Meiotic activation of rat pachytene spermatocytes with okadaic acid: the behaviour of synaptonemal complex components SYN1/SCP1 and COR1/SCP3

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SUMMARY

The phosphatase inhibitor okadaic acid accelerates meiotic events in rodent germ cells in culture. Isolated pachytene spermatocytes treated with okadaic acid proceed to a metaphase I arrest in a few hours as opposed to the similar process in vivo, which requires several days. Leptotene/zygotene spermatocytes cannot be activated in this way, suggesting that okadaic acid enables cells to bypass a sensor of the meiotic progression, which is pachytene specific. We monitored the chromosome behaviour accompanying the transition to metaphase I in rat spermatocytes with antibodies against COR1/SCP3, a component of the meiotic chromosome cores, and against the synaptic protein, SYN1/SCP1. Okadaic acid induced a rapid synaptonemal complex dissolution and bivalent separation, followed by chromosome condensation and chiasmata formation, similar to the succession of events in untreated cells. The similarity between meiosis I induced with okadaic acid and the meiosis I events in vivo extends to the dissolution of the nuclear membrane and the disappearance of the microtubule network at the onset of metaphase I. This cell culture system provides a model for the in vivo transition from pachytene to metaphase I and therefore can be used in the study of this transition at the molecular level. The effect of okadaic acid is most likely mediated by the activation of tyrosine kinases, as addition of genistein, a general tyrosine kinase inhibitor, completely abolishes the observed effect of okadaic acid on chromosome metabolism. The okadaic acid-induced progression to the metaphase I arrest is not affected by the inhibition of protein synthesis. However, pachytene spermatocytes incubated in the presence of protein synthesis inhibitors for 6 hours show loss of synapsis which is abnormal in that it is not accompanied by chiasmata formation. The two meiosis-specific proteins, SYN1/SCP1 and COR1/SCP3, are efficiently phosphorylated in vitro by extracts from isolated pachytene cells. Extracts from cells that have reached metaphase I upon okadaic acid treatment, with concomitant displacement of SYN1/SCP1 and COR1/SCP3 from their chromosomes, do not have this capability. These data support the hypothesis that phosphorylation of SYN1/SCP1 and COR1/SCP3 targets their removal from the chromosomes and that activity of the kinases involved correlates with the presence of these two proteins on the chromosomes.

Key words: Meiosis, Synaptonemal complex, Spermatocyte, Okadaic acid, Immunocytochemistry

INTRODUCTION

The characterization of spermatogenesis at the molecular level has been complicated by the large number of different cell types in the mammalian testis. The methods for testicular cell fractionation such as sedimentation and centrifugal elutriation (Meistrich, 1977; Heyting et al., 1985) give sufficient levels of purification to allow biochemical and cytological analyses of certain meiotic prophase I stages. It remains difficult, however, to analyse progressive changes because of the impossibility to separate homogenous populations of cells in closely related stages. A novel approach for culturing mid- to late pachytene spermatocytes and rapidly inducing them to proceed to metaphase I with okadaic acid (OA) has recently been reported

(Handel et al., 1995). This system offers the advantage that the morphological changes accompanying the progression through prophase I can be easily monitored and their molecular basis characterized (Wiltshire et al., 1995).

OA is a potent inhibitor of the ATP-Mg^{2+}-dependent (AMD or type 1) and polycation-stimulated (PCS or type 2A) protein phosphatases (Bialojan and Takai, 1988; reviewed by Cohen et al., 1990). OA induces oocytes in culture to exit the prophase I arrest (the dictyate stage) in a variety of species, a process that is morphologically manifested by chromatin remodelling and nuclear envelope breakdown. It is believed that OA acts primarily by activating the maturation promoting factor (MPF; also called the M-phase promoting factor; reviewed by Hunt, 1989 and Nurse, 1990). MPF is a heterodimer of a B-type

cyclin and the p34^{cdc2} kinase (Norbury and Nurse, 1992; Murray and Hunt, 1993). p34^{cdc2} activity is regulated by an equilibrium between phosphorylation and dephosphorylation at specific residues (Cyert and Kirschner, 1988; Dumphy and Newport, 1989; Atherton-Fessler et al., 1993) and it is possible that OA interferes with this equilibrium, rendering a constitutively active MPF.

We are interested in chromosome behaviour during meiotic prophase I in rodent spermatocytes. Events such as homologous chromosome pairing, synapsis and recombination, essential for the successful completion of meiosis, occur at this stage (reviewed by Roeder, 1997). At pachytene the synaptonemal complex (SC), a structure composed of proteins and nucleic acids, is fully assembled along the length of the paired homologs (for review, see von Wettstein et al., 1984; Moens, 1994). The SC has a tripartite structure with two lateral regions, which establish contacts with the DNA of the sister chromatids of each homolog, and a central region, which connects the two lateral regions through an array of transverse filaments. The transverse filaments mediate synapsis and contain a 125 kDa protein, termed SYN1 in hamster (synapsis; Dobson et al., 1994) or SCP1 in rat/mouse (synaptonemal complex protein 1; Meuwissen et al., 1992; Moens et al., 1992; Sage et al., 1995). The C termini of SYN1/SCP1 have been shown to localize in the lateral elements of the SC, while the N termini are localized in the central region of the SC (Dobson et al., 1994; Schmekel et al., 1996; Liu et al., 1996). The N termini of SYN1/SCP1 have been shown to interact in a two-hybrid system (Liu et al., 1996), consistent with a model of synapsis being mediated by connections of the transverse filament ends in the central region of the SC. SYN1/SCP1 is recruited to the SC at zygotene and it is fully assembled at pachytene, when complete synapsis is detected. By late pachytene, the gradual displacement of SYN1/SCP1 from the chromosomes initiates, marking the beginning of SC dissolution (Dobson et al., 1994; Moens and Spyropoulos, 1995).

The first identified component of the axial elements (cores) of the lateral regions of the SC was termed SCP3 in rat/mouse (synaptonemal complex protein 3; Heyting et al., 1987; Moens et al., 1987; Lammers et al., 1994) and COR1 in hamster (cores; Dobson et al., 1994). Two-hybrid analyses showed that the interaction between COR1/SCP3 molecules is mediated by the coiled-coil domains present in the carboxyl half of the molecule (Tarsounas et al., 1997). It is likely that COR1/SCP3 is involved with the attachment of the DNA to SCs, and based on its persistence along the separated chromosomal axes after dissolution of synapsis, it has been postulated that it is involved in sister chromatid cohesiveness at meiosis I (Dobson et al., 1994). At metaphase II, COR1/SCP3 localizes to the sister kinetochores, suggesting a role in their cohesiveness until the final meiotic segregation (Moens and Spyropoulos, 1995). A second component of the meiotic chromosome cores, termed SCP2 in rat and human, has been recently identified (Offenberg et al., 1998).

An interesting aspect related to the dynamics of these two proteins on the meiotic chromosomes is the precise timing of their departure from the chromosomes: SYN1/SCP1 displacement coincides with the repulsion of homologues at diplotene, while COR1/SCP3 disappears at metaphase II

synchronously with sister chromatid separation. This suggests that the displacement and degradation of SYN1/SCP1 and COR1/SCP3 are precisely programmed. The interaction of both proteins with the ubiquitin-conjugating enzyme Ubc9 in a two-hybrid system (Tarsounas et al., 1997) indicates that ubiquitination could be the signal for initiation of their degradation.

In this study we use an immunocytological approach to characterize the general cell morphology and, in particular, the behaviour of meiotic chromosomes in rat pachytene spermatocytes that progress to a metaphase I arrest point upon treatment with OA. Chromosomal configuration during the pachytene-metaphase I transition was determined with antibodies against the synaptic protein SYN1/SCP1, and against the chromosome core component COR1/SCP3. These two proteins are considered molecular markers for the meiotic prophase I stages and sub-stages, and therefore they are instrumental in the characterization of the OA-induced transition in unprecedented detail. A monoclonal anti-β tubulin antibody was used for visualization of microtubules. We conclude that the meiotic progression induced with OA follows the conventional pattern normally observed in testicular cells. This system therefore represents a powerful tool for studying various aspects of meiosis I, as it allows cytological and biochemical analyses of the meiotic events in a 'fast forward' mode. In this system we characterize the effect of inhibition of tyrosine kinases and of inhibition of protein synthesis on chromosome metabolism. We also provide evidence that phosphorylation of the two meiosis specific proteins SYN1/SCP1 and COR1/SCP3 occurs at pachytene and discuss its relevance for SC dissasembly and meiotic progression.

MATERIALS AND METHODS

Spermatocyte isolation

Pachytene spermatocytes were isolated from testes of 30- to 40-day-old rats by centrifugal elutriation. For testis preparation we used a modified version of the procedure described by Heyting et al. (1985). Testes were removed, decapsulated and transferred to 20 ml of TIM (testis isolation medium; 0.1 M NaCl, 0.05 M KCl, 1.2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 6 mM Na$_2$HPO$_4$, 0.7 mM KH$_2$PO$_4$, 0.1% glucose, 0.04% L-glutamine and 0.001% Phenol Red; Dietrich et al., 1983) supplemented with 1 mM sodium pyruvate, 6 mM sodium-DL-lactate and antibiotics. The seminiferous tubules were dispersed by incubation for 55 minutes at 31°C with 2 mg/ml collagenase in TIM, then washed in TIM and sedimented. Following four washes in TIM, the tubules were resuspended in TIM containing 0.35 mg/ml trypsin and incubated for 15 minutes at 31°C. Cells were pelleted by centrifugation at low speed and resuspended in TIM with 0.1% BSA, trypsin inhibitor and 1 μg/ml DNase I. Cells were drawn up and down 100× through the large end of a 10 ml plastic pipette and filtered using a 70-mesh nylon screen.

Separation of the spermatocyte fraction by elutriation was performed following the procedure described by Grabske et al. (1975) and Meistrich (1977). The pachytene fraction was collected at a flow rate of 25-30 ml/minute. A total of 10^8-10^9 testicular cells in suspension usually yields 10^7-10^8 cells in the pachytene spermatocyte fraction. About 90% of these cells contain SCs, as observed in subsequent analyses by immunofluorescence. A fraction of small cells in early prophase I stages (leptotene/zygotene) was also collected at a flow rate of 15-20 ml/minute.

Short-term spermatocyte culture and [^{35}S]methionine labelling

Cell culture was performed as described by Wiltshire et al. (1995). The collected cells were counted and resuspended in TIM. Cells were plated at a concentration of 5×10^6 cells/ml in 35×10 mm culture dishes for cell suspension (Sarstedt) and cultured at 32ºC with 5% CO$_2$ in air. The culture medium was supplemented with 2-5 µM OA (Sigma) from 1 mM stock in 100% ethanol or with an equal volume of the solvent in control samples. The cells incubated in ethanol are morphologically indistinguishable from untreated cells. For immunocytological assays, 40 µl of culture was removed at various times and spread on glass slides as described below.

For metabolic labelling, [^{35}S]methionine (Amersham) was added to the culture medium before incubation to a final concentration of 0.1 mCi/ml, sometimes along with the appropriate drugs. The cells were washed five times in PBS and lysed in 2× SDS sample buffer (4 M urea, 10% SDS, 0.25 M Tris-HCl, pH 6.8, 20% glycerol, 0.015% Bromophenol Blue, 1 mM EDTA, 10% v/v β-mercaptoethanol). Equal numbers of cells were used for each treatment, and equal volumes of the lysates were loaded into the protein gel.

Antibodies

The anti-SC-component antibodies used in both slide and blot immunostaining were previously described (Dobson et al., 1994). The CREST serum with specificity for centromeres was described by Moens et al. (1987). The anti-β tubulin antibody was a mouse monoclonal (Cedarlane). All primary antibodies were used at 1/500 or 1/1,000 dilution. The anti-COR1/SCP3 antibody was raised in mice against the full-length, bacterially expressed, recombinant His-tagged protein, which was purified on an Ni-NTA column (Qiagen). The anti-SYN1/SCP1 antibody was generated in rabbit against a protein fragment expressed from clone HSC15 (Moens et al., 1992). This serum was used as such or was affinity-purified on a Syn1C-immobilized Sepharose column (Pharmacia Biotech). The Syn1C protein represents the C-terminal half of mouse SYN1/SCP1 (amino acids 499-994; Sage et al., 1995). A 1,663 bp fragment of the mouse *Syn*1/*Scp*1 full-length cDNA in pBluescript was PCR-amplified (*Pfu*, Stratagene) using SCP1416F (5′-GCC GGA TCC ATG AAA ACT GAG-3′) and T7 promoter (5′-TAA TAC GAC TCA CTA TA-3′) primers. Following digestion with restriction enzymes *Bam*HI and *Xho*I, the fragment was cloned in the corresponding sites of pET28a (Novagen) to produce an in-frame N-terminal fusion with the (His)$_6$ tag.

Immunocytology

A 40 µl sample containing approximately 2×10^5 cells was used for slide preparation from each time point. Glass slides were washed, immersed in a solution of 2% BSA in distilled water and air-dried. The spreading procedure was that described by Peters et al. (1997). Briefly, slides were dipped in 2% paraformaldehyde, pH 8.5, 1% Triton X-100 in distilled water. A drop of the cell suspension was placed on the upper left corner of the slide and allowed to run down along the slide in one direction, and then in the perpendicular direction. Slides were dried briefly and washed three times in 0.4% Kodak Photo-Flo, pH 8.0, in water. After another short air-drying period, slides were either stored overnight at –40°C or processed directly for immunostaining as previously described (Dobson et al., 1994).

The following variations from this procedure were also used: (1) for spindle staining, cells were fixed in methanol at room temperature for 15 minutes and spread as above; (2) for cytoplasmic staining, cells were fixed in 2% paraformaldehyde, pH 8.5, in PBS in the absence of Triton X-100.

Electron microscopy

Cultured spermatocytes (5×10^5 cells) were pelleted, fixed in 2% glutaraldehyde in PBS and postfixed in 1% osmium tetroxide in PBS.

Following overnight dehydration in 100% ethanol, cells were embedded in Epon and sectioned using an ultramicrotome equipped with a diamond knife. The sections were mounted on Formvar-coated single-hole grids, stained and examined with the electron microscope.

Cell fractionation

Cultured spermatocytes were pelleted and resuspended in 50-100 µl of lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.01% NaN$_3$, 1 mM PMSF, 0.1% Triton X-100, 0.5 mM DTT, 150 mM NaCl). Following 5 minutes incubation at room temperature, the nuclei were pelleted by centrifugation at 5,000 *g*. The supernatant was cleared by centrifugation at 30,000 *g* at 4°C for 15 minutes and used in the λ-phosphatase assay or for SDS-PAGE. The nuclear pellet was solubilized in 50-100 µl of buffer containing 8 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl, pH 8.0. 50 µg total protein from each fraction was mixed with 2× SDS sample buffer, boiled for 5 minutes and analysed by SDS-PAGE (Laemmli, 1970). 10-12% SDS-polyacrylamide gels were run for 3-4 hours at 4°C and 40 mA per gel.

Immunoblotting

Protein fractions were separated by SDS-PAGE and transferred to nitrocellulose filters as described by Sambrook et al. (1989). After 1 hour blocking at room temperature in blocking buffer (5% non-fat dried milk in TBST: 10 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 0.05% Tween-20) the filters were incubated overnight at 4°C in primary antibodies diluted 1:500 or 1:1,000 in blocking buffer. Following three washes in blocking buffer, they were incubated in alkaline phosphatase-conjugated secondary antibodies (Cedarlane Laboratories) diluted 1:1,000 or 1:10,000 in blocking buffer. The filters were washed again three times in TBST and proteins were detected with NBT/BCIP (Promega) in alkaline phosphatase buffer (0.1 M Tris, pH 9.0, 0.1 M NaCl, 0.05 M MgCl$_2$). To detect changes in the mobility of the 125 kDa SYN1/SCP1, electrophoresis was through 6% SDS-polyacrylamide gels for 10-12 hours at 4°C and 10 mA per gel.

λ-phosphatase treatment

10 µl of each cytoplasmic fraction was incubated for 1 minute at 30°C in λ-phosphatase buffer (New England Biolabs). 2000 i.u. of λ-phosphatase (New England Biolabs) were added per reaction and the incubation was continued for 2 hours at 30°C. Control reactions were performed in phosphatase buffer, but in the absence of the enzyme. 2× SDS sample buffer was added to stop the reaction, and proteins were separated by SDS-PAGE and immunoblotted for SYN1/SCP1.

In vitro protein kinase assay

The protein kinase activity of cell extracts was determined using a modified version of the method described by Felix et al. (1989). 10^6 cells were washed in PBS and resuspended in kinase buffer (80 mM β-glycerophosphate, 20 mM EGTA, pH 7.3, 15 mM MgCl$_2$, 1 mM DTT, 1 mM PMSF, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 5 µg/ml aprotinin, 0.5 µg/ml chymostatin, 0.5 µg/ml antipain). Following two freeze-thaw cycles, cells were sonicated and the soluble fraction separated by centrifugation for 1 hour at 50,000 *g*.

The target proteins were expressed as follows: the coding region of *Cor*1/*Scp*3 cDNA was subcloned into the *Eco*RI-*Xho*I sites of pET29b, which rendered a C-terminal fusion with the (His)$_6$ tag. The full-length coding region of *Syn*1/*Scp*1 cDNA was PCR-amplified (*Pfu*, Stratagene) using SYNSTART (5′-GCC GGA TCC ATG GAG AAG CAA-3′) and T7 promoter (5′-TAA TAC GAC TCA CTA TA-3′) primers from a construct in pBluescript and cloned in the *Bam*HI-*Xho*I sites of pET28a, rendering a protein (His)$_6$-tagged at the N terminus. The proteins expressed in *Escherichia coli* BL21(DE3) were affinity-purified under native conditions on Ni-NTA agarose (Qiagen) according to manufacturer instructions. As a control, Ni-NTA agarose beads were incubated with a protein extract of induced *E. coli* BL21(DE3) containing the pET28a vector without any insert. For

storage, the beads were treated as described by Fuchs et al. (1997). Briefly, following protein binding the beads were washed 3× in sonication buffer (50 mM NaH$_2$PO$_4$, pH 8.0, 300 mM NaCl) with 50 mM imidazole, and 2× in storage buffer (40 mM Hepes, pH 7.6, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 0.2% Nonidet P-40) to block the free Ni-binding sites. Bead-bound proteins were then resuspended in 50% glycerol and stored at −20°C.

For each reaction, 10 µg of protein bound to the beads were used. The beads were washed in kinase buffer, and incubated with 0.5 mg of the cell extract, 0.1 mM ATP and 1 µCi of [γ-^{32}P]dATP (Amersham) for 20 minutes at 20°C. The beads were washed 5× in kinase buffer with 50 mM imidazole to remove non-specifically bound proteins, and further used for thrombin cleavage or for elution of proteins by boiling in 2× SDS sample buffer. For thrombin cleavage, the beads were washed twice in thrombin buffer (40 mM Tris-HCl, pH8.5, 150 mM NaCl, 2.5 mM CaCl$_2$) and incubated in twice the bead volume of the same buffer with human thrombin (Sigma; 0.25 i,u,/10 µg protein) for 20 minutes at room temperature with occasional mixing. The beads were washed in thrombin buffer and the bound protein eluted by boiling in 2× SDS sample buffer. Radioactively labelled proteins were separated by SDS-PAGE on 10% or 12% gels and visualized using instant autoradiography with a Canberra-Packard Instant Imager.

Drugs

All the drugs used in this study were purchased from Sigma. Stock solutions were at a concentration of 1 mM in 100% ethanol for OA, 100 mg/ml in 100% ethanol for cycloheximide (CHX), 10 mg/ml in 100% ethanol for chloramphenicol (CHM) and 0.05 M in dimethylsulfoxide (DMSO) for genistein (GEN). The solvents were shown in control experiments to have no effect when the spermatocytes were cultured in their presence for 6 hours.

RESULTS

Spermatocyte immunocytology upon okadaic acid treatment follows the patterns observed in vivo

Following fractionation of testicular cells, the composition of the pachytene spermatocyte fraction was assayed with anti-SYN1/SCP1 and -COR1/SCP3 antibodies. This fraction contained 90% pachytene spermatocytes, as determined by the presence of solid staining along the chromosomes with an antibody against the synaptic protein SYN1/SCP1 (Fig. 1A). No diplotene or metaphase I cells were detected. The cells were cultured in the presence or absence of the phosphatase inhibitor OA, or in the presence of ethanol. OA has been previously reported to induce the rapid transition of pachytene spermatocytes to metaphase I (Handel et al., 1995). Cells harvested immediately after OA treatment (0 hours; Fig. 1A), ethanol-treated cells (not shown) and the cells cultured in the absence of any treatment (Fig. 1B) gave staining patterns with anti-COR1/SCP3 (green), anti-SYN1/SCP1 (red) and anti-centromere (red) antibodies identical to those previously reported in pachytene spermatocytes (Moens et al., 1987; Dobson et al., 1994; Moens and Spyropoulos, 1995). This indicates that the cells were not induced to exit pachytene in the presence of ethanol or by the culture conditions alone. Our observations follow the pattern described by Wiltshire et al. (1995) in that after only 1 hour treatment with OA a heterogenous cell population containing late pachytene, diplotene and early metaphase I stages was observed. The number of cells that exit pachytene increased with the time of incubation in OA. After treatment with 5 µM OA for 6 hours,

approximately 90% of the cells were in metaphase I. A similar experiment was performed with isolated leptotene/zygotene cells (data not shown). This fraction was purified by elutriation at a flow rate of 15-20 ml per minute. It contained only leptotene/zygotene cells even after 6 hours of OA treatment, suggesting that the phosphatase inhibitor has no effect on these early prophase I cells.

The changes accompanying the transition from pachytene to metaphase I were visualized by removing a sample of cells at various times after OA addition and determining the presence of COR1/SCP3, SYN1/SCP1 and centromeric proteins by immunofluorescence. In meiotic cells in vivo, SC dissolution starts at the end of pachytene and is accompanied by a gradual dislocation of SYN1/SCP1 from the chromosomes. The few remaining sites of cohesion are characterized by the presence of stretches of SYN1/SCP1 (Moens and Spyropoulos, 1995). At the end of diplotene, all SYN1/SCP1 is lost and the COR1/SCP3 staining is still compact on the separating cores.

In most of the OA-treated cells, the SYN1/SCP1 protein started to dissociate from the chromosomes during the first hour of treatment (Fig. 1C). Immunostaining with an anti-SYN1/SCP1 antibody (white) and anti-COR1/SCP3 antibody (green) demonstrates the presence of a discontinuous, almost punctuated SYN1/SCP1 staining pattern at the sites of cohesion between the homologs. The arrowheads in Fig. 1C point at a desynapsed region which is continuously stained with anti-COR1/SCP3, but lacks SYN1/SCP1 staining. This indicates that SYN1/SCP1 is gradually removed from the chromosomes and that during desynapsis it persists at the sites of cohesion, consistent with its postulated role as one of the glue proteins that keeps the homologs together at synapsis. After 2 hours OA treatment, SYN1/SCP1 is completely removed from the cells that proceed into diplotene (Fig. 1D). The red staining marks the centromeres. SYN1/SCP1 departure coincides with desynapsis, consistent with previous observations in untreated cells that SYN1/SCP1 removal is required for separation of the cores of homologous chromosomes (Dobson et al., 1994; Moens and Spyropoulos, 1995).

In cells treated with OA for 4 hours (Fig. 1E) the traditional metaphase I configuration is observed with DAPI and anti-COR1/SCP3 antibody staining. No SYN1/SCP1 is detected at this stage. The homologs are connected at the sites of crossing-over, demonstrating that the OA treatment has allowed chiasmata formation. COR1/SCP3 becomes discontinuous in the separating axes at the next stage (5 hours OA), characterized by further condensation of the chromosomes which is shown in Fig. 1F. At metaphase I, a punctate pattern indicated that COR1/SCP3 is still present on the axes in some of the cells (Fig. 1G, early), while others have completely lost COR1/SCP3 from their chromosomes (Fig. 1G, late). Due to difficulty in spreading cells treated with OA, we could not ascertain if there was any COR1/SCP3 present at the centromeres of metaphase I-arrested cells. In untreated cells, COR1/SCP3 is normally detected at the metaphase I centromeres, and also between the separating sister kinetochores at anaphase II (Moens et al., 1995).

The pattern of SYN1/SCP1 and COR1/SCP3 distribution in the X-Y body of OA-treated cells is different from that in the autosomes. SYN1/SCP1 and COR1/SCP3 departure appears retarded on these chromosomes (Fig. 1C-G; arrows). Residual

SYN1/SCP1 staining and strong COR1/SCP3 staining are present even at metaphase I on the sex chromosomes, indicating that their behaviour at meiosis is probably governed by a distinct regulatory mechanism.

OA effect is abolished by the addition of genistein, a general tyrosine kinase inhibitor

Since OA is a phosphatase inhibitor, we attempted to analyse the effect of a tyrosine kinase inhibitor such as GEN on
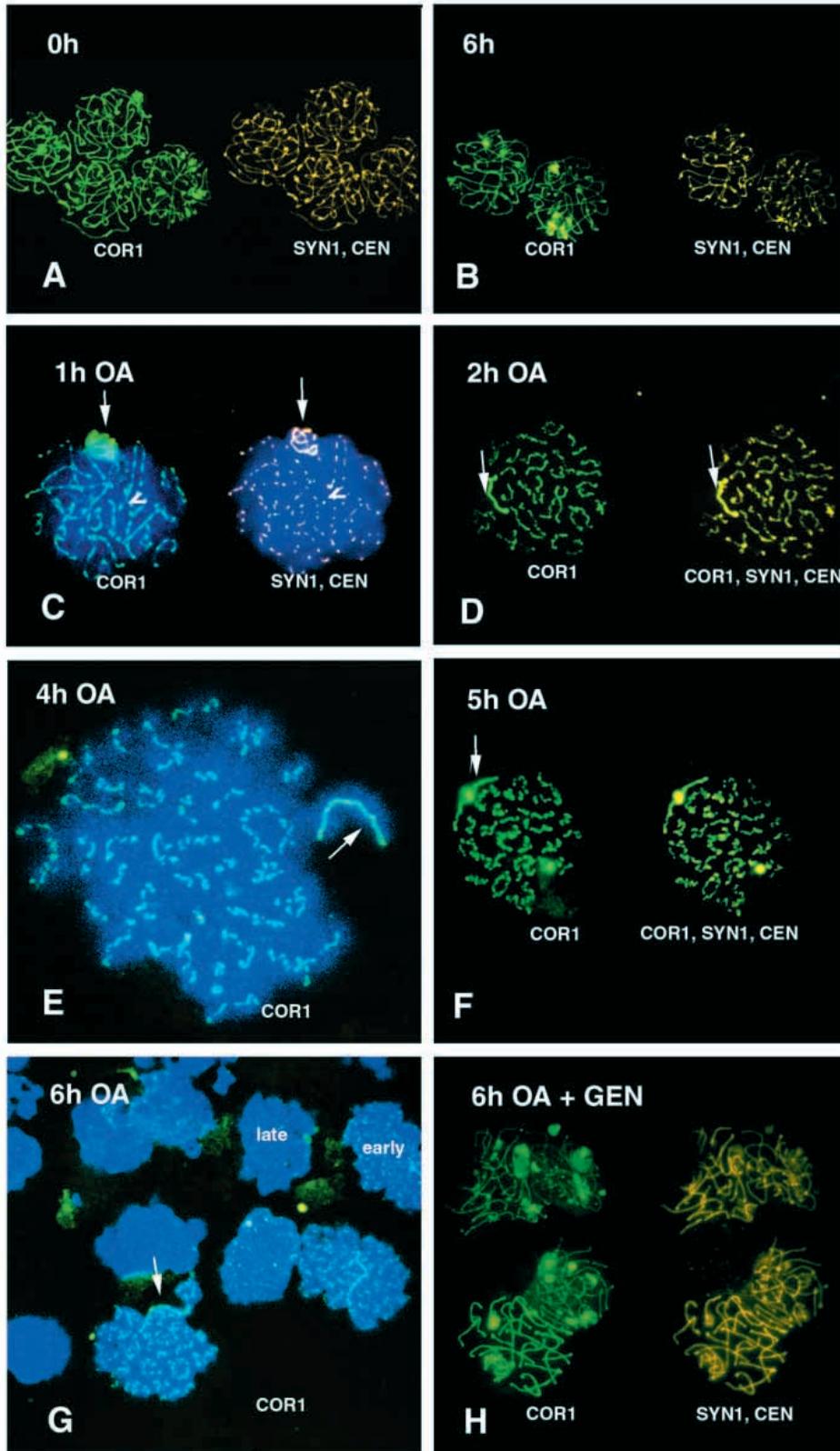

Fig. 1. The distribution of SYN1/SCP1 and COR1/SCP3 in normal and OA-treated rat spermatocytes. The same cell or group of cells were stained as follows: COR1/SCP3 was labelled with FITC (green; left); SYN1/SCP1 and centromeres were stained with rhodamine (red; right); blue DAPI staining was occasionally used to visualize the chromosomes. Arrows are pointing to the X-Y chromosomes stained at high levels throughout the pachytene to metaphase I transition. (A) At 0 hours OA treatment, 90% of the cells have the pachytene configuration. The bivalents are stained along their length with anti-COR1/SCP3 and anti-SYN1/SCP1 antibodies, indicative of full synapsis. (B) Cells incubated in the absence of any treatment for 6 hours maintain the pachytene appearance. (C) After 1 hour of OA treatment, a heterogenous population of cells is detected, the majority of which have advanced to diplotene, as has the one shown here. The bivalents are in the process of desynapsis, as demonstrated by the loss of SYN1/SCP1. The COR1/SCP3 protein (green) is detected along the separating cores of the homologs (e.g. the region marked by the open arrowheads), while SYN1/SCP1 is present only at the sites of connection between the cores. (D) After 2 hours OA, COR1/SCP3 staining is continuous along the homologous cores, now released from the pachytene cohesion. SYN1/SCP1 is absent from the autosomes. The XY body (arrow) shows intense SYN1/SCP1 and COR1/SCP3 staining. The connections between homologs are visible as chiasmata at this stage. (E,F) At 4 and 5 hours OA treatment the nuclei progress even further towards the classical metaphase I configuration, as indicated by chromosome condensation and displacement of COR1/SCP3 from the chromosomal axes. The anti-COR1/SCP3 (green) and anti-centromere (yellow) staining are merged in D and F. (G) After 6 hours of OA treatment, most of the metaphase I nuclei have completely lost the COR1/SCP3 staining. The chromosomes are now condensed and visible as separate entities with DAPI staining. (H) Pachytene cells simultaneously exposed to OA and GEN (a tyrosine kinase inhibitor) for 6 hours do not have the ability to override the pachytene arrest, as shown by the maintenance of intact SCs.
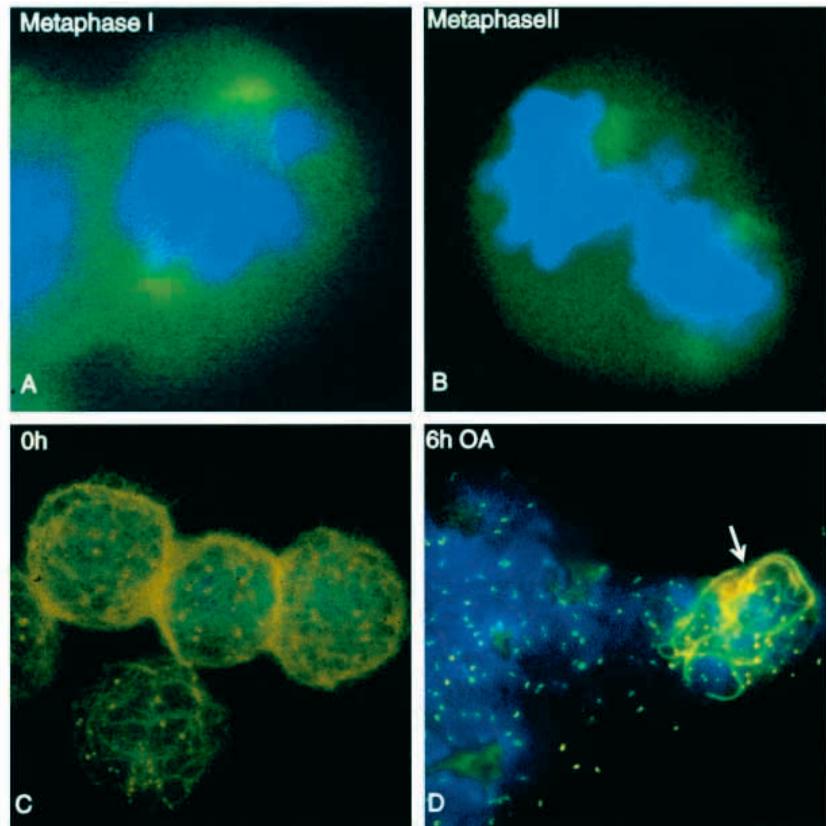
Fig. 2. Staining of spermatocytes fixed in methanol with an anti-β tubulin antibody (FITC). In a heterogenuous population of untreated testicular cells, this antibody detected metaphase I (A) or metaphase II (B) spindles. The condensed metaphase chromosomes are stained with DAPI. (C) In pachytene cells, the cytoplasmic microtubule network (green) surrounds the nucleus. Paired centromeres are stained with rhodamine. (D) No microtubules are present following treatment with OA for 6 hours at a stage corresponding to metaphase I. Irregular filaments, possibly collapsed spindles (arrow), are occasionally detected. The centromeres of this group of cells are stained with a human CREST serum and goat anti-human rhodamine-conjugated secondary antibody.

spermatocytes in culture. When GEN alone is added to the culture medium no morphological changes are detected in the pachytene spermatocytes after a 6 hour incubation. When these cells are incubated in the presence of OA and GEN for 6 hours (Fig. 1H), no progression to later prophase I stages is detected and the SCs remain intact. This indicates that inhibition of tyrosine kinases counteracts the effect of OA. Moreover, when GEN was added to samples of cells incubated with OA for 1, 2 and 4 hours and the incubation was continued to a total of 6 hours, the morphology of the cells observed before the addition of GEN was conserved (data not shown). This demonstrates that tyrosine kinase activity is required at several stages during the OA-induced meiotic progression.

The effect of OA treatment on microtubules

We have also analysed the effect of OA on microtubules using an anti-β tubulin monoclonal antibody visualized with FITC (Fig. 2; yellow). Chromosomes were localized with DAPI staining (blue) and centromeres with CREST (Fig. 2C,D; orange). In spreads of untreated testicular nuclei, anti-β tubulin stains metaphase I and II spindles (Fig. 2A and B, respectively). At pachytene, arrays of microtubules are present in the cytoplasm surrounding the nucleus (Fig. 2C). No such structures are detected in cells that reach the metaphase I configuration following treatment with OA (Fig. 2D). Occasionally, disorganized filaments are found in the chromosome mass that are strongly stained with the anti-β tubulin antibody (arrow) similarly to those reported by de Vantery et al. (1996) in OA-treated mouse oocytes. It has been previously shown in rat oocytes (Zernicka-Goetz et al., 1997) that one of the effects of OA is disruption of microtubules and

inhibition of the metaphase I spindle assembly. Consistent with this, we did not detect any spindle formation in cells treated with OA.

OA induces changes in the subcellular localization of the COR1/SCP3 and SYN1/SCP1 proteins

Following culture times of 0, 3 and 6 hours in the presence or absence of OA, the cells in each sample were fractionated by lysis and centrifugation. The pellet contained the nuclear fraction and the supernatant was the cytoplasmic fraction. Equal amounts of nuclear and cytoplasmic protein from 0, 3 and 6 hours OA treatment and 6 hours solvent treatment were separated by SDS-PAGE and analysed by western blotting with anti-SYN1/SCP1 and -COR1/SCP3 antibodies (Fig. 3A).

Immediately after OA treatment (0 hours; lanes 1 and 5) and at 6 hours solvent treatment (control; lanes 4 and 8), the cells correspond to normal pachytene, where SYN1/SCP1 and COR1/SCP3 are chromosomal proteins that are not detectable in the cytoplasm. OA treatment of pachytene spermatocytes led to the translocation of some SYN1/SCP1 and COR1/SCP3 proteins into the cytoplasm, as indicated by their immune detection in the cytoplasmic fraction of cells treated with OA for 3 and 6 hours (Fig. 3A, lanes 6 and 7). The change in cellular localization occurs concomitantly with the dislocation of these proteins from chromosomes illustrated in Fig. 1C-G (the cytoplasmic compartment has not been preserved in these preparations). The levels of these two proteins in the nucleus remain much higher than the amount of protein translocated into the cytoplasm during the OA treatment. Therefore no decrease in the nuclear levels of SYN1/SCP1 and COR1/SCP3 was detected during this re-localization by western blotting

(Fig. 3A, lanes 2 and 3). The reduction in the amount of cytoplasmic COR1/SCP3 and SYN1/SCP1 at 6 hours OA treatment observed in the western blot could be due to their degradation in the cytoplasm. Redistribution of SYN1/SCP1 and COR1/SCP3 between the nuclear and cytoplasmic compartments is reinforced by observations made in preparations of OA-treated cells that allow staining of cytoplasmic components. A diffuse COR1/SCP3 (green) and SYN1/SCP1 (red) immunofluorescent staining becomes visible in the cytoplasm of the metaphase I cells (Fig. 3B,C). The nuclei are visualized with blue DAPI staining and are not accessible to the antibodies due to the mode of fixation used in these preparations.

We investigated whether the presence of SYN1/SCP1 and COR1/SCP3 in the cytoplasm is due to degradation of the nuclear envelope upon OA treatment. Pachytene spermatocytes treated with OA (Fig. 3E,F) and untreated controls (Fig. 3D) were analysed by electron microscopy. In cells at 0 hours OA

treatment (Fig. 3D) and control cells cultured for 6 hours in the presence of the solvent (not shown), the nuclear envelope appears intact. Sections of these pachytene spermatocytes show short stretches of SCs (Fig. 3D, arrows). Following OA treatment the nuclear membrane starts to disintegrate and large holes in the membrane become visible (Fig. 3E, arrows; inset). In the large number of cells that have reached metaphase I after 6 hours of exposure to OA, the chromosomes (Fig. 3F, CH) appear as separate entities and the nuclear membrane is no longer detectable, as it is the case in normal metaphase I cells. It is therefore possible for nuclear proteins like SYN1/SCP1 and COR1/SCP3 that have been displaced from the chromosomes to diffuse freely into the cytoplasm.

Protein synthesis is not required for the pachytene to metaphase I transition, but it is required for maintenance of synapsis and chiasmata formation

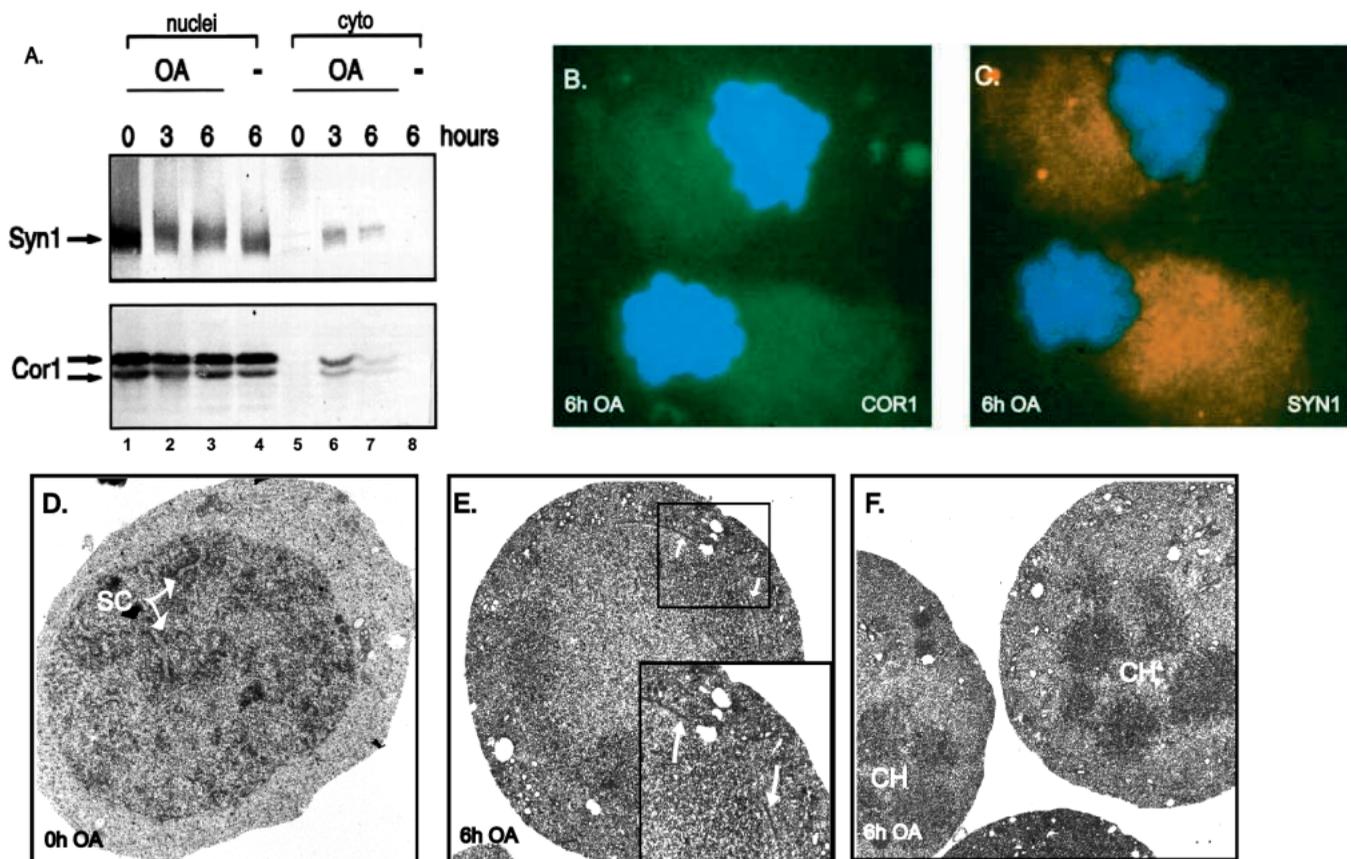To further characterize the pachytene to metaphase I transition

Fig. 3. (A) Immunodetection of SYN1/SCP1 and COR1/SCP3 in nuclear (lanes 1-4) and cytoplasmic (lanes 5-8) extracts of OA-treated and untreated rat spermatocytes. Equal amounts of protein extracts were loaded in each lane of the 10% SDS-PAGE gels for protein separation. SYN1/SCP1 (top) and COR1/SCP3 (bottom) are detected in the nuclear extracts at all times during OA treatment and in the nuclei of untreated cells. Rat COR1/SCP3 is detected as a doublet (30/33 kDa) as previously reported (Dobson et al., 1994; Lammers et al., 1994). Immediately after the OA treatment (0 hours) and at 6 hours solvent treatment no SYN1/SCP1 or COR1/SCP3 are present in the cytoplasm (lanes 5 and 8), consistent with their role as structural proteins associated with the chromosomes and with the inability of untreated cells to exit pachytene. After 3 hours of OA treatment, both SYN1/SCP1 and COR1/SCP3 appear in the cytoplasmic fraction (lane 6). At 6 hours OA they are also present in the cytoplasm (lane 7) but at lower levels, probably due to degradation. (B,C) Immunostaining of OA-treated cells spread under mild conditions that preserve the cytoplasm, indicates the presence of COR1/SCP3 (green) and SYN1/SCP1 (red) in the cytoplasm. Chromosomes visualized with DAPI (blue) are obviously condensed. (D-F) Electron micrographs of rat spermatocytes at 0 and 6 hours exposure to OA treatment. In normal pachytene spermatocytes (D) the nuclear envelope is intact and the nuclei contain SCs (arrows). Nuclei of cells treated with OA for 6 hours show occasional fragmentation (E; arrows in the inset mark a hole) and more often total disintegration of the nuclear envelope (F). In these advanced stages, compacted metaphase I chromosomes (CH) are present as distinct dark bodies in the cytoplasm.
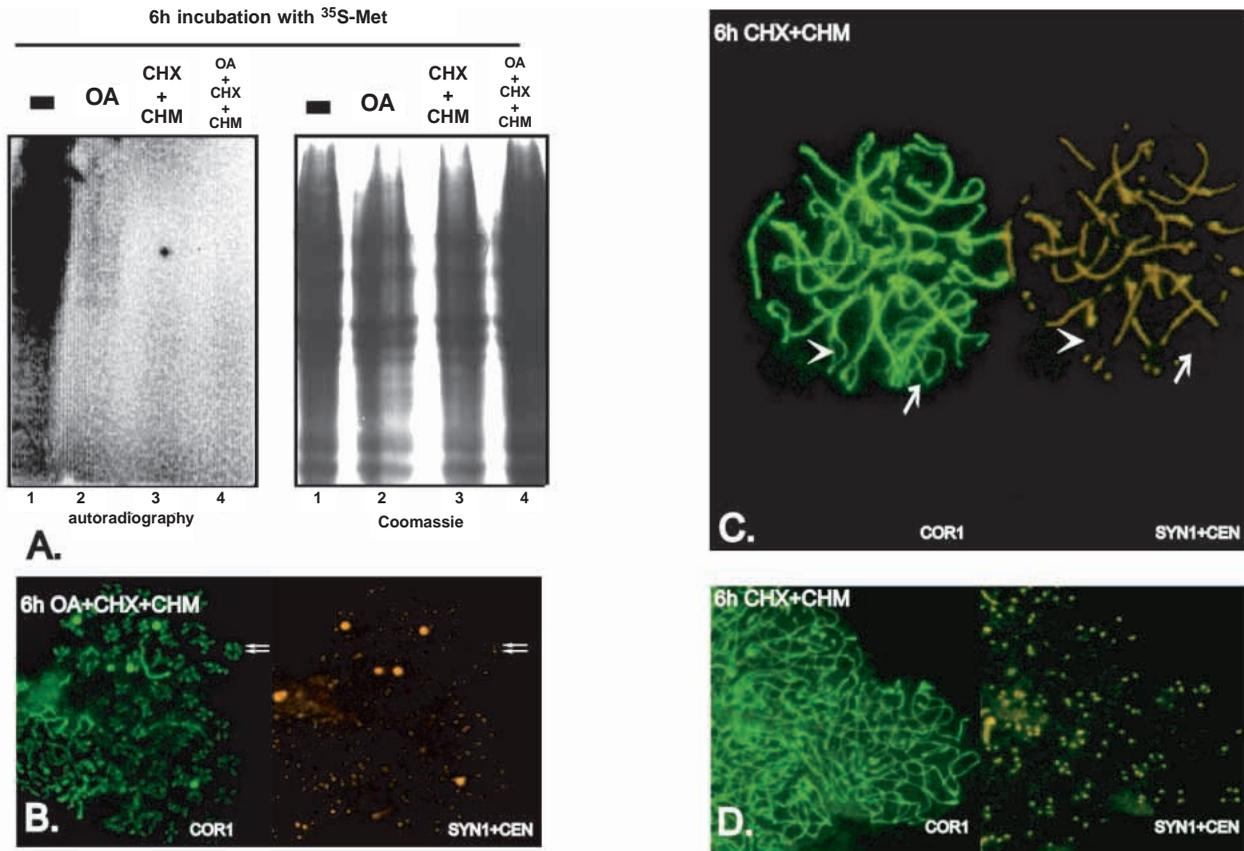
Fig. 4. The effect of protein synthesis inhibitors (CHX and CHM) on the pachytene spermatocytes. (A) Cells incubated for 6 hours in the presence of [^{35}S]methionine exhibit incorporation of the label in total protein (lane 1). Treatment with OA reduced the label incorporation (lane 2) and treatment with CHX+CHM (lane 3) or with CHX+CHM in combination with the OA (lane 4) abolished it. (B) Treatment of pachytene spermatocytes with OA, CHX and CHM allowed the transition to metaphase I to occur similarly to the cells treated with OA alone in the sense that chiasmata are generated, chromosome condensation occurs and SYN1/SCP1 is completely removed form the chromosomes. Nuclei with a metaphase I configuration are stained with anti-COR1/SCP3 antibody (FITC; left) and anti-SYN1/SCP1 and anti-centromere antibodies (rhodamine; right). The two arrows mark the two closely positioned centromeres in a chiasmatic bivalent. (C) Treatment with protein synthesis inhibitors CHX+CHM induce desynapsis of the pachytene chromosomes. COR1/SCP3 (green) stains the cores of homologous chromosomes and SYN1/SCP1 (red) only the synapsed regions. Arrowheads indicate a desynapsed portion at the end of a chromosome that contains the core component COR1/SCP3, but lacks the SYN1/SCP1 protein. Antibody staining of the sex chromosomes (arrows) is markedly diminished (anti-COR1/SCP3) or totally abolished (anti-SYN1/SCP1) in the presence of the protein synthesis inhibitors, as opposed to the cells in pachytene-diplotene. (D) The majority of the pachytene cells incubated for 6 hours with CHX+CHM adopt this 'desynapsed' configuration. The cores of the homologous chromosomes (left) are separated and SYN1/SCP1 is not detected (right); however the chromosomes lack the chiasmata configurations normally present in diplotene spermatocytes (see Fig. 1D).

in the rat spermatocytes, we tested the effects of protein synthesis inhibitors, extending the results reported by Wiltshire et al. (1995). Equal number of pachytene cells were incubated in the presence of [^{35}S]methionine for 6 hours. OA, cycloheximide (CHX) and chloramphenicol (CHM) were added alone or in the combinations shown at the same time as the [^{35}S]methionine, and the cells were incubated for 6 hours. To ensure that the label had entered the cells and had become incorporated in newly synthesized proteins, we prepared cell lysates and separated them by SDS-PAGE.

Cells arrested at pachytene exhibit active protein synthesis, as shown by incorporation of [^{35}S] methionine into total protein during a 6 hour incubation (Fig. 4A, lane 1). We inhibited protein synthesis by adding a mixture of 0.1 mg/ml CHX and 0.01 mg/ml CHM as described by Page and Orr-Weaver (1997). Addition of CHM, a prokaryotic translation inhibitor, is effective in abolishing the CHX-resistant

mitochondrial protein synthesis. OA is also an effective inhibitor of protein synthesis, as only a residual (approximately 2% label) incorporation was detected in spermatocytes incubated in its presence (Fig. 4A, lane 2). CHX and CHM treatment completely abolished protein synthesis (Fig. 4A, lane 3). We asked whether these drugs induced any morphological changes in the meiotic chromosomes. Treatment with OA, CHX and CHM results in the OA-specific chromosome morphology (Fig. 4B): chromosomes are compacted in the classical metaphase I configuration with COR1/SCP3 (green) along the cores and SYN1/SCP1 (red) completely removed. Paired centromeres (red; arrows) and chiasmata are clearly visible. Based on these immunocytological observations, we conclude that the OA-induced transition from pachytene to metaphase I is not dependent on protein synthesis.

When pachytene cells are cultured in the presence of CHX and CHM for 6 hours synapsis between homologous

chromosomes is lost in 70% of the cells (Fig. 4D). The degree of loss of cohesion varies among the cells. Immune staining indicates that SYN1/SCP1 is removed from the chromosomes from the ends towards the middle (Fig. 4C; arrowheads), and when its loss is complete, the cores (green) are totally separated. The characteristic of desynapsis here is that it is not accompanied by crossing over as in the case of normal or OA-treated cells. The cores are not connected by chiasmata (compare Figs 4D, 1D), possibly due to the inhibition of recombination intermediate maturation in the absence of protein synthesis. Another consequence of the CHX+CHM treatment is the lack of staining of the X-Y chromosomes by the anti-SYN1/SCP1 antibody and a reduction in staining by the anti-COR1/SCP3 antibodies (Fig. 4C, arrows).

Phosphorylation of SYN1/SCP1 and COR1/SCP3 at pachytene

The diffuse appearance of SYN1/SCP1 on western blots (Fig. 3A; Meuwissen et al., 1992) raises the possibility that SYN1/SCP1 exists as a phosphorylated protein in normal pachytene cells. To investigate this, we treated the cytoplasmic extract of the 6-hour OA-treated cells with λ-phosphatase (Fig. 5A). Following immunoblotting, a significant increase in the electrophoretic mobility of SYN1/SCP1 is observed as compared to the control reaction in which the protein is incubated with buffer (Fig. 5A, lane 2). The M_r of SYN1/SCP1 following λ-phosphatase treatment (lane 1), is less than that of nuclear SYN1/SCP1 in the absence of OA treatment (lane 3) and of nuclear SYN1/SCP1 after 6 hours of OA treatment (lane

4). This indicates that SYN1/SCP1 is normally phosphorylated at pachytene and that OA treatment maintains this condition. We could not perform an analogous λ-phosphatase assay on the nuclear fractions due to the insolubility of the SYN1/SCP1 protein in these fractions.

We have tried a similar approach to demonstrate phosphorylation of COR1/SCP3 at pachytene, but no difference in the electrophoretic mobility was detected (data not shown). Consequently, we used an in vitro approach to test whether COR1/SCP3 is phosphorylated efficiently by pachytene extracts (Fig. 5B). Extracts of pachytene spermatocytes were prepared as described by Kubiak et al. (1993). To test whether the kinases phosphorylating COR1/SCP3 are testis specific, we used a rabbit reticulocyte extract (RRE) in a parallel reaction. Both the pachytene extract and the RRE phosphorylated COR1/SCP3 in vitro in the presence of $[\gamma\text{-}^{32}P]\text{dATP}$, as indicated by the detection of ^{32}P label in the protein following the reaction (lanes 1, 2, 4, 5). The radioactive label can be removed by treatment with λ-phosphatase (data not shown) indicating that ^{32}P is covalently attached to the protein. No phosphorylation is detected when COR1/SCP3 is incubated under similar conditions in the absence of any extract (lane 3), or when the reaction is conducted with proteins encoded by the vector, in the absence of any insert (lanes 6 and 7; see Materials and methods).

The COR1/SCP3 protein was expressed in *E. coli* with a $(His)_6$ tag at the C terminus and a 40-amino-acid addition at the N terminus, resulting from transcription from the first methionine of the pET29 vector. To show that the COR1/SCP3
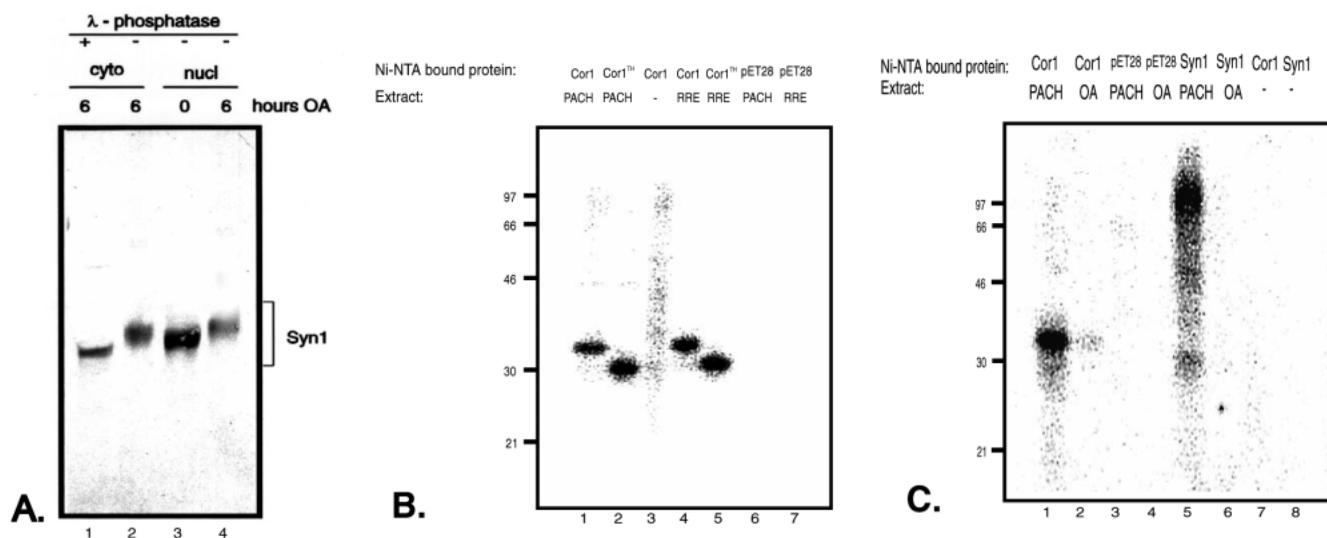

Fig. 5. (A) In cytoplasmic extracts treated with λ-phosphatase (lane 1) the electrophoretic mobility of the SYN1/SCP1 protein is greater than in the cytoplasmic extract incubated with the phosphatase buffer only (lane 2). This shows that SYN1/SCP1 present in the cytoplasm of OA-treated cells is phosphorylated. The nuclear extracts at 0 (lane 3) and 6 hours (lane 4) OA treatment also contain the phosphorylated form of SYN1/SCP1. Proteins were separated in a 6% polyacrylamide gel (the calculated M_r for SYN1/SCP1 is 125) and immunodetection was done with an affinity-purified polyclonal anti-SYN1/SCP1 antibody. (B) Bacterially expressed COR1/SCP3 protein (M_r 30/33) is phosphorylated in vitro in the presence of a pachytene cell extract (PACH; lanes 1 and 2) or a rabbit reticulocyte extract (RRE; lanes 4 and 5). Control reactions with COR1/SCP3 in the absence of any extract (lane 3) or with vector proteins in the presence of both extracts (lanes 6 and 7) show no incorporation of label. To show that the 40-amino-acid heterologous peptide present at the N terminus of COR1/SCP3 is not phosphorylated, we cleaved this peptide with thrombin. COR1/SCP3 (M_r 30) was efficiently phosphorylated by both the pachytene and the rabbit reticulocyte extracts (lanes 2 and 6). (C) Bacterially expressed COR1/SCP3 and SYN1/SCP1 proteins are efficiently phosphorylated by extracts from pachytene spermatocytes (lanes 1 and 5). Extracts prepared in parallel from spermatocytes treated with OA for 6 hours do not phosphorylate SYN1/SCP1 (lane 6) and exhibit low ability to phosphorylate COR1/SCP3 (lane 2). No phosphorylation is detected in the absence of target proteins (lanes 3 and 4) or of the spermatocyte extract (lanes 7 and 8).

protein is the real substrate for the kinase reaction, we removed this 40-amino-acid peptide by thrombin treatment. The remaining full-length COR1/SCP3 protein (M_r 30,000) is phosphorylated by both the pachytene extract and the RRE (Fig. 5B, lanes 2 and 5), indicating that the presence of the 40 amino acids at the N terminus does not affect the kinase reaction.

We asked whether the ability of the pachytene spermatocyte extracts to phosphorylate SYN1/SCP1 and COR1/SCP3 in vitro is maintained following treatment of these cells with OA. We prepared extracts from cells treated with OA for 6 hours, which morphologically correspond to early metaphase I cells, and tested them in kinase reactions in the presence of [γ-^{32}P]dATP (Fig. 5C). Phosphorylation of both COR1/SCP3 and SYN1/SCP1 does occur in the presence of pachytene spermatocyte extracts (lanes 1 and 5). The radical decrease (lane 2) or absence (lane 6) of radioactive label incorporation shows that OA treatment reduces the ability of cellular kinases to phosphorylate COR1/SCP3 and completely abolishes SYN1/SCP1 phosphorylation. The controls with proteins expressed from the vector without an insert (lanes 3 and 4) confirmed that the incorporation is due to phosphorylation of the COR1/SCP3 or SYN1/SCP1 protein. The lack of phosphorylation in the absence of cell extracts (lanes 7 and 8) indicates that a protein kinase present in these extracts is required for the phosphorylation.

DISCUSSION

A model system for studying the meiotic progression from pachytene to metaphase I

A culture system in the presence of OA that allows pachytene spermatocytes to efficiently progress to metaphase I has been previously described (Wiltshire et al., 1995). The main events taking place during this transition, SC dissolution and chiasmata formation, were monitored by Wiltshire et al. (1995) using bright-field and electron microscopy techniques. Here we have extended the characterization of this process with an immunocytochemical analysis of general aspects of cell morphology. In particular, we followed the dynamics of the homologous meiotic chromosomes visualized with antibodies against the chromosome-associated proteins SYN1/SCP1 and COR1/SCP3.

The majority of testicular cells isolated by elutriation as described here are in mid and late pachytene and comparatively few cells are in early pachytene. In all isolated cells, SYN1/SCP1 and COR1/SCP3 staining overlap along the length of the synapsed chromosomes (Fig. 1A), as previously shown (Dobson et al., 1994). OA induces the entry of pachytene cells into diplotene during the first hour of treatment, considerably faster than in animals where pachytene lasts several days (Handel, 1987). This transition is accompanied by the total displacement of SYN1/SCP1 from the chromosomes. The consequence of this event is that the bivalents separate rapidly and completely at late diplotene (Fig. 1D) and their separation is preceded by a stage at which SYN1/SCP1 is detected only at the sites of cohesion between the homologs (Fig. 1C). This evidence supports the postulated role of SYN1/SCP1 as the 'glue' holding homologs together at pachytene. COR1/SCP3 displacement form the chromosomes

occurs at a much lower rate than that of SYN1/SCP1 in the OA treated cells, consistent with the previously reported observations in spermatocytes in vivo (Dobson et al., 1994) that suggest a possible role for COR1/SCP3 in sister chromatid cohesion at prophase I. We concluded that the behaviour of these two proteins during the OA-induced transition follows the pattern reported for the in vivo meiosis.

OA-treated cells remain arrested at metaphase I, as identified by DAPI staining, the position of the centromeres and the residual COR1/SCP3 staining in the chromosome axis (Fig. 1E). We could not ascertain whether COR1/SCP3 staining is present at the centromeres of metaphase I chromosomes, as it is the case in untreated spermatocytes. A characteristic of the OA-treated cells is the solid SYN1/SCP1 and COR1/SCP3 staining in the sex vesicle at all stages including metaphase I, suggesting that the dynamics of the sex chromosomes at meiosis I is governed by a distinct regulatory mechanism which can be only partially suppressed by OA (Fig. 1C-G, arrow).

Gavin et al. (1991) have demonstrated that in OA-treated mouse oocytes the metaphase I arrest is partially caused by the inhibition of spindle formation. Two OA-sensitive phosphatases have been shown to be directly involved in the activation of the pericentriolar material into microtubule organizing centres (Alexandre et al., 1991), which supports the view that OA inhibits spindle assembly. Our results in OA-treated rat spermatocytes are consistent with these observations: an anti-β tubulin antibody labels the metaphase I (Fig. 2A) and II (Fig. 2B) spindles in isolated spermatocytes, but not in OA-treated cells. These cells lack meiotic spindles, although the morphology of the chromosomes corresponds to an early metaphase I, when spindle formation is initiated. Highly disorganized structures are commonly found in these cells (Fig. 2D, arrow), which represent most likely collapsed spindles and confirm the previously reported effect of OA in microtubule disruption (de Vantery et al., 1996). The similarity with the in vivo progression of meiosis relies in the microtubule network being present at pachytene (Fig. 2C), and disappearing (Fig. 2D) at OA-induced metaphase I.

Following OA treatment, SYN1/SCP1 and COR1/SCP3 proteins are detected in the cytoplasm by western blotting (Fig. 3A) or immunological staining of intact cells (Fig. 3B,C). Based on the similarity of the OA-induced system and meiosis in vivo, we postulate that this re-localization occurs in untreated spermatocytes as well, but it cannot be visualized due to the limitations of the meiotic studies discussed above. An active transport of these proteins across the nuclear membrane, following their displacement from the chromosomal sites, cannot be excluded. However, our electron microscopy observations indicate that the changes in intracellular localization are probably facilitated by dissolution of the nuclear envelope upon OA treatment (Fig. 3D-F). The germinal breakdown of the nuclear envelope (GVBD) has been previously reported to accompany the OA-induced maturation of mouse oocytes in vitro (Rime and Ozon, 1990; Alexandre et al., 1991).

The pleiotropic effect of OA on pachytene spermatocytes is most likely the consequence of the disruption of the normal intracellular balance between kinases and phosphatases. The fact that OA effects are abolished by genistein (Fig. 1H), which is a potent tyrosine kinase inhibitor (O'Dell et al., 1991), suggests the possibility that OA acts primarily on a

phosphatase that normally inhibits the activation of a tyrosine kinase. Suppression of this phosphatase activity by OA renders an active tyrosine kinase that allows the meiotic pathway to proceed. Phosphorylation at a tyrosine residue has been shown to activate MAP kinase (Jessus et al., 1991), which is important for meiotic resumption in mouse oocytes.

OA cannot induce the ability to progress to later stages in the leptotene/zygotene spermatocytes from normal animals (the fraction isolated by elutriation at a flow rate of 15-20 ml per minute); however, it does so very efficiently with wild-type pachytene cells. We conclude that the effect of the phosphatase inhibitor OA during the meiotic prophase I is primarily to bypass a pachytene-specific sensor of the meiotic progression, resulting in the rapid degradation of the SYN1/SCP1 protein, subsequent desynapsis and progression to metaphase I.

We were interested in the effect of protein synthesis inhibitors on the OA-induced meiotic advancement. OA itself causes a drastic reduction in the levels of protein synthesis, and protein synthesis inhibitors CHX and CHM have a more pronounced effect (Fig. 4A). Addition of OA+CHX+CHM to pachytene spermatocytes causes the OA-characteristic progression to a metaphase I configuration, characterized by desynapsis, chromosome condensation and the presence of chiasmata (Fig. 4B), indicating that protein synthesis is not required for the completion of these events (Wiltshire et al., 1995). However, addition of CHX+CHM to pachytene cells results in loss of synapsis without chiasmata formation in about 70% of the cells (Fig. 4C,D). In this case, the resolution of recombination intermediates into chiasmata seems to be obstructed by the addition of protein synthesis inhibitors. A similar case of desynapsis without chiasmata formation was reported in lily microsporocytes treated with CHX (Parchman and Stern, 1969). These contradictory results can be reconciled assuming that OA activates an alternative pathway for maturation of recombination intermediates or enables the cells to bypass the requirement for protein synthesis.

SYN1/SCP1 and COR1/SCP3 phosphorylation patterns during the pachytene to metaphase I transition

Loss of synapsis at late pachytene-diplotene could be mediated by phosphorylation of specific SC components. In the system characterized here, the departure of SYN1/SCP1 is accompanied by desynapsis and sets the stage for the subsequent chromosome disjunction at the end of meiosis I. Phosphorylation might induce a structural modification that causes SYN1/SCP1 to lose contact with the core components, facilitating the removal of SYN1/SCP1 from the chromosomes. In a λ-phosphatase assay we show that SYN1/SCP1 is normally phosphorylated at pachytene (Fig. 5A). Also our in vitro experiments (Fig. 5C, lanes 5 and 6) show that extracts prepared from pachytene cells are able to phosphorylate bacterially expressed SYN1/SCP1, while cells treated with OA for 6 hours do not possess this ability. This indicates that some SYN1/SCP1 phosphorylation occurs at pachytene and, according to immuncytological data, it is followed by the displacement of SYN1/SCP1 from the chromosomes. After 6 hours of OA treatment, when cells lack SYN1/SCP1, SYN1/SCP1 phosphorylation is no longer detectable. Therefore the activity of the kinases involved correlates with the presence of their substrate on the chromosomes at the various meiotic stages induced with OA treatment. A candidate for such a kinase

was proposed by Meuwissen et al. (1992) to be the MPF kinase. This assumption is based on the presence of a potential $p34^{cdc2}$ phosphorylation site (Langan et al., 1989) in the SYN1/SCP1 amino acid sequence, and on the fact that pachytene cells are unique in exhibiting MPF kinase activity (Chapman and Wolgemuth, 1994).

Similar to SYN1/SCP1, the COR1/SCP3 protein shows gradual displacement from the chromosomes during OA treatment and phosphorylation in the presence of a pachytene cell extract (Fig. 5B,C). Our results support data previously reported by Lammers et al. (1995) showing that COR1/SCP3 is phosphorylated at pachytene. Interestingly, extracts from cells cultured in the presence of OA for 6 hours exhibit a much lower ability to phosphorylate COR1/SCP3 than the pachytene extracts. This correlates with the presence of COR1/SCP3 in low amounts on the chromosomes. It is therefore possible that changes in the COR1/SCP3 distribution on the chromosomes involve phosphorylation-dependent mechanisms. The fact that a rabbit reticulocyte extract was equally effective in phosphorylating COR1/SCP3 in vitro suggests that some of the kinases involved are not necessarily meiosis specific. These results set the stage for identification of the meiotic kinases acting on SYN1/SCP1 and COR1/SCP3, which is essential for understanding the molecular basis of SC dissolution and chiasmata formation. The precise timing of these events and their correct accomplishment are critical for the proper chromosome segregation at meiosis.

In summary, our data show that the OA treatment of pachytene spermatocytes allows the meiotic progression to metaphase I, similar to the in vivo advancement. Critical parameters such as chromosome morphology, distribution of the meiotic specific proteins SYN1/SCP1 and COR1/SCP3, microtubule staining and nuclear envelope integrity indicate that the events induced by OA treatment correspond to those observed in testicular cells. Minor differences or deficiencies observed in the cells arrested at metaphase I could be due to secondary effects of OA at this stage accompanying the block of meiotic progression. This cell culture system can therefore prove useful for characterization of the meiotic pathway progression in vivo. Events such as homologous chromosome desynapsis and resolution of recombination intermediates into chiasmata can be studied at the molecular level in this system. Studies to decipher the effect of OA on the meiotic pathway machinery can also be performed.

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