

Immunofluorescent Localization of Cyclic Nucleotide-dependent Protein Kinases on the Mitotic Apparatus of Cultured Cells

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ABSTRACT Cyclic nucleotides and cyclic nucleotide-dependent protein kinases have been implicated in the regulation of cell motility and division, processes that depend on the cell cytoskeleton. To determine whether cyclic nucleotides or their kinases are physically associated with the cytoskeleton during cell division, fluorescently labeled antibodies directed against cyclic AMP, cyclic GMP, and the cyclic nucleotide-dependent protein kinases were used to localize these molecules in mitotic PtK₁ cells. Both the cyclic GMP-dependent protein kinase and the type II regulatory subunit of the cyclic AMP-dependent protein kinase were localized on the mitotic spindle. Throughout mitosis, their distribution closely resembled that of tubulin. Antibodies to cyclic AMP, cyclic GMP, and the type I regulatory and catalytic subunits of the cyclic AMP-dependent protein kinase did not label the mitotic apparatus. The association between specific components of the cyclic nucleotide system and the mitotic spindle suggests that cyclic nucleotide-dependent phosphorylation of spindle proteins, such as those of microtubules, may play a fundamental role in the regulation of spindle assembly and chromosome motion.

Three distinct fibrous systems have been identified in the cytoplasm of eukaryotic cells in culture: microtubules, microfilaments, and intermediate filaments. These cytoskeletal structures play a role in cell motility, the maintenance of cell shape, intracellular transport and secretion, and cell division (13). All three cytoskeletal systems undergo rapid rearrangement as cultured cells progress through the division cycle. The highly organized microfilament bundles and the arrays of individual and clustered intermediate filaments give way to much smaller aggregates as cells enter mitosis (7, 12). Microtubules, which exist during interphase as a network of long cytoplasmic fibers, disassemble and are replaced by the microtubules of the mitotic spindle before the movement of chromosomes during cell division (8, 35).

There is some evidence to suggest that cyclic nucleotides are involved in the regulation of microtubule assembly and in the rearrangement of other cytoskeletal fibers that occur as the cell undergoes morphological changes. Cyclic AMP (cAMP) affects

several behavioral characteristics of cells that depend on the cytoskeleton. These include the growth rate, motility, and cell morphology (16, 24). Cyclic nucleotide levels are known to fluctuate during the cell cycle. In many cell types, cAMP levels are depressed throughout mitosis (26). In Novikoff hepatoma cells, cyclic GMP (cGMP) levels rise as cAMP levels fall in cells entering mitosis—a trend that is reversed as the cells pass out of metaphase (39). The morphological changes that occur in fibroblasts after exposure to dibutyryl cAMP, i.e., flattening and elongation, are associated with a redistribution of the microtubules and microfilaments (38).

The only known mechanism of cyclic nucleotide action in the cell is the activation of specific protein kinases (14, 18, 19). The target proteins of cyclic nucleotide-dependent protein kinases are, by and large, unknown. However, certain regulatory molecules associated with microtubules are specifically phosphorylated *in vitro* by cAMP kinases (5, 21, 29, 30, 32). These observations, together with the effects of cyclic nucle-

tides on cell morphology and growth, suggest a fundamental relationship between the cytoskeleton and the cyclic nucleotide system.

Immunocytochemical studies with antibodies to cyclic nucleotides have demonstrated the specific tissue distribution of cyclic nucleotides and emphasized their possible role in cell regulation (33). Antibodies to components of the cytoskeleton, including microtubules and actomyosin cables, have been used to map the distribution of these structures in cultured nonmuscle cells (8, 20, 22). To better understand the role that cyclic nucleotides might play in the regulation of the cell cytoskeleton, we have begun to investigate whether the cyclic nucleotide-dependent protein kinases are physically associated with cytoskeletal structures, and whether these associations change as the structural proteins perform different functions during cell motility or cell division. We report here the use of fluorescence immunocytochemistry to localize cyclic nucleotides and the cyclic nucleotide-dependent protein kinases during mitosis in PtK₁ cells. We find that the cGMP-dependent protein kinase (cGMP-kinase) and the type II regulatory subunit (R^{II}) of the cAMP-dependent protein kinase (cAMP-kinase) are specifically associated with the mitotic apparatus.

MATERIALS AND METHODS

Cell Culture

A cell line well suited for study of the cytoskeleton of mitotic cells is the PtK₁ line derived from the kidney epithelium of the rat kangaroo (*Potorus tridactylis*). PtK₁ cells remain attached to the substrate during mitosis. Although they round up slightly, they remain sufficiently well spread to allow one to view the condensed chromosomes and the spindle in the various stages of mitosis. PtK₁ cells were obtained from the American Type Culture Collection, Rockville, Md., and were grown as monolayers in minimal essential medium (MEM) supplemented with nonessential amino acids, sodium pyruvate, and 10% fetal calf serum (Grand Island Biological Co., [GIBCO] Grand Island, N.Y.). The cells were seeded at 5×10^5 /ml onto 18×18 -mm glass coverslips individually contained in the 26×33 -mm chambers of Lux tissue culture multiplates and were given fresh medium 18 h before their use. In experiments requiring Colcemid, 0.1 μ g/ml of Colcemid (GIBCO) was added to the cultures for the last 2 h of incubation.

Immunofluorescence

Immunofluorescent staining was essentially as described previously (22). The coverslips were immersed in 3.7% formaldehyde in phosphate-buffered saline (PBS: 8.5 g NaCl, 2.14 g Na₂HPO₄ · 7 H₂O and 0.32 g NaH₂PO₄/liter) at room temperature. After 15–20 min, the coverslips were rinsed in PBS and placed in methanol at –20°C for 4 min followed by acetone at –20°C for 1 min to permeabilize the cells. The coverslips were then briefly rinsed in PBS, drained, and incubated with nonimmune IgG for 2 h in a moisture chamber at 37°C. Before incubation in the primary antibody, the coverslips were again briefly rinsed in PBS. All antisera were centrifuged at 1,600 g for 20 min before use. 30 μ l of primary antiserum was applied at dilutions of 1:10–1:40 ammonium sulfate-fractionated IgG (0.6–1.3 mg/ml, as determined by the Lowry protein assay). After a 90-min incubation at 37°C in primary antibody, the coverslips were rinsed in four changes of PBS for 5 min each. 30 μ l of fluorescein-conjugated goat anti-rabbit or rabbit anti-goat (N. L. Cappel Laboratories Inc., Cochranville, Pa.) at a 1:75 dilution in PBS was applied to the coverslips, and the incubation was continued at 37°C for 30–45 min. The coverslips were rinsed in four changes of PBS for 5 min each and mounted in Elvanol (20 g of polyvinyl alcohol [Monsanto Research Corp., Dayton, Ohio], 80 ml of PBS heated to 70°C, cooled, 40 ml of glycerol added [11]). After 2 h, slides were viewed on a Leitz Orthoplan epifluorescent microscope. All photographs were taken with a $\times 63$ oil immersion objective with Kodak Tri-X film and were developed in Microdol \times (1:3) for 16 min at 75°C.

To demonstrate the specificity of the staining patterns observed, the R^{II} and cGMP-kinase antibodies were preabsorbed with their antigens before staining. Liquid-phase absorption was carried out by incubating the antibodies with their respective antigens at 37°C for 30 min and then at 4°C overnight. The antisera were diluted as usual in PBS and centrifuged for 20 min at 1,600 g before use in staining. The optimal ratios of antibody:antigen required to block staining were determined by titration of antigen in antibody. The R^{II} IgG was blocked

maximally with 9 μ g of purified R^{II} antigen (90 μ g). The 18 μ g of cGMP-kinase was blocked maximally with 0.34 μ g of purified antigen.

Antibodies

Purified type I regulatory subunit (R^I) and the catalytic subunit (C) of the cAMP-kinase were prepared from bovine skeletal muscle according to the method of Beavo et al. (2). The R^{II} was prepared from bovine heart, as described previously (15, 28). Bovine lung was the source of the cGMP-kinase, which was prepared as described by Gill et al. (10).

Antisera to the R^I and C subunits and to the cGMP-kinase were obtained from rabbits by repeated injections of the antigen emulsified in Freund's or H37 Ra complete adjuvant (Difco Laboratories, Detroit, Mich.). Antibodies to R^{II} were prepared in goats by Antibodies, Inc., Davis, Calif. Antibodies to cAMP and cGMP were prepared in rabbits by injection of 2'-O-succinyl nucleotide derivatives conjugated to either bovine serum albumin or keyhole limpet hemocyanin (36). Tubulin antibody was raised in rabbits against homogeneous bovine brain tubulin as described previously (22). IgG fractions were obtained from each serum by repeated fractionation with 40% ammonium sulfate. The precipitates were dialyzed against PBS and stored at –20°C.

Immunodiffusion analysis has shown that the R^I antiserum cross-reacts with purified R^I and type I holoenzyme but not R^{II} or C. The R^{II} antiserum cross-reacts with purified R^{II} and type II holoenzyme but not R^I or C. The C subunit cross-reacts with purified C and both type I and type II holoenzymes but not with purified R^I or R^{II}. The antibody to cGMP-kinase also formed a single precipitin line in double immunodiffusion against a crude extract of PtK₁ cells, which fused with the precipitin line formed against the purified antigen. Although the R^I antibody did not form a precipitin line against a crude extract of PtK₁ cells, it did form a single precipitin line against a partially purified preparation of R^I prepared from PtK₁ cells. This line formed a line of identity with the precipitin line formed between the R^{II} antibody and the antigen from which it was made. Complete details on the preparation and the characterization of the antisera will be reported elsewhere.¹

RESULTS

cAMP-kinase are composed of regulatory subunits (R) and C (15). Upon binding of cAMP to R, the subunits dissociate, freeing the C to phosphorylate specific proteins. Two cAMP-kinases have been identified, R^I and R^{II}, which differ in their R (6). cGMP-kinase is composed of an R and a C that do not dissociate upon binding of cGMP (10). Antibodies directed against cAMP, cGMP, cGMP-kinase, and the subunits of the type I and type II cAMP-kinases were used in indirect immunofluorescence to localize these molecules in mitotic PtK₁ cells. The staining patterns of these antibodies were compared with those of an antibody against tubulin, which have been documented previously (22).

Prophase and Prometaphase

The prophase and prometaphase fluorescent patterns seen with anti-R^{II} or anti-cGMP kinase in mitotic PtK₁ cells are generally similar to those visualized with antitubulin (Fig. 1). During interphase (not shown), PtK₁ cells labeled with antibodies to the R^{II} show cytoplasmic but no nuclear staining. As the cells enter prophase and the nuclear envelope breaks down, cytoplasmic staining is low but the condensing chromosomes become surrounded by a bright fluorescence (Fig. 1 a). By prometaphase, a single area of bright fluorescence can be seen located in the midst of the chromosomes (Fig. 1 b). As spindle formation begins, individual fluorescent fibers can be seen.

Fluorescent localization of cGMP-kinase antibodies in inter-

¹ Beavo, J. A., P. J. Bechtel, T. Martin, Y. Koide, W. A. Spruill, C. L. Kapoor, W. J. Yount, and A. L. Steiner. Cyclic nucleotide-dependent protein kinases: production and characterization of antibodies to type I bovine skeletal muscle regulatory subunit, catalytic subunit, holoenzyme, and type II bovine heart muscle regulatory subunit. Manuscript submitted for publication.

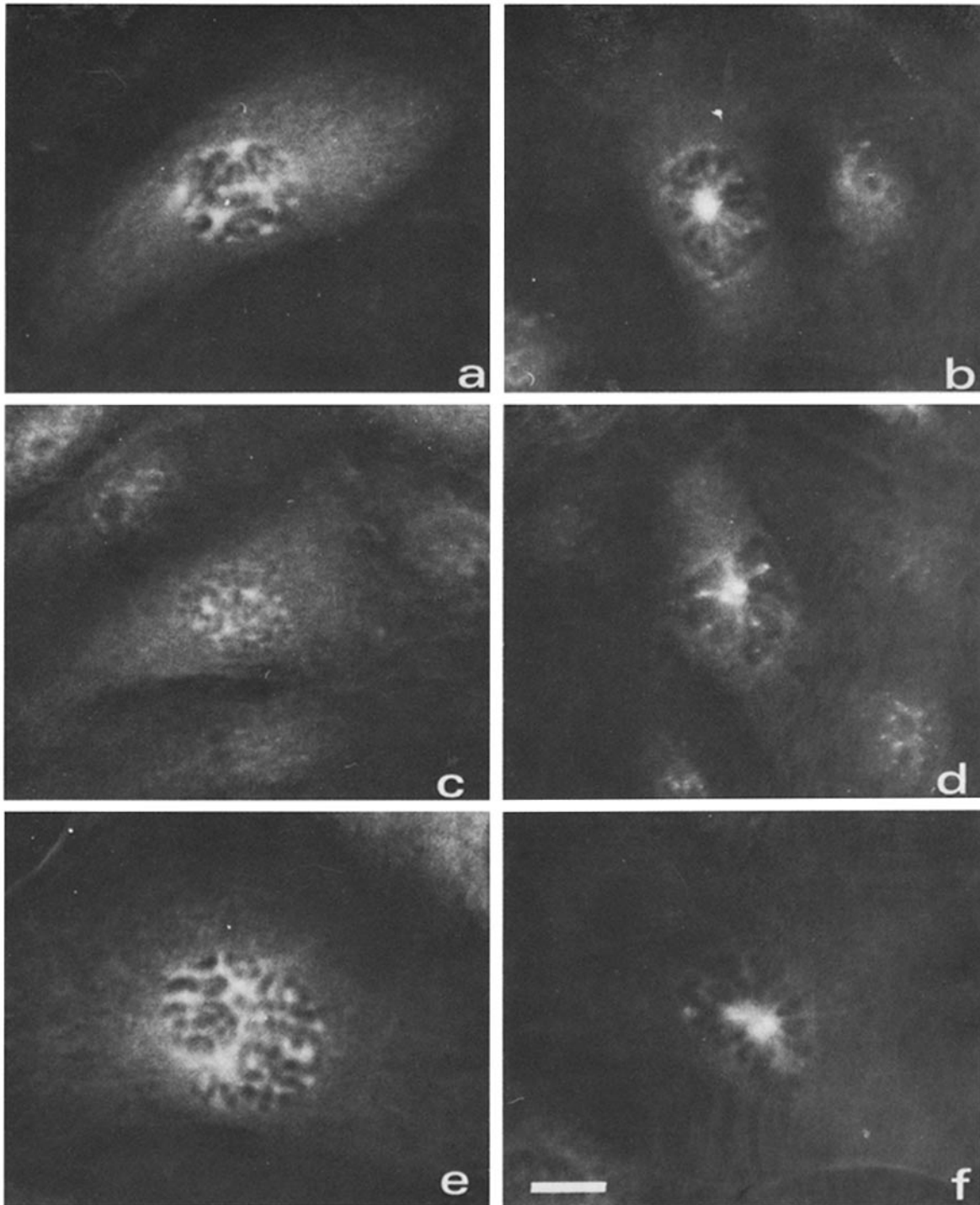


FIGURE 1 Comparison of the immunofluorescence distribution of R^{II} , cGMP-kinase, and tubulin during prophase and prometaphase. (a) R^{II} , prophase; (b) R^{II} , prometaphase; (c) cGMP-kinase, prophase; (d) cGMP-kinase, prometaphase; (e) tubulin, prophase; (f) tubulin, prometaphase. Bar, 10 μ m.

phase cells shows very little nuclear and only diffuse cytoplasmic staining (not shown). As the cells move into prophase, diffuse fluorescence appears in the area of the condensing chromosomes (Fig. 1 c), although the fluorescence is not so pronounced as that seen with R^{II} antibodies. During prometaphase, cGMP-kinase staining also reveals a single bright area in the center of the condensing chromosomes (Fig. 1 d).

Metaphase and Anaphase

During metaphase, R^{II} antibodies stain the mitotic spindle

very clearly (Fig. 2 a). In some cases, the fluorescent spindle fibers seem to extend not only from the chromosomes to the poles but also between and above the chromosomes on what appear to be interpolar fibers. Whereas the pattern of staining of metaphase cells with R^{II} antibody is virtually indistinguishable from that with antitubulin (Fig. 2 e), the intensity of staining is not so great. In anaphase, the individual fibers of the spindle can no longer be so clearly distinguished with anti- R^{II} as they are with antitubulin. However, the area between the separating chromosomes still fluoresces strongly (Fig. 2 b).

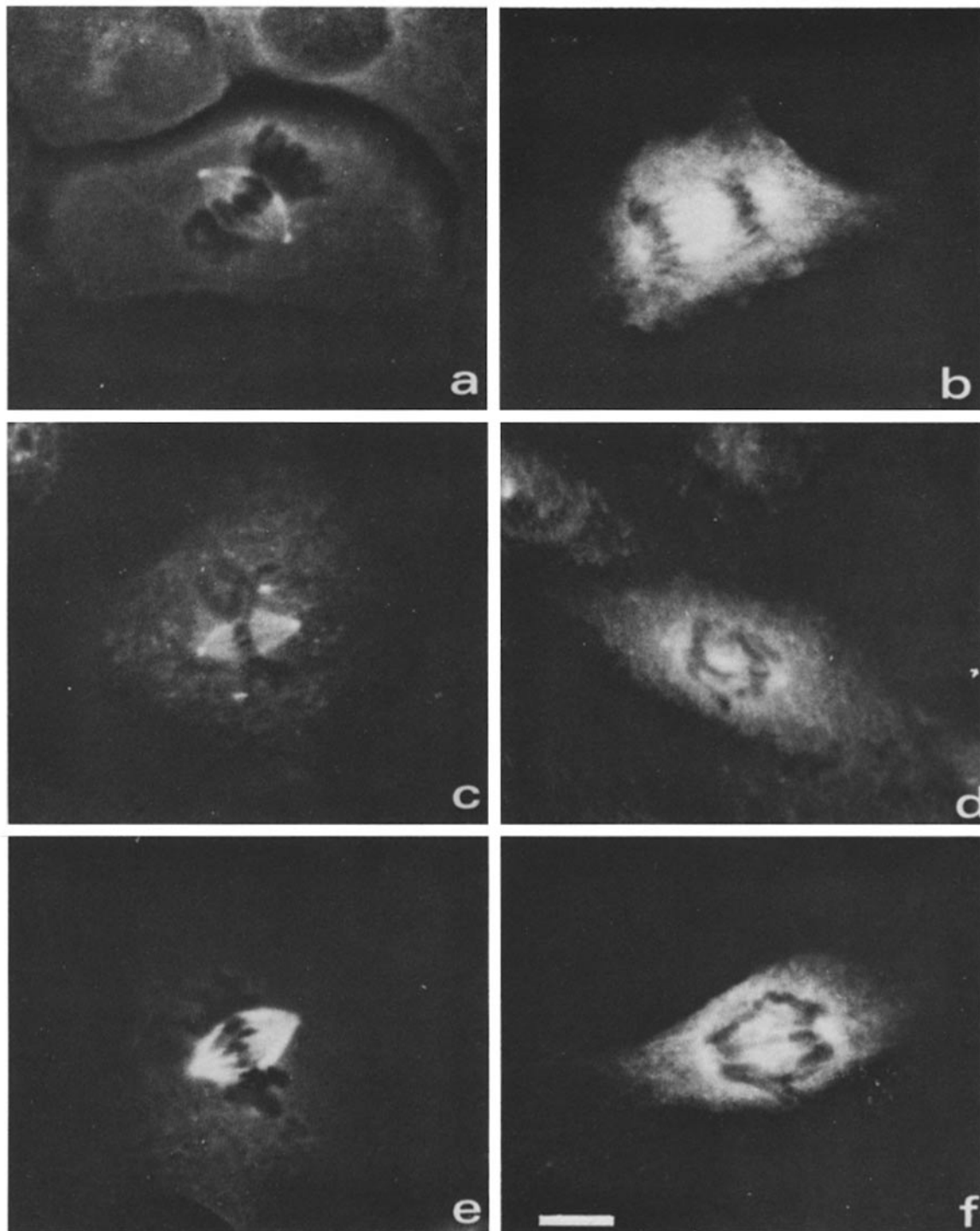


FIGURE 2 Comparison of the immunofluorescence distributions of R^{II} , cGMP-kinase, and tubulin during metaphase and anaphase. (a) R^{II} , metaphase; (b) R^{II} , anaphase; (c) cGMP-kinase, metaphase; (d) cGMP-kinase, anaphase; (e) tubulin, metaphase; (f) tubulin anaphase. Bar, 10 μ m.

In late anaphase, bright staining is observed between the kinetochores and the poles. Often, the chromosomes are surrounded by a halo of fluorescence, which is particularly pronounced between the chromosomes and the poles.

Antibodies to cGMP-kinase also label the metaphase spindle (Fig. 2 c). Although the fluorescence is less distinct than that of R^{II} staining and individual spindle fibers may be difficult to distinguish, the fibers again appear to run over and between the chromosomes. This suggests that the cGMP-kinase is also associated with the interpolar and the kinetochore fibers of the

spindle. The cGMP-kinase fluorescence in the spindle becomes more diffuse as the chromosomes begin to separate (Fig. 2 d). In anaphase, individual spindle fibers cannot be observed, but the area between and around the separating chromosomes is still brightly stained.

Neither the R^{II} nor the cGMP-kinase antibodies stain metaphase spindles as consistently as antitubulin. Of 100 metaphase cells stained with antitubulin, every cell shows distinct spindle fluorescence (Table I). Of >100 metaphase cells stained with antibodies to R^{II} or cGMP-kinase, 20–30% of the cells do

not show specific spindle fluorescence. In about half of the metaphase cells that do not show spindle fluorescence, there is a faint diffuse fluorescence in the spindle area but the spindle itself is not stained. The remaining cells have no fluorescence above background levels (Fig. 3). The centriolar staining frequently seen in such cells (Fig. 3) is also present in preimmune sera, although it is not visible in preimmune serum-stained cells photographed at the shorter exposure times compared

with the automatic exposure of highly stained mitotic spindles (cf. Fig. 7f).

Telophase and Midbody Formation

As the cells enter telophase, R^{II} antibody staining (Fig. 4a) often shows fluorescence between the chromosomes and the forming contractile ring similar to, but less intense than that seen with antitubulin (Fig. 4e). The contractile ring itself does not fluoresce. Midbody staining differs from that of tubulin antibodies. Instead of the small, well-defined region of fibrillar staining in the midbody area seen with antitubulin, bright but diffuse fluorescence emanates outward from the midbody well into the cytoplasm of the two daughter cells (Fig. 4b). Although a tubulinlike midbody pattern (Fig. 4f) is not seen, it may be obscured by the overlying, more extensive fluorescence.

Antibodies to the cGMP-kinase do not stain PtK₁ cells in telophase so consistently or so brightly as do tubulin antibodies. Occasionally, diffuse fluorescence considerably brighter than background is seen extending between the chromosomes (Fig. 4c). The contractile ring (not shown) is unstained. cGMP-kinase fluorescence in the area of the midbody (Fig. 4d) is similar to, although much less intense than, that of antitubulin (Fig. 4f). A small localized wedge of fluorescence is often

TABLE I
Percentages of Fluorescently Labeled Mitotic Spindles in Metaphase Cells

	No. of metaphase cells	No. of fluorescent spindles	No. of metaphase cells without fluorescent spindles	Percent of spindle staining
Tubulin	100	100	0	100
R ^{II}	104	83	21	79.8
GMP-kinase	126	98	28	78.2

The percentages of mitotic spindles stained in metaphase PtK₁ cells with fluorescently labeled antibodies to tubulin, the R^{II} of cAMP-kinase, and cGMP-kinase are compared.

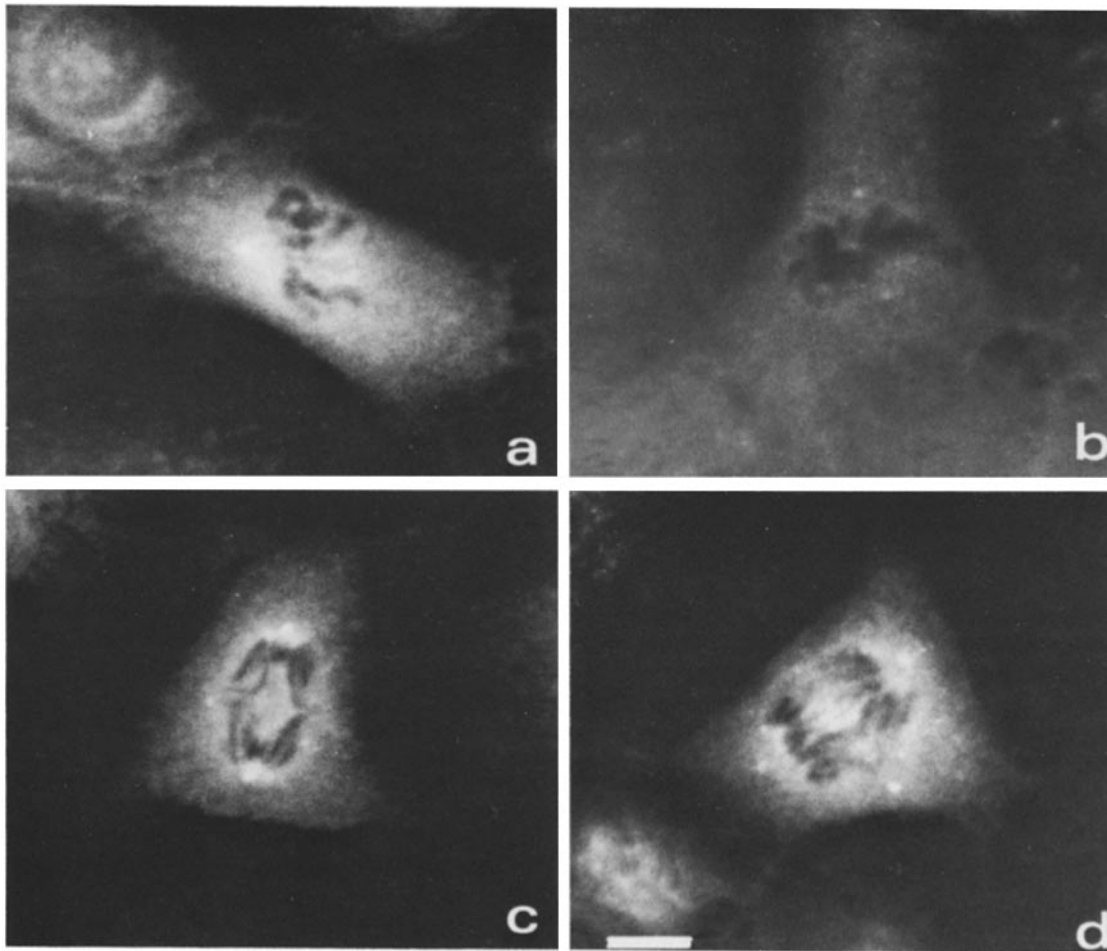


FIGURE 3 Other distributions of R^{II} and cGMP-kinase during metaphase and anaphase. Appearance of 21% of metaphase cells stained with R^{II} antibody (a) and appearance of 28% of metaphase cells stained with cGMP-kinase antibody (b) showed little or no spindle fluorescence. Staining of anaphase cells with R^{II} (c) and cGMP-kinase (d) was also often inconclusive. Bar, 10 μ m.

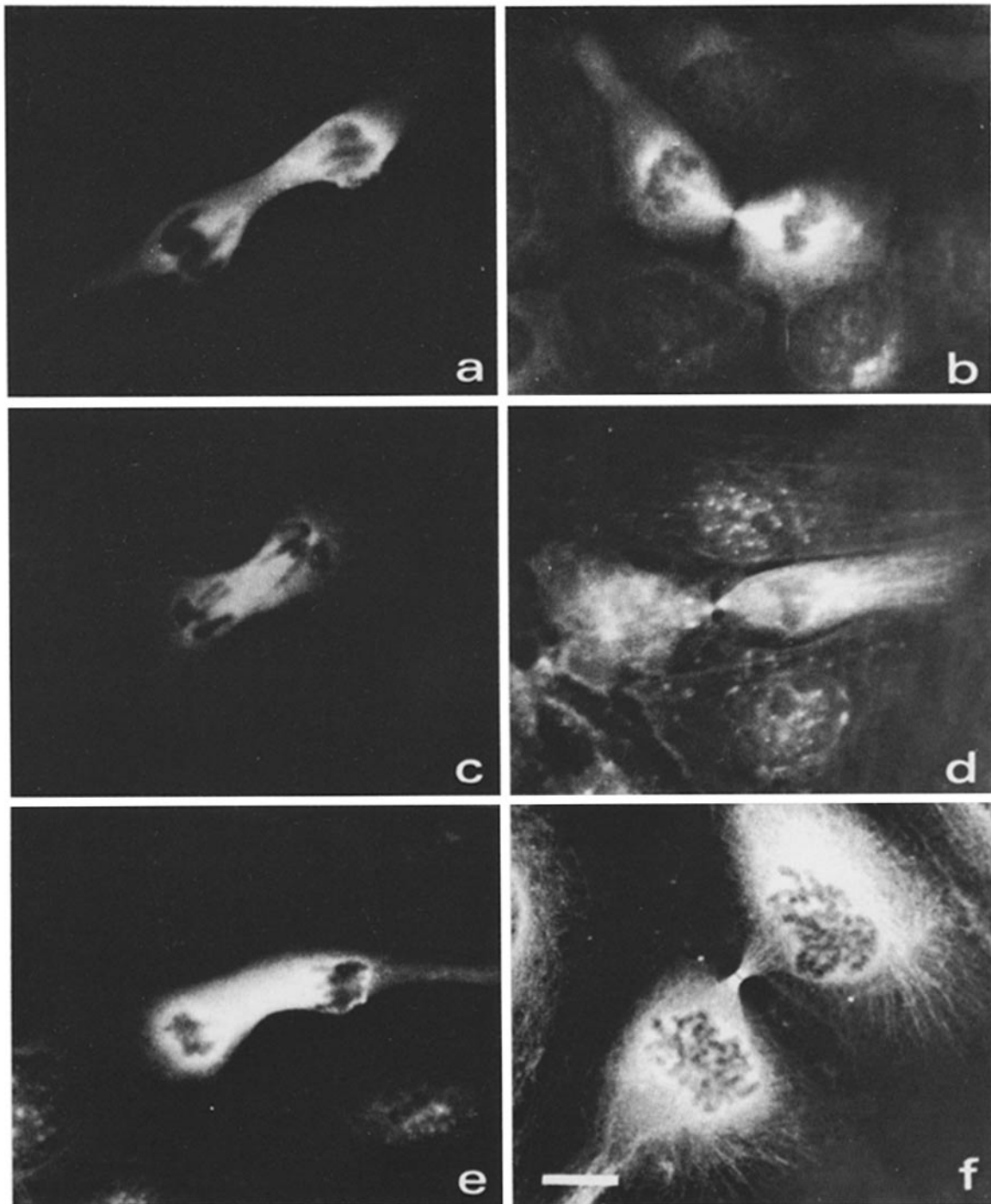


FIGURE 4 Comparison of the immunofluorescence distributions of R^{II} , cGMP-kinase, and tubulin during telophase and midbody formation. (a) R^{II} , late telophase; (b) R^{II} , cytokinesis; (c) cGMP-kinase, early telophase; (d) cGMP-kinase cytokinesis; (e) tubulin, early telophase; (f) tubulin, cytokinesis. Bar, 10 μ m.

present in the midbody area, but the staining is fairly diffuse and individual microtubule fibers cannot be distinguished.

The localization of R^{II} and cGMP-kinase in a pattern similar to that of tubulin suggested that these molecules might be associated with microtubules in mitotic cells. Further evidence for this was found by disruption of microtubules by incubation of the cells at 4°C or in 0.1 μ g/ml of Colcemid for 2 h before fixation. Both cold and Colcemid exposure eliminate the characteristic tubulinlike staining pattern observed in mitotic cells with antibodies to R^{II} and cGMP-kinase (Fig. 5).

R^I and C of cAMP-Kinase

During prophase, staining with R^I antibodies results in brightness around the condensing chromosomes, but no specific staining of the developing spindle is observed. In metaphase, usually only a faint diffuse fluorescence can be seen in the region of the spindle (Fig. 6 a). In a limited number of cells, this faint fluorescence appears to be localized on the spindle, but it is very rare that individual fibers can be distinguished. Frequently, cells in metaphase show no staining at all in the

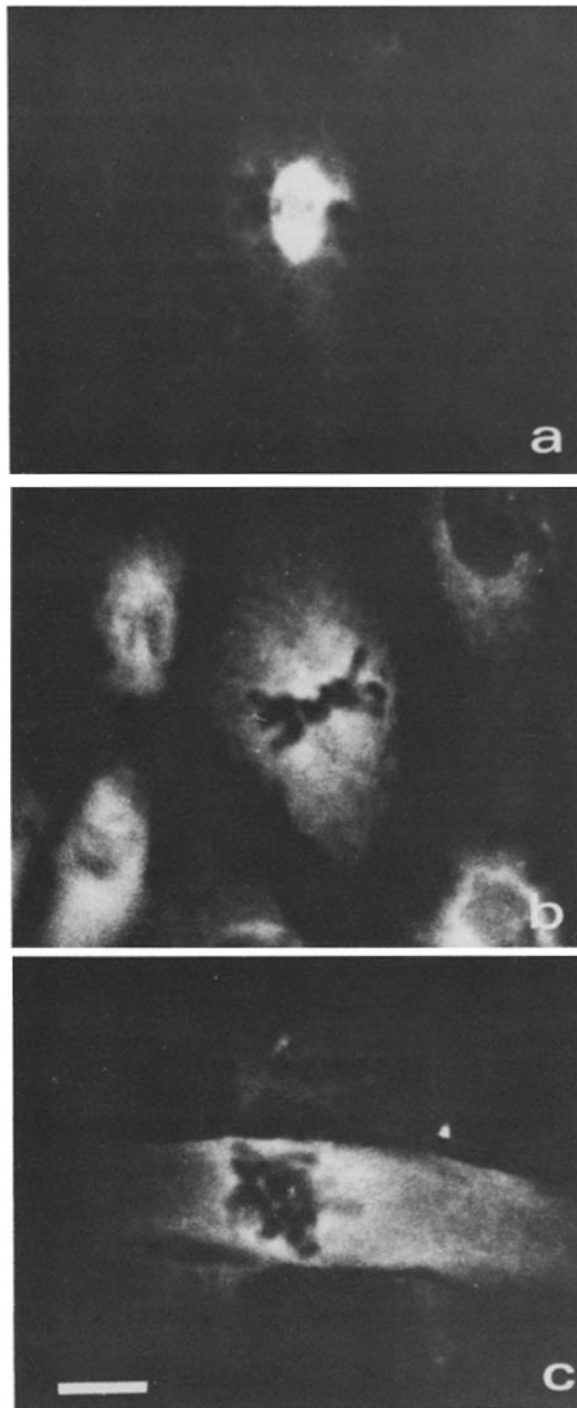


FIGURE 5 Effect of Colcemid and cold on staining of mitotic cells by anti-R^{II}. (a) R^{II} staining of control cells. (b) Metaphase cells incubated at 4°C for 1 h before staining with antiserum to R^{II}. (c) Disrupted chromosomes of mitotic PtK₁ cell exposed to 0.1 μg/ml of Colcemid for 2 h before staining with antiserum to R^{II}. Bar, 10 μm.

spindle region when stained with antibodies to R^I. The staining patterns of R^I antibodies during anaphase and telophase do not differ significantly from those of the nonimmune sera. There is no detectable staining of the remnant of the spindle, of the midbody, or of the contractile ring.

Antibodies to C of the cAMP-kinases stain mitotic cells the

same as the R^I antibodies (Fig. 6 b). As the cells progress through prophase, the fluorescence around the chromosomes is diffuse. In some cells, there is faint staining in the spindle region during metaphase (Fig. 6 b), but this fluorescence does not seem to be localized specifically on the spindle. No localized fluorescence is observed in anaphase or telophase, or as the cell undergoes cytokinesis. Antibodies to cAMP and cGMP also do not show specific staining of the mitotic spindle (not shown). Although bright centriolar fluorescence and a faintly fluorescent outline of the spindle are often seen with the cyclic nucleotide antibodies, these are also present in cells stained with the preimmune sera from the animals in which the antibodies were made.

Antibody Specificity

A number of experiments provide evidence that the pronounced spindle staining of metaphase cells observed with anti-R^{II} or anti-cGMP-kinase is the result of a specific antibody-antigen reaction. The secondary antibodies, either fluorescently labeled goat anti-rabbit or rabbit anti-goat, do not stain the mitotic apparatus of PtK₁ cells when used without previous exposure of the cells to the primary antibody. Comparison of the cGMP-kinase staining pattern (Fig. 7 b) with that of the preimmune serum from the same animal (Fig. 7 f) shows that antibodies specific to spindle fiber components were not present before immunization. No preimmune serum was available from the animal from which the R^{II} antibodies were obtained. However, incubation of either cGMP-kinase or R^{II} antibodies with their specific antigens for 30 min at 37°C and then overnight at 4°C before fixation and staining results in nearly complete blocking of staining on structural components of the cells (R^{II}, Fig. 7 c; cGMP-kinase, Fig. 7 e). The ability of the R^{II} antigen to completely block antibody staining is a strong argument for antibody specificity. We have also considered whether staining of the spindle by either R^{II} or cGMP-kinase antibodies is the result of cross-reactivity between the R^{II} antibody and the cGMP-kinase, or vice versa. Preincubation of cGMP-kinase with R^{II} antibodies (Fig. 7 g) or R^{II} with cGMP-kinase antibodies (Fig. 7 h) before staining does not result in any reduction of the spindle staining properties of either of the antibodies. If the staining of either antibody is the result of a cross-reactivity with the other antigen, fluorescence should be partially or completely eliminated by the cross-reacting antigen.

To rule out the possibility that the immunized animals might have had autoantibodies to tubulin, the cross-reactivity of the anti-R^{II} IgG fraction with tubulin antigen was tested by double immunodiffusion. There was no apparent cross-reactivity. In addition, purified tubulin was used to try to block the R^{II} antibody staining. Although tubulin blocked antitubulin staining of the spindle nearly completely and R^{II} antigen blocked anti-R^{II} staining of the spindle, there was no diminution of anti-R^{II} fluorescence on spindles after preincubation with tubulin (Fig. 8).

DISCUSSION

Generally, cyclic nucleotides act by controlling the phosphorylation of specific proteins by cyclic nucleotide-dependent protein kinases, with a consequent alteration in the function of

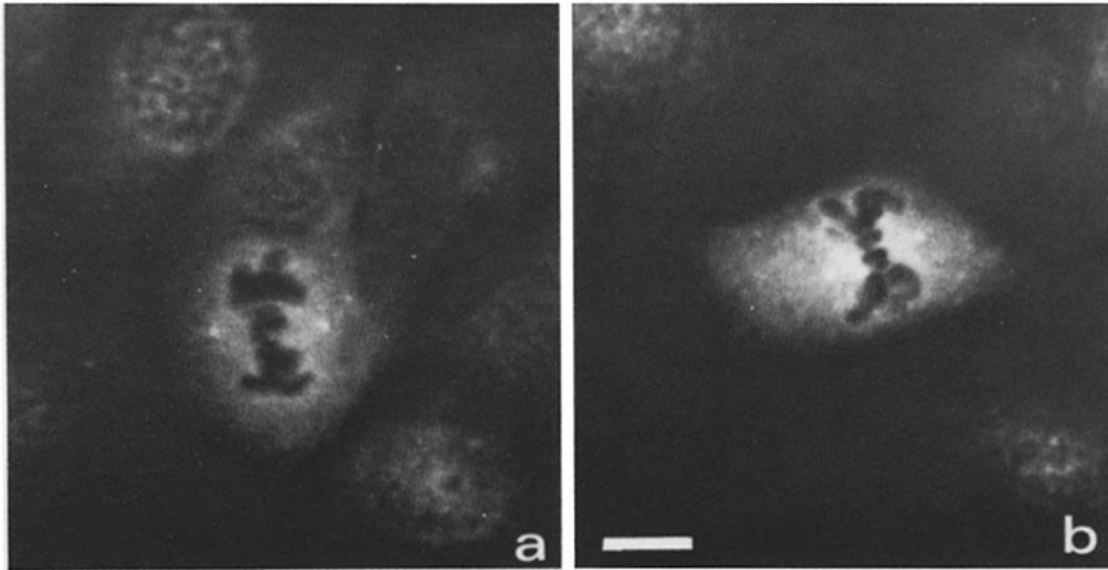


FIGURE 6 Immunofluorescent localization of R^I and C during metaphase. Bar, 10 μm .

the phosphorylated protein (14). Although cyclic nucleotides have been implicated in the regulation of cell motility and division, no physical association between the cyclic nucleotide system and the cytoskeleton has been demonstrated *in vivo*.

In this paper, we have used immunofluorescence to show that the R^{II} of cAMP-kinase and the cGMP-kinase holoenzyme are associated with the mitotic apparatus of dividing PtK₁ cells. Staining patterns of R^{II} and cGMP-kinase are very similar and follow closely those observed for tubulin. Although tubulin fluorescence is much brighter, this can be attributed to the fact that tubulin is the major component of both the spindle and midbody.

Neither R^I nor C is localized on the mitotic apparatus. Although the R^I is not localized on the spindle, its presence in PtK₁ cells has been detected by the measurement of two peaks of cAMP binding activity after DEAE-cellulose fractionation (9, 27, unpublished observations). In preliminary experiments, it also appears that neither cAMP nor cGMP is localized in the mitotic spindle or midbody. The localization of R^{II} and cGMP-kinase on the spindle without the concomitant presence of either of the cyclic nucleotides raises the question of whether these molecules might also be associated with the spindle in the cell but are lost during fixation. The immunocytochemical techniques used in this study most likely detect only fixed or bound molecules. Unfixed or soluble proteins and nucleotides may be extracted. Immunofluorescence will also not detect proteins whose antigenic sites are masked. It is possible that both C and R^I are associated with the spindle but are bound in such a way that their antigenic sites are not available for antibody binding.

It is also possible that R^I is not associated with the spindle. If this is the case, the presence of R^{II} on the mitotic apparatus suggests a functional and biochemical difference between the two R subunits. For example, R^{II} may have additional binding sites specific for microtubule proteins. Because C is also not detected on the spindle, it is possible that R^{II} could bind independently to the spindle and perhaps function as an allosteric regulator.

R^{II} and cGMP-kinase appear to be not only on chromosomal

spindle fibers but also on interpolar fibers. During cytokinesis, they are associated with the midbody rather than the contractile ring. This distribution suggests an association with microtubules rather than actin in mitotic cells. It is also possible, however, that the cyclic nucleotide-dependent protein kinases could be associated with the vesicular system found throughout the spindle (25). In contrast to the consistent metaphase and anaphase staining of the mitotic spindle with antitubulin, a fraction of both metaphase and anaphase spindles is not stained by either R^{II} or cGMP-kinase antibodies. This observation may simply be a reflection of the specificity of the antibodies or the accessibility of the antigens. On the other hand, it may indicate a transitory association between the kinases and the spindle.

There is biochemical evidence that a cAMP-kinase copurifies with tubulin isolated from rat brain (29). This protein kinase phosphorylates certain microtubule-associated proteins that have been implicated as regulators of microtubule assembly (5, 22, 32). By analogy, the presence of cGMP-kinase and the R^{II} of cAMP-kinase on the mitotic spindle suggests that cyclic nucleotide-dependent phosphorylation of specific proteins in the mitotic apparatus may be fundamentally involved in the regulation of spindle assembly and chromosome motion.

The calcium-dependent regulator protein (calmodulin), a calcium-binding protein structurally similar to troponin C, has also been localized by immunofluorescence on the mitotic spindles of 3T3 and PtK₁ cells (1, 37). Calcium ions can depolymerize microtubules *in vitro*, and recent evidence suggests that they may regulate spindle microtubule polymerization *in vivo* (17, 23, 36). It has been hypothesized that calmodulin, through its calcium-binding properties, may play a functional role in spindle microtubule assembly and disassembly (37). Calmodulin is also involved in the regulation of cyclic nucleotide metabolism. It is known to activate 3'5' cyclic nucleotide phosphodiesterase and adenyl cyclase, and to stimulate specific protein kinases (3, 4, 31). The simultaneous presence on the mitotic spindle of both the cyclic nucleotide-dependent protein kinases and the calcium-dependent regulator protein suggests that the two systems may interact reciprocally in the control of mitosis.

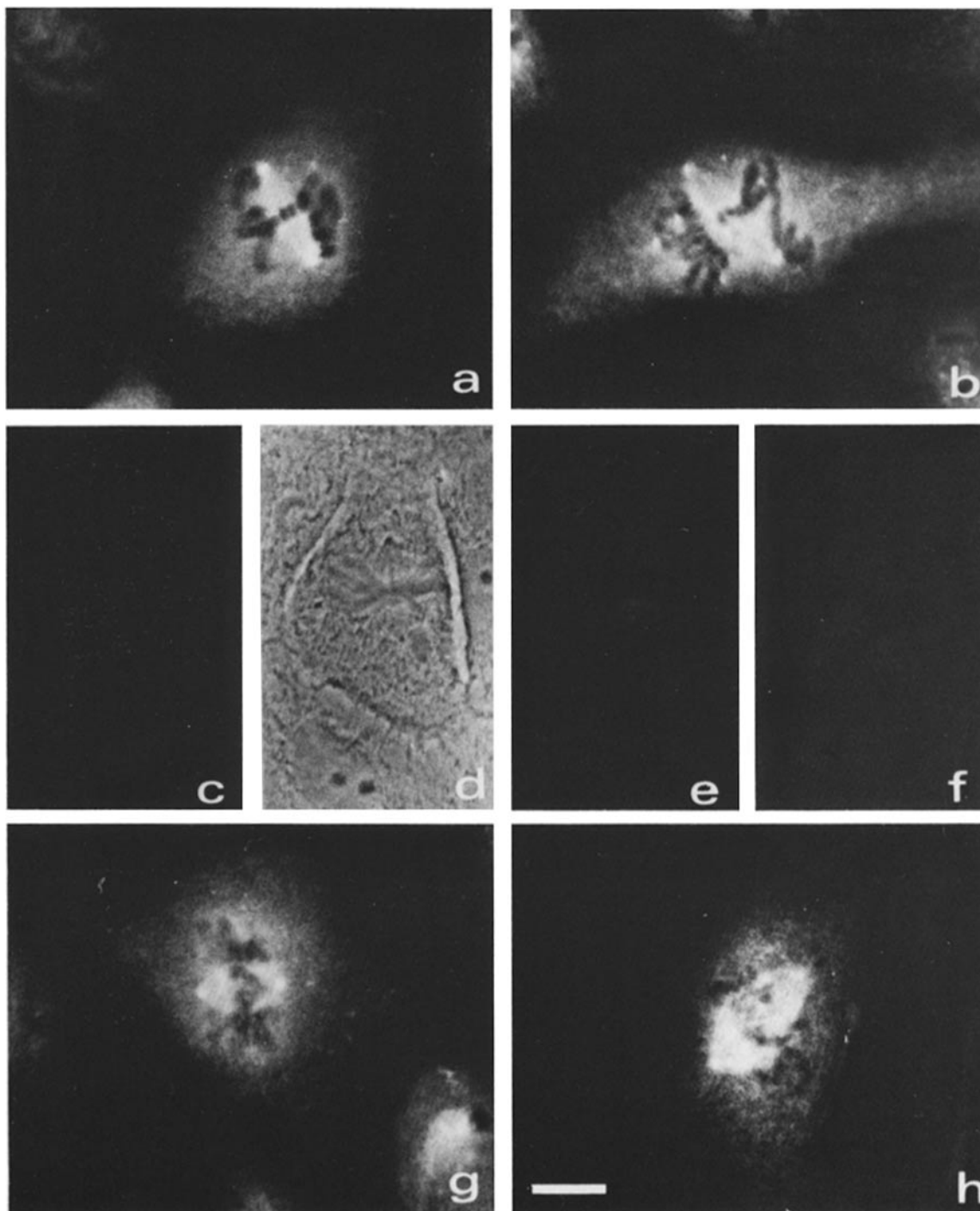


FIGURE 7 Specificity of the R^{II} and cGMP-kinase antibody staining patterns of metaphase cells as demonstrated by antigen preabsorption, preimmune serum staining, and lack of cross-reactivity. (a) R^{II} ; (b) cGMP-kinase; (c and d) R^{II} antibody preabsorbed with R^{II} antigen, fluorescence, and phase contrast; (e) cGMP-kinase antibody preabsorbed with cGMP-kinase antigen; (f) cGMP-kinase preimmune serum; (g) R^{II} antibody preabsorbed with cGMP-kinase antigen; (h) cGMP-kinase antibody preabsorbed with R^{II} antigen. Bar, 10 μ m.

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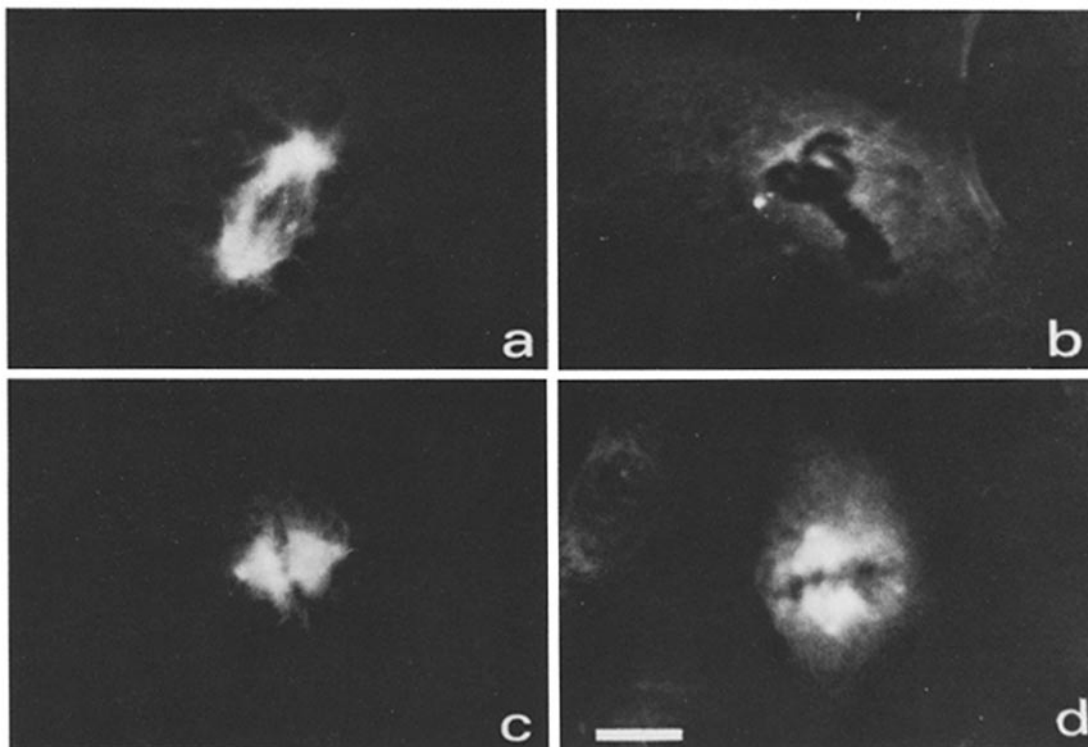


FIGURE 8 Lack of cross-reactivity between R^{II} and tubulin. (a) Tubulin staining of a forming spindle fiber; (b) tubulin antibody preabsorbed with tubulin antigen, metaphase; (c) R^{II}, metaphase; (d) R^{II} antibody preabsorbed with tubulin antigen, metaphase. Bar, 10 μ m.

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