Temporal progression of recombination in human males

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To date, immunocytochemistry has been used in humans to detect a limited number of meiotic proteins: components of the synaptonemal complex (SCP1 and SCP3) and some proteins known to participate in recombination events, such as MLH1 or RAD51. However, the colocalization or coexistence of proteins known to participate during the different stages of human meiosis remains largely unstudied, and these studies could provide important clues about the mechanics of recombination. This work reports the relative timing and localization of five different meiotic proteins that have previously been implicated in human homologous recombination [RAD51, replication protein A (RPA), MSH4, MLH1 and MLH3]. MSH4 foci appear concurrently with synopsis initiation at zygotene, shortly after the first RAD51 foci are detected. The presence of RPA in MSH4 foci was noted, suggesting that these two proteins may act co-operatively. Both RPA and MSH4 foci reach maximal numbers at the end of zygotene, when synopsis is concluding. From this point, RPA foci all but disappear by the end of pachytene, whereas MSH4 foci decline to a stable number at mid-pachytene, where they localize with MLH1/MLH3 recombination sites. We discuss a possible role for MSH4 in synopsis initiation and/or maintenance.

Key words: human/meiotic recombination/MSH4/RAD51/synapsis

Introduction

In mammals, homologous recombination is essential for meiosis. It generates reciprocal exchanges of genetic material between two homologues, ensuring both genetic diversity and the proper segregation of homologous chromosomes at meiosis I. The events leading to homologous recombination (or crossover) start with the generation of double strand breaks (DSBs) that produce 3’ single-stranded overhangs (Zenvirth et al., 2003). These overhangs may progress to single-end invasions (Hunter and Kleckner, 2001), which in turn conduct a search for homology and the formation of Holliday junctions. The resolution of this structure results, in yeast, in homologous recombination (Börner et al., 2004).

A number of meiotic proteins, operating at different stages of the recombination process, have been identified in lower organisms and orthologous proteins are present in mammals. DSBs are known to be directed by SPO11 (Keeney et al., 1997), single-end invasion and homology search in mammals are catalyzed by two RecA homologues, RAD51 and DMC1 (Shinohara and Shinohara, 2004), and are promoted by other proteins such as replication protein A (RPA) (Sung et al., 2003). Holliday junctions are resolved by several proteins of the mismatch repair system (Hoffmann and Borts, 2004). The mismatch repair family in eukaryotes is formed by multiple orthologues of bacterial mutS and mutL proteins, which act as heterodimers in replication error repair. In mitotic cells, mutS orthologues are responsible for the detection of mismatched nucleotides that occur during the synthesis of new DNA and for the subsequent recruitment of a heterodimer of the mutL orthologue to the mismatch site. However, during meiosis, these proteins are implicated in different events. The heterodimer formed by proteins MSH4 and MSH5, for instance, apparently does not have a role in mismatch repair in yeast (Ross-Macdonald and Roeder, 1994; Hollingsworth et al., 1995). This heterodimer is found primarily during meiosis in yeast and also in mammals (Paquis-Fluckinger et al., 1997; Bocker et al., 1999) and is essential for chromosome pairing (de Vries et al., 1999; Edelmann et al., 1999; Knietz et al., 2000). Additional roles that have also been suggested for this heterodimer include regulation of the physical distance between adjacent recombination events (crossover interference) (Zalevsky et al., 1999; Novak et al., 2001) and stabilization of Holliday junction structures in vitro (Snowden et al., 2004). The heterodimer formed by mutL orthologues MLH1 and MLH3 has been associated with crossover regulation and resolution. In mammals, MLH1 foci number and distribution correlate with chiasmata (Barlow and Hultén, 1998; Anderson et al., 1999; Hassold et al., 2004), and both MLH1 and MLH3 have been found to colocalize with chiasmata precociously induced with okadaic acid (Marcon and Moens, 2003).

Current knowledge about the behaviour of proteins implicated in the meiotic process has emerged from studies in yeast and mice. Analysis of mutant organisms shows that mutations of any of these proteins can alter the normal progression of meiosis, causing a reduction in fertility or an increase in the frequency of chromosome abnormalities (Svetlanov and Cohen, 2004). Recently, immunocytogetic and electronic microscopy analyses have allowed the analysis of meiotic structures such as the synaptonemal complex. Moreover, through the precise pinpointing of different meiotic proteins, the use of these techniques provides information about the time frame and the location of specific meiotic events (Plug et al., 1998; Moens et al., 2002; Lenzi et al., 2005).
In this study, we report the time course of meiotic protein expression during recombination in human spermatocytes for the first time. By using immunocyto genetics techniques with antibodies directed against synaptonemal complex elements (SYN1/SCP3), centromeres (CREST), RAD51, RPA and three different mismatch repair proteins MSH4, MLH1 and MLH3, we have been able to establish a temporal frame and localization for each of these proteins, and the possible roles for each protein are discussed.

Materials and methods

Testicular tissue was analysed from patients undergoing orchitectomy for testicular cancer (n = 3; Calgary, Alberta, Canada) and vasosvastosity for fertility (n = 3; University of California San Francisco, San Francisco, CA, USA). Testicular tissue was kept in phosphate-buffered saline (PBS) until use and transferred on ice to Calgary by air courier where appropriate. We have previously demonstrated that cold storage of testicular tissue does not effect recombination frequencies (Sun et al., 2004b). Patients gave informed consent, and the study was approved by Institutional Review Boards in Calgary and at the University of California San Francisco.

After meiotic spreads were obtained (Sun et al., 2004a), blocking reagent (4–5% donkey serum in PBS with 0.1% Triton X) was applied to the slides to prepare spermatocytes for immunolabelling. The primary antibodies used in this work were mouse COR1 (1 : 200 dilution, a gift from P. Moens, York University), human centromeres (CREST) (1 : 100 dilution, a gift from M. Fritzler, University of Calgary), rabbit MLH1 (1 : 100 dilution, Oncogene, San Diego, CA), rabbit MSH4 (1 : 100 dilution, a gift from C. Her, Washington State University), goat MLH3, rabbit RAD51 and goat RPA (all at 1 : 100 dilution, all from Santa Cruz technologies, Inc, Santa Cruz, CA, USA), goat SCP3 (1 : 250 dilution, a gift from T. Ashley, Yale University), and mouse SYN1 (1 : 1000 dilution, a gift from P. Moens, York University). Primary antibodies were diluted in PBS, and individual testis tissue specimens were incubated with various combinations of these antibodies as outlined in Table I. Several different secondary fluorescent antibodies were used to detect the primary antibodies. Lateral and central structural elements of the synaptonemal complex and kinetochores were detected with combinations of aminomethyl coumarin acetic acid (AMCA)-labelled donkey anti-mouse immunoglobulin G (IgG), -goat IgG and -human IgG secondary antibodies (1 : 50 dilution, Jackson Immuno-research, West Grove, PA). Various protein components of the recombination sites were detected using the appropriate combination of Alexa488-labelled donkey anti-rabbit, anti-mouse and anti-goat secondary antibodies, and Alexa555-labelled donkey anti-rabbit, anti-mouse and anti-goat secondary antibodies (1 : 250 dilution, Molecular Probes, Inc., OR, USA). When needed, to colocalize a second protein utilizing an already used primary antibody host (rabbit), a second round of primary antibodies was applied, followed by a corresponding second round of secondary antibodies (Roig et al., 2004).

Image analysis

Slides were scanned with a Zeiss Axioshot fluorescent photomicroscope equipped with appropriate filters. Images of prophase nuclei (leptotene to pachyten e) were captured and processed using Applied Imaging Cytovision 3.1 software (Applied Imaging Corporation, Santa Clara, CA, USA). Spermatocytes were staged according to the number of CREST signals (24–45 at zygotene, 24 at pachytene) and to the presence of synapsis (through the detection of SCP1). The pachytene stage was established according to sex body morphology (Codina-Pascual et al., 2005; Solari, 1980).

Table I. Antibody combinations used in this study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Antibody combinations</th>
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<tr>
<td>h72s</td>
<td>MLH1/MLH3 MLH1/MSH4</td>
</tr>
<tr>
<td>h73s</td>
<td>MSH4/RPA MLH1/MSH4</td>
</tr>
<tr>
<td>h78s</td>
<td>MLH1/MLH3 MSH4/RPA</td>
</tr>
<tr>
<td>h69</td>
<td>MLH1/MLH3 MSH4/MLH3</td>
</tr>
<tr>
<td>h71s</td>
<td>MLH1/RAD51 RAD51/RPA</td>
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<tr>
<td>h89</td>
<td>MLH1/RAD51 RAD51/RPA</td>
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Results

RAD51

RAD51 was first seen at late leptotene-early zygotene, forming distinct linear arrays of fluorescent foci attached primarily to lateral elements, although some foci were located on what appeared to be newly synapsed synaptonemal complex segments (confirmed by the presence of SCP1). The mean number of foci at this stage was 104.3 ± 28.1. The number of foci remained constant from early to mid-zygotene (Figure 1), at which time numbers began to decline, until very few foci (11.6 ± 14.7) were seen on autosomal synaptonemal complexes at the beginning of pachytene. By mid-late pachytene, the mean number of RAD51 foci was 2.4 ± 3.9. Conversely, heavy concentrations of foci remained along the axial elements of the X chromosome and the pseudoautosomal region, indicating the presence of SPO11-generated DSBs on these regions (Mahadevaiah et al., 2001). These foci persisted until late pachytene. The decrease in the number of RAD51 foci coincided temporally with the progressive increase in synapsed segments. At pachytene, RAD51 was also found in asymmetric regions in some bivalents.

RAD51 colocalized briefly with RPA and MSH4 at zygotene (Figure 2a and b). The mean number of foci positive for RPA or MSH4 and RAD51 proteins at this stage was low (18.4 ± 9.8 and 20.3 ± 6.9, respectively, less than a fifth of the total RAD51 foci, Figure 3a) and appeared adjacently located. There was never more than one positive focus per nucleus for RAD51/MLH1. This very low colocalization frequency may be responsible for the observed lack of coimmunoprecipitation between these two proteins in mice (Kolas and Cohen, 2004).

RPA

RPA-positive foci were seen at early stages of zygotene (Figure 2b), when the mean focus number was 86.6 ± 33.8. The peak was observed at late zygotene, coincident with the end of synapsis between homologous chromosomes (mean 195.7 ± 30.6, Figure 2c). At early pachytene, the number of foci began to decrease, so by late pachytene very few foci remained on autosomal bivalents (mean 6.6 ± 4.7). RPA was also detected on asynapsed segments at zygotene and early pachytene.

RPA foci colocalized with more than half of observed MSH4 foci at zygotene (mean focus number 77.7 ± 12.2, Figures 2c and 3b). This...
number decreased as pachytene progressed (mean 17.6 ± 10.8 by mid-pachytene, Figures 1 and 3b). At pachytene, very few RPA foci were found colocalizing with MLH1 (mean focus number 8.8 ± 2.3).

As found in RAD51, RPA was heavily loaded onto the axis of the X chromosome beginning at early pachytene (Figure 2d), but it remained longer than RAD51, as shown by the fact that RPA (but not RAD51) foci were found on the sex body of some late pachytene spreads. Similar to the pattern observed for autosomal bivalents, these two proteins did not completely colocalize in the sex body.

**MSH4**

At zygotene, MSH4 and RPA foci exhibited very similar patterns, first appearing at early zygotene, peaking at late zygotene, and starting to decrease at the beginning of pachytene (Figure 1). The peak number of MSH4 foci found at late zygotene–early pachytene was similar to the number of RPA-positive foci found at the same stage (193.1 ± 24.6 and 195.7 ± 30.6, respectively). Diverging from the RPA foci pattern, the number of Msh4-positive foci remained stable from mid-pachytene until late pachytene (mean 55.8 ± 6.2), and most MSH4-positive foci found at this time were also positive for both MLH1 and MLH3 (50.3 ± 4.3 and 48.8 ± 5.1, respectively, Figures 2c and f, 3c and d).

Interestingly, MSH4 foci were also detected on the sex body at pachytene. This pattern has not been reported in other mammalian models. In early pachytene spreads, MSH4 antibody could be clearly seen loaded on the axes of both the X and Y chromosomes. Even in the presence of sex univalents, MSH4-positive foci were found on the axes of both sex chromosomes (Figure 2e). MSH4 foci were also detected occasionally on the lateral elements of asynapsed regions of autosomal bivalents at pachytene (Figure 2e). The association between MSH4 and asynapsed regions is not clear, because not all asynapsed regions at pachytene had MSH4 foci.

**MLH3/MLH1**

In humans, MLH3 began to localize on meiotic nodules at early pachytene, coinciding with the decrease in MSH4 foci. At this stage, the mean number of MLH3 foci was 11.8 ± 9.1, and all MLH3 foci colocalized with MSH4 (Figure 3d). Foci positive for MLH3 and MLH1 appeared shortly after the first appearance of MLH3 foci, confirming that MLH3 is able to attach to the recombination nodule in the absence of MLH1 (Lipkin et al., 2002), and also indicating that the MLH1 protein is recruited immediately after the first MLH3 foci appear. As pachytene progressed, the presence of foci positive for only MLH3 was observed (mean 2.5 ± 2.4 by mid-late pachytene). In the pseudoautosomal region located in the short arm of chromosome Y, an MLH3/MSH4 focus can be detected at the PAR region on the short arm of chromosome Y. Images were viewed at 1000 × magnification.
Discussion

When comparing the progression of the different proteins analysed in this study, the most intriguing temporal expression is that observed for the MSH4 protein. Indeed, the lasting presence of MSH4 foci throughout meiotic prophase that we observed in humans has also been reported in the mouse (Santucci-Darmanin et al., 2000) and suggests that MSH4 might play different roles during meiosis. Moreover, the interaction of MSH4 with RAD51, RPA, MLH1 and MLH3 at different stages of the process in humans and in mice (Santucci-Darmanin et al., 2000, 2002; Moens et al., 2002; Neyton et al., 2004), also raises the possibility that MSH4 exerts diverse functions during meiosis.

The fact that Msh4 mutant yeast (Saccharomyces cerevisiae) show recombination with no apparent interference has been interpreted to mean that MSH4 has a role in the crossover interference process during early recombination (Novak et al., 2001). A recent study proposed that the MSH4/MSH5 heterodimer is responsible for driving Holliday junctions towards a crossover outcome during meiosis (Snowden et al., 2004). According to this model, crossover interference results from the way in which the MSH4/MSH5 heterodimer binds to meiotic recombination intermediates. As shown previously in the mouse (Kneitz et al., 2000), the number of MSH4 foci during early meiotic prophase exceeded the final number of crossover events by a factor of 2–3. If the crossover interference model proposed by Snowden et al. (2004) is true, then the question arises: what controls the MSH4/MSH5 heterodimer binding pattern to allow only a subset of MSH4/MSH5 heterodimers (among the hundreds concurrently present in the cell) to generate crossover products?

The coexistence of RAD51 and MSH4 at very early stages of zygotene implies that the MSH4/MSH5 heterodimer is acting locally at sites of emerging recombination events. Because spermatocytes from msh5 mutant mice have frequent nonhomologous synapsis, it is tempting to think that MSH4/MSH5 plays a role in the homology search promoted by RAD51. This may simply be a consequence of the stabilizing role proposed for these two proteins (Snowden et al., 2004), thus, facilitating the homology search through the stabilization of the emerging Holliday junction.

In yeast, the MSH4 protein is part of a synapsis initiation complex, and MSH4 mutation has been related to synapsis delay and with failure of interference establishment (Novak et al., 2001). Although a synapsis initiation complex has never been described in humans, the presence and temporal distribution of MSH4 at sites of nascent synapsis (almost simultaneous with SCP1, peaking at the end of zygotene) suggests a role for MSH4 in directing or maintaining the synapsis process.

Results observed in msh4 mutant mice also indicate that MSH4 is essential for proper chromosome synapsis: such mice can initiate, but not maintain and complete synapsis (Kneitz et al., 2000). The fact that MSH4 can be detected on axial elements of asynapsed regions at pachytene (Figure 2e), also support the idea of a structural role for MSH4.

Small numbers of RAD51/RPA foci have also been observed in the mouse, and it has been suggested that the coexistence of the two proteins exhibits a rapid changeover, probably immediately after synapsis is achieved (Moens et al., 2002). This observation is consistent with the fact that RPA has a positive effect on RAD51-mediated strand exchange (Sung et al., 2003). In yeast, the RAD51 pairing intermediate is stabilized by RPA, which sequesters the displaced strand away from the D-loop generated during the initiation of single-end invasion (Eggler et al., 2002). The presence of SCP1 filaments, indicating synapsis, and the fact that RPA foci arrays appear adjacent to RAD51 arrays may indicate a similar role for RPA in humans.

Figure 3. Evolution of MSH4 during meiotic prophase. Bar graphs show the evolving percentage of MSH4 relative to the other four proteins from early zygotene (1z) to late zygotene (3z) and through the different pachytene stages (1p–5p): (a) RAD51 (n = 40), (b) replication protein A (RPA ) (n = 89), (c) MLH1 (n = 55) and (d) MLH3 (n = 60).
It is not known whether the observed colocalization between RPA and MSH4 is a consequence of actual co-operation between these proteins. As suggested above, an alternative role for early MSH4 foci may relate to stabilization of the synaptonemal complex. Because the temporal and spatial distribution of RPA and MSH4 are very similar, and peak during the end of synapsis, RPA may also have a role guaranteeing synopsis. Because a mature synaptonemal complex is essential for successful resolution of recombination (Sym et al., 1993; Tung and Roeder, 1998), RPA and MSH4 stabilization of synopsis would ensure successful recombination.

Unlike MSH4 and RPA, MLH1 and MLH3 do not appear to play a role in synopsis maintenance. Mice mutant for MLH1 or MLH3 achieve synopsis, indicating that synaptonemal complex stability does not depend on these proteins. The presence of MSH4 in recombination foci until the onset of diploteine, colocalized with MLH1 and MLH3, suggests a role for this protein in the resolution of crossovers. This role of MSH4 in recombinant nodules, however, could be more structural, rather than recombination support (Snowden et al., 2004). By this theory, the sliding clamps formed by MSH4/MSH5 heterodimers would stabilize recombination sites, subsequently attracting MLH1/MLH3. Colocalization analysis of MLH3, MLH1 and MSH4 suggests that recombination sites first recruit MLH3, which then attracts MLH1 to form the heterodimer necessary for the resolution of crossovers. This idea is supported by studies in mice mutant for MLH1 or MLH3: mlh1 mutant mice have some MLH3-positive foci (Kolas and Cohen, 2004), but mlh3 mutant mice have no MLH1-positive foci, indicating that in the absence of MLH3, MLH1 is unable to attach to recombination sites (Lipkin et al., 2002). The presence of MLH3-only foci (lacking MSH4 and MLH1) is difficult to explain, but three possible theories have been offered (Kolas and Cohen, 2004): (i) these foci are detected just before recruitment of MLH1, (ii) MLH3 is binding certain targets as a homodimer and (iii) MLH3 is interacting with a protein other than MSH4 or MLH1. Nevertheless, crossover resolution is completely dependent on the presence of both proteins, suggesting that the MLH1/MLH3 heterodimer is the substrate recognized by the yet-to-be-identified mutH protein that completes the recombination process.

When comparing our results on human spermatocytes with results published in human oocytes (Roig et al., 2004; Lenzi et al., 2005), several differences can be noted. First, the number of RAD51 foci in human oocytes (Lenzi et al., 2005) is almost three-fold higher than the number observed in human spermatocytes. In addition, high numbers of RAD51 foci can be seen at early pachytene in human oocytes (Lenzi et al., 2005), whereas in human spermatocytes, RAD51 foci are barely present at the beginning of pachytene. These results suggest that less DSBs are generated in human male meiosis than in human female meiosis, and that protein turnover in DSB repair occurs faster in human spermatocytes than in human oocytes. Indeed, colocalization results of γ-H2AX and RPA in human oocytes (Roig et al., 2004) also indicate that DSBs are repaired at a slower pace in the female than in the male. A second difference is that in late pachytene spermatocytes, almost all MLH1 foci are also positive for MSH4. This is not the case in human oocytes (Lenzi et al., 2005), where the number of MSH4 foci at late pachytene is almost half the number of MLH1 foci, indicating that the mutS–mutL complex may be more stable in males than in females. The stability of this complex has been deemed essential for the proper segregation of homologous chromosomes (Novak et al., 2001; Kolas and Cohen, 2004). Thus, a higher stability in males could explain the lower frequency of nondisjunction observed in human male meiosis compared to female meiosis.

In summary, we propose a dynamic model for meiotic recombination in human spermatocytes as follows: after buckling at a RAD51 bridge, a single-end invasion is triggered and a Holliday junction is formed. The Holliday junction structure attracts MSH4/MSH5 heterodimers that stabilize it and either launch synopsis through the deposition of SCP1 or help maintain synopsis at the sites where it has been initiated. Simultaneously, the presence of ssDNA at the sites of single-end invasion attracts RPA, which acts in coordination with MSH4 to maintain synopsis. Once synopsis has been achieved, both proteins are released, and MSH4 remains at recombination sites, stabilizing the Holliday junction. Finally, MSH4 attracts MLH3 and then MLH1, which participate as a heterodimer in the resolution of crossovers.

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