Association of mammalian SMC1 and SMC3 proteins with meiotic chromosomes and synaptonemal complexes

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SUMMARY

In somatic cells, the heterodimeric Structural Maintenance of Chromosomes (SMC) proteins are involved in chromosome condensation and gene dosage compensation (SMC2 and 4), and sister chromatid cohesion and DNA recombination (SMC1 and 3). We report here evidence for an involvement of mammalian SMC1 and SMC3 proteins in meiosis. Immunofluorescence analysis of testis sections showed intense chromatin association in meiotic prophase cells, weaker staining in round spermatids and absence of the SMC proteins in elongated spermatids. In spermatocyte nuclei spreads, the SMC1 and SMC3 proteins localize in a beaded structure along the axial elements of synaptonemal complexes of pachytene and diplotene chromosomes. Both SMC proteins are present in rat spermatocytes and enriched in preparations of synaptonemal complexes. Several independent experimental approaches revealed interactions of the SMC proteins with synaptonemal complex-specific proteins SCP2 and SCP3. These results suggest a model for the arrangement of SMC proteins in mammalian meiotic chromatin.

Key words: SMC protein, Meiosis, Synaptonemal complex, SCP protein, Testis

INTRODUCTION

The evolutionarily well conserved eukaryotic Structural Maintenance of Chromosomes (SMC) protein family, with four family members named SMC1 to SMC4, is involved in several key DNA and chromatin dynamic processes in somatic cells (for recent reviews see Koshland and Strunnikov, 1996; Heck, 1997; Jessberger et al., 1998; Hirano, 1998, 1999; Strunnikov and Jessberger, 1999). In higher eukaryotes, SMC proteins have been found almost exclusively as either SMC1/3 or SMC2/4 heterodimers, most often occurring in large multiprotein complexes, of which at least four have been described. One of these complexes is the SMC2/4 heterodimer-based condensin, which is necessary for mitotic chromosome condensation in the yeasts Saccharomyces cerevisiae (Strunnikov et al., 1995) and Schizosaccharomyces pombe (Sutani and Yanagida, 1997), and in Xenopus laevis extracts (Hirano et al., 1997). Besides the SMC proteins, the frog condensin contains at least three other polypeptides, among them a protein homolog to the Drosophila melanogaster protein Barren (Bhat et al., 1996). The MIX-1 and DPY-27 proteins of Caenorhabditis elegans, proteins homologous to the SMC2 and SMC4 proteins, are present in a multiprotein complex that regulates gene dosage compensation on the X chromosomes of the hermaphrodite nematode (Chuang et al., 1996; Lieb et al., 1998).

The other heterodimer, SMC1/3, is component of two protein complexes with different functions. Genetic studies in S. cerevisiae revealed a requirement for the SMC1/3 proteins in mitotic sister chromatid cohesion (Guacci et al., 1997; Michaelis et al., 1997). A corresponding protein complex, called cohesin, was found in X. laevis egg extracts (Losada et al., 1998), and contains at least three other polypeptides, one of which is homologous to the S. pombe Rad21 and S. cerevisiae Mcd1/Scc1p proteins required for DNA repair and sister chromatid cohesion during mitosis (Birkenbihl and Subramani, 1992; Guacci et al., 1997; Michaelis et al., 1997). In meiosis, a protein paralogous to Rad21 and Mcd1/Scc1p, Rec8, is important for sister chromatid cohesion and recombination (Molnar et al., 1995; Klein et al., 1999; Watanabe and Nurse, 1999). The SMC1/3 heterodimer has also been identified as constituent of the recombination complex RC-1, which was isolated from bovine thymus and is present in a variety of somatic cells (Jessberger et al., 1996; Stursberg et al., 1999). This complex catalyzes SMC protein-dependent cell-free transfer of duplex DNA molecules, which can lead to recombinational repair of gaps and deletions (Jessberger et al., 1993, 1996). The presence of the SMC1/3 heterodimer in these multiprotein complexes together with different non-SMC subunits and different activities furthered speculations about an SMC-mediated relationship between sister chromatid cohesion and recombinational repair (Jessberger et al., 1998; Hirano, 1998; Strunnikov and Jessberger, 1999).

At the molecular level, SMC proteins are only at the
beginning of being understood. Their characteristic protein structure with coiled-coil domains flanked by globular N- and C-terminal domains, and divided in the central region by a flexible hinge domain, has served to classify SMC proteins as potential motor proteins, crossstays, or scaffolding proteins involved in chromatin dynamics (Peterson, 1994; Gasser, 1995). It is likely that the energy that is probably required for such functions is derived from ATP hydrolysis, because SMC proteins contain an N-terminal Walker A motif and a C-terminal Walker B or DA box. Melby et al. (1998) demonstrated for two prokaryotic homodimeric SMC proteins that the two arms of SMC molecules could move around the central hinge. These dimers are antiparallel, bearing an N and C terminus at each end. Thus, an SMC dimer may bridge and even move two DNA duplex molecules. Possibly, chromosome C terminus at each end. Thus, an SMC dimer may bridge and even move two DNA duplex molecules. Possibly, chromosome recombination pathways require moving of DNA, which could be facilitated by these unique properties of SMC proteins. The C-terminal, but not the N-terminal domains of several different SMC proteins bind DNA with a strong preference for double-stranded DNA, and for DNA rich in A/T and/or secondary-structure such as stem-loops (Ahkmmedov et al., 1998).

In mitosis, the role of cohesins includes the direction of recombination repair towards the sister chromatid rather than the homologous chromosome (if there is one) (Kadyk and Hartwell, 1993). Mutations in the S. pombe RAD21 gene (which is homologous to SCC1 of S. cerevisiae) cause a hyperrecombination phenotype, i.e. mitotic interchromosomal recombination is more frequent in rad21 mutants than in wild type (Grossenbacher and Thuriaux, 1981). In meiosis, recombination and sister chromatid cohesion are essential, but the relation between the two processes has been modified in such a way that reciprocal recombination between homologous chromosomes (homologues) and reductive segregation of homologues are ensured (reviewed by van Heemst and Heyting, 1999). During meiotic prophase, a characteristic, zipperlike protein structure, the synaptonemal complex (SC), is formed between homologues, which plays a pivotal role but largely unclarified role in adapting recombination and cohesion for meiosis (see reviews by Hawley and Arbel, 1993; Roeder, 1995; Heyting, 1996; Hasenkampf, 1996; Kleckner, 1996). SCs consist of two axial elements (AEs), which are connected by numerous transverse filaments along their length. Each AE supports the two sister chromatids of one homologue. In budding yeast, SMC3 colocalizes with an AE component during meiotic prophase (Klein et al., 1999). Moreover, SMC3 is essential for meiotic recombination and sister chromatid cohesion (Klein et al., 1999). These observations suggest an interaction of AE-components with proteins involved in cohesion, but no experimental evidence has been obtained for this.

In mammals, two AE components have been identified, SCP2 (Offenberg et al., 1998) and SCP3 (Lammers et al., 1994), which are specifically expressed in meiotic prophase. We have investigated SMC1 and SMC3 proteins in relation to SCP2 and SCP3 in mammalian meiotic tissue, cells and protein preparations. We compared the occurrence and localization of SMC proteins with those of SCP2 and SCP3, and looked for interactions between SMC and SCP proteins. Here, we show that both SMC1 and SMC3 colocalize with the AEs of the chromosomes in diplotene and pachytene, and demonstrate their interaction with the synaptonemal complex and its meiosis-specific components SCP2 and SCP3.

**MATERIALS AND METHODS**

**Antibodies, immunoblotting and immunoprecipitation**

Monoclonal anti-SMC antibodies were elicited by standard hybridoma techniques against full-length bovine SMC1 (bSMC1) or bSMC3 proteins, obtained after overexpression in E. coli, partial purification, and elution as a single band from SDS polyacrylamide gels in a non-denaturing buffer. C-terminal parts of bSMC1 (491 C-terminal amino acids) or bSMC3 (312 C-terminal amino acids; named SMC3-C) were overexpressed as his-tagged molecules in E. coli, and purified to >95% homogeneity by chromatography on Ni-agarose and gel filtration columns. Polyclonal anti-SMC antibodies were generated by injection of the C-terminal domains into rabbits, and the antibodies were affinity purified on C-terminal domains attached to CNBr-sepharose (Sigma Inc.). For immunofluorescence labeling of the AEs of SCs, we used a mouse monoclonal anti-SCP3 antibody (mAb I52F10; Heyting et al., 1987; Offenberg et al., 1998), or a rabbit polyclonal anti-SCP2 antisera (serum 493; Offenberg et al., 1998). Anti-RAD21 antibodies were generated by injection of the mouse RAD21 protein C-terminal peptide TQEPPSYDIAITPGRPFFHI (molecular mass 2285 Da) (McKay et al., 1996) into rabbits. The antibody was affinity-purified on a peptide-CNBr-sepharose column. We performed immunoblotting using the ECL system (Amersham Pharmacia Biotech Inc.) according to standard protocols. In immunoprecipitations, 0.08 mg extract protein in 300 mM NaCl and 0.75% Brij58 were incubated at 4°C for 16 hours with 10 μg polyclonal, affinity purified anti-SMC3-C antibodies. Protein A-beads, preadsorbed with BSA, were added, the mixture was further incubated for 3 hours at 4°C, and thereafter the beads were washed five times with a buffer containing 300 mM NaCl. Bead-bound material, including antibodies and antigen, was analyzed by SDS-gel electrophoresis, silver staining of the gels and/or immunoblotting.

**Nuclear spreads**

Spermatocytes were purified from rat testis and spread by the agar filtration procedure as described (Heyting and Dietrich, 1991). We performed indirect immunofluorescence double-labeling experiments on the resulting preparations and on frozen sections of the rat testis according to published procedures (Heyting and Dietrich, 1991), using secondary antibodies conjugated to fluorescent isothiocyanate (FITC) or Texas Red (TR) for the detection of anti-SCP and anti-SMC antibodies. As negative controls for the polyclonal antisera we used the preimmune sera, and for the monoclonal antibodies we used a control hybridoma supernatant containing antibodies against fish brain tubulin.

**Tissue sectioning and staining**

Testes from 37-day-old rats were frozen in liquid nitrogen, 10 μm cryostat sections prepared, briefly air-dried, frozen and stored at -70°C, and thawed before fixation. The sections were fixed in methanol:acetone (4:1) or methanol:acetone:chloroform (1:1:1) for 10 minutes on ice, followed by three wash steps with PBS. Methanol:acetone fixed sections were permeabilized by incubation with 0.1% Triton X-100 in 0.1% sodium citrate, pH 6.0, for 5 minutes on ice, followed again by three wash steps with PBS. Sections were blocked for 15 minutes at room temperature (RT) with a solution containing 3% fat-free milk powder, 10% goat serum and 0.1 mM PMSF in PBS. Primary antibody incubations were performed in blocking buffer with protein A-purified anti-SMC1-C polyclonal antibody at 3.5 μg/ml and with anti-SCP3 monoclonal antibody (I52F10), diluted 1:1. Incubation was for 1-2 hours at RT, or 1 hour...
at 37°C, followed by 12-48 hours at 4°C. After three washes with PBS, sections were blocked for 15 minutes at RT and incubated with goat anti-rabbit-FITC (Jackson ImmunoResearch laboratories, Pennsylvania, USA) and goat anti-mouse-TR (Jackson) for 1 hour at 37°C. After three washes in PBS, all preparations were counterstained with 4',6-diamino-2-phenylindole (DAPI, Boehringer Mannheim, Germany) and mounted in Vecta Shield (Vector Laboratories Inc., Burlingame, CA, USA) antifade solution. We analyzed the preparations using a Zeiss Axiosplan research microscope equipped with epifluorescence illumination and Plan-Neofluar optics. We photographed selected images directly on a 400 ISO color negative film using single band-pass emission filters (for DAPI, FITC and Texas Red fluorescence) with separated excitation filters. Negatives were scanned at high resolution and their computer images were processed and combined by means of the Corel Draw Photopaint software package.

Extracts, chromatography and immunoaffinity purification

Spermatocyte nuclei were prepared from purified rat spermatocytes by the hypotonic lysis/Triton method (Meistrich, 1975). Synaptonemal complexes were isolated from rat testes as described (Heyting and Dietrich, 1991).

For tissue nuclear extracts, between 10 g and 100 g of bovine thymus, spleen or testis were washed and homogenized, and the nuclei were prepared as described (Jessberger et al., 1993). The cytoplasm-free nuclei were extracted by incubation for 45 minutes on ice in a buffer containing 500 mM NaCl, 5 mM MgCl2, 5 mM KCl, 0.1 mM EDTA, 20 mM EPPS, pH 7.4, and the complete protease inhibitor set (Boehringer Mannheim). Thereafter, the extracted chromatin was pelleted by centrifugation in a Ti-45 ultracentrifuge rotor (40,000 r.p.m., 30 minutes, 4°C). The nuclear extract supernatant, containing between 0.8 and 3.5 mg/ml protein, was collected, and either directly subjected to column chromatography, or adjusted to 10% glycerol and frozen at −70°C. For gel filtration, 10 mg of nuclear extract protein were loaded onto a 240 ml BioGel A15m column, preequilibrated with buffer T (300 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA, 10 mM EPPS, pH 7.4, 5% glycerol, and the protease inhibitors). The column was developed at a flow rate of 0.2 ml/minute. All molecular masses are approximate values for globular proteins.

Immuno-affinity chromatography was performed with polyclonal affinity-purified anti-SMC3-C antibodies bound to CNBr-Sepharose 4B beads (1 mg protein/ml resin). 5-10 mg tissue nuclear extract protein were incubated in batches for 18 hours at 4°C with the antibody-beads in the presence of 500 mM NaCl and either 0.1% NP-40 or 0.75% Brij58, with little difference in outcome. The resin was poured into a column, washed extensively with PBS including the detergent, and eluted in two steps of increasing stringency. First, with 0.1 M glycine-HCl, pH 2.3, and second with tridistilled, sterile water. The eluates were analyzed by gel electrophoresis followed by either silver staining or immunoblotting.

RESULTS

Stage-specific expression of SMC1 protein in meiotic tissue

To determine whether the mammalian SMC1 protein is expressed in vivo in meiotic cells, we performed immunofluorescence of rat testis sections. Fig. 1 shows sections of seminiferous tubules of the rat after immunofluorescence double labeling of SMC1 and SCP3. If frozen sections were not treated with permeabilization buffer, SMC1 was detected in high abundance throughout the nucleoplasm in pachytene and diplotene nuclei, whereas it was detected in smaller amounts in early zygotene nuclei (Fig. 1A-D). If the sections had been permeabilized, SMC1 was detected mainly along the AEs in pachytene and diplotene spermatocytes (Fig. 1E-H), and in smaller amounts in leptotene and zygotene spermatocytes (not shown). Thus, SMC1 is present throughout meiotic prophase nuclei, but is preferentially retained along SCs upon treatment with buffers containing Triton X-100 (see Materials and Methods). Fig. 1E,G furthermore show that nuclei of round spermatids still contain detectable amounts of SMC1. Nuclei of cells in the latest stage of differentiation, the elongated spermatids, do not yield a significant signal with the SMC1 antibody, and can be seen only in the DAPI stain (Fig. 1D,H). Thus, while most of the SMC protein is present along the chromosome cores in prophase I, some remains present on the chromatin well beyond meiosis II.

Localization of SMC1 and SMC3 proteins along AEs of SCs in spread spermatocytes

The selective persistence of SMC1 along synaptonemal complexes upon the permeabilization treatment suggested a possible interaction of part of the SMC1/3 heterodimers with SC-components. This was intriguing also, because SCs are thought to be involved in meiotic sister chromatid cohesion (Maguire, 1991; Bickel and Orr-Weaver, 1996). In particular, it has been proposed that components of the AEs are essential for the establishment and/or maintenance of meiotic sister chromatid cohesion (Maguire, 1991; Dobson et al., 1994). Therefore, we further investigated the localization of the two SMC proteins relative to the AEs of SCs in spermatocytes, which had been lysed in the presence of Triton X-100 and spread by the agar-filtration procedure (Heyting and Dietrich, 1991). We visualized SMC1 or SMC3 together with the SC AE components SCP2 or SCP3 by immunofluorescence labeling, using combinations of monoclonal and polyclonal antibodies (Figs 2, 3). The SMC1 and SMC3 proteins were localized in numerous dots in a characteristic bead-like arrangement along the AEs of the SCs (Figs 2, 3). This pattern was observed from late zygotene up to and including diplotene. In the pachytene stage, when the two AEs of SCs are tightly apposed along their length (‘synapsed’), and cannot be resolved by light microscopy, two rows of dots could be observed along some of the SCs (Figs 2D,G, 3F). Along most pachytene SCs, we could not discern two rows of dots, probably because most SCs in agar filtrate preparations are twisted (Heyting et al., 1985), which will obscure the arrangement of dots in rows. Possibly, these rows of dots mark the chromosomal axes of the two homologous chromosomes. In the stages of meiotic prophase that immediately precede or succeed pachytene, the individual AEs of SCs can be discerned in those SC segments where synopsis has not yet been established (zygotene), or has been resolved (diplotene). Along these individual AEs and along the unsynapsed AE segments of the XY bivalent (Fig. 2B), we always found single rows of SMC1 and SMC3 dots (Fig. 2A,C,E). Incidentally, we found isolated pairs of dots along unsynapsed AEs (Fig. 3E), and in one instance two paired SMC1 dots on the telomeric end of an unsynapsed AE (Fig. 2H), but we found no double rows. In early zygotene nuclei (Fig. 2A), the SMC1 protein appears to colocalize less tightly with AEs than in pachytene and diplotene nuclei. Most of the SMC1 dots lie along AEs, but in addition, these nuclei...
contain larger, brightly staining aggregates, which are not clearly associated with AEs (Fig. 2A). Diplotene chromosomes, which have partially (Fig. 3B) or entirely (Fig. 2C) desynapsed, also show numerous SMC1 and SMC3 dots along the AEs. The polyclonal anti-SMC1 antiserum (Fig. 2G) generally yielded a brighter staining, but also a slightly higher background than the monoclonal anti-SMC1 antibodies. In some instances, the SMC proteins appeared to be detached from the AEs of SCs as they lie in a string-like structure next to them, clearly different from the low background staining observed throughout the image (Fig. 2B,C; see arrows), as if a linear structure containing the SMC1 dots had been torn away from the AE by the spreading procedure. This suggests that the SMC1 and SMC3 proteins are not integral components of AEs, but of a structure that runs along AEs and associates with them. We think that this structure may be the still-elongated and undivided axis of the chromosome, which would explain that chromatin loops appear to emanate from the SCs (Heng et al., 1994). It remains to be established whether SMC1 and SMC3 are directly attached to the AEs of SCs and whether there are other proteins contributing to the connection.

Immunofluorescence labeling of spread spermatocytes with anti-RAD21 antibodies revealed dots irregularly distributed over the entire nucleus (not shown). There was no indication, however, for a concentration of RAD21 dots similar to those formed by the SMC proteins along the SCs, which sets the mammalian meiotic protein complex(es) containing SMC1 and 3 apart from the mitotic cohesin (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998). This is in accordance with observations by Klein et al. (1999) and Watanabe and Nurse (1999) of a replacement of Rad21p by Rec8p in budding and fission yeast meiosis. A similar substitution may occur in mammalian meiosis (Parisi et al., 1999).
SMC1 and SMC3 proteins in meiosis

Presence of SMC1 and SMC3 proteins in isolated synaptonemal complexes

In initial immunoblotting experiments using mono- or polyclonal antibodies specific for either SMC1 or SMC3, we found expression of the proteins in testis and ovary nuclear extracts from mouse and calf at levels comparable to that of certain other tissues, e.g. spleen, thymus, lung, kidney and liver (Stursberg et al., 1999). Generally, tissues containing actively proliferating cells showed higher levels of SMC1 and SMC3 proteins than those which consist to a larger extent of arrested cells, e.g. brain.

We then set out to determine whether SMC1 and SMC3 proteins are present in preparations of spermatocyte nuclei and synaptonemal complexes (for reviews see von Wettstein et al., 1984; Heyting, 1996; Kleckner, 1996; Roeder, 1997). Equal amounts of protein prepared from rat spermatocyte nuclei (Meistrich, 1975) and synaptonemal complexes (SCs) (Heyting and Dietrich, 1991) were analysed (Fig. 4). The SC preparations, which consist of >80% SCs,
contain about ten major protein bands besides several minor polypeptides; they have been described in detail by Heyting et al. (1985). While SMC protein signals from spermatocyte nuclei were relatively weak, strong signals for both SMC1 and SMC3 were seen in the SC preparations (Fig. 4).

We also looked for the presence of RAD21 in preparations of spermatocyte nuclei and SCs, using affinity-purified polyclonal anti-mouse RAD21 antibodies. Although the antibodies readily detect the RAD21 protein in fractions derived from total bovine testis or thymus extracts, and can be specifically blocked by the antigenic peptide (not shown), only weak signals were obtained, mostly visible after prolonged exposure in spermatocyte nuclei and SCs (Fig. 4). This indicates that RAD21 is underrepresented relative to the SMC proteins in spermatocyte nuclei, which are enriched in preparations of SCs. This is in agreement with related observations in yeast cells (Klein et al., 1999; Watanabe and Nurse, 1999).

Association of SMC1 and SMC3 with synaptonemal complex proteins

Among the synaptonemal complex-specific proteins identified so far in higher eukaryotes are the Synaptonemal Complex Proteins 2 and 3 (SCP2, Offenberg et al., 1998; SCP3, Lammers et al., 1994). These proteins of molecular mass 173 and 30/33 kDa, respectively, constitute parts of the AEs of SCs, and we asked whether interactions of these proteins with the SMC proteins could be observed at the molecular level. Immunoprecipitations from bovine testis nuclear extracts were performed with polyclonal anti-SMC3 antibodies attached to protein A-beads, and the bound material was analysed by immunoblotting with anti-SCP3 or anti-SCP2 antibodies (Fig. 5A,B). The anti-SMC3 antibody precipitated not only the SMC1/SMC3 heterodimer as predominant polypeptides (not shown), but also both the SCP3 (Fig. 5A) and SCP2 (Fig. 5B) proteins. The SCP2 signal appeared weaker, perhaps as result of the lower degree of evolutionary conservation between species (Offenberg et al., 1998; Schalk et al., 1999), or less efficient transfer of the large protein onto the nitrocellulose membrane. In control immunoprecipitations, the SCP proteins were not detected if extracts from thymus or spleen were used (Fig. 5A), or if the anti-SMC3 antibody was omitted from the experiment (Fig. 5B). In another approach, immunoaffinity chromatography using anti-SMC3 antibodies, which were covalently bound to a sepharose resin, yielded corresponding results (Fig. 5C,D). Testis nuclear extracts were loaded in the presence of 500 mM NaCl and detergent, and the resin was then washed with PBS including detergent. Bound material was eluted in two steps of increasing stringency, the first at low pH (pH 2.8) and the second with distilled water. More than 30 polypeptides eluted in the low pH, and about 10 polypeptides in the water fractions, as judged by silver staining of analytical polyacrylamide gels (Fig. 5D). Although the SMC1 and three proteins are prominent in these fractions, bands corresponding to the position of SCP3 are visible. SCP3 protein was detected by immunoblotting in both the low pH and the water-eluted fractions. No anti-SCP antibody signal was observed in control proteins.
experiments, where the anti-SMC3 antibodies had been omitted (Fig. 5C), or where thymus extract has been used (not shown). The eluate immunoblots did not yield a signal upon testing with a variety of other antibodies specific for nuclear proteins, including nucleophosmin, poly(ADP)ribose polymerase, PCNA, Rad51, Rad52, all five histones and p53 (not shown).

In another approach, independent of immunorecognition, we coupled recombinant SCP3 protein to sepharose 4B and used it as an affinity chromatography resin. For controls, sepharose 4B without the SCP3 protein was used in parallel (Fig. 5E). Nuclear extracts from testes were loaded, and bound material eluted stepwise with increasing KCl concentrations. The resulting fractions were analyzed by gel electrophoresis and probing with anti-SMC1 antibodies. While there was no signal in the sepharose 4B control, the SMC1 protein was detected to elute in the 500 mM KCl fraction from the SCP3-sepharose column.

Together, these results indicate interactions between protein components of the synaptonemal complex such as SCP2 and SCP3 with SMC1 and SMC3 proteins.

**Chromatographic analysis of SMC protein complexes in testes extracts**

In addition to these interaction experiments, we chromatographically analysed total nuclear protein extracts from bovine testes or thymus tissue for the presence of SMC protein complexes (Fig. 6). Identically prepared extracts were subjected to gel filtration in a 300 mM NaCl-buffer on a BioGelA15m column, and fractions tested by immunoblotting for the presence of SMC1, SMC3, DNA ligase III and SCP3. In consecutive thymus and testes extract fractions the major portion of SMC1 and SMC3 proteins were detected at positions between 0.3 and 3 MDa (Fig. 6A-D). The complexes RC-1 (0.5 MDa) and cohesin (2-3 MDa) migrate within this range (Jessberger et al., 1993, 1996; Losada et al., 1998; Jessberger et al., unpublished results). SMC1 and SMC3 proteins present in lower molecular weight fractions (0.3 MDa) probably represent the free heterodimer (Losada et al., 1998), or partially dissociated complexes. In testes fractions, additional smaller amounts of both, SMC1 and SMC3 were observed at the 6-9 MDa position (Fig. 6C,D), but none at higher positions. Thymus extracts did not or only weakly display SMC protein signals at this position (Fig. 6A,B). Immunoprecipitations confirmed these observations, as polyclonal antibodies specific for the C terminus of SMC3 precipitated both SMC3 and SMC1 from the 0.5, 2-3 and 6-9 MDa fractions, with the 6-9 MDa yielding a signal about one-fifth as intense as that of the 2-3 MDa fraction (not shown). DNA ligase III (Fig. 6E) elutes in the lower molecular mass range, consistent with its association with the 550 kDa complex RC-1 (Jessberger et al., 1993). Standard salt extraction procedures only release a minor fraction of the total SCP3 from the chromatin. Of this soluble fraction, almost all SCP3 was found to elute at a very low molecular mass position in the exclusion volume (Fig. 6F), corresponding to its molecular mass of 30/33 kDa. A small portion of the protein, however, was consistently found to elute at high molecular mass positions between 3 and 5 MDa, and some at around 2 MDa (Fig. 3F), indicating the potential to form higher order complexes. Probably due to the resistance of SCP3 to moderate salt-extraction and instability of the complexes, we found it difficult, however, to obtain larger amounts of these complexes.

Absorbance measurements at 260 and 280 nm indicated that little if any nucleic acids were present in these fractions; the crude extracts contained small amounts of nucleic acids, which eluted primarily around the void volume of 15 MDa, where no SMC1 and 3 proteins were detectable. Inclusion of DNAse I in the gel filtration chromatography or the immunoprecipitation assays did not alter these results.
Although mammalian SMC proteins and their multiprotein complexes have so far been only described for somatic cells, the biological functions of the SMC complexes rendered it likely that the proteins would also be present in meiotic cells. This hypothesis was recently supported by observations in S. cerevisiae, where Smc3p was found to be required for meiotic sister chromatid cohesion and meiotic recombination (Klein et al., 1999). We undertook an investigation in mammalian meiotic cells of SMC1 and SMC3 proteins, which are components of the protein complexes cohesion (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998) and RC-1 (Jessberger et al., 1996; Stursberg et al., 1999). In particular, we determined the expression of SMC1 in testicular cells, the specific association of SMC1 and SMC3 with meiotic chromatin, the enrichment of the SMC proteins in synaptonemal complexes, and their interaction with SC-specific proteins.

The bead- or focus-like pattern observed in pachytene and diplotene spermatocyte chromosome spreads (Figs 2,3) indicates accumulation of SMC1 and SMC3 proteins at distinct sites along the AEs of the meiotic chromosomes. The labeling patterns on frozen sections, however (Fig. 1A,C) suggest that the SMC1/3 heterodimer is also present on the chromatin loops. Possibly, the agar filtration spreading technique causes preferential loss of SMC1 and SMC3 from the periphery of the chromatin loops. Given the previously reported strong preference of SMC proteins to bind to DNA secondary structures, A/T-rich sequences (Akhmedov et al., 1998), and to distinct sites on mitotic chromosome III of S. cerevisiae (Blat and Kleckner, 1999), the bead-pattern may reflect the existence of such structures and sites, bound by SMC proteins, along the meiotic chromosomes. Though meiotic and mitotic sister chromatid cohesion differ in several respects, the uneven pattern may also generally resemble mitotic sister chromatid cohesion, in that both are not entirely uniform along the sister chromatids (Miyazaki and Orr-Weaver, 1994; Bickel and Orr-Weaver, 1996). Close association of the SMC proteins with the SCs was most easily shown in diplotene and pachytene. In early zygote (Fig. 2A), the SMC proteins do not seem to be as closely associated with the AEs, as the staining is predominantly green (i.e. does not overlap with the anti-SCP label). Close inspection, however, reveals that almost all SMC staining localizes next to the AEs, which are stained by anti-SCP2. It is possible that in early zygote, the SMC/SCP structure has not yet been completely assembled, and that this structure is not finished before pachytene (Figs 1-3). Accordingly, we found both SMC proteins to be highly enriched in preparations of SCs (Fig. 4), which originate mainly from pachytene and diplotene, and which contain only a very limited number of proteins (Heyting et al., 1985). Co-immunoprecipitation, co-immunoaffinity purification and direct affinity interactions of two specific components of the SCs, SCP2 and SCP3, with the SMC proteins also argue for a direct interaction of the SMC proteins with the SCs (Fig. 5).

Small amounts of high molecular mass complexes containing SCP3 were detected in gel filtration experiments along with SMC1 and SMC3, which elute in these but also in many other fractions (Fig. 6). Probably due to the ability of SMC proteins to form a variety of protein complexes from the heterodimer to large, several MDa complexes, both SMC proteins are not confined to the SCP3-containing fractions, but elute in a wide range from about 0.2 to up to 9 MDa. DNA ligase III elutes in the lower molecular mass range, consistent with its association with the 550 kDa complex RC-1 (Jessberger et al., 1993).

Protein RAD21, of which homologs are associated with the SMC1/3 heterodimer in the mitotic sister chromatid cohesion complex from yeast and frog (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998), was neither found to be enriched in SC preparations, nor chromosome-associated in the bead-like fashion as the SMC1 and SMC3 proteins. While the RAD21 protein clearly is expressed in total testis (McKay et al., 1996; Parisi et al., 1999), it may, as in yeast (Molnar et al., 1995; Klein et al., 1999; Watanabe and Nurse, 1999), be partially replaced in meiotic cells by a meiotic counterpart, Rec8p.

Based on our observations, we propose the model given in Fig. 7 for the arrangement of SMC1 and SMC3 on mammalian meiotic chromatin. One or several SMC1/3-containing meiotic complex(es) are likely to be associated with both the chromatin loops and the AEs of the SCs. Here, they enter into limited interactions with protein components of the SCs such as SCP2 and SCP3. Currently, it is not known whether all meiotic sister
chromatid cohesion in mammalian cells is generated by the multiprotein complex harboring SMC1/SMC3/Rec8, or whether RAD21 protein replaces Rec8 in some instances, e.g. at specific chromosomal locations. One possibility is that the fraction of SMC1/SMC3-complexes which contain Rec8 protein are selected for association with the chromosomal axis and participation in meiotic homologous recombination, similar to the situation in yeast (Klein et al., 1999; Watanabe and Nurse, 1999). In any scenario, is very likely that SMC1 and SMC3 codetermine the structure of the AEs of SCs, and thereby of the SCs themselves. It is also likely that the SMC proteins participate in formation of the chromatin loop structures all along the loops themselves, and along the SCs, perhaps by constituting the bases of the loops at the AEs. Similar models have been proposed for SMC proteins in mitotic cells (Gasser, 1995), and would be in concordance with the observations made in yeast (Klein et al., 1999). The strong preference of SMC proteins to bind DNA with secondary structures (Akhmedov et al., 1998) may support specific binding to these regions, which by analogy to the mitotic SARs (Laemmli et al., 1992) may be A/T- and/or secondary structure-rich.

The presence of the two SMC proteins along the AEs of SCs suggests a role related to that of cohesion in mitotic cells. After replication and entry into meiotic prophase I, the two sister chromatids are closely held together from leptotene until and including diplotene along the AEs of SCs (von Wettstein et al., 1984; Bickel and Orr-Weaver, 1996; Heyting, 1996; Kleckner, 1996; Roeder, 1997), which are thought to be essential for meiotic sister chromatid cohesion (Magain, 1991; Dobson et al., 1994). The localization of SMC1 and SMC3 along the AEs of the SCs throughout meiotic prophase suggests that meiotic sister chromatid cohesion is at least co-mediated by the SMC1 and SMC3 proteins. Sister chromatid cohesion proximal to chiasmata is maintained throughout the first meiotic division to ensure proper segregation of the sister chromatids at metaphase/anaphase of meiosis II. If the SMC1/3 heterodimer is (co)responsible for meiotic sister chromatid cohesion, it should be present throughout meiosis I, and a fraction of it should remain on the chromatids into meiosis II. Our testis section staining results (Fig. 1) are consistent with this, for there remains a lower level of SMC1/3 proteins in and possibly beyond meiosis II.

Finally, the involvement of yeast Smc3p in meiotic recombination (Klein et al., 1999) and of the SMC1/3 heterodimer in the mammalian protein complex RC-1, which has recombination activities (Jessorger et al., 1993; Achmedov et al., 1996), renders it likely that the SMC proteins are also involved in mammalian meiotic recombination. Possible roles for the SMC proteins may be in providing the structural frame for DNA recombination by aligning the chromatids and supporting formation of the AEs of the SC. It is also possible that SMC proteins play a more active role in recombination through support of DNA strand pairing and eventually strand exchange, since similar activities have been observed with isolated SMC1/3 heterodimers (Jesserger et al., 1996). The number and localization of SMC1 and SMC3 dots are reminiscent of the Rad51 foci, as detected by immunofluorescence along leptotene-early pachytene AEs of human, mouse and rat (Barlow et al., 1997; Moens et al., 1997), albeit that the Rad51 foci disappear in pachytene while the

SMC dots persist. It would be of interest to compare the localization of foci containing known recombination proteins like Rad51 with that of the SMC dots described here. Furthermore, it remains to be determined whether there exists a meiotic complex similar to RC-1.

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