Identification of a 52-kD Calmodulin-binding Protein Associated with the Mitotic Spindle Apparatus in Mammalian Cells

Richard C. Brady, Fernando Cabral, and John R. Dedman

Departments of Internal Medicine and Physiology/Cell Biology, The University of Texas Medical School at Houston, Houston, Texas 77225

Abstract. A pool of 10 calmodulin-binding proteins (CBPs) was isolated from Chinese hamster ovary (CHO) cells via calmodulin (CaM)–Sepharose affinity chromatography. One of these ten isolated CBPs with a molecular mass of 52 kD was also found to be present in isolated CHO cell mitotic spindles. Affinity-purified antibodies generated against this pool of isolated CBPs recognize a single 52-kD protein in isolated CHO cell mitotic spindles by immunoblot analysis. Immunofluorescence examination of CHO, 3T3, NRK, PTK-2, and HeLa cells resulted in a distinct pattern of mitotic spindle fluorescence. The localization pattern of this 52-kD CBP directly parallels that of CaM in the spindle apparatus throughout the various stages of mitosis. Interestingly, there was no association of this 52-kD CBP with cytoplasmic microtubules. As is the case with CaM, the localization pattern of the 52-kD CBP in interphase cells is diffuse within the cytoplasm and is not associated with any discrete, cellular structures. This 52-kD CBP appears to represent the first mitotic spindle–specific calmodulin-binding protein identified and represents an initial step toward the ultimate determination of CaM function in the mitotic spindle apparatus.

CALMODULIN (CaM)[1] is the major Ca^{2+} receptor in smooth muscle and nonmuscle cells and has been demonstrated to confer Ca^{2+} sensitivity upon a number of cellular processes and enzyme systems (26). Upon binding Ca^{2+}, CaM undergoes a conformational shift, thus exposing a hydrophobic domain which is capable of interacting with a complimentary hydrophobic domain on the proteins and enzymes which it activates. Our laboratory refers to the proteins which bind CaM in a Ca^{2+}-dependent manner as calmodulin-binding proteins (CBPs). It appears to be the CBPs which are the actual discriminators of CaM function within various cell types. Since CaM is a ubiquitous protein, the specific physiologic response that is elicited in a given cell type in response to an increase in cytosolic free Ca^{2+} is most likely due to the inherent repertoire of CBPs present within a particular cell type.

Early studies entailing the indirect immunofluorescence localization of CaM in cultured cells indicated that CaM is highly concentrated within the mitotic spindle apparatus of dividing cells (38, 39). The physiological role of CaM in the regulation of mitotic spindle function and integrity, however, is unclear. CaM has been demonstrated to confer an increased sensitivity to Ca^{2+}-induced depolymerization of bovine brain microtubules in vitro (24). Furthermore, effects of Ca^{2+} on spindle microtubule integrity have been reported in in vivo studies (20), and in studies with isolated mitotic spindles (19, 33). Thus, one possible role for CaM in mitosis is the regulation of microtubule assembly/disassembly within the mitotic apparatus.

In addition to effects on microtubules, another conceivable role for CaM in mitosis is the regulation of the ATP-dependent Ca^{2+} sequestration and consequently, free Ca^{2+} levels in the mitotic spindle apparatus. CaM has been shown to regulate intracellular ATP-dependent calcium transport in a variety of tissues through the activation of membrane ATPases (6, 7, 9, 10, 18, 21). Ca^{2+}-ATPase activity has also been demonstrated in the mitotic spindle (29, 30). Furthermore, an extensive membrane system has been demonstrated in the mitotic apparatus (13) and physiological studies with isolated mitotic spindles indicate that these mitotic membranes are capable of sequestering Ca^{2+} (35). Thus, CaM could perform multiple functions during mitosis.

CaM does not appear to associate with the mitotic apparatus by directly binding to tubulin. Purified tubulin does not bind to CaM–Sepharose affinity matrices in a Ca^{2+}-dependent manner (31) nor does it bind ^{125}I-CaM on gel overlay analysis (Brady, R. C., F. Cabral, and J. R. Dedman, unpublished observation). Accordingly, the identification of specific CBPs within the spindle apparatus and the subsequent biochemical characterization of possible inherent enzymatic activities should provide valuable information regarding the role of CaM in the regulation of mitotic spindle function and integrity.

1. *Abbreviations used in this paper:* CaM, calmodulin; CBP, CaM-binding protein; CHO, Chinese hamster ovary; MAP, microtubule-associated protein.

The present report describes the identification of CBPs in the isolated mitotic apparatus of Chinese hamster ovary (CHO) cells and the production of antibodies which recognize a 52-kD mitotic spindle CBP whose pattern of localization within the spindle directly correlates with that of CaM throughout the various stages of mitosis.

Materials and Methods

CBP Isolation and Antibody Production

Approximately 4×10^8 CHO cells were grown in 150-mm dishes (Falcon Labware, Oxnard, CA) and scraped from the substratum with a rubber policeman. The cells were then pelleted by centrifugation at 800 g for 5 min in a clinical centrifuge and the pellet was washed by gently resuspending in PBS and again centrifuged at 800 g for 5 min. All subsequent steps were performed at 4°C. The washed pellet was resuspended in 2 vol of 0.02 M Tris, pH 7.4, 0.5% aprotinin, 2 mM EGTA, 0.2 M NaCl (homogenization buffer), and then homogenized with 90 strokes of a Dounce homogenizer and centrifuged at 20,000 g for 20 min. The supernatant was then adjusted to 1 mM Ca^{2+} with a $CaCl_2$ standard solution (Orion) and applied to a CaM-Sepharose column constructed as in reference 5. Once the sample was applied, the column was washed with homogenization buffer containing 1 mM $CaCl_2$ followed by homogenization buffer containing 1 mM $CaCl_2$, and 0.5 M NaCl. The absorbance of the eluate was monitored at 280 nm until it reached zero and the protein absorbed to the column in a Ca^{2+}-dependent manner was eluted with homogenization buffer containing 2 mM EGTA. The protein peaks from several runs were pooled, lyophilized, and emulsified with complete Freund's adjuvant. The immunization regimen consisted of injecting 5 mg of pooled protein subcutaneously into a male sheep on day 1 followed by an injection of 2 mg on day 14 and a third injection of 2 mg protein on day 28. Animals were bled at 7 and 20 d after the third injection. Approximately 600 ml of serum was obtained at each bleed. The resultant antiserum was affinity purified as in reference 5 over a column consisting of isolated CHO cell CBPs which were immobilized to cyanogen bromide-activated Sepharose.

Cell Maintenance and Spindle Isolation

The CHO cell line used in these studies and the conditions for its growth have been previously described (4). To obtain a synchronous population cells were incubated in alpha modification of minimal essential medium (α MEM) (Gibco, Grand Island, NY) containing 5 mM thymidine for 16 h in order to block in S phase. They were then washed with fresh α MEM and incubated for 4 h before adding 0.035 μg/ml Nocodazole. After 3 h, ~80% of the cells were blocked in prometaphase as evidenced by their round morphology and condensed chromosome organization. Spindles were isolated from these cells via a modification of the procedure of Mullins and McIntosh (27). Briefly, mitotic CHO cells blocked in prometaphase were shaken from the substratum, centrifuged at 800 g for 5 min in a clinical centrifuge, gently resuspended in fresh αMEM and incubated at 37°C in a water bath in order to allow them to progress to metaphase. This routinely occurred within 12–15 min and was determined by examining lysed aliquots of the cells with phase-contrast microscopy at various times during the reversal period. Cells were then centrifuged for 3 min at 800 g and resuspended in 10 ml of swelling buffer (1 M hexylene glycol, 2 mM Pipes [pH 6.8], and 0.5 mM $MgCl_2$). The cells were immediately centrifuged for 3 min at 800 g and the pellet was lysed in 10 ml of lysis buffer (1 M hexylene glycol, 2 mM Pipes [pH 6.8], 1 mM EGTA and 1% Nonidet P-40) by mixing for 12 s at setting 5 on a Vortex mixer. 4 ml of post-lysate buffer (1 M hexylene glycol, 50 mM 2-(N-morpholino)ethane sulfonic acid, pH 6.3) were added and the solution was gently mixed and maintained on ice for 10 min. Spindles were pelleted by centrifugation at 770 g for 10 min through a 5-ml cushion of 40% glycerine, 50 mM 2-(N-morpholino)ethane sulfonic acid (pH 6.3).

SDS Gel Electrophoresis

SDS PAGE was performed according to Laemmli (22) using 9% gels. Protein samples were solubilized in hot SDS sample buffer and boiled for 5 min before electrophoresis. Protein determinations were performed according to Bradford (1).

Immunoblot Analysis

Isolated spindles and CBPs were subjected to SDS PAGE and electrophoretically transferred to nitrocellulose paper (32, 37). The transfer was performed at room temperature for 4 h at 200 milliamps constant current. After transfer, the nitrocellulose paper was dried overnight at room temperature between two layers of Whatman 3 MM filter paper. For immunoreaction, the dried transfer was incubated with 20 mM Tris (pH 7.4), 0.9% NaCl (TS) containing 3% bovine serum albumin (BSA) for 1 h at room temperature, washed for 1 h in TS, and then incubated for 1 h at room temperature with a 1:5,000 dilution of affinity-purified sheep anti-CHO cell CBPs in TS (containing 0.5% BSA and 0.05% Triton X-100). The transfer was then washed for 1 h with TS and then incubated for 1 h with a 1:1,000 dilution of peroxidase-conjugated rabbit anti-sheep IgG in TS containing 5% rabbit serum. After washing, reaction product was visualized according to Hawkes et al. (12) by incubating the transfer in a developing solution consisting of 0.05 mg/ml 4-chloro-1-naphthol and 0.01% hydrogen peroxide in TS.

Indirect Immunofluorescence

Immunofluorescence was performed as described by Brady et al. (3). Briefly, cells were grown on glass coverslips and then fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. PTK-2, NRK, 3T3, and HeLa cells on coverslips were the generous gift of Dr. Bill Brinkley and were similarly fixed. The cells were washed with PBS for 30 min and then incubated with affinity-purified sheep anti-CHO CBPs for 1 h at 37°C. Next, cells were washed for 30 min in PBS and then incubated at 37°C for 30 min with fluorescein-conjugated rabbit anti-sheep IgG (Cappel Laboratories, Cochranville, PA) at a dilution of 1:20. After this incubation, the cells were washed and mounted on glass slides in a solution of 1:9 PBS in glycerine and viewed on a Nikon Optiphot microscope equipped with epifluorescence optics.

Results

In an effort to biochemically characterize cellular CBPs and produce antibodies which would aid in the ultimate elucidation of their cellular function, localization, and turnover, CHO cell CBPs were isolated via CaM-Sepharose affinity chromatography and the pool of isolated CBPs was injected into sheep. To optimize the yield of isolated CBPs, CHO cells were grown in large quantities and were harvested for CBP isolation in log phase growth. As can be seen in Fig. 1, there were ~7–10 CBPs with molecular masses ranging from 52–200 kD present in the soluble fraction of CHO cell homogenates which adsorbed to the CaM-Sepharose affinity matrix in a Ca^{2+}-dependent manner. Interestingly, the CBP with a molecular mass of 52 kD (star, Fig. 1) co-migrates with a major 52-kD spindle CBP identified by CaM gel overlay analysis (Brady, R. C., M. J. Schibler, J. R. Dedman, and F. Cabral, manuscript submitted for publication).

The antibodies generated against the isolated CBPs were then affinity purified and used in immunoblot analyses of isolated spindles. Although all of the injected CBPs seen in Fig. 1 reacted with the affinity-purified antibodies upon immunoblot analysis (not shown) only a single protein with a molecular mass of 52 kD reacted when isolated mitotic spindles were subjected to immunoblot analysis (Fig. 2).

To confirm the biochemical evidence for the presence of this 52 kD CBP within the CHO cell mitotic spindle and determine its species distribution, PTK-2, NRK, CHO, 3T3, and HeLa cells were subjected to indirect immunofluorescence examination with these affinity-purified antibodies. The localization pattern of this 52-kD CBP was compared to that of CaM and tubulin within the spindle apparatus throughout the various stages of mitosis in each of the above cell types. As is the case with CaM localization, interphase cells incubated with the anti-CBP antibodies exhibited only
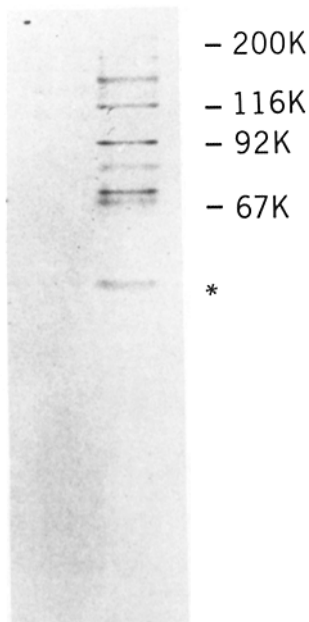
Figure 1. SDS PAGE of isolated CHO cell CBPs. Approximately 10 proteins are contained in the peak eluted from the CaM–Sepharose affinity column. 50 μg of protein were applied to this gel. The asterisk indicates the 52-kD CBP.

Figure 2. Immunoblot of isolated CHO cell spindles which were incubated with a 1:5,000 dilution of affinity-purified sheep anti–CHO cell CBPs. Note the single, immunoreactive band at 52 kD. 100 μg of protein was applied to the gel.

a diffuse pattern of fluorescence throughout the cytoplasm which did not appear to be associated with any discrete, subcellular structures (Fig. 3). In mitotic cells, however, a discrete pattern of spindle fluorescence was observed (Fig. 4, *a–d*). Cells in prometaphase exhibited fluorescence within the centrosomes (Fig. 4 *a*). During metaphase, the pole-to-kinetochore fibers of the spindle were labeled (Fig. 4 *b*). Lack of staining of the interpolar microtubules was observed in anaphase cells where again, only the kinetochore microtubules were visible (Fig. 4 *c*). At telophase the midbody was labeled (Fig. 4 *d*). As can be seen, the localization pattern of the 52-kD spindle CBP (Fig. 4, *a–d*) directly correlates with that of CaM (Fig. 4, *e–h*) throughout the various stages of mitosis and is distinct from that of tubulin (Fig. 4, *i–l*) especially in its inability to stain interpolar microtubules during anaphase. Also, the localization pattern of tubulin antibodies (Fig. 4 *l*) within the midbody reveals staining of the centralmost portion which is lacking in the patterns derived from the CaM and 52-kD CBP antibodies (Fig. 4, *d* and *h*). This pattern of spindle fluorescence was observed in all cell types examined (PTK-2, NRK, CHO, 3T3, and HeLa). In addition, none of these cell types exhibited cytoplasmic microtubule fluorescence when incubated with the anti–CBP antibodies.

The SDS PAGE migration of the 52-kD CBP is similar to that reported for a 51-kD autophosphorylatable subunit of CaM-kinase II (25). This enzyme is a large complex with a native molecular mass of approximately 550,000 D and is composed of subunits with molecular masses of 51 and 60 kD. Ca^{2+}–CaM has been demonstrated to activate this kinase with subsequent phosphorylation of a variety of substrates in vitro (28). Furthermore, both the 51 and 60 kD subunits of CaM Kinase II have been shown to bind CaM in gel overlays (11, 25). To examine the possibility that the 52-kD spindle CBP detected by our antibodies might represent the 51-kD calmodulin-binding subunit of CaM kinase II, an immunoblot of purified CaM kinase II (generously provided by Dr. Fred Gorelick, Yale University School of Medicine) was performed using our affinity-purified anti-
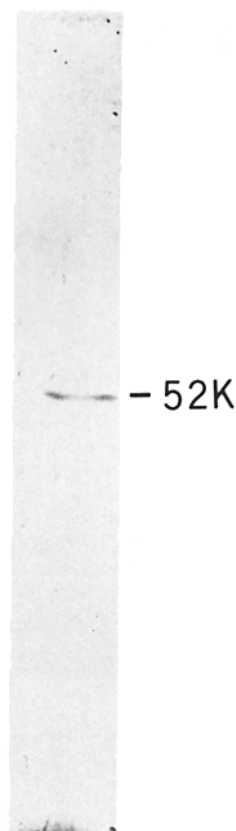
bodies. No reactivity of our antibodies with purified CaM kinase II was observed (data not shown).

Discussion

Since the demonstration by Welsh et al. (39) that CaM is localized within the mitotic spindle apparatus, little information has been generated regarding the molecular nature or the functional significance of this association. The direct binding of CaM to tubulin appears to be an unlikely mechanism. Rebhun et al. (31) have demonstrated that specific, Ca^{2+}-dependent binding of 6S tubulin to CaM–Sepharose affinity matrices does not occur. Furthermore, these workers report that CaM has no effect on the Ca^{2+} sensitivity of purified tubulin which is free of microtubule-associated proteins (MAPs). On the other hand, evidence does exist for the interaction of CaM with MAPs (23, 31, 36). The present report describes the identification of a 52-kD calmodulin-binding protein which may be involved in mediating the association of calmodulin with the mitotic apparatus. This 52-kD protein appears to be a spindle-specific, microtubule-associated CBP since antibodies against this 52-kD protein do not react with cytoplasmic microtubules when cultured cells are subjected to indirect immunofluorescence examination.

However, even though the 52-kD CBP appears to associate specifically with mitotic spindle microtubules as opposed to cytoplasmic microtubules it is clearly not a mitosis-specific protein. The pool of CBPs used for antibody production were isolated via CaM affinity chromatography from predominantly interphase CHO cells. Thus, the 52-kD CBP is apparently already present in CHO cells before mitosis, and must
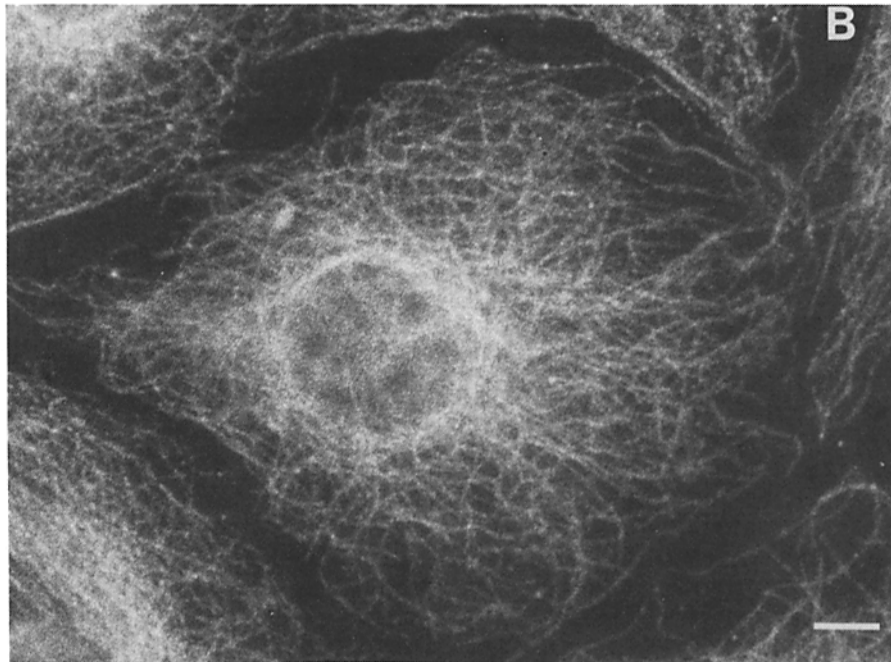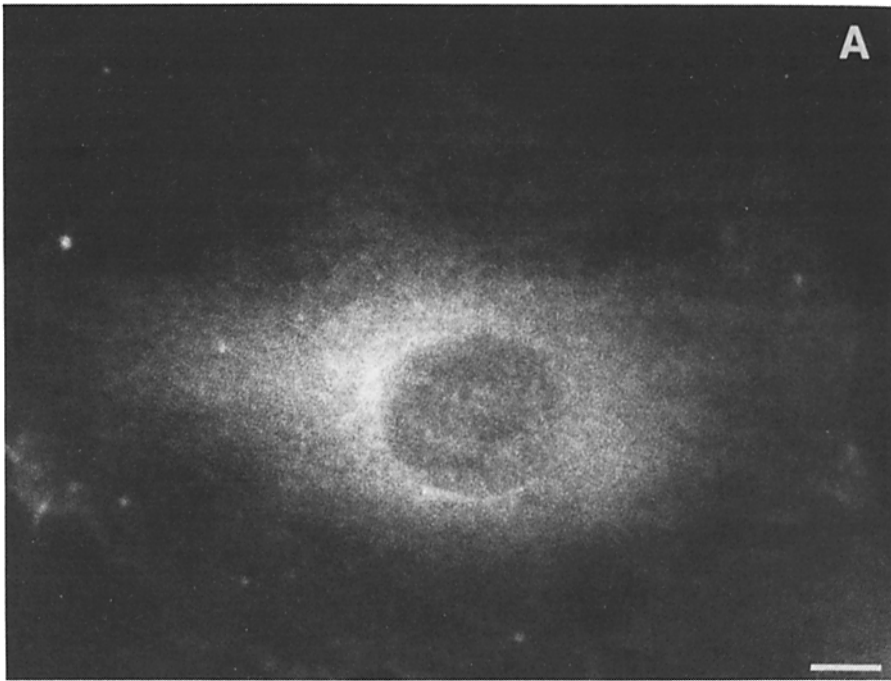
Figure 3. Fluorescence micrographs of interphase PTK-2 cells incubated with anti–CHO cell CBPs (*A*) and anti–tubulin (*B*). Note the diffuse fluorescence in the cytoplasm of *A* and the lack of cytoplasmic microtubule fluorescence, as opposed to the extensive network of cytoplasmic microtubules observed in *B* when the cells are incubated with anti-tubulin. Bars, 10 μm.

associate with the spindle apparatus as it forms. The geometric configuration of the microtubule–52-kD CBP complex is presently unclear. It is possible that the 52 kD CBP might associate with other mitotic spindle CBPs detected by gel overlay analyses (Brady, R. C., M. J. Schibler, J. R. Dedman, and F. Cabral, manuscript submitted for publication) forming an oligomeric complex which functions as a unit. On the other hand, the 52-kD CBP might interact directly with spindle microtubules. If this were the case, however, one would not expect the observed differential association of the 52-kD CBP with mitotic spindle as opposed to cytoplasmic microtubules. Another possibility is that the 52-kD CBP interacts with microtubules by binding to a MAP which is

specific for spindle microtubules and does not bind to cytoplasmic microtubules. Such a MAP could be produced in a mitosis-specific manner or sequestered during interphase and thus, would be unavailable for binding to interphase microtubules. Alternatively, it might interact with a subset or specific configuration of microtubules present only within the mitotic apparatus. The existence of other such spindle-specific MAPs has been reported (14, 15).

It is noteworthy that the affinity-purified antibodies used in this study were generated against a pool of at least ten soluble CBPs, all of which were found to react positively upon immunoblot analysis (data not shown). In spite of this, their use as a specific probe was permitted by the isolation of an
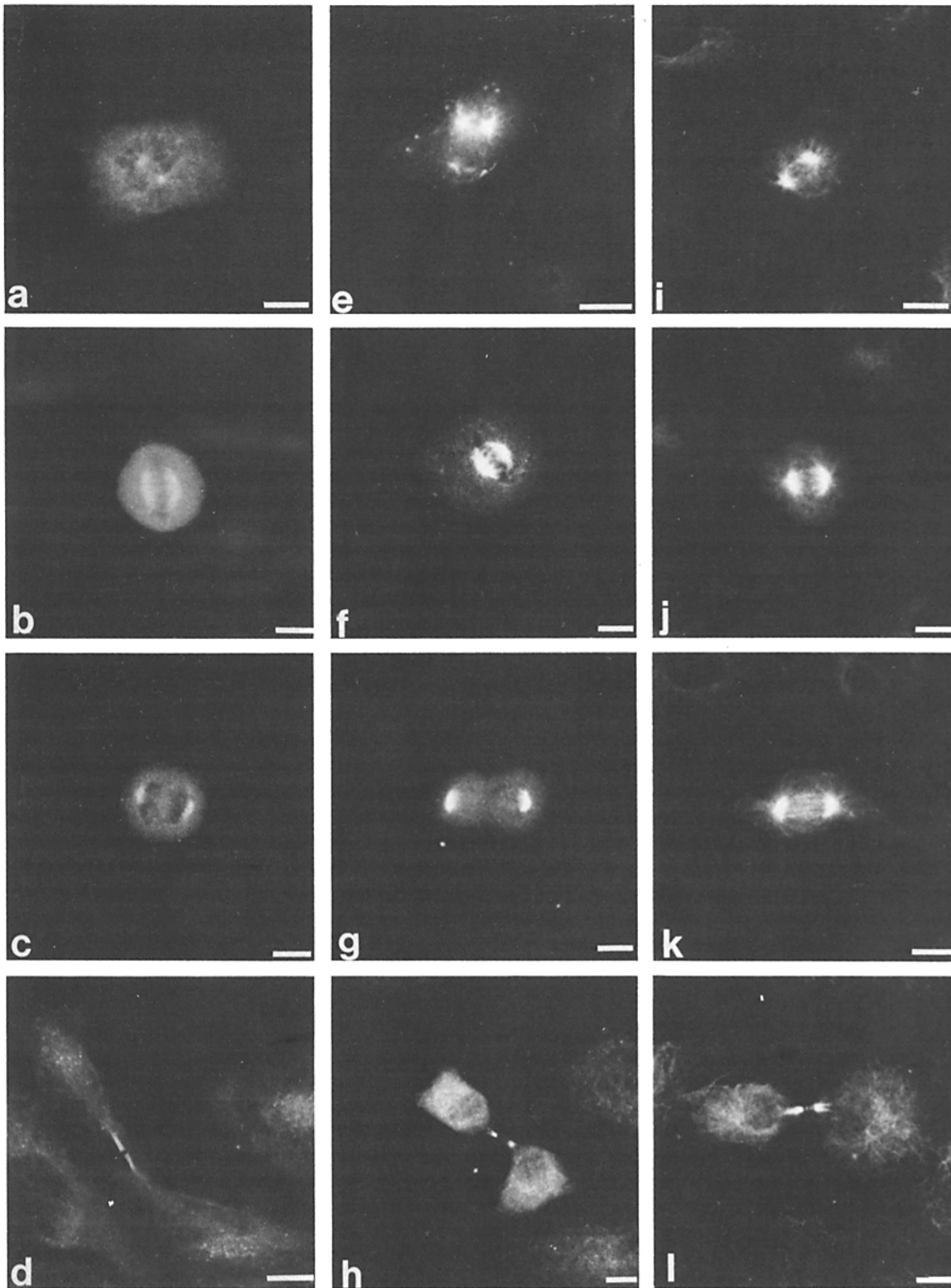
Figure 4. Fluorescence micrographs of PTK-2 cells in each stage of mitosis indicating the localization pattern of the 52-kD CBP (*a–d*), CaM (*e–h*), and tubulin (*i–l*). (*a, e,* and *i*) Prometaphase; (*b, f,* and *j*) metaphase; (*c, g,* and *k*) anaphase; and (*d, h,* and *l*) midbodies. Note the similarity in localization pattern of the 52-kD CBP and CaM within the spindle at each stage of mitosis and how this localization pattern contrasts with that of tubulin at anaphase and in the midbody. Bar, 10 μm.

individual cellular organelle, the mitotic spindle. The fact that only one of these 10 immunoreactive proteins is present in the spindle apparatus, according to immunoblot analysis, indicates that it is specifically the 52-kD CBP which is responsible for the immunofluorescence pattern observed within the mitotic spindle. Consequently, even though the antibodies generated in this study were directed against a pool of proteins, the isolation of a specific cellular structure

resulted in the identification and localization of a specific CBP.

This 52-kD CBP appears to represent the first spindle-specific CBP to be reported. The function of this protein is of paramount interest and remains to be determined. One possibility is that it represents a Ca^{2+}-CaM-activated enzyme or a structural protein which mediates the interaction of CaM with spindle microtubules. The 52-kD CBP could, for example, be a calmodulin-activated kinase whose activity might lead to phosphorylation and inactivation of mitotic spindle MAPs with concomitant decreased stability and subsequent depolymerization of spindle microtubules. Indeed, in vitro studies have shown that the phosphorylation of MAPs prevents them from functioning to promote microtubule assembly, thus, resulting in disassembly or increased flux of microtubules (16, 17). Furthermore, Schulman (34) has reported that the phosphorylation of MAP-2 is stimulated by a Ca^{2+}-CaM-dependent kinase in vitro. Thus, a precedent for such a mechanism in the mitotic spindle exists. This CaM-dependent kinase (referred to as CaM–kinase II) contains a CaM-binding subunit of 51 kD (25). However, the fact that our antibodies did not react with purified CaM-kinase II on immunoblots suggests that the 52-kD CBP identified in the mitotic spindle is similar in molecular mass to the 51-kD CaM-binding subunit of CaM kinase II but is most likely a different protein. This does not however, exclude the possibility that the 52-kD spindle CBP might represent another, as yet unidentified CaM-dependent protein kinase. We are currently examining the inherent kinase as well as phosphatase activity in isolated CHO cell mitotic spindles and preliminary results indicate that these enzymatic activities and endogenous substrates exist in these preparations (2).

In conclusion, this report describes the first CaM-binding protein to be identified in the mitotic spindle apparatus of mammalian cells. Localization studies suggest it is a spindle-specific protein which appears to be capable of mediating the association of CaM with the mitotic apparatus. Further studies will be necessary to ultimately determine the specific function of this protein and thus, the actual role of CaM in mitosis.

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