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Calmodulin Is Associated With Microtubules Forming in PTK_1 Cells Upon Release From Nocodazole Treatment

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To investigate the association of calmodulin (CaM) with microtubules (MTs) in the mitotic apparatus (MA), the distributions of CaM and tubulin were examined in cells in which the normal spindle organization had been altered. A fluorescent CaM conjugate with tetramethylrhodamine isothiocyanate (CaM-TRITC) and a dichlorotriazinyl aminofluorescein conjugate with tubulin (tubulin-DTAF) were injected into cells that had been treated with the MT inhibitor nocodazole. With moderate nocodazole concentration (0.3 μg/ml, 37°C, 4 h) in live cells, CaM-TRITC and tubulin-DTAF concentrated identically on or near the centrosomes and kinetochores. In serial sections of these cells, small MT segments were observed by transmission electron microscopy (TEM) in the regions where fluorescent protein had concentrated. When a higher drug concentration was used (3.0 μg/ml, 37°C, 4 h), no regions of CaM-TRITC or tubulin-DTAF localization were observed, and no MTs were observed when serial sections were examined by TEM. However, following release from the high-concentration nocodazole block, CaM-TRITC colocalized with newly formed MTs at the kinetochores and centrosomes. Later in the recovery period, when chromosome-to-pole fibers had formed, CaM association with kinetochores diminished, ultimately attaining its normal pole-proximal association with kinetochore MTs in cells that progressed through mitosis. We interpret these observations as supporting the hypothesis that in the MA, CaM attains a physical association with kinetochore MTs and suggest that CaM-associated MTs may be inherently more stable.

Key words: mitosis, spindle, kinetochore, centrosome

INTRODUCTION

The ubiquitous Ca^{++}-binding protein calmodulin (CaM) was shown to be a component of the mitotic apparatus (MA) a decade ago [Welsh et al., 1978]. Since that time many investigators have speculated on its role there [for review see Welsh and Sweet, 1988]. Despite a significant body of published work, however, no model for the action of CaM in the MA has been universally accepted.

The pattern of localization of CaM in the MA is similar but not identical to the microtubule (MT) distribution. For this reason, much work has focused on possible interactions of CaM with MTs. Several groups have sought to demonstrate a structural association of CaM with MTs. Welsh et al. [1979] showed that CaM is associated with the kinetochore MT (kMT) subclass and that removal of kMTs would eliminate CaM localization in the MA. DeMey and coworkers [1980] used the peroxidase–antiperoxidase method with transmission electron microscopy (TEM) to demonstrate the presence of CaM on or near spindle MTs. The appearance of labeled MTs was indistinguishable from that obtained when tubulin antibody was used. Similarly, Vantard et al. [1985], using immunogold staining and TEM, found CaM near kMTs of *Haemanthus* endosperm cells. CaM has also been found associated with the focal regions of

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asters induced by taxol treatment [DeMey et al., 1981; DeBrabander et al., 1986] and with interphase MTs polymerizing at the centrosomes after cold or hypotonic treatment [Deery et al., 1984]. Nonetheless, all of this evidence is circumstantial; although CaM can be shown to bind to tubulin in vitro, it does so with relatively low affinity [Kumagai and Nishida, 1979; Davies and Klee, 1981; Sobue et al., 1981]. Others have reported that CaM will bind with higher affinity to microtubule-associated proteins such as tau [Sobue et al., 1981], STOP proteins [Job et al., 1982], or a 52-kD protein [Brady et al., 1986], but in each of these cases the binding of CaM to the target protein is Ca^{++} dependent. We have shown that the association of CaM with the MA can occur in the absence of Ca^{++} [Sweet et al., 1987, 1988] and would therefore predict that a CaM–tubulin "bridge" protein would bind to CaM independently of Ca^{++}. Thus, the evidence for an association of CaM with MTs remains inconclusive.

We have studied the proximity of CaM to MTs in the MA under conditions in which the normal spindle MT organization has been significantly altered. We hypothesize that CaM will continue to colocalize with MTs under these conditions, suggesting a structural association of CaM with mitotic MTs.

In this report we present evidence to support this hypothesis. A fluorescent CaM conjugate with tetramethylrhodamine isothiocyanate (CaM-TRITC) and a dichlorotriazinyl aminofluorescein conjugate with tubulin (tubulin-DTAF) were injected into mitotic cells that had been treated with the MT inhibitor nocodazole. At high nocodazole levels (3.0 μg/ml, 37°C, 4 h), CaM-TRITC and tubulin-DTAF did not show any region of concentration. At lower concentrations of nocodazole (0.3 μg/ml, 37°C, 4 h), CaM-TRITC and tubulin-DTAF concentrated identically on or near the kinetochores and centrosomes. Following release from the polymerization block, CaM-TRITC first colocalized with newly polymerizing MTs at the centrosomes and kinetochores, then ultimately attained its normal pole-proximal distribution in cells that progressed through mitosis. We interpret these observations as supporting the hypothesis that in the MA, CaM maintains an intimate association with kMTs.

MATERIALS AND METHODS

Cell Culture

PtK_1 cells were a gift from Dr. J. Richard McIntosh, University of Colorado, Boulder. They were grown in Ham's F12 medium supplemented with 10% defined fetal bovine serum (Hy-Clone, Logan, VT) in a 37°C humidified incubator with a 5% CO_2, 95% air atmosphere. Culture medium was changed daily.

Protein Preparation

CaM was purified from bovine testis by Ca^{++}-dependent phenyl-Sepharose affinity chromatography [Gopalakrishna and Anderson, 1982]. TRITC (Research Organics, Cleveland, OH) was coupled to CaM (CaM-TRITC) as described by Welsh [1983].

Dichlorotriazinyl aminofluorescein (Molecular Probes, Eugene, OR) was conjugated to bovine brain tubulin (tubulin-DTAF) as described by Leslie et al. [1984].

Microinjection

For microinjection, cells were grown on 25-mm diameter #1 thickness glass coverlsips affixed with a silicone adhesive to the underside of a 35-mm diameter plastic tissue culture dish (Falcon, Oxnard, CA) in which a 20-mm diameter hole had been bored. With this construction, we were able to perform microinjection and imaging without remounting the coverslip.

Micropipettes were pulled from self-filling capillary tubes (cat. #1B100F, World Precision Instruments, New Haven, CT) using a Sutter Instruments Sachs-Flaming PC-84 micropipette puller. Micropipettes were positioned with a Leitz micromanipulator. Injection was accomplished by applying pressure to a 50-ml syringe connected to the micropipette via Silastic™ tubing (cat. #602-175, Dow Corning, Midland, MI).

Imaging

Cells were viewed through a Leitz 63× 1.4NA phaco 4 plan apo fluorescence objective mounted on a Leitz Diavert microscope equipped for epifluorescence illumination. The microscope is mounted on a Micro-G vibration isolation table to minimize movement during microinjection.

Images of cells were obtained with a DAGE/MTI ISIT video camera coupled to the microscope through a Leitz Vario-Orthomat zoom lens adapter. Video output from the camera was digitized by an International Imaging Systems Model 75 image processor interfaced to a Masscomp DP500 computer. All images consisted of a sliding average over 16 video frames, with an out-of-focus image interactively subtracted to enhance contrast and reduce background. Processed images were photographed either directly from a Mitsubishi 13″ monitor or with a Matrix Instruments recorder equipped with a 35-mm film back.

Image Processing

Fluorescence distributions were compared using the addition and subtraction capabilities of the image processor. First, a binary image (in which the regions of most intense fluorescence are white and other areas are

black) was interactively created from each fluorescence image. For comparison of tubulin-DTAF with CaM-TRITC, the CaM binary image was subtracted from the tubulin-DTAF image and the difference image was added to a lower threshold binary image in order to delineate the cell margins. In the resulting image, regions containing only tubulin-DTAF fluorescence appear white, regions containing only CaM-TRITC appear black, and the regions of intersection, which contained both fluorochromes, appear light gray.

For comparison of fluorescence distributions with phase contrast images, a binary image representing the fluorescence distribution was superimposed onto the phase contrast image. In the resulting image, regions of fluorescence localization appear as bright white spots.

Drug Treatments and Experimental Protocol

For nocodazole treatment, nocodazole (Sigma, St. Louis, MO) was dissolved in DMSO (Sigma) at 100 μg/ml. Aliquots of this stock solution were added directly to the culture medium to achieve the desired concentrations.

For all of the experiments described, cells were treated with either 0.3 or 3.0 μg/ml of nocodazole for 4 h at 37°C. Immediately prior to microinjection, the culture dish was removed from the incubator and placed on the microscope stage. A mixture of tubulin-DTAF (1 mg/ml) and CaM-TRITC (1 mg/ml) was injected into 50–100 cells during a period of 20–30 min, and the culture dish was returned to the incubator. After 30 min, the medium containing nocodazole was replaced with fresh, nocodazole-free medium. The medium was changed every 10 min thereafter. Following a recovery period of 0 to 60 min, the culture dish was removed from the incubator and, for dishes that were to be processed for TEM, the medium was replaced with a fixative consisting of 2.5% glutaraldehyde, 3% formaldehyde in PBS. The dish was taken immediately to the fluorescence microscope, and microinjected cells were relocated. Fluorescence and phase contrast images of several cells in the dish were obtained, and cells in fixative were processed for electron microscopy as described below.

Correlative Electron Microscopy

Treated cells were located for electron microscopy in the following manner: an injected cell was located after fixation by the presence of fluorescent analog. A square was scribed around this cell with a micropipette, and fluorescence and phase contrast video images were taken. Prior to embedding for TEM, the cell location was recorded using the "cell list" function of an ACAS 470 (Meridian Instruments, Okemos, MI). After embedding, cells were relocated on the ACAS 470. A thin film of ink was placed on the bottom surface of the plastic, and the laser of the ACAS 470 was used at sufficient power output to ablate a region of the ink circumscribing the regions in the plastic containing each cell of interest. These regions could be visually located without magnification and were then cut out and remounted for serial sectioning.

Cells were prepared for TEM by standard techniques and serially sectioned. Regions of fluorescent protein localization were identified in the serial sections by comparison with fluorescence and phase contrast images. Centrosomes and kinetochores were relocated in successive sections to facilitate correlation in three dimensions; representative sections are shown in the figures.

RESULTS

When cells were treated with 0.3 μg/ml of nocodazole for 4 h at 37°C, the only microtubules that remained were short MT segments at or near the kinetochores and centrosomes (not shown). Mitotic cells (mitotic in the sense that the chromosomes were condensed) displayed no evidence of spindle structure (Fig. 1c, compare Fig. 2c). In cells that were injected with a mixture of CaM-TRITC and tubulin-DTAF however, fluorescence images revealed concentration of both tubulin-DTAF (Fig. 1a) and CaM-TRITC (Fig. 1b) in a punctate pattern among the condensed chromosomes. This distribution was entirely different from the distributions of CaM and tubulin in normal mitotic cells (Fig. 2a,b).

We compared the CaM-TRITC and tubulin-DTAF distributions in 0.3 μg/ml nocodazole-treated cells with the aid of an image processor. Superposition of the fluorescence images onto each other (Fig. 3a) indicated that the regions of CaM-TRITC concentration appeared to coincide with the regions of tubulin-DTAF concentration. Superposition of the CaM-TRITC fluorescence image onto a phase contrast image of the cell (Fig. 3b) suggested that the regions of fluorescence concentration were at or on the kinetochores. Although the hardware and software for "rubber-sheeting" are available on our image processing system, no attempts were made to use this capability to improve the registration of the fluorescence and/or phase contrast images.

When cells were treated with 3.0 μg/ml of nocodazole for 4 h at 37°C, MTs were completely removed. When kinetochores were followed through serial sections with TEM, no evidence of MTs was found (Fig. 4c). Similarly, centrosomes were present, but MTs were not detected in their vicinity (Fig. 4d). In cells that were injected with a mixture of CaM-TRITC and tubulin-DTAF, fluorescence images did not reveal any particular

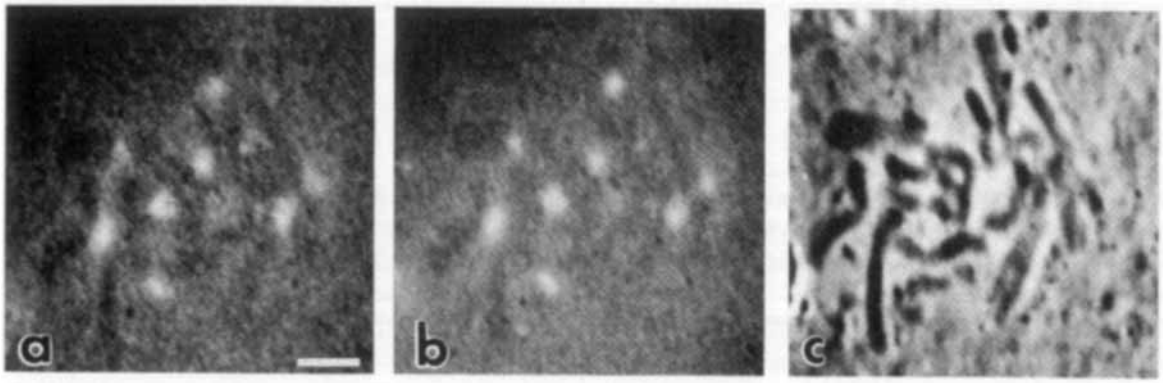

Fig. 1. PtK_1 cell treated with nocodazole (0.3 μg/ml, 4 h, 37°C) and injected with tubulin-DTAF and CaM-TRITC. **a:** Tubulin-DTAF fluorescence image; bar, 2.5 μm. **b:** CaM-TRITC fluorescence image. **c:** Phase contrast image.

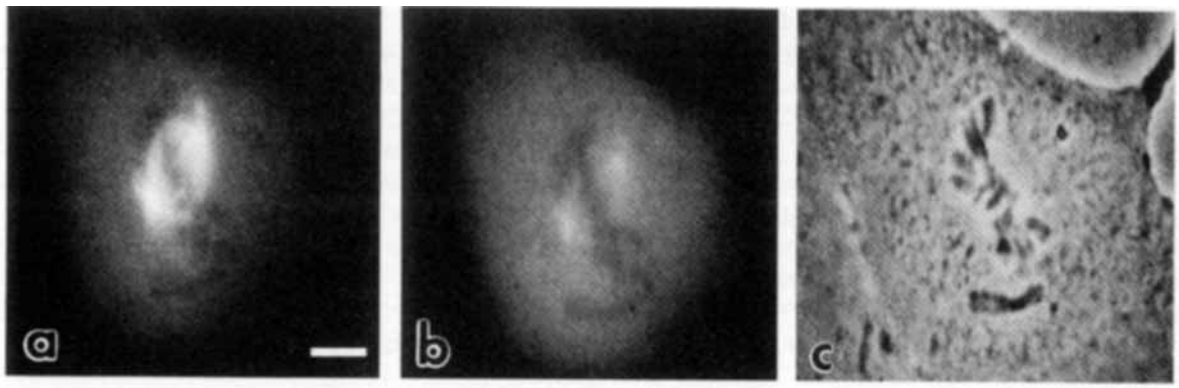

Fig. 2. Metaphase PtK_1 cell injected with tubulin-DTAF and CaM–TRITC. **a:** Tubulin–DTAF fluorescence image; bar, 5μm. **b:** CaM–TRITC fluorescence image. **c:** Phase contrast image.

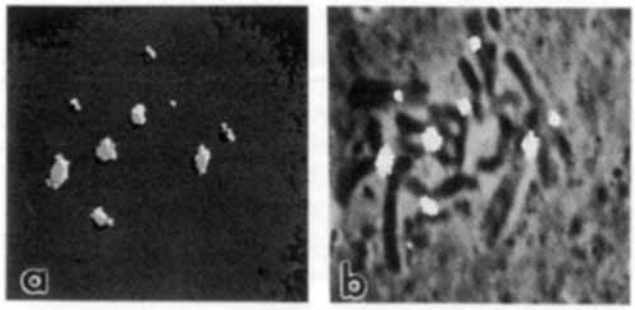

Fig. 3. Comparison images for the PtK_1 cell shown in Figure 1. No attempt was made to register the raw images. **a:** Comparison of tubulin-DTAF and CaM-TRITC distributions. In this image, white regions indicate the presence of only tubulin-DTAF, black regions indicate the presence of only CaM-TRITC, and gray regions of intersection indicate the presence of both fluorochromes. **b:** Comparison of CaM-TRITC fluorescence distribution with phase contrast image. In this image, white regions in the vicinity of the chromosomes indicate the presence of fluorescent protein.

regions of concentration of fluorescent proteins (Fig. 4a,b).

Previous work had shown that removal of nocodazole from mitotic cells is followed by rapid recovery of spindle structure [DeBrabander et al., 1980, 1981b]. We began with cells that had been treated with 3.0 μg/ml nocodazole at 37°C for 4 h and compared CaM-TRITC and tubulin-DTAF distributions after short (10 min), intermediate (30 min), and long (60 min) recovery periods.

Short Recovery (10 min)

In nocodazole-treated cells (3.0 μg/ml, 37°C, 4 h) that were allowed to recover for 10 min at 37°C prior to fixation, we observed a fluorescence pattern that was identical to the pattern observed in microinjected cells that had not been released from 0.3 μg/ml nocodazole treatment. The distributions of tubulin-DTAF (Fig. 5a) and CaM-TRITC (Fig. 5b) appeared for the most part to coincide. When serial sections of these cells were examined with TEM, MTs were observed near kinetochores

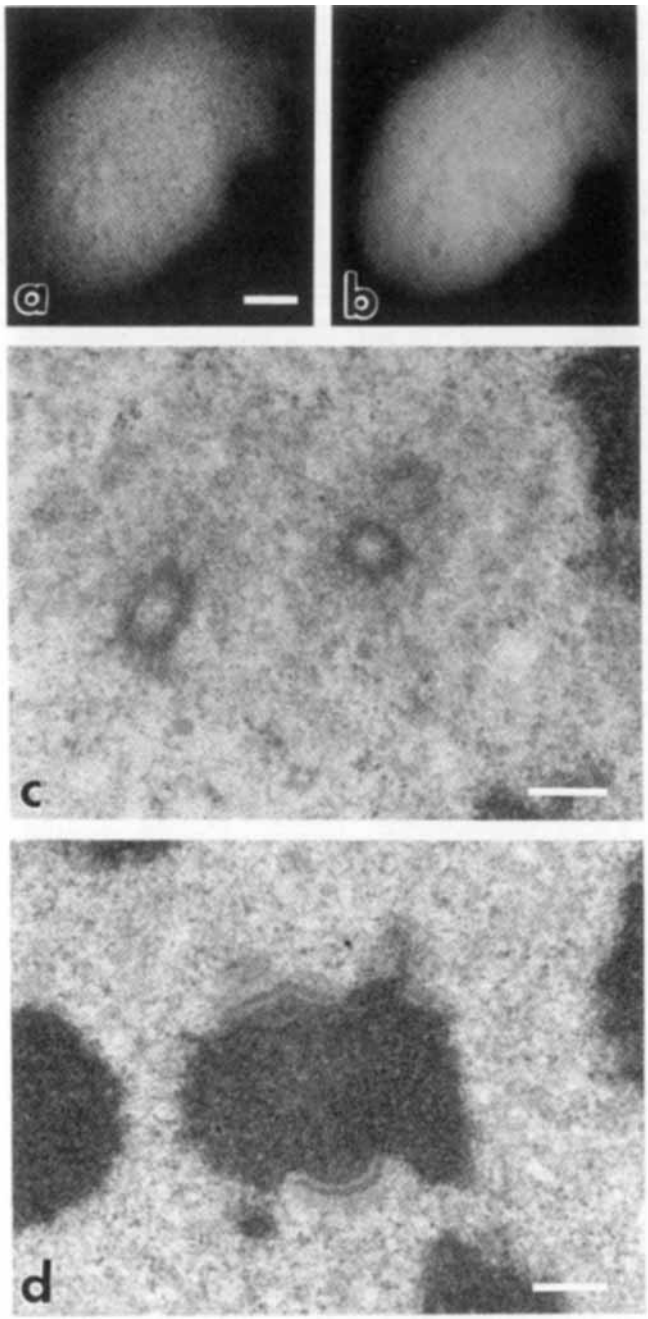

Fig. 4. PtK_1 cell treated with nocodazole (3.0 μg/ml, 4 h, 37°C) and injected with tubulin-DTAF and CaM-TRITC. **a:** Tubulin-DTAF fluorescence image; bar, 5 μm. **b:** CaM-TRITC fluorescence image. **c:** TEM image of one serial section showing a kinetochore (from a set of serial sections comprising the entire volume of the cell; bar, 0.25 μm. **d:** TEM image from a serial section of a centrosome; bar, 0.33 μm.

(Fig. 5c) and centrosomes (Fig. 5d). Comparison of TEM sections with fluorescence and phase contrast images indicated that regions of concentration of tubulin-DTAF and CaM-TRITC corresponded with regions containing MTs (the regions shown by TEM in Fig. 5c and d correspond to the boxed regions in a).

Intermediate Recovery (30 min)

In nocodazole-treated cells (3.0 μg/ml, 37°C, 4 h) that were allowed to recover for 30 min at 37°C prior to fixation, the distribution of tubulin-DTAF (Fig. 6a) and CaM-TRITC (Fig. 6b) did not always completely coincide. In the TEM image of the cell shown, MTs appear to traverse between chromosomes and a focus of MTs that surrounds a region of electron-dense material, which may be a pole (the region shown by TEM in Fig. 6c corresponds to the boxed region in a; the electron-dense material is indicated by an arrow in c). The appearance of the CaM-TRITC fluorescence distribution suggests that CaM may be somewhat more concentrated near the pole region.

Long Recovery (60 min)

Cells that were fixed 60 min following removal of nocodazole were found in various stages of recovery. Although the most common finding was an appearance similar to late prometaphase or metaphase (with multipolar spindles somewhat more common), cells were observed in anaphase, telophase, and after completion of mitosis. In all cases, the distribution of CaM and tubulin were identical to those found in untreated cells: CaM was concentrated in pole-proximal regions (Fig. 7a,b). When these cells were relocated and examined by serial sectioning and TEM, kinetochores and centrosomes that appeared normal were observed (Fig. 7c,d).

DISCUSSION

After microinjection into mitotic cells that had been treated with a moderate dose of nocodazole (0.3 μg/ml, 37°C, 4 h) or in microinjected cells immediately following release from treatment with a high dose of nocodazole (3,0 μg/ml, 37°C, 4 h), CaM-TRITC and tubulin-DTAF were observed to be concentrated in identical patterns on or near the kinetochores and centrosomes. The regions of fluorescence localization corresponded with areas where MTs were observed by TEM. In cells that had been released from polymerization blockade, as the cells resumed what appeared to be a normal mitotic progression, CaM-TRITC ultimately assumed its usual distribution in spindles that appeared normal when examined by TEM. We conclude that CaM-TRITC colocalizes with MTs that are growing from kinetochores and centrosomes. This observation supports a model of the MA in which CaM maintains a physical association with kMTs.

Our conclusions rely on several assumptions. Be-

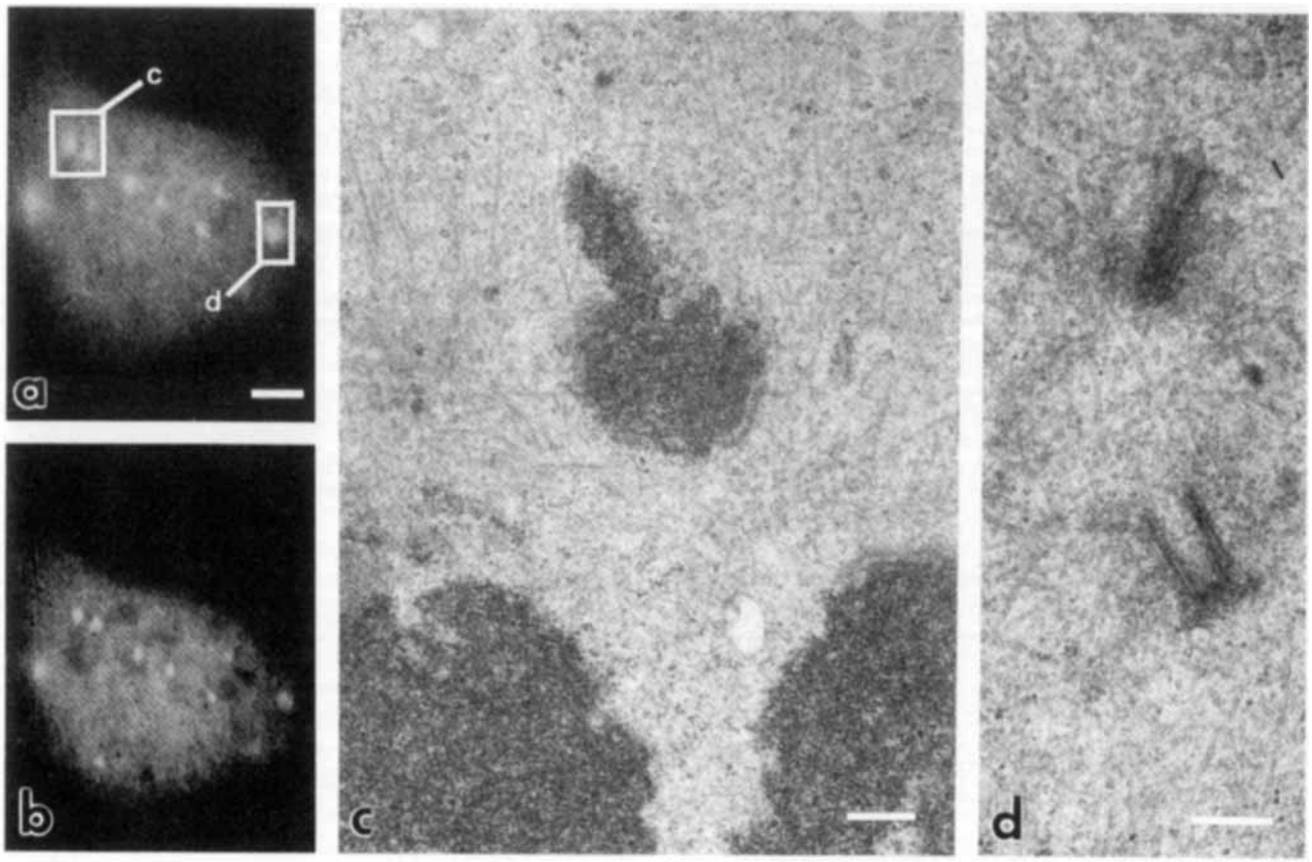

Fig. 5. PtK_1 cell treated with nocodazole (3.0 μg/ml, 4 h, 37°C), injected with tubulin-DTAF and CaM-TRITC, then allowed to recover from nocodazole treatment for 10 min at 37°C. **a:** Tubulin-DTAF fluorescence image. Area in rectangle labeled **c** is shown in panel c and area in rectangle labeled **d** is shown in panel d. Bar, 5 μm. **b:** CaM-TRITC fluorescence image. **c:** TEM image from a serial section of the region indicated in a, showing a kinetochore with growing MTs; bar, 0.25 μm. **d:** TEM image from a serial section of the region indicated in a showing a centrosome; bar, 0.33 μm.

yond the assumption that we have not overlooked fixation or microscopy artifacts, the most significant assumption is that MTs that are forming upon release from nocodazole treatment are equivalent in composition and structure to normal spindle MTs. Nocodazole is presumed to work by binding to free tubulin dimers, thus lowering the free tubulin concentration and inhibiting first nucleation and then, at higher concentrations, polymerization [Hoebeke et al., 1976]. When mitotic cells are released from nocodazole treatment by washing out the drug with fresh, drug-free medium, microtubules begin to grow in the regions near the centrosomes and kinetochores [DeBrabander et al., 1980, 1981b]. These MTs elongate, ultimately forming fibers that traverse between a kinetochore and a centrosome or between a kinetochore and a "pseudopole" (a focus of MTs which does not contain a centriole). After 30 to 60 min in nocodazole-free medium, mitotic cells attain an apparently normal metaphase configuration and progress from metaphase through telophase [DeBrabander et al., 1980, 1981b]. We believe that because such cells can complete mitosis, there is sufficient evidence to warrant the assumption that the MTs formed upon nocodazole recovery are equivalent to normal spindle MTs. Although these cells, as a result of nocodazole exposure, do not take the normal route to traverse mitosis, we feel that they provide an adequate alternative system in which one can examine the association of CaM with MTs in the MA.

Using this experimental system, we observed localization of tubulin-DTAF in the MA of cells in the presence of a moderate concentration (0.3 μg/ml) of nocodazole (Fig. 1), but not at a higher concentration (3.0 μg/ml) (Fig. 4). These observations are consistent with those of DeBrabander et al. [1980, 1981b]. Although it is clear in these cells that most MT polymerization was completely blocked, at the lower concentration some kinetochores were seen with short MT segments nearby (not shown). This indicates, as suggested by DeBrabander et al. [1980, 1981b], that the

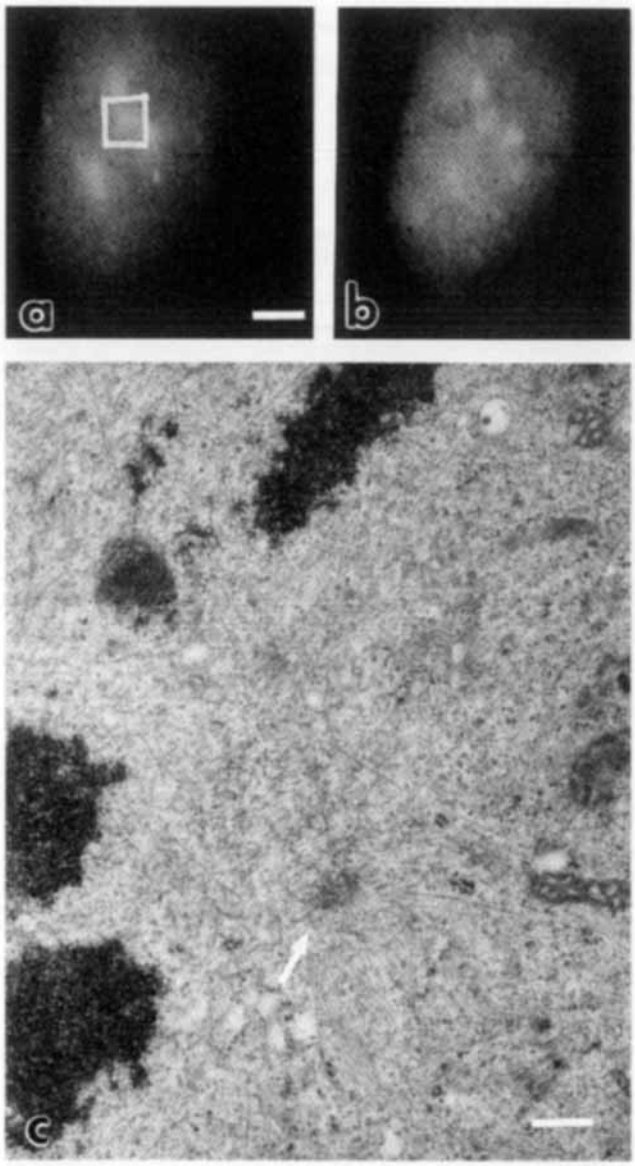

Fig. 6. PtK_1 cell treated with nocodazole (3.0 μg/ml, 4 h, 37°C), injected with tubulin-DTAF and CaM-TRITC, then allowed to recover from nocodazole treatment for 30 min at 37°C. **a:** Tubulin-DTAF fluorescence image. Area in box is shown in panel c. Bar, 5 μm. **b:** CaM-TRITC fluorescence image. **c:** TEM image from one of a set of serial sections of the region indicated in a. Arrow indicates electron-dense region that may be a centrosome; bar, 0.5 μm.

environment near the kinetochores may permit MT nucleation at a significantly lower free tubulin concentration than elsewhere in the MA. Microinjected tubulin-DTAF may increase the free tubulin concentration in these cells and thus contribute also to MT polymerization near kinetochores. We feel, however, that the microinjected tubulin-DTAF was not solely responsible for the presence of these MTs, because we saw similar MT segments on kinetochores in uninjected cells. It is also possible that tubulin-DTAF is concentrating in structures not recognizable ultrastructurally as MTs. Indeed, in the cell shown in Figure 4a, which was treated with the high concentration of nocodazole (3.0 μg/ml) and in which no MTs were observed during TEM of serial sections, there are a few areas in which tubulin-DTAF shows slight concentration.

We also observed that the CaM-TRITC distribution coincides with all areas of tubulin-DTAF concentration before and during initial recovery from nocodazole treatment. This implies that there is an intimate association between CaM and tubulin, presumably in the form of MTs; it is difficult to conceive of a simple MT-independent scheme that would account for the appearance of CaM near a kinetochore (CaM is not normally observed concentrated near kinetochores) concurrent with the appearance of MTs. These observations are consistent with those reported in abstract form by DeMey et al. [1981], using the immunogold and peroxidase–antiperoxidase methods. However, their report of CaM association with newly forming MTs in cells released from nocodazole treatment has not been published in full.

In the initial stages of spindle reformation following release of cells from drug treatment, CaM was distributed identically to MTs near the kinetochores. However, as recovery progressed, CaM colocalization with MTs became more concentrated in regions near poles and pseudopoles. This apparent redistribution of CaM, from an association with both kinetochores and poles early in recovery to a pole-proximal association with pole-to-chromosome MTs later in recovery, has at least two potential explanations. It is possible that the association of CaM with the kMTs is restricted to the nucleation phase and that CaM dissociates from MTs once significant elongation occurs; later in recovery, as a functional MA is formed, CaM would become most prevalent near the poles because it is there that the majority of MT nucleation is thought to occur. The second possibility is that CaM is continuously associated with MTs at their nucleation site, but MTs that nucleate at the kinetochores early in recovery are not retained as the cell reenters mitosis, perhaps as a result of these MTs having the incorrect polarity. In either case it is clear that, early in recovery, CaM is associated with MTs at their nucleation sites whether nucleation occurs near the kinetochores or the centrosomes.

The association of CaM with MTs at their site of nucleation suggests that CaM-associated MTs (CaM^+-MTs) might be inherently more stable. In cells treated with the moderate concentration of nocodazole (0.3 μg/ml), the only MTs observed appeared to be CaM-associated. Because these cells had almost certainly entered mitosis in the presence of nocodazole [Debrabander

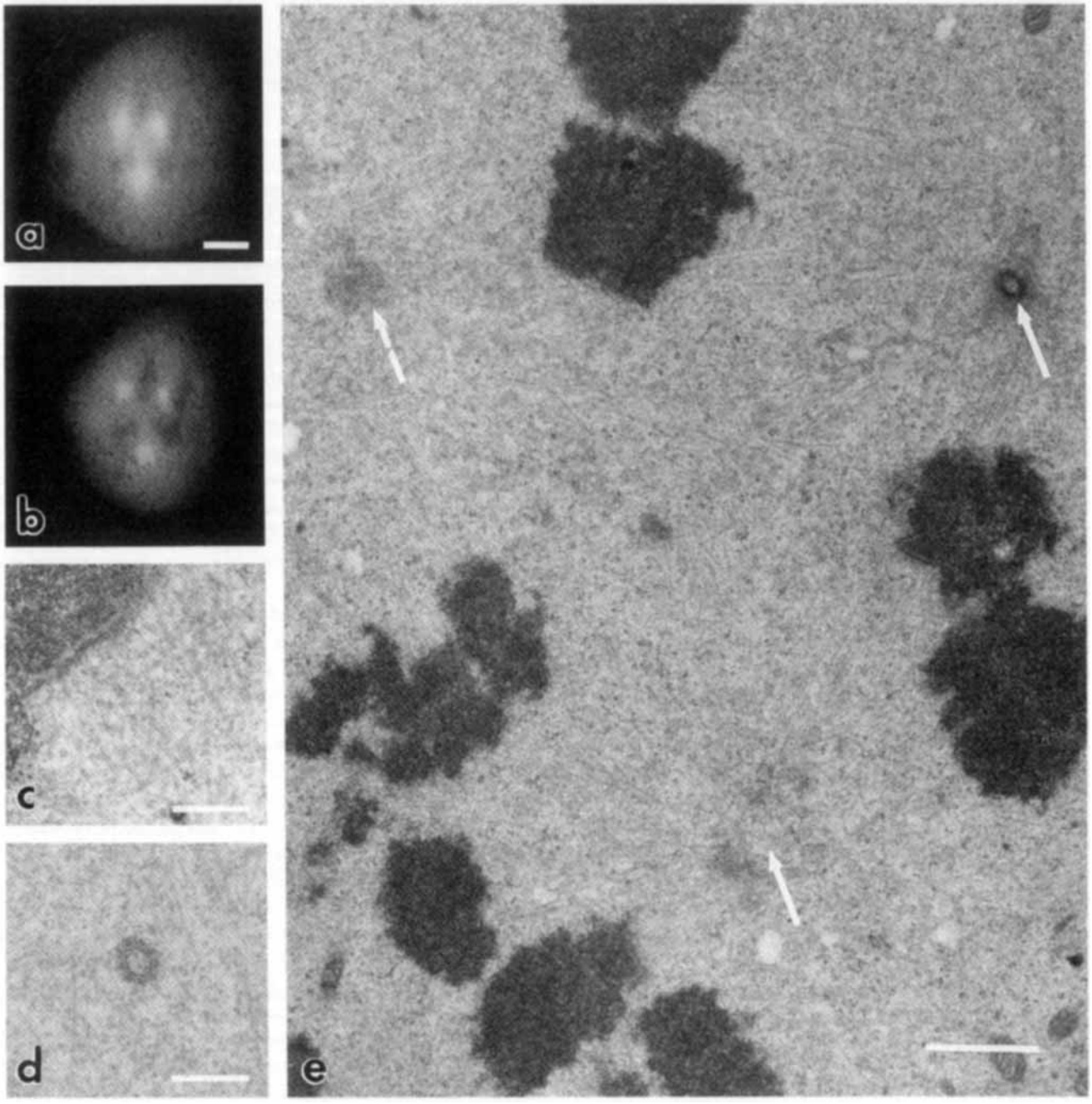

Fig. 7. PtK_1 cell treated with nocodazole (3.0 μg/ml, 4 h, 37°C), injected with tubulin-DTAF and CaM-TRITC, then allowed to recover from nocodazole treatment for 60 min at 37°C. **a:** Tubulin-DTAF fluorescence image; bar, 5 μm. **b:** CaM-TRITC fluorescence image. **c:** TEM image of a kinetochore from one of a set of serial sections; bar, 0.25 μm. **d:** TEM image of a centrosome from one of a set of serial sections; bar, 0.33 μm. **e:** Serial section TEM image of the entire MA. Arrows indicate centrosomes. Bar, 1 μm.

et al., 1981a,b] and were able to incorporate tubulin-DTAF into MTs, the observed MTs would have polymerized in the presence of the drug. In addition, the first MTs that appeared upon removal of nocodazole from cells treated with the higher drug concentration (3.0 μg/ml) were CaM associated. Our interpretation of this observation is that the presence of CaM in association with these MTs serves to enhance the MT stability, allowing polymerization in the presence of nocodazole. This is consistent with our recent observation that CaM can act to stabilize MTs in the absence of Ca^{++} [Sweet and Welsh, 1986; Sweet et al., 1988], but somewhat in con-

flict with the long-standing hypothesis that CaM will promote MT disassembly in a Ca^{++}-dependent fashion [Marcum et al., 1978; Job et al., 1981; Keith et al., 1983].

Although the concept that CaM might contribute to MT stability [Sweet and Welsh, 1986; Sweet et al., 1988] is not widely held, the idea that CaM^+-MTs are more stable is well documented. Indeed, in virtually every study that has demonstrated a colocalization of CaM with MTs, CaM was found in association with an MT subclass that could be defined as "stable" (to cold: Welsh et al. [1979], Deery et al. [1984]; to nocodazole: Sweet and Welsh [1985]; taxol stabilized: DeMey et al. [1981], DeBrabander et al. [1986]). Moreover, the hypothesis that CaM contributes to MT stability is not unreasonable because other nontubulin protein components of MTs have been shown to influence MT stability. For example, it has been reported that addition of MT-associated proteins to MTs in vitro can increase their stability [Murphy and Borisy, 1975; Sloboda et al., 1976; Job et al., 1985].

We thus propose the following model to account for our observations: early during recovery from nocodazole, conditions in the cell require CaM association for MT assembly. It is likely that the "CaM^+" configuration is dictated by the nucleation sites; CaM^+-MTs are only found in the vicinity of centrosomes and kinetochores. One can speculate that taxol, which will induce CaM^+-MT arrays [DeMey et al., 1981; DeBrabander et al., 1986], may mimic the centrosome/kinetochore nucleation site. This ability of CaM^+-MTs to polymerize in the MA, when CaM-free MTs do not, may explain why spindle MTs are able to form during prophase, while at the same time the MTs of the cytoplasmic MT complex are disappearing; the MTs formed at the centrosome during this period are CaM^+-MTs. The reorganization of CaM to its usual pole-proximal localization later in the recovery period most likely reflects the cell regaining its normal ability to regulate MT assembly and rebuild a functional spindle.

Finally, the association of CaM with MTs forming near kinetochores suggests that the MT binding/nucleation sites in the vicinity of the kinetochore may be specific for CaM^+-MTs. If this were the case, it might explain how CaM becomes preferentially associated with kMTs at metaphase; during prometaphase, kinetochores might only be able to nucleate or capture (depending on which model of mitosis one uses) MTs that are CaM-associated.

In summary, we have found that CaM will colocalize with MTs repolymerizing in cells recovering from nocodazole treatment. This observation supports the hypothesis that CaM maintains a physical association with kMTs in the MA and suggests that the CaM^+-MT configuration may be inherently more stable.

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