Actin and myosin inhibitors block elongation of kinetochore fibre stubs in metaphase crane-fly spermatocytes

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running head: A Forer et al.: Actin, myosin and tubulin flux

ABSTRACT

We used an ultraviolet microbeam to cut individual kinetochore spindle fibres in metaphase crane-fly spermatocytes; then we followed the growth of the "kinetochore stubs", the remnants of kinetochore fibres that remain attached to kinetochores. Kinetochore stubs elongate with constant velocity by adding tubulin subunits at the kinetochore, and thus elongation is related to flux of tubulin in the kinetochore microtubules. Stub elongation was blocked by cytochalasin D and latrunculin A, actin inhibitors, and by butanedione monoxime, a myosin inhibitor. We conclude that actin and myosin are involved in generating elongation and thus in producing flux of tubulin in kinetochore microtubules. We suggest that actin and myosin act in concert with a spindle matrix to propel kinetochore fibres poleward thereby causing stub elongation and generating anaphase chromosome movement in non-irradiated cells.

Key words: chromosome movement, spindle function, crane-fly spermatocytes, butanedione monoxime, cytochalasin, latrunculin, UV microbeam

INTRODUCTION

The mechanism(s) that generate anaphase chromosome movements remain elusive. Recent experiments suggest that the force transmitted to the kinetochore is generated by the "flux" of tubulin subunits through the microtubules (MTs) of the kinetochore fibre (review in Rogers et al., 2005), flux being either an inherent property of the MTs themselves (e.g., Margolis and Wilson 1978, 1981; Chen and Zhang, 2004) or caused by MT motors associated with other MTs or associated with a stationary spindle matrix (Mitchison, 1989; review in Scholey et al., 2001).

We have argued that flux of tubulin in kMTs is derived from forces generated by a spindle matrix, that these forces cause compression of kMTS at the pole and elongation at the kinetochore, and that they utilise actin and myosin (Forer and Wilson, 1994; Pickett-Heaps et al., 1997; Spurck et al., 1997). Evidence that actin and myosin are involved in generating flux is indirect; for example, a gap in acetylation of kMTs at the kinetochores in non-treated cells is indicative of tubulin flux (Wilson et al., 1994; Wilson and Forer, 1997), and actin and myosin inhibitors eliminate this gap (Silverman-Gavrila and Forer, 2000). In the present experiments we have directly tested actin and myosin involvement by ascertaining whether actin and myosin inhibitors block the elongation of kinetochore stubs. We know from previous experiments that kinetochore stubs elongate with constant velocity in crane-fly spermatocytes (Forer, 1965, 1966; Wilson and Forer, 1989a) and in other cells (Czaban et al., 1993; Maiato et al., 2004, 2005). We also know from previous experiments that tubulin is incorporated at the kinetochore and that subunits flux toward the severed end as the kinetochore stub elongates during metaphase (Maiato et al., 2004; Wilson and Forer, 1989b). In the present experiments we created kinetochore stubs during metaphase using an ultraviolet microbeam; then we added drugs that interfere with actin and myosin. If flux derives from actin and myosin associated with the spindle matrix as we suggest, then interfering with actin

or myosin should block or slow elongation of the kinetochore stub. As reported herein, anti-actin drugs cytochalasin D (CD) and latrunculin A (LatA) and the anti-myosin drug butanedione monoxime (BDM) indeed block the elongation of kinetochore stubs.

METHODS

We placed crane-fly spermatocytes on quartz coverslips in a fibrin clot, put the coverslip into a perfusion chamber and perfused the cells initially with insect Ringers solution (Forer and Pickett-Heaps, 2005). At selected times we perfused the cells with drugs (in insect Ringers solution), and sometimes washed out the drugs later. Butanedione monoxime (BDM), from Sigma, was dissolved as a stock solution in insect Ringers solution. Latrunculin A (LatA) and cytochalasin D (CD), both from Calbiochem, were dissolved as stock solutions in DMSO. BDM, used as an inhibitor of myosin function (review in Forer and Fabian, 2005), was at a final concentration of 20 mM. CD and LatA were used as inhibitors of actin (discussion in Forer and Pickett-Heaps, 1998). CD was added at a concentration of 40μM. LatA was added at a concentration of 2.5 μM. On the day of the experiment the stock solutions were diluted by at least 1000 fold into insect Ringers solution to make the final perfusion solutions. We perfused the cells with insect Ringers solution at irregular intervals (roughly once per 10-15 minutes). Neither putting the cells in a clot, nor perfusion with Ringers solution, nor perfusion with #0.1% DMSO affects the behaviour of the kinetochore stub (KT-stub) or chromosome movement or anything else we can see, as is the situation in other cells (Forer and Pickett-Heaps, 2005).

We observed the cells as described previously (Forer et al., 2003), using a polarising microscope, at high resolution (condensor NA = 1.2, objective NA = 1.3), and recording images on optical disks in time lapse. We irradiated the cells with monochromatic ultraviolet light from a mercury-arc lamp (generally using light of wavelength 290 nm), analysed the images using custom software to measure distances, plotted the data using commercial software (SlideWrite), and prepared individual images for presentation using Adobe Photoshop, adjusting only brightness and contrast. For confocal microscopy, irradiated cells were lysed with NP40 in a cytoskeleton

stabilising buffer, fixed with glutaraldehyde, stained, located on the coverslip, and viewed using a Leica confocal microscope.

In analysing the movements of kinetochores and the elongations of KTstubs, we viewed individual frames, visually identified the ends of the stubs (or positions of kinetochores), marked those positions with a cursor, entered the times of the frames, and then plotted positions versus time. We generally measured 5-10 images per minute. One difficulty in this analysis is that kinetochore stubs move up and down (i.e., in and out of focus) as well as laterally (Forer et al., 1997; Spurck et al., 1997). Thus we needed to judge for individual frames whether the tip of the stub in question was in focus. When we followed two stubs in a cell, we sometimes could follow only one at a time because of the continuous shifts in focus level of the stubs. To deal with issues such as these, different observers made independent judgements; we sometimes needed to re-graph individual cells before reaching final agreement as to the result for that cell.

RESULTS

KT-stubs produced in metaphase crane-fly spermatocytes are freed from connection to the pole: their poleward ends change angle rapidly and irregularly as the KT-stubs elongate (Forer et al., 1997), and they often elongate at an angle to the pole (Spurck et al.,1997; Forer et al., 1997, 2003), as in *Drosophila* S2 cells (Maiato et al., 2004). In metaphase cells KT-stubs elongate at a constant rate, near that of anaphase chromosome movement, while the associated chromosomes remain at the equator (Figure 1), as described previously for this and other cell types (Forer 1965; Wilson and Forer, 1989a; Czaban et al., 1993; Maiato et al., 2004, 2005). Irradiated cells sometimes enter anaphase before the KT-stubs reach the poles (Forer, 1966; Sillers and Forer, 1983; Spurck et al.,1997) and the movements of the associated chromosomes can be normal.

To determine whether actin and myosin are involved in generating tubulin flux in kMTs we tested whether actin and myosin inhibitors block elongation of the KT-stub. CD, LAT and BDM each blocked KT-stub elongation, in a total of 23/29 KT-stubs (Table I). When we added drugs after irradiation, elongation stopped (e.g., Figure 2), and when we added drugs before irradiation elongation did not take place until the drugs were removed. Table I masks some variability, however. Most cells had 1 KT-stub but 8 cells had 2: we saw no differences in behaviour for 1 or 2 stubs per cell. Some KT-stubs (15) were formed before drug addition whereas others (14) were formed after: there may be differences between these two situations, as seen in Table II, but this possibility requires further experimental testing. For the anaphase irradiations, cells were treated beforehand with drugs which stopped chromosome movements. Then each cell was irradiated to produce one KT-stub. These KT-stubs acted similarly to those formed by irradiation during metaphase. Four cells irradiated in metaphase entered anaphase while they still had KT-stubs (e.g., Figure 3). These KT-stubs were not exceptional in their behaviour (i.e., they behaved like stubs in

cells that remained in metaphase throughout): elongation resumed at washout, or at anaphase prior to washout, or prior to washout but after anaphase started. Despite all these variations, the main conclusion holds: anti-actin and anti-myosin drugs generally blocked elongation of the KT-stub.

The effects of the drugs were all-or-none: KT-stub elongation either was not affected or it stopped. KT-stubs that had stopped elongating in CD did sometimes resume elongation while still in CD (Table I, Figure 2), but other KT-stubs treated with CD and KT-stubs treated with other drugs resumed elongation only after the drug was washed out (e.g., Figure 3). The cessations of elongation were not due to non-specific toxicity because anaphase sometimes started when the KT-stub elongation was blocked. When KT-stub elongation resumed after the drugs were washed out, elongation rates were the same as (if not faster than) before addition of the drug (Table III).

DISCUSSION

In our experiments two actin inhibitors (CD, LAT) and one myosin inhibitor (BDM) blocked elongation of 23/29 KT-stubs (Table I). Since the KT-stub incorporates tubulin at the kinetochore end (Chen and Zhang, 2004; Maiato et al., 2004;, Wilson and Forer, 1989b), the same end as in not-irradiated kMTs, elongation of the KT-stub is a measure of tubulin flux. Thus the inhibitors block flux. Our present data confirm in a direct way previous indirect experiments on crane-fly spermatocytes (Silverman-Gavrila and Forer, 2000) that indicated that anti-actin (CD, LatB, Swinholide) and anti-myosin (BDM) drugs eliminated the gap in kMT acetylation just poleward from the kinetochore (Wilson et al., 1994; Wilson and Forer, 1997). We conclude that actin and myosin functions are required for generating flux.

Some published data would seem to contradict our conclusion: Ganem et al. (2005) concluded that kinesin Kif2a, situated at spindle poles, is required for kMT flux. Whereas a depolymerising agent at the pole may be necessary for flux to occur when kMTs are attached to the pole, flux still takes place in KT-stubs that are not attached to spindle poles: tubulin subunits still incorporate at the kinetochore and then move away from the kinetochore (Chen and Zhang, 2004; Maiato et al., 2004; our data). Flux thus is not necessarily driven by a kinesin at the pole. In other published data, Miyamoto et al. (2004) concluded that the protein Eg5 drives flux in *Xenopus* extract spindles; but, as they also note, flux in kMTs might have different mechanisms than flux in interpolar MTs, which is what they studied. In these regards, our conclusion that actin and myosin are involved in generating flux does not rule out contributions of other components such as Eg5, or other MT motors, or CLASP (Maiato et al., 2005). Since actin and myosin inhibitors blocked elongation of only 23 of 29 KT-stubs, this suggests to us that different mechanisms may contribute

to flux (as also suggested by Cameron et al., 2006), and that the inhibitors have no effect when the force machinery uses an alternate, non-actin-myosin force production method. Further, blocked KT-stub elongation sometimes resumed before the drug was washed out (in 6 of 23 KT-stubs), so the block to elongation can be overcome even in the continued presence of the drug, further indicating that the cell sometimes can switch to other mechanisms. Thus we consider that flux requires actin and myosin most of the time, but that other mechanisms also may be utilised.

How might actin and myosin be involved in generating flux? Many models of spindle function link flux to force production for chromosome movement (e.g., LaFountain et al., 2004; Chen and Zhang, 2004; Rogers et al., 2004; Maiato et al., 2005), but we rather consider flux to be a *consequence* of forces that act on the kinetochore, that flux results from other forces and does not in itself produce the force. We have argued that forces generated by the spindle matrix propel kMTs poleward, even in the absence of attached chromosomes (Zhang and Nicklas, 1996); that compressive forces on kMTs at the pole drive depolymerisation at the pole; that stretching forces on kMTs at the kinetochore drive polymerisation at the kinetochore; and that compressive forces on kMTs at the kinetochore drive depolymerisation at the kinetochore (Forer and Wilson 1994; Pickett-Heaps et al., 1996; Fabian and Forer, 2005). To extend that argument to the experiments described in this article, we think that matrix forces cause flux during metaphase by tending to stretch kMTs at their kinetochore ends and to compress them at their pole ends; and that after the chromosomes disjoin at the start of anaphase the same forces contribute to propelling kMTs to the poles. After creation of a KT-stub in metaphase, actin and myosin associated with the spindle matrix would act on the KT-stub to propel it poleward; the resultant stretching tension at the kinetochore induces tubulin polymerisation at the kinetochore. Actin and myosin inhibitors block forces acting on the KT-stub, thereby blocking polymerisation at the kinetochore and consequent KT-stub

elongation. We have no solid data on how actin and myosin might contribute to flux (and to KT-stub elongation) and only can speculate on how the matrix-derived forces might utilise actin and myosin. To consider only a few possibilities: actin is associated with kMTs (Table I of Forer et al., 2003), so myosin in the matrix might act on kinetochore fibre actin; or actin and myosin might act together in a matrix to support MT motors that act on kMTs; or myosin anchored to a matrix might act on kMTs directly or via a linking component (discussed in Fabian and Forer, 2005).

The key point is that our data point to the involvement of actin and myosin in production of flux in kMTs.

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TABLE I:

EFFECTS OF DRUGS ON KT-STUB ELONGATION

TREATMENT		NUMBERS OF TREATMENTS AFTER WHICH		
	Total number of stubs	Stubs do not elongate		No effect
Drug		Elongation resumes before washout	Elongation resumes at washout	Stubs elongate in presence of drug
CD	11	6	2*	3
LAT	12	0	10* +	2*
BDM	6	0	5*	1

*: one cell was in anaphase at the time of irradiation

+: in one metaphase cell the KT-stub did not elongate for 12 minutes in LAT, after which the observations were terminated, so we do not know for sure when the stub would have resumed elongation.

TABLE II

COMPARISON OF TIMING OF DRUG ADDITION, BEFORE OR AFTER IRRADIATION, LUMPING THE THREE DRUGS TOGETHER

DRUGS ADDED AFTER IRRADIATION, AS THE STUBS ELONGATED		DRUGS ADDED PRIOR TO IRRADIATION	
Number of stubs that stopped elongating	Number of stubs that continued to elongate	Number of stubs that did not elongate	Number of stubs that elongated normally
14	1*	9**	5***

*: This cell was treated with CD

**: includes 5 stubs that were the second stubs produced in a single cell.

***: includes 3 stubs that were the second stubs produced in a single cell.

TABLE III:

RATES OF KT-STUB ELONGATION BEFORE AND AFTER STOPPAGE

Average initial KT-stub elongation rates [number of KT-stubs] (± standard deviation)*	Average KT-stub elongation rates after washout for KT-stubs that stopped elongating [number of stubs] (±standard deviation)	Ratio of elongation rates (after wash/initial) for KT-stubs that stopped elongating [number of ratios] (± standard deviation)**
0.32 µm/min [n=20] (± .18)	0.42 µm/min [n=19] (± .23)	1.51 [n=11] (±.72)

*: including stubs that were not affected by drugs, excluding those irradiated in the presence of drugs that had initial elongation rates of zero

**: excluding those stubs produced in the presence of drug that had initial elongation rate of zero

FIGURE LEGENDS

Figure 1: *Figure 1A* illustrates a control irradiation that produced a KT-stub at 13:33:20. A white arrow indicates the poleward end of the KT-stub. The bar indicates 10µm. *Figure1B* is a graph versus time (abscissa) of the length of the KT-stub (ordinate), i.e., the measured length between the poleward end of the KT-stub and its kinetochore, for the same cell. The line is the software-generated line of best fit to the points. The stub elongated at a rate of about 0.67 µm/min. Times are in hrs:min:sec.

Figure 2: *Figure 2A* illustrates a cell in which a KT-stub was produced at 12:30:05, and to which CD was added and then washed out. The white arrows indicate the poleward end of the KT-stub. Illustrations labelled CD indicate that the cell was in CD at that time; those not labelled CD were either prior to addition of CD (12:34:10) or after washout of CD (12:41:30). The bar indicates 10µm. *Figure 2B* is a graph versus time (abscissa) of the length of the KT-stub (ordinate) for the same cell. The lines are software-generated lines of best fit to the indicated points. The KT-stub initially elongated at a rate of 0.38 µm/min ($r = 0.92$); elongation stopped (or was drastically reduced in rate) after addition of CD, for about 5 minutes, and resumed prior to washout (and continued after washout) at a rate of 0.63 µm/min ($r = 0.96$), faster than the original elongation rate.

Figure 3: *Figure 3A* illustrates a cell that was irradiated twice to produce two KT-stubs, on partner half-bivalents; BDM was added between the irradiations and washed out after the second irradiation. The cell entered anaphase a few minutes after washout. The first KT-stub was produced at 16:42:15 and the second at 16:49:10. Illustrations labelled BDM

indicate that the cell was in BDM at that time; those not labelled BDM were either before addition of BDM (16:45:50) or after washout (16:57:30). The white arrows indicate the poleward ends of the KT stubs. The upper arrow indicates the first KT-stub, the lower arrow the second. The black arrowheads in the illustration at 17:05:17 indicate the kinetochores on the partner half-bivalents associated with the two KT-stubs, to indicate normal anaphase separations of the partner half-bivalents. The bar indicates 10μm. The panel at 17:06:31 illustrates the same cell after addition of lysis buffer. The final panel (labelled *tubulin*) illustrates the confocal image of 4 superposed planes of focus after the cell was stained with antibodies against tubulin, illustrating that the KT-stubs remained without MT connection to the poles throughout their anaphase movements. *Figure 3B* is a graph versus time (abscissa) of the length of the two KT-stubs (left ordinate) and of interkinetochore distance for the half-bivalents associated with the two KT-stubs (right ordinate, solid purple diamonds), for the same cell. The half-bivalents with KT-stubs separated normally during anaphase. The lines are software-generated lines of best fit to the indicated points. The first stub elongated initially at a rate of 0.34μm/min (r = 0.89); elongation stopped (or was drastically reduced in rate) in the presence of BDM. Elongation resumed after washout at a rate of 0.63 μm/min (r = 0.91), and it continued during anaphase. The second KT-stub, associated with the partner half-bivalent of the first KT-stub, was produced while the cell was in BDM; it did not elongate while in BDM, but elongated after washout at a rate of 0.23 μm/min (r = 0.89). Elongation continued in anaphase. Anaphase disjunction occurred 2-3 minutes after washout; the partner half-bivalents associated with the two KT-stubs moved normally, with separation velocities of 1.2 μm/min (r = 0.99).

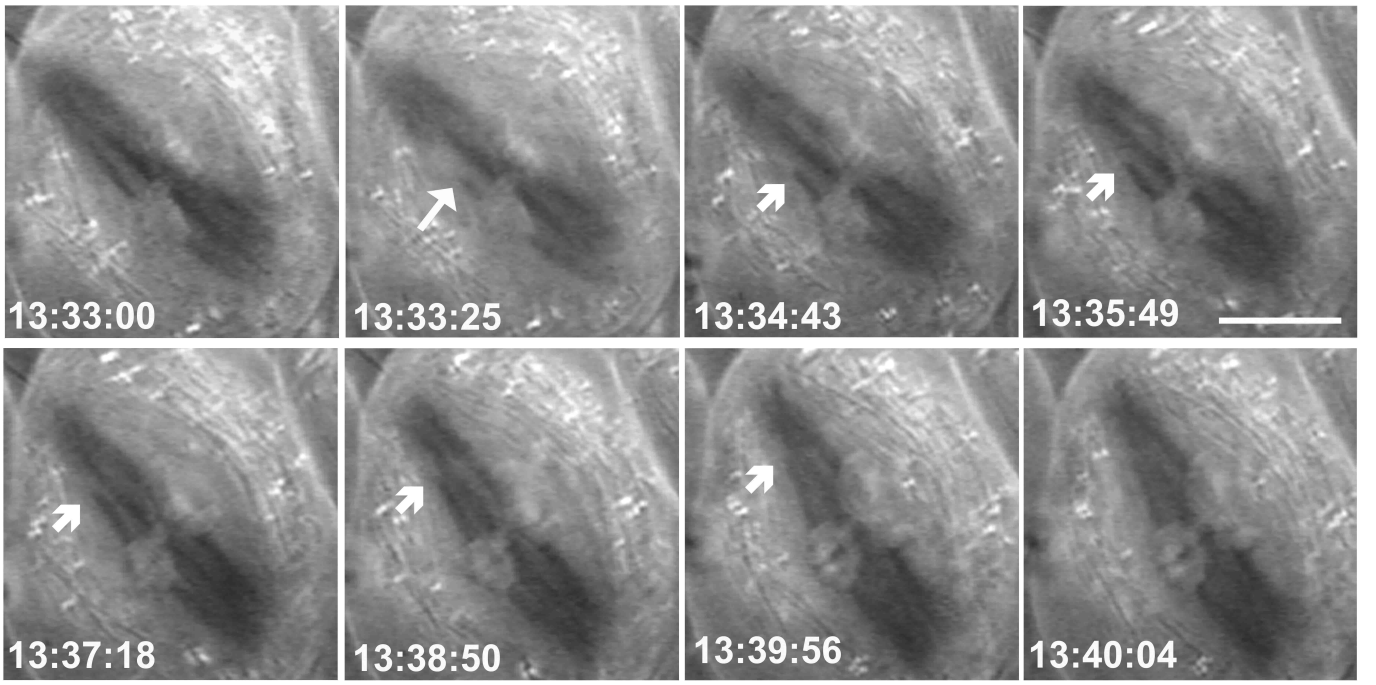

FIGURE 1A

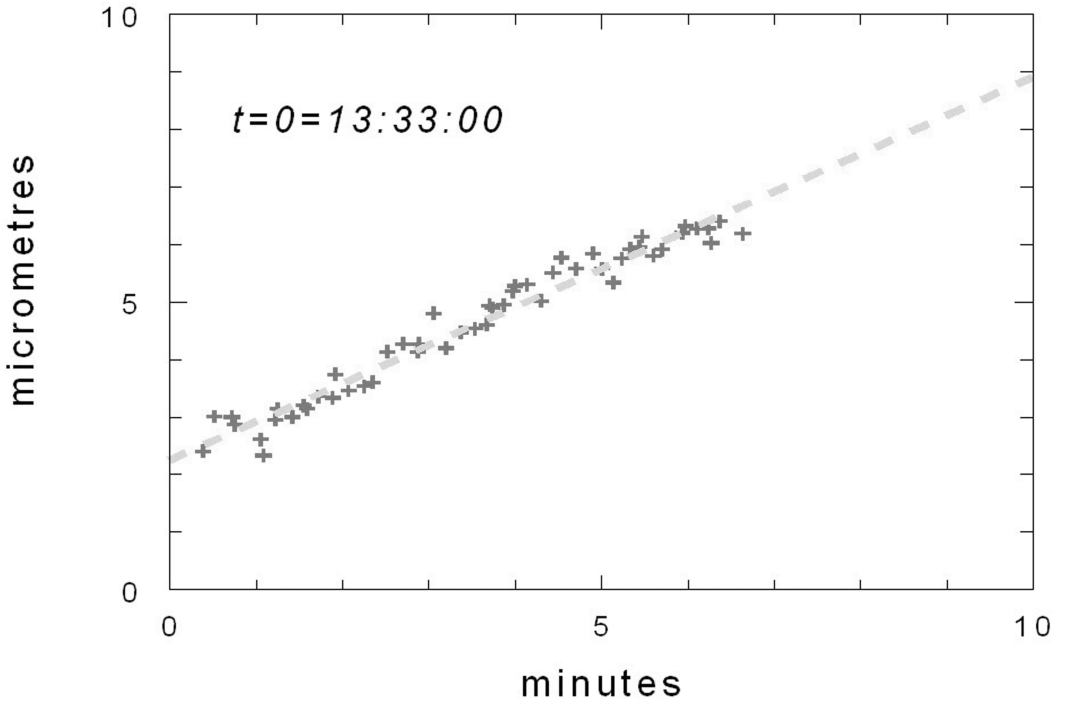

FIGURE 1B

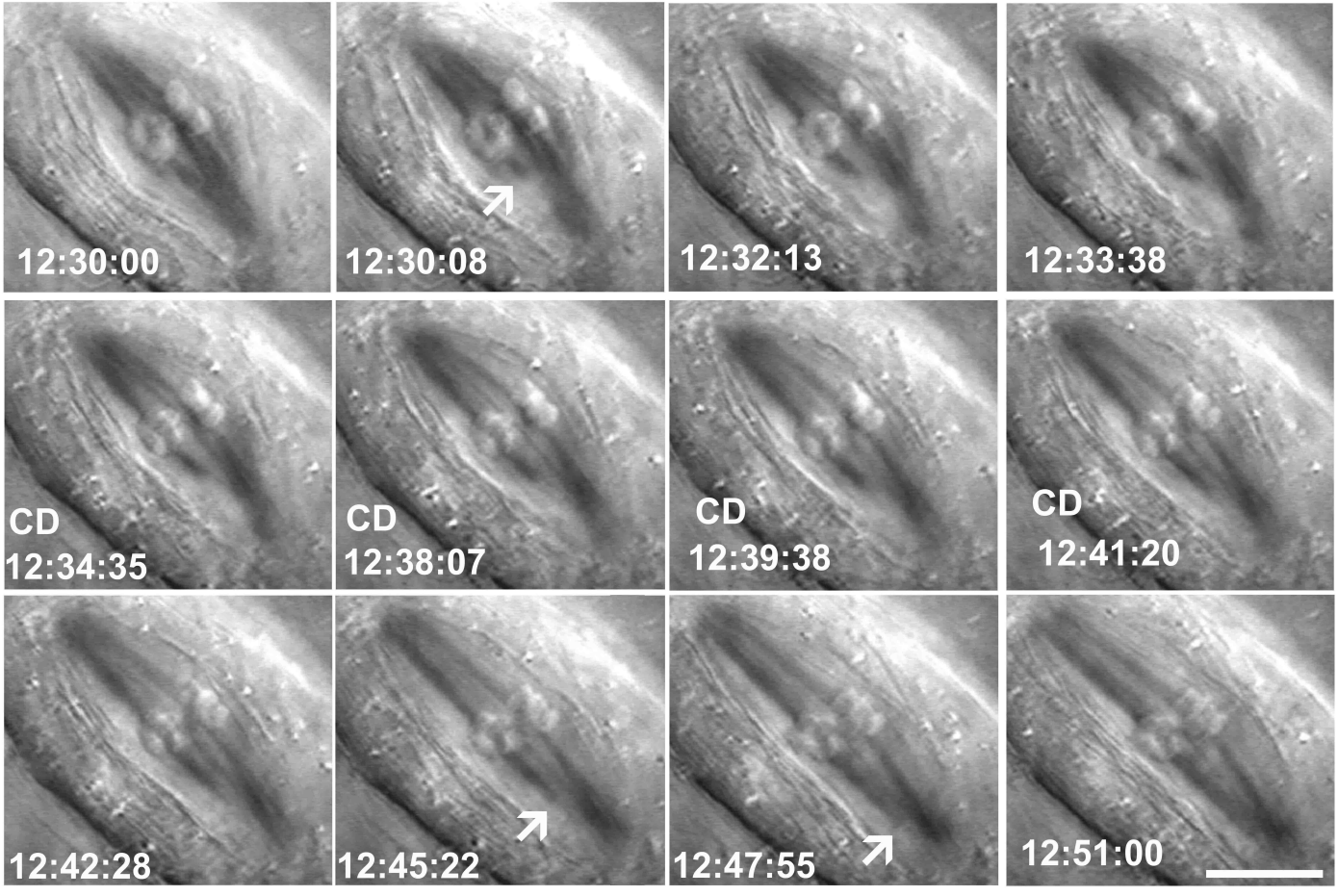
12:30:00
12:30:08
12:32:13
12:33:38
CD
12:34:35
CD
12:38:07
CD
12:39:38
CD
12:41:20
12:42:28
12:45:22
12:47:55
12:51:00

FIGURE 2A

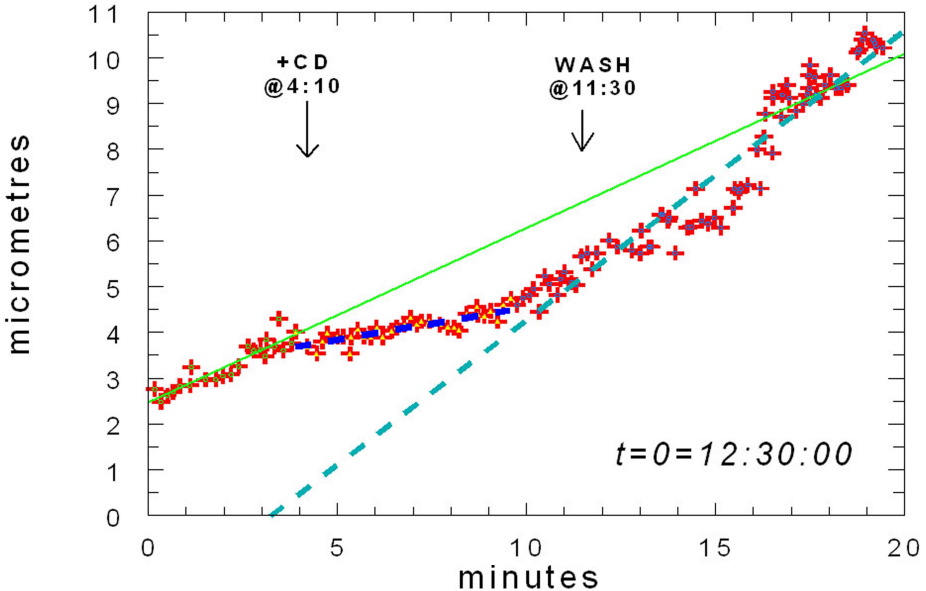
micrometres
minutes
+CD
@4:10
WASH
@11:30
t=0=12:30:00
0
1
2
3
4
5
6
7
8
9
10
11
0
5
10
15
20

FIGURE 2B

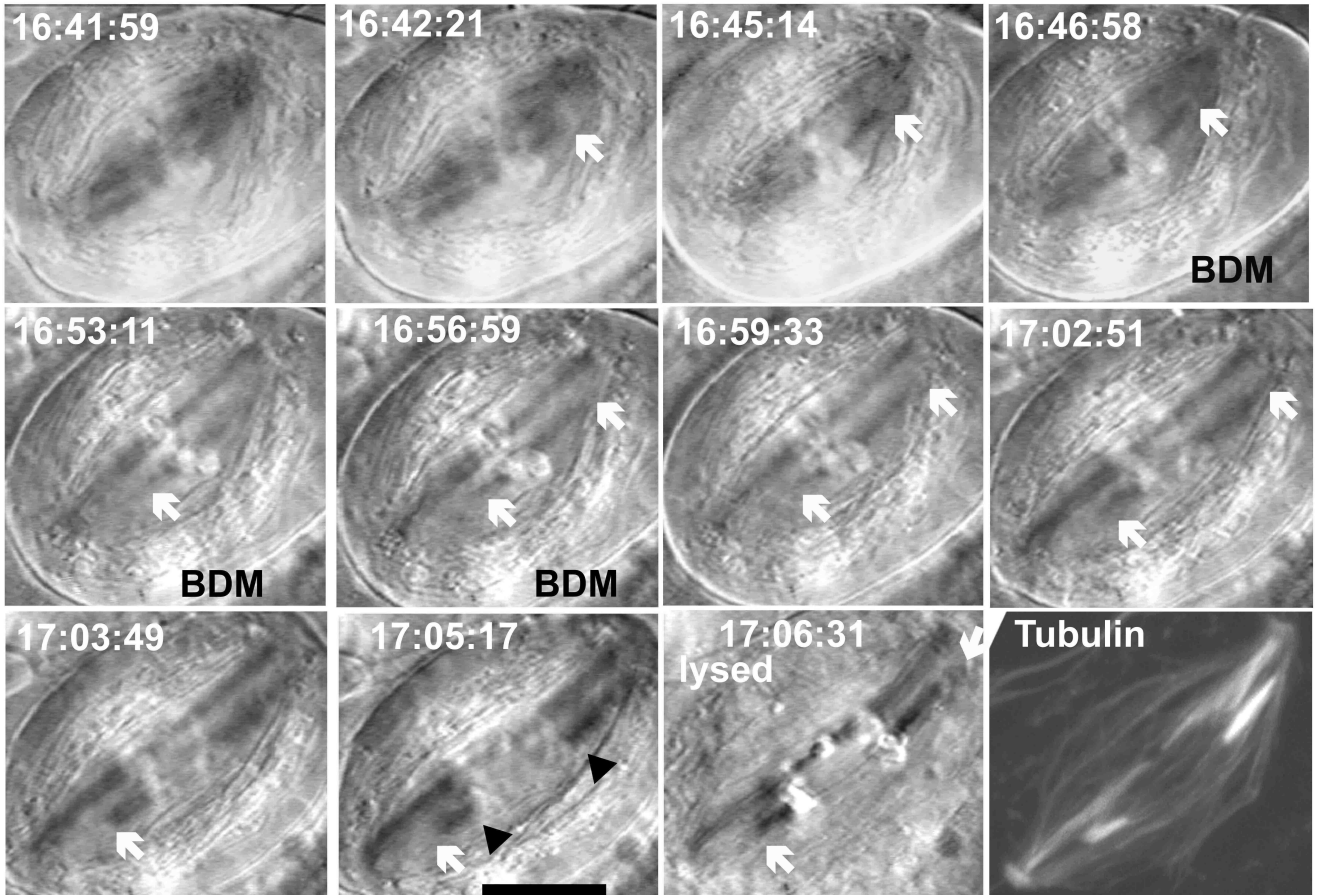
16:41:59
16:42:21
16:45:14
16:46:58
BDM
16:53:11
BDM
16:56:59
BDM
16:59:33
17:02:51
17:03:49
17:05:17
17:06:31
lysed
Tubulin

FIGURE 3A

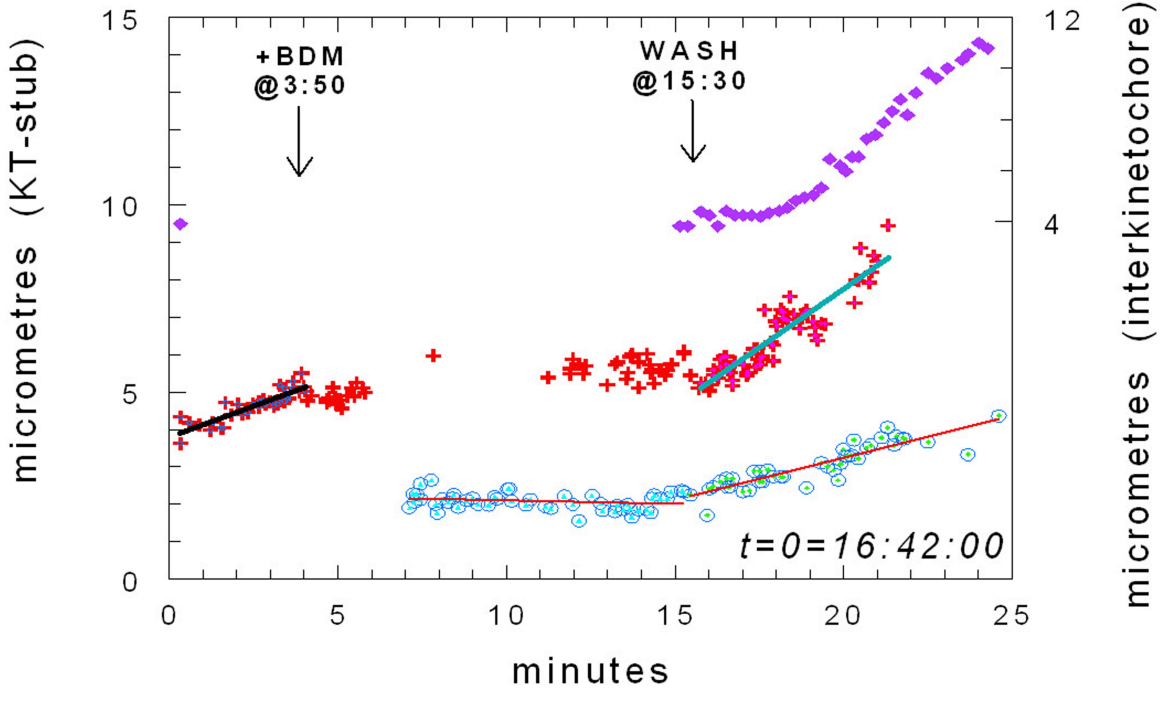
+BDM
@3:50
WASH
@15:30
t=0=16:42:00
micrometres (KT-stub)
micrometres (interkinetochore)
minutes
0
5
10
15
20
25
0
5
10
15
4
12

FIGURE 3B