested by other groups.

Materials and methods

Plasmid construction and DNA sequencing

E. coli strains DH5 α (BRL), TOP10 (Invitrogen), and SCS110 (Stratagene) were used for plasmid propagation. Yeast-*E. coli* shuttle vectors, used for cloning purposes were pRS vectors (Sikorski and Hieter 1989; Christianson et al. 1992), YCplac111 (Gietz and Sugino 1988), and pTI15 (Icho and Wickner 1988). pT7blue (Novagen) was used as a cloning vector for PCR-generated fragments. Plasmid p54 (Shirahige et al. 1993) containing the *EcoRI* fragment of chromosome VI (Riles et al. 1993) was

Figure 7. Hypothetical models for the arrangement of SMC protein molecules in vivo. The coiled-coil regions are shown with the arrows, and the amino termini are shown bound to an NTP molecule (ellipse). The differences between the Smc2-like and Smc1-like molecules are not incorporated into the diagrams. Dimers are drawn in parallel orientation as the vast majority of the coiled-coil interactions exhibit this orientation. (a) Homodimer and heterodimer formation; (b) a filament formed by the association of primary dimers; (c) a filament made from overlapping monomers. An attractive feature of the filament models is that they suggest a function for the DA box in nucleotide binding by placing it next to the NTP binding site (b,c). The DA box motif has a subset of acidic residues analogous to the DEAD box of helicases (Gorbaleyna et al. 1989; Linder et al. 1989), which is part B of the nucleotide-binding site (Dever et al. 1987). An alternative hypothesis has been proposed for bringing the DA box close to the NTP-binding domain (Saitoh et al. 1994). The filament made from overlapping monomers has the attractive feature of demanding symmetry between the first and the second coiled-coil domains, which is the case for all SMC proteins (Notarnicola et al. 1991; Strunnikov et al. 1993; Chuang et al. 1994; Hirano and Mitchison 1994; Saitoh et al. 1994; Saka et al. 1994).

obtained from K. Shirahige (Osaka University, Japan). The *XbaI*–*SalI* fragment (Fig. 1a) corresponding to the assumed position of *SMC2* was subcloned into pBluescript (Stratagene), giving pAS280. The pAS280 plasmid was used to construct 5' and

3' nested deletions of *SMC2* (Henikoff 1984). Resulting plasmids were sequenced using the fluorescent ddNTP cycle-sequencing method and the DNA sequencer (370A; Applied Biosystems). Both strands of *SMC2* were sequenced. Conflicting regions were resequenced using the custom primers.

The pAS285 plasmid was constructed by inserting the *URA3* gene into pAS280 digested with *HindIII*. This creates a disruption of the very 3' end of the *SMC2* ORF. The full-length disruption of *SMC2* (pAS297) was made by digesting pAS285 with *SmaI* (*URA3*) and *MluI*, filling the *MluI*-created end with Klenow enzyme and self-ligation. pAS404/1 used for plasmid shuffling was made via insertion of the *SMC2* gene (*XbaI*–*SalI* fragment) into YCplac111 (pAS404) and removal of the *SacI* site from the polylinker by digestion with *EcoRI*, *XbaI*, and self-ligation. pAS406 and pAS405 were constructed similarly to pAS404 but using pTI15 and pRS425, respectively, as a backbone. pAS428, a 2 μ -based *SMC2::ha* plasmid, was built by inserting the *XbaI*–*XbaI* fragment containing three copies (3 $\times$ ha) of the HA tag (contribution of M. Rose, Princeton University, NJ) into the unique *SpeI* site of pAS405. The *XbaI* sites flanking the HA tag fragment compatible with the translation phase of *SMC2* were generated by PCR. pAS447 (*CEN*, *SMC2::ha*) is pRS317 with the *SalI*–*XbaI* fragment of pAS428. c-myc-Tagged *SMC2* (*SMC2::myc*) was constructed by cloning the *XbaI*–*XbaI* 6 $\times$ c-myc fragment (Roth and Gall 1989; Strunnikov et al. 1993) into the *SpeI* site of pAS280, resulting in pAS299. The *SalI*–*XbaI* fragment of pAS299 was inserted into YCplac111 (giving pAS408; *CEN*, *SMC2::myc*) and into pRS423 (giving pAS443; 2 μ , *SMC2::myc*). The *SMC1::myc* construct has been described (Strunnikov et al. 1993). pAS258 (2 μ , *SMC1::myc*) was produced via cloning of the *XhoI*–*NheI* piece of pAS211 (Strunnikov et al. 1993) into *XhoI* and *SpeI* of pRS426.

Yeast strains and genetic techniques

S. cerevisiae strains used are listed in Table 2. All strains were of S288C background. Media, incubation conditions and standard genetic manipulations were performed according to published protocols (Sherman et al. 1986; Jones et al. 1992).

Deletion alleles of *smc2* were constructed by transforming the diploid strain AS260 (Strunnikov et al. 1995) with either pAS285 (cut with *SalI* and *XbaI*) or pAS290 (cut with *SalI* and *XbaI*), giving the strains heterozygous in *smc2*- $\Delta 1$ (AS263) and *smc2*- $\Delta 2$ (AS268), respectively. After confirmation of disruption by Southern analysis, AS263 and AS268 were sporulated and the asci dissected to obtain the haploid progeny. Haploid strains bearing *SMC2* plasmid in the background of *smc2*- $\Delta 1$ or *smc2*-

Table 2. *S. cerevisiae* strains

Strain	Genotype	Source
AS260	<i>MATa/MATα ade2 his3 leu2 lys2 trp1/TRP1 ura3</i>	Strunnikov et al. (1995)
AS263	<i>MATa/MATα ade2 his3 leu2 lys2 trp1/TRP1 (ura3) SMC2/smc2-$\Delta 1$</i>	this study
AS268	<i>MATa/MATα ade2 his3 leu2 lys2 trp1/TRP1 (ura3) SMC2/smc2-$\Delta 2$</i>	this study
6aAS268	<i>MATa ade2 his3 leu2 lys2 (ura3) smc2-$\Delta 2$</i>	this study
6dAS268	<i>MATα ade2 his3 leu2 lys2 (ura3) smc2-$\Delta 2$</i>	this study
2aAS283	<i>MATα ade2 his3 leu2 lys2 ura3 smc2-6</i>	this study
2bAS283	<i>MATa ade2 his3 leu2 lys2 ura3 smc2-6</i>	this study
3aAS283	<i>MATα ade2 his3 leu2 lys2 ura3 smc2-6</i>	this study
1cAS287	<i>MATa ade2 his3 (leu2) ura3 bar1::LEU2 smc2-6</i>	this study
3aAS273	<i>MATα ade2 his3 (leu2) lys2 ura3 smc1-2::LEU2</i>	this study
AS248	<i>MATa/MATα leu2 ura3 smc1-$\Delta 2$</i>	Strunnikov et al. (1993)
YPH102	<i>MATα ade2 his3 leu2 lys2 ura3</i>	Sikorski and P. Hieter (1989)
YPH499	<i>MATa ade2 his3 leu2 lys2 trp1 ura3</i>	Sikorski and P. Hieter (1989)

$\Delta 2$ deletions were recovered only when AS263 or AS268 was transformed with a *SMC2* plasmid prior to sporulation. All subsequent plasmid shuffling was done in strains 6aAS268 or 6dAS268, and in the *Ura*⁻ derivative 1-6dAS268. A temperature-sensitive allele of *smc2* was introduced into the *smc2*- $\Delta 2$ strains via transformation with pAS425/6 (*smc2*-6) cut with *Xba*I and *Sal*I. Selection was based on the replacement of *URA3* marking *smc2*- $\Delta 2$ by the temperature-sensitive allele of *smc2*.

Mutagenesis of SMC2 in vitro and screening for temperature-sensitive mutants

Mutations in the *SMC2* gene were generated using the mutagenic polymerase chain reaction followed by gap repair in vivo. Primers corresponding to nucleotides 3983–4000 (antisense) and 2831–2848 (sense) of the *SMC2* fragment (Fig. 1b) were used to amplify this region of *SMC2* under the condition of distorted nucleotide concentration and in the presence of 5 mM MnCl₂ during the last 5 cycles of PCR (30 cycles total). The PCR products were mixed in equimolar ratio with pAS404/1 digested with *Sac*I, and the mixture was denatured by heat and transformed into 1-6dAS268/pAS406. Resulting Leu⁺ transformants were cured of pAS406 using 5-fluoro-orotic acid (5-FOA) selection. Six hundred *Ura*⁻ clones were screened for temperature sensitivity. Plasmid DNA from 12 independent clones (pAS404/11–pAS404/112) was rescued in *E. coli*, and the *smc2* fragments were recloned into the integrative vector.

Cytological methods, antibodies, and FISH

The indirect immunofluorescence staining of yeast cells was performed as described (Kilmartin and Adams 1984). Formaldehyde (3.7%) fixation was used routinely. Yeast nuclear DNA was visualized with DAPI present in mounting media (Mowiol). Microtubules were detected with the mouse monoclonal antibody YOL1/34 (1:200) (Kilmartin et al. 1982) and the goat anti-mouse antibodies conjugated to rhodamine (Cappel). Mouse monoclonal anti-c-myc antibody 9E10 (Evan et al. 1985) and mouse monoclonal anti-HA antibody 12CA5 (BAbCO, Richmond CA) were used to monitor tagged Smc1p and Smc2p on Western blots.

The protocol and probes for FISH were described previously (Guacci et al. 1994). The distance between fluorescent signals was measured for at least 100 nuclei per each experiment. For each strain/condition combination the hybridization was repeated at least three times. The median and mean values were determined for data generated in every independent experiment and subsequently averaged for each strain/condition combination. To obtain values for the interphase cells, YPH102 and 2bAS283 were subjected to starvation (Strunnikov et al. 1993) for 24 hr at 23°C and then released into YEPD medium. After 50% of the population generated a small bud (3 hr at 23°C), the samples were assayed by FISH.

Immunoprecipitation

The strain of interest was grown to a density of 10⁸ cells/ml (100 ml of selective media). The cell pellet was placed on ice and resuspended in 2 ml of immunoprecipitation (IP) buffer (50 mM Tris-HCl at pH 8.0, 0.2 M NaCl, 0.1% Triton X-100, protease inhibitor mix). Two grams of glass beads was added, and the tube was shaken vigorously by vortex bursts (10× for 1 min). The suspension was collected into two tubes and centrifuged at 18,000g for 10 min at 4°C. The supernatant was transferred into new tubes, which were spun at 110,000g for 30 min at 4°C (TLA-100.3 rotor, Beckman). The supernatant was removed

carefully, avoiding contamination with the floating material. This procedure yielded 1.2 ml of clarified extract; 0.5 ml was used for each IP reaction with 50 μ l of antibody–Sephacose (6 hr at 4°C). After completion of the IP reaction the supernatant was concentrated by TCA precipitation. The beads were placed into an empty Centricon column (Princeton Sep.) and washed three times with the IP buffer. Subsequently the material bound to beads was eluted with the sample-loading buffer (without β -mercaptoethanol) (15 min at room temperature), and the eluate was separated from the beads by a brief spin. To avoid the overloading of polyacrylamide gels by the supernatant samples, the supernatant and precipitate fraction were loaded in 1:10 ratio, judging from the cell equivalents.

Four types of antibodies were used for the IP experiments: Monoclonal anti-HA tag (12CA5, BAbCO), monoclonal anti- τ 7 tag (Novagen), affinity-purified rabbit anti-Smc1p (Strunnikov et al. 1993), and rabbit serum (protein A-purified) depleted of anti-Smc1p antibodies (the last flowthrough of affinity purification scheme). In a typical cross-linking reaction, 200 μ g of antibody was coupled to 1 ml of CNBr Sepharose beads (Pharmacia), followed by the standard washing steps. The antibody–Sephacose was stored in PBS/sodium azide at 4°C. The aliquots of beads were washed two times with IP-buffer prior to each reaction. The anti-c-myc (9E10) monoclonal antibody (Evan et al. 1985) or 12CA5 antibodies were used to develop Western blots.

Computational methods

Protein and DNA homology searches were done using the BLAST (Altschul et al. 1990) server at National Center for Biotechnology Information (NCBI) (Bethesda, MD) and the GCG sequence analysis package (v. 7.2) at the National Cancer Institute/Frederick Cancer Research Facility (NCI/FCRF) VAX computer (Frederick, MD). Phylogenetic analysis was done according to published recommendations (Hillis et al. 1993) using the following programs of PHYLIP package for Power Mac (Felsenstein 1989). The KITCH program was used to build the phylogenetic tree (Fig. 2c) based on data generated by the PROTDIST application (PAM matrix). The graphic presentation of the tree was performed with the DRAWGRAM program. The branching pattern of the best tree was validated by bootstrap procedure (Felsenstein 1985) employing the SEQBOOT and CONSENSE applications. The bootstrap procedure was used to generate 120 resampling sets, for each of which 13 random-entry matrices were calculated. The frequencies of branching pattern calculated for the consensus tree are incorporated into Figure 2c.

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