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Differential staining of actin in metaphase spindles with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin and fluorescent DNase: Is actin involved in chromosomal movement?

(phallacidin/phalloidin/mitosis/fluorescence)

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ABSTRACT **The distribution and polymerization state of actin in metaphase rat kangaroo cells was studied by fluorescence microscopy. Formaldehyde-fixed, acetone-extracted cells were labeled with either of two types of actin probes. The first, 7-nitrobenz-2-oxa-1,3-diazole-phallacidin, has high affinity for F actin and does not bind monomeric G actin. The second was a conjugate of DNase I labeled with either tetramethylrhodamine or fluorescein. DNase binds with high affinity to G actin and with lesser affinity to F actin. The polymerization state of actin was deduced by comparing the fluorescence distribution of the phallacidin derivative with that of the fluorescent DNase. The results indicate that the pole-to-chromosome region of the metaphase spindle contains G actin but little if any conventional F actin. F actin is found concentrated in a diffuse distribution outside the spindle region in metaphase cells and returns to the interzone area between the chromosomes by early telophase. These results exclude spindle models for chromosomal movement that require more than about five F actin filaments per chromosome, support the hypothesis that F actin is involved in force generation for cell cleavage, and are not inconsistent with the possibility that actin outside the spindle may be involved in chromosomal movement.**

The mechanism responsible for chromosomal movement during mitosis has not been established. Most investigators have attempted to show that either actin or tubulin in the mitotic spindle is involved in the force production required for chromosomal movement (1–5). The evidence for and against each protein includes biochemical, electron microscopic, and fluorescence microscopic results.

Strong evidence exists that tubulin is a spindle component, although there remains some uncertainty regarding the amount present (5–7). Electron micrographs show microtubules inserted into chromosomal kinetochores, structures thought to be tubulin-nucleation centers (5). Each chromosome has one such site of attachment. This has led to speculation that tubulin participates directly in generating force for chromosomal movement (for review, see ref. 5).

Biochemical evidence shows that actin is a component of nonmuscle cells (8). However, it is difficult to determine the subcellular location of actin by biochemical techniques. Some experiments have indicated the presence of actin in nuclear fractions (9, 10), but others have indicated that actin in these fractions may be a cytoplasmic contaminant. By using isolated nuclei, Comings and Harris found that actin and myosin constitute at most a very small fraction of nonhistone proteins (6).

Electron microscopy has provided evidence for the existence of spindle actin (11–14). These experiments have identified actin by the appearance of characteristic arrowhead complexes formed from the binding of heavy meromyosin (HMM) or its nuclease S1 subfragment to actin filaments (15). Glycerination has been a standard step in the preparation of these specimens, and it has been suggested that cytoplasmic actin may contaminate the spindle during this procedure (16). It has been reported (17) that the glycerination procedures (2, 18) used in these experiments permit the redistribution of tropomyosin and myosin and change the antigenic properties of the mitotic spindle toward myosin antibody in rat kangaroo cells. Evidence against cytoplasmic actin contamination of the spindle comes from experiments in which glutaraldehyde fixation, which should prevent gross transfer of actin, was used to fix specimens. These experiments also reported the presence of actin in mitotic spindles (18–20). Further complications arise from the report of actin filaments in close proximity to microtubules (18). This observation suggests that actin filaments in the spindle might be obscured by microtubules, thus accounting for the apparent lack of spindle actin filaments as reported (1, 21, 22).

The fluorescence data regarding metaphase spindle actin are conflicting. In some experiments (23–29), fluorescent anti-actin antibody, HMM, or nuclease S1 applied to cells appear localized in filamentous structures in the metaphase spindles. However, results with affinity-purified anti-actin antibody or myosin fragments and fixation procedures avoiding glycerination indicate no increased actin concentration in the spindle (17).

In summary, experiments to date conclusively demonstrate tubulin as a spindle component whereas the actin results are open to dispute. The studies involving fluorescence labeling of metaphase spindle actin are the most positive yet the most controversial of all the actin localization results.

Localization alone is not enough to establish function of actin in chromosomal movement. Actin-based models of such movement generally postulate some form of actin sliding filament contraction as the force generator. These models require that spindle actin exists in a filamentous form. Hence, not only the localization but the form of actin in the spindle is important.

Our approach to the question of actin involvement in chromosomal movement uses a highly specific fluorescent F-actin label—7-nitrobenz-2-oxa-1,3-diazole-phallacidin (NBD-Ph) (30–33). NBD-Ph binds to F-actin polymers but not to monomeric G actin. It produces no detectable nonspecific staining of cellular components. To complement NBD-Ph staining, we also produced and characterized both rhodamine (Rh) and fluorescein (Fl) conjugates of DNase I (Rh-DNase I and Fl-DNase I). These probes preferentially bind to G actin but also bind to F actin with a lower affinity (34). Use of these two types of probes, one highly specific for F actin and the other preferential for G actin, allows us to deduce the polymerization state of cel-

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Abbreviations: NBD-Ph, 7-nitrobenz-2-oxa-1,3-diazole-phallacidin; HMM, heavy meromyosin; Rh, rhodamine; Fl, fluorescein.

lular actin from the fluorescence. In addition, results obtained with these probes provide an independent test of results obtained with anti-actin antibody or fluorescent HMM.

In this paper, we present results of labeling metaphase PtK_2 cells with NBD-Ph and fluorescent DNase. To show that our fixation methods produce results consistent with earlier ones, we include results of labeling metaphase cells with fluorescent HMM or antitubulin. Our data show the presence of G actin in the spindle, place an upper bound on the amount of F actin in the spindle, and exclude any actin sliding filament structure within the mitotic spindle that involves more than about five F-actin filaments per chromosome.

MATERIALS AND METHODS

The preparation and characterization of NBD-Ph have been described (31).

Electrophoretically purified DNase I (bovine pancreas, Sigma) was conjugated with tetramethylrhodamine isothiocyanate or fluorescein isothiocyanate. The procedure was essentially as described (35); modifications included the use of celite-absorbed dye, rather than powdered dye, in the reaction mixture and of a linear NaCl gradient, rather than a step gradient, to elute the purified product from the DEAE-cellulose column. The purified conjugates, Rh-DNase and Fl-DNase, were assayed for DNase activity as described (36) and for inhibition of DNase activity by actin as described (37).

Rat kangaroo cells (PtK_2; American Type Culture Collection) on glass coverslips were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum (GIBCO) and used for labeling at 70–80% confluency. In preliminary experiments, cells were prepared for labeling by using the Triton lysis method with tubulin (27), the formalin fixation method (23), or fixation in 5% formaldehyde in phosphate-buffered saline for 10–15 min, followed by washing three times in the same saline and acetone extraction for 4–6 min at −20°C. All three procedures gave equivalent results; the data presented here were obtained by using the formaldehyde fixation procedure.

After fixation and extraction, the cells were incubated with 200 μl of 150 nM NBD-Ph for 20 min at 22°C, Fl-HMM at 10–20 μg/ml for 40 min at 37°C, or Fl-DNase or Rh-DNase at 40 μg/ml for 40 min at 22°C. Cells were incubated with affinity-purified rabbit antitubulin antibody for 40 min at 37°C, washed three times in phosphate-buffered saline, and finally labeled for 40 min at 37°C with rhodamine goat anti-rabbit antibody (Cappel Laboratories, Cochranville PA), which had been purified on DEAE-Sephadex to remove overcharged molecules.

RESULTS

Preliminary experiments showed that nonspecific staining of PtK_2 cells by fluorescent DNase I conjugates increased when the dye/protein ratio was >1. Nonspecific nuclear staining was particularly evident with Rh-DNase conjugates having larger dye/protein ratios. The results reported here were obtained with conjugates having dye/protein ratios of 0.8 (Rh-DNase) or 1.0 (Fl-DNase). The DNase activity of these conjugates was

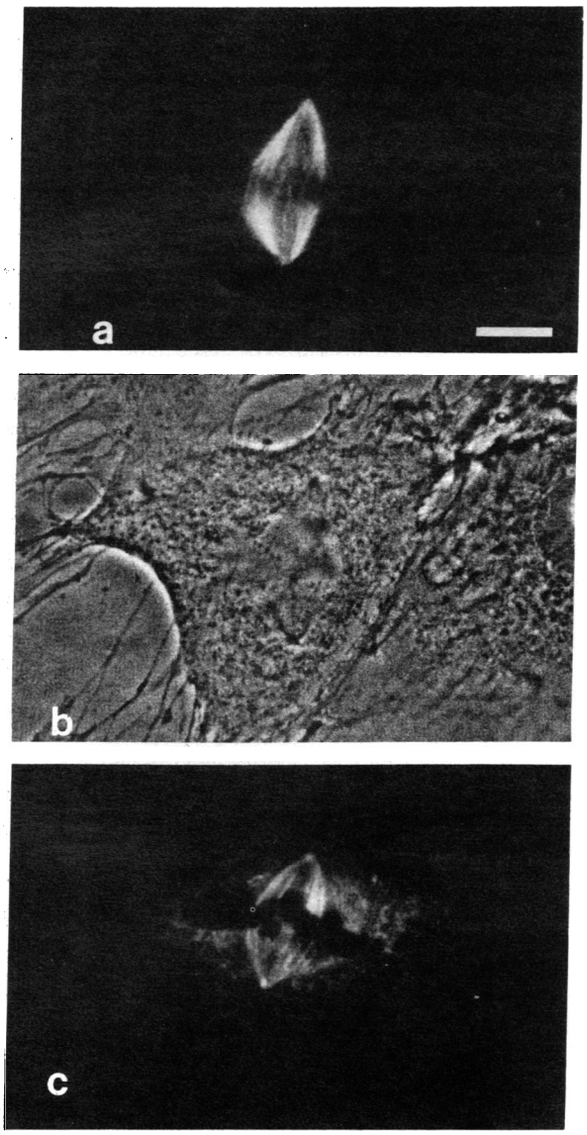

FIG. 1. Metaphase rat kangaroo cells. (*a*) Spindles as seen by indirect immunofluorescence using affinity-purified rabbit antitubulin antibody and rhodamine-labeled goat anti-rabbit antibody. (*b*) Phase-contrast image of *a*. (*c*) Spindle structures as seen with Fl-HMM. (Bar = 8 μm; ×1200.)

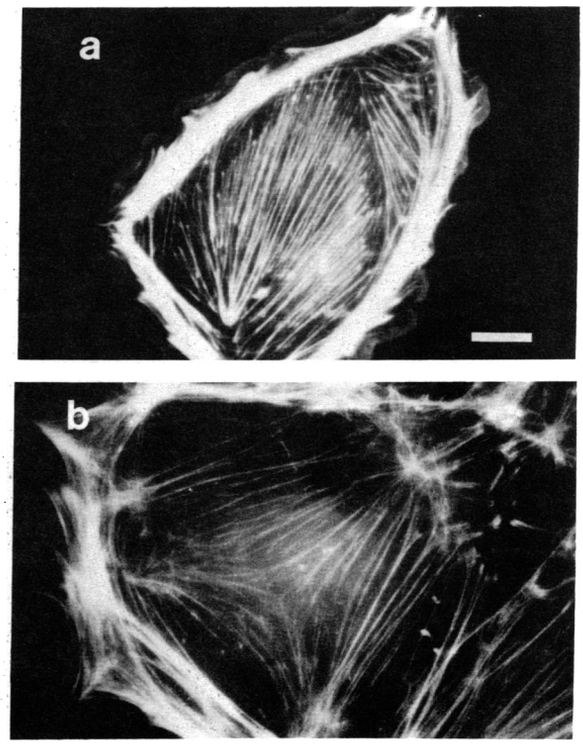

FIG. 2. Interphase rat kangaroo cells. (*a*) NBD-Ph fluorescence. (*b*) Fl-DNase fluorescence. (Bar = 8 μm; ×900.)

≈44% (Rh-DNase) or 70% (Fl-DNase) of the activity of the unlabeled starting DNase I. When measured in the presence of equimolar actin, the DNase activities of Rh-DNase, Fl-DNase, and unlabeled DNase decreased to 39%, 60%, and 26% of the respective uninhibited rates.

Fig. 1 *a* and *b* shows Rh-antitubulin indirect immunofluorescence and phase-contrast micrographs of a mitotic spindle in a metaphase PtK_2 cell. The correspondence between the fluorescent fibrous microtubules and the phase dark poles with associated spindles is evident. Double-labeling experiments (not shown) using Rh-antitubulin confirmed the existence of spindle microtubules in metaphase cells that were coincubated with Fl-DNase or NBD-Ph. The fluorescence staining patterns of the Fl-DNase and NBD-Ph in the double-labeled cells were identical to the patterns observed in cells stained with only Fl-DNase or NBD-Ph as described below.

Fig. 1*c* shows Fl-HMM staining in a filamentous pattern in the metaphase spindle. This result duplicates earlier Fl-HMM labeling of mitotic spindles (23, 26) and shows that our fixation procedure allows staining of these filamentous structures. The chromosomes appear as distinct shadows surrounded by a diffuse fluorescent background. Note that fibrous structures do not cross the chromosomal bodies.

In interphase PtK_2 cells labeled with either NBD-Ph (Fig. 2*a*) or Fl-DNase (Fig. 2*b*), most of the fluorescence is associated with stress fibers bordered by a thick rim of actin microfilaments. The result with Fl-DNase confirms the work of Wang and Goldberg (35) and shows that fluorescent DNase will stain stress fibers and microfilaments. In double-labeling experiments on interphase cells, Rh-DNase and NBD-Ph colabeled filaments (results not shown). Although nuclear staining with fluorescent DNase was generally low level, it was almost always greater than that observed with NBD-Ph. NBD-Ph staining of nuclei in interphase cells was never large enough to permit the identification of nuclei by fluorescence localization. In all cell types we have stained with NBD-Ph, including L6 myoblasts; NRK, human, and mouse fibroblasts; and BHK, we have seen no nuclear fluorescence in spread cells.

NBD-Ph staining of metaphase cells exhibits the characteristic fluorescence distribution shown in Fig. 3*a*. The area between the chromosomes and the poles is distinguished by a lack of fluorescent staining. The excluded area encompasses the entire spindle; compare the accompanying phase-contrast photograph (Fig. 3*b*). The spindle fluorescence is so low in this central area that perpendicularly oriented structures are visible from basal regions of the cell. This photograph is typical of meta-

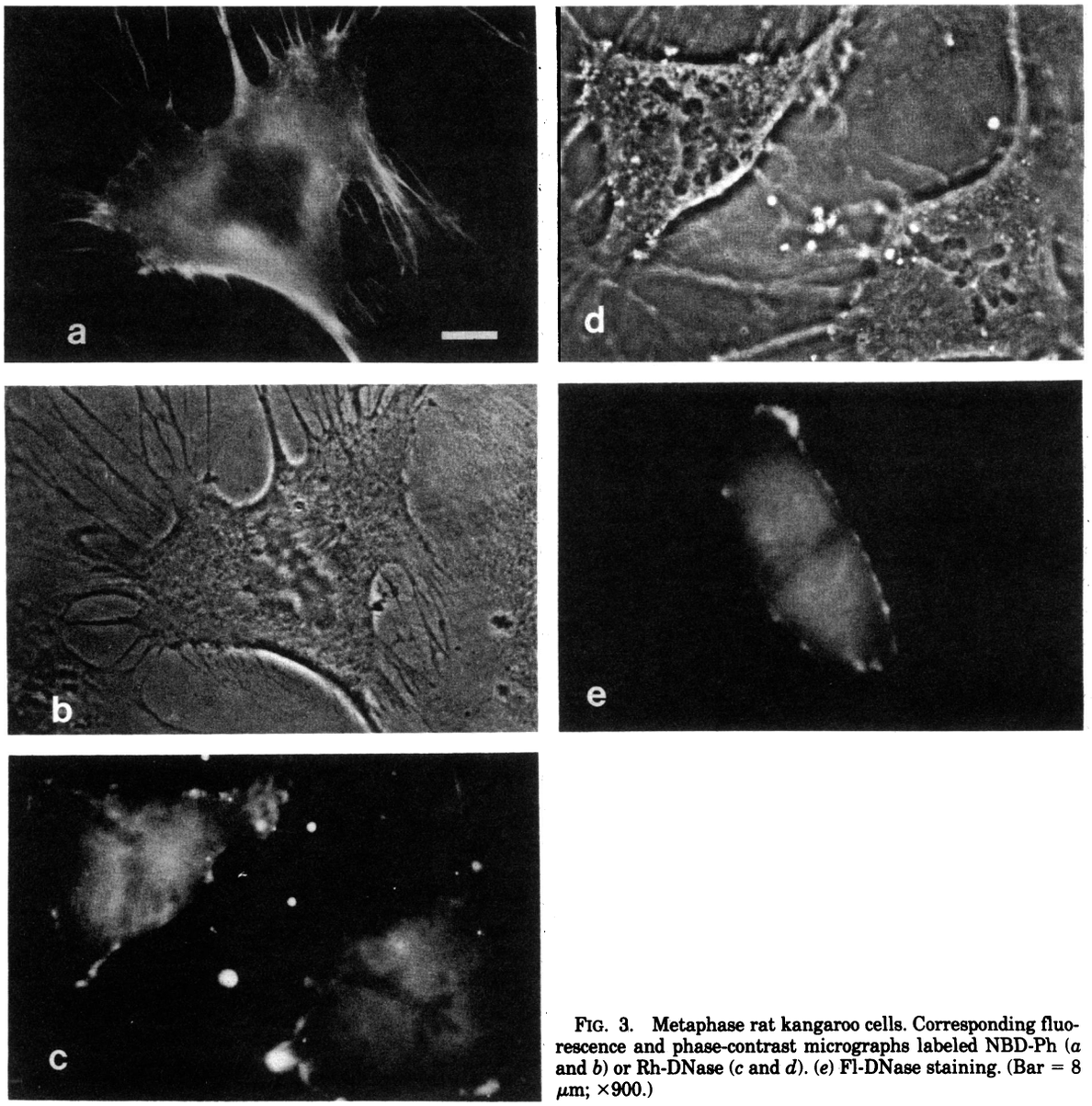

FIG. 3. Metaphase rat kangaroo cells. Corresponding fluorescence and phase-contrast micrographs labeled NBD-Ph (*a* and *b*) or Rh-DNase (*c* and *d*). (*e*) Fl-DNase staining. (Bar = 8 μm; ×900.)

phase staining by NBD-Ph. In no cases did we see fibrous structures emanating from the polar region.

In contrast to the staining pattern of NBD-Ph, Fl-DNase and Rh-DNase extensively stained the pole-to-chromosome region (Fig. 3 *c* and *e*). Unlike Fl-HMM, this staining is totally diffuse. We were unable to find fibrous structures between poles and chromosomes in metaphase cells using these probes. The fluorescent DNase conjugates were similar to NBD-Ph in that all three probes labeled retraction fibers, processes extending from the cell periphery. Preincubation of the Fl-DNase with a 20-fold molar excess of actin blocked virtually all staining in metaphase cells, indicating a lack of nonspecific labeling (not shown).

The final pair of photographs (Fig. 4 *a* and *b*) shows NBD-Ph staining and phase of the interzonal cleavage region in late anaphase or early telophase cells. In an attempt to quantitate the relative amounts of F actin in the cleavage region and the laterally adjacent cytoplasm, the grain density was measured on the negative film image of this cell. After correction for the characteristic response of the film, the fluorescence intensity of the cleavage region was found to be four times greater than that of the laterally adjacent cytoplasm.

DISCUSSION

Previous studies of the distribution of actin in the mitotic spindle have been subject to two general criticisms. The first involves probe specificity. It has been suggested that antibodies and myosin fragments may bind nonspecifically to spindle components other than actin (5). There is also considerable evidence showing that myosin and tubulin bind to each other in stoichiometric ratios (38–40). The second criticism suggests possible actin redistribution during fixation, lysis, or labeling (5, 17, 22). Even if glutaraldehyde fixation is used, it is difficult to rule out protein rearrangement or denaturation in subsequent steps.

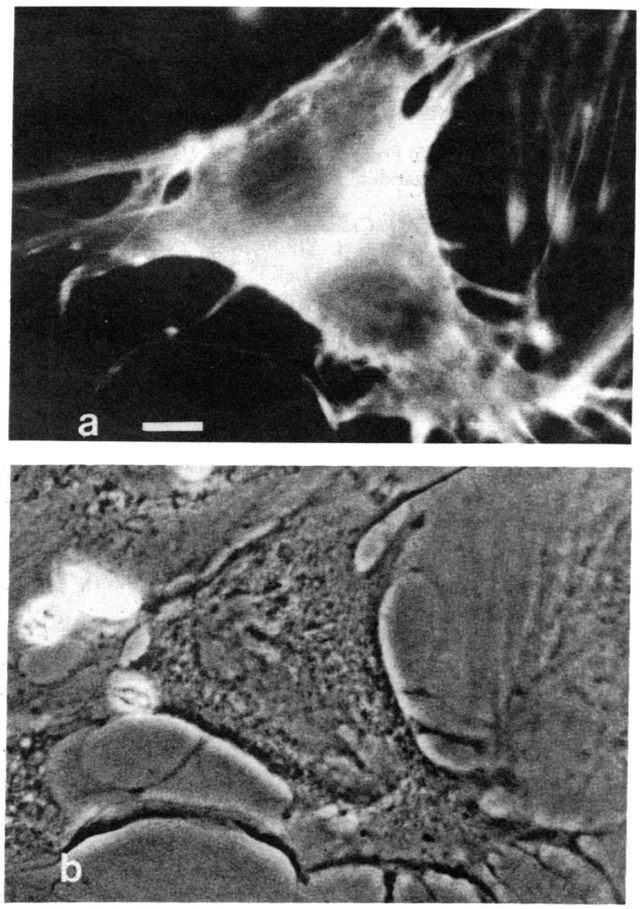

FIG. 4. Late anaphase or early telophase rat kangaroo cell. (*a*) NBD-Ph fluorescence and (*b*) phase-contrast images. The printing conditions of the fluorescence micrograph (*a*) are adjusted to highlight the fluorescence from the interzonal actin in the telophase cell. The fluorescence from the cell in the lower right corner of *b* is no longer visible under these conditions. (Bar = 6 μm; ×1200.)

The results presented here must be interpreted subject to these same criticisms. We have used two new probes to stain actin in metaphase cells. The first probe, NBD-Ph, is highly specific for F actin and shows exceptionally low levels of nonspecific staining (30, 31). In particular, nonspecific nuclear staining is not detected (30). The second probe, fluorescent DNase, has high affinity for G actin and lesser affinity for F actin (34, 35). As frequently observed with other Fl- and, particularly, Rh-conjugated probes, nonspecific staining with fluorescent DNase can be significant. By selecting conjugates of low dye/protein ratio, nonspecific staining with this probe can be reduced to negligible levels.

The criticism regarding actin redistribution during staining is particularly relevant for the two probes used in this study. Phallotoxins are known to stabilize F actin and enhance polymerization (41), and NBD-Ph will stabilize stress fibers against 0.6 M KI (31). These properties of phallotoxins suggest that actin filaments in the spindle should be stabilized by NBD-Ph and perhaps even enhanced by additional polymerization of G actin from neighboring cytoplasm. In view of these expectations, the complete lack of spindle fluorescence shown in Fig. 3*a* is particularly striking. Note that pretreatment with unlabeled HMM does not induce subsequent NBD-Ph staining in the spindle (results not shown). Hence HMM-induced polymerization of G actin, known to occur *in vitro* (42, 43), does not seem to occur in the spindle of formaldehyde-fixed cells.

DNase I is known to depolymerize F actin (34), so it might be argued that the fluorescent DNase conjugates disrupted any F actin located in the spindles. Although we cannot rule out this possibility, the observation that stress fibers and microfilaments remain intact after the same treatment (see Fig. 2*b*) argues against gross filament disruption by fluorescent DNase.

NBD-Ph is specific for polymerized actin and will not stain G actin. Fluorescent DNase stains both F actin and G actin. Due to the absence of spindle staining by NBD-Ph (see Fig. 3*a*) and the intense staining by fluorescent DNase (see Fig. 3 *c* and *e*) we conclude that the mitotic spindle region contains appreciable amounts of G actin but, at most, nondetectable amounts of F actin. Aubin *et al.* stained metaphase cells with both anti-actin antibody and fluorescent HMM and saw no structures resembling filaments in the spindle (17). Our results verify this observation and extend it by classifying the type of actin present in this region. The absence of NBD-Ph staining implies that the metaphase spindle lacks even significant amounts of diffuse oligomeric actin. Various other workers (23–29) have stained metaphase cells with anti-actin antibody or myosin fragments and observed fluorescence staining of fibrous structures in the spindle. It is important to note that our fixation procedures do allow Fl-HMM staining of fibrous spindle structures (see Fig. 1*c*). Thus, the absence of spindle staining in our NBD-Ph and DNase experiments cannot be attributed to extraction of spindle structure during our fixation procedure.

If the mitotic spindle does contain appreciable amounts of actin organized into fibrous structures, then either no binding sites for NBD-Ph or fluorescent DNase exist or nearly all the binding sites are blocked. An absence of binding sites would indicate that the spindle contains either an unusual actin molecule (17) or a typical actin molecule that forms atypical polymeric structures. Blockage of both NBD-Ph and fluorescent DNase would be required to occur at two different areas on the actin molecule because NBD-Ph will label cells after pretreat-

ment with fluorescent DNase (not shown). The binding site for DNase can be blocked by pretreatment of F actin with HMM (34). Pretreatment with unlabeled HMM does not prevent NBD-Ph staining of stress fibers (not shown), so a hypothetical spindle myosin could not account for lack of NBD-Ph staining.

As shown by the staining of cleavage furrow actin (see Fig. 4 *a* and *b*), NBD-Ph staining does reflect the redistribution of actin during the mitotic process. The distribution of fluorescence seen in this micrograph confirms the electron microscopic observations of Schroeder (1) that F actin appears in the interzone or cell cortex between the chromosomes by telophase. Because the enhancement of NBD-Ph fluorescence in the cleavage region over that in the laterally adjacent cytoplasm is so large (four times), it seems unlikely that it could be due to simply an increased cell thickness in the cleavage region, as has been suggested (25). Rather, there appears to be an actual increase in the concentration of F actin in this region.

The present experiments cannot exclude the possibility that the spindle contains F actin in amounts too small to be detected by fluorescence microscopy. According to Forer (5) and Nicklas (44), an actin myosin couple with only one actin filament per chromosome could provide sufficient force to drive chromosomal movement. If this assertion is correct, then the actin required to move a chromosome 10 μm would be $\approx$2000 actin molecules, or about 200 actins per micrometer. As F actin can bind as much as 1 mol of phallotoxin per mol of actin (41), a single chromosomal actin filament stained with NBD-Ph could bind as many as 200 fluorophores per micrometer. In our experience, this fluorophore density approximates the minimum required to produce sufficient contrast for visibility above cellular autofluorescence. Therefore, actin filament bundles containing, for example, five or more filaments per chromosome would certainly be detected in these experiments, although fewer filaments per bundle might go undetected.

A somewhat larger amount of F actin organized as single filaments dispersed in the spindle could escape detection against the background fluorescence outside the spindle. However, it is not known whether either such dispersed F-actin structures or bundles containing fewer than five filaments could develop force by sliding filament contraction. If they cannot, our results exclude actin-based sliding filament contraction within the spindle as the motive force for chromosomal movement.

These results do not preclude extraspindle actin-based models of chromosomal or centriolar movement. The dense network of F actin that surrounds the spindle may serve to anchor the spindle poles. The alignment of prophase chromosomes along the equator between the poles requires movements perpendicular to the axis of the spindle microtubules, and the force driving this spreading may orginate in the F-actin network surrounding the spindle. The mechanism of force generation for this movement, like the mechanism of polar migration, remains to be established.

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