Protoplasma (2000) 212: 56–71

PROTOPLASMA

Printed in Austria

Chromosome attachment to the spindle in crane-fly spermatocytes requires actin and is necessary to initiate the anaphase-onset checkpoint

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Received August 19, 1999
Accepted January 10, 2000

Summary. We investigated the possible involvement of actin in the attachment of chromosomes to spindles in crane-fly primary spermatocytes. In a previous study, cytochalasin D, an inhibitor of actin polymerisation, prevented bivalent attachment to microtubules when applied at prophase, but did not cause the detachment of already attached bivalents. We were able to detach the already attached bivalents by first treating prometaphase cells with an antitubulin drug, nocodazole, to disrupt spindle microtubules. 2 min after nocodazole addition, we added cytochalasin D, to disrupt actin filaments; then 2 min later nocodazole was removed, and the cells were kept in cytochalasin D until the time of normal anaphase. Double treatment with nocodazole and cytochalasin D blocked reattachment of bivalents to the spindle. Single treatment with nocodazole alone caused chromosome detachment but did not prevent reattachment when nocodazole was washed out. Extended treatment with cytochalasin D alone starting in prometaphase did not cause bivalents to detach from the spindle. These data suggest that actin is needed for attachment of bivalents to spindle microtubules. This protocol is relevant to the anaphase-onset checkpoint. From previous experiments it was argued that the anaphase-onset checkpoint recognises unattached chromosomes only after those chromosomes first interact with (become attached to) the spindle. Our experiments showed that anaphase disjunction occurred at normal times when bivalents were prevented from attaching to the spindle (by adding cytochalasin D in prophase), while anaphase disjunction was greatly delayed when previously attached bivalents were detached (with nocodazole) and then prevented from re-attaching (with cytochalasin D) in the double treated cells. Thus the anaphase-onset checkpoint recognises only those unattached bivalents that previously were attached to the spindle. Other results provided further indication that actin–microtubule interactions are important in spindle organisation. Nocodazole treatment for 4 min caused most microtubules to disappear: bivalents aggregated around remnant microtubules. When cytochalasin D treatment followed nocodazole treatment, remnant spindle microtubules were not seen, suggesting that actin interactions help stabilise those microtubules.

Keywords: Chromosome attachment; Actin; Anaphase-onset checkpoint; Cytochalasin D; Kinetochore microtubule; Nocodazole; Spindle.

Abbreviations: CD cytochalasin D; NMBD nuclear-membrane breakdown; NOC nocodazole.

Introduction

Proper attachment of chromosomes to the spindle in prometaphase and their subsequent alignment at the spindle equator in metaphase are prerequisites for equal distribution of genetic material in normal cell division. Chromosomes attach to the spindle in prometaphase through their kinetochore, which establishes an initial interaction with the kinetochore microtubules growing outward from the pole each kinetochore faces (Hayden et al. 1990, Merdes and De Mey 1990, Nicklas and Ward 1994). Microtubule capture by the kinetochore involves the cooperation of a battery of kinetochore, microtubule motor and accessory proteins (K. Brown et al. 1996, Thrower et al. 1996, Schaar et al. 1997, Wood et al. 1997, Yao et al. 1997). After their initial interaction with microtubules, chromosomes may move rapidly poleward along the surface of a single microtubule (Rieder and Alexander 1990), a pattern of movement which facilitates the capture of additional microtubules and which occurs with a kinetics that matches that of the minus end motor molecule dynein (Alexander and Rieder 1991). Both dynein and its associated protein complex dynactin are found in kinetochores in prometaphase (Steuer et al. 1990, Pfarr 1990, Echeverri et al. 1996) and have microtubule binding sites (Schafer et al.

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1994, Waterman-Storer et al. 1995). Dynactin is thought to anchor dynein to the kinetochore and maintain the attachment of dynein to microtubules (Echeverri et al. 1996).

Chromosome attachment to spindle fibres might require actin–microtubule interaction: actin inhibitors cytochalasin D (CD) (S. Brown and Spudich 1979, Cooper 1987, Urbanik and Ware 1989) and latrunculin B (Spector et al. 1989) prevent chromosome attachment when applied to crane-fly spermatocytes in prophase (i.e., prior to chromosome attachment) but do not cause chromosome detachment when applied in prometaphase, when the chromosomes already are attached to microtubules (Forer and Pickett-Heaps 1998a). That attachment is blocked indicates that actin might be involved in attachment; that detachment does not occur could be because actin is no longer needed after attachment or could be because actin and kinetochore microtubules bind tightly together, this interaction protecting actin from antiactin drugs. Actin also has been implicated in chromosome attachment in other cells, where cytochalasins cause chromosome detachment (Sampson et al. 1996, Snyder and Cohen 1995) or where actin is implicated in spindle microtubule organisation (Wrench and Snyder 1997) and kinetochore organisation (Snyder and Cohen 1995, La Fountain et al. 1992).

Antiactin drugs that block attachment may do so by interacting with spindle fibre actin. Kinetochore fibres in crane-fly spermatocytes contain actin filaments in addition to microtubules (Forer and Behnke 1972; Czaban and Forer 1992, 1994; Silverman-Gavrila and Forer 2000) as also has been reported in other cells (Gawadi 1974; Forer et al. 1979; Sanger 1975; Seagull et al. 1987; Traas et al. 1987, 1989; Van Lammeren et al. 1989; Bednara et al. 1990; Schmit and Lambert 1990; Staiger and Cande 1991; Czaban and Forer 1992; Dinis and Mesquita 1993; Cleary 1995; Kengen et al. 1995). Actin also colocalises with astral spindle microtubules in *Xenopus laevis* egg cell-free lysates (Sider et al. 1999).

The drugs might also or instead interact with kinetochore-associated dynactin (Echeverri et al. 1996). The core of the dynactin complex contains an actin-related protein, ARP1 (Schafer et al. 1994), which has different properties from conventional actin, in being resistant to CD and not binding phalloidin (Holleran et al. 1996). As to conventional actin, its presence in the dynactin complex is not certain: Schafer et al. (1994) say it is present, while Holleran et al. (1996) say it is not. Thus it is not clear whether the effects of actin inhibitors on attachment are due to effects on spindle actin only or also or instead on actin present in the dynactin complex.

We used crane-fly spermatocytes to investigate the role of actin in chromosome attachment by using a combined treatment (see Fig. 1). We first allowed chromosomes to attach to spindle microtubules; then we added for a short time an antitubulin drug, nocodazole (NOC) (Hoebecke et al. 1976, Vasquez et al. 1997), to disrupt microtubules and to detach the chromosomes (Spurck et al. 1986). After this, in the absence of kinetochore microtubule–chromosome interactions, we treated the cells with the actin-disrupting drug CD. Then the NOC was washed out and the cells were kept in CD until the time they were expected to enter anaphase. Our data, presented herein, show that the double treatment blocked chromosome attachment and thus that interaction of microtubules with actin is necessary for chromosome attachment to the spindle.

The same experiments allowed us to study also the role of chromosome attachment in triggering the anaphase-onset checkpoint. Most previous work has considered that "unattached kinetochores . . . release an inhibitory signal that delays anaphase until they properly attach to the spindle" (Waters et al. 1998: p. 1181). However, experiments on crane-fly spermatocytes suggest that more than "an inhibitory signal" is involved, that chromosomes must first attach to spindle microtubules before they are recognised by the anaphase-onset checkpoint: when chromosomes are prevented from attaching to the spindle (by being treated in prophase with CD or latrunculin B), anaphase disjunction nonetheless occurs at the normal time (Forer and Pickett-Heaps 1998b). This suggests that an initial chromosome–spindle interaction is required to activate the anaphase-onset checkpoint. An alternate interpretation of those data might be that crane-fly spermatocytes do not have an anaphase-onset checkpoint, as LeMaire-Adkins et al. (1997) consider to be the situation in human oocytes. We tested the two interpretations by detaching already attached chromosomes with the antitubulin drug NOC and then preventing bivalent reattachment with the antiactin drug CD. If prior attachment is required to activate the anaphase-onset checkpoint, anaphase should be delayed until all chromosomes attach to the spindle when CD is washed out, whereas if there is no anaphase-onset checkpoint disjunction should occur at

the usual time. Our results, reported herein, show that anaphase onset was delayed until the CD was washed out, indicating that the checkpoint is not activated by unattached chromosomes until those chromosomes interact with the spindle.

Material and methods

Solutions

We used for cell preparations: halocarbon oil (Halocarbon Products Corp., River Edge, N.J.); insect Ringer's solution (IR; 0.13 M NaCl, 0.005 M KCl, 0.001 M $CaCl_2$, 6 mM Sørensen's phosphate buffer, final pH 6.9); fibrinogen (Calbiochem, La Jolla, Calif.), 10 mg/ml in IR; thrombin (Sigma Chemical Co., St. Louis, Mo.), 50 units/ml IR; and valap (vaseline-lanolin-paraffin, 1 : 1 : 1).

Cytochalasin D (Calbiochem) and nocodazole (Sigma) were dissolved in dimethyl sulfoxide (DMSO); measured aliquots were stored as stock solutions at –80 °C; for use, they were thawed and diluted with IR to a final concentration of 20 μM for CD and 10 μM for NOC. The final concentrations of DMSO in the diluted drug solutions were 0.2% for CD and 0.1% for NOC.

Crane-fly spermatocyte preparations

Living crane-fly (*Nephrotoma suturalis* [Loew]) spermatocytes were prepared as described by Forer and Pickett-Heaps (1998a). In brief, IV-instar larvae were dissected and the testes removed under halocarbon oil; testes were washed in a drop of IR and were pierced in a drop of fibrinogen on previously flamed coverslips. The cells were attached to coverslips by adding thrombin to form a fibrin clot. The coverslips were sealed with valap onto perfusion-chamber slides (Pickett-Heaps and Spurck 1982), were perfused with IR, and, during experiments, were perfused with various solutions.

Phase-contrast microscopy

Phase-contrast images were taken with a Nikon inverted optiphot microscope using a Nikon or Zeiss ×100 oil-immersion objective (numerical aperture, 1.3). The signal passed through a Panasonic video-camera, a date-time generator, and an LKH-100 Real-time Image Processor (L. K. Hawke Inc., Research Triangle Park, N.C.) before being recorded with a Sony video-casette recorder. Anaphase chromosome velocities were measured from the taped images as described by Wilson and Forer (1989). In brief, on individual frames we marked kinetochore positions with a cursor, the pixel positions being transferred by a computer program into a data file. The kinetochore positions were converted to interkinetochore distances which were plotted versus time, and the points fitted to a "best-fit" line by commercially available software. Selected images from tapes were printed with a Mitsubishi or Sony thermal printer.

Cell treatments

For every treated cell we first followed control cells from the same preparations or from the contralateral testis to ensure that cells proceeded normally through the first meiotic division, from prophase to telophase (60–120 min; Forer and Pickett-Heaps 1998b). Control cells were perfused every 10 min with IR or with the maximal DMSO concentration (0.2%) used in drug solutions. All experiments were performed at room temperature. Not all treated cells were studied with immunofluorescence staining; for those that were, the positions of the cells were marked on the coverslips with a diamond scribe so they could be located again after staining. The cells were generally followed from different times in prophase until at least 180 min after nuclear-membrane breakdown (NMBD).

Fluorescence staining for acetylated and for tyrosinated α-tubulin

In each session of staining we stained both control and treated cells. We double stained for acetylated and tyrosinated α-tubulin (Wilson et al. 1994) by lysing cells for 10 min in a lysis buffer [100 mM piperazine-N,N′-bis(2-ethanesulfonic acid), final pH 6.9; 10 mM EGTA; 5 mM $MgSO_4$; 5% DMSO; 1% Nonidet P-40], fixing them for 9 min in 0.25% glutaraldehyde in PBS (phosphate-buffered saline: 0.13 M NaCl, 6 mM phosphate buffer, final pH 7), and then incubating them for 20 min in 1 mg of sodium borohydride ($NaBH_4$) per ml of PBS. After rinsing with PBS we stored the coverslips in a mixture of PBS and glycerol (1 : 1) at 4 °C until they were immunostained. Prior to staining, we rinsed the coverslips with PBS. We first stained the cells with 6-11B-1 (Sigma-Aldrich), a mouse monoclonal antibody specific for acetylated α-tubulin, diluted 1 : 300. As secondary antibody we used rat-adsorbed fluorescein isothiocyanate(FITC)-conjugated goat anti-mouse immunoglobulin G (Caltag Laboratories, San Francisco, Calif.) diluted 1 : 50. Then we stained the cells with the rat monoclonal antibody YL1/2, specific for tyrosinated α-tubulin (a gift from Dr. J. Kilmartin), diluted 1 : 500. As secondary antibody we used mouse-adsorbed Texas Red-conjugated goat anti-rat immunoglobulin G (Caltag Laboratories), diluted 1 : 100. All antibody dilutions were made with PBS.

Confocal microscopy

Fluorescence images were obtained with a krypton-argon laser Bio-Rad MRC-600 confocal scanner (Mississauga, Canada) attached to a Nikon Optiphot 2 microscope with a Nikon Fluor oil immersion ×60 objective lens (numerical aperture, 1.4). The two fluorochromes (FITC and Texas Red) were visualised simultaneously by a split-screen dual detection mode. Optical sections were taken 0.8–1.2 μm apart and were saved on optical disks from which micrographs were printed with a Sony video-graphic printer. The cells that were followed live were identified after lysis and staining from their phase-contrast microscopy images. We also studied other cells from the same slides that we did not follow while live.

Results

We determined whether attached chromosomes which were detached by depolymerising the kinetochore microtubules with the antitubulin drug NOC would reattach to the spindle when the microtubules were allowed to recover in the presence of the antiactin drug CD. The design of the experiment is shown in Fig. 1, and the rational is as follows. We tested the effects of the two drugs individually, prior to assessing the effects of the combined treatment. Doses and durations of NOC treatment that caused spindle breakdown (as seen in phase-contrast microscopy) were higher for metaphase cells than for cells in early prometaphase. Likewise, immunofluorescence observations after NOC treatments showed that in earlier stages the microtubules were more easily depoly-

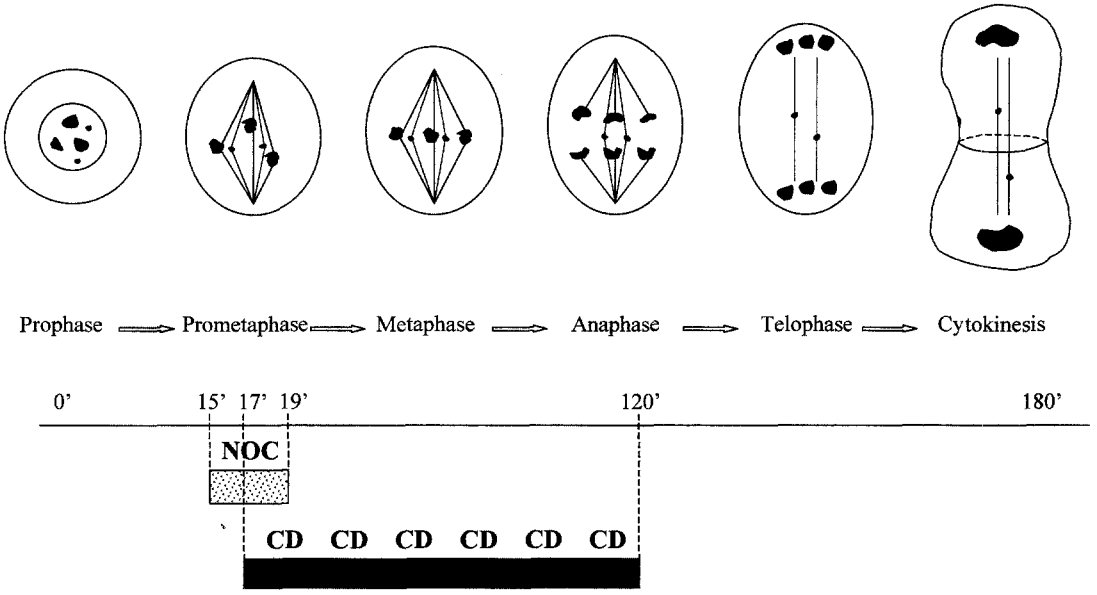


Fig. 1. Schema of double treatment with NOC and CD. Time is given in minutes; 0', time of NMBD

merised than in later ones and that kinetochore microtubules were the most resistant. Therefore we added NOC for 4 min at a specific time (15 min) after NMBD to minimise the dose and duration of NOC treatment.

For the double treatment we treated the cells first with NOC at 15 min after NMBD. 2 min later we replaced the NOC with a solution containing both NOC and CD. We added CD simultaneously with NOC to ensure that CD was present before microtubules reformed. Then 2 min later we washed the NOC out, by adding CD alone. The cells were kept in CD until 120 min after NMBD, the latest time of anaphase onset in control cells.

We describe first control cells, then cells treated for 4 min with NOC alone, then with CD alone, and finally cells treated doubly with NOC and CD.

Control cells

Anaphase onset in control cells rinsed with IR or with DMSO occurred from 57 to 121 min after NMBD; the average time was 91.3 ± 15.74 min (mean with standard deviation; n = 37) after NMBD. One such cell is illustrated in Fig. 2. After NMBD, in early prometaphase, the autosomal bivalents move toward and away from the poles until they establish a stable bipolar attachment to the newly formed spindle which is seen as an area clear of cytoplasmic granules in the middle of the cell, delimited by mitochondria (Fig. 2B). Shortly before anaphase onset, the spindle changes its shape from being ellipsoidal to being rhombic (Fig. 2C, D), as described by La Fountain (1971, 1972). In late prometaphase the bivalents congress to the spindle equator: after all bivalents arrived at the equator in metaphase (Fig. 2C), they disjoin (Fig. 2D), and the half-bivalents move poleward (Fig. 2E). After the autosomes reach the poles the univalent sex-chromosomes each move to opposite poles, as cytokinesis starts (Fig. 2F).

We studied microtubule distributions in cells at the time of our experimental interventions (Fig. 3A), at about 20 min later (Fig. 3B), and in anaphase (Fig. 3C, D). Control cells lysed at 15–19 min after NMBD (Fig. 3A) had two flagella at each pole, astral microtubules radiating from the centrioles, kinetochore microtubules extending from the poles to their newly attached chromosomes, and nonkinetochore microtubules not associated with chromosomes. At this stage, most spindle microtubules were nonkinetochore microtubules and were not acetylated. Acetylated microtubules were seen only in the flagella, in some astral microtubules, and in the kinetochore microtubules (Fig. 3A, left). At about 40 min after NMBD the kinetochore microtubules were more prominent; the spindle was completely formed and the kinetochore microtubules were in thicker bundles and were more acetylated than previously (Fig. 3B). In metaphase the prominent kinetochore microtubule

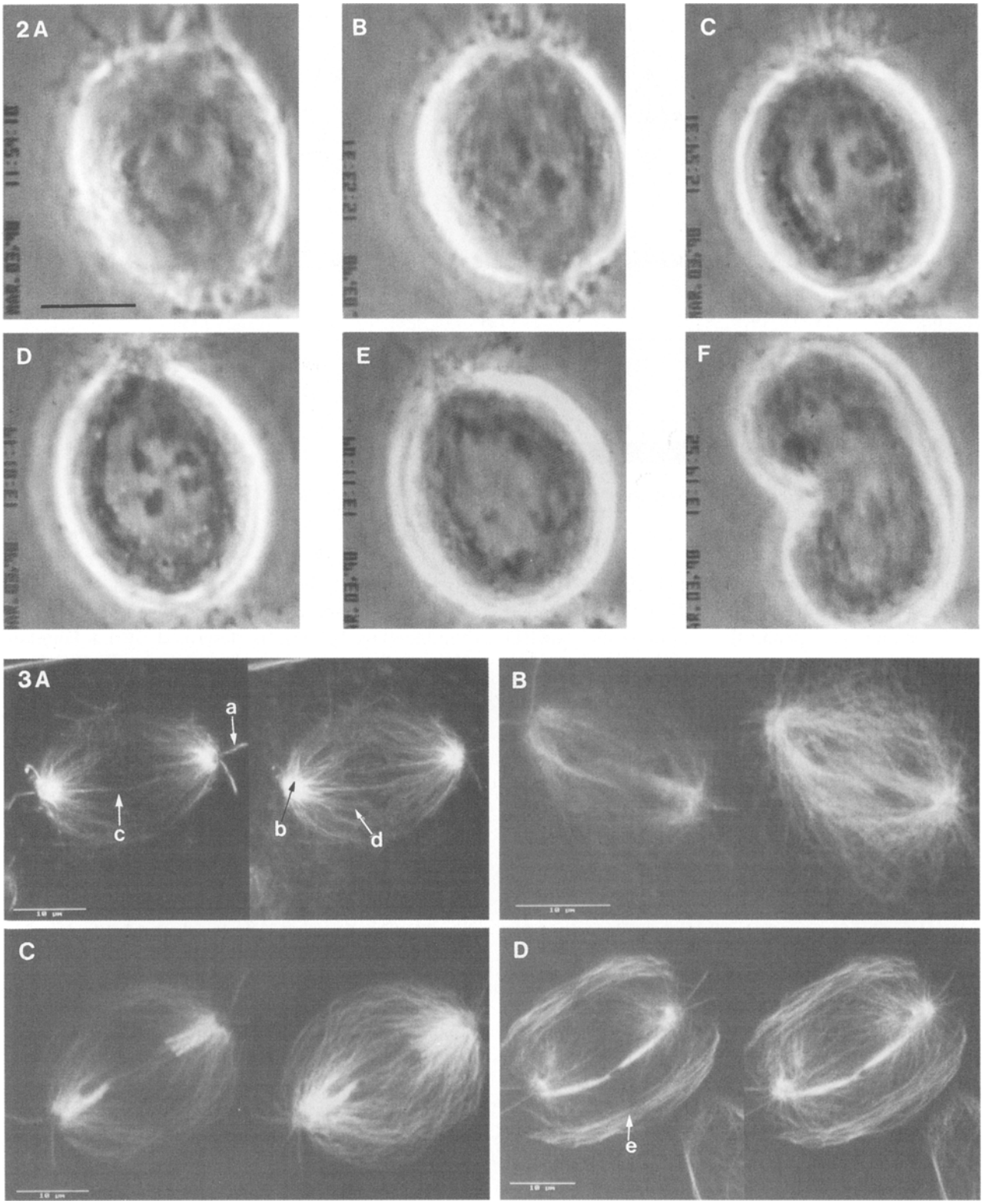


Fig. 2A–F. Phase-contrast images of control primary spermatocyte during the first meiotic division. **A** Prophase: just before NMBD. **B** Prometaphase. **C** Metaphase. **D** Anaphase onset: 60 min after NMBD. **E** Later anaphase. **F** Cytokinesis. Bar: 10 μm

Fig. 3A–D. Confocal microscopy images of control primary spermatocytes double stained for acetylated α-tubulin (left panels) and tyrosinated α-tubulin (right panels). *a* flagellum, *b* astral microtubules, *c* kinetochore microtubules, *d* nonkinetochore spindle microtubules, *e* cytoplasmic microtubules. **A** Cell lysed at 15 min after NMBD. **B** Cell lysed at 40 min after NMBD. **C** Cell lysed during autosomal anaphase. **D** Cell lysed during autosomal telophase. Bars: 10 μm

bundles were yet thicker and, aside from the flagella, were the only microtubules seen in the acetylated α-tubulin channel. The general appearance was similar in anaphase with the addition of some cytoplasmic microtubules which were acetylated (Fig. 3C). In late anaphase, when the autosomes reached the poles, the sex chromosomes began to move poleward and their kinetochore microtubule bundles were prominent in the interzone: the sex chromosome kinetochore fibres were acetylated, as were some cytoplasmic microtubules (Fig. 3D).

Nocodazole treatment in prometaphase

NOC was added for 4 min starting at 15 min after NMBD. NOC treatment (Fig. 4A–F) caused the disappearance of the mitochondrial outline around the spindle and invasion of cytoplasmic granules into the spindle at about 3.7 ± 1.9 min (mean with standard deviation; n = 6) after the start of treatment, indicating the complete breakdown of the spindles (Fig. 4C). Chromosomes that were stretched and had been moving regularly before treatment became shorter and began to move rapidly and "chaotically" after treatment (Fig. 4C). Confocal images of cells treated with NOC and lysed immediately after the 4 min treatment showed that most spindle microtubules had disappeared. The only microtubules remaining were those of the flagella and what we presume from their acetylation to be kinetochore microtubules (Fig. 5); these were gathered in the middle of the cells in a band that extended from pole to pole, while, as deduced from phase-contrast images, the chromosomes were near the band of resistant microtubules.

The NOC effects were reversible. After washing out NOC, a clear spindle area outlined by mitochondria reappeared at 18 ± 8.4 min (mean with standard deviation; n = 7) later, indicating that the spindle had reformed; by that time the previously contracted chromosomes regained their initial length and movements (Fig. 4D). In fluorescence images of cells lysed after 20 min of recovery from NOC treatment, the spindle microtubules were normal, as in controls, except for the absence of astral microtubules. By metaphase almost all chromosomes reattached: 50 out of 51 bivalents (in 17 cells) arrived at the metaphase plate and disjoined at 99 ± 19.8 min (mean with standard deviation) after NMBD, a value not statistically different ($t = 0.48$, $P = 0.64$) from the control cells. After disjunction (Fig. 4F), the half-bivalents moved normally to the spindle poles except for 4 out of 17 cells in which 1, 2, or all 3 pairs of half-bivalents moved part-way and then stopped permanently. In the only one of the 17 cells in which 1 bivalent did not reach the equator, that bivalent was at the pole when NOC was added, and it remained there throughout the experiment; the other two bivalents congressed to the equator and disjoined at 113 min after NMBD. (In the control cell, anaphase started at 107 min after NMBD.) The half-bivalents of the two disjoined bivalents moved poleward, while the chromosome at the pole did not disjoin.

By telophase, treated cells (Fig. 6A–F) had fully recovered microtubules, including astral microtubules (Fig. 6F).

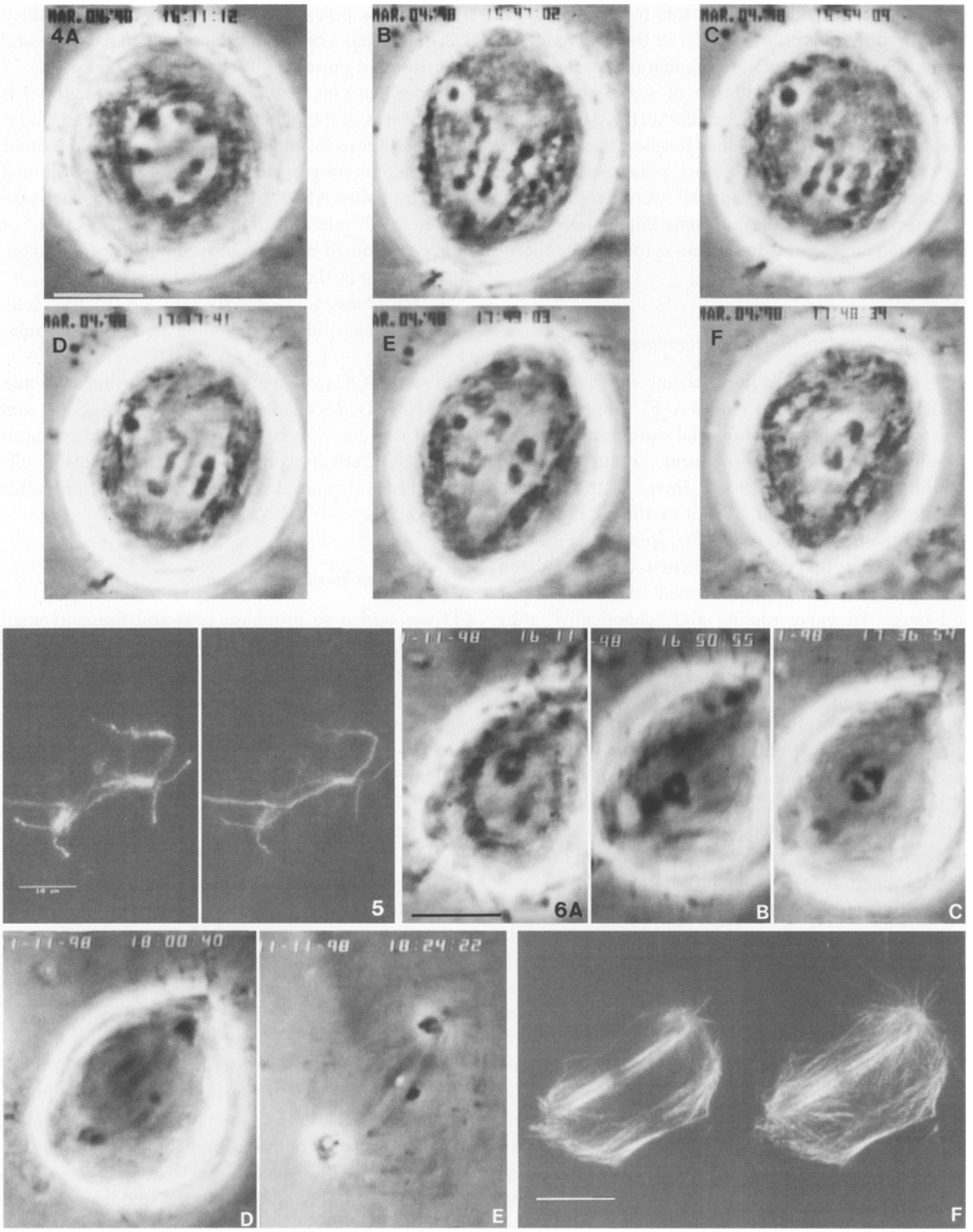
In sum, NOC treatment for 4 min starting at 15 min after NMBD disorganised spindle microtubules and caused the detachment of already attached chromosomes. NOC effects were generally reversible: the cells recovered and entered anaphase at normal times after NMBD.

CD treatment starting at prophase

CD was added to prophase cells and the cells were kept in CD until anaphase. The depolymerising effect of CD on actin was confirmed in live cells by the collapse shortly after CD perfusion of the actin fingers (or "pompons"; La Fountain et al. 1992) present near each pole, as described by Forer and Pickett-Heaps (1998a).

CD added prior to NMBD blocked the attachment to the spindle of 20 out of 24 bivalents in 8 cells. CD was added 8 to 73 min before NMBD. We considered a bivalent as being attached when the bivalent congressed to the metaphase plate, disjoined, and the two half-bivalents moved poleward. We considered the bivalents as being not attached to the spindle by the criteria described by Forer and Pickett-Heaps (1998a): when they did not congress; when they remained motionless in the spindle or were pushed outside the spindle or to the spindle periphery; when half-bivalents did not separate further either after initial anaphase disjunction or after washing out the drug; and when the direction of disjunction was not parallel to the pole-to-pole spindle axis.

No bivalents were attached to the spindle in 6 of the 8 cells: the spindles became disorganised by the time of normal anaphase onset. Two bivalents were not attached in 1 of the 8 cells. All bivalents were attached in 1 cell.


4A
B
C
D
E
F
5
6A
B
C
D
E
F

The unattached bivalents had variable positions: they either were outside (2), at the periphery of (10), or inside (8) the former spindle area. They also had variable behavior: at anapahase they either completely disjoined (12), or had a chiasma present between the half-bivalents (4), or attempted to disjoin (4). Disjunction occurred at 100 ± 8.7 min (mean with standard deviation; n = 7) after NMBD, a time not significantly different (t = 1.711, P = 0.14) from control cells in the sister testes, in which anaphase onset occurred at 89.2 ± 8.6 min (mean with standard deviation; n = 7) after NMBD. We washed out the CD at 120 min after NMBD. The spindles did not reorganise for the next hour, the minimum time for which the cells were followed, and disjoined half-bivalents did not move further. These data confirmed that the bivalents were not attached.

Bivalents were attached in 2 cells. In one cell one bivalent congressed normally and its two half-bivalents moved poleward, while the other 2 bivalents were not attached. Both unattached bivalents disjoined at anaphase, but the half-bivalents did not move further, even after washing out the CD at 120 min after NMBD. In the other cell all 3 bivalents congressed, disjoined at 114 min after NMBD and the half-bivalents, moving slowly poleward, sped up after the CD was washed out at 10 min after anaphase onset.

We followed a live cell treated with CD from prophase until 110 min after NMBD and we lysed it 7 min after washing out the CD (Fig. 7 A–D). The attachment of all 3 bivalents was judged as blocked (Fig. 7 C), in that the bivalents were situated at the periphery of the former spindle area and none were attached to microtubules (Fig. 7 E). In another cell from the same slide, treated also in prophase, but followed only occasionally, one bivalent was attached to kinetochore microtubules and its two half-bivalents separated at a time comparable with normal anaphase onset (Fig. 7 F–H). The other 2 bivalents were at the spindle periphery and disjoined, but the half-bivalents did not separate further and were not associated with kinetochore microtubules (Fig. 7 G, H).

In sum, we confirm the previous result (Forer and Pickett-Heaps 1998b) that CD added in prophase can block attachment of bivalents, but that anaphase onset is not affected because unattached and attached bivalents disjoined at the normal time.

CD treatment starting at prometaphase

CD was added to prometaphase cells starting at 17 min after NMBD; the cells were kept in CD until anaphase. Chromosome behavior was not altered in prometaphase or metaphase. CD did not cause the detachment of already attached bivalents as judged from living cells: 38 out of 39 bivalents (in 13 cells) remained attached and disjoined at 85.7 ± 20 min (mean with standard deviation) after NMBD (Fig. 8 A–E). Anaphase onset was not significantly different from control cells (t = 0.2, P = 0.85). In one of these 13 cells one bivalent was at the spindle periphery and only one of the 2 chiasma disjoined, while the other two bivalents at the cell equator disjoined and moved poleward after the CD was washed out.

The fact that chromosomes were not detached by CD treatment was confirmed by fluorescence images of microtubules in cells treated with CD from prometaphase to anaphase, lysed in anaphase, and stained. The tubulin fluorescence of the cells followed live (Fig. 9 A–D) and lysed in anaphase showed that spindle microtubules were present in the usual microtubule pattern (Fig. 9 E) seen in control cells.

CD affected anaphase chromosome movement as described previously (Forer and Pickett-Heaps 1998a): 32 bivalents moved slowly and 5 did not move poleward after disjunction. After washing out the CD at 10 min after anaphase onset 23 out of 32 bivalents sped up and 3 out of 5 bivalents resumed movement.

Fig. 4 A–F. Phase-contrast microscopy images of a primary spermatocyte treated with NOC from 16:48 to 16:52 starting at 15 min after NMBD. **A** Before NMBD. **B** Before NOC treatment. **C** Shortly after NOC treatment; the spindle is disorganised. **D** 25 min after washing out NOC; the spindle is reorganised. **E** Anaphase onset for 2 chromosome pairs: 80 min after NMBD. **F** Anaphase onset for the third chromosome pair: a different focal plane. Bar: 10 μm

Fig. 5. Confocal microscopy image of a primary spermatocyte treated with NOC at 15 min after NMBD, lysed 4 min later, and double stained for acetylated α-tubulin (left panel) and tyrosinated α-tubulin (right panel). Bar: 10 μm

Fig. 6 A–F. Primary spermatocyte treated with NOC at 15 min after NMBD for 4 min, from 16:25 to 16:29. **A** Before NMBD. **B** After washing out the NOC. **C** Anaphase onset. **D** Autosomal telophase. **E** Image of the same cell after lysis in telophase. **F** Confocal microscopy images of the same cell as in **E**, double stained for acetylated α-tubulin (left panel) and tyrosinated α-tubulin (right panel). Bar: 10 μm

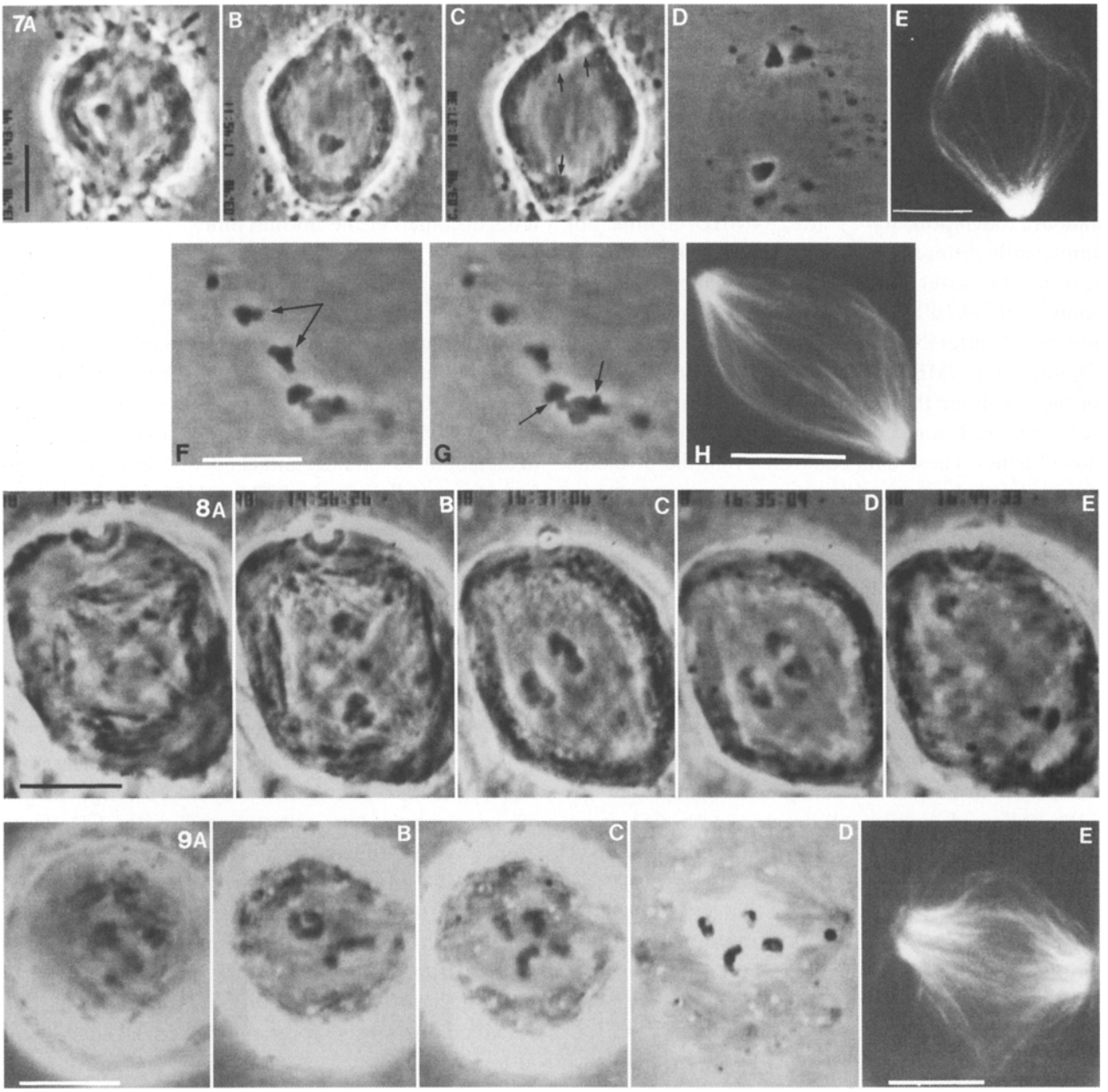


Fig. 7 A–H. Primary spermatocytes treated with CD. **A–D** Cell treated from prophase, at about 40 min before NMBD, to 110 min after NMBD (treated from 16:03 to 18:30). **A** Just before NMBD. **B** After NMBD. **C** After washing out the CD, and just prior to lysis: arrows indicate unattached bivalents. **D** After lysis (7 min after washing out the CD). **E** Confocal microscopy image of the same cell as in **D** stained for tyrosinated α-tubulin. **F** Another cell from the same slide treated similarly and lysed in anaphase: arrows indicate separating half-bivalents of the attached bivalent. **G** Another focal plane of the same cell as in **F**: arrows indicate the two unattached bivalents. **H** Confocal microscopy images of the same cell as in **F**, stained for tyrosinated α-tubulin. Bars: 10 μm

Fig. 8 A–E. Phase-contrast microscopy images of a primary spermatocyte treated with CD from prometaphase starting, at about 17 min after NMBD, until about 10 min after anaphase onset. **A** Before NMBD. **B** Before CD treatment. **C** Metaphase. **D** Anaphase onset at 110 min after NMBD. **E** Autosomal telophase. Bar: 10 μm

Fig. 9 A–E. Primary spermatocyte treated with CD from prometaphase (starting at about 17 min after NMBD) until mid-anaphase. **A** Before NMBD. **B** During CD treatment. **C** Anaphase onset at 80 min after NMBD. **D** Same cell as in **C**, lysed in anaphase. **E** Confocal microscopy image of the same cell as in **D**, stained for tyrosinated α-tubulin. Bars: 10 μm

In sum, when cells were treated with CD at 17 min after NMBD and kept in CD until anaphase, there was no detachment of already attached chromosomes, anaphase onset was not affected, but anaphase chromosome movement was reversibly perturbed.

Double treatment with NOC and CD

Attachment

Double treatment with NOC and CD (following the protocol illustrated in Fig. 1) blocked bivalent reattachment in 24 out of 26 cells as summarised in Table 1. In 2 (out of 26) cells a normal-looking spindle formed after washing out the NOC and while the cells were in CD; all 3 bivalents congressed to the spindle equator and all disjoined at 90 and 110 min after NMBD, respectively, while still in CD. These times are similar to those in control cells from sister testes ($t = 0.049$, $P = 0.96$). Thus, in these 2 cells the double treatment did not block the reattachment of the bivalents to the spindle.

Reattachment of all 3 bivalents was blocked in the other 24 (out of 26) cells (e.g., Fig. 10). After NOC treatment the spindles were kept disorganised, as after treatment with NOC alone. Later, after NOC was removed, but the cells were kept in CD, a spindle area appeared at the same place as before the treatment. This area appeared after variable times and with variable degrees of recovery for different cells. The bivalents moved slowly, or not at all, while the cells were in CD, and the clear spindle area was invaded by granules at about the normal time of anaphase onset. In 21 of the 24 cells the bivalents did not disjoin by 120 min after NMBD (Table 1), the latest time of anaphase onset seen in control cells. At that time, 120 min after NMBD, the bivalents were either outside (8), at the periphery of (3), or inside (61) the former spindle area. The lack of congression, the absence of clear spindle region, and the peripheral positions of bivalents are all different from control treatments with either drug alone and, we assume, are the result of the bivalents not being attached. In the other 3 of these 24 cells (Table 1) all bivalents disjoined, at respectively 60, 60, and 80 min after NMBD. The direction of disjunction with respect to the former pole-to-pole axis was different for all 3 bivalents in each cell, and the half-bivalents did not move further after washing out the CD at about 10 min after disjunction, so we think the bivalents in these 3 cells indeed were not attached.

Overall, double treatment with NOC and CD (illustrated in Fig. 1) blocked chromosome attachment to the spindle in 24 of 26 treated cells: in 3 of these cells chromosomes disjoined at the normal time, but in 21 cells chromosomes did not disjoin (Table 1).

Table 1. Bivalent and spindle behaviour in cells double treated with NOC and CD

Condition and behaviour of spindle or bivalents	Nr. of cells
Before CD washout	
Bivalents attached and disjoined	2[a]
Bivalents not attached but disjoined	3[b]
Bivalents neither attached nor disjoined	21
After CD washout	
Spindle reformed, bivalents disjoined	7/21
Spindle reformed, bivalents not disjoined	9/21
Spindle not reformed	5/21

[a] Half-bivalents moved poleward in CD and moved faster when the CD was washed out

[b] Half-bivalents did not move poleward either in CD or after the CD was washed out

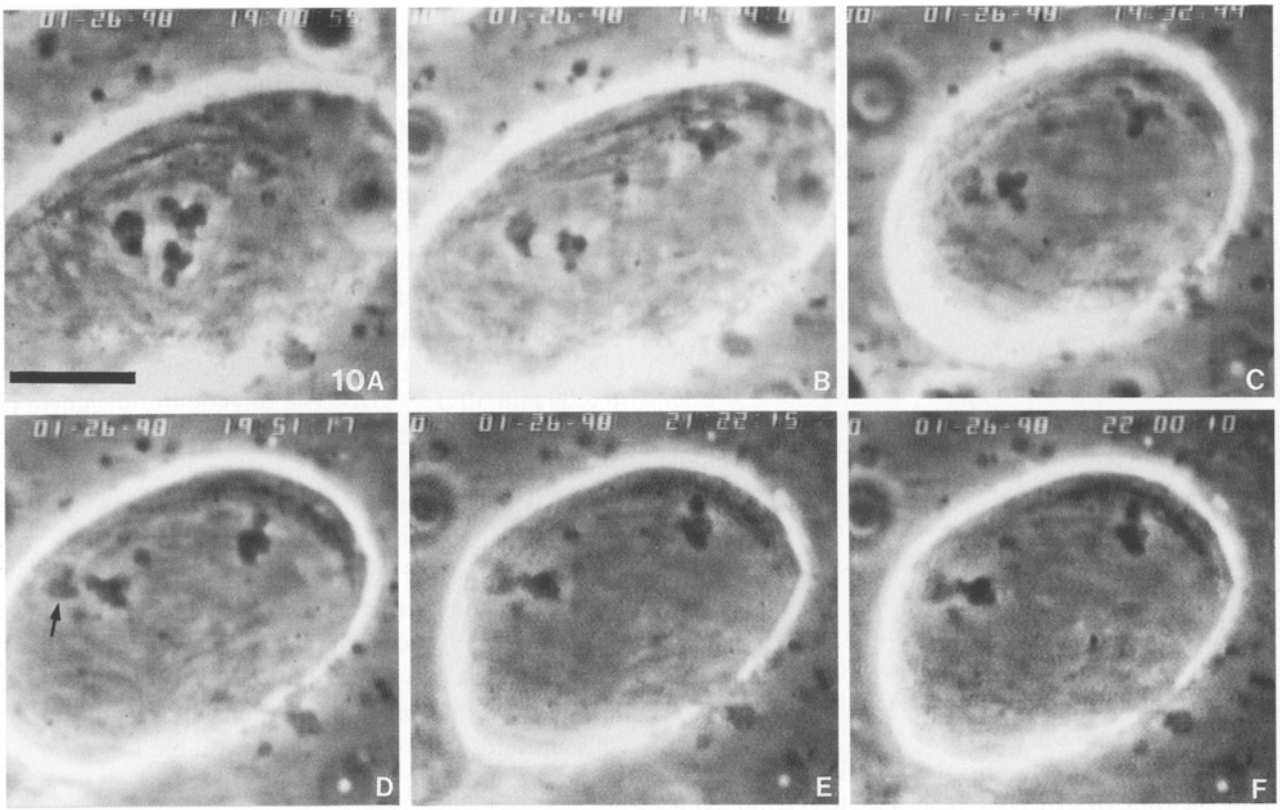
Disjunction

We washed out the CD at 120 min after NMBD, the latest time anaphase onset occurred in control cells, to see if spindles would reform, and, if so, if chromosomes would reattach to the spindle and disjoin.

Spindles reappeared in 16 of the 21 cells in which the attachment and disjunction of all 3 bivalents was blocked (Table 1). In the other 5 (of the 21) cells, spindles did not reappear within 1 h, the minimum time period that they were followed.

In the 16 cells in which spindles reappeared, bivalents that were immobile or moved slowly during CD treatment started to move as clear spindle areas appeared, and some of the bivalents congressed toward a metaphase plate. Chromosome disjunction took place in 7 of these cells (Table 1): in each cell all 3 bivalents disjoined (e.g., Fig. 11). Disjunction occurred from 130 to 180 min after NMBD (10 to 60 min after washing out the CD), on average at 157.7 ± 18.64 min (mean with standard deviation; $n = 7$) after NMBD, considerably delayed ($t = 10.5$, $P = 0$) compared to control cells from sister testes, none of which disjoined later than 102 min after NMBD.

Spindles reformed in the other 9 (of the 16) cells (Table 1), and some bivalents moved within the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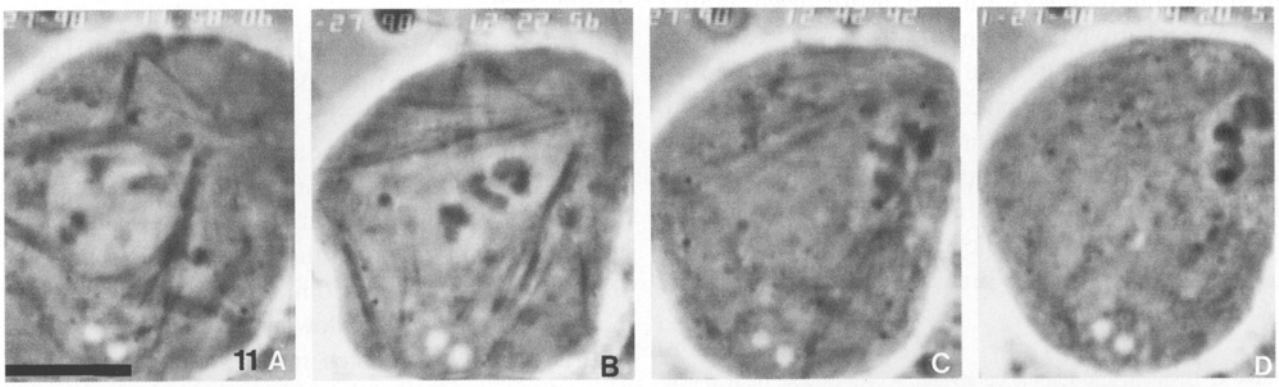
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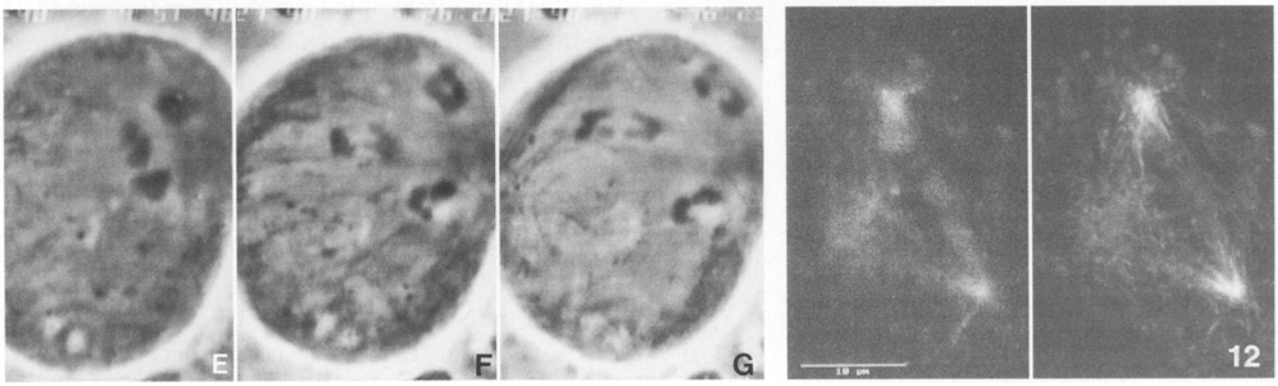
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12

spindle and thus seem to be reattached. There was no disjunction for as long as we followed the cells (up to 4 h in some cases). In at least 3 of the cells one bivalent remained within the granules, outside the clear spindle area.

Overall, our data indicate that after detached bivalents were prevented from reattaching to the spindle by CD treatment, spindles formed in about three-quarters of the cells once the CD was washed out, bivalents attached to the spindle, and in about half of these cells a delayed anaphase took place. In the other half anaphase may have been delayed until all bivalents could reattach to the spindle, beyond the time we followed the cells.

Actin–microtubules interactions

Immunofluorescence data show that microtubule stability was influenced by CD. In cells lysed after the end of NOC application during the double treatment, the band of acetylated microtubules seen after treatment with NOC alone (Fig. 5) was no longer present (Fig. 12). The only microtubules that were present were in flagella and few cytoplasmic microtubules near the poles.

Discussion

Our experiments provide several lines of evidence that actin interacts with microtubules during chromosome attachment to the developing spindle: (a) the attachment of bivalents was blocked when the antiactin drug CD was added starting from prophase; (b) the reattachment of bivalents was prevented when extended CD treatment followed a short treatment with the microtubule-depolymerising drug NOC; and (c) treatment with NOC itself did not depolymerise kinetochore microtubules, while double treatment with NOC and CD did.

Disruption of actin filaments with CD in prophase, prior to spindle formation, blocked the attachment of bivalents to spindle microtubules. Previous experiments using CD and latrunculin B, another antiactin drug, showed that both drugs had similar effects on chromosome attachment: treatments started from prometaphase did not cause chromosome detachment from the spindle, while treatments started from prophase prevented one or more bivalents from attaching to the spindle (Forer and Pickett-Heaps 1998a, b). Since the two drugs have different structures and action mechanisms, the similarity of effects on chromosome attachment leads to the conclusion that these effects are a direct consequence of actin filament disruption and not a secondary side effect, it being improbable that both antiactin drugs have identical side effects. A recent report showed that nuclei contain G-actin and that CD treatment in prophase alters chromosome positioning at the nuclear envelope in crane-fly spermatocytes (La Fountain et al. 1999); it is possible that the G-actin must polymerise for bivalents to attach stably to microtubules and that CD blocks this polymerisation. Alternatively, it might be that CD blocks the actin from the dynactin complex from functioning at the kinetochore (Echeverri et al. 1996), thereby blocking attachment.

Treatment with CD in early prometaphase, after chromosomes were already attached, did not cause their detachment. This result differs from other cells where cytochalasins cause chromosome detachment (Snyder and Cohen 1995, Sampson et al. 1996). One reason for the difference might be that spindle actin in crane-fly spermatocytes is protected from the drugs by its interaction with kinetochore microtubules; other data rule this possibility out, however. At the concen-

Fig. 10 A–F. Cell treated with NOC from 15 to 19 min after NMBD (from 19:26 to 19:30) and with CD from 17 min after NMBD to 128 min after NMBD (from 19:28 to 21:19). **A** Just before NMBD. **B** Before NOC application. **C** Cell incubated in CD. **D** Cell incubated in CD; the arrow indicates a bivalent at the spindle periphery. **E** Just after CD was washed out; the spindle is disorganised. **F** 30 min after washing out the CD and nearly 3 h after NMBD the bivalents still have not disjoined. Bar: 10 μm

Fig. 11 A–G. Phase-contrast microscopy of a primary spermatocyte treated with NOC from 15 to 19 min after NMBD (from 12:37 to 12:41) and with CD from 17 min after NMBD until 120 min after NMBD (from 12:39 to 14:22). **A** Before NMBD. **B** After NMBD, before NOC application. **C** Cell incubated in CD: all bivalents are at the spindle periphery. **D** Just before CD was washed out; the spindle is disorganised. **E** After CD was washed out, the spindle started to reorganise. **F** 2 bivalents disjoin at about 180 min after NMBD. **G** The last bivalent disjoined 8 min later. Bar: 10 μm

Fig. 12. Confocal microscopy of a primary spermatocyte treated with NOC at 15 min after NMBD, with NOC and CD at 17 min after NMBD, and lysed at 19 min after NMBD and double stained for acetylated α-tubulin (left panel) and tyrosinated α-tubulin (right panel). Bar: 10 μm

tration of CD and duration of treatment we used, we know that most actin filaments in the spindle were depolymerised, since a 10 min treatment of metaphase cells with CD caused depolymerisation of the actin associated with kinetochore microtubules and caused actin fragments to aggregate into brightly stained spikes (Silverman-Gavrila and Forer 2000); similar effects were seen in prometaphase cells on the same slides. Thus protection of actin by kinetochore microtubules does not seem to be the reason that CD does not cause detachment in crane-fly spermatocytes. We do not know why CD causes chromosome detachment only in some cell types, but perhaps there are clues from the effects of double treatment, that reattachment is blocked in crane-fly spermatocytes when CD treatment follows NOC treatment. Our data suggest that microtubules must interact with actin filaments during the very first stages of chromosome attachment, and that in spermatocytes, at least, the actin is less important after attachment is stabilised. Actin filaments embedded at the kinetochore might act together with kinetochore microtubules to generate initial tension at the kinetochore, tension which has been shown to stabilise correct bipolar chromosome attachment (Dietz 1956, Nicklas and Ward 1994, Li and Nicklas 1997). Consistent with this idea is data that in epithelial cells actin is involved in translocating microtubules in lamellipodia (Waterman-Storer and Salmon 1997). Two lines of evidences suggest that spindle actin might similarly translocate kinetochore microtubules poleward and thereby generate tension. In one, actin inhibitors block anaphase chromosome movements (Forer and Pickett-Heaps 1998a), while in the other a variety of actin inhibitors and the myosin inhibitor BDM inhibit "flux" of tubulin along kinetochore microtubules (Silverman-Gavrila and Forer 2000). Thus it might be that the initial "stabilising" force for chromosome attachment requires actin, to provide "tension" on kinetochore microtubules, and that once chromosome connections to microtubules are stabilised attachment might be able to be maintained even after inhibitors remove or reduce that force.

There are other possible ways that actin might be involved. It might be that actin is a required component of a spindle matrix (Pickett-Heaps et al. 1996) and that the matrix is required for proper organisation of spindle microtubules to which chromosomes can attach. Data from other systems are consistent with this idea: disruption of actin filaments with cytochalasins affects the organisation of spindle

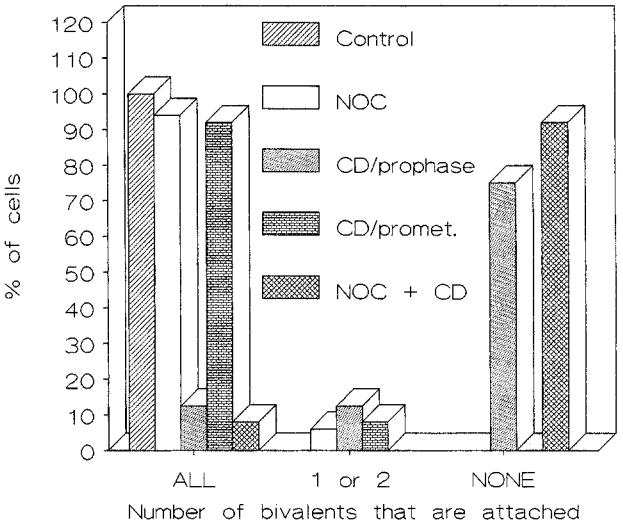

Fig. 13. Effect of treatments on bivalent attachment. Control cells: in all 37 cells all 3 bivalents were attached. Cells treated with NOC in prometaphase: in 16 of the 17 cells all bivalents were attached and in one cell 1 bivalent was not attached. Cells treated with CD in prophase: in 6 of the 8 cells no bivalents were attached, in one cell 2 bivalents were not attached, and in one cell all bivalents were attached. Cells treated with CD from prometaphase: in 12 of the 13 cells all bivalents were attached and in one cell 1 bivalent was not attached. Cells treated doubly, with NOC and CD: in 24 of the 26 cells all bivalents were not attached and in two cells all bivalents were attached

microtubules (Traas et al. 1989, Wrench and Snyder 1997), induces modification of microtubule dynamics and microtubule-dependent organelle translocation (Krendel et al. 1998), and prevents the organisation of cortical microtubules (Hasezawa et al. 1998). Thus it might be that actin–microtubule interactions are needed for spindle organisation. Yet another possibility is that actin is necessary for kinetochore organisation (Snyder and Cohen 1995, La Fountain et al. 1992), that actin is involved in the initial recognition of kinetochore by spindle microtubules. Irrespective of the mechanism, our data (summarised in Fig. 13) show that chromosome attachment to the spindle requires actin.

Our data strongly suggest that the anaphase-onset checkpoint recognises only chromosomes that previously have interacted with the spindle. When previously attached bivalents that were detached by NOC alone treatment were allowed to reattach to the spindle by washing out the drug, bivalent disjunction occurred after all the bivalents reattached to the reorganised spindle, at times similar to control cells. When never-attached bivalents were not attached at

the expected time of anaphase, because of CD treatment that started in prophase, there was no delay in anaphase disjunction, confirming the results of Forer and Pickett-Heaps (1998b). However, when previously attached bivalents were not attached at the expected time of anaphase (because of the double treatment with NOC and CD), anaphase disjunction occurred in only 3 out of 24 cells (Table 1). When the CD was washed out, spindles formed again in 16 of the 21 cells (Table 1) and anaphase disjunction ensued in 7 of these 16 cells, much delayed compared to normal anaphase. We assume that in the other 9 cells in which spindles reformed, anaphase was delayed until after we stopped following the cells. Thus, delays in onset of anaphase, due to the anaphase-onset checkpoint, occurred only when previously attached bivalents were prevented from reattaching; delays did not occur when never-attached bivalents remained not attached. Thus the checkpoint is "activated" only when bivalents become attached.

With respect to the 3 out of 24 cells in which there was disjunction (Table 1), it may be that we added NOC before all the bivalents were attached to the spindle and that CD prevented chromosome attachment, as it was reported that CD treatment shortly after NMBD sometimes prevents bivalent attachment (Forer and Pickett-Heaps 1998a).

If our interpretation is correct, that attachment "triggers" the anaphase-onset checkpoint, it might be that attachment triggers a molecular change in the kinetochore, and that, only after that change, does the kinetochore emit the inhibitory signal discussed by Waters et al. (1998). This idea might be tested by comparing the molecular structure of kinetochores in never-attached bivalents with that of previously attached bivalents.

Other of our data indicate that there are interactions between actin and the forming spindle: some spindle microtubules remain after brief treatment with NOC (Fig. 5), but not if CD also is present (Fig. 12). Others have seen interactions between kinetochore microtubules and kinetochore fiber actin (Forer and Behnke 1972, Forer et al. 1979), and between astral microtubules and actin (Sider et al. 1999), the latter being mediated through microtubule-associated proteins. Microtubule-associated proteins in other systems also interact with actin (e.g., Correas et al. 1990, Fujii et al. 1993, Tögel et al. 1998), MAP2 protecting actin filaments from CD destabilisation as well as assuring microtubule stabilisation (Ferhat et al. 1996). There are also actin-associated proteins that can bind also to microtubules (Fach et al. 1985). Our data are consistent with the in vitro studies and suggest that interaction between microtubules and actin filaments are important in vivo in setting up spindles.

Acknowledgments

We are grateful for the support of grants from the Natural Sciences and Engineering Research Council of Canada (to A.F.) and to an Ontario Graduate Scholarship in Science and Technology (to R.V.S.G.).

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