Dynamic relocalization of the chromosomal passenger complex proteins inner centromere protein (INCENP) and aurora-B kinase during male mouse meiosis

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Summary

INCENP and aurora-B kinase are two chromosomal passenger proteins that are thought to play key roles in coordinating chromosome segregation with cytokinesis in somatic cells. Here we have analyzed their subcellular distribution, and that of phosphorylated histone H3, and the timing of their relative appearance in mouse spermatocytes during both meiotic divisions. Our results show that in mitotic spermatogonial cells, INCENP and aurora-B show the same pattern of distribution as they do in cultured somatic cells. INCENP labels the synaptonemal complex central element from zygotene up to late pachytene when it begins to relocalize to heterochromatic chromocentres. Aurora-B first appears at chromocentres in late diplotene before the initial phosphorylation of histone H3. INCENP and aurora-B concentrate at centromeres during diakinesis and appear during metaphase I as T-shaped signals at their inner domains, just below associated sister kinetochores. During late anaphase I both proteins relocalize to the spindle midzone. Both proteins colocalize at a connecting strand traversing the centromere region and joining sister kinetochores, in metaphase II centromeres. This strand disappears at the metaphase II/anaphase II transition and relocalizes to the spindle midzone. We discuss the complex dynamic relocalization of the chromosomal passenger complex during prophase I. Additionally, we suggest that this complex may regulate sister-chromatid centromere cohesion during both meiotic divisions.

Key words: INCENP, Aurora-B kinase, Phosphorylated histone H3, Meiosis, Centromere, Sister-chromatid cohesion

Introduction

The centromere is the chromosomal region that interacts with the spindle microtubules and holds sister chromatids together until their segregation at anaphase (Craig et al., 1999; Choo, 2001). Three domains have been defined at the centromere: the kinetochore domain, the central domain and the pairing domain (Earnshaw and Rattner, 1989; Pluta et al., 1990). The kinetochore domain is a proteinaceous structure located along the outer surface of the centromere that interacts with the spindle microtubules. This domain contains different constitutive proteins, as well as microtubule motor proteins, which are involved in chromosome alignment during prometaphase and chromatid segregation at anaphase, and mitotic spindle checkpoint proteins, which are implicated in the regulation of the metaphase/anaphase transition (Craig et al., 1999). The central domain is located beneath the inner kinetochore plate and was defined by the presence of the constitutive centromere protein CENP-B (Cooke et al., 1990). Finally, the pairing domain is the region along the inner surface of the centromere that represents the site of interaction between sister chromatids at metaphase. This domain was first defined by the location of the CLiPs (chromatid linking proteins) (Rattner et al., 1988), and later by INCENP (inner centromere protein), a chromosomal 'passenger' protein (Cooke et al.,

1987). Although much is known about the role of different kinetochore proteins, the role of the pairing domain proteins remains poorly characterized.

INCENP is a protein that is conserved from budding yeast to humans (Ainsztein et al., 1998; Kim et al., 1999; Saffery et al., 1999; Adams et al., 2000; Adams et al., 2001b; Kaitna et al., 2000; Oegema et al., 2001; Leverson et al., 2002). This protein, as a chromosomal passenger protein, distributes dynamically during mitosis (Adams et al., 2001c; Terada, 2001). INCENP concentrates at the pairing centromere domain, also called the inner centromere domain, during metaphase, transfers to the spindle midzone and to the cell cortex at the cleavage furrow during anaphase, and then concentrates at the midbody during telophase (Cooke et al., 1987; Earnshaw and Cooke, 1991; Mackay and Earnshaw, 1993). Expression of truncated INCENP proteins, or of a CENP-B–INCENP chimeric protein in transfected cells, has demonstrated that INCENP is required for prometaphase chromosome congression, sister chromatid disjunction at anaphase and the completion of cytokinesis (Eckley et al., 1997; Mackay et al., 1998). These processes are severely affected in INCENP-knockout mouse embryos, thus demonstrating that INCENP is essential for cell division (Cutts et al., 1999). INCENP forms a chromosomal passenger

complex and colocalizes with survivin, and with the aurora-B serine-threonine protein kinase, in all organisms studied, from budding yeast to humans (Kim et al., 1999; Adams et al., 2000; Adams et al., 2001a; Adams et al., 2001b; Kaitna et al., 2000; Speliotes et al., 2000; Uren et al., 2000; Morishita et al., 2001; Wheatley et al., 2001; Leverson et al., 2002). It has been proposed that INCENP targets survivin to centromeres (Wheatley et al., 2001) and that INCENP and survivin are essential for the targeting of aurora-B to chromosomes (Adams et al., 2000; Adams et al., 2001b; Rajagopalan and Balasubramanian, 2002). This chromosomal passenger complex has been implicated in coordinating chromosome segregation with cytokinesis in somatic cells.

Aurora-B kinase is overexpressed in many human cancer cell lines (Giet and Prigent, 1999), as is INCENP (Adams et al., 2001a), and has also been implicated in both chromosome congression and segregation, and efficient completion of cytokinesis (Terada et al., 1998; Schumacher et al., 1998; Kaitna et al., 2000; Leverson et al., 2002; Murata-Hori et al., 2002; Kallio et al., 2002). Several substrates for aurora-B have been identified. In budding yeast the single aurora kinase Ipl1p phosphorylates the kinetochore protein Ndc10p (Biggins et al., 1999) and is required to allow an accurate biorientation of mitotic chromosomes (Tanaka et al., 2002). Aurora-B also phosphorylates the human centromeric protein CENP-A, a histone H3-like kinetochore protein (Zeitlin et al., 2001). In this sense, it has been shown in rat cells that the kinase activity of aurora-B is required for the localization of different microtubule motor proteins at kinetochores and for chromosome biorientation and then congression (Murata-Hori and Wang, 2002; Murata-Hori et al., 2002). Aurora-B kinase, and their homologues, also phosphorylates histone H3 at serine 10 in different organisms (Hsu et al., 2000; Speliotes et al., 2000; Adams et al., 2001b; Giet and Glover, 2001; Crosio et al., 2002; MacCallum et al., 2002).

Recently, different observations have led us to propose that INCENP and aurora-B are implicated in sister-chromatid centromere cohesion. In stable dicentric human chromosomes, INCENP is present at the inactive centromere when sister chromatids appear joined, whereas it is no longer detectable when centromere cohesion is lost (Vagnarelli and Earnshaw, 2001). Additionally, the abnormalities observed in *Drosophila* cells undergoing anaphase after abrogation of INCENP and aurora-B function by RNA interference suggest that these proteins are implicated in centromere cohesion (Adams et al., 2001b). The abnormal distribution of INCENP at centromeres in chicken cells with the mutated cohesin subunit Rad21/Scc1 further support a relationship between INCENP and centromere cohesion (Sonoda et al., 2001). Although numerous studies have analysed the subcellular distribution and pattern of expression of the chromosomal passenger complex in somatic cells from different systems, information concerning their distribution in meiotic cells is scarce. Recently, the subcellular localization of aurora-B has been studied during meiosis in *C. elegans*. Depletion of aurora-B prevents the release of the meiosis-specific cohesin subunit Rec8 from chromosomes. It has been proposed that aurora-B phosphorylates Rec8, and therefore promotes the release of sister-chromatid cohesion during both meiotic divisions (Rogers et al., 2002).

Owing to the absence of studies on INCENP and aurora-B

kinase expression during mammalian meiosis, we analysed their subcellular distribution throughout meiosis in squashed mouse spermatocytes. For comparative purposes, we additionally studied INCENP distribution in mitotic spermatogonial cells and 3T3 cultured mouse cells. We surprisingly found that in prophase I INCENP appears at the central element of the synaptonemal complex and redistributes to the heterochromatic chromocentres during late pachytene. Then, INCENP and aurora-B concentrate at centromeres showing peculiar T-shaped signals at their inner domain in metaphase I. Both proteins are also found at a connecting strand traversing the centromere region, and joining sister kinetochores, at metaphase II centromeres. We discuss the complex dynamic relocalization of the chromosomal passenger complex during prophase I. Additionally, we suggest that this complex regulates sister-chromatid centromere cohesion during both meiotic divisions.

Materials and Methods

Cell culture

For this study, cultured 3T3 mouse cells and HeLa cells were used. Mouse 3T3 cells were grown on coverslips, and HeLa cells in flasks, in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 1% 0.2 M L-glutamine (all from Imperial Laboratories), at 37°C in a humidified atmosphere containing 5% CO_2. Mouse 3T3 cells were fixed in freshly prepared 2% formaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) containing 0.1% Triton X-100 (Sigma) and washed in PBS.

Electrophoresis and immunoblotting

HeLa cells were harvested and washed twice with PBS. Then, they were lysed in boiling SDS sample buffer (50 mM Tris-HCl pH 6.8, 3% SDS, 2 mM EDTA, 15% sucrose, 9% β-mercaptoethanol, 0.005% bromophenol blue).

Testes from adult male C57BL/6 mice were removed and placed in 2 ml of SDS solubilization solution (50 mM Tris-HCl pH 6.8, 5 mM EDTA, 3% SDS, 1% protease inhibitor cocktail). Then, testes were homogenized on ice in a Potter homogenizer. The extract was placed in a boiling water bath for 5 minutes. The appropriate quantity of extract was diluted with 5× SDS-lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) and boiled for 5 minutes.

SDS-PAGE was carried out in 10% polyacrylamide gels. Gels were electrically transferred to Trans-Blot sheets (Bio-Rad) for 1.5 hours at 4°C and 310 mA. Sheets were blocked for 1 hour with 4% non-fat dry milk in PBS, followed by an overnight incubation at 4°C with pAb1186 serum against INCENP at 1:500 dilution in 4% non-fat dry milk in PBS. Immunoreactive bands were visualized by incubation for 1 hour at room temperature with horseradish-peroxidase-conjugated donkey anti-rabbit Ig (Amersham Life Science) at a 1:5000 dilution in PBS and subsequently developed using an ECL (Enhanced Chemiluminescence) detection system (Amersham) according to the manufacturer instructions.

Squashing and spreading of spermatocytes

Adult male C57BL/6 mice were killed by cervical dislocation. The testes were then removed and detunicated, and seminiferous tubules processed for either squashing or spreading. For squashing, we followed the technique previously described (Page et al., 1998; Parra et al., 2002). Briefly, seminiferous tubules were fixed in freshly prepared 2% formaldehyde in PBS containing 0.1% Triton X-100.
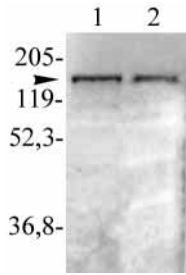
Fig. 1. Immunoblot analysis of HeLa cell extracts (lane 1) and mouse testes extracts (lane 2) probed with pAb1186 serum. The positions of molecular mass markers are indicated by numbers, and the arrowhead indicates the INCENP bands.

After 5 minutes, several seminiferous tubules fragments were placed on a slide coated with 1 mg/ml poly-L-lysine (Sigma) with a small drop of fixative, and the tubules were gently minced with tweezers. The tubules were then squashed and the coverslip removed after freezing in liquid nitrogen. For spreading of spermatocytes, we followed the drying-down technique of Peters et al. (Peters et al., 1997).

Immunofluorescence microscopy

After fixation, the slides and coverslips were rinsed three times for 5 minutes in PBS and incubated for 45 minutes at room temperature with primary antibodies. To detect INCENP we used a polyclonal rabbit serum (pAb1186) raised against chicken INCENP, but also recognising human INCENP (Eckley et al., 1997), at a 1:100 dilution in PBS. Centromeres were detected with the GS human anti-centromere autoantibody (ACA) that recognises CENP-A, -B and -C (Earnshaw and Cooke, 1989) at a 1:10,000 dilution in PBS. To detect SCP3 we used a polyclonal guinea pig serum [kindly provided by Ricardo Benavente (Alsheimer and Benavente, 1996)], at a 1:100

dilution in PBS. To detect SCP1 we used a polyclonal rabbit serum (A2) that recognises SCP1 [kindly provided by Christa Heyting (Meuwissen et al., 1992)] at a 1:200 dilution in PBS. To detect aurora-B kinase, we employed the mouse monoclonal AIM-1 antibody (Transduction Labs) at a 1:30 dilution in PBS. To detect survivin, we employed three polyclonal rabbit sera against human survivin (Novus Biologicals, Calbiochem, and Autogenbioclear) at a 1:30 dilution. Phosphorylated histone H3 (pH3) was detected with a polyclonal rabbit serum (Upstate Biotechnology) at a 1:1000 dilution. Following three washes in PBS, the slides were incubated for 30 minutes at room temperature with secondary antibodies. A combination of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson) at a 1:150 dilution in PBS with Texas-Red-conjugated goat anti-human IgG (Jackson) at a 1:150 dilution in PBS, a Texas-Red-conjugated goat anti-guinea pig IgG (Jackson) at a 1:150 dilution in PBS or a Texas-Red-conjugated goat anti-mouse IgG (Jackson) at a 1:150 dilution in PBS was used for simultaneous double immunolabelling. The slides were subsequently rinsed in PBS and counterstained for 3 minutes with 2 µg/ml DAPI (4′,6-diamidino-2-phenylindole). After a final rinse in PBS, the slides were mounted in Vectashield (Vector Laboratories) and sealed with nail varnish. In double immunolabelling experiments, primary antibodies were incubated simultaneously except for the double localization of INCENP and SCP1. In this case slides were first incubated with the rabbit serum against INCENP for 1 hour at room temperature, rinsed in PBS and incubated overnight at 4°C with an FITC-conjugated goat Fab' fragment anti-rabbit IgG (Jackson) at a 1:100 dilution in PBS. Afterwards, slides were rinsed six times for 5 minutes in PBS, incubated with the rabbit serum against SCP1 for 1 hour, rinsed three times for 5 minutes in PBS and incubated with a Texas-Red-conjugated goat anti-rabbit IgG (Jackson) at a 1:150 dilution. A control with the preimmune serum from rabbit 1186 was made and resulted in no staining.

Fig. 2. Expression of INCENP in spermatogonial mitosis (INCENP, green; ACA serum, red; DAPI, blue). (A,B) Partial projection of two focal planes throughout a spermatogonial interphase showing bright INCENP signals at heterochromatic chromocentres (arrows) highlighted with DAPI. ACA signals are located inside chromocentres. (C-E) Partial projection of three focal planes throughout a spermatogonial prophase. Bright fuzzy INCENP signals are located at centromeres (arrow in C,E) where pairs of ACA dots, representing sister kinetochores (arrows in D), are found. Inserts show a larger magnification of the arrowed centromere. A faint INCENP labelling is also found on condensing chromosomes. (F,G) Partial projection of five focal planes throughout an end-on view of a spermatogonial metaphase. A faint INCENP labelling is found on chromosome arms, whereas bright fuzzy signals are found at centromeric regions. The insert shows a centromeric region with its two sister kinetochores. (H,I) Partial projection of two focal planes throughout a spermatogonial anaphase. INCENP only labels some thin threads (arrows) at the spindle midzone. (J,K) Partial projection of two focal planes throughout a spermatogonial telophase where INCENP is present at two small and very bright signals (arrowheads) at both sides of the midbody. Bar, 10 µm.
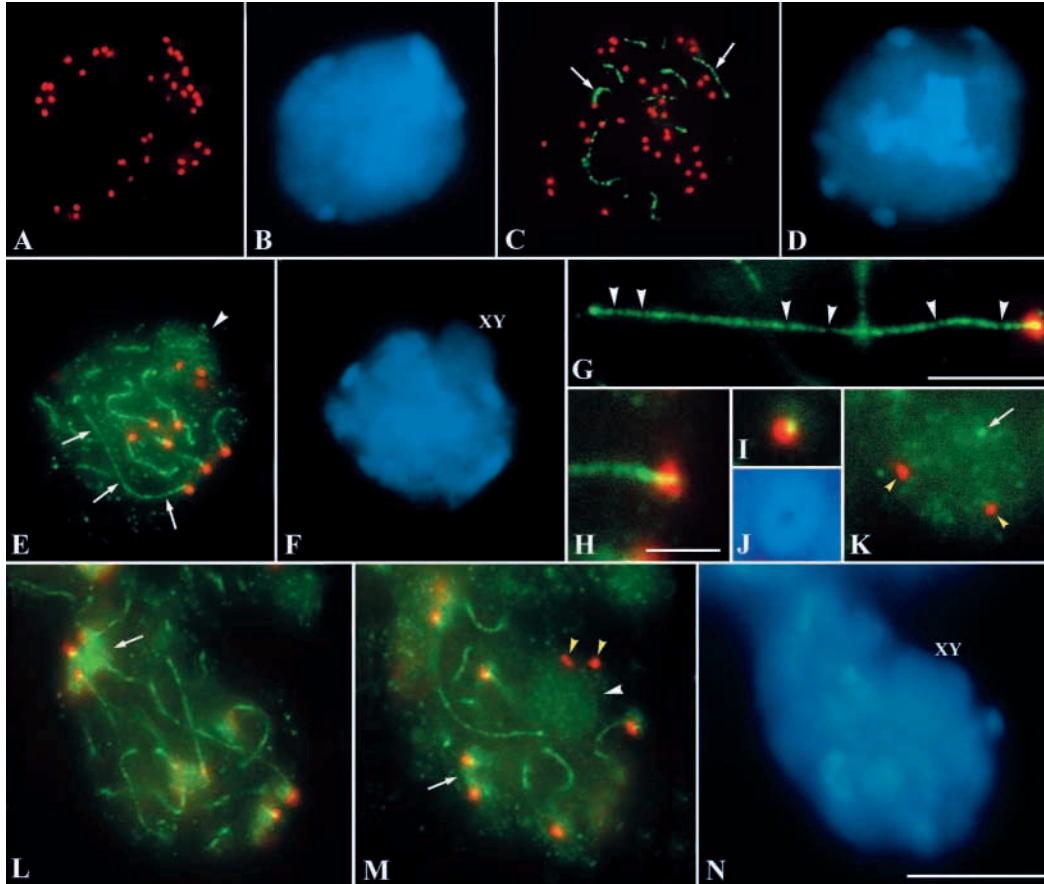
Fig. 3. INCENP distribution in early prophase I spermatocytes (INCENP, green; ACA serum, red; DAPI, blue). (A,B) Partial projection of 21 focal planes throughout an early prophase I spermatocyte. No INCENP signals are discerned; instead only ACA signals representing unpaired centromeres are seen. (C,D) Partial projection of 15 focal planes throughout an early prophase I spermatocyte. Some short and discontinuous INCENP threads (arrows) are observed in the nucleus. (E,F) Partial projection of three focal planes throughout a pachytene spermatocyte. Long INCENP threads (arrows) showing a heterogeneous thickness are detected in the nucleus. The sex body (XY) shows a faint INCENP labelling (arrowhead). (G) Projection of two focal planes of an INCENP-labelled thread at pachytene. The thread presents narrower regions (arrowheads) along its length. (H) Side view of the proximal end of an INCENP thread at pachytene. The single ACA signal, representing synapsed homologous centromeres, surrounds the end of the thread. (I,J) End-on view of the proximal end of an INCENP thread at pachytene. The spot representing the INCENP signal is lateral to the ACA signal. (K) Partial projection of three focal planes throughout the pachytene sex body that appears slightly labelled with INCENP. The unpaired sex centromeres (yellow arrowheads), and the INCENP-labelled pseudoautosomal region (arrow), are observed. (L-N) Two focal planes throughout a late pachytene spermatocyte. Chromocentres appear labelled (arrows), whereas INCENP labelling at nuclear threads is fainter. The sex body (XY) appears faintly labelled (white arrowhead in M). The centromeres of both sex chromosomes (yellow arrowheads in M) are separated. (A-F,L-N) Bar in N, 10 μm. (G,K) Bar in G, 5 μm. (H-J) Bar in H, 2 μm.

Observations were performed using an Olympus BH-2 microscope equipped with epifluorescence optics, and the images were recorded with an Olympus DP50 digital camera. Digital images were then treated using the Adobe PhotoShop 6.0 software.

Results

Immunoblot assay

To test the specificity of the pAb1186 serum we performed an immunoblot analysis with mouse testes and HeLa cell extracts. We observed that this anti-INCENP pAb specifically recognised the same protein band in testes and HeLa cells extracts that migrated at a position representing approximately M_r 150,000 (Fig. 1) (Adams et al., 2001a). Thus, pAb1186 serum also recognises mouse INCENP.

The distribution of INCENP in spermatogonial mitosis is similar to that found in cultured somatic cells

The squashing technique we employed allowed us to observe in the same preparations mitotic spermatogonial cells and spermatocytes at different developmental stages. To compare INCENP expression during mouse spermatogenesis with that found during mouse mitosis, we first analysed its distribution in spermatogonial mitosis (Fig. 2). In interphase spermatogonia, INCENP appeared as large bright nuclear signals corresponding to the heterochromatic chromocentres observed after DAPI staining (Fig. 2A,B). Some faint nucleoplasmic staining was also observed. After a double immunolabelling of INCENP and centromeres with the ACA serum, it became evident that centromeres appeared as discrete foci immersed in the INCENP-labelled chromocentres (Fig. 2A,B). In prophase spermatogonia a bright INCENP labelling was observed at
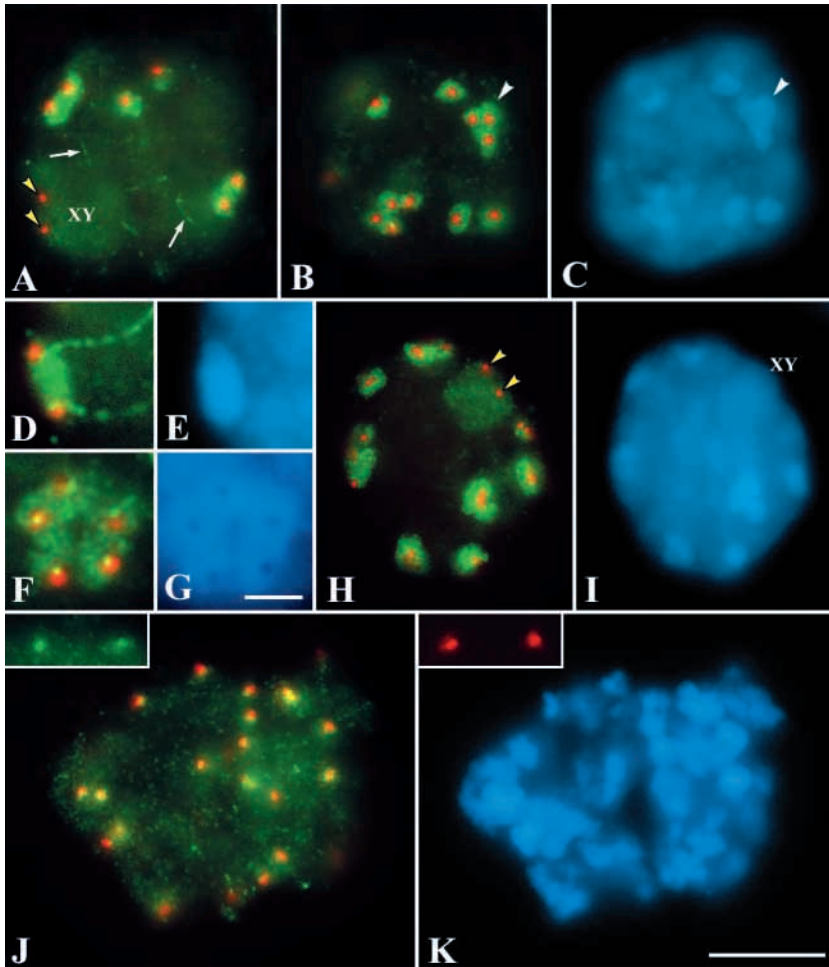
Fig. 4. INCENP expression in diplotene and diakinesis spermatocytes (INCENP, green; ACA serum, red; DAPI, blue). (A-C) Two partial projections from the same diplotene spermatocyte. INCENP labels the chromocentres, which appear very bright (arrowheads in B,C), the sex body (XY), which is fainter, and the short and faint nuclear threads (arrows). The unsynapsed sex centromeres (yellow arrowheads) are observed in the sex body. (D,E) Side view of the insertion of the proximal ends of two INCENP threads into the nuclear envelope in an early diplotene spermatocyte. The centromeric heterochromatin of these two threads appears joined as a single bright chromocentre. (F,G) End-on view of the insertion of five autosomal centromeric regions into the nuclear envelope in an early diplotene spermatocyte. Note that the INCENP-labelled heterochromatic regions are larger than the ACA signals representing synapsed homologous centromeres. (H,I) Partial projection of four focal planes throughout a late diplotene spermatocyte. No INCENP threads are observed. INCENP labels intensely the chromocentres, and dimly the sex body (XY) where the centromeres (yellow arrowheads) are indicated. (J,K) Partial projection of three focal planes throughout a diakinesis spermatocyte. INCENP signals partially colocalize with ACA ones. Inserts show a high magnification of two centromeres. (A-C,H-K) Bar in K, 10 µm. (D-G) Bar in G, 5 µm.

centromeric regions, and a diffuse staining on condensing chromosomes was also seen (Fig. 2C-E). The centromeric INCENP signals were bright and fuzzy, and colocalized with the brightly labelled centromeric heterochromatin detected with DAPI (Fig. 2C-E). By contrast, the ACA serum recognised pairs of dots, representing sister kinetochores, that appeared at opposing surfaces of the INCENP signals (insert in Fig. 2C,D). During metaphase, the INCENP labelling was similar to that found during prophase. Thus, bright and fuzzy centromeric signals were observed, and chromosome arms showed a faint staining along their lengths (Fig. 2F,G). The INCENP labelling changed dramatically during anaphase. In early anaphase, INCENP completely disappeared not only from centromeres but also from chromatid arms and was only detected as thin threads at the spindle midzone (Fig. 2H,I). During telophase, two brightly labelled regions flanking the midbody were observed (Fig. 2J,K). Interestingly, the same pattern of expression was observed for the aurora-B kinase (data not shown). We also studied the INCENP expression in somatic 3T3 cultured mouse cells (data not shown) in order to compare it with its distribution in spermatogonial mitosis. Our results showed that INCENP was similarly distributed in both kinds of mouse mitotic cells, and its expression pattern is likewise similar to that described previously in chicken (Cooke et al., 1987), *Xenopus* (Adams et al., 2000) and human somatic cells (Eckley et al., 1997).

INCENP distributes as nuclear threads in early prophase I spermatocytes

In squashed seminiferous tubules, and after DAPI staining and centromere labelling with the ACA serum, leptotene and zygotene spermatocytes showed about 40 signals corresponding to the unpaired autosomal and sex centromeres (Fig. 3A-D). In some of these early prophase I spermatocytes no INCENP staining was detected (Fig. 3A,B). However, in other ones, INCENP was detectable as short and thin discontinuous nuclear threads (Fig. 3C,D). Pachytene spermatocytes were easily recognized because 21 centromere signals, nineteen corresponding to paired autosomal centromeres and two additional ones corresponding to unpaired sex centromeres, were detected throughout their nuclear volume, and the sex body was discerned at the nuclear periphery (Fig. 3E,F). In these pachytene spermatocytes, INCENP-labelled nuclear threads appeared longer and brighter than in early prophase I spermatocytes, and their ends reached the nuclear periphery (Fig. 3E,F). One of the ends of these threads colocalized with centromeres (Fig. 3E,F). Additionally, the entire sex body showed a faint INCENP staining that was brighter than the nucleoplasmic background (Fig. 3E,F,K). In some favourable focal planes full-length threads were observed (Fig. 3G). It was apparent that these threads did not show a homogeneous thickness along their length but that they presented some narrower regions (arrowheads in Fig. 3G).
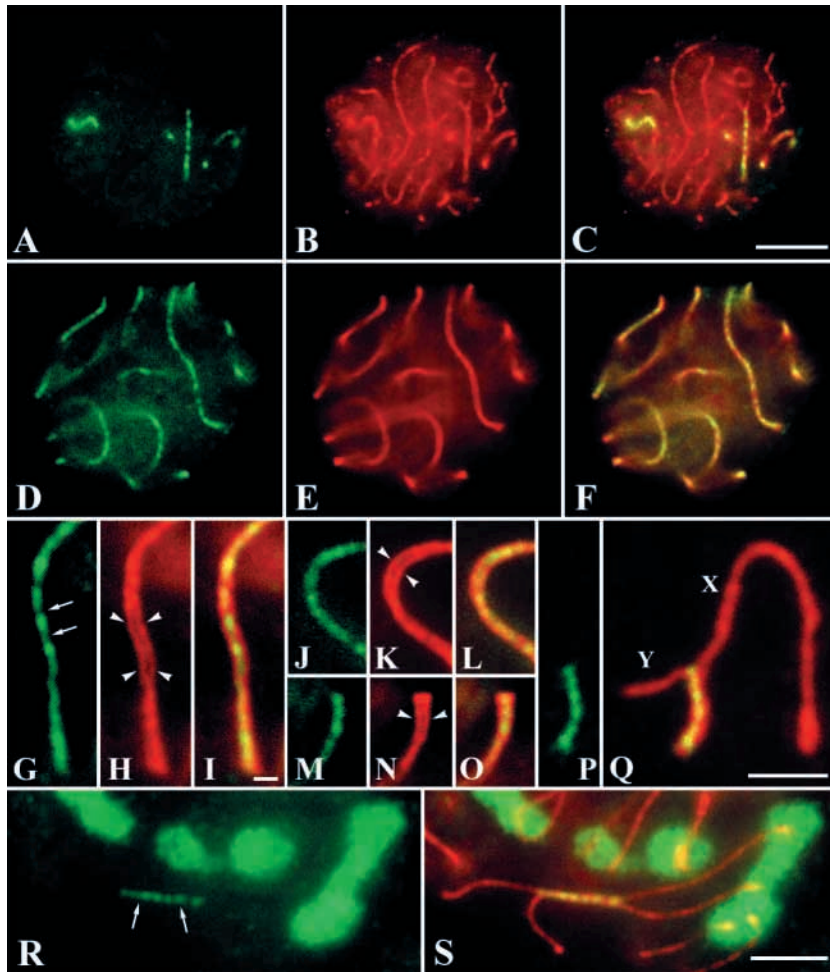
Fig. 5. Double immunolabelling of INCENP and SCP3 in either squashed (A-O,R,S) or spread (P,Q) prophase I spermatocytes (INCENP, green; SCP3, red; regions of colocalization, yellow). (A-C) Single focal plane of a zygotene spermatocyte. Some short INCENP threads colocalize with SCP3 threads where homologous chromosomes have synapsed. (D-F) Partial projection of two focal planes throughout a pachytene spermatocyte. The long INCENP threads colocalize with SCP3-labelled synaptonemal complexes (SCs). (G-L) Selected pachytene SC fragments where the two lateral elements (LEs) (arrowheads) are clearly individualised. The INCENP threads, presenting narrow regions along their lengths (arrows in G), are located in between the LEs. (M-O) End of a pachytene SC showing that the INCENP thread also appears in between LEs (white arrowheads). (P,Q) Selected sex bivalent from a spread of an early pachytene spermatocyte. INCENP only labels the pseudoautosomal region. The unsynapsed sex axial elements (X,Y) are indicated. (R,S) Selected diplotene bivalent. A short INCENP thread (arrows) colocalizes with the region where LEs are still synapsed. Chromocentres show a bright INCENP labelling. (A-F) Bar in C, 5 μm. (G-O) Bar in I, 0.7 μm. (P,Q) Bar in Q, 2 μm. (R-S) Bar in S, 3.3 μm.

When side views of the insertion of the threads to the nuclear envelope were observed, it became evident that one of their ends was always surrounded by ACA labelling (Fig. 3H). When these proximal ends were viewed end-on, INCENP appeared as a dot located either inside or lateral to the ACA labelling (Fig. 3I,J). Interestingly, in some focal planes throughout the pachytene sex body a small thread or a dot was detected. This INCENP signal never colocalized with the two ACA signals corresponding to the unpaired sex centromeres (Fig. 3K). The extent and location of this INCENP labelling inside the sex body was reminiscent of the pseudoautosomal region, the site of pairing and recombination between sex chromosomes.

INCENP is found at the central element of the synaptonemal complex

The results described above suggest that INCENP labels a synaptonemal complex (SC) component. To test whether the INCENP labelling at putative SCs was specific we made a control with the preimmune serum from rabbit 1186 that resulted in no staining (data not shown). To precisely determine the early prophase I stage when INCENP became first detectable, and to ascertain whether INCENP really labelled SCs, we made a double immunolabelling of INCENP and SCP3, a structural component of the SC lateral elements (LEs)

(Moens and Spyropoulos, 1995). In leptotene spermatocytes, no INCENP labelling was detected, but during zygotene the short INCENP threads colocalized with some SCP3-labelled fragments that appeared thicker than those where colocalization was not found (Fig. 5A-C). Thus, INCENP colocalized with regions where homologous LEs were synapsed. During pachytene the INCENP threads colocalized with SCs (Fig. 5D-F). In squashed spermatocytes, the guinea pig anti-SCP-3 antibody allowed us to resolve both LEs along the entire SCs length (white arrowheads in Fig. 5H,K,N). As a rule, SCs appeared twisted along their length, and consequently the two LEs could only be accurately discerned in short regions. When such regions were observed at higher magnification it became evident that the INCENP threads were located in between the LEs (Fig. 5G-L). This pattern of localization was also observed at the SC ends (Fig. 5M-O). To corroborate these results we double immunolabelled INCENP and SCP3 on spread spermatocytes. Results confirmed that INCENP threads colocalized with SCs. Interestingly, in sex bivalents the unsynapsed axial elements did not show INCENP labelling, which was only observed in the pseudoautosomal region (Fig. 5P,Q).

The findings described above suggest that INCENP labels the SC central element (CE). To verify this proposal, we double immunolabelled INCENP and SCP1, a component of the CE, on squashed and spread spermatocytes. In zygotene (Fig. 6A-
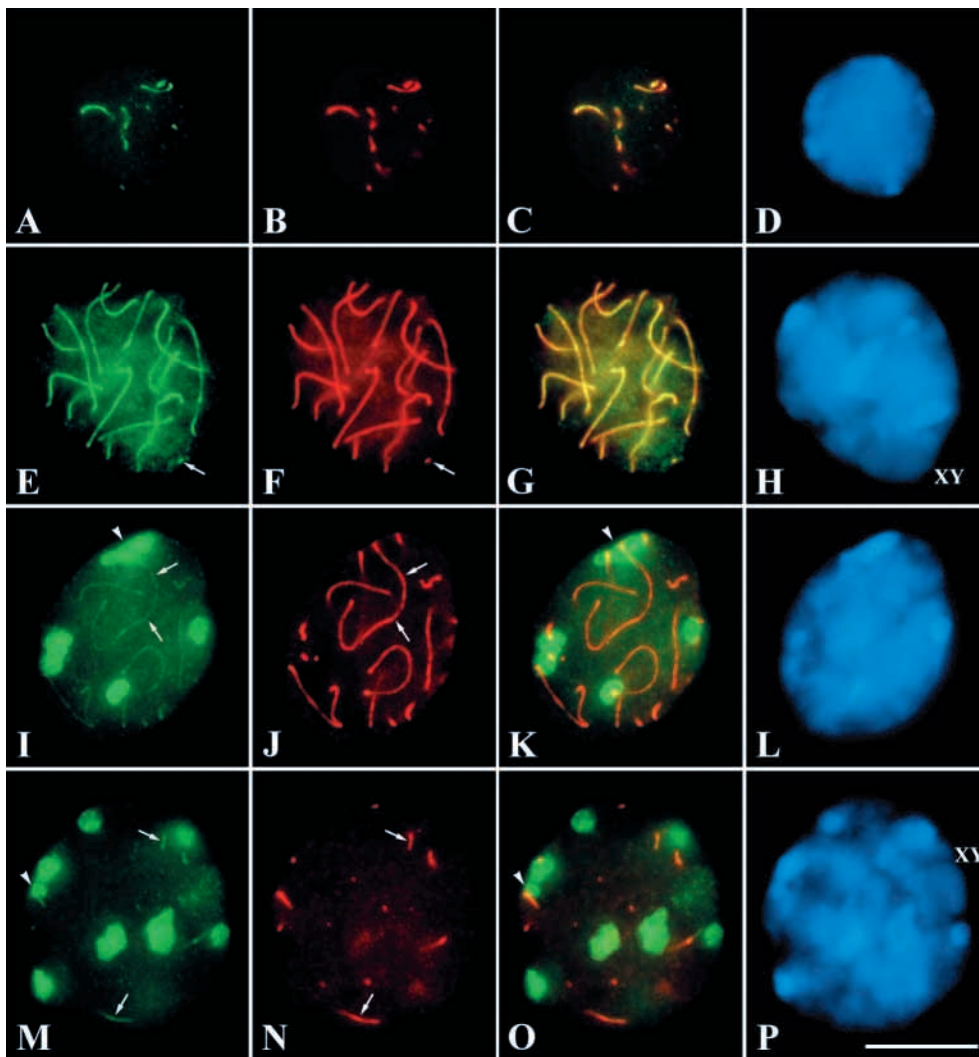
Fig. 6. Double immunolabelling of INCENP and SCP1 in prophase I squashed spermatocytes (INCENP, green; SCP1, red; DAPI, blue). (A-D) Partial projection of four focal planes throughout a zygotene spermatocyte. Short INCENP threads colocalize with short SCP1 segments representing SC portions with central element where homologous chromosomes have synapsed. (E-H) Partial projection of two focal planes throughout a pachytene spermatocyte. INCENP threads and SC central elements along autosomal bivalents colocalize. The peudoautosomal region in the sex bivalent (XY) is labelled by both proteins (arrows). (I-L) Partial projection of three focal planes throughout an early diplotene spermatocyte. INCENP threads (arrows in I) and remaining SC central elements in desynapsing autosomal bivalents (arrows in J) colocalize. Note that the INCENP threads are fainter than the corresponding SCP1 fragments. Chromocentres (arrowhead) are brightly labelled only with INCENP. (M-P) Partial projection of two focal planes throughout a late diplotene spermatocyte. Short INCENP threads (arrows in M) are very faint in comparison with the corresponding SCP1 fragments (arrows in N). Chromocentres (arrowhead) are brightly labelled with INCENP. A faint INCENP labelling of the sex body (XY) is also observed. Bar in P, 10 µm.

D) and pachytene (Fig. 6E-H) squashed spermatocytes INCENP and SCP1 signals perfectly matched, although the INCENP threads appeared more discontinuous than the SCP1 threads. As expected, the pseudoautosomal region of sex chromosomes appeared to be labelled with both antibodies during pachytene (arrow in Fig. 6E,F). Altogether, these results demonstrate that INCENP labels the synaptonemal complex CE, but that its behaviour during prophase I is different from that shown by SCP1.

INCENP relocalizes from synaptonemal complex CEs to heterochromatic chromocentres from late pachytene up to late diplotene

In late pachytene spermatocytes the INCENP threads appeared to be slightly fainter than those found in earlier pachytene nuclei. This loss of fluorescence intensity at the threads was simultaneous with a faint INCENP staining that began to be recognised at the heterochromatic chromocentres found at the nuclear periphery (Fig. 3L-N). These chromocentres, which represent clustered centromere heterochromatic regions of autosomes, appeared brighter than the labelling at the sex body

(Fig. 3L-N). Interestingly, the centromeric regions of the sex chromosomes did not appear to be labelled (Fig. 3M).

During early diplotene, the INCENP-labelled chromocentres appeared brighter than during late pachytene and matched with the heterochromatic chromocentres revealed with DAPI (Fig. 4A-C). Inside some chromocentres a single centromere signal was observed, whereas several centromere signals were observed in other chromocentres (Fig. 4A-G). The sex body appeared fainter than chromocentres (Fig. 4A-C). Additionally, a few short and faintly labelled INCENP threads were present either in the nuclear interior (arrows in Fig. 4A) or associated with the chromocentres (Fig. 4D,E). When early diplotene spermatocytes where observed after a double immunolabelling of INCENP and SCP3, the short INCENP threads colocalized with the still synapsed regions in desynapsing autosomal bivalents (Fig. 5R,S). Moreover, these INCENP threads colocalized with SCP1 ones, but whereas SCP1 threads were bright, INCENP ones were fainter (Fig. 6I-L). In these early diplotene spermatocytes, heterochromatic chromocentres were brightly labelled with INCENP but not with either SCP3 (Fig. 5R,S) or SCP1 (Fig. 6I-L).
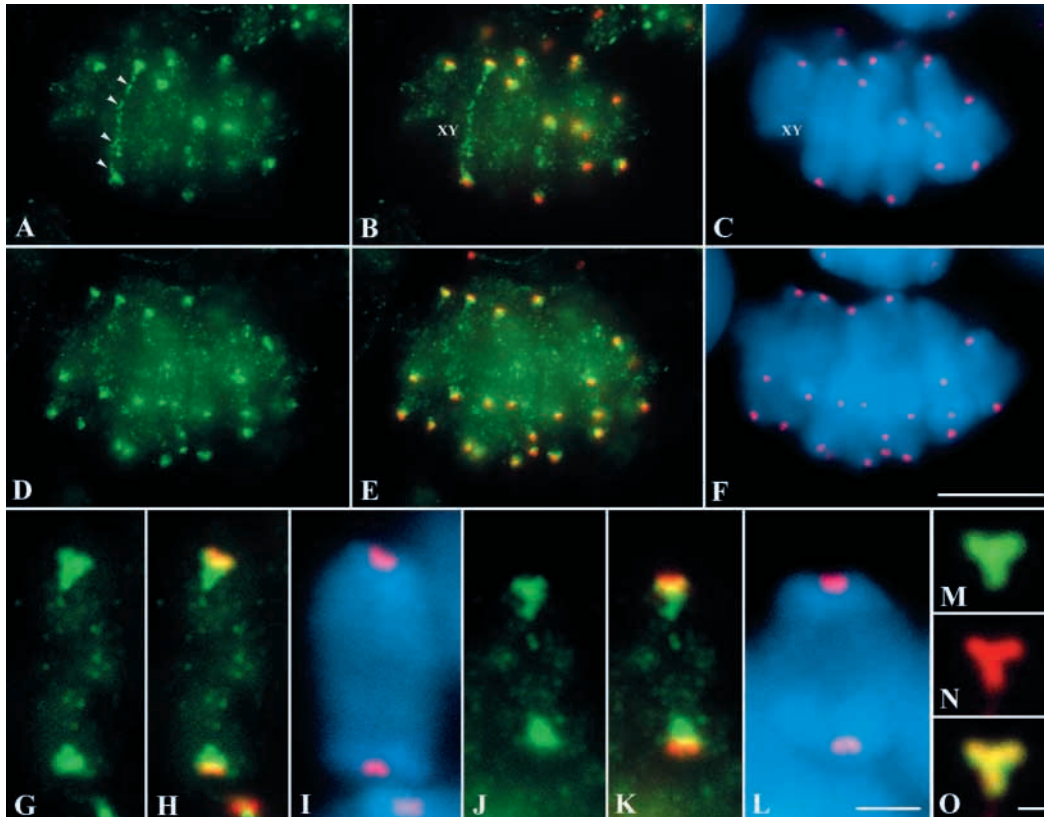
During late diplotene, INCENP threads became

Fig. 7. INCENP distribution in metaphase I spermatocytes (INCENP, green; ACA serum, red in B, C, E, F, H, I, K and L; SCP3, red in N and O; DAPI, blue). (A-F) Two partial projections, each one from three focal planes, throughout the same metaphase I spermatocyte. INCENP labels the centromeres, as detected with the ACA serum. A diffuse faint labelling is observed on bivalents, and a bright signal is discerned at the interchromatid domain (arrowheads) in the sex (XY) bivalent. (G-L) Two selected metaphase I autosomal bivalents. INCENP labels a T-shaped structure beneath the kinetochore labelling obtained with the ACA serum, although both signals partially colocalize. (M-O) Selected metaphase I centromere. INCENP and SCP3 colocalize at a T-shaped structure. (A-F) Bar in F, 10 µm. (G-L) Bar in L, 2 µm. (M-O) Bar in O, 0.5 µm.

progressively fainter and shorter until they finally disappeared whereas bright chromocentres and the fainter sex body appeared to be labelled (Fig. 4H,I). It is worth noting that although the fluorescence intensity of INCENP threads gradually decreased, short segments of SCP1 were still brightly labelled (Fig. 6M-P). Thus, INCENP is lost from SC fragments before SCP1. The pattern of distribution of INCENP from late pachytene up to late diplotene suggests that there is dynamic relocalization of INCENP from synaptonemal complex CEs to the heterochromatic chromocentres.

During diakinesis, the INCENP labelling at the sex body disappeared, chromocentres were no longer identified and a faint labelling was observed over the condensing chromosomes (Fig. 4J,K). A bright INCENP staining was observed at small regions partially colocalizing with the centromere signals, although INCENP domains were slightly larger (Fig. 4J,K). This result suggests that the ongoing chromosome condensation promotes the loss of INCENP from the centromeric heterochromatin.

INCENP shows an unexpected labelling at metaphase I centromeres

During metaphase I a diffuse INCENP labelling was observed on the condensed chromatin both in autosomal and sex bivalents (Fig. 7A-F). Interestingly, the sex bivalent showed a bright staining at the interchromatid domain, the contact surface between sister chromatids (Suja et al., 1999), that was not discerned in the autosomal bivalents (Fig. 7A-C). However, brighter INCENP signals were detected at centromeres (Fig. 7). These signals showed an unexpected T-shape when viewed from the side (Fig. 7G,J). These signals were smaller than the region occupied by the centromeric heterochromatin revealed with DAPI (Fig. 7G-L). Thus, although from late pachytene up to late diplotene the INCENP signals at chromocentres colocalized with the centromeric heterochromatin detected with DAPI, during diakinesis and metaphase I INCENP signals only occupy a small region at the centromeres. This result indicates that INCENP progressively concentrates at the centromere region during late prophase I stages. To precisely determine the location of these signals we double immunolabelled centromeres with INCENP and the ACA serum GS, which mainly recognises proteins found at the inner kinetochore plate (Earnshaw and Cooke, 1989). We observed that most of the T-shaped INCENP labelling was found beneath kinetochore signals, although some degree of colocalization was detected (Fig. 7G-L). Thus, INCENP is mostly located beneath homologous kinetochores.
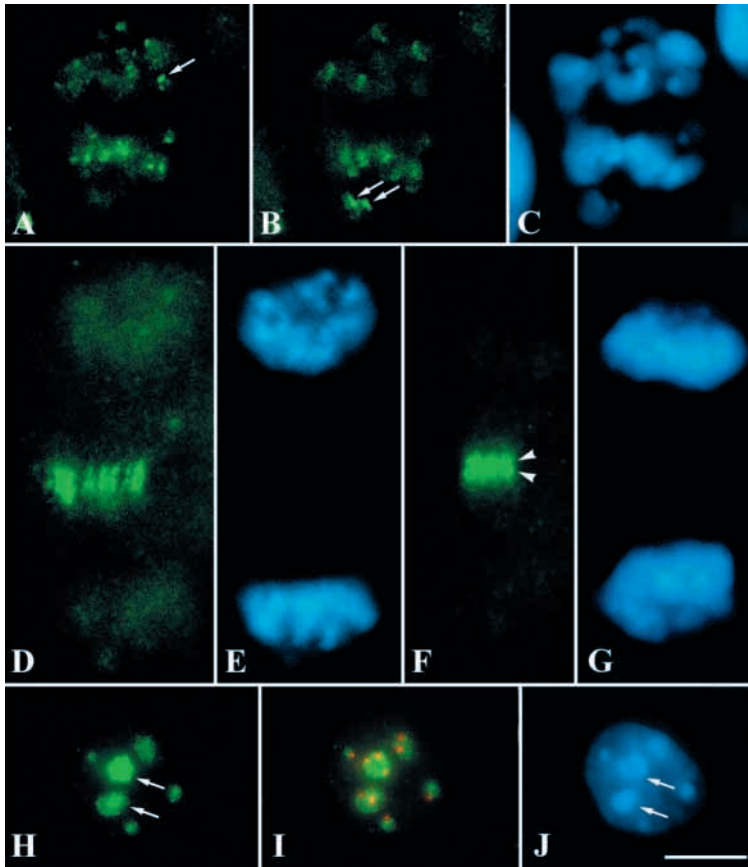
Fig. 8. INCENP expression from anaphase I up to interkinesis (INCENP, green; ACA serum, red in I; DAPI, blue). (A-C) Two focal planes throughout an early anaphase I spermatocyte. The only INCENP signals are at centromeres (arrows). (D-E) Single focal plane of a late anaphase I spermatocyte. A bright INCENP labelling is detected at the spindle midzone. Some faint and dispersed INCENP labelling is observed at centromeres. Since a short exposure time was used to accurately visualize the midzone signal, labelling at centromeres is very low. (F,G) Single focal plane of a telophase I spermatocyte. INCENP only labels the spindle midzone (arrowheads). (H-J) Partial projection of two focal planes throughout an interkinesis spermatocyte. A bright INCENP labelling is observed at chromocentres (arrows). Bar, 10 μm.

Similar T-shaped signals have been previously described in metaphase I centromeres with anti-SCP3 antibodies (Prieto et al., 2001). In order to verify whether INCENP colocalizes with SCP3 we used double immunolabelling. Results showed that both proteins appeared as T-shaped structures at centromeres and that they colocalized (Fig. 7M-O).

INCENP is retained at centromeres throughout anaphase I and relocalizes to the spindle midzone during late anaphase I

During early anaphase I INCENP signals were only found at centromeres (Fig. 8A-C). These centromere signals were fuzzy and fainter than those previously found during metaphase I (compare Fig. 7A,D with Fig. 8A,B). At late anaphase I a bright INCENP labelling was detected at the spindle midzone, although a fainter and more diffuse labelling at centromeres was still visible (Fig. 8D,E). However, at telophase I this faint centromere labelling disappeared and only two brightly labelled INCENP signals were observed at the midbody (Fig. 8F,G). These results show, in contrast to what was found during mitosis, that INCENP remains at centromeres throughout anaphase I and that the labelling at the spindle midzone only appears at late anaphase I.

INCENP appears at a connecting strand between sister kinetochores in metaphase II centromeres

In spermatocytes undergoing interkinesis, INCENP was detected at chromocentres that colocalized with those observed after DAPI staining (Fig. 8H-J). All centromere signals were observed inside those chromocentres (Fig. 8I). This result is thus comparable to that observed in interphase spermatogonia (compare Fig. 2A,B with Fig. 8H-J). In metaphase II spermatocytes INCENP was not observed on condensed chromatin but only at centromeres (Fig. 9A-C). Two INCENP spots were observed on opposite faces of the centromere region. These spots partially colocalized with sister kinetochores revealed by the ACA serum, although most of the INCENP spots were beneath kinetochores (Fig. 9A-C). Surprisingly, INCENP also labelled an irregular strand between sister kinetochores that crossed the entire centromere (arrowheads in Fig. 9D-G), which is remarkably similar to the CLiP staining previously described in mitotic chromosomes (Rattner et al., 1988). At the onset of anaphase II, just when sister chromatids began to separate, the strand appeared to break in the middle region (arrow in Fig. 9H,I). During early anaphase II, however, INCENP was still detected at centromeres (Fig. 9J,K). At late anaphase II, as is the case during late anaphase I, INCENP labelling was detected at the spindle midzone, finally concentrating at the midbody (Fig. 9L,M). During telophase II, in addition to midbody labelling, clear INCENP signals were observed to colocalize with kinetochores (Fig. 10A-C). These INCENP signals became larger and fainter during late telophase II (Fig. 10D-F). In early round spermatids one or two large INCENP signals were found at the nuclear interior colocalizing with the heterochromatic chromocentres revealed after DAPI staining (Fig. 10G,H). In
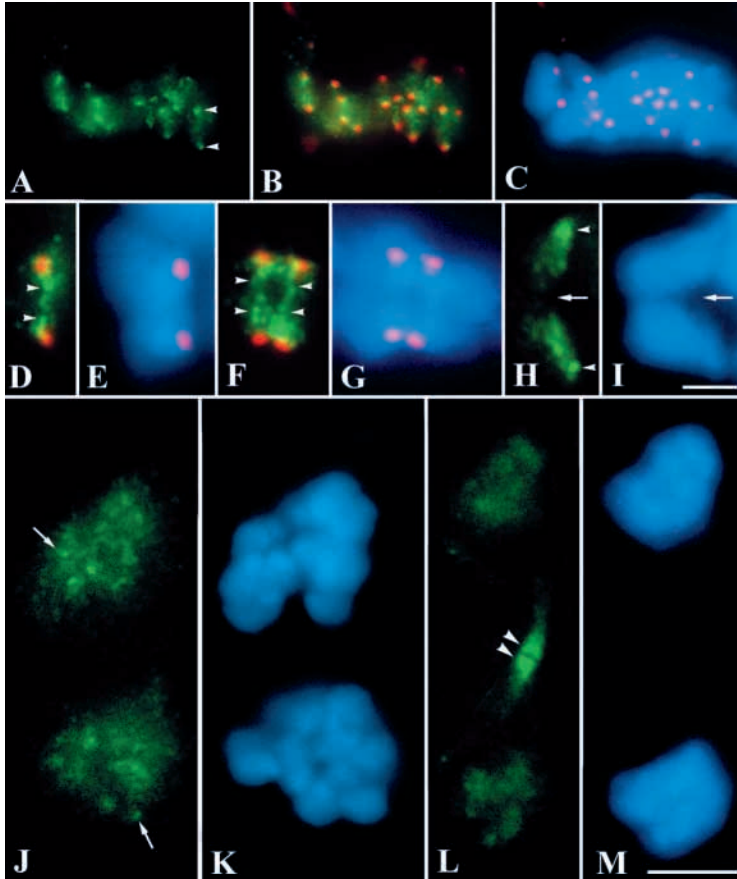
Fig. 9. INCENP labelling from metaphase II up to telophase II (INCENP, green; ACA serum, red; DAPI, blue). (A-C) Partial projection of five focal planes throughout a metaphase II spermatocyte. INCENP signals appear at centromeres and are concentrated in two spots (arrowheads) partially colocalizing with sister kinetochores as revealed with the ACA serum. (D-G) Selected metaphase II chromosomes. INCENP is concentrated below sister kinetochores and at an irregular connecting strand that joins them. (H,I) Early anaphase II chromosome. INCENP is concentrated at sister kinetochores (arrowheads), and some faint interrupted labelling at the connecting strand (arrow) is visible. (J,K) Partial projection of two focal planes throughout an anaphase II spermatocyte. A diffuse INCENP labelling at centromeres (arrows) is observed. (L,M) A single focal plane of a late anaphase II spermatocyte. The INCENP labelling is discerned as two parallel plates (arrowheads) at the midbody. (A-C,J-M) Bar in M, 5 µm. (D-I) Bar in I, 2 µm.

more advanced spermatids the INCENP labelling was no longer found at chromocentres, although the centromere signals were still observed (Fig. 10I,J).

Aurora-B kinase appears at chromocentres after INCENP association, and both proteins colocalize in metaphase I and metaphase II centromeres

In somatic cells INCENP targets aurora-B to chromosomes. We therefore performed a double immunolocalization of both proteins to test whether this also occurred in mouse meiosis. Aurora-B was first detected in diplotene spermatocytes. Surprisingly, and in contrast to INCENP, aurora-B did not label the synaptonemal complex CE in previous prophase I stages. In diplotene spermatocytes that still showed a faint INCENP labelling at SC remnants, and bright staining at chromocentres, no aurora-B labelling was observed (Fig. 11A-D). However, in late diplotene spermatocytes, when no SC remnants were visible with INCENP, aurora-B colocalized with INCENP at chromocentres (Fig. 11E-H). These results suggest that INCENP also targets aurora-B in mouse spermatocytes. From diakinesis onwards, INCENP and aurora-B colocalized. Interestingly, the localization of both proteins perfectly matched at the T-shaped structure at metaphase I centromeres (Fig. 11I-L), and they also appeared to overlap at the connecting strand between sister kinetochores at metaphase II centromeres (Fig. 11M-P). We also tried to localize survivin with three different antibodies; however, none of them labelled meiotic centromeres.

Aurora-B appears at diplotene chromocentres before phosphorylated histone H3

It has been demonstrated that in somatic cells the aurora-B kinase is responsible for histone H3 phosphorylation (pH3). To test this in meiosis we performed a double immunolocalization of aurora-B and pH3. Some diplotene spermatocytes showed aurora-B at chromocentres but no pH3 staining (Fig. 11Q-T). However, both proteins colocalized at chromocentres in some other late diplotene (Fig. 11U-X) and early diakinesis spermatocytes. During diakinesis, aurora-B appeared to be concentrated at individualized centromeres, as is the case with INCENP, whereas pH3 labelled all condensing bivalents (data not shown). The first appearance of phosphorylated H3 in late diplotene spermatocytes agrees with previous localizations (Cobb et al., 1999). Our results thus suggest that the presence of aurora-B may be necessary for the initial phosphorylation of histone H3 at heterochromatic chromocentres.

Discussion

In this study we have analysed for the first time the subcellular distribution of the chromosomal passenger complex proteins INCENP and aurora-B kinase during mammalian meiosis. We unexpectedly found that INCENP, but not aurora-B, colocalizes with the SC. Co-detection of INCENP with SCP3 and SCP1, components of the synaptonemal complex LEs and CE, respectively (for a review, see Zickler and Kleckner, 1999), demonstrates that INCENP is present at the CE from zygotene up to diplotene. We observed that INCENP begins to relocalize
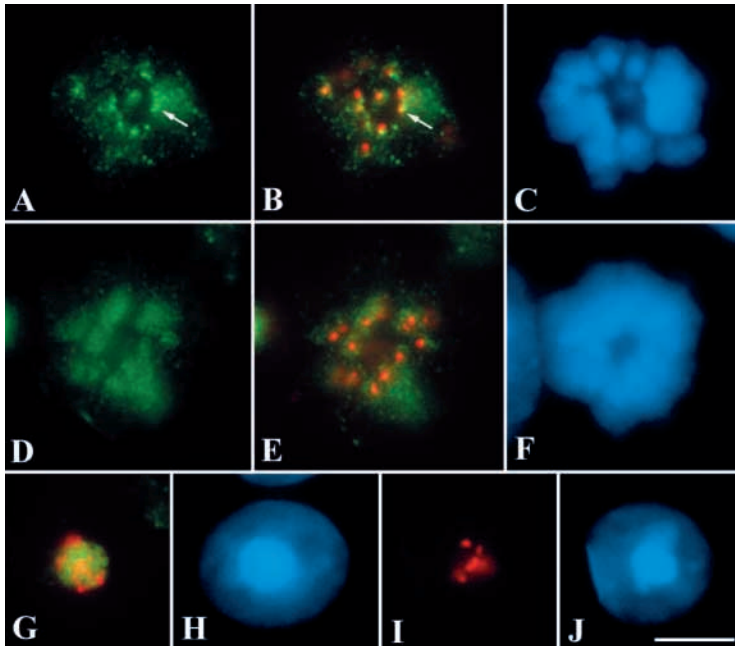
Fig. 10. INCENP labelling at telophase II poles and round spermatids (INCENP, green; ACA serum, red; DAPI, blue). (A-C) Partial projection of two focal planes throughout an end-on view of an early telophase II pole. INCENP signals colocalize with kinetochores (arrows). (D-F) Partial projection of two focal planes throughout an end-on view of a late telophase II pole. INCENP signals appear as diffuse and faintly labelled clouds surrounding kinetochores. (G,H) Single plane of an early round spermatid. INCENP localizes at a centrally located and large brightly labelled chromocentre containing centromeres. (I,J) Single plane of a late round spermatid where only centromere signals are observed. Bar, 5 μm.

to heterochromatic chromocentres during late pachytene, and that it disappears from CEs during diplotene whereas SCP1 is still present at remaining SC regions. Thus, the pattern of disassembly of INCENP and SCP1 from the CE is different, and, consequently, INCENP and SCP1 are unlikely to form a functional complex, at least during late pachytene and diplotene. The presence of INCENP at CEs suggests a novel undefined function for this protein during mammalian meiosis. It is tempting to speculate that INCENP, like SCP1, mediates and maintains the association between both LEs from zygotene up to diplotene. Since INCENP and SCP1 appear at the same time it is unlikely that one protein could recruit the other to CEs. Obviously, more ultrastructural and biochemical studies are needed to understand the role of INCENP in the SC, as well as its dynamic relocalization from SCs to the heterochromatic centromere regions during late pachytene and diplotene.

Aurora-B first appears during late diplotene at heterochromatic chromocentres that are already labelled by INCENP. This result suggests that, as occurs in somatic cells (Adams et al., 2000; Adams et al., 2001b), INCENP may also target aurora-B to the chromocentres during the first meiotic prophase. It is not obvious why, during male mouse meiosis, INCENP targeting is first detected in zygotene spermatocytes at least a week before aurora-B is first observed at diplotene. One possibility is that INCENP does not target aurora-B during meiosis. Secondly, it may be that aurora-B is not expressed in zygotene spermatocytes. Aurora-B expression might occur during diplotene once INCENP has completely disappeared from CEs. Thirdly, it is possible that these differences in targeting arise from meiosis-specific differences in survivin or another as yet unknown subunit of the chromosomal passenger complex. Histone H3 phosphorylated on serine[10] is first detected during late diplotene at chromocentres that are already labelled by INCENP and aurora-B. This sequence of appearance of aurora-B kinase and pH3 supports previous reports suggesting that aurora-B phosphorylates histone H3 in somatic cells (Hsu et al., 2000; Speliotes et al., 2000; Giet and

Glover, 2001; Zeitlin et al., 2001; Crosio et al., 2002; MacCallum et al., 2002).

INCENP and aurora-B colocalize throughout heterochromatic chromocentres during diplotene and concentrate at individual centromeres during diakinesis in parallel with ongoing chromosome condensation and separation. However, during metaphase I both proteins appear as T-shaped signals that do not occupy all the heterochromatic region, as detected by DAPI, and that are located beneath associated sister kinetochores as detected with the ACA serum. Thus, INCENP and aurora-B are progressively concentrated from centromeric heterochromatin to the T-shaped centromere domain between diakinesis and metaphase I. As these proteins are located at the inner centromere domain in mitotic chromosomes, we propose that this T-shaped domain corresponds to the inner centromere domain of metaphase I chromosomes. A similar T-shaped distribution was not described when mammalian metaphase I centromeres were stained for other centromeric components. Interestingly, we previously reported that SCP3 appears as T-shaped signals at metaphase I centromeres (Prieto et al., 2001). Here we demonstrate by a double immunolabelling that INCENP colocalizes with SCP3 at the inner centromere domain. Moreover, the cohesin subunit Rad21/Scc1 also colocalizes with INCENP and SCP3 in this domain (J.A.S., unpublished). Thus, the inner centromere domain in mouse metaphase I centromeres contains not only the chromosomal passenger complex, with at least INCENP and aurora-B, but also some subunits of the cohesin complex. Taking into account the presence of INCENP and aurora-B at the inner centromere domain during metaphase I, their colocalization with the cohesin subunit Rad21, and their release from centromeres during anaphase I, it is tempting to speculate that INCENP and aurora-B are present at the right place and time for a function in regulating sister-chromatid centromere cohesion. This is supported by the observation that inactivation of INCENP and aurora-B by RNAi interferes with the disjunction of sister
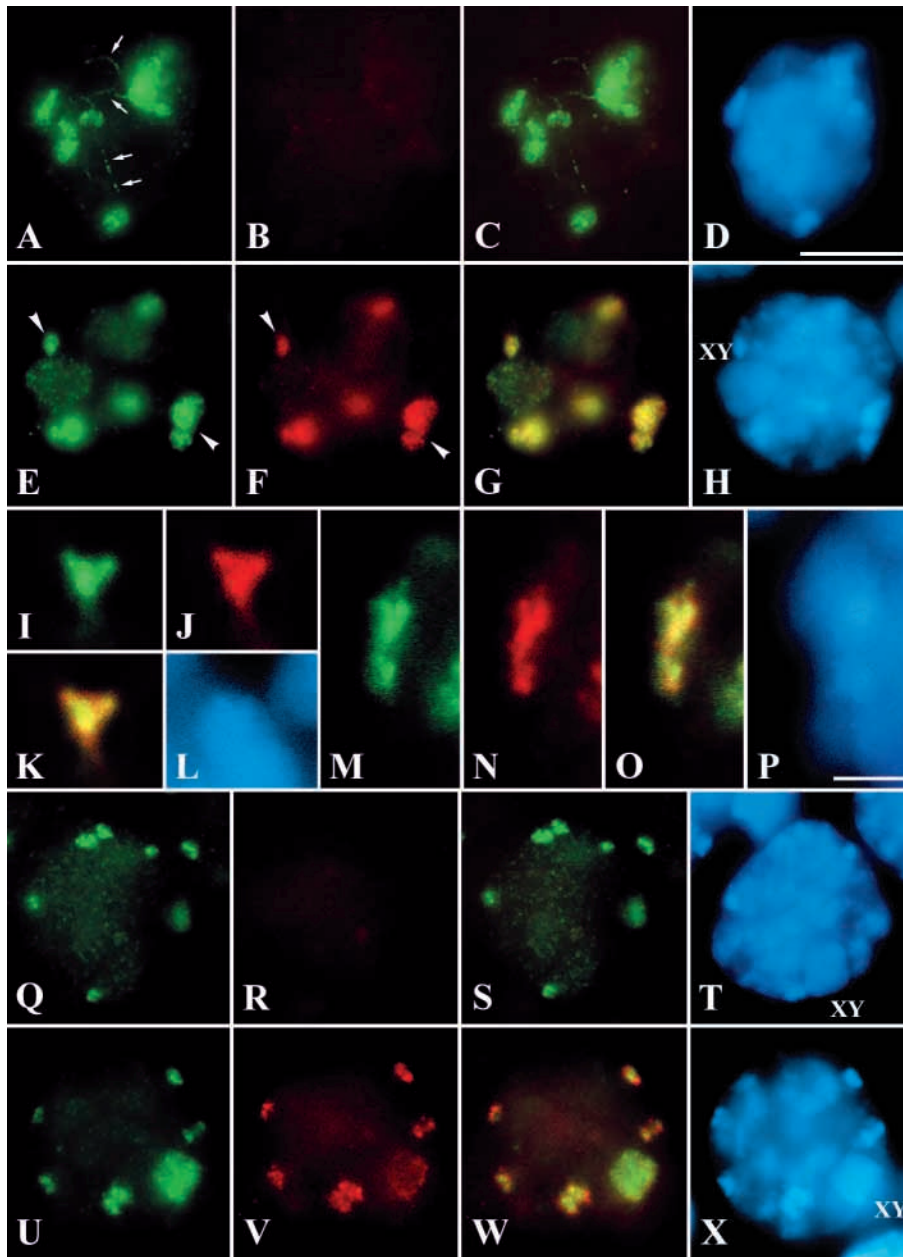
Fig. 11. Double immunolabelling of INCENP and aurora-B kinase (A-P), or aurora-B and phosphorylated histone H3 (pH3) (Q-X), in squashed spermatocytes (INCENP, green in A-P; aurora-B kinase, red in A-P, green in Q-X; pH3, red in Q-X; DAPI, blue). (A-D) Partial projection of three focal planes throughout a diplotene spermatocyte. Chromocentres and short and faint nuclear threads (arrows) are labelled with INCENP, but not with aurora-B. (E-H) Partial projection of two focal planes throughout a late diplotene spermatocyte. Chromocentres (arrowheads) are labelled by both INCENP and aurora-B. The sex body (XY) appears faintly labelled with INCENP. (I-L) Selected metaphase I centromere. INCENP and aurora-B colocalize at a T-shaped structure. (M-P) Selected metaphase II centromere. The connecting strand traversing the centromere shows colocalization of INCENP and aurora-B. (Q-T) Late diplotene spermatocyte. Chromocentres only appear labelled with aurora-B. (U-X) Late diplotene spermatocyte. Chromocentres and the sex body (XY) are labelled with aurora-B and pH3. (A-H,Q-X) Bar in D, 10 μm. (I-P) Bar in P, 2 μm.

kinetochores in *Drosophila* cells undergoing anaphase (Adams et al., 2001b). Additionally, it has been recently proposed that the cohesin subunit Rad21 is necessary for the accurate targeting of INCENP to the inner centromere domain (Sonoda et al., 2001). Moreover, aurora-B phosphorylates the cohesin subunit Rec8, a meiosis-specific variant of Rad21, and therefore promotes the release of sister-chromatid cohesion during both meiotic divisions in *C. elegans* (Rogers et al., 2002).

Another interesting result is the appearance of INCENP and aurora-B in a connecting strand traversing the centromere region, and joining sister kinetochores, in metaphase II centromeres. This strand disappears at the metaphase II/anaphase II transition, and these proteins then relocalize to the spindle midzone. A similar labelling has not been previously observed in mammalian metaphase II centromeres.

However, a connecting strand has been reported after silver staining in grasshopper metaphase II centromeres (Rufas et al., 1989; Suja et al., 1992). In somatic cells, CLiPs (Rattner et al., 1988), DNA topoisomerase IIα (Rattner et al., 1996), the mitotic centromere-associated kinesin (MCAK) (Maney et al., 1998) and Mei-S332 in *Drosophila* (Blower and Karpen, 2001) have been detected at a similar connecting strand and proposed to be somehow involved in centromere cohesion. Similarly, we propose that INCENP and aurora-B could regulate centromere cohesion during mammalian meiosis II, possibly through action of aurora-B on one of the other components. Interestingly, we have recently shown that aurora-B can phosphorylate DNA topoisomerase IIα in vitro (C. Morrison and W.C.E., unpublished). We have observed that neither the cohesin subunits Rad21 (J.A.S., unpublished) nor STAG3 (Prieto et al., 2001) is present at the connecting strand during

metaphase II. However, it has been reported that the cohesin subunits Smc1β and Smc3 appear at metaphase II centromeres (Revenkova et al., 2001). Taking into account these results, it is possible that INCENP and aurora-B regulate centromere cohesion by acting on different cohesin complexes during meiosis I and meiosis II. It remains to be tested whether INCENP colocalizes with Smc1β, Smc3 and Rec8, the meiosis-specific variant of Rad21, in metaphase II centromeres, and whether any of these cohesin subunits is a target of aurora-B phosphorylation. Obviously more studies are needed to ascertain whether during meiosis the chromosomal passenger complex plays a critical role in coordinating chromosome separation by regulating centromere cohesion and cytokinesis.

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