Calmodulin-Microtubule Association in Cultured Mammalian Cells

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ABSTRACT A Triton X-100-lysed cell system has been used to identify calmodulin on the cytoskeleton of 3T3 and transformed SV3T3 cells. By indirect immunofluorescence, calmodulin was found to be associated with both the cytoplasmic microtubule complex and the centrosomes. A number of cytoplasmic microtubules more resistant to disassembly upon either cold (0–4°C) or hypotonic treatment, as well as following dilution have been identified. Most of the stable microtubules appeared to be associated with the centrosome at one end and with the plasma membrane at the other end. These microtubules could be induced to depolymerize, however, by micromolar Ca^{++} concentrations. These data suggest that, by interacting directly with the microtubule, calmodulin may influence microtubule assembly and ensure the Ca^{++}-sensitivity of both mitotic and cytoplasmic microtubules.

Since the discovery that Ca^{++} inhibits microtubule assembly in vitro (36), subsequent studies have indicated that several factors can influence the degree of Ca^{++}-sensitivity. These findings have implicated intracellular Ca^{++} in the regulation of microtubule assembly in vivo. Tubulin possesses high intrinsic sensitivity to micromolar Ca^{++} concentrations (1, 16) and this sensitivity can decrease to the millimolar range for tubulin-containing microtubule-associated proteins (MAPs)[1] that co-purify with tubulin following cycles of assembly-disassembly (20, 26). Assembly in crude brain extracts exhibits high Ca^{++}-sensitivity (12, 29) and addition of crude extract to cycle-purified tubulin restores the sensitivity (25).

A class of proteins that co-purifies with microtubules from rat brain and that greatly increases microtubule stability in vitro (14, 21, 35) (stable tubule-only proteins [STOPs]), has recently been identified. Polymerized microtubules containing concentrations of STOPs substoichiometric to that of tubulin are stable at 0–4°C or in the presence of millimolar Ca^{++}, conditions that normally would rapidly depolymerize microtubules (13). However, addition of ATP or the Ca^{++}-dependent regulatory protein, calmodulin, renders these microtubules labile to cold or Ca^{++}, respectively.

The decreased Ca^{++}-sensitivity of microtubules stabilized by MAPs and STOPs, for example, must be modulated by some factor(s) present in crude extracts. Calmodulin is one candidate because it was initially demonstrated that calmodulin antibodies specifically stain cold-stable kinetochore-to-pole and interzonal microtubules in the mitotic apparatus (38). In addition, it was suggested that Ca^{++}/calmodulin could mediate microtubule disassembly in vitro, albeit at high calmodulin to tubulin molar ratios (19, 20, 24, 27). Further support for this hypothesis comes from the observations that MAP2 (28) and STOPs (14) will bind to a calmodulin affinity column in a Ca^{++}-dependent fashion. Finally, cold-stable microtubules resistant to Ca^{++} alone, readily depolymerize at substoichiometric calmodulin concentrations in vitro (13). However, despite the above findings, to date, the presence of calmodulin on interphase cytoplasmic microtubules has not been demonstrated.

Following incubation of cells in Triton X-100, most of the plasma membrane is solubilized and unpolymerized tubulin is readily diluted and extracted, creating a permeabilized cell system for studying the microtubule-subunit dissociation reaction (2, 30). The cells are also well extracted for subsequent immunofluorescent studies. In the present report, we have employed such a Triton-lysed cell system to study the localization of calmodulin upon disassembly of the cytoplasmic microtubule complex (CMTC) and following microtubule reassembly. We observe a number of cytoplasmic microtubules that do not disassemble upon either cold treatment or various dilution conditions but do disassemble in the presence of micromolar Ca^{++} concentrations. Indirect immunofluorescence studies indicate that calmodulin is associated with cytoplasmic microtubules and the centrosomes.

[1] *Abbreviations used in this paper:* CMTC, cytoplasmic microtubule complex; MAPs, microtubule-associated proteins; PEG, polyethylene glycol; RB, reassembly buffer; STOPs, stable tubule-only proteins.

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MATERIALS AND METHODS

Cell Culture and Lysis: Swiss mouse fibroblast cells (from American Type Tissue Culture Collection) were grown on glass coverslips for 24–48 h to 90–95% confluence in plastic petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% (for 3T3 cells) or 5% (for SV40-3T3) fetal calf serum at 10% CO_2 and were used in all experiments. Immediately before lysis, cells on coverslips were removed from medium and either washed for 10 s at 25°C in 0.08 M PIPES buffer 6.9 containing 10 mM EGTA, 1 mM $MgCl_2$, and 0.1 mM guanosine triphosphate (extraction buffer) essentially as described by Schliwa et al. (30) or in extraction buffer containing 4% polyethylene glycol (PEG). Cells were either lysed in extraction buffer containing 0.05% Triton X-100 for 1.5 min followed by a 30-s wash in extraction buffer to remove the detergent or in 0.5% Triton and washed in PEG buffer. An additional 30–90 s wash in 1 mM EGTA buffer was carried out to reduce the EGTA concentration. Microtubule disassembly was then studied by inverting the cells on coverslips over a drop (0.15 ml) of microtubule reassembly buffer (RB) containing 0.08 M PIPES pH 6.9, 1 mM EGTA-$MgCl_2$, and 0.1 mM guanosine triphosphate at the indicated temperatures. When Ca^{++} was present, the free Ca^{++} concentration was calculated using the Ca^{++}-EGTA buffer system described previously (20, 27).

Immunofluorescent Staining and Microscopy: Either immediately after cells were lysed and washed or after their exposure to various microtubule-depolymerizing buffer conditions, cells were fixed for 30 min at 25°C with 3% formaldehyde in PIPES-RB containing 1% DMSO and processed for indirect immunofluorescence using sheep antibody to tubulin or calmodulin and fluorescein-tagged rabbit-anti-sheep IgG as described previously in detail (5). Monospecific antibody to calmodulin was prepared in sheep against native rat testis protein and was purified on a calmodulin affinity column (7).

When double antibody staining was performed, fixed cells were incubated at 37°C with mouse IgG_1 monoclonal antibody followed by polyclonal rabbit-antivimentin for 1 h each. Cells were then incubated with a mixture of fluorescein-tagged goat-anti-mouse and rhodamine goat-anti-rabbit for 45 min. The mouse monoclonal antibody (#4-22.6) was generated against the α-subunit of *Chlamydomonas* flagellar tubulin. Polyclonal antibody against purified Chinese hamster ovary vimentin was prepared in rabbits (6) and the raw serum diluted prior to use. Cells were examined with a Leitz Orthoplan microscope equipped with epiillumination and photographs were recorded on Tri-X Pan film.

RESULTS

Localization of Calmodulin on Microtubules

A complete cytoplasmic microtubule complex (CMTC) was observed (Fig. 1 *A*) when cells were first fixed in formaldehyde and followed by lysis in cold acetone as described previously (4). Earlier studies from this laboratory have found calmodulin to be associated predominately with a cold-stable population of microtubules of the mitotic apparatus (38). The failure to observe anticalmodulin staining of any cytoplasmic microtubules in previous studies may have been at least partially related to the protocol of fixation prior to lysis, since this generally yields high fluorescent background and diffuse staining throughout the cytoplasm of interphase cells (Fig. 1 *B*). However, when cells were extracted before fixation, a more definitive immunofluorescent staining pattern was obtained. When cells were lysed in 0.5% Triton-10 mM EGTA-RB for 90 s and subsequently washed in 4% PEG to stabilize microtubules, the CMTC was stained by calmodulin antibody (Fig. 1 *D*), and was similar to that observed with antitubulin (Fig. 1 *C*). Microtubule staining was not obtained with antibody that did not bind to the calmodulin-affinity column (Fig. 1 *E*), or if anticalmodulin was preabsorbed for 1 h at 25°C and overnight at 2°C with a 60-fold molar excess of purified calmodulin (Fig. 1 *F*).

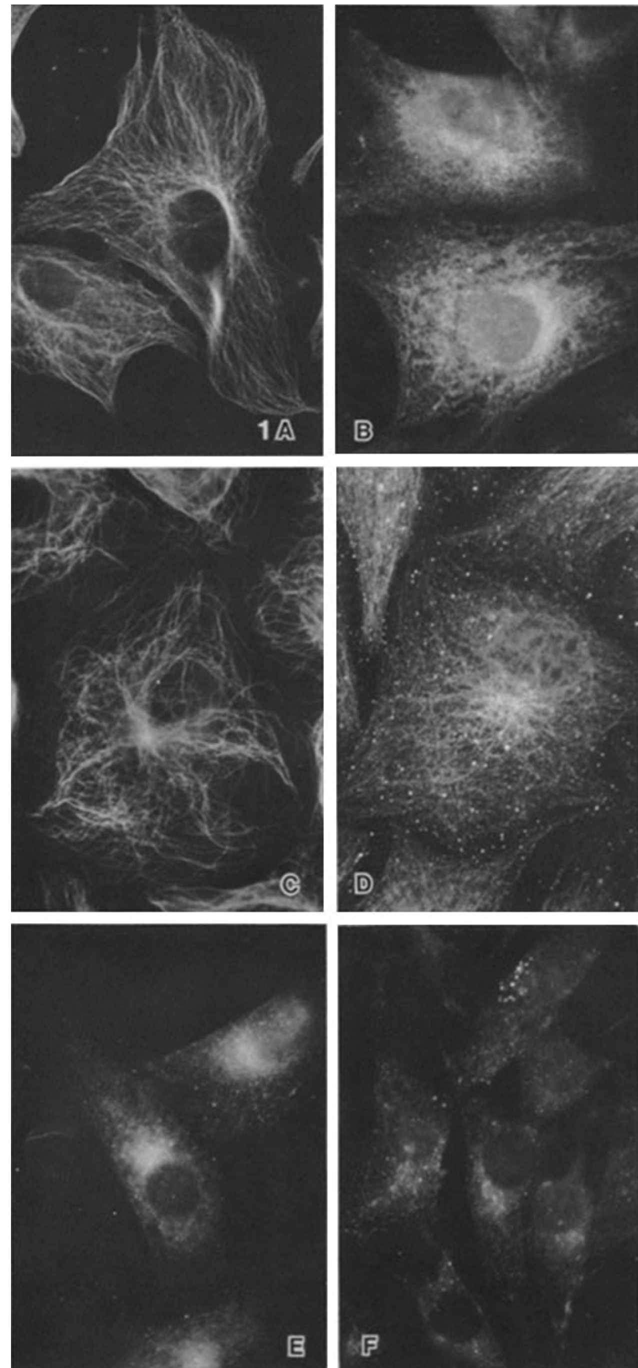

FIGURE 1 Anticalmodulin staining of 3T3 interphase microtubules. Cells were fixed with 3% formalin in PBS and lysed for 6.5 min in −20°C acetone followed by either antitubulin (*A*) or anticalmodulin (*B*) immunofluorescence. *C* and *D* show cells lysed in 0.5% Triton-10 mM EGTA-RB for 90 s, washed in 4% PEG-RB, and prepared for antitubulin and anticalmodulin staining, respectively. Cells were lysed as in *C* and *D*, followed by antibody staining using either antibodies that did not absorb to the calmodulin affinity column during purification (*E*) or anticalmodulin preabsorbed with purified calmodulin (*F*). × 465 (*A*); × 480 (*B*); × 550 (*C*); × 550 (*D*); × 445 (*E*); × 385 (*F*).

It is possible that the lysis procedure employed could result in a redistribution and nonspecific association of proteins with microtubules or other cytoskeletal components. Therefore, lysed cells were double-stained with antibodies to tubulin and vimentin to determine whether these proteins, for example, cross-react with their respective polymers. Fig. 2 shows cells that were lysed as described in Fig. 1, *C* and *D* and double stained with tubulin and vimentin antibodies. The antitubulin-microtubule staining pattern observed at the fluorescein

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wavelength (Fig. 2*A*) was distinct from the antivimentin-intermediate filament staining seen at the rhodamine wavelength (Fig. 2*B*). Distinctive staining patterns were also observed in cells that were lysed in 0.05% Triton without PEG treatment (not shown).

Behavior of CMTC to Depolymerizing Conditions

Cold-stable microtubules associated with the mitotic apparatus have been observed previously by electron microscopy (3, 11, 17) and indirect immunofluorescence (38). Thus, the stability properties of the CMTC were examined at low temperatures. When cells were lysed at room temperature and subsequently incubated for 10–15 min in RB at 0–4°C, some microtubules appeared to depolymerize (Fig. 3*A*). However, numerous microtubules were resistant to the cold dilution and seemed to be closely associated with the plasma membrane at their distal ends as viewed by phase-contrast microscopy (data not shown). A similar pattern was observed if living cells were first incubated in medium at 0–4°C for 40 min followed by fixation, or lysis then fixation. It is interesting to note here that upon incubation of lysed cells in ATP-RB, cytoplasmic microtubules become more sensitive to cold or warm dilution (data not shown). A similar ATP effect has also been reported by others (2, 21). When lysed cells containing only the cold-resistant microtubules were prepared for anticalmodulin immunofluorescence, the staining pattern appeared the same as that observed with antitubulin (Fig. 3*B*).

Another way to examine a dilution effect on the CMTC is to subject living cells to hypotonic swelling (4). When cells were treated with hypotonic medium (3 H_2O:1 medium) for 15–20 min followed by fixation and lysis or lysis in Triton-RB then fixation, many cytoplasmic microtubules were found to be disrupted, although some persisted (Fig. 3*C*). The hypotonic-resistant tubules (and the centrosomes) also stained with anticalmodulin (Fig. 3*D*) and were similar in number to those diluted after lysis. Recently others (39) have also found calmodulin to be localized in the centrosome.

The CMTC can be effectively disrupted by treatment of living cells for several hours with the microtubule inhibitor drug, colcemid (for example, see reference 5). Following incubation of living cells with colcemid, essentially all the cytoplasmic microtubules depolymerized; frequently, a few tubules could be found associated with the centrosome (Fig. 3*E*). When cells treated in this fashion were incubated with anticalmodulin, resistant microtubules and the centrosomes were decorated in addition to some diffuse cytoplasmic staining (Fig. 3*F*). Thus, these disassembly experiments provide evidence that the calmodulin antibody staining was indeed associated with microtubules, as indicated by the sensitivity of the staining pattern to three different depolymerizing conditions.

Calmodulin-Microtubule Association Following Microtubule Reassembly

If calmodulin is involved in Ca^{++}-regulated microtubule assembly (8, 20, 22), calmodulin might be expected to associate with microtubules and the centrosome (microtubule organizing center) at early times during CMTC formation. This possibility was examined by allowing microtubules to

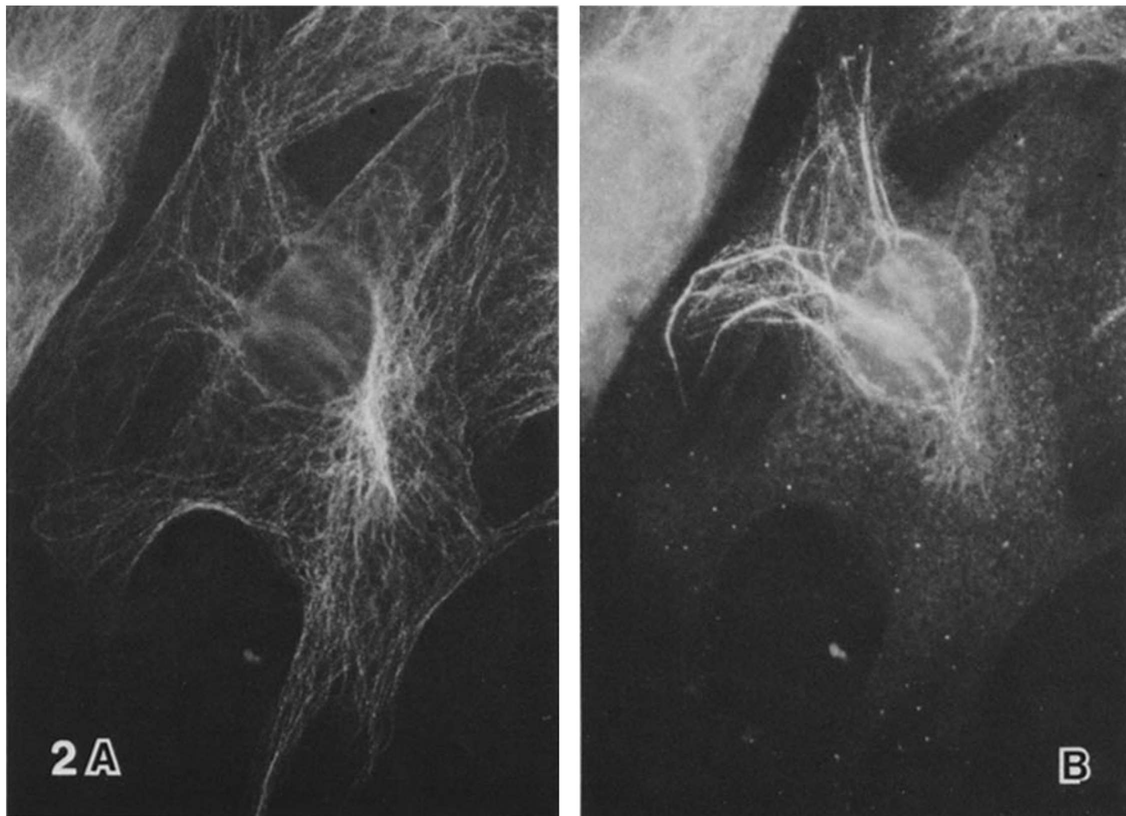

FIGURE 2 Antitubulin and antivimentin staining of 3T3 interphase microtubules and intermediate filaments. Cells were lysed in 0.5% Triton-10 mM EGTA-RB for 90 s, washed in 4% PEG-RB, and prepared for double antibody staining with mouse antitubulin and rabbit antivimentin. Microtubules were stained by fluorescein-conjugated goat-anti-mouse (*A*), whereas intermediate filaments were stained by rhodamine-conjugated goat-anti-rabbit (*B*). × 850.

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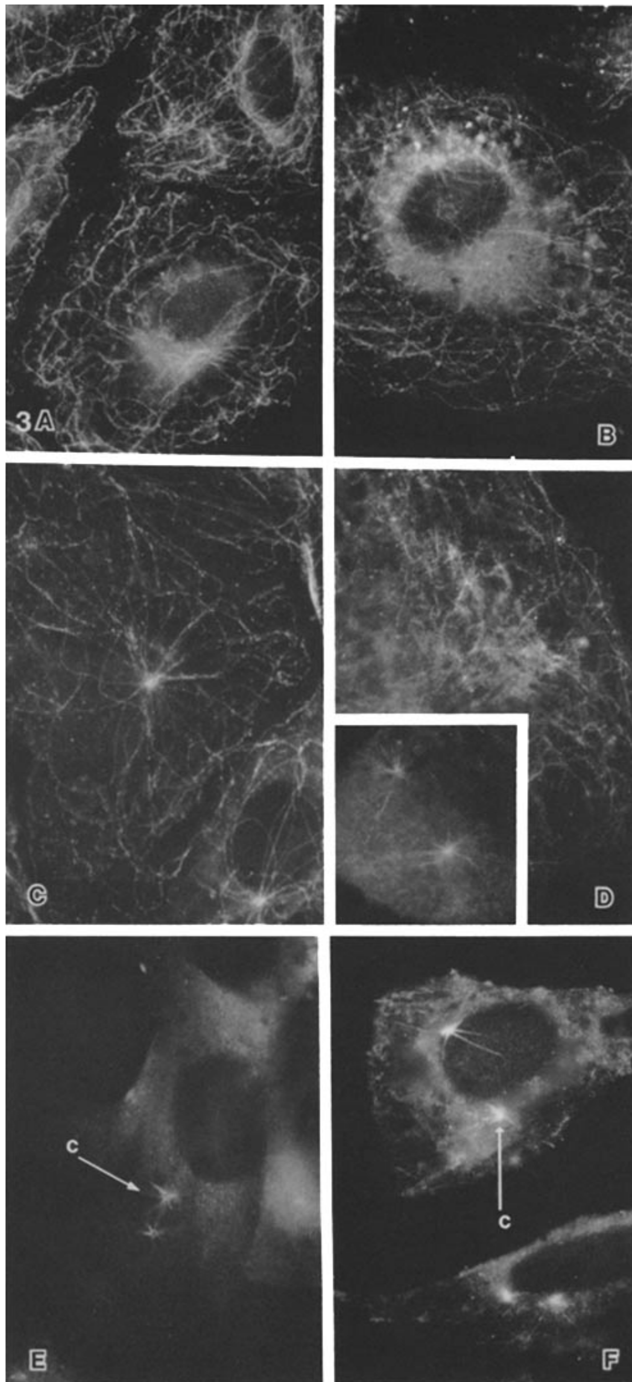

FIGURE 3 Anticalmodulin staining of 3T3 interphase microtubules resistant to cold/dilution or hypotonic treatment. Cells were lysed in Triton-10 mM EGTA-RB for 90 s, incubated in RB at 2°C for 12 min, fixed, and stained with antitubulin (*A*) or anticalmodulin (*B*). Note the presence of microtubules at the cell membrane. *C* and *D* show cells exposed for 20 min to 1 medium: 3 H_2O at 37°C, lysed, and stained with either antitubulin or anticalmodulin, respectively. Note the staining of centrosomes (*D, inset*). Colcemid (0.3 μM) was added to cells in medium for 2.5 h and cells were then prepared for either antitubulin (*E*) or anticalmodulin (*F*) staining. Centrosomes (c) are clearly stained with anticalmodulin. × 600 (*A*); × 705 (*B*); × 735 (C); × 740 (*D*); × 735 (*E*); × 705 (*F*).

reassemble from the centrosome during recovery from either hypotonic or cold treatment.

Hypotonic-sensitive microtubules were disassembled during the treatment described in Figs. 3, *C* and *D*. In this case, we show the results using the transformed SV40-3T3 cell line. Compared with 3T3 cells, the cytoplasm of SV3T3 cells appeared to contain a similar number of microtubules resistant to cold (not shown) or hypotonic treatment (Fig. 4*A*) and these tubules were also stained by anticalmodulin (Fig. 4*B*). After a 20-min recovery period in fresh isotonic medium, microtubule reassembly was apparent around the centrosomal region (Fig. 4*C*). These newly formed microtubules also stained with anticalmodulin (Fig. 4*D*).

Microtubules were also allowed to reassemble from the centrosome during recovery from cold treatment. The cold-sensitive cytoplasmic microtubules were first depolymerized by incubating cells in medium at 2°C for 40 min, after which time a reduced CMTC was observed as shown in Fig. 3*A*. This was followed by incubation in medium at 37°C for 5 min, at which time the CMTC had essentially reformed as determined by antitubulin staining (Fig. 4*E*). As shown in Fig. 4*F*, the reassembled microtubules stained with anticalmodulin and the pattern was similar to that observed prior to cold treatment (Fig. 1*D*).

Response of Cytoplasmic Microtubules to Ca^{++}

If intracellular levels of Ca^{++} are involved in the regulation of microtubule assembly-disassembly, then the CMTC should be sensitive to Ca^{++} concentrations in the micromolar range, particularly if calmodulin is associated with the tubules (13, 20). After lysis in Triton-RB and incubation at 25–37°C in reassembly buffer containing 11 μM free Ca^{++}, the CMTC was disrupted and disassembly in many cases was nearly complete within 7 min (Fig. 5*A*). This sensitivity was similar to previous findings of Schliwa et al. (30). Consistent with in vitro studies (15, 33, 37), depolymerization appeared to occur from the polymer ends, starting in many cases at the distal ends at the cell periphery and proceeding toward the centrosome. However, a number of free-ended microtubule segments could be observed in the cytoplasm, apparently a result of disassembly from both ends. Although disassembly along the polymer wall cannot be ruled out in some cases, depolymerization was more extensive at 60 μM Ca^{++} and disassembly from opposite ends was more pronounced (Fig. 5*C*). It is important to note that the extent of disassembly at all Ca^{++} concentrations that were studied varied from cell to cell. However, at 110 μM Ca^{++}, disassembly was essentially complete in most cells (Fig. 5*E*). As was observed under the various disassembly conditions shown in Fig. 3, upon Ca^{++} disassembly, the anticalmodulin staining pattern followed the antitubulin staining of microtubules (Fig. 5, *B*, *D*, and *F*).

DISCUSSION

The data presented in this paper indicate that calmodulin is associated with both cytoplasmic microtubules and the centrosomes. In addition to 3T3 cells, antibodies to calmodulin also stain cytoplasmic microtubules of several mammalian cell lines including PtK, bovine kidney (MDBK), African Green monkey kidney (BSC-1), HeLa, and human lung (WI-38) (data not shown). The indirect immunofluorescent staining pattern observed using anticalmodulin is sensitive to various microtubule depolymerizing conditions. Furthermore, calmodulin is localized on microtubules reassembling upon recovery from these conditions. The CMTC can be disrupted when exposed to micromolar Ca^{++} concentrations and our data as well as others (13, 20, 27, 28, 30, 38) suggest

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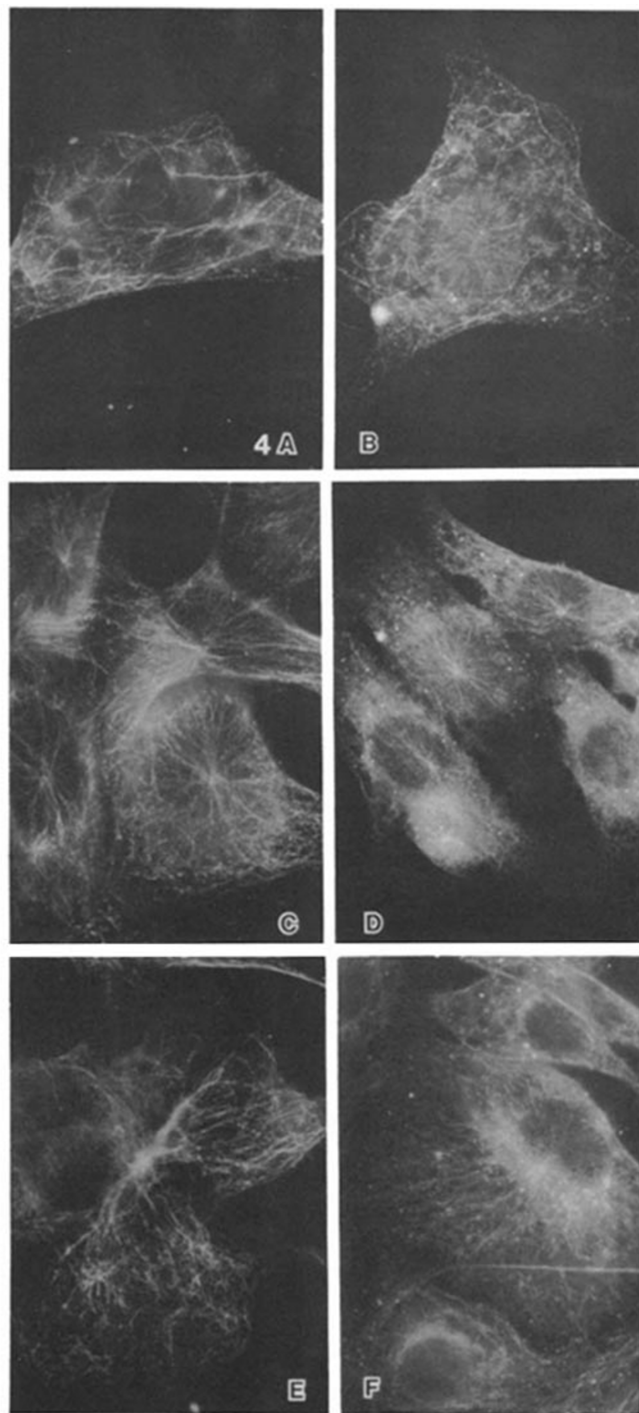

FIGURE 4 Anticalmodulin staining of interphase microtubules reassembled upon recovery from hypotonic or cold treatment. Transformed SV40-3T3 cells were hypotonically treated as in Fig. 3 C and prepared for either antitubulin (*A*) or anticalmodulin (*B*) staining. Cells were hypotonically treated as in *A* and *B* then incubated in isotonic medium for 20 min followed by antitubulin (*C*) or anticalmodulin (*D*) staining. *E* and *F* show 3T3 cells incubated for 40 min in medium at 2°C followed by incubation at 37°C for 5 min, lysis, and fixation and staining with antitubulin or anticalmodulin, respectively. × 760 (*A*); × 760 (*B*); × 760 (*C*); × 480 (*D*); × 565 (*E*); × 525 (*F*).

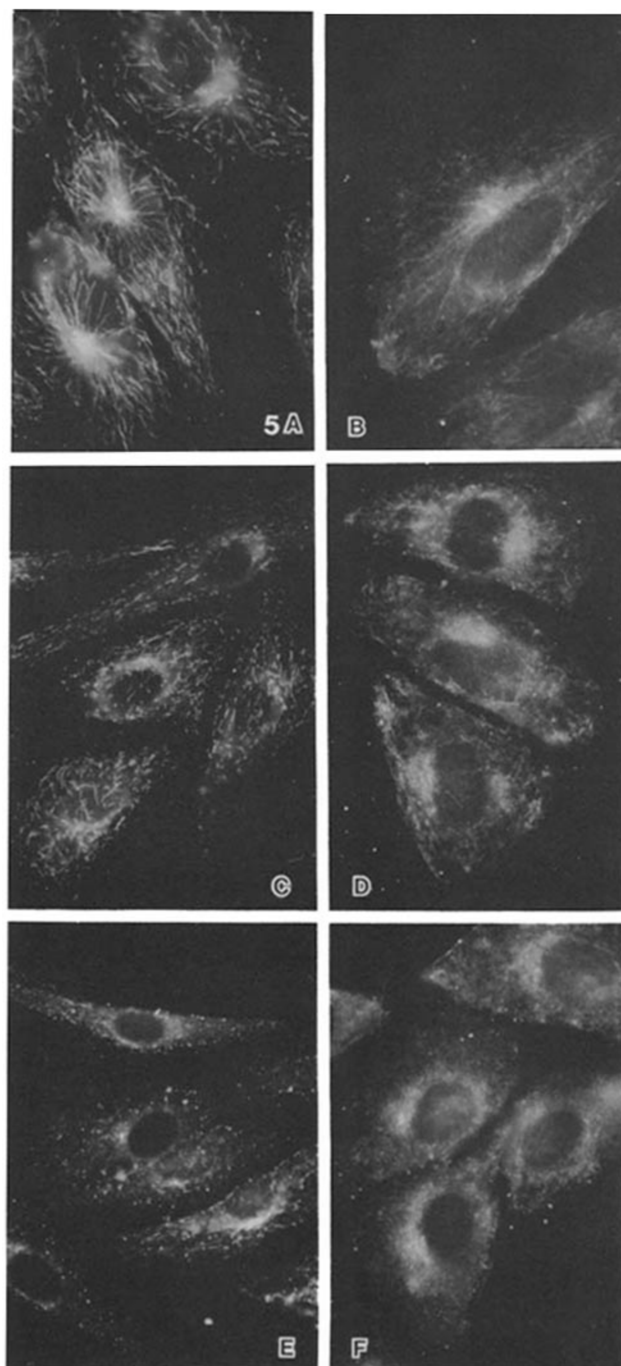

FIGURE 5 Calcium-induced disassembly of CMTC. Cells were lysed in Triton-10 mM EGTA-RB for 90 s, washed for 30 s in 1 mM EGTA, then 11 μM Ca^{++}-RB each, and then incubated for 7 min in RB solutions containing either 11 (*A* and *B*), 60 (*C* and *D*), or 110 μM (*E* and *F*) free Ca^{++}. Antitubulin staining is shown in *A*, *C*, and *E*; anticalmodulin is *B*, *D*, and *F*. × 480 (*A*); × 445 (*B*); × 355 (*C*); × 555 (*D*); × 355 (*E*); × 555 (*F*).

that this sensitivity may be mediated by calmodulin since an antibody to calmodulin stains these microtubules.

The use of a lysed cell model to evaluate localization of molecules on intracellular structures must be carefully controlled. Whereas it is advantageous to remove soluble material such as the bulk of "soluble" calmodulin, the protocol can also lead to artifactual redistribution of proteins. Although such a possibility can never be entirely ruled out, several reasons suggest that this is not the case in our experiments. First, calmodulin is found on microtubules following stabilization of these structures with PEG (Fig. 1) or in the presence of EGTA (Figs. 3 and 4). Second, calmodulin remains asso-

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ciated with some cytoplasmic microtubules during induced disassembly (Fig. 5). In this case lysis has already occurred. Third, Fig. 2,*A* and *B* show that the lysis procedure does not, for example, alter the distribution of vimentin. Finally, most calmodulin binding protein interactions result from a Ca^{++}-induced conformational change in calmodulin that exposes a hydrophobic surface (18, 34). For this reason EGTA releases >90% of total cell calmodulin (10, 23). The calmodulin associated with microtubules appears to be the result of a Ca^{++}-independent reaction since it is found in a qualitatively similar manner when lysis is affected by EGTA or PEG.

We have also observed that some cytoplasmic microtubules are relatively insensitive to dilution and cold-induced disassembly. Stability of microtubules to cold temperatures in vivo and in vitro has been previously reported. A number of microtubules whose ends are associated with the kinetochores and the poles (centrosomes) as well as interzonal tubules in the mitotic apparatus of cultured cells resist disassembly at 0–4°C (3, 11, 38). These microtubules also stain with calmodulin antibodies. A similar stability has been observed for a population of microtubules isolated from rat brain (14, 21, 35). The stability observed in vitro does not appear to be due to different tubulin dimer species but rather results from the interaction of a nontubulin protein(s) (STOPs) with the microtubule. Whether similar factors are responsible for cold-stable spindle and cytoplasmic microtubules remains to be elucidated. However, the lack of sensitivity to disassembly conditions such as dilution and/or cold indicates that at least two types of microtubules may co-exist within the same cytoplasm, and one class can exhibit very low subunit dissociation and turnover under certain conditions. Since many of these microtubules appear to be associated with the centrosome and either the kinetochore at mitosis (3) or the plasma membrane during interphase (4), it is conceivable that they may be specifically involved in chromosome movements as well as cell shape changes and membrane receptor mobility.

Although it is clear that calmodulin can increase the sensitivity of microtubules to Ca^{++} in certain cases in vitro (13, 19, 20, 24), its target of action and the mechanism by which it induces disassembly remain an enigma. Calmodulin may mediate disassembly through an interaction with proteins that associate with microtubules, for example, MAPs (28) or STOPs (13, 14), since tubulin has not been clearly shown to bind calmodulin. Calmodulin also does not increase the intrinsic Ca^{++}-sensitivity of microtubules assembled in the absence of MAPs (19). As previously mentioned, the association of calmodulin with microtubules does not appear to require Ca^{++}, since anticalmodulin stains microtubules after lysis and incubation in buffer containing 10 mM EGTA. A low Ca^{++} requirement is practical in that it allows calmodulin to associate with microtubules without necessarily inducing disassembly. This idea is further supported by the observation that calmodulin is found associated with microtubules reassembling after cold or hypotonic-induced disassembly. Precedent exists for the association of calmodulin with other proteins in a Ca^{++}-independent fashion. Calmodulin is the δ subunit of phosphorylase kinase and cannot be removed from the enzyme with EGTA (9). Yet phosphorylase kinase can be activated by micromolar Ca^{++} upon binding of this ion to the δ subunit. In addition, phosphorylase kinase can be further activated by the Ca^{++}-dependent binding of a second mole of calmodulin. This latter effect can be mimicked by troponin C and can be abolished by preincubation with anticalmodulin drugs such as trifluoperazine (31). It is interesting to note that these drugs do not prevent the Ca^{++} activation of the enzyme due to the δ subunit. Marcum et al. (20) revealed that troponin C could also substitute for calmodulin in the Ca^{++}-dependent depolymerization of microtubules in vitro.

Studies from our laboratory have shown that anticalmodulin drugs do not consistently affect the Ca^{++}-dependent depolymerization of microtubules in situ or the Ca^{++}-dependent disassembly reaction in vitro. A lack of consensus also appears in the literature concerning drug inhibition. Previous studies by Schliwa et al. (30) using lysed cells indicated that various anticalmodulin drugs inhibit Ca^{++}-induced disassembly whereas observations of Perry et al. (27) and Job et al. (13) using tubulin purified in vitro contrast the former study. Together these data suggest that calmodulin may associate with microtubules in a Ca^{++}-independent manner but only affect the state of polymerization in the presence of micromolar Ca^{++}. Such a mechanism has also been proposed by Sobue and co-workers (32) for the calmodulin regulation of actin-myosin interaction in the presence of caldesmon and myosin light chain kinase.

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