Temporally and spatially selective loss of Rec8 protein from meiotic chromosomes during mammalian meiosis

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Summary

Sister chromatid cohesion is maintained from DNA replication to metaphase-to-anaphase transition by multi-subunit protein complexes called cohesin, which include at least four proteins, SMC1α, SMC3, Rad21 and either SA1 or SA2, in mammalian somatic cells. We report here the first evidence of the involvement of Rec8 protein, a mammalian homolog of yeast Rec8p, in meiosis-specific chromosome behavior in mammals. In immunoblotting and immunohistochemical analysis using specific antibodies against mouse Rec8, we found that Rec8 was expressed in the testis but not in the kidney or liver; more precisely, it was expressed in spermatocytes and spermatids but not in spermatogonia or other somatic cells. We also found that Rec8 is present in both phosphorylated and dephosphorylated states in vivo. Immunoprecipitation analyses revealed that Rec8 associates with other cohesin proteins, SMC1β (meiosis-specific protein) and SMC3 and with a component of synaptonemal complexes, SCP3, but not with SMC1α. In meiotic chromosome spreads, Rec8 was localized along the axial/lateral elements of the synaptonemal complexes in meiotic prophase from the leptotene to diplotene stages. At later stages, diakinesis and metaphase I, Rec8 was localized along the interstitial axes of chromosomes, including both centromere and arm regions of chromosomes. However, concomitantly with separation of homologous chromosomes in anaphase I, Rec8 was no longer detected along the arm regions, while it persisted on centromere regions up to metaphase II. In anaphase II, the centromeric signals were diminished. We propose from these results that mammalian Rec8 protein, in association with SMC3 and SMC1β but not SMC1α, is involved in meiosis-specific chromosome behavior, and that homologous chromosome separation is triggered by selective loss of Rec8 from chromosome arms in meiosis I, while sister chromatid cohesion is maintained until metaphase II/anaphase II transition by centromeric Rec8 during mammalian meiosis.

Key words: Rec8 protein, Chromosome cohesion, Sister chromatid, Homologous chromosome, Mammalian meiosis

Introduction

In eukaryotes, maintenance of sister chromatid cohesion is a prerequisite for correct bipolar attachment of the chromosome to the spindle in prometaphase, and then loss of the cohesion triggers the segregation of sisters to opposite poles in anaphase. Thus, sister chromatid cohesion from DNA synthesis to anaphase onset is essential for generation of two daughter cells with exactly the same complement of chromosomes. Recently, multisubunit protein complexes called cohesin have been found to be responsible for sister chromatid cohesion in eukaryotes, and most of the components are well conserved from yeast to human. In the budding yeast Saccharomyces cerevisiae, cohesin contains at least four subunits: Scc1p (sister chromatid cohesion) [also called Mcd1p (mitotic chromosome determinant) or Rad21p (radiation-sensitive mutants) in the fission yeast Schizosaccharomyces pombe], Scc3p, Smc1p (structural maintenance of chromosomes), and Smc3p (Michaelis et al., 1997; Tóth et al., 1999). Scc1p accumulates in early G1 phase, binds to chromosomes during S phase, and dissociates from them at the metaphase-to-anaphase transition (Michaelis et al., 1997). Dissociation of Scc1p from the chromosomes is caused by its cleavage by separin, a cysteine protease related to caspases (Uhlmann et al., 1999; Uhlmann et al., 2000). At the metaphase-to-anaphase transition, proteolytic activity of separin is turned on by the destruction of its inhibitor, called securin, through its ubiquitination by anaphase-promoting complex (APC) (Ciosk et al., 1998). In Xenopus, two distinct cohesins are present; a 14S complex termed x-cohesinSA1, which contains XSMC1, XSMC3, XRAD21 and XSA1, and a 12.5S complex termed x-cohesinSA2, which contains XSMC1, XSMC3, XRAD21 and XSA2 (Losada et al., 1998; Losada et al., 2000). Both XSA1 and XSA2 belong to the SA family of mammalian proteins and exhibit similarity to yeast Scc3p (Losada et al., 2000). In contrast to yeasts, cohesin in vertebrate cells dissociates from the chromosomes in two steps, at first from arm regions in early prometaphase and then from centromere regions at the metaphase-to-anaphase transition (Losada et al., 1998). It has been proposed in vertebrates that a separin-independent pathway removes cohesin from chromosome arms, whereas a

separin-dependent pathway cleaves centromeric cohesin (Waizenegger et al., 2000).

Meiosis is an essential step in sexual reproduction to produce gametes that have a reduced number of chromosomes from diploidy to haploidy. The reduction of chromosome number is conducted by two rounds of meiotic division following a single round of DNA replication. During prophase in meiosis I, homologous chromosomes pair, recombine, and crossover with their partners, yielding junctional sites called chiasmata between non-sister chromatids in a bivalent chromosome. As a result, homologous chromosomes are aligned at the metaphase plate and separate at anaphase in meiosis I without separation of sister chromatids. A pair of sister chromatids in a homologous chromosome remains attached through meiosis I until they finally separate at the onset of anaphase in meiosis II. Therefore, meiotically dividing cells must be equipped with special molecules that ensure these specific behaviors of meiotic chromosomes. It has been shown that a meiosis-specific cohesin subunit in yeast, Rec8p, replaces a mitotic cohesin subunit, Scc1p/Rad21p, during meiosis and that this replacement is needed for preventing sister chromatids from separating precociously in meiosis I (Klein et al., 1999; Watanabe and Nurse, 1999). Homologous chromosome separation in meiosis I is promoted by the proteolytic cleavage of Rec8p by separin (Buonomo et al., 2000). In mammals, putative homologs of the *rec8* gene have been isolated, and high levels of mRNA expression of mouse *rec8* has been found in both male and female germ line cells (Parisi et al., 1999; Lee et al., 2002). In addition, meiosis-specific cohesin proteins, STAG3 and SMC1β, whose yeast homologs have not been found in the database, have been found on mammalian meiotic chromosomes (Prieto et al., 2001; Revenkova et al., 2001). However, there has been no report on mammalian Rec8 protein so far.

In this study, we investigated the protein expression of mammalian Rec8 during meiosis in the male mouse. For this purpose, specific antibodies against mouse Rec8 were raised in the mouse and rabbit and used for immunoprecipitation, immunoblotting, and immunohistochemistry. We found that mammalian Rec8, which associated with SMC3 and SMC1β but not with SMC1α, was expressed from the pre-leptotene stage and was localized along the axial/lateral element (AE/LE) of synaptonemal complexes from leptotene to diplotene stages in prophase I. During diakinesis and metaphase I, Rec8 was localized in both centromeres and arm regions of chromosomal interstices. At the metaphase I-to-anaphase I transition, Rec8 dissociated from the arms but remained associated with centromere regions until metaphase II. In anaphase II, Rec8 was no longer detected on chromosomes. The localization and the step-wise dissociation of Rec8 from meiotic chromosomes suggests that Rec8 is the protein that is responsible for both cohesions between homologs and sisters and that its dissociation from arms and centromeres causes the separation of homologous chromosomes and that of sister chromatids, respectively.

Materials and Methods

Preparation of antibodies

According to the known sequences (DDBJ/EMBL/GenBank accession numbers: *rec8*, AF262055; *scp3*, AF181473S6), the C-terminal domain of mouse *rec8* and the full length of mouse *scp3* were amplified by PCR using primer sets: for *rec8*, 5′-GGAATTCCTGCTTCACTACCACTGGATG-3′ (5′ primer in which an *Eco*RI site, indicated by an underline, has been introduced) and 5′-GTGCTCGAGGGGGAATTTGGGTCCAG-3′ (3′ primer in which an *Xho*I site, indicated by an underline, has been introduced); for *scp3*, 5′-CGGAATTCAGATGCTTCGAGGGTGTGGG-3′ (5′ primer in which an *Eco*RI site, indicated by an underline, has been introduced) and 5′-TTTCTCGAGGAATAACATGGATTGAAGAGA-3′ (3′ primer in which an *Xho*I site, indicated by an underline, has been introduced). The amplified PCR products of *rec8* and *scp3* were subcloned into the *E. coli* expression vectors pET-21c (Novagen, Madison, WI) and pGEX-KG (Guan and Dixon, 1991), respectively. The produced recombinant proteins of Rec8 and SCP3 were purified according to the methods described previously (Hirai et al., 1992). Rabbit and mouse polyclonal antisera were raised against the recombinant Rec8 protein and then affinity-purified with the antigenic proteins electroblotted onto an Immobilon membrane (Millipore, Tokyo, Japan). Mouse polyclonal antisera were raised against the recombinant SCP3 protein.

Concurrently with the investigation of mammalian Rec8, we have been investigating cohesin proteins in fish. We have cloned a medaka (Oryzias latipes) homolog of mammalian smc1α from a cDNA library constructed from the testis, and we have produced a mouse polyclonal antibody against the recombinant C-terminal 337-amino-acid sequence of medaka SMC1α (DDBJ/EMBL/GenBank accession number, AB097255). The antigenic C-terminal amino acid sequence of medaka SMC1α shows 89% and 59% homology to the corresponding regions of mouse SMC1α and SMC1β, respectively. Hence, the anti-medaka SMC1 antibody has crossreactivity to both mouse SMC1α and SMC1β in western blotting and immunoprecipitation analyses, although it is less reactive to mouse SMC1β than to SMC1α in immunoprecipitation analysis.

Goat polyclonal anti-SMC3 antibody and mouse monoclonal anti-α-tubulin antibody (DM1A) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma (St Louis, MO), respectively.

Preparation of extracts

Nuclear extracts from tissues were prepared as follows. Tissues from 6-8-week-old C57BL/6 mice were minced with a surgical blade and homogenized by a Teflon homogenizer in nine times volume (w/v) of 0.25 M sucrose-containing TKM solution (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$). The homogenized solution was filtered through a 70 μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) and centrifuged for 10 minutes at 600 ***g***. The pellet was resuspended in 0.25 M sucrose-containing TKM solution. Then 2× volume of 2.3 M sucrose-containing TKM solution was added to the cell suspension. After adding 150 μl of 2.3 M sucrose-containing TKM solution to each 1.5 ml tube, 600-800 μl of cell suspension and 200 μl of 0.25 M sucrose-containing TKM solution were loaded in that order to the top. Nuclei were purified by centrifugation for 30 minutes at 12,000 ***g***. The nuclear pellet was resuspended in 0.25 M sucrose-containing TKM solution and then centrifuged for 5 minutes at 2000 ***g***. The pellet was extracted in RIPA buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% Na deoxycholate, 0.1% SDS, 50 mM NaF, 5 mM 2-mercaptoethanol] containing Protease-Inhibitor-Cocktail (Roche, Mannheim, Germany). After sonication, the extraction buffer was centrifuged at 18,000 ***g*** for 1 hour. Then the supernatant was recovered as nuclear extract.

Whole extracts of testes from 0-, 1-, 2-, 3- and 5-week-old mice were also prepared by extracting them in RIPA buffer containing Protease-Inhibitor-Cocktail. Briefly, after testes had been decapsulated, they were homogenized, sonicated in the buffer, and centrifuged at 18,000 ***g*** for 1 hour at 4°C. Then the supernatant was recovered as whole testis extracts.

Western blotting and immunoprecipitation

In immunoprecipitations, testis nuclear extracts were incubated with either of the antibodies (anti-Rec8, anti-SMC1 and anti-SMC3 antibodies) in the presence of Protein G Sepharose (Amersham Biosciences, Piscataway, NJ) at 4°C overnight with rotor agitation. Then, the sephoroses were washed six times in the RIPA buffer, and the immunoprecipitates were analyzed by SDS-PAGE followed by western blotting. As a control, testis extracts were incubated without the primary antibody or with control preimmune serum in the presence of Protein G Sepharose, and the immunoprecipitates were analyzed as above. For the treatment of Rec8 with protein phosphatases (PPases), immunoprecipitates obtained with anti-Rec8 antibody were washed five times in RIPA buffer and twice in alkaline PPase buffer (50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$) or in 1× λ-PPase buffer equipped with the λ-PPase, then incubated with 0.25 U/μl calf intestine alkaline PPase (Takara, Tokyo, Japan) or 20 U/μl λ-PPase (New England BioLabs, Beverly, MA) for 30 minutes at 30°C. As controls, the immunoprecipitates were incubated without PPases in the buffers.

The immunoprecipitates, nuclear extracts, and whole testis extracts were separated by SDS-PAGE with 7.5% or 12.5% gels. After blotting onto an Immobilon membrane, the proteins were probed with primary antibodies. The antigen-protein complex was detected with alkaline phosphatase (AP)-conjugated secondary antibodies [AP-goat anti-mouse IgG (American Qualex International, San Clemente, CA), AP-goat anti-rabbit IgG (Zymed Laboratories,San Francisco, CA), AP-rabbit anti-goat IgG (Chemicon International, Temecula, CA)] and visualized by incubation in a color-substrate solution (0.2 mM nitoroblue tetrazolium, 0.2 mM 5-bromo-chloro-3-indolyl phosphate, 100 mM Tris-HCl, pH 9.5, 5 mM $MgCl_2$).

Tissue staining

Testes from 6-8-week-old mice were frozen in liquid nitrogen, and 10-μm-thick cryostat sections were prepared. The sections were air-dried on Vectabond (Vector Laboratories, Burlingame, CA)-coated slides and fixed in cold 1% paraformaldehyde in PBS for 15 minutes. After washing in PBS twice, sections were blocked in 10% goat serum (Sigma) in PBS for 1 hour and incubated with the primary antibodies at appropriate dilutions in the blocking buffer at 4°C overnight. After washing three times in PBS, the Rec8 and SCP3 signals were detected with secondary antibodies [Alexa 488-conjugated anti-rabbit IgG antibody, Alexa 488-conjugated anti-mouse IgG antibody, Alexa 546-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR)]. DNA was counterstained with propidium iodide in the single-labeled sections.

Preparation of nuclear spreads and immunocytochemistry

Preparation of meiotic nuclear spreads was performed according to the methods for surface spreading of meiotic chromosomes described previously (Moens and Pearlman, 1991) with some modifications described below. Testicular cell suspension was prepared according to the method described by Heyting and Dietrich (Heyting and Dietrich, 1991), and the cells were put on poly-L-lysin-coated coverslips. The cells on the coverslips were placed in 85 mM NaCl for 3 minutes, transferred to 1% paraformaldehyde solution (pH 8.2 with 0.01 M sodium borate) containing 0.03% SDS for 3 minutes, and then to 1% paraformaldehyde solution (pH 8.2) without SDS for 3 minutes. The coverslips were rinsed three times for 1 minute each in 4% (v/v) Photo-Flo (Kodak, Rochester, NY) in distilled water (pH 8.0) and air-dried overnight. For preservation of chromatin loops in the nuclear spreads, the cells on coverslips were incubated in 75 mM KCl solution for 3 minutes instead of 85 mM NaCl solution. Further, for the preservation of meiotic chromosome shape, the operation to diffuse chromatin by SDS during fixation was omitted. Instead, after the incubation in 85 mM NaCl for 3 minutes, the cells were fixed in 1% paraformaldehyde in PBS for 15 minutes.

According to the labeling methods described by Heyting and Dietrich (Heyting and Dietrich, 1991), the coverslips were placed for 10 minutes in PBS, for 20 minutes in 1 μg/ml DNase I (Sigma) in PBS, for 10 minutes in detergent (5 mM EDTA, 0.25% gelatin, and 0.05% Triton X-100 in PBS), for 10 minutes in PBS, and for 30 minutes in blocking buffer (3% BSA, 10% goat serum, 0.05% Triton X-100 in PBS). The coverslips were incubated with the first antibodies at appropriate dilutions in the blocking buffer at 4°C overnight. After washing for 10 minutes in PBS, for 10 minutes in detergent and for 10 minutes in PBS, the coverslips were incubated with the secondary antibodies at appropriate dilutions in the blocking buffer. After washing three times in PBS, DNA was counterstained with propidium iodide in single-labeled samples. The samples were mounted with Vectashield Mounting Medium (Vector Laboratories) and observed under a Bio-Rad MicroRadiance confocal microscope.

Results

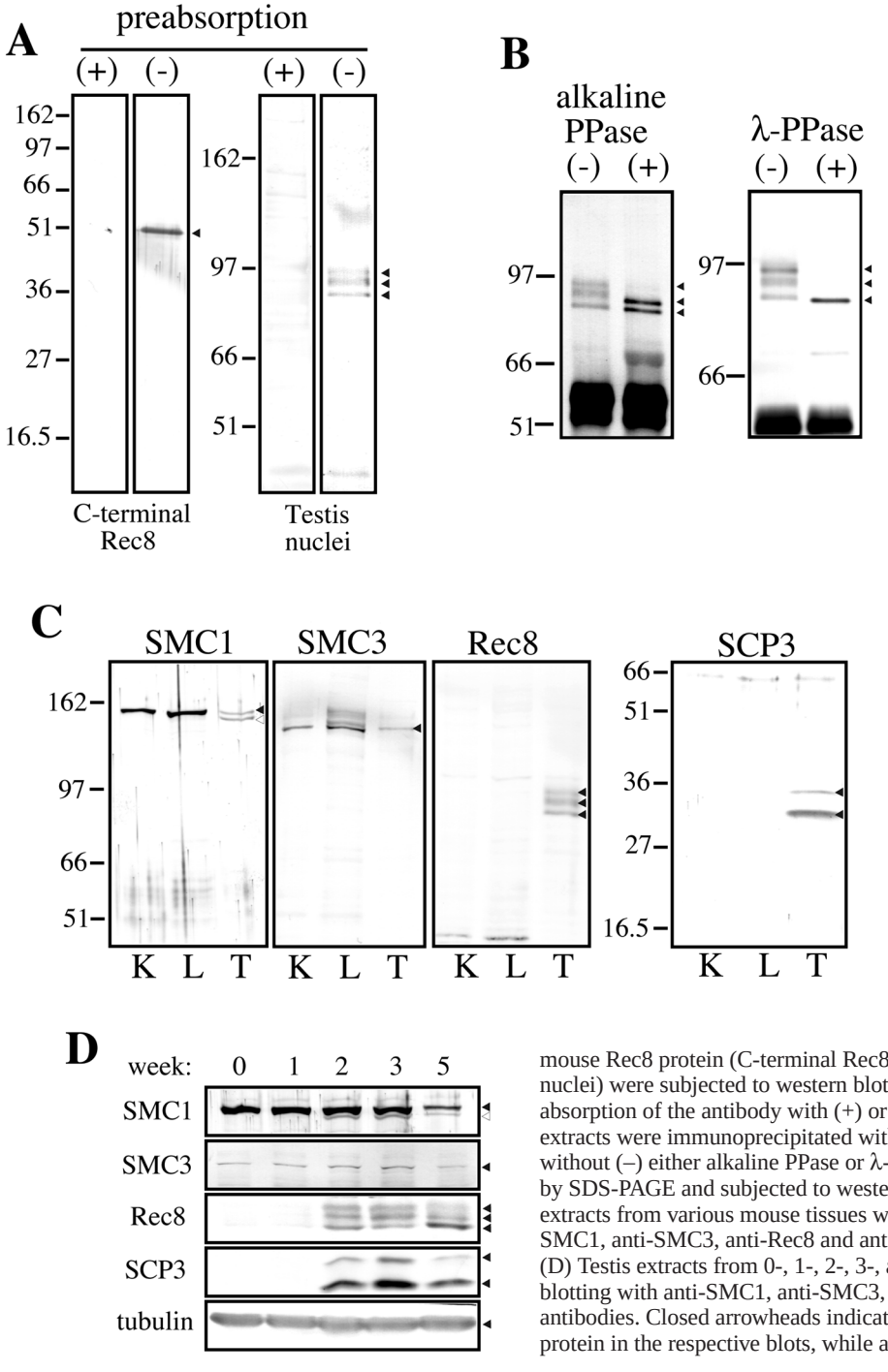
Specific expression of Rec8 in meiotic cells

For the analysis of mammalian Rec8 protein, rabbit and mouse polyclonal antibodies were raised against the C-terminal 342-amino-acid sequences of mouse Rec8 protein expressed in E. coli. Both mouse and rabbit polyclonal anti-Rec8 antibodies recognized the antigenic recombinant protein in western blot analysis (only the results obtained with the rabbit polyclonal antibody are shown in Fig. 1A). In testis nuclear extract, both antibodies recognized three bands ranging from 82 to 95 kDa in SDS-PAGE. The absorption of antibody with the antigenic protein prior to western blotting gave no signals of either recombinant Rec8 or endogenous proteins (Fig. 1A). In the mouse genome, there are two genes showing sequence similarity to yeast *rec8*; mouse *rad21* and *rec8* have 20.2% and 21.3% homology to S. pombe *rec8* and 16.7% and 19.8% homology to S. cerevisiae *rec8* in amino acid sequence, respectively. Since mammalian Rad21 protein has been detected as a 120 kDa band in SDS-PAGE (Losada et al., 2000), the three bands did not represent mouse Rad21. Yeast Rec8p has been reported to be phosphorylated during meiosis (Watanabe and Nurse, 1999). It is very likely that the three bands recognized by anti-Rec8 antibody in the present study represent different phosphorylated states of mammalian Rec8. To verify this possibility, anti-Rec8 immunoprecipitates from testis nuclear extracts were treated with either alkaline PPase or λ-PPase. Incubation in their buffers in the absence of PPases did not affect the mobility of the three bands in SDS-PAGE (Fig. 1B). Treatment with alkaline PPase affected the mobility and diminished the upper band. Treatment with λ-PPase diminished the upper and middle bands, in accordance with the increase in the lower band's intensity (Fig. 1B). Therefore, we concluded that the three bands detected specifically with anti-Rec8 antibodies represent mouse Rec8 protein and that the upper and middle bands are phosphorylated forms of Rec8, whereas the lower band is a dephosphorylated form of Rec8.

Using the anti-Rec8 antibody, we first examined the expression of Rec8 protein in various mouse tissues. Nuclear extracts from kidney, liver and testis were analysed by western blotting with the rabbit polyclonal anti-Rec8 antibody (Fig. 1C). We also examined the expressions of other cohesin subunits (SMC1 and SMC3) and a component of synaptonemal complexes (SCP3) to guarantee that cohesin proteins and synaptonemal complex proteins were extracted properly in those extracts. SMC3 was observed in all of the extracts as a

145-kDa band. SMC1α was detected as a 160-kDa band in kidney, liver and testis extracts, whereas SMC1β protein was detected as a 155-kDa band only in testis extract as has been previously reported (Revenkova et al., 2001). SCP3 protein was detected as 30/33-kDa bands only in testis extract as has been previously reported (Heyting et al., 1987; Lammers et al., 1994). Thus, proteins of mitotic and meiotic cohesin and synaptonemal complexes were extracted properly in each extract. Of these extracts, anti-Rec8 antibody detected three bands (82 to 95 kDa) only in testis nuclear extracts (Fig. 1C). Essentially the same result was obtained with mouse polyclonal anti-Rec8 antibody (data not shown).

Next, we examined when Rec8 protein is expressed in mouse testis. Testis extracts were prepared from 0-, 1-, 2-, 3- and 5-week-old mice (Fig. 1D). In male mice, meiosis starts between 1 and 2 weeks of age. Tubulin protein, as a loading control, was expressed in extracts from 0- to 5-week-old mice at a similar level. SMC1α and SMC3 were also expressed in extracts from 0-5-week-old mice. By contrast, protein expression of Rec8 began at 2 weeks of age. Other meiosis-specific proteins, SMC1β and SCP3, were also expressed in extracts from 2-week-old mice. These results suggest that Rec8 is a meiosis-specific protein that is expressed only when spermatogenesis starts in the testis.



Association of Rec8 protein with other cohesin proteins

To examine the association of Rec8 protein with other cohesin proteins, we immunoprecipitated testis nuclear extracts with the antibodies against cohesin proteins and analyzed the immunoprecipitates by western blotting. Anti-Rec8 immunoprecipitates contained Rec8, SMC1β, SMC3, and SCP3 but not SMC1α (Fig. 2). Immunoprecipitates with anti-SMC1 or anti-SMC3 antibodies contained Rec8, SMC1, SMC3 and SCP3-reactive bands, although the intensities of Rec8 bands were very faint in anti-SMC1 immunoprecipitates, probably due to weaker crossreactivity to SMC1β than to SMC1α in immunoprecipitation analysis (compare the relative band intensity of SMC1β to SMC1α in testis nuclear extracts in Fig. 1C with that in anti-SMC1 immunoprecipitates in Fig. 2). No band was detected in immunoprecipitates without the primary antibodies or with control preimmune serum (data not shown). These results demonstrate that Rec8 protein associates with other cohesin proteins, SMC1β and SMC3, and a synaptonemal complex protein, SCP3 (30

Fig. 1. Specific expression of Rec8 protein in meiosis-undergoing testis, as revealed by an anti-mouse Rec8 antibody. (A) The anti-mouse Rec8 antibody was raised in a rabbit against the bacterially expressed C-terminal fragment of mouse Rec8 protein. The antigenic C-terminal mouse Rec8 protein (C-terminal Rec8) and nuclear extracts from mouse testis (Testis nuclei) were subjected to western blotting with the anti-Rec8 antibody after pre-absorption of the antibody with (+) or without (–) the antigen. (B) Testis nuclear extracts were immunoprecipitated with anti-Rec8 antibody and treated with (+) or without (–) either alkaline PPase or λ-PPase. The immunoprecipitates were separated by SDS-PAGE and subjected to western blotting with anti-Rec8 antibody. (C) Nuclear extracts from various mouse tissues were subjected to western blotting with anti-SMC1, anti-SMC3, anti-Rec8 and anti-SCP3 antibodies. K, kidney; L, liver; T, testis. (D) Testis extracts from 0-, 1-, 2-, 3-, and 5-week-old mice were examined in western blotting with anti-SMC1, anti-SMC3, anti-Rec8, anti-SCP3 and anti-tubulin antibodies. Closed arrowheads indicate SMC1α, SMC3, Rec8, SCP3 and tubulin protein in the respective blots, while an open arrowhead indicates SMC1β protein.

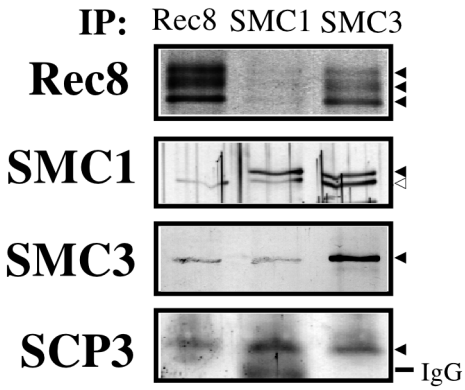


Fig. 2. Association of Rec8 with other cohesin proteins in the mouse. Testis nuclear extracts were immunoprecipitated with either of three antibodies, anti-Rec8, anti-SMC1 or anti-SMC3 antibody. The immunoprecipitates were subjected to SDS-PAGE followed by western blotting with the same antibodies plus anti-SCP3 antibody.

kDa), but not with SMC1α. The results also demonstrate that the phosphorylation state of Rec8 protein does not affect its association with SMC1β and SMC3, since both phosphorylated and dephosphorylated Rec8 proteins were coimmunoprecipitated with these proteins (Fig. 2).

Localization of Rec8 on synaptonemal complex in meiotic prophase

To examine the expression pattern of Rec8 protein in mouse testis cells, frozen sections of mouse testis were subjected to indirect immunofluorescence labeling with anti-Rec8 antibody or anti-SCP3 antibody or both (Fig. 3). SCP3 is one of the major components of the axial/lateral element (AE/LE) of synaptonemal complexes (Lammers et al., 1994). In the sections, SCP3 was detected as several lines, which represented the synaptonemal complexes in spermatocytes. Similarly, Rec8 was mainly detected along the synaptonemal complexes, but the signal was dotty lines in spermatocytes. In contrast to SCP3, Rec8 was also detected in round and elongated spermatids although the signals were weak compared to those in spermatocytes. No labeling was essentially observed in spermatogonia and other somatic cells. Immunofluorescent double labeling with anti-Rec8 antibody and anti-SCP3 antibody showed that most signals of Rec8 and SCP3 overlapped with each other. However, some spermatocytes showed Rec8 labeling but hardly showed SCP3 labeling (indicated by arrows in the lower panels in Fig. 3), indicating that Rec8 protein expression starts at an earlier stage of meiosis than does the expression of SCP3 in spermatocytes, probably from pre-leptotene stage. In control sections incubated with anti-Rec8 antibody preabsorbed with antigenic proteins or with secondary antibodies alone, no specific signals were observed (data not shown).

To examine the localization of Rec8 on synaptonemal complexes in detail, we performed immunofluorescence double labeling of Rec8 and SCP3 on nuclear spreads of mouse testis cells. Anti-SCP3 immunocytochemistry showed the presence of short and fine AEs of synaptonemal complexes in leptotene spermatocytes (Fig. 4A). In this stage, Rec8 was detected as dotty signals along the AEs. In the zygotene stage, when AEs have become longer and have started to form synapsis (AEs are now called LEs), Rec8 signals were more concentrated and detected as dotty lines along the AE/LEs (Fig. 4B). The dotty lines of Rec8 signal were also detected along LEs of synapsed chromosomes and unsynapsed XY chromosomes (indicated by an arrow) in the pachytene stage (Fig. 4C) and along LEs of desynapsed chromosomes in the diplotene stage (Fig. 4D).

In the above observations, chromatin was mostly dispersed except for the proximal to synaptonemal complexes (Fig. 5A). Hence, if Rec8 protein existed on chromatin loops expanding from the axis of synaptonemal complexes, the signal would not be observable. By treatment of cells with KCl instead of NaCl prior to fixation in the presence of SDS, we

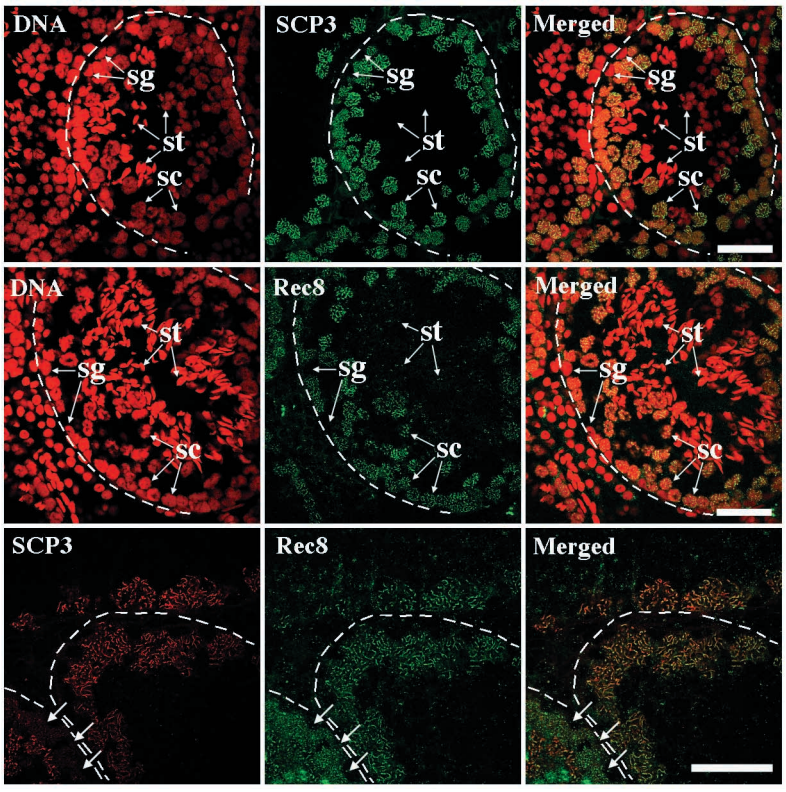


Fig. 3. Coexpression of Rec8 protein with SCP3 protein in mouse testis. Frozen sections were incubated with either a mouse polyclonal anti-SCP3 antibody or rabbit polyclonal anti-Rec8 antibody, or both antibodies, and detected with Alexa 488 (green)-conjugated or Alexa 546 (red)-conjugated, anti-mouse or rabbit IgG secondary antibodies. DNA was stained with propidium iodide in single-labeled sections. Broken lines indicate the basement membrane of seminiferous tubules. Spermatogonia (sg) lie next to the basement membrane. Spermatocytes (sc) are larger cells and lie away from the basement membrane. Spermatids (st) are farther from the membrane and have a smaller nuclei. Arrows in lower panels indicate the cells in which Rec8 protein was expressed but in which SCP3 signal was hardly detected. Bar, 50 μm.

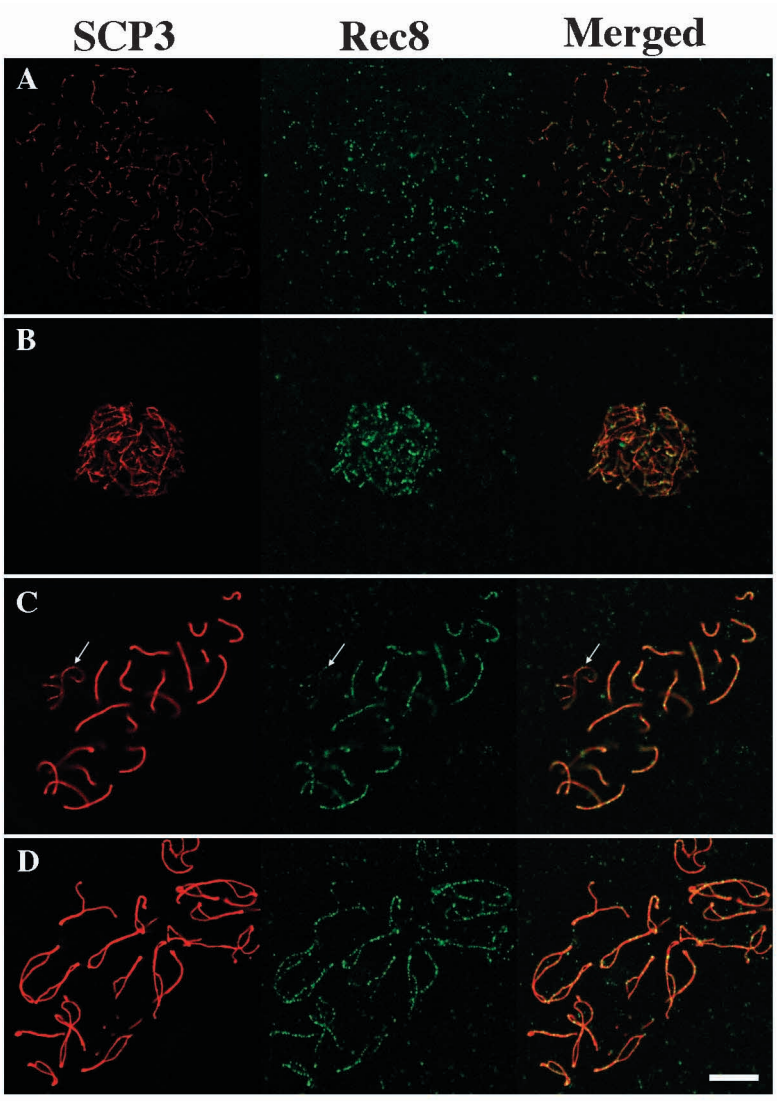


Fig. 4. Localization of Rec8 protein on synaptonemal complexes during meiotic prophase. Nuclear spreads from testicular cells were prepared as described in Materials and Methods and subjected to immunofluorescent double labeling with anti-Rec8 and anti-SCP3 antibodies. (A) Leptotene; (B) zygotene; (C) pachytene; (D) diplotene. An arrow indicates an unsynapsed XY bivalent. Bar, 10 μm.

found that chromatin diffusion was suppressed and chromatin loops could be seen (Fig. 5B,C). Using this method, we examined the localization of Rec8 on chromatin loops. Rec8 existed on the portion of chromatin loops that came in contact with synaptonemal axes, but it was hardly detected along chromatin loops extending from the synaptonemal axes, implying that Rec8 is involved in the conjunction of chromatin loops with the synaptonemal axes and that Rec8-mediated cohesion of sister chromatids is limited to the sites of chromatin adjacent to synaptonemal complexes.

Localization of Rec8 on meiotic chromosomes

To examine the localization of Rec8 protein on meiotic chromosomes, chromosome spreads were prepared by the method described in Materials and Methods to prevent dispersion of chromatin by SDS during fixation, thereby preserving chromosome shape. In meiotic prophase I at the pachytene stage (Fig. 6A), Rec8 was detected as lines of dotty signals, representing synaptonemal complexes. At later stages, however, the intensity of the Rec8 signal tended to decline as meiosis proceeded. In diakinesis and metaphase I stages (Fig. 6B,C,I), a considerable amount of Rec8 signal was still detected along the chromosomal axes (indicated by arrows), which covered both the regions of the centromere and the chromosome arm proximal to chiasmata. In these stages, Rec8 was also observed along chromosome arms distal to chiasmata (indicated by blue arrowheads in Fig. 6B,C) or on the sites at which the homologs were seen overlapped (indicated by an white arrowhead in Fig. 6I). After metaphase I-to-anaphase I transition (Fig. 6D,E,J), homologous chromosomes separated from each other, whereas sister chromatids were still attached to each other at their centromere regions. In these stages, the Rec8 signals along chromosome arms were no longer detected, while the signals were detected at the conjunction sites of sisters (centromere regions) (indicated by arrows in Fig. 6J). The centromeric Rec8 signals often observed as doublets. The reason why centromeric Rec8 signals were observed as doublets is not clear, but we speculate that it may reflect the antibody's inability to access deep into the inner centromere regions. The Rec8 signals at centromere regions were detected up to metaphase II (Fig. 6F). In anaphase II, however, the signals of centromeric Rec8 were diminished (Fig. 6G). The Rec8 signal was not observed on mitotic chromosomes in spermotogonia (Fig. 6H). The localization and selective loss of Rec8 from meiotic chromosomes suggest that mammalian Rec8 protein plays pivotal roles in chromosome cohesion and separation in both meiosis I and II.

Discussion

Cohesin complexes in mammalian meiosis

It has been reported that *rec8* mRNA is specifically expressed in germ line cells, spermatocytes and oocytes (meiotic cells) and spermatids (post-meiotic cells), in mice and also in the thymus in human (Parisi et al., 1999; Lee et al., 2002). However, protein expression of Rec8 has not been examined in mammals. In accordance with the results of previous reports, the present study showed that mammalian Rec8 protein was specifically expressed in germ line cells, spermatocytes and spermatids. The biological meaning of the existence of Rec8 in spermatids is difficult to speculate, since spermatids do not need to divide any more. It might be merely a reminiscent of meiosis.

The possible components of mitotic and meiotic cohesin complexes in budding yeast and mammals, based on accumulated knowledge from the previous and present studies, are illustrated in Fig. 7. In budding yeast, mitotic cohesin complexes consist of at least Scc1p, Scc3p, Smc1p and Smc3p, and only the Scc1p subunit is replaced by Rec8p during

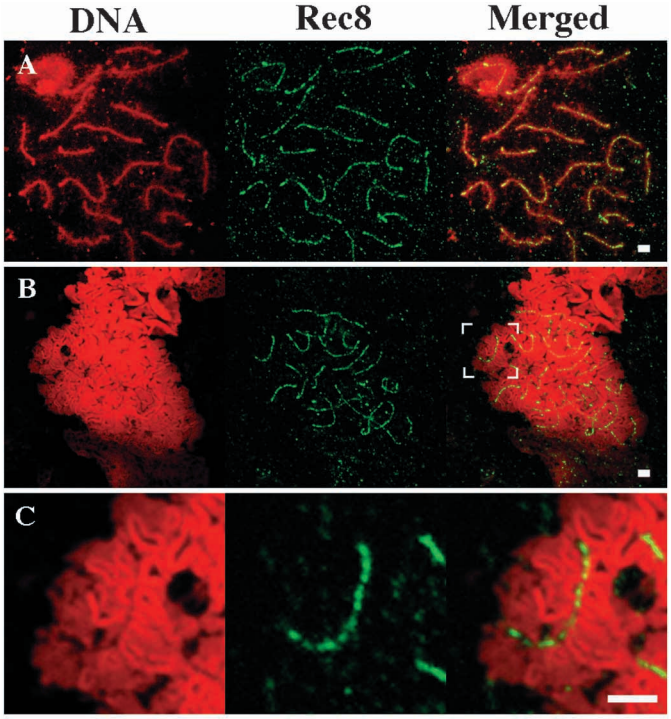


Fig. 5. Localization of Rec8 on chromatin loops in meiotic prophase. Nuclear spreads from mouse testicular cells were prepared by treatment of cells with 85 mM NaCl (A) or 75 mM KCl (B) before fixation in 1% paraformaldehyde with SDS, as described in Materials and Methods. The samples were immunofluorescently stained with anti-Rec8 antibody. DNA was counter-stained with propidium iodide. (C) Magnification of the region indicated in panel B. Bar, 2 μm.

meiosis. In fission yeast, two Scc3p homologs, Psc3p and Rec11p, have been identified, and Rec11p is required for chromatid cohesion in meiosis (Krawchuk et al., 1999; Tomonaga et al., 2000). Therefore, Rad21p and Psc3p are probably replaced by Rec8p and Rec11p during meiosis, respectively. Vertebrate cohesin complexes consist of, at least, SMC1, SMC3, SCC1/Rad21 and either one of SA1 and SA2 in mitosis (Losada et al., 2000; Sumara et al., 2000). In addition, mammalian meiotic cells express two other cohesin proteins, SMC1β and STAG3 (SA3), of which homologs have not been found in yeasts (Prieto et al., 2001; Revenkova et al., 2001). Our immunoprecipitation study revealed that mammalian Rec8 associates with SMC1β and SMC3 but not with SMC1α, suggesting that mammalian meiotic cohesin consists at least of Rec8 (Rad21 isoform), SMC1β (SMC1

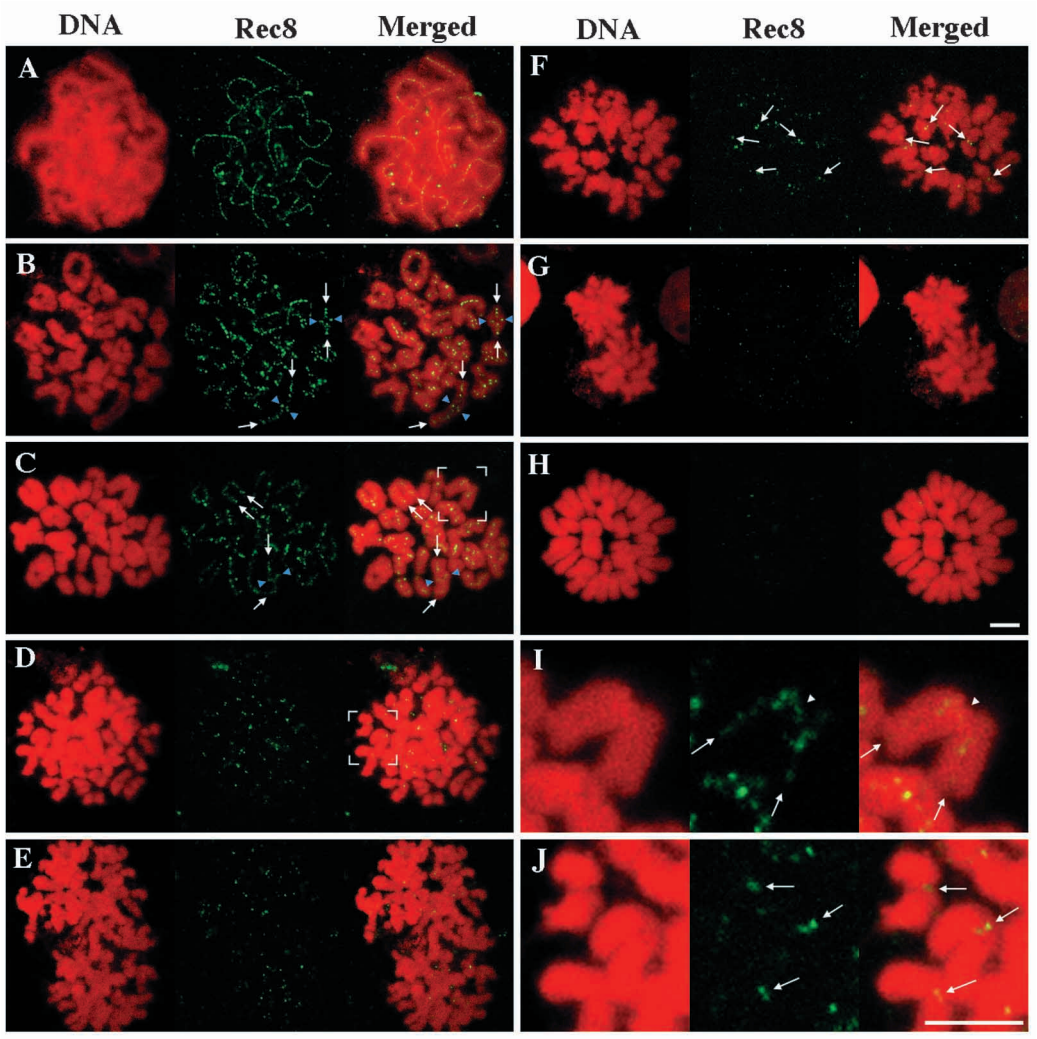


Fig. 6. Localization of Rec8 protein on chromosomes in meiosis I and II. Meiotic chromosome spreads were prepared to prevent chromatin dispersion by SDS during fixation. The chromosome spreads were immunofluorescently stained with anti-Rec8 antibody, and DNA was counter-stained with propidium iodide. (A) Pachytene; (B) diakinesis; (C) metaphase I; (D) early anaphase I; (E) late anaphase I; (F) metaphase II; (G) anaphase II; (H) mitosis. The regions indicated in C and D are magnified in I and J, respectively. Arrows indicate Rec8 signals on the interstices between sister chromatids. White arrowheads indicate Rec8 signals on the overlapped sites of two homologous chromosomes. Blue arrowheads indicate Rec8 signals on the interstitial axes between homologous chromosomes (chromosome arm regions distal to chiasmata). Bar, 5 μm.

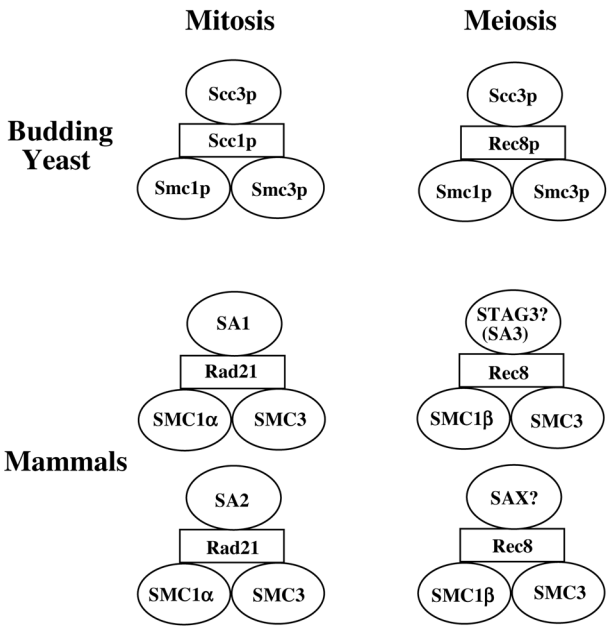


Fig. 7. Possible mitotic and meiotic cohesin complexes in yeast and mammals. In budding yeast, one of the mitotic cohesin subunits, Scc1p, is replaced by Rec8p in meiosis. In mammals, by contrast, two types of cohesin complex exist in mitosis; their components are SMC1α, SMC3, Rad21 and either one of SA1 and SA2. In meiosis, three proteins, SMC1α, Rad21 and SA1/SA2, are likely to be replaced by SMC1β, Rec8 and SA3/SAX (unidentified meiotic SA homolog) in the meiotic cohesin complexes, respectively.

isoform) and SMC3. However, close investigations into localizations of SMC1β and Rec8 in the previous and the present studies shed light on the difference that dissociation of SMC1β from chromosome arms occurs earlier than that of Rec8 in meiosis I. SMC1β dissociates from the chromosome arms in late prophase and diakinesis stages, and very little SMC1β, if any, is localized on the arms at metaphase I, whereas a considerable amount of Rec8 is still localized along chromosome arms at metaphase I. Therefore, Rec8 may associate with other as-yet-unidentified meiotic isoforms of SMC1 in addition to SMC1β. It is also possible that the difference between dissociation time of Rec8 and that of SMC1β from chromosome arms was caused by the difference in fixations utilized in the previous and present studies. STAG3 is known to be a meiotic isoform of SA1 and SA2 and to associate with SMC1 and SMC3, although it is not clear whether the STAG3-associated SMC1 is SMC1α or SMC1β or both (Prieto et al., 2001). In contrast to Rec8 and SMC1β, STAG3 is localized only in the arm regions of inter-chromatids in metaphase I. Furthermore, in late anaphase I and thereafter, STAG3 is not detected (Prieto et al., 2001). Therefore, even if Rec8 and SMC1β associate with STAG3, the association should be limited spatially and temporally to the arm regions of chromosomes until metaphase I. As has been previously proposed (Prieto et al., 2001), it is very likely that two or more mammalian cohesin complexes participate in meiosis. Furthermore, it has been shown recently that the mitotic cohesins, STAG2 and Rad21, are localized on axial elements during diplotene stage, but do not exist on the chromosome axes thereafter in meiosis (Prieto et al., 2002). Mitotic cohesins may also participate in meiotic chromosome behavior at some restricted time. In all cases, Rec8 is probably a common and essential component of meiotic cohesin complexes from yeast to human.

Rec8 is a component of AE/LEs of synaptonemal complexes in meiotic prophase I

The present study showed that Rec8 protein is expressed prior to the expression of SCP3 in spermatocytes, suggesting that Rec8 expression starts earlier than leptotene stage. Although it is not clear exactly when mammalian Rec8 synthesis starts, yeast Rec8p starts to be expressed from pre-meiotic DNA synthesis (Klein et al., 1999). After pre-meiotic DNA synthesis, chromosomes are arranged along proteinaceous axes called AEs, to which chromatin loops are attached during early meiotic prophase. As meiotic prophase proceeds, the AEs become aligned in parallel and incorporated in zipper-like structures (synaptonemal complexes), which are now called LEs. The mature synaptonemal complexes are a tripartite structure, two LEs and a central element (CE). Results of genetic and cytological studies suggest that synaptonemal complexes play roles in maintenance of homolog adhesion during meiotic prophase and possibly in facilitation of meiotic exchange (Walker and Hawley, 2000). In mammals, three proteins have been identified as the components of synaptonemal complexes; SCP1 (Syn1) is a component of CE, and SCP2 and SCP3 (Cor1) are components of LEs (Dobson et al., 1994; Lammers et al., 1994; Offenberg et al., 1998). SCP3 is a main determinant of AEs of synaptonemal complexes since *SCP3*-deficient spermatocytes fail to form AEs and thus synaptonemal complexes (Yuan et al., 2000). Recently, cohesin proteins, Rec8p and Smc3p, have been found along the AEs in yeast, and these proteins have been proved to be essential for the formation of synaptonemal complexes, since AEs are not formed in *rec8*-deleted or *smc3*-point mutants in budding yeast (Klein et al., 1999). In mammals, SMC1α, SMC1β, SMC3 and STAG3 are localized along AEs throughout prophase I from leptotene to diplotene stages (Eijpe et al., 2000; Prieto et al., 2001; Revenkova et al., 2001). In the present study, we found that mammalian Rec8 was also localized along the AE/LEs throughout meiotic prophase I. Therefore, all cohesin proteins so far examined are parts of components of AE/LEs of synaptonemal complexes. The role of cohesin complexes in AE/LEs is not clear, but it has been proposed that the cohesin core recruits recombination proteins and promotes synapsis between homologous chromosomes, since cohesin-containing chromosomal cores are formed and they are synapsed in meiotic nuclei in *SCP3*-deficient spermatocytes (Pelttari et al., 2001). In addition, Rec8 was localized only on the sites of chromatin loops adjacent to synaptonemal axes (Fig. 5C), suggesting that Rec8-containing cohesin cores determine the positions on chromatin loops at which synaptonemal axes are conjugated with the replicated DNAs.

Cohesion and separation of homologous chromosomes in meiosis I and of sister chromatids in meiosis II

In most organisms, the chiasmata and the arm cohesion distal to chiasmata link homologous chromosomes together, allowing them to align on the spindle in meiosis I. Cohesion along

chromosome arms is lost during meiosis I, while sister chromatids remain associated at centromeres until the onset of anaphase II. Loss of arm cohesion is required for the resolution of chiasmata and thus for separation of the homologs in meiosis I, whereas maintenance of cohesion at centromeres is needed for sister chromatids to separate properly in meiosis II (Miyazaki and Orr-Weaver, 1994). In mammals, localizations of SMC1β and STAG3 have been investigated to account for the meiosis-specific chromosome behavior. STAG3, from its localization as above mentioned, can possibly account for the selective loss of cohesion along chromosome arms in meiosis I but can not account for the maintenance of cohesion between sister centromeres until anaphase II. Conversely, SMC1β remains chromatin-associated at the centromeres up to metaphase II, but its dissociation from chromosome arms occurs so early, in diplotene stage, that SMC1β cannot maintain the cohesion between homologs until metaphsae I (Revenkova et al., 2001). In the present study, we showed that mammalian Rec8 is released along chromosome arm regions both proximal and distal to chiasmata, when homologs separate from each other in anaphase I, while Rec8 on inter-sister centromere regions is maintained until anaphase II (Fig. 8). This spatially and timely selective loss of Rec8 from chromosomes is concomitant with the two-step separation of chromosomes during meiosis. Thus, Rec8 is the only molecule so far examined in mammals that can, for itself, account for the meiosis-specific chromosome behavior in both meiosis I and II.

Then, how is the selective loss of Rec8 from chromosome arms in meiosis I and from centromeres in meiosis II regulated? In budding yeast, Rec8p is cleaved by separin, and this cleavage is essential for progression into anaphase I, since noncleavable mutations in Rec8p's potential separin cleavage sites, as well as mutations in separin itself, arrest cells at metaphase I (Buonomo et al., 2000). Furthermore, securin, the inhibitor of separin, is destroyed at the onset of anaphase in both meiosis I and II, probably through APC/Cdc20 pathway (Salah and Nasmyth, 2000). In contrast to the reports for yeast, however, APC activity and securin destruction are not required for the first meiotic division but are required for the second meiotic division in Xenopus oocytes, because microinjection of antibodies against APC activator or APC core subunit or microinjection of destruction-box peptide or methylated ubiquitin, does not affect progression through meiosis I and arrests the oocytes at metaphase II (Peter et al., 2001). Therefore, it is assumed that segregation of homologous chromosomes and that of sister chromatids are differentially regulated in higher eukaryotes. In somatic cells of vertebrates, most of the arm regions of sister chromatids are separated as chromosome condensation proceeds in prophase. Concomitantly, much of cohesin dissociates from the arm regions, although a small amount of cohesin persists predominantly in centromere regions until metaphase (Losada et al., 1998; Waizenegger et al., 2000). The dissociation of cohesin from chromosomes in prophase is independent of proteolytic cleavage, although the remaining cohesin at centromeres is finally destroyed by proteolytic cleavage of the subunit SCC1/Rad21 by separin at the metaphase-to-anaphase transition (Sumara et al., 2000; Waizenegger et al., 2000). Instead, the dissociation of cohesin from chromosome arms in prophase seems to be regulated by phosphorylation of cohesin proteins by mitotic kinases, Cdk1 kinase or polo-like kinase (Losada et al., 2000; Alexandru et al., 2001). The two-step separation of chromosomes in the first and second meiotic divisions is reminiscent of the two-step separation in prophase and anaphase in mitosis. Therefore, the selective loss of Rec8 during meiosis may be caused by a mechanism similar to that in mitotic division. However, it is notable that loss of Rec8 from chromosome arms occurs at a different time from that of mitotic cohesin in the cell cycle; Rec8 is released in anaphase during meiosis I, while SCC1/Rad21 is released in prophase during mitosis. Therefore, in mammals, it is possible that selective loss of Rec8 from the meiotic chromosomes depends on the partner(s) in the cohesin complex; for example, STAG3 (SA3)-associated Rec8 is released from chromosome arms at the onset of anaphase I, while SAX (unidentified SA isoform)-associated Rec8 on centromeres is maintained until the onset of anaphase II. Conversely, centromeric Rec8 may be protected from dissociation by (an) unidentified molecule(s) such as Mei-S332, which has been found on centromeres of meiotic chromosomes in Drosophila (Kerrebrock et al., 1995). These hypotheses are merely speculative in vertebrates and remain to be clarified.

In conclusion, we propose that Rec8 is an essential meiosis-specific cohesin and that its role in chromosome cohesion during meiosis is conserved from yeasts to mammals even though there may be several differences among species in its associated partners in cohesin complexes and in the

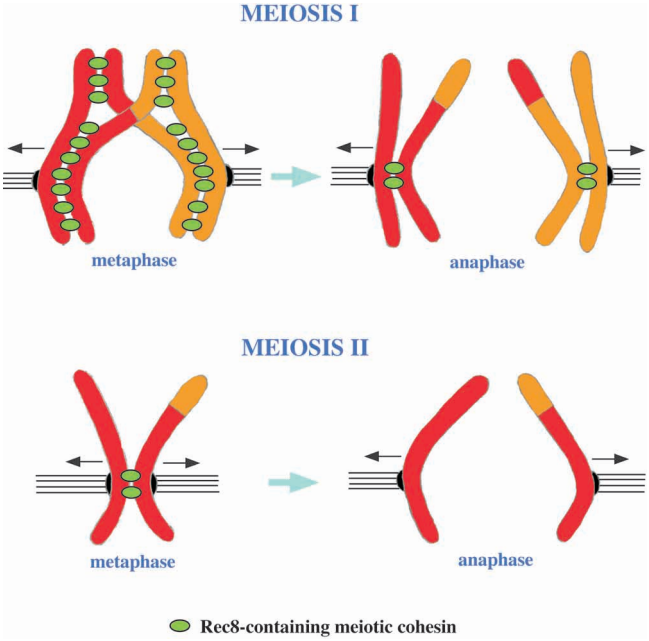


Fig. 8. Localization of meiotic cohesin complexes during mammalian meiotic divisions. Rec8-containing meiotic cohesin is localized along the chromosomal axes between sister chromatids, including the centromere regions, the arm regions both proximal and distal to chiasmata at metaphase I. At this stage, the arm cohesion distal to chiasmata is responsible for uniting homologous chromosomes together, and the resolution of them triggers separation of the homologs at anaphase I. Rec8 is released from the arm regions concomitantly with the separation of homologs. In metaphase II, Rec8-containing meiotic cohesin is localized at centromere regions at which sister chromatid cohesion is maintained. Release of Rec8 from the centromere regions coincides with the separation of sister chromatids in anaphase II.

mechanisms underlying its dissociation from chromosomes. The possible roles of Rec8 in the behavior of meiotic chromosomes are (1) maintenance of cohesion between homologous chromosomes until metaphase I by its association with chromosome arms distal to chiamata; (2) separation of homologous chromosomes in anaphase I by its selective dissociation from the chromosome arms; (3) maintenance of cohesion between sister chromatids until metaphase II by its association with centromere regions; and (4) separation of sister chromatids in anaphase II by its final dissociation from the centromere regions.

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