SUPPLEMENTARY RESULTS

Characterization of RAD21/SCC1 antibodies

We generated two antibodies raised against different regions of human RAD21/SCC1 (Supplementary Fig 1A). RAD21 Ab1 was raised in sheep against a recombinant protein corresponding to an internal part (amino acid 195-280) of human RAD21/SCC1, which is poorly conserved with mouse REC8. RAD21 Ab2 was produced in rabbits using a 14-mer synthetic peptide (YSDIIATPGPRFHII), which corresponds to the C-terminus of RAD21/SCC1 and has five amino acids identical to REC8 (Supplementary Fig 1A). Both of these regions are 99% identical to mouse RAD21/SCC1 (McKay *et al.*, 1996). Both antibodies recognized the 120 kDa product of *in vitro* translated human RAD21/SCC1 but not the control DNA product, suggesting that the antibodies are specific to RAD21/SCC1 protein (Supplementary Fig 1B). We then tested the specificity of these antibodies using HeLa cell extracts. Both antibodies reacted strongly with a protein band of approximately 120 kDa, which corresponds with the size of RAD21/SCC1 protein on SDS-PAGE, as reported in several previous studies (Supplementary Fig 1C, Hauf *et al.*, 2001; Hoque and Ishikawa, 2001). Additional bands with reduced signal intensity were also observed, particularly with the C-terminal antibody, RAD21 Ab2 (Supplementary Fig 1C). We noted that the intensity of the 43-45 kDa protein band appeared to increase as the signal intensity of the 120 kDa band decreased, suggesting that the 43-45 kDa polypeptide is likely a proteolytic cleavage fragment of RAD21/SCC1. Other bands that reacted weakly with the antibodies may represent the proteolytic cleavage products of RAD21/SCC1 protein or cross-reactive epitopes of other proteins. Similar results were obtained when mouse testicular extracts were probed with the antibodies (Supplementary Fig 1D).

We next evaluated the ability of the rabbit polyclonal antibody, RAD21 Ab2, which recognizes the C-terminus of RAD21/SCC1, to immunoprecipitate SMC proteins from extracts of testicular and somatic (mouse embryonic fibroblast) cells. The presence of RAD21/SCC1 in the immunoprecipitates was confirmed by probing the same blot with both RAD21/SCC1 antibodies (Supplementary Fig 2A). As expected, SMC1 was recovered from the immunoprecipitates of testes and somatic cells (Supplementary Fig 2A). Since this SMC1 antibody reacts with both SMC1*a* and SMC*b*, the signal detected in the immunoprecipitates of testicular extracts is likely to represent both forms of SMC1. However, we could not resolve the two proteins given that they have a very similar molecular mass (Revenkova *et al.*, 2001). SMC3, which is present in both mitotic and meiotic cohesin complexes (Jessberger, 2002), was co-immunoprecipitated with RAD21/SCC1 from both somatic and testicular cell extracts (Supplementary Fig 2A). The antibodies did not react with apparent bands of 80-100 kDa in the immunoprecipitates (Supplementary Fig 2B), indicating that they have no or negligible cross-reactivity with REC8 proteins (Eijpe *et al.*, 2003; Lee *et al.*, 2003). Collectively, these data demonstrated that the antibodies produced are specific for the RAD21/SCC1 protein.

Characterization of RAD21/SCC1 antibodies in HeLa cells

We further examined the specificities of the antibodies in somatic HeLa cells by indirect immunofluorescence. Controls were performed in parallel by either omitting primary antibodies or using pre-immune sera (Supplementary Fig 3A-B and Supplementary Fig 4A-B). RAD21 Ab1 displayed various degrees of nuclear staining, ranging from very strong to barely detectable in interphase cells (Supplementary Fig 3C). However, it was excluded from nucleoli in all cells. A weak cytoplasmic staining was observed in all cells examined. In mitotic cells, staining was absent from condensed chromosomes and was most intense outside chromosomes (Supplementary Fig 3D). This pattern of staining by RAD21 Ab1 closely resembles that of other published reports for SCC1-Myc (Waizenegger et al, 2000) and RAD21 (Hoque *et al.*, 2001). Staining of cells arrested at metaphase by colcemid showed patchy areas of intense staining on chromosomes (Supplementary Fig 3E). RAD21 Ab2 stained nucleoli while it did not bind to any other parts of nuclei during interphase, displaying a pattern of nuclear staining complementary to RAD21 Ab1 (Supplementary Fig 4C). It bound patchily to areas of chromosomes along the metaphase plate in metaphase cells and to centromeres of metaphase-like chromosomes in colcemid treated-cells (Fig 1A and 1B). No obvious chromosome staining was detected in anaphase (Fig 1C). Similar to RAD21 Ab1, a weak staining was detected outside nuclei throughout the cell cycle. The differential patterns of nuclear staining for the two antibodies may reflect the accessibility of epitopes. It appears that RAD21 Ab2 may have higher affinity to centromeric RAD21/SCC1.

Analysis of RAD21/SCC1 antibodies in mouse testicular cells

Since a previous study using *in situ* hybridization showed that *Rad21*/*Scc1* expression was confined to spermatogonia in mouse testis (Lee *et al.*, 2002), we tested both antibodies against testis cell fractions. Spermatogonia (mitotic cells) were enriched from 7 day-old mice. Early meiotic prophase mixed leptotene and zygotene spermatocytes, and early pachytene spermatocytes were enriched from 17 day-old mice, and pachytene spermatocytes (predominately mid- to late- stage) were enriched from adult mice. The cell purity of each fraction was higher than 80% as assessed by microscopy. The presence of spermatogonia in the fractions enriched for meiotic cells is considered to be unlikely since they are very different in size and difficult to isolate from testes of mice more than eight day-old used for isolating meiotic cells. Furthermore, the enrichment of pachytene spermatocytes was verified using an anti-SCP3 (Supplementary Fig 5A, right panel).

RAD21 Ab1 reacted strongly to the RAD21 band (approximately 120 kDa) in spermatognia and leptotene/zygotene spermatocytes but less strongly in pachytene spermatocytes (Supplementary Fig 5A, left panel). In all cell types a slightly faster migrating band was detected (Supplementary Fig 5A, left panel). A 43-kDa band, which was detected in HeLa cell extracts, was present in all three types of enriched cells (Supplementary Fig 5A, left panel). In a parallel experiment, RAD21 Ab2 showed strong reactivity to the 120 kDa RAD21 band in cells enriched for spermatogonia and leptotene/zygotene spermatocytes (Supplementary Fig 5B). The signal intensity for the 120-kDa band decreased dramatically in cells enriched for pachytene spermatocytes (Supplementary Fig 5B). Judging by the appearance of the band, it appeared that several faster migrating bands reacted weakly with this antibody. These fast migrating bands may represent the different phosphorylation state of RAD21 protein. Our results are consistent with a previous report which showed a low level of RAD21/SCC1 in the synaptonemal complexes isolated from rat spermatocytes (Eijpe *et al.*, 2000). The negative staining in mouse spermatocytes reported by Lee *et al* (2002) is presumably due to the sensitivity of the detection method used.

Since pachytene spermatocytes enriched from adult mice represent primarily mid- to late pachytene cells, we further tested early pachytene spermatocytes enriched from prepubertal (17 day-old) mice using RAD21 Ab2 in an independent experiment. The result confirmed that RAD21SCC1 protein is present in all cell types examined (Fig 2B). Collectively, the results of both experiments were consistent, demonstrating the presence of RAD21/SCC1 in meiotic prophase cells.

Analysis of RAD21/SCC1 antibodies on meiotic chromosome spreads by indirect immunofluorescence

We next tested the specificities of RAD21/SCC1 antibodies on chromosome spreads of spermatocytes by indirect immunofluorescence. Controls performed in parallel by omitting primary antibodies, as well as using pre-immune sera, did not show any specific signal on meiotic chromosomal cores although cross-reactivities to sperm-heads and tails by both anti-sheep and anti-rabbit secondary antibodies were observed (Supplementary Fig 6). To ensure the signal specificity we documented the staining pattern of both antibodies separately (Supplementary Fig 7 and 8). The third antibody raised to human SCC1 (Weizenegger *et al.*, 2000) was used to further verify the specificity (Supplementary Fig 9). The number of cells documented for each meiotic stage varied according to the abundance of each type of cell in the testis. In typical meiotic chromosome spreads from adult mice, we obtain 2% leptotene, 11% zygotene, 54% pachytene, 25% diplotene, 0.6% metaphase I and 6.2% anaphase II cells. At least 50 cells for the more abundant pachytene spermatocytes were examined, 20 cells for diplotene spermatocytes, 5 cells for leptotene/zygotene and metaphase/anaphase I spermatocytes, and 3–5 cells for anaphase II. Metaphase II cells are extremely rare in our chromosome spread preparation and we were able to find only two metaphase II cells for RAD21 Ab1. Anaphase II cells were not recorded for RAD21 Ab2.

All three antibodies exhibited an identical pattern of staining in the key stages of meiosis, including leptonema, zygonema, pachynema, diplonema, metaphase I and anaphase II (Supplementary Fig 7, 8 and 9). In general, RAD21 Ab1 displayed a similar signal intensity to anti-SCC1, whereas a slightly weaker staining on chromosome arms by RAD21 Ab2 was observed. As RAD21/SCC1 shares low-level homology with REC8, the question arises whether the signal detected by these antibodies might represent REC8. We consider this possibility unlikely. Firstly, our antibodies fail to detect any protein bands of 80-100 kDa which display the characteristics of REC8, as reported, on Western blot analyses of total testis extract (Supplementary Fig 1D), enriched spermatocytes (Supplementary Fig 5) and immunoprecipitates (Supplementary Fig 2B). Secondly, the pattern of staining in several key meiotic stages is distinct from that reported for REC8 (Eijpe *et al.*, 2003; Lee *et al.*, 2003). For example, our results showed that the antibodies stained strongly in centromeric and telomeric regions in late diplonema (Supplementary Figs 7D, 8D and 9D). This is not observed for REC8 (Eijpe *et al.*, 2003; Lee *et al.*, 2003). REC8 was detected along chromosome arms but not in the centromeric regions in metaphase I, whereas intensive labelling was observed at the centromeres in metaphase I using these RAD21/SCC1 antibodies (Fig 4A, Supplementary Fig 7E, 8E and 9E). At metaphase II/anaphase II REC8 appeared as two dots flanking the SCP3 signal (Eijpe *et al.*, 2003; Lee *et al.*, 2003). In contrast, our antibodies produced a rod-shaped signal, overlapping with SCP3 (Fig 4D). Third, staining of chromosome spreads from spermatocytes of Rec8-deleted mice revealed that the RAD21/SCC1 signal intensity in leptonema and zygonema is similar to that of wild-type mice (our unpublished data). Taken together, we concluded that the signal detected by all three RAD21/SCC1 antibodies represents the distribution of RAD21/SCC1.

Apart from staining meiotic cells, we noted that all three antibodies strongly stained a sub-population of cells in our preparation (Supplementary Figs 7 and 8, images not displayed for anti-SCC1). We have not yet determined whether these cells are spermatogonia or pre-leptotene (pre-meiotic S phase) cells, as both cell types are very similar in size (Bellve, *et al.*, 1977). Cohesins are loaded onto sister chromatids during DNA replication in mitosis (Nasmyth, 2001). Several mammalian cohesins including SMC3, SMC1*a*, SMC1*b* and REC8 were detected in pre-leptotene cells (Eijpe *et al.*, 2003).

Further analysis using cell-specific markers should allow us to determine the identity of these cells.

SUPPLEMENTARY METHODS

Generation of RAD21/SCC1 antisera

Two polyclonal antibodies to human RAD21/SCC1 were generated (Supplementary Fig 1A). RAD21 Ab1 was raised against a recombinant protein corresponding to an internal region of human RAD21/SCC1 (amino acid 195 to 280) in sheep. RAD21 Ab2 was raised in rabbit against a synthetic peptide (YSDIIATPGPRFHII), which corresponds to the C-terminus of human RAD21/SCC1. Both antisera were purified using affinity columns.

Western blot analyses

Total protein from HeLa cells was extracted in buffer containing 10 mM $NaCl_2$, 10 mM Tris pH 7.5, 0.5% Nonidet P-40, 1% SDS and protease inhibitors (Roche). Testicular proteins were extracted in buffer containing 10 mM Hepes pH 8.0, 1.5 mM $MgCl_2$, 10 mM $NaCl_2$, 0.5 mM DTT, 10 mM Sodium Metabisulfite and protease inhibitors (Roche). Protein concentrations were determined using the BCA protein assay (Pierce). The protein extracts were separated on 7.5% SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with the primary polyclonal antibodies at 10 μg/ml. Signal was detected by chemiluminscence.

Immunoprecipitation

Protein extracts were prepared from testes of 18- to 25 day-old mice and from MEFs as described above. For immunoprecipitation, Protein A Sepharose CL-4B (Pharmacia Biotech) was incubated with 20 μg of anti-RAD21 antibodies. RAD21 Ab2 (rabbit polyclonal) was primarily used for immunoprecipitation and similar results were obtained using RAD21 Ab1 (sheep polyclonal). Beads were incubated with 400 μg of protein extracts in binding buffer (10 mM Tris pH 7.5, 100 mM $NaCl_2$, 10 mM $MgCl_2$, 2 mM EDTA, 1% NP40, 1 μg/ml DNase I and protease inhibitors), washed extensively with 200 mM $NaCl_2$ and 100 mM $NaCl_2$. The immunoprecipitates were separated by 6% SDS–PAGE with 50 μg of total protein extracts as positive controls. Proteins were transferred to PVDF membrane (NEN Polyscreen). The blots were probed with an anti-SMC1 antibody (Abcam). The blot was stripped and re-probed with anti-RAD21 Ab1 and anti-RAD21 Ab2. A parallel blot was probed with an anti-SMC3 antibody (a gift from Dr Jessberger).

RNA and Western blot analysis of enriched meiotic cells

Cell fractions enriched for spermatogonia and meiotic prophase spermatocytes were obtained essentially as described (Bellve *et al.*, 1977). RNA blot analysis was performed as previously described (Shannon *et al.*, 1999). To prepared nuclear extracts, frozen cell pellets were lysed in Krebs-Ringer Bicarbonate (KRB) buffer containing 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μl/ml Dnase I on ice for 5 minutes. SDS sample buffer was added. The cell suspension was boiled and 50 μg of proteins was loaded onto 10% SDS–PAGE and Western blot analysis performed as described above.

Immunostaining

HeLa cells grown on coverslips were fixed in 4% paraformaldehyde buffered with PBS (phosphate-buffered saline) for 15 minutes at room temperature. Cells were permeabilized for 5 minutes in PBS containing 0.1% Triton X-100 and 0.5% BSA. Cells were blocked in 4% BSA in PBS for 10 minutes and incubated with primary antibodies diluted at appropriate concentrations in 4% BSA overnight at 4 °C. Signal was detected using Alexa 488 anti-sheep IgG or Alexa 488 anti-rabbit IgG (Molecular Probes) diluted at 1:500 in 4% BSA. Cells were counter-stained with PI (propidium iodine). Metaphase chromosomes were obtained by incubating cells in the presence of 10 μg/ml colcemid for 4-6 hours. Cells were then incubated in 0.075 M KCl for 10 minutes and transferred onto slides by cytospin. Fixation and immunostaining were performed as described above.

Chromosome spreads were prepared from the testis of adult mice as described (Peters *et al.*, 1997). Spreads were stored at –20 °C until use. Slides were incubated with ADB (3% BSA, 10% goat serum and 0.05% Triton-X 100) for 1 hour with gentle shaking. Primary antibodies were added at appropriate dilutions in ADB and incubated overnight at 4 °C. After 3x15 minute washes in 10% ADB, the slides were incubated with the secondary antibodies. The slides were mounted in anti-fading mounting medium and observed primarily using a Bio-Rad MRC 1000 confocal microscope. A Zeiss microscope equipped with epifluorescence was used to capture images displayed in Supplementary Figure 6A–D.

ADDITIONAL REFERENCES

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