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Astral and spindle forces in PtK₂ cells during anaphase B: a laser microbeam study

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SUMMARY

Rat kangaroo kidney epithelium (PtK₂) cells develop prominent asters and spindles during anaphase B of mitosis. It has been shown that severing the spindle at early anaphase B in living PtK₁ cells results in a dramatic increase in the rate of pole-pole separation. This result suggested that the asters pull on the spindle poles, putting tension on the spindle, while the spindle acts as a governor, limiting the rate of pole separation. To further test these inferences, we used a UV-laser microbeam to damage one of the two asters in living PtK₂ cells at early anaphase B and monitored the effects on individual spindle pole movements, pole-pole separation rates and astral microtubules (MTs). Irradiation at the estimated position of a centrosome greatly reduced its array of astral MTs and nearly stopped the

movement of the irradiated pole, whereas the sister pole retained its normal array of astral MTs and actually accelerated. Control irradiations, either close to the estimated position of the centrosome or beside the spindle at the equator, had little or no effect on either spindle pole movements or astral MTs. These results support the inferences that during anaphase B in living PtK cells, the central spindle is under tension generated by pulling forces in the asters (presumably MT-mediated) and that the spindle generates counterforces that limit the rate of pole separation. The results also suggest that the central spindle in living PtK cells may be able to generate a pushing force.

Key words: aster, laser microbeam, microtubule, mitosis, spindle

INTRODUCTION

Mitotic anaphase commonly occurs in two stages: during anaphase A, the sister chromatids migrate toward the spindle poles, and during anaphase B the central spindle elongates as pole-pole separation continues (Inoué and Ritter, 1975). Asters undergo a regular cycle of increasing and decreasing size in conjunction with the animal cell mitotic cycle (McIntosh, 1983; Vandre et al., 1984). Although maximum size typically occurs during anaphase, it has often been assumed that the asters do not take an active role in separating the chromosomes (Cande and Hogan, 1989; Fuge, 1977; Mazia, 1961; McIntosh and McDonald, 1989).

In fungi, there is now compelling evidence that mitotic asters pull on the spindle poles and help to elongate the central spindle during anaphase B (Aist et al., 1991; Aist and Berns, 1981). There is also considerable evidence that asters in animal cells have a similar role in anaphase B. This evidence has come from five different species and a variety of approaches, including descriptive cytology of normal and aberrant mitoses (Bajer et al., 1980), destruction of microtubules (MTs) by the application of heat

(Bergan, 1960), treatment with MT-altering drugs (Daub and Hauser, 1988; Hiramoto et al., 1986) and micromanipulation (Hiramoto and Nakano, 1988; Kronebusch and Borisy, 1982).

Until now, the only experimental evidence that asters pull on the spindle poles of PtK cells was that reported by Kronebusch and Borisy (1982). They severed the central spindle of PtK₁ cells at early anaphase B using a microneedle and found that the spindle poles not only continued to separate without an intact central spindle, but that they separated at 2-6 times the rate measured in control cells with intact central spindles. Observations and data were presented to show that this effect was not a physical artefact of the micromanipulation procedure. The authors inferred that the asters pull on the poles of the spindle and that the spindle governs the rate of pole separation by producing counterforces. To further test this inference, we conducted the present experiments to specifically damage one of the two arrays of astral MTs in PtK₂ cells and see if by doing so we could diminish the putative astral pulling force. Our approach was based on the well-documented, localized destruction of MTs in general (Snyder et al., 1991; Tao et

al., 1988) and of astral MTs in particular (Berns et al., 1977; Hyman, 1989; Koonce et al., 1984; Spurck et al., 1990) by UV microbeams and presumably involved selective impairment of the ability of the centrosome to polymerize astral MTs.

MATERIALS AND METHODS

Cultures

PtK₂ cells (rat kangaroo kidney epithelium) were maintained and prepared for UV irradiation through quartz optics, as described by Tao et al. (1988).

Video microscopy and quantitative data acquisition and analysis

Video microscopy using phase-contrast optics, image processing, motion analysis, data analysis and photography from the video monitor were done as before (Aist and Bayles, 1988; Aist et al., 1991), except that the original recordings were made on a Panasonic Model AG-6030 VHS time-lapse recorder operated at 5 frames s⁻¹ and then transferred to the U-Matic format before further processing and analysis.

The resolving power and precision of our video microscopy system were more than adequate to monitor faithfully the movement of the spindle poles. The 100× quartz objective had a NA of 0.85, giving a calculated resolving power for the microscope of 0.24 μm with illumination of 530 nm wavelength. To make measurements from the videotape sequences, we used a mouse-driven cursor (see Fig. 7 of Aist et al., 1991), Imagemasure 1200 software (Microscience Division, Phoenix Technology, Inc., Seattle, WA) with video overlay, and several custom-made programs for data manipulation. The video overlay and cursor eliminated the problem of visual parallax. Imagemasure subdivides the video image into 512 × 512 pixels. According to our calculations, at the magnification we used, the resolving power of the optics (0.24 μm) was represented by 2-3 pixels on the monitor, and one pixel represented 0.1 μm² in the specimen. Because the center of the cursor was represented by 1 pixel, it was technically possible to measure distances in the specimen to within 0.1 μm. In practice, however, there was greater error introduced by the need to estimate visually the position of the centrosome for each set of measurements, as described below. We found that two trained operators measured ten different spindle lengths to within 0.18 μm in seven cells and to within 0.46 μm in the other three. To further reduce operator error and variation among experiments, one of us (J.R.A.) personally conducted all of the measurements that were used for the final data sets. Each of these measurements was made two or three times to assure that a reproducible result was obtained. The validity of these procedures was confirmed by the generation of reasonable and interpretable curves from the data sets (Fig. 4).

We used several precautions and procedures to be certain that what we measured as spindle pole movement really did represent movement of the pole within the cell. Firstly, we verified visually that the chromosomes actually moved relative to stationary organelles that we had outlined on the video monitor. Secondly, we checked carefully to ensure that there was no stage movement or cell migration, by comparing the positions of stationary, extracellular particles or cell borders, respectively, that we outlined on the video monitor at the beginning and the end of each sequence. Thirdly, we drew outlines of the chromosomes on the monitor at the beginning and the end of each sequence to verify that the chromosome sets actually did or did not move. And fourthly, we used a custom-made software program that established a single

pixel as a stationary, fixed, invisible reference point in relation to which the subsequent pole movements were measured. This reference point was established for each video sequence by placing the cursor near the center of the spindle at the beginning of the sequence and entering its coordinates into the computer by clicking the mouse.

Plots were made using Sigma Plot (Jandel Scientific, Corte Madera, CA) and drawn using an IBM Color Plotter (type 7372). Data points were obtained every 30 s over the course of each experiment. A mild smoothing function was applied, and the curves passed directly through every data point.

Irradiations

UV irradiations at 266 nm wavelength were performed as described earlier (Aist et al., 1991), with two exceptions. Firstly, a picosecond laser was used instead of the nanosecond laser. This new laser system consisted of a picosecond Nd-YAG laser (Coherent model Antares 76-YAG) amplified through a Continuum model RGA 60-10 regenerative amplifier, producing a pulse rate of 10 Hz. The output was frequency-converted to a wavelength of 266 nm. Secondly, 5 shots instead of 2 were fired in rapid succession (0.5-1.0 s apart) for each irradiation. The power level of each laser pulse was approx. 0.11-0.14 μJ at the specimen plane, where the theoretical beam diameter was 0.3 μm. For irradiations intended to damage the aster, the position of the (usually invisible) centrosome was estimated visually, based on the configuration of the associated set of chromosomes. To improve our accuracy in making this estimation, we first examined published micrographs of mitotic PtK cells and the few of our videotape sequences in which the centrosomes were visible, and found that the longer chromosome arms had a strong tendency to point toward a site in the aster where the centrosome was, at early anaphase B. When we targeted that site (Fig. 3), we usually were successful in slowing the movement of the irradiated pole. Cytoplasm-irradiated controls were irradiated beside the spindle, at the equatorial plane (Fig. 1). Aster-irradiated controls were irradiated in the aster about 1.4 μm from the estimated position of the centrosome (Fig. 5). The purpose of these controls was to test for non-target effects of irradiation, such as loss of spindle MTs or gelation of the astral cytoplasm, that could influence the results.

Cell selection

To select cells that were suitable for these experiments, several different morphological criteria were applied to all treatment categories. In PtK cells, some astral MTs extend to the cell borders, and cell morphology tends to change from greatly flattened to a more spherical configuration during prophase and metaphase. Since we were studying astral migration, one important requirement was to be sure that each aster had ample room to migrate; thus, only the more flattened cells that were longer and wider than average during anaphase were included in the data sets. Preliminary analyses showed that in both irradiated and non-irradiated categories, the short, narrow, or pointed cells that had rounded up considerably during metaphase had a strong tendency to have slower moving poles, presumably because of physical restraints imposed on the asters by the cell borders. Therefore, only cells were included that were 10.8 μm wide at a point in each aster that was 13.8 μm from the equator of the spindle (in a direction that was parallel to the spindle) at the end of the experiment. Another criterion was that the chromosome sets had to be oriented in a more-or-less side view, so as to facilitate reliable estimations of the centrosome position (Figs 1-3). We noticed also that at these power levels the irradiation would sometimes induce a bubble or hole to form momentarily at the plasma membrane. Even though this artefact usually had no apparent effect on the outcome of the experiment, we discarded all such experiments. More than ten,

otherwise successful, centrosome-targeted experiments were discarded because of these stringent morphological and experimental criteria. Thus, although nine centrosome-targeted experiments were included in the tabular data, the same results were obtained in a total of at least 20 centrosome-targeted cells. We could not detect any other artefacts, such as cytoplasmic lesions or swollen organelles, resulting from these irradiations.

Because we could see the centrosomes in only a few of the cells, due partly to the relatively low NA (0.85) of our 100× quartz objective, we had no convenient way to determine visually whether or not our targeting of the centrosomes had been accurate, as we did have with the earlier spindle irradiations (Aist and Berns, 1981). Yet we noticed that when the critical parameters of power level, focusing, chromosome orientation and targeting were all carefully controlled, irradiation at the estimated location of the centrosome produced a drastic reduction in the migration rate of the irradiated pole in about 80% of the cells, whereas non-irradiated poles never moved that slowly. Based on these results, we assumed that in about 80% of these experiments we were successful in hitting near enough to the centrosome to severely hamper movement of the spindle pole and that about 20% of the time we barely missed the centrosome. Because the purpose of the experiment was to damage a pulling mechanism in the aster, if there was one, we included only those centrosome-targeted cells in which the irradiated pole migrated more slowly than all but the slowest pole of the 50 non-irradiated poles in the pool of non-irradiated and cytoplasm-irradiated controls, i.e. about 0.37 μm/min. In this selection process, the rate of movement of the non-irradiated, sister poles was ignored. By this procedure we virtually eliminated the possibility that apparently poorly targeted poles would be included among the centrosome-targeted cells. Because it is highly unorthodox to use as a selection criterion the very parameter being measured, we have done extensive analyses (Tables 2 and 3) to show that the effects we observed must have been a result of spatially selective laser damage to the astral motility system and not to our use of this selection criterion.

Tubulin immunocytochemistry

The fixation and staining protocols used initially for tubulin immunofluorescence were similar to those described by Tao et al. (1988). We used three different fluorochromes (fluorescein, rhodamine and Texas Red) during the course of the study, and the following protocol with Texas Red gave the best results. 1–2 min after irradiation, the cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min. After three washes in PBS for 3 min each, they were permeabilized in 0.1% Triton X-100 in PBS for 10 min. Following three, 3 min washes in PBS, the cells were treated twice with 1% sodium borohydrite in PBS for 10 min each. Then the cells were washed in PBS three times, 5 min each, and treated for 30 min with mouse monoclonal anti-tubulin (Sigma Chemical Company, St. Louis, MO), at 1:500 dilution. Next, the cells were washed three times, 3 min each, in PBS and treated for 30 min with biotinylated goat anti-mouse IgG (Pierce, Rockford, IL) at 1:5,000 dilution. Following three, 3 min washes in PBS, the cells were stained with Texas Red-conjugated streptavidin (Pierce, Rockford, IL) at 1:100 dilution for 30 min. The cells were again rinsed with PBS (3 × 3 min each), then mounted in FluorSave Reagent (Calbiochem, San Diego, CA) and stored in the dark in a refrigerator until examined the following day.

For observation and imaging of MTs, we used a BioRad Laser Scanning Confocal Microscope, model MRC 600, attached to a Nikon Optiphot light microscope. Specimens were observed using a 60× Plan Apo objective. The filter block used was dichroic reflector 540 LP and barrier filter 550 LP. Each mitotic cell was first observed at different focal planes to get an overall assess-

ment of the extent of astral MT arrays and to determine the optimal plane of focus to visualize the two centrosome regions. Then, images were recorded in two different focal planes, at and near the optimal focal plane. Thus, we made sure that the micrographs faithfully represented the state of the two astral MT arrays as they appeared in each of the cells. Following analog enhancement of the contrast, images were enhanced for signal-noise ratio by averaging several scans. Photographs were taken of the screen images with an Olympus 35 mm camera using Kodak Plus-X or Tri-X film.

RESULTS

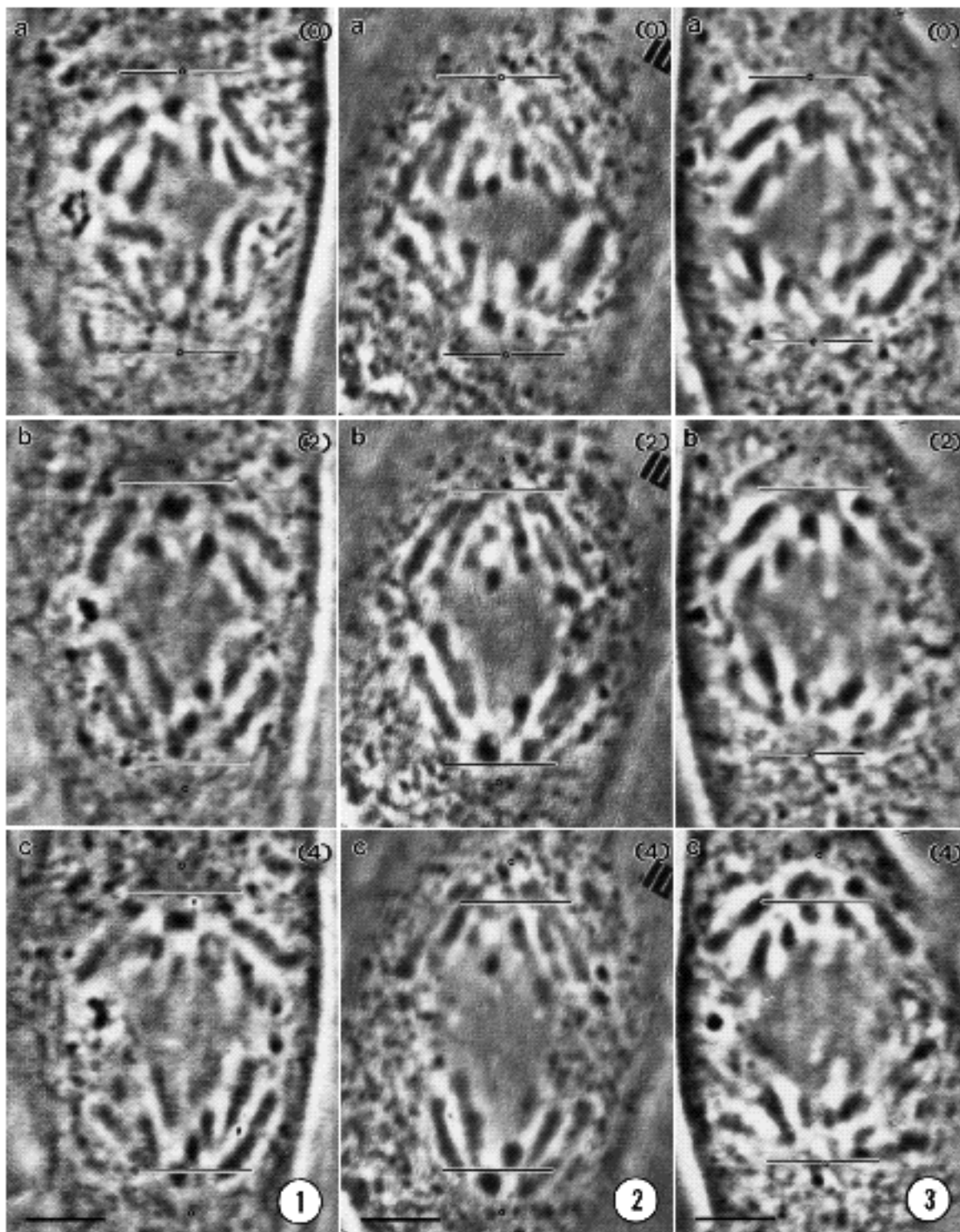
Irradiations

The effects of irradiation are illustrated by representative examples in Figs 1–3. Irradiation of the cytoplasm beside the spindle (Fig. 1a, asterisk) had little or no effect on the progress of mitosis relative to the non-irradiated cell (Fig. 2). In contrast, an apparently successful targeting of the centrosome (Fig. 3) almost stopped the movement of the irradiated pole, while the non-irradiated pole continued its movement unabated. Representative results were plotted (Fig. 4). In the centrosome-targeted cell, the irradiated pole (asterisk) virtually stopped for the first 5 min after irradiation; then, it resumed movement at a rate comparable to normal anaphase B rates. Such resumption of movement was noted in several of the successfully centrosome-targeted cells. Of interest, too, is the fact that both the individual pole movements and pole-pole separation rates were not always constant throughout anaphase B in either irradiated or non-irradiated cells, but sometimes they were punctuated by pauses of 1–2 min duration (Fig. 4). Both of these latter results are similar to results obtained with mitotic fungal nuclei (Aist and Bayles, 1988; Aist et al., 1991).

A summary of the combined results from all of the cells meeting the selection criteria is presented in Table 1. Comparisons between non-irradiated and cytoplasm-irradiated cells showed that there were no statistically significant differences in the rates of either spindle pole movement or pole-pole separation. Both parameters were, however, slightly slower, on average, in the cytoplasm-irradiated cells, suggesting that there may have been a small amount of non-target effect of irradiation. On the other hand, in the centrosome-targeted cells, the movement of the irradiated poles was reduced to less than one-third of that in either of the other two kinds of control cells. Surprisingly, however, there was no concomitant statistically significant reduction in the rate of pole-pole separation in the centrosome-targeted cells (Table 1), although a slight reduction, on average, was apparent. This high rate of pole-pole separation was brought about by a corresponding increase in the rate of movement of the non-irradiated pole in the same cells. The decrease shown by the irradiated poles was very highly significant statistically ($P < 0.001$), even when compared to any of the other categories of spindle poles in Table 1. The increase shown by the non-irradiated poles was significantly different from the average rate for all poles in both non-irradiated and cytoplasm-irradiated cells ($P < 0.05$), but it was only marginally different, statistically,

from the "faster" poles ($P = 0.079$ and 0.056 , respectively). This combination of deceleration of irradiated poles and acceleration of non-irradiated poles in the centrosome-tar-

geted cells led to a five-fold difference in the average rates of the sister poles, whereas assigning the two poles in each of the non-irradiated or cytoplasm-irradiated cells to



Figs 1-3. Time-lapse video micrographs of living PtK₂ cells during the first 4 min of anaphase B in three representative experiments. Small, open circles mark the estimated positions of the centrosomes, asterisks show where UV-laser microbeam irradiations were targeted (left of the spindle equator in 1a and at the estimated position of the lower centrosome in 3a) and thin horizontal lines mark the initial, estimated positions of the spindle poles, for reference. Elapsed time (in min) is given in parentheses in the upper-right corner of each frame. Note that the poles in the cytoplasm-irradiated (Fig. 1) and the non-irradiated (Fig. 2) cells moved considerably, whereas in the centrosome-targeted cell (Fig. 3) the non-irradiated pole moved fast, but the irradiated pole barely moved. Bars, 5 μ m.

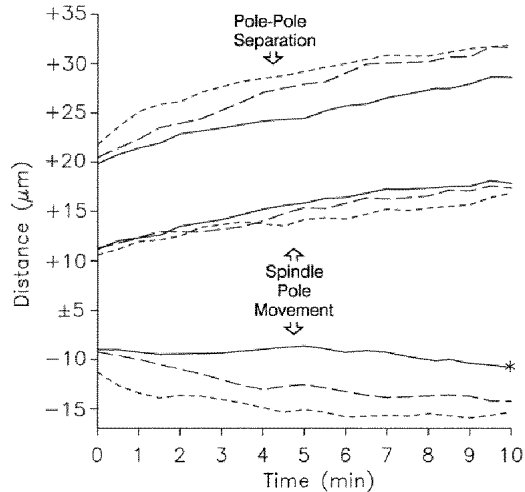


Fig. 4. Plots showing rates of pole-pole separation and spindle pole movements in three representative experiments. Lines with long dashes represent a non-irradiated control; those with short dashes, a cytoplasm-irradiated control; those with solid lines, a centrosome-targeted cell. The irradiated pole (asterisk) was virtually stationary for the first 5 min, then it moved at a normal rate. Note that occasional, 1-2 min pauses occurred in both pole-pole separation and spindle pole movement. The y-axis was collapsed by 5 μm on both sides of zero to conserve space.

“slower” or “faster” categories yielded a much smaller, yet statistically significant, difference between these two categories (Table 1).

Although the above comparison may suggest that the effects seen in centrosome-targeted cells are real, the fact that sister poles in control cells moved at significantly dif-

Table 1. Effects of UV-laser microbeam irradiations on the subsequent rates of spindle pole movement and pole-pole separation in living PtK₂ cells during the first 4 min of anaphase B

Cell category	Pole movement ($\mu\text{m}/\text{min} \pm \text{s.d.}$)	(n)	Pole-pole separation ($\mu\text{m}/\text{min} \pm \text{s.d.}$)	(n)
Non-irradiated				
Slower poles	0.63 \pm 0.13 a*	(14)		
Faster poles	0.83 \pm 0.20 b,d	(14)		
All poles	0.73 \pm 0.19 a,d	(28)		
			1.45 \pm 0.31 a	(14)
Cytoplasm-irradiated				
Slower poles	0.55 \pm 0.16 a	(11)		
Faster poles	0.81 \pm 0.19 b,d	(11)		
All poles	0.68 \pm 0.22 a,d	(22)		
			1.34 \pm 0.27 a	(11)
Centrosome-targeted				
Irradiated poles	0.22 \pm 0.11 c	(9)		
Non-irradiated poles	1.08 \pm 0.34 b	(9)		
			1.26 \pm 0.42 a	(9)

Slower poles, the slower of the two poles in each cell. Faster poles, the faster of the two poles in each cell.

n, sample size.

*Values in a column that are not followed by the same letter are significantly different by the two-sample *t*-test ($P < 0.05$). All possible, pairwise comparisons were made.

ferent rates, on average, was at first troublesome because we had to select centrosome-targeted cells for inclusion in the study on the basis of the slow rate of movement of the irradiated pole. To demonstrate that this potentially biased selection criterion could not have generated the effects we are attributing to centrosome targeting, we conducted further analyses. Firstly, we constructed artificial categories of unusually “slow” and “fast” poles from the pool of 50 poles in the non-irradiated and cytoplasm-irradiated cells to see if such “slow” poles are naturally and commonly paired with such “fast” poles and vice versa (Table 2). No such pairing was found: the sisters of the “slow” poles, the sisters of the “fast” poles and a third category of “all poles in other cells” all moved at similar average rates that were not significantly different ($P = 0.42$) from each other. In fact, in only two of these 25 control cells did a “slow” and a “fast” pole occur together. Moreover, the centrosome-targeted poles moved at only one-half the rate of even the selected “slow” poles ($P = < 0.0001$), suggesting that there does not exist among these control cells a sizeable subset of naturally slow-moving poles that could account for the observed deceleration of centrosome-targeted poles. Interestingly, the non-irradiated poles in centrosome-targeted cells were accelerated to a rate at least as high as the rate of the selected “fast” poles (Table 2), indicating that this was a very marked acceleration indeed and that most spindle poles have the capability to move faster than they do when both asters are left fully active. Secondly, we conducted another type of irradiated-control experiment in which the aster was intentionally irradiated near (about 1.4 μm from), instead of at, the estimated position of the centrosome (Table 3). When the centrosome was purposely, but barely, missed in this manner, there was only a small, statistically nonsignificant ($P = 0.24$) difference in rates of movement between the irradiated and the non-irradiated poles. Because we never saw a marked effect of such aster irradiation on the rate of pole movement, and to avoid unnecessary biasing of the results, we did not apply a rate

Table 2. Rates of pole movement among various categories of spindle poles in living PtK₂ cells during the first 4 min of anaphase B

Category	Rate ($\mu\text{m}/\text{min} \pm \text{s.d.}$)	(n)
Irradiated poles	0.22 \pm 0.11 a*	(9)
“Slow” poles	0.43 \pm 0.05 b	(8)
Sisters of “slow” poles	0.74 \pm 0.21 c	(8)
Non-irradiated poles	1.08 \pm 0.34 d	(9)
“Fast” poles	1.04 \pm 0.11 d	(8)
Sisters of “fast” poles	0.66 \pm 0.20 c	(8)
All poles in other cells†	0.69 \pm 0.10 c	(22)

“Slow” poles, spindle poles, in non-irradiated and cytoplasm-irradiated cells, that moved at $< 0.50 \mu\text{m}/\text{min}$.

“Fast” poles, spindle poles, in non-irradiated and cytoplasm-irradiated cells, that moved at $> 0.90 \mu\text{m}/\text{min}$.

Non-irradiated poles are the sister poles of the irradiated poles.

*Values not followed by the same letter are significantly different by the two-sample *t*-test ($P < 0.05$). All possible, pairwise comparisons were made. n, sample size.

†Includes only poles in non-irradiated and cytoplasm-irradiated cells having neither a “slow” pole nor a “fast” pole.

criterion to these poles but included all cells that met the morphological and experimental criteria. Moreover, all of these aster-irradiated poles moved faster than did the fastest irradiated pole in the centrosome-targeted cells. Taken together, these data (Tables 2 and 3) show that the results we obtained with centrosome-targeted cells must have been due to irradiation effects and not to artefacts created by selecting, for the data set, only those cells whose irradiated poles were greatly decelerated by natural causes. The results with aster-irradiated controls strongly suggest also that a non-target effect, such as a gelation of astral cytoplasm that would immobilize the irradiated pole and prevent its being pushed along by the elongating spindle, did not occur in the centrosome-targeted cells.

To address further the possibility that the effects of centrosome targeting may have been caused by a gelation of astral cytoplasm, we carefully reexamined the videotape sequences twice for any indication of visible differences between the irradiated and the non-irradiated poles in the centrosome-targeted cells. None was found. No visible laser lesions were ever seen, and Brownian motion was seen clearly and was similarly common in both astral regions in each cell.

Tubulin immunocytochemistry

To detect effects of irradiation on astral MTs, we visualized MTs with tubulin immunofluorescence and confocal microscopy (Fig. 5). We found that targeting the centrosome usually eliminated its associated MT staining (Fig. 5c). Furthermore, in all of the five stained cells in which the astral MTs were visualized well enough to make a reliable assessment, centrosome targeting decreased the astral MT array relative to the non-irradiated sister pole, within 1-2 min (Fig. 5b, c). In contrast, irradiation of one aster, near the estimated location of the centrosome, had no discernible effect on the array of MTs in either aster (Fig. 5d). None of the irradiations appeared to diminish the interzone MTs in the spindle, although this aspect was often hard to assess fully in early anaphase B spindles because the chro-

mosomes partially absorbed the fluorescence in the interzone (Fig. 5b-d). In mid-to-late anaphase B cells that had been centrosome-targeted earlier, the mid-zone staining was not visibly diminished (not illustrated). Spindle MTs were never seen to be extended into the astral region of centrosome-targeted poles, confirming that the spindle did not elongate, under its own power, farther or faster than the position and arrangement of the daughter chromosome clusters had led us to assume during analysis of the videotape sequences.

DISCUSSION

Targeting of one centrosome at early anaphase B in living PtK₂ cells almost stopped the movement of the irradiated pole for 4 min, whereas the movement of the non-irradiated pole in the same cell was accelerated. This combination of deceleration and acceleration resulted in a remarkable five-fold difference in the rates of movement of the two poles of the same mitotic apparatus. These results appear to be consistent with either of two basic interpretations. According to one interpretation, the asters would not generate a pulling force, and targeting of one centrosome would somehow anchor and immobilize that pole. The spindle, being unaffected, would continue to elongate under its own power at an essentially normal rate, pushing against the anchored, irradiated pole and thereby accelerating the movement of the non-irradiated pole. Alternatively, during normal anaphase B, the asters would be pulling on the spindle poles, putting the spindle under tension. Consequently, when the MTs of one aster are diminished by irradiation, its pulling force would be so impaired that forward movement of that spindle pole would almost cease. The undamaged aster, on the other hand, would now be free of the full restraining force that was formerly produced by the damaged aster and transmitted through the spindle, and would now accelerate under its own power to a higher than normal rate of movement. Meanwhile, the spindle would elongate on its own at an essentially normal rate, thus preventing the accelerating, non-irradiated pole from dragging the entire mitotic apparatus with it. The observed acceleration of the non-irradiated pole would indicate that most spindle poles are apparently capable of moving faster than they do when both asters are left fully active, and it would be this capability that would put the spindle under tension in non-irradiated cells. A spindle under tension would, necessarily, produce counterforces that would govern the rate at which the asters would be permitted to separate the spindle poles.

The key point that can distinguish between these two interpretations is whether or not the irradiated pole remains mobile. Were the irradiated pole to remain mobile and the asters unable to pull, then spindle pushing forces would be expressed equally at both poles as equivalent rates of pole movement. But, if the asters do pull on the spindle poles, then the results that we actually observed would be predicted. Apart from interpreting the results themselves as evidence that the irradiated pole was immobile, we have been unable to identify any independent evidence or documentable argument to support such an assumption. But, there are several reasons to believe that the irradiated pole

Table 3. Effects of UV-laser microbeam irradiation, either at (centrosome-targeted) or near (aster-irradiated) the estimated position of the centrosome, on the subsequent rates of spindle pole movement, in living PtK₂ cells during the first 4 min of anaphase B

Category	Pole movement ($\mu\text{m}/\text{min} \pm \text{s.d.}$)	(<i>n</i>)
Aster-irradiated cells		
Irradiated poles	0.53 \pm 0.09 a*	(5)
Non-irradiated poles	0.64 \pm 0.17 a	(5)
Centrosome-targeted cells		
Irradiated poles	0.22 \pm 0.11 b	(9)
Non-irradiated poles	1.08 \pm 0.34 c	(9)

Aster-irradiated cells were purposely irradiated in one astral region, about 1.4 μm from the estimated position of the centrosome, as another type of irradiated control.

*Values not followed by the same letter are significantly different by the two-sample *t*-test ($P < 0.05$). All possible, pairwise comparisons were made.

n, sample size.

remained mobile, and was not anchored by gelation or coagulation of the astral cytoplasm. Firstly, it is characteristic of laser microbeam microsurgery that the direct effects of irradiation are confined to a remarkably small target area (Berns et al., 1981). In the present experiments, this area would be no more than 0.3 μm in diameter. It would be

extremely uncharacteristic in such experiments for the effect to spread over an area as large as the entire astral region. Secondly, by using a low level of energy per pulse and spacing the pulses 0.5-1.0 s apart, we avoided the production of the visible "laser lesions" that occur when laser energy is converted to heat that coagulates a very limited

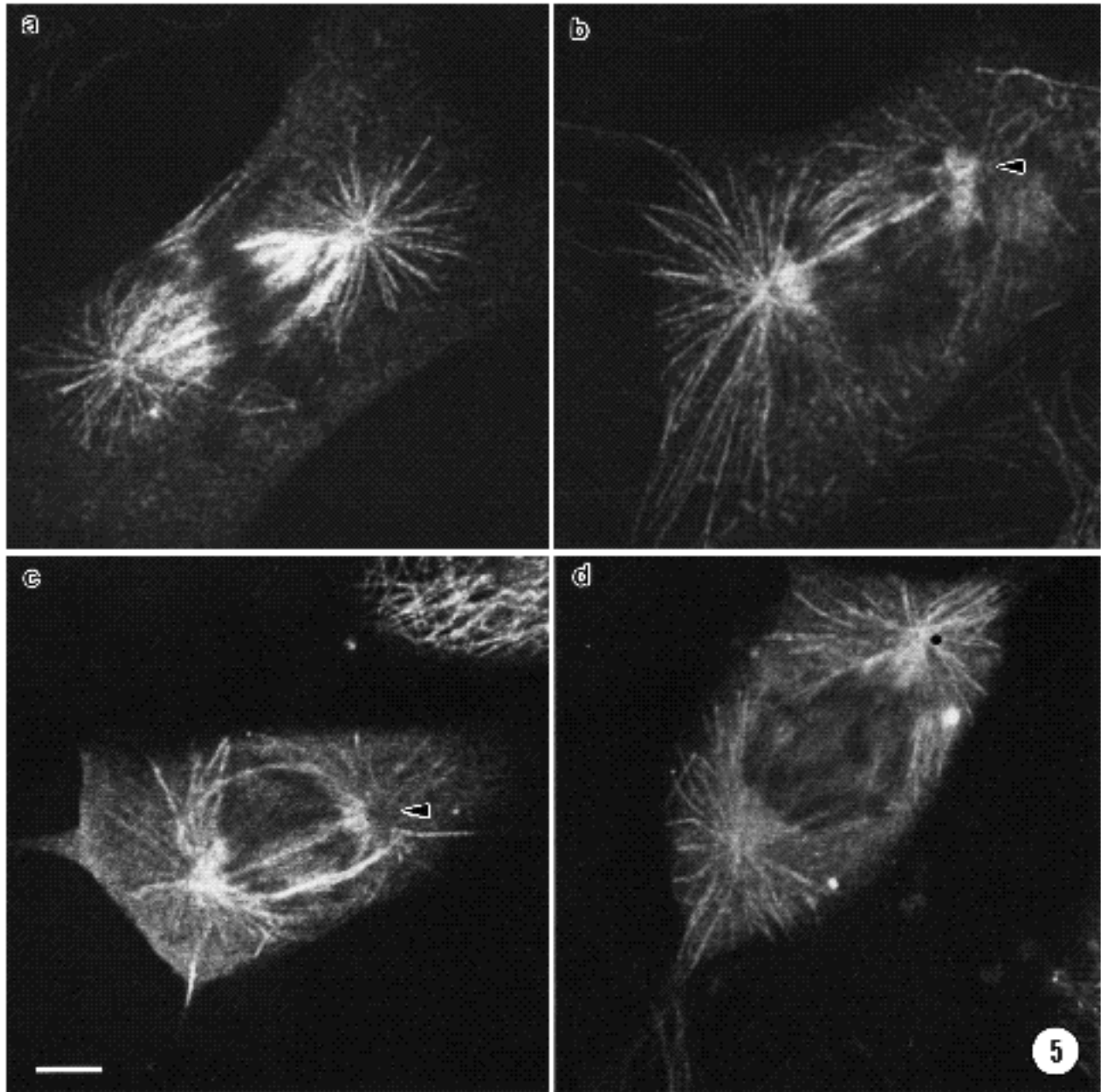


Fig. 5. Confocal micrographs of PtK₂ cells fixed during mitosis and processed for tubulin immunofluorescence. The cell in (a) is at early anaphase A; the others, early anaphase B. (a) A non-irradiated control. Both of the spindle poles are associated with a well-developed array of astral MTs. The chromosomes absorbed the fluorescence from spindle MTs, producing a dark central region in the spindle. (b) Fixed about 1.5 min after centrosome targeting (at the arrowhead). Numerous astral MTs are associated with and convergent at the non-irradiated centrosome, whereas relatively few are associated with the targeted centrosome and some do not converge at it. (c) Centrosome-targeted, also about 1.5 min before fixation. In this case too, the array of astral MTs has been diminished and MT staining has been eliminated in a large area centered on the centrosomal region (arrowhead). (d) An aster-irradiated control that was irradiated at the black circle near the upper centrosome and fixed about 1.5 min later. A complete array of astral MTs is present at both spindle poles. Bar, 5 μm .

area (0.25-1.0 μm diameter) of the target (Berns et al., 1981). Under these experimental conditions, it seems highly unlikely that the entire astral region would be coagulated by the irradiation. Thirdly, if there were a gelation of cytoskeletal elements in the astral region, then one might expect to see this artefactual condition reflected as a detectable reduction in Brownian motion in the aster of the targeted centrosome. Although the videotapes were carefully examined, no difference in such organelle motion was apparent in the asters of irradiated versus non-irradiated poles. Thus, the astral cytoplasm did not show visible signs of being gelled as a result of centrosome targeting. Fourthly, it could be postulated that destruction of MTs in the centrosome-targeted aster may have caused the cytokeratin cytoskeleton to collapse around the irradiated pole and form a cage that immobilized it. But, this postulate seems highly unlikely, because such a collapse does not occur in either epithelial cells in general (Klymkowsky et al., 1989) or in PtK₂ cells in particular (Osburn et al., 1980). And fifthly, if irradiation in the vicinity of the spindle pole region were to render the pole immobile as a routine side effect, then both the control aster irradiations that were intentionally targeted beside the centrosome (Table 3) and the unintentional, apparent misses of the centrosome that occurred in 20% of the otherwise well-executed, centrosome-targeted irradiations should also have nearly stopped those irradiated poles and accelerated (due to the putative spindle pushing forces) the non-irradiated poles. But they had little or no effect on the movement of either of the poles. Instead, deceleration of the irradiated pole and acceleration of the non-irradiated pole occurred only, and frequently, when the centrosome was purposely targeted, indicating that these effects were extremely site-specific. These results imply strongly that the centrosome-targeted poles were still moveable. Moreover, the effects of irradiation on astral MTs suggested that the irradiated poles had a damaged motility mechanism, namely, the aster.

Another possible explanation of the results was addressed experimentally. The data in Table 2 show that it is virtually impossible for the cell selection criteria to have created the observed effects artificially.

When all of the evidence and related, pertinent information discussed above is taken into consideration, it appears most likely that the irradiated poles did remain mobile. Thus, we infer that during anaphase B in PtK cells (1) the asters pull on the spindle poles, putting the spindle under tension, (2) the spindle produces counterforces that resist the pull of the asters, and (3) the spindle can produce a pushing force in vivo that can elongate the spindle essentially as fast as the normal in vivo rate, when the pulling force of one of the asters is impaired.

Our tubulin immunofluorescence results showed that laser microbeam-induced inhibition of spindle pole movement was associated with spatially selective diminution of astral MTs at the irradiated poles. This apparently centrosome-specific effect was probably due to laser damage to the pericentriolar material, impairing its ability to nucleate MTs, but without direct evidence of centrosome damage we cannot draw a definite conclusion on this point. Neither the spindle MTs nor the MTs of the non-irradiated aster appeared to be substantially affected. Moreover, irra-

diation of the aster, near but not at the estimated position of the centrosome, did not diminish the astral MTs, presumably because the pericentriolar material was not damaged, thus leaving intact the centrosome's MT-nucleating ability. These results suggest, but do not prove, that astral MTs could be part of the astral force-generating mechanism. Such a role for astral MTs would be consistent with the results of a number of other studies (Aist et al., 1991; Berlin et al., 1990; Hiramoto et al., 1986; Hyman, 1989; Koonce et al., 1984) in which destruction of astral MTs affected the direction or rate of astral or spindle pole motility. Additional studies would be required, however, before such a role for astral MTs per se could be established for PtK cells.

Whereas Kronebusch and Borisy (1982) severed the central spindle in PtK cells and observed a marked acceleration in the separation rates of the two poles, we damaged one aster and observed a marked deceleration in the rate of movement of the damaged pole. As they pointed out a decade ago: "Experiments have yet to be performed which specifically interrupt astral MTs.... In order to fully embrace this attractive mechanism, future experiments must show that disruption of astral MTs stops anaphase B movements in cases where disruption of interzone MTs does not" (Kronebusch and Borisy, 1982). With the present results, substantial experimental evidence is now available to suggest that during anaphase B in living PtK cells, the asters pull on the spindle poles and the central spindle acts as a governor that limits the rate of pole separation.

Our results bring to a total of four the number of species in which in vivo experiments on both asters and spindles have produced results and conclusions that are similar to those we have presented here. Hiramoto et al. (1986) used spatially selective, near-UV irradiation to activate asters and spindles in colcemid-treated sand dollar eggs and found that the spindle was elongated only when the asters were activated. Experiments with the filamentous fungus, *Nectria haematococca*, in which laser microbeams were used to inactivate spindles (Aist and Berns, 1981) and asters (Aist et al., 1991), demonstrated astral pulling forces and spindle counterforces. And recent genetic experiments with mutants of the yeast fungus, *Saccharomyces cerevisiae*, have confirmed this role of the asters: a mutant without asters produced only a short, thick, unelongated spindle (Berlin et al., 1990), and another mutant, this one without one of the half-spindles but with two full sets of astral MTs, managed to fully separate the two spindle pole bodies without a central spindle (Winey et al., 1991). Taken together with the experimental evidence for astral pulling in other animal species (Bergan, 1960; Daub and Hauser, 1988; Hiramoto and Nakano, 1988; Lutz et al., 1988), the evidence is now substantial that astral pulling may be both common and widespread, although not necessarily universal, among species with astral mitosis. Since several of these studies (Aist et al., 1991; Hiramoto et al. 1986; Berlin et al., 1990) have provided direct, experimental evidence indicating that astral pulling helps to elongate the central spindle during anaphase B in vivo, it would seem appropriate to incorporate such forces, along with the accompanying spindle counterforces, into comprehensive models of the forces involved in astral mitosis.

Such models have already been presented for both fungal (Aist and Berns, 1981) and animal (Kronebusch and Borisy, 1982) cells. Interactions of astral MTs with either the plasma membrane or a filamentous cytoskeleton or matrix, such as F-actin, are possible candidates for force generation. Structural and experimental evidence suggestive of such putative interactions has been published (Aist and Bayles, 1991; Aist and Berns, 1981; Daub and Hauser, 1986; Hyman, 1989; Lutz et al., 1988), although the micro-manipulation experiments of Carlson (1952) and the ultrastructural analysis of Jensen et al. (1991) have raised serious doubts as to the functional significance of astral MT-plasma membrane interactions in mitotic force generation in some cases. Another possibility for the mechanism of astral pulling in animal cells is that astral MTs from each centrosome that are oriented back toward the opposite aster may interact laterally to produce sliding forces that would be translated to the centrosomes as forces pulling on the spindle poles (Bergan, 1960; Sheldon and Wadsworth, 1990). Yet a third possibility was outlined in general terms by Kronebusch and Borisy (1982) and in detail by Enos and Morris (1990): an anterograde MT motor, such as kinesin, if attached to the spindle pole body (or the centrosomes of animal cells), could reel in the astral MTs and generate a pulling force directed toward distal sites of astral MT anchorage. This suggestion seems especially attractive for PtK cells, since recent observations by Sheldon and Wadsworth (1990) showed that astral MTs elongate rapidly at their distal (plus) ends during anaphase B, even as the centrosome is drawing closer to the cell periphery. Thus, some mechanism to shorten the astral MTs at the centrosome appears to be necessary. Moreover, Spurck et al. (1990) showed that when astral MTs in PtK cells are cut by a UV microbeam, their centrosome-attached pieces shorten toward the centrosome, a result that is consistent with the postulated reeling-in mechanism. Furthermore, Neighbors et al. (1988) found that kinesin was localized only in the centrosomes of mitotic PtK cells, where it could be involved in reeling in the astral MTs. It is just such a mechanism – one that requires dynamic astral MTs in order to operate – that can explain why taxol treatment blocked pole-pole separation in PtK cells even though continuity of the central spindle was lost (Amin-Hanjani and Wadsworth, 1991).

Regarding the apparent counterforces in the spindle, they are most likely generated by microtubule-associated proteins (MAPs), as discussed by Jensen et al. (1991). In the presence of spindle tension induced by the pulling force of the asters, either structural MAPs such as MAP2, or motor MAPs such as dynein or kinesin, could, if located in the spindle, produce a net counterforce. One candidate molecule, a kinesin-like protein (Nislow et al., 1992), has recently been shown to be localized exclusively in the central overlap region of the spindle interzone during anaphase B in PtK₁ cells (Nislow et al., 1990); this region is precisely where the apparent counterforces would most likely be generated. Interestingly, injection of antibodies to this MAP did not prevent anaphase B (Nislow et al., 1990), an expected result if this MAP can function normally to slow spindle pole separation when the spindle is under aster-generated tension.

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