EMBO reports

scientific report

STAG2 and Rad21 mammalian mitotic cohesins are implicated in meiosis

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Received January 11, 2002; revised March 22, 2002; accepted March 28, 2002

STAG/SA proteins are specific cohesin complex subunits that maintain sister chromatid cohesion in mitosis and meiosis. Two members of this family, STAG1/SA1 and STAG2/SA2,‡ are classified as mitotic cohesins, as they are found in human somatic cells and in *Xenopus laevis* as components of the cohesin^{SA1} and cohesin^{SA2} complexes, in which the shared subunits are Rad21/SCC1, SMC1 and SMC3 proteins. A recently reported third family member, STAG3, is germinal cell-specific and is a subunit of the meiotic cohesin complex. To date, the meiosis-specific cohesin complex has been considered to be responsible for sister chromatid cohesion during meiosis. We studied replacement of the mitotic by the meiotic cohesin complex during mouse germinal cell maturation, and we show that mammalian STAG2 and Rad21 are also involved in several meiosis stages. Immunofluorescence results suggest that a cohesin complex containing Rad21 and STAG2 cooperates with a STAG3-specific complex to maintain sister chromatid cohesion during the diplotene stage of meiosis.

INTRODUCTION

Sister chromatid cohesion is a key event in chromosomal segregation during the cell cycle; it is maintained by a multisubunit protein complex termed cohesin (Michaelis *et al.*, 1997; Losada *et al.*, 1998; Sumara *et al.*, 2000). Regulation of this essential process differs in mitosis and meiosis. Whereas sister chromatids are separated during a single metaphase–anaphase transition in mitosis, in meiosis, sister arm cohesion and centromeric cohesion are subsequently lost in two division steps (for reviews, see Cohen-Fix, 2001; Lee and Amon, 2001; Lee and Orr-Weaver, 2001; Nasmyth, 2001; Uhlmann, 2001). This difference in chromosome behavior is reflected in the composition of the cohesin complex. In budding yeast *Saccharomyces cerevisiae* (Klein *et al.*, 1999) and in fission yeast *Schizosaccharomyces pombe* (Parisi *et al.*, 1999; Watanabe and Nurse, 1999), the mitotic cohesin Scc1 is replaced by a meiotic form, Rec8. In yeast meiosis, as for Scc1 in mitosis, proteolytic cleavage of Rec8 removes the cohesin complex from the chromatid arms, triggering anaphase I, while some Rec8 is maintained near the centromere, allowing correct segregation of the homologs (Buonomo *et al.*, 2000). Centromeric cohesion is lost during the transition from metaphase to anaphase II, and the sister chromatids segregate to give rise to two nuclei. Fission yeast have both Rec8 and an Scc3 homolog, Rec11, which is also the specific meiotic form of Scc3 (Krawchuck *et al.*, 1999). We reported recently that a mammalian Rec11 homolog, STAG3 (Pezzi *et al.*, 2000), is a cohesin specific to sister chromatid arm cohesion during meiosis I and is not present in meiosis II (Prieto *et al.*, 2001). Nonetheless, little is known of the putative participation of the so-called mitotic cohesin subunits in meiosis.

Our work on the role of cohesins in meiosis (Prieto *et al.*, 2001) led us to study the spatio-temporal replacement of the specific cohesin subunits. Anti-STAG1 and anti-STAG2 antibodies did not detect STAG1 and STAG2 in meiotic cells (Prieto *et al.*, 2001). To study their role in meiotic chromatid cohesion, we produced new anti-hSTAG1 and anti-hSTAG2 antibodies to different regions of these molecules. As Rad21 is a

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‡The previous *SA* nomenclature for the genes encoding this protein family has been replaced recently by *STAG* in the DDBJ/EMBL/GenBank database. Sequence accession numbers are NM005862, NM006603 and NM012447 for human STAG1, STAG2 and STAG3, respectively, and NM009282, NM021465 and NM016964 for mouse Stag1, Stag2 and Stag3, respectively.

partner of STAG1 and STAG2 in mitotic cohesin complexes, we generated anti-hRad21 antibodies to study its meiotic expression pattern.

RESULTS AND DISCUSSION

Changes in the cohesin complexes during early prophase I

We analyzed the reactivity pattern of a panel of anti-hSTAG1, anti-hSTAG2 and anti-hRad21 antibodies. A selected anti-hSTAG2 antibody showed an immunofluorescence signal in HEp-2 (human epithelial cell type 2) cells, concurring with the predicted behavior of a mitotic cohesin. It showed a speckled nuclear, non-nucleolar pattern during interphase, when the signal is most intense in G_2 (Figure 1A). In prophase (during DNA condensation), the STAG2 signal is released from chromatin (Figure 1B). After prophase, the STAG2 signal decreases and is found outside the chromosomes, as seen in metaphase (Figure 1C). Throughout the cell cycle, both the nuclear signal and the behavior of a selected anti-hRad21 antibody were similar to that of the anti-hSTAG2 antibody (Figure 1D–F).

In western blot analysis, anti-hSTAG2 and anti-hRAD21 antibodies specifically recognized bands of apparent molecular weights that concur with those reported for human STAG2 (Losada *et al.*, 2000) and Rad21 (Hoque and Ishikawa, 2001) (see Supplementary figure 1A available at *EMBO reports* Online). The presence of two STAG2 bands may be due to the different phosphorylation stages described for human STAG2 (Losada *et al.*, 2000). Anti-STAG2 antibody was specific and did not cross-react with STAG3 (see Supplementary figure 1B). We were unable to obtain specific anti-hSTAG1 antibodies suitable for these experiments.

We characterized STAG2 and Rad21 expression and localization in mouse spermatogenesis relative to the STAG3 distribution pattern. STAG2 immunofluorescence was intense in Sertoli and other somatic cells. STAG2 was also detected in premeiotic cells (spermatogonia), in which variable STAG2 and STAG3 expression levels were found. Some spermatogonia showed intense STAG2 immunofluorescence only (Figure 2A), whereas STAG2 and STAG3 were clearly visible in others, including a few cells in which STAG3 is the most abundant signal (Figure 2B).

STAG2 and STAG3 are coexpressed in pre-leptotene cells; a certain organization is already apparent at this stage, in which the signals do not overlap and begin to adopt a defined punctate distribution (Figure 2D). In zygotene, the STAG3 signal delineated the axial elements (AEs) of synaptonemal complexes (SCs), as reported previously (Prieto *et al.*, 2001), but STAG2 was not observed (Figure 2F). Similar results were obtained in pachytene spermatocytes, in which the STAG3 signal marked SCs (Figure 2G; Prieto *et al.*, 2001), whereas STAG2 was not observed. STAG2 staining was apparent in Sertoli cells in the same preparation (Figure 2G). The Rad21 pattern in spermatogonia (Figure 2C) is similar to that of STAG2 in the same cells (Figure 2B) and partially colocalizes with STAG3 (Figure 2C). Rad21 is visualized in leptotene as speckled nuclear staining (Figure 2E); in contrast, the signal is very low during zygotene and pachytene and is excluded from chromatin (Figure 2H).

The presence of STAG3 in some spermatogonia, which were characterized by morphological criteria (see Supplementary figure 2), permits speculation that STAG3 expression represents commitment to initiation of meiosis by these spermatogonia, which are producing the proteins required for subsequent stages. Concurring with this hypothesis, it is reported that neither SCC1 (Uhlmann and Nasmyth, 1998) nor REC8 (Watanabe *et al.*, 2001) are functional when expressed after DNA replication. The STAG3 signal remains during later maturation stages, until its disappearance at the transition from metaphase to anaphase I (Prieto *et al.*, 2001). We propose that, in the pre-meiotic S phase, both mitotic cohesin$^{STAG2/SA2}$ and meiotic cohesin^{STAG3} complexes bind to chromatin. Subsequent structural changes induce release of the cohesin$^{STAG2/SA2}$ complex, maintaining the cohesin^{STAG3} complex to generate a structure compatible with subsequent SC formation. STAG3 would thus have a critical role in specific chromosomal organization events during the pre-leptotene stage.

STAG3 and REC8 colocalize along the chromosome arms in pachytene

The cohesin REC8 replaces Rad21/SCC1 during meiosis in yeast (Parisi *et al.*, 1999; Buonomo *et al.*, 2000) and *Caenorhabditis elegans* (Pasierbek *et al.*, 2001). Using various anti-hREC8 antibodies, we detected REC8 immunofluorescence in pachytene mouse spermatocytes as linear structures that marked the chromosome axes (Figure 3A), similar to the reported STAG3 immunofluorescence signal (Prieto *et al.*, 2001). Detailed analysis of the REC8 signal showed clear differences with STAG3. Both STAG3 and REC8 signals colocalize along the chromosome axis but not at chromosome ends, in which only REC8 is observed (Figure 3). We reported previously that STAG3 colocalized only partially with the inner portion of the centromere signal (Prieto *et al.*, 2001). These results concur with specific STAG3 involvement in chromatid arm cohesion and suggest a role for mammalian REC8 in arm and centromeric cohesion, described to date only for yeast Rec8 cohesin (Klein *et al.*, 1999).

STAG2 and Rad21 mitotic cohesins are also present in diplotene stage

Homolog desynapsis and SC breakdown characterizes the end of pachytene and the beginning of diplotene. In diplotene cells, fragments of desynapsed AEs of SCs are seen by STAG3 immunofluorescent staining (Prieto *et al.*, 2001). Figure 4A shows a diplotene cell, surrounded by pachytene cells, in which the STAG2 signal coincides with decondensed chromatin but not with the chromosomal axis. In the XY pair, in which synapsis and desynapsis are not synchronous relative to autosomes (Moses, 1980), the axis is clearly labeled by STAG3, whereas STAG2 is not observed. Rad21 is also observed on diplotene cell chromosomes, but in this case we found Rad21 in both decondensed DNA and the chromosome axis of desynapsed SCs (Figure 4B). Comparison of STAG3 and Rad21 immunofluorescence along the desynapsed diplotene chromosomes showed Rad21 in those regions in which the STAG3 signal is weak or absent (Figure 4C). In many organisms, the beginning of diplotene is referred to as the diffuse stage and compared to G_2 in a mitotic

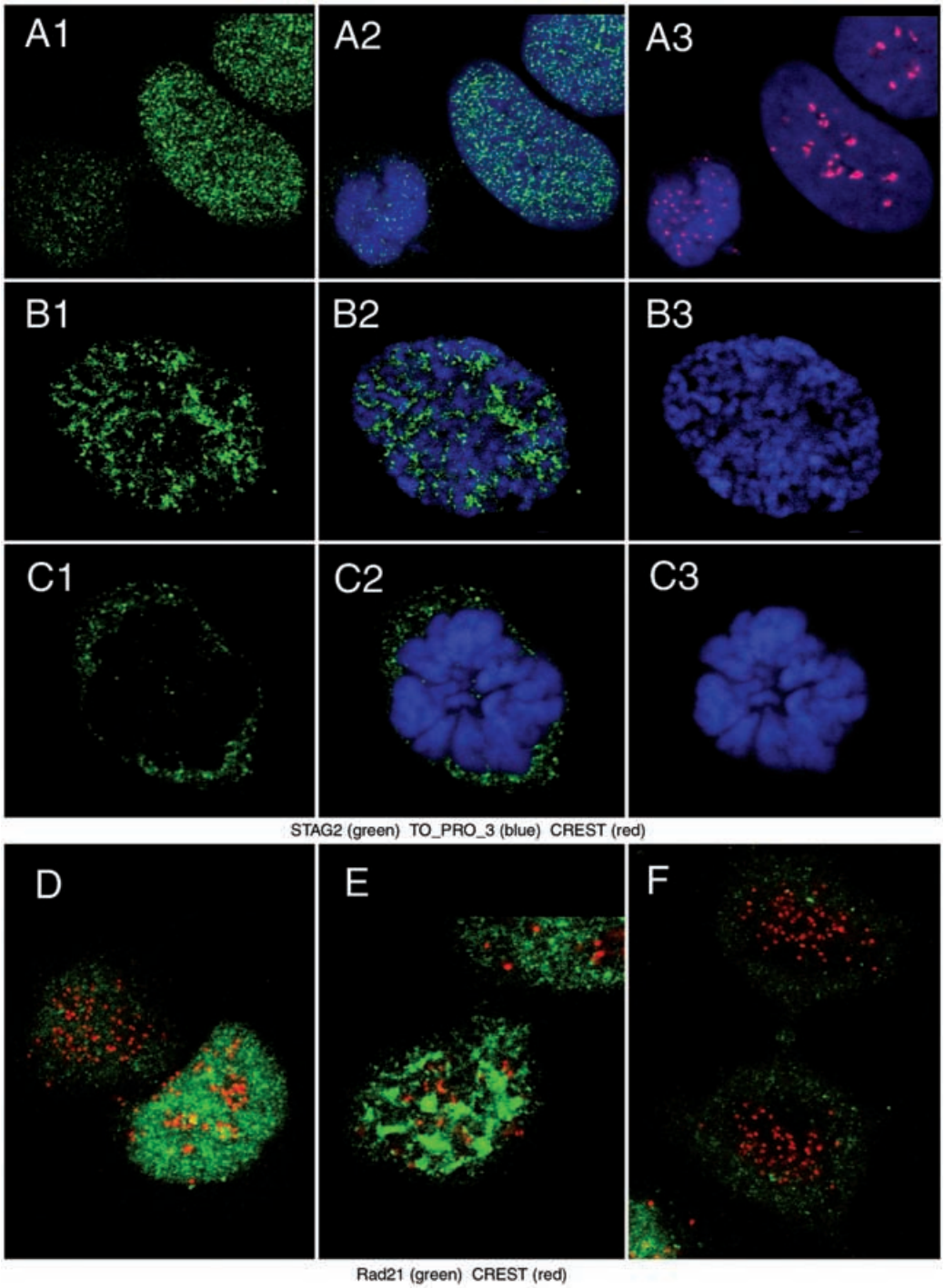

Fig. 1. STAG2 and Rad21 expression patterns during the human somatic cell cycle. HEp-2 cells were stained for STAG2 or Rad21 cohesins (green), centromeres (red) and DNA (blue). (**A**) Confocal layers of HEp-2 cells in G_1 (left) and G_2 (right). Staining of STAG2 (A1), STAG2 and DNA (A2) and centromeres and DNA (A3). (**B**) Confocal layer of a prometaphase cell stained for STAG2 (B1) , STAG2 and DNA (B2) or DNA alone (B3). (**C**) Confocal layer of a metaphase cell stained for STAG2 (C1), STAG2 and DNA (C2) or DNA alone (C3). (**D–F**) Rad21 and centromere staining of interphase (D), prophase (E) and telophase (F) stages.

cell cycle. It has been suggested that new proteins may be added at this stage to maintain sister chromatid cohesion (Stack and Anderson, 2001). Mitotic cohesin complex binding to STAG3-free regions may thus be needed to maintain chromatid arm cohesion during desynapsis in diplotene.

In prometaphase I and metaphase I, STAG3 was found between sister chromatids (Figure 4D–F; Prieto *et al.*, 2001); STAG2 (Figure 4D and E) and Rad21 (Figure 4F) signals were also observed outside the chromosomes. During anaphase I, STAG2 and Rad21 showed similar behavior. At early anaphase I, STAG2

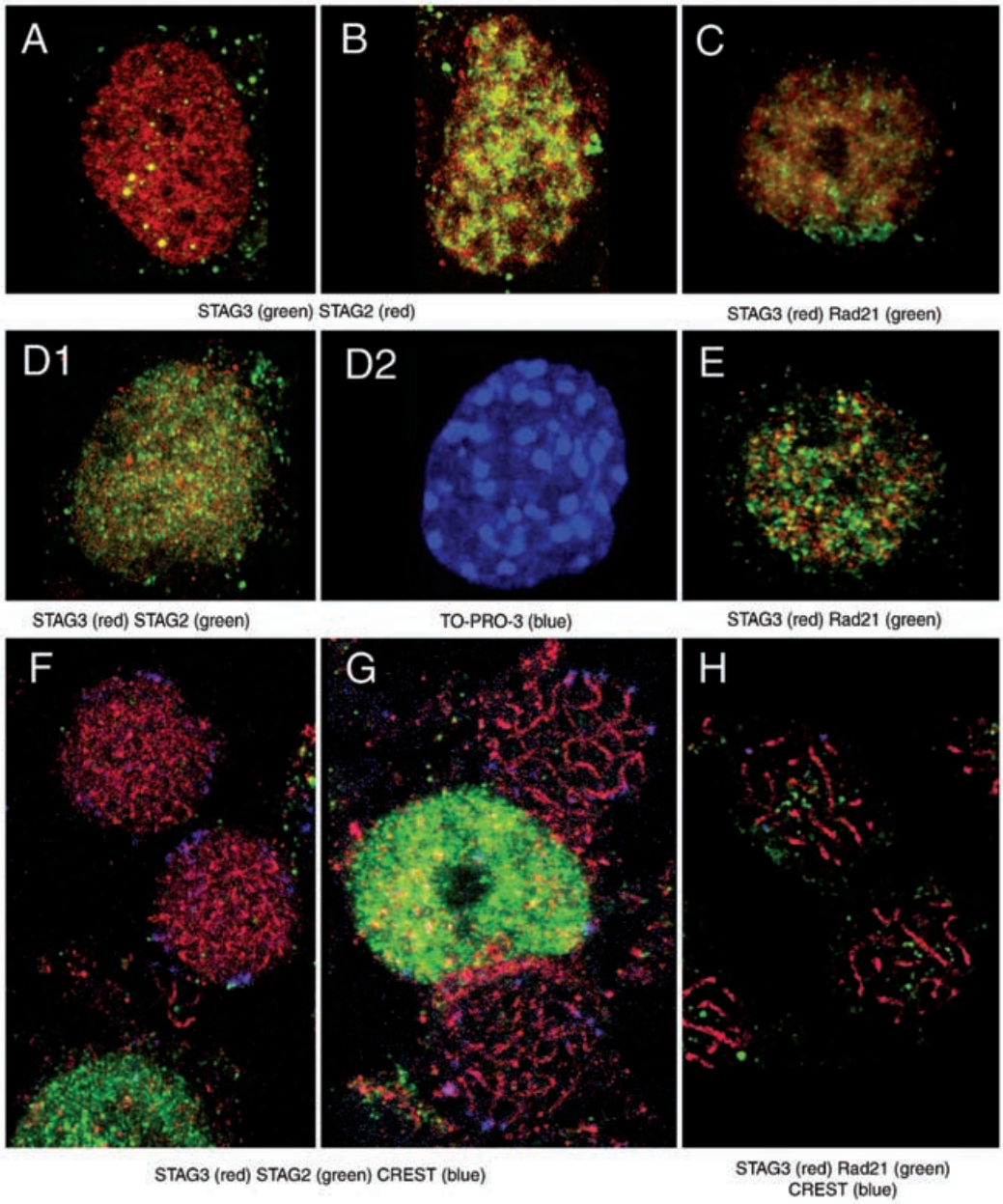

Fig. 2. Expression of mitotic and meiotic cohesins in spermatogonia and cohesin replacement during early mouse prophase I. (**A** and **B**) Differential staining of spermatogonia with STAG3 (green) and STAG2 (red). (**C**) Spermatogonium stained with STAG3 (red) and Rad21 (green). (**D**–**E**) Staining of a pre-leptotene cell for STAG3 (red) and STAG2 (green) (D1), DNA (blue) (D2) and for STAG3 (red) and Rad21 (green) (E). (**F**) Zygotene spermatocytes stained for STAG3 (red), STAG2 (green) and centromeres (blue). Part of a Sertoli cell is shown at the bottom. (**G**) Pachytene spermatocytes stained as in (F), with a Sertoli cell between them. (**H**) Pachytene spermatocytes stained with Rad21 (green) rather than STAG2. As a control, red and green dyes were interchanged for all three cohesins (data not shown).

immunofluorescence was not associated with chromatin (Figure 4G) and STAG3 was seen only near the centromeric region (Figure 4G, inset). In late anaphase I, neither STAG3 nor Rad21 signals colocalized with DNA (Figure 4H).

The most distinctive feature of meiosis compared to mitosis is the formation and destruction of a specific structure, the SC, in prophase I. This original structure regulates recombination and supports crossing-over and DNA repair; new cohesin molecules, such as REC8 and STAG3, are thus required to maintain sister chromatid cohesion. In fact, the replacement of a meiotic cohesin (REC8) with its mitotic version (SCC1) allows sister chromatid cohesion but cannot support SC formation (Nasmyth, 2001).

Regarding the other components of the cohesin complex (SMC proteins), a meiosis-specific SMC1 isoform, termed SMC1β, has been described (Revenkova *et al.*, 2001). Biochemical and immunocytological studies showed that SMC1, now termed SMC1α, and the newly characterized SMC1β, have distinct SC localizations. Two complexes containing different SMC1–SMC3 cores have been proposed in meiotic cells (Revenkova *et al.*, 2001). SMC1α–SMC3 appears to be associated with the AEs,

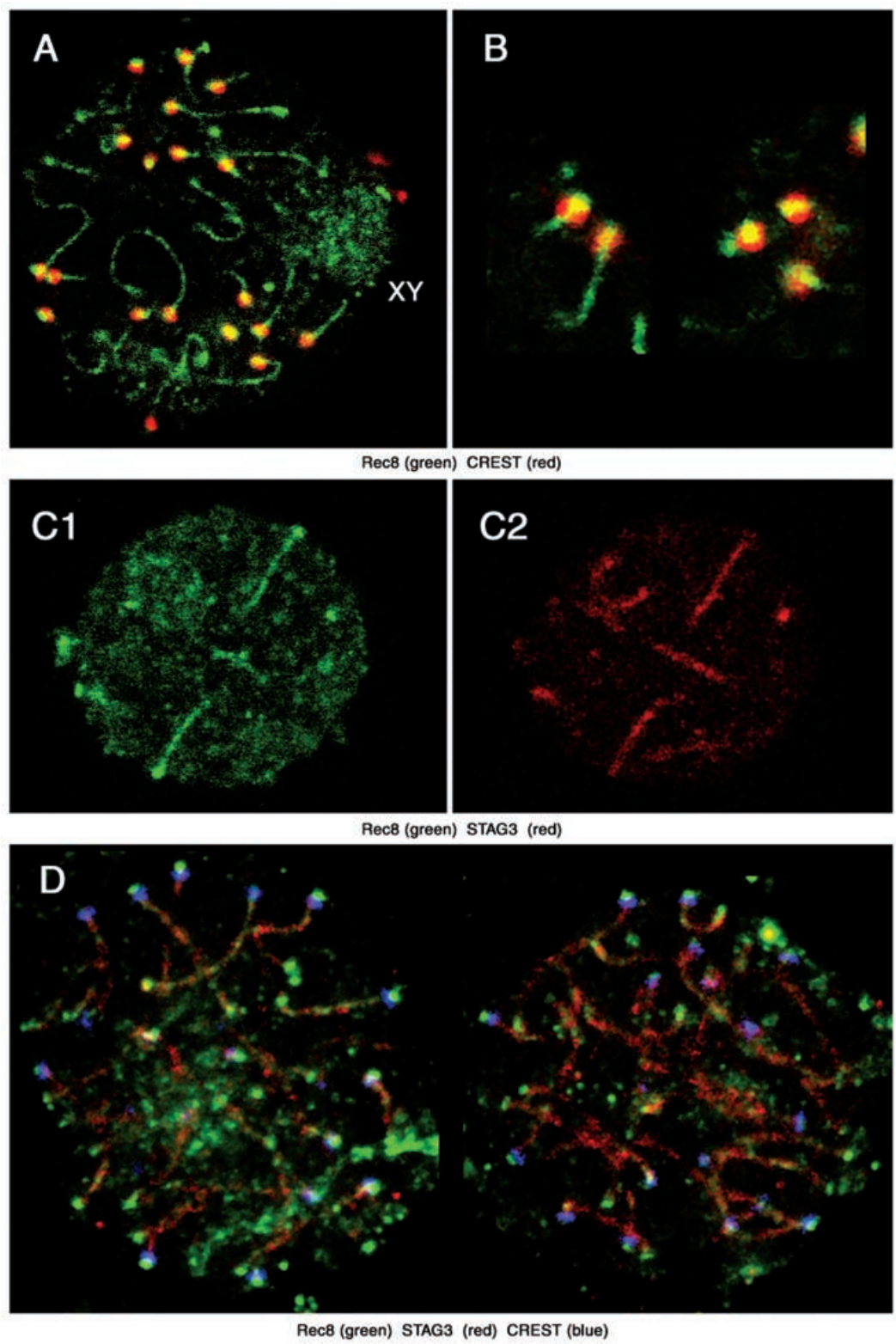

Fig. 3. Colocalization of REC8 and STAG3 along sister chromatid arms but not at chromosome ends in mouse pachytene spermatocytes. (**A**) Mouse pachytene spermatocyte stained for REC8 (green) and centromeres (red). The XY pair shows diffuse staining. (**B**) REC8 staining in the centromeric region is shown at a higher magnification. (**C**) Confocal layer stained with REC8 (green) (C1) and STAG3 (red) (C2). (**D**) Pachytene mouse spermatocytes stained with anti-REC8 (green), STAG3 (red) and centromere (blue) antibodies. STAG3 staining reaches the inner portion of the centromeric region, whereas REC8 also stains the chromosome end.

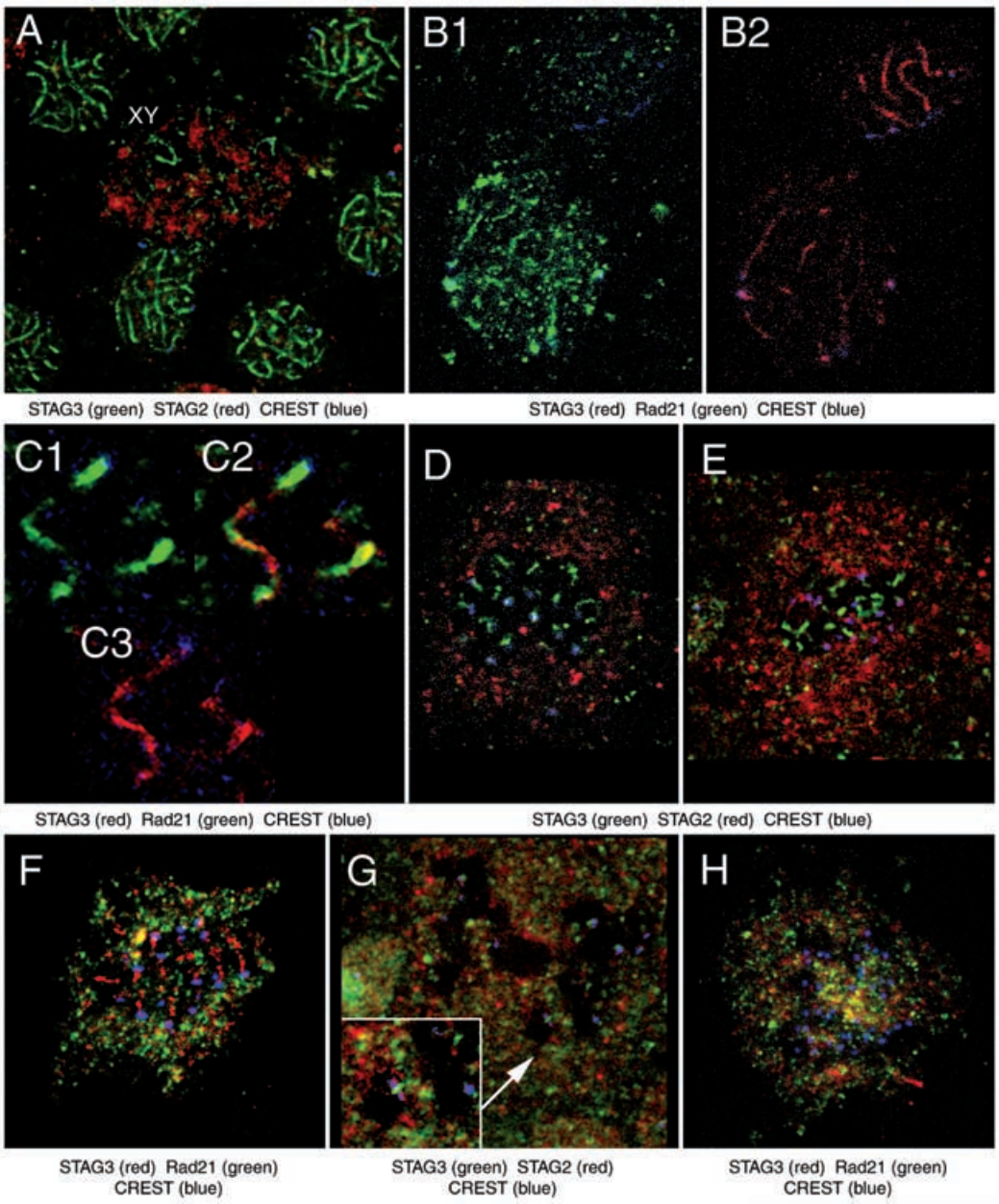

Fig. 4. STAG2 and Rad21 appear again in diplotene spermatocytes. (**A**) Confocal layer of a late diplotene cell stained for STAG3 (green) and STAG2 (red), surrounded by pachytene cells. The XY asynchronous pair shows STAG3 but not STAG2 staining. In all panels, centromeres are blue. (**B**) Layer of a diplotene (bottom) and a pachytene (top) cell stained with Rad21 (green) (B1) or STAG3 (red) (B2). (**C**) Enlarged detail of AEs of desynapsed chromosomes from a diplotene spermatocyte stained with Rad21 (green) (C1), STAG3 (red) (C3) or merged (C2). (**D**) Prometaphase I and (**E**) metaphase I. Confocal layers of cells stained with STAG3 (green) and STAG2 (red). In both stages, only STAG3 remains associated with chromosomes, whereas STAG2 is visualized outside the chromatin. (**F**) A metaphase cell stained with STAG3 (red) or Rad21 (green). As for STAG2 (E), no Rad21 signal was observed on the DNA. (**G**) Early anaphase I. A confocal layer stained with anti-STAG3 (green) and STAG2 (red) antibodies; only a residual centromere-associated STAG3 signal is shown (inset). (**H**) Anaphase I. A layer stained with anti-Rad21 (green) and STAG3 (red) antibodies. DNA-associated cohesin signals are not detected.

where it is seen as a punctate pattern, as well as in the chromatin loops, whereas SMC1β–SMC3 is closely localized along the AEs of the SC.

Speculation

Although this work is essentially cytological, and conclusions on localization/functional relationships are thus limited, our studies suggest involvement of the STAG2 and Rad21 mitotic cohesins in sister chromatid cohesion in meiosis and support participation by several multiprotein cohesin complexes in meiosis I. The Rad21/STAG2/SMC1α/SMC3 mitotic protein complex, which maintains cohesion in mitosis until the last gonial stages, is replaced by a specific meiotic cohesin complex putatively composed of REC8/STAG3/SMC1β/SMC3, which is bound to

chromatin during pre-meiotic S phase and later visualized as fibers during AE formation. We speculate that the cohesin complex containing STAG3 associates with discrete chromatin domains to form a specific structure in which components of AEs of the SC, such as SCP3 protein, assemble. Pelttari *et al.* (2001) recently reported that the cohesin core can maintain a chromatin structure sufficient for recruiting recombination and transverse filament proteins in the absence of AEs in *Scp3*-deficient mouse spermatocytes.

When the SC is formed, STAG3 binding domains on chromatin are brought together and the STAG3 signal is visualized as a linear structure (Prieto *et al.*, 2001), whereas neither Rad21 nor STAG2 were detected in association with the SC. Concurring with these results, Eijpe *et al.* (2000) reported that Rad21 is under-represented in SCs and mouse spermatocyte nuclear preparations. After pachytene, both SC desynapsis and chromatin decondensation occur. A new chromatin condensation process then takes place in the formation of the metaphase chromosome. During these processes, the discontinuous STAG3 signal may be produced by release of part of the STAG3, spacing out of STAG3 binding domains, or both. Our results suggest that in diplotene, following the course of desynapsis, a Rad21/STAG2 core complex assembles to chromatin by binding mainly to chromatin loops. Cohesion is known to be functional only if established during S phase (for a review, see Nasmyth, 2001); thus, either Rad21 and STAG2 are not working as cohesins in diplotene or these proteins cooperate in cohesion at this stage by an S-phase-independent mechanism. In the transition to metaphase I, the chromosome is condensed by condensin action and the Rad21/STAG2 core complex is removed from DNA. Sister chromatid arm cohesion in metaphase I is maintained by the remaining REC8/STAG3/SMC1β/SMC3 located at the interchromatid domain. This complex is finally released during the transition from metaphase I to anaphase I. Centromeric cohesion until anaphase II, necessary for correct chromatid segregation, is maintained by a complex containing REC8/SMC1β/SMC3, concurring with the persistence of mammalian REC8 (Nasmyth, 2001) and SMC1β (Revenkova *et al.*, 2001) at the centromere in meiosis II.

Emerging data suggest that different combinations of cohesin subunits may form various functional cohesin complexes that act in distinct stages of mitosis and meiosis. This is the first evidence of a role for mitotic non-SMC cohesins in mammalian meiosis and contributes to the characterization of regulatory mechanisms in mitosis and meiosis.

METHODS

Primary and secondary antibodies. Rabbit K828 anti-STAG2 antibody was raised against a synthetic peptide of the final 16 amino acids of the human STAG2 protein sequence (Carramolino *et al.*, 1997). A mouse polyclonal antibody (m228) was raised against a human STAG3 fragment (hSTAG3 ORF amino acids 626–757) expressed in *Escherichia coli* (Pezzi *et al.*, 2000). Rabbit K854 anti-Rad21 antibody was raised against amino acids 546–565 of human Rad21 (MacKay *et al.*, 1996) and rabbit K775 anti-REC8 antibody was raised against the last 143 amino acids of human REC8 protein (Parisi *et al.*, 1999). Specificity of anti-STAG2 and anti-Rad21 antibody was confirmed by competition assays with the appropriate synthetic peptide. As a control, centromeres were visualized using human CREST scleroderma serum (del Mazo *et al.*, 1986). All secondary antibodies were from goat. Anti-mouse IgG Alexa 488 and anti-rabbit IgG Alexa 488 were from Molecular Probes (Eugene, OR). Anti-mouse IgG Cy3, anti-human IgG Cy3 or Cy5 and anti-rabbit IgG Cy3 antibodies were obtained from Jackson Laboratories (West Grove, PA). For HEp-2 immunofluorescence experiments, rabbit antibodies were diluted 1:500 and human anti-centromere serum 1:3000. Primary antibodies were diluted in PBS with 5% goat serum (Gibco-Life Technologies, Gaithersburg, MD). Secondary antibodies were used in the same conditions, at a 1:500 dilution. For the squash procedure, rabbit and mouse polyclonal antibodies were diluted 1:50 and human anti-centromere serum 1:1000. Primary antibodies were diluted in PBS containing 10% goat serum. Secondary antibodies were used in the same conditions, at a 1:200 dilution.

Immunofluorescence on HEp-2 slides. Paraformaldehyde-fixed HEp-2 slides (BIOS) were used for immunofluorescence analysis of STAG2 and Rad21 distribution during the cell cycle. Samples were incubated (15 min) in PBS with 10% goat serum (Gibco), washed twice with PBS and incubated (45 min) with primary antibodies. After two washes in PBS, antibodies were detected by incubation (45 min) with secondary antibodies. Cells were washed twice in PBS and mounted with Vectashield anti-fading medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). All steps were performed at room temperature. TO-PRO-3 iodide (1 μM, Molecular Probes) was used for DNA staining. Samples were analyzed under a Leitz DMIRB epifluorescence microscope and a Leica TCSNT confocal laser scanning microscope. Images were noise-filtered, corrected for background and processed using Adobe Photoshop.

Immunofluorescence on squashed spermatocytes. To analyze cohesin distribution in mouse spermatocytes, we used a previously described squash procedure (Page *et al.*, 1988). Briefly, mouse testes were removed and seminiferous tubules cleared (10 min) in PBS. Selected tubule sections were fixed (10 min) in freshly prepared 2% formaldehyde (Merck, Darmstadt, Germany) in PBS containing 0.05% Triton X-100 (Sigma, St Louis, MO). Several seminiferous tubules were placed on a 1 mg/ml poly-L-lysine (Sigma)-coated slide with a drop of fixative and squashed, and the coverslip was removed after freezing in liquid nitrogen. Slides were rinsed in PBS (twice, 5 min each) and processed as for HEp-2 samples.

Western blotting and immunoprecipitation. Nuclear extracts from mouse organs and human cells were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce, Rockford, IL). Nuclear extract supernatants contained 1–1.5 mg/ml protein. For western blotting, proteins were resolved by SDS–PAGE and blotted with anti-STAG2 (K828), anti-Rad21 (K854) and anti-STAG3 (m228) antibodies (all at a 1:1000 dilution).

Immunoprecipitation experiments were performed as described previously (Prieto *et al.*, 2001). For western blotting, proteins were resolved by SDS–PAGE and probed with indicated sera (all at 1:1000 dilution). Volumes loaded into immunoprecipitate wells were four times higher than those loaded into the extract wells.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

ACKNOWLEDGEMENTS

We thank A. Valdeolmillos, J.S. Rufas and J.A. Suja for helpful discussions of the experimental work and C. Mark for editorial assistance. The Department of Immunology and Oncology was founded and is supported by the Spanish Council for Scientific Research (CSIC) and by the Pharmacia Corporation.

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DOI: 10.1093/embo-reports/kvf108