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Control of microtubule assembly-disassembly by calcium-dependent regulator protein

(immunofluorescence/calcium-binding proteins/mitosis)

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ABSTRACT **The Ca^{2+}-dependent regulator (CDR) protein of cyclic nucleotide phosphodiesterase is a low molecular weight, acidic, Ca^{2+}-binding protein which has been implicated in a number of Ca^{2+}-dependent enzymatic functions. Indirect immunofluorescence has revealed that CDR is specifically associated with the chromosome-to-pole region of the mitotic apparatus during metaphase-anaphase in a pattern distinctly different from that of similar cultured cells stained with anti-tubulin. This characteristic localization in the mitotic half-spindle suggested a role for CDR in the control of microtubule assembly-disassembly during mitosis. Thus, CDR was examined for its effects on microtubule polymerization *in vitro*. It was determined that stoichiometric concentrations of CDR and a homologous Ca^{2+}-binding protein, skeletal muscle troponin C, both inhibited and reversed microtubule assembly in a Ca^{2+}-dependent manner. CDR-dependent inhibition of *in vitro* microtubule assembly occurred at physiological Ca^{2+} concentrations (~10 μM) that, in the absence of CDR, caused only a slight reduction in polymerization. At Ca^{2+} concentrations in the low physiological range (<1 μM), no inhibition was observed. These biochemical results, together with the immunofluorescent localization of CDR in the mitotic half-spindle, provide evidence that Ca^{2+} is an endogenous regulator of microtubule disassembly through the activity of CDR.**

Recent work in our laboratories has focused on the properties of the Ca^{2+}-dependent regulator (CDR) of cyclic nucleotide phosphodiesterase. This protein is low molecular weight, thermostable, and binds Ca^{2+} with high affinity at physiological concentrations. The isolation and characterization of rat testis CDR have been documented (1), and the primary sequence of the protein has been determined (2). A comparison of the sequence of CDR with that of the Ca^{2+}-binding component of skeletal muscle, troponin C, demonstrates that these proteins are similar, sharing 50% direct sequence homology (2). In addition, although CDR and troponin C possess limited biological crossreactivity, they are different proteins (3). In contrast to troponin C, CDR is an abundant (1–20 μM) and ubiquitous intracellular protein that regulates a number of enzymatic functions, including phosphodiesterase activation (4) and actomyosin ATPase activity (5, 6), and has been implicated in microfilament organization (7). The development of monospecific antibodies to CDR has allowed the study of its distribution and localization by indirect immunofluorescence (8,*). Several of these results indicated that CDR was concentrated in the chromosome-to-pole region of the metaphase-anaphase mitotic apparatus in all cell lines examined (8). This observation, coupled with the fact that Ca^{2+} depolymerizes microtubules *in vitro* (9–13) and has been implicated in microtubule regulation *in vivo* (14–16), suggested that CDR might be involved in Ca^{2+}-dependent depolymerization of microtubules during anaphase chromosome movement. Additionally, the recent observation that the Ca^{2+} sensitivity of microtubule assembly *in vitro* could be modulated by an unspecified factor(s) (17), together with the immunofluorescent localization of CDR in the mitotic half-spindle, provided a strong rationale for examining the effect of this protein on microtubule polymerization *in vitro*. We here describe the Ca^{2+}-dependent regulation of microtubule assembly-disassembly by CDR and other Ca^{2+}-binding proteins.

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MATERIALS AND METHODS

Immunofluorescence. Antibodies to rat testis CDR were produced in goats and were isolated, purified, and characterized.* Anti-tubulin antibodies were produced in rabbits and isolated as described by Fuller *et al.* (18). Swiss mouse 3T3 cells were grown on 11 × 22 mm glass coverslips in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a humidified 10% CO_2/90% air atmosphere. Coverslips were processed for indirect immunofluorescence according to Brinkley *et al.* (19). Fluorescein-labeled rabbit anti-goat IgG and goat anti-rabbit IgG were obtained from Melloy Laboratories, Springfield, VA.

Protein Preparation. *Microtubule Protein.* Microtubule protein was isolated from rat brains by a modification of the temperature-dependent assembly-disassembly scheme described by Borisy *et al.* (20). Samples were homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, NY) at a ratio of 1 g of brain to 1.3 ml of buffer [100 mM Pipes (1,4-piperazinediethylsulfonic acid)/1 mM EGTA (ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid)/0.1 mM $MgSO_4$/0.1 mM GTP, pH 6.94 at 24°]. Purification was continued in the same buffer containing 1.0 mM GTP until the second pellet of microtubules was obtained, at which time the protein pellets were frozen and stored in liquid N_2. For the inhibition experiments (Fig. 3 and Table 1) the frozen pellets were resuspended in 1.1× assay buffer (111 mM Pipes/1.11 mM EGTA/0.11 mM $MgSO_4$) containing 0.1 mM GTP. A further depolymerization-cold centrifugation, polymerization-warm centrifugation cycle was performed and the microtubule pellet was resuspended in 1.1× assay buffer containing no added GTP. This material was incubated on ice and centrifuged in the cold to yield 3× microtubule protein. The protein concentration was determined as described (21); the protein preparation was stored on ice until used in an assay. For experiments involving the reversal of microtubule assembly (Fig. 4) 3× microtubule protein was prepared as above except that the 1.1× assay buffer contained 1.11 mM GTP.

Calcium-Binding Proteins. CDR was isolated from rat testis by the procedure of Dedman *et al.* (1); rabbit skeletal muscle

Abbreviations: CDR, calcium-dependent regulator protein; Pipes, 1,4-piperazinediethylsulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

* Dedman, J. R., Welsh, M. J. & Means, A. R. (1978) *J. Biol. Chem.*, in press.

troponin C was the generous gift of J. D. Potter. Parvalbumin was isolated from carp muscle by a modification of the procedure of Kretsinger and Nockolds (22). This modification involved the addition of $^{45}Ca^{2+}$ prior to ion exchange chromatography, which allowed the identification of two Ca^{2+}-binding protein peaks. Gel electrophoresis [15% polyacrylamide/0.1% sodium dodecyl sulfate ($NaDodSO_4$) gels] showed that both Ca^{2+}-binding peaks represented pure proteins that migrated with mobilities similar to parvalbumin. The proteins were identified as parvalbumins on the basis of their Ca^{2+}-binding, mobility on gels, and spectral properties as compared with an authentic parvalbumin standard kindly supplied by R. H. Kretsinger.

Polymerization Assays. All preparations of Ca^{2+}-binding proteins were exhaustively dialyzed against 1.1× assay buffer containing 1.11 mM $CaCl_2$. This dialysis insured that the final free Ca^{2+} concentration in each Ca^{2+}-binding protein sample was that of the dialysis buffer ($1.1–1.3 \times 10^{-5}$ M) and was thus independent of the Ca^{2+}-binding properties of the individual proteins. Samples of microtubule protein resuspended in assay buffer lacking GTP were diluted with water and/or a stock $CaCl_2$ solution to yield a concentration of 2.53 mg/ml in 0.495 ml of solution. For those samples to which exogenous Ca^{2+} was added, the concentration ratio of Ca^{2+}:EGTA was maintained at unity. Exogenous Ca^{2+} was added to balance the EGTA contribution of the 3× microtubule protein buffer and maintain a constant Ca^{2+}:EGTA ratio of 1. Turbidity at 320 nm was monitored with a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH) equipped with a water-jacketed cuvette chamber. Temperature was maintained at 37° with a Lauda Model K2 constant-temperature water circulator (Sargent–Welch Scientific Company, Skokie, IL). After a 15-min incubation at 37°, microtubule polymerization was initiated by addition of 5 μl of 100 mM GTP. The change in OD_{320} with time was recorded on a Varian Aerograph Model 20 strip chart recorder (Varian Aerograph, Walnut Creek, CA).

For experiments involving the reversal of polymerization (Fig. 4), 3× microtubule protein prepared in 1.1× assay buffer containing 1.11 mM GTP was mixed with 1.1× assay buffer or the Ca^{2+}-binding protein solutions to a concentration of 2.75 mg/ml in a volume of 0.45 ml at 0°. This solution was diluted with water and the GTP concentration was adjusted to 1 mM, resulting in 0.5 ml of reaction volume. The mixture was placed in a cuvette and microtubule polymerization was initiated by incubation at 37° in the spectrophotometer. After 10 min of incubation the samples had attained maximal OD_{320} values, at which time 2–3 μl of 100 mM $CaCl_2$ was added to yield a final 1:1 concentration ratio of Ca^{2+}:EGTA, and the change in turbidity of the samples was continuously monitored. Viscometric experiments were conducted in a similar fashion with Ostwald type capillary viscometers (Cannon Instruments, State College, PA). The final concentrations (±1%) of the components in the assay mixtures for all experiments were 100 mM Pipes/1.0 mM EGTA/1.0 mM GTP/0.10 mM $MgSO_4$, with $CaCl_2$ concentrations ranging from 0 to 1.0 mM.

EGTA/Ca^{2+} Buffer System. Free Ca^{2+} concentrations were calculated from the association constants for metals (Ca^{2+} and Mg^{2+}) to the ligands (EGTA and GTP) at various pH values by the computer program described by Perrin and Sayce (23) and Potter and Gergely (24). The association constants for proton and metal binding to EGTA were obtained from Potter and Gergely (24), and the constants for GTP/metal binding from Olmsted and Borisy (12). All exogenous Ca^{2+} added was Orion Ca^{2+} standard (0.1000 mol/liter).

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed by the method of Laemmli (25) on gel columns (6 × 80 mm) at 2 mA/gel. The gels were fixed overnight in 40% methanol/7% acetic acid and stained for 2–3 hr at 37° in the same solution containing 0.05% Coomassie blue. Gels were partially destained electrophoretically, with complete destaining obtained by diffusion in 7% acetic acid.

RESULTS

The immunofluorescent localization of CDR in the mitotic spindle of an anaphase 3T3 cell is shown in Fig. 1*a*. The entire cell displays diffuse fluorescence; however, there is preferential decoration of the chromosome-to-pole region of the mitotic spindle (8). CDR appears to be most highly concentrated at the spindle poles, with an uneven distribution of bright fluorescence throughout the half-spindle. This staining pattern is distinctly different from anti-tubulin immunofluorescent images of similar mitotic cells (Fig. 1*b*) (18, 19). In contrast to anti-tubulin, anti-CDR staining is absent from the interzone region of the spindle at anaphase and appears not to be associated with spindle asters. The distinctive localization of CDR in the mitotic half-spindle where microtubule depolymerization occurs during anaphase chromosome movement (26) suggested an involvement of this regulatory molecule in the control of the state of microtubule assembly during mitosis. In order to test this possibility, CDR and other Ca^{2+}-binding proteins were examined for their effects on the assembly of microtubule protein purified by three assembly–disassembly cycles (3× microtubule protein) from rat brain. A gel of this material is shown in Fig. 2, gel a. The protein composition of microtubule protein from rat brain is quite similar to that previously described for microtubule protein isolated from porcine brain (20) consisting of approximately 70–75% tubulin and 15–20% high molecular weight proteins, with the remaining 5–10% of the staining distributed throughout the gel in a number of minor bands. Also shown in Fig. 2 are 15% polyacrylamide gels of troponin C isolated from rabbit skeletal muscle (gel b), CDR purified from rat testis (gel c), and carp muscle parvalbumin (gel d). These gels demonstrate the purity of the protein preparations and their differences in electrophoretic mobility.

The effects of Ca^{2+}, CDR, and troponin C on microtubule assembly *in vitro* are shown in Fig. 3. Polymerization of solutions of microtubule protein in the presence and absence of Ca^{2+} and calcium-binding proteins was initiated by the addition of GTP at an added concentration of 1.0 mM after a 15-min preincubation at 37°. Microtubule assembly in the absence of free Ca^{2+} occurred with a very short lag after GTP addition, and turbidity developed in a characteristically sigmoidal fashion, reaching a stable plateau approximately 10 min after initiation. The addition of free Ca^{2+} at 3.9×10^{-7} M in the absence or presence of CDR (2.5 mg/ml) resulted in only a very slight reduction in turbidity development. When the free Ca^{2+} concentration was raised to 1.1×10^{-5} M in the absence of Ca^{2+}-binding proteins, microtubule polymerization occurred

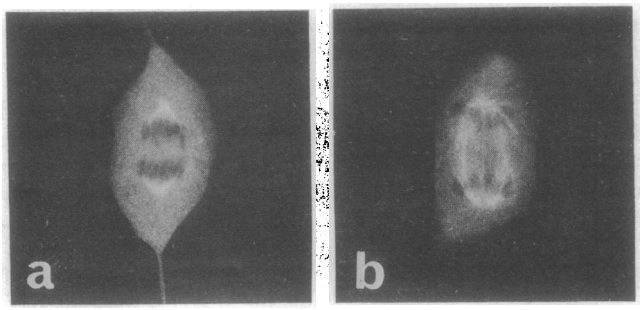

FIG. 1. Immunofluorescent photographs of anaphase 3T3 cells stained with (*a*) anti-CDR and (*b*) anti-tubulin. (×500.)

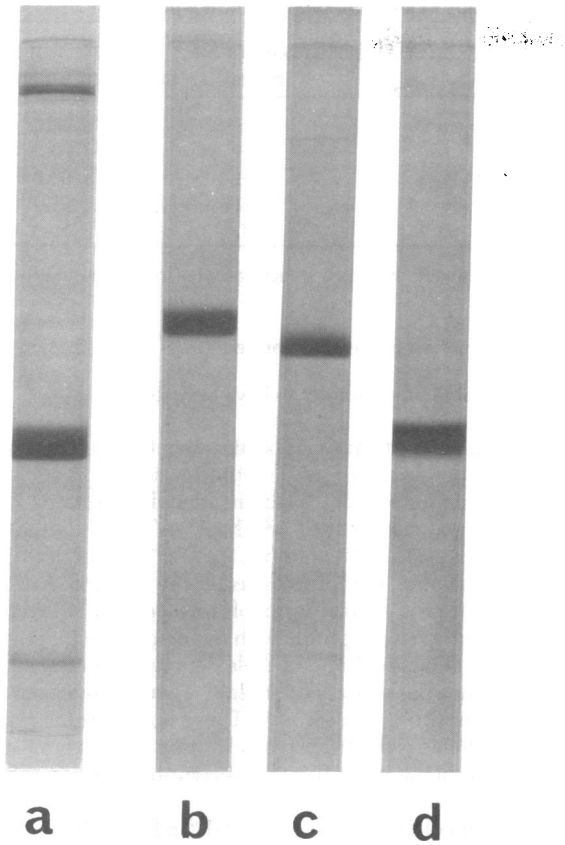

FIG. 2. Polyacrylamide disc gels of the protein samples used in the microtubule polymerization assays. A 7.5% polyacrylamide/0.1% $NaDodSO_4$ gel of 3× microtubule protein isolated from rat brain is shown in gel a; 25 μg of protein was applied to the gel. The Ca^{2+}-binding proteins were run on 15% polyacrylamide/0.1% $NaDodSO_4$ gels: (gel b) rabbit skeletal muscle troponin C, 15 μg; (gel c) rat testis CDR, 15 μg; and (gel d) carp muscle parvalbumin, 15 μg.

with a slightly increased lag time and a reduction in maximum OD_{320} of approximately 15%. However, inclusion of either CDR (2.5 mg/ml) or troponin C (0.6 mg/ml) at the same free Ca^{2+} concentration (1.1×10^{-5} M) resulted in complete inhibition of microtubule assembly. These results clearly indicate that both CDR and troponin C can inhibit microtubule assembly in a Ca^{2+}-dependent manner.

We next asked whether CDR and troponin C would depolymerize intact microtubules at similar Ca^{2+} concentrations. Samples of microtubule protein were polymerized by incubation at 37° until plateau turbidity was obtained (Fig. 4). Addition of Ca^{2+} (1.1×10^{-5} M) alone resulted in a slight drop in ΔOD_{320} followed by a long period of linear decrease in turbidity which eventually reached a plateau at a ΔOD_{320} value 15–25% less than the plateau before Ca^{2+} was added. The results were quite different when either CDR (3.0 mg/ml) or troponin C (0.75 mg/ml) was included in the reaction mixture. Prior to Ca^{2+} addition, turbidity development of 3× microtubule protein in the presence of the Ca^{2+}-binding proteins was similar to that of the control reaction. The slight reduction in maximum ΔOD_{320} displayed by these samples can be accounted for by the concentrations of free Ca^{2+} present in the original reaction solutions due to the dialysis of the Ca^{2+}-binding proteins (7.5×10^{-7} M for troponin C, 4.4×10^{-7} M for CDR). Increasing Ca^{2+} to a final concentration of 1.1×10^{-5} M for the troponin C sample and 2.2×10^{-5} M for the CDR-containing sample resulted in an immediate drop in turbidity of approximately 20–35% followed by a short period of recovery and a long, slow decrease in ΔOD_{320}. These samples eventually

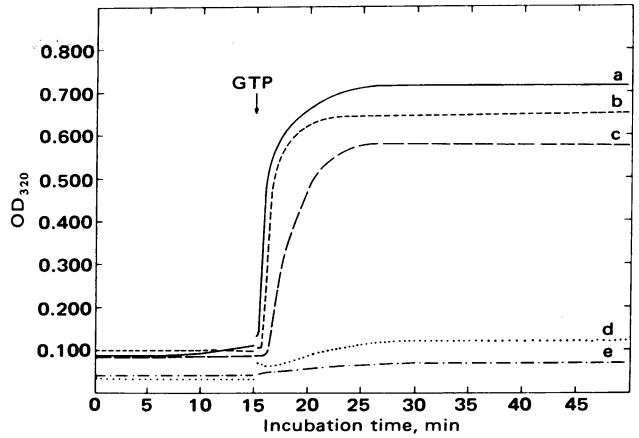

FIG. 3. Ca^{2+}-dependent inhibition of microtubule assembly by CDR and troponin C. The time course of microtubule assembly at 37° for samples containing rat brain microtubule protein (2.5 mg/ml) was monitored by light scattering at 320 nm. The samples represented above contained: (a) microtubule protein; (b) microtubule protein + 3.9×10^{-7} M free Ca^{2+} + 2.5 mg of CDR per ml (the same curve was obtained in the absence of CDR); (c) microtubule protein + 1.1×10^{-5} M free Ca^{2+}; (d) microtubule protein + 0.6 mg of troponin C per ml + 1.1×10^{-5} M free Ca^{2+}; and (e) microtubule protein + 2.5 mg of CDR per ml + 1.1×10^{-5} M free Ca^{2+}.

reached a stable plateau of $\Delta OD_{320} > 0$. However, samples from other experiments at slightly higher free Ca^{2+} or Ca^{2+}-binding protein concentrations did reach ΔOD_{320} values of zero, indicating total microtubule depolymerization. The biphasic nature of the turbidity decrease was confirmed by viscometric measurements (data not shown). Thus, CDR and troponin C, in addition to inhibiting microtubule assembly in a Ca^{2+}-dependent manner, also act to depolymerize intact microtubules at physiological Ca^{2+} concentrations.

A comparison of CDR, troponin C, and carp parvalbumin and their effects on microtubule assembly are shown in Table 1. All three proteins are homologous, have low molecular weights, are highly acidic, and bind divalent cations. These proteins also share the property of inhibiting microtubule polymerization in a Ca^{2+}-dependent manner. However, the

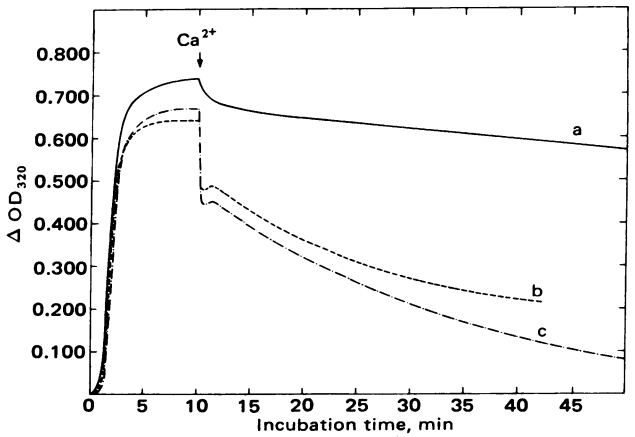

FIG. 4. Ca^{2+}-dependent reversal of microtubule assembly by CDR and troponin C. Microtubule protein from rat brain (2.5 mg/ml) was polymerized by incubation at 37° and monitored by the change in optical density at 320 nm. When plateau turbidity was attained (10 min), $CaCl_2$ was added and the decrease in light scattering was followed with time. After Ca^{2+} addition the samples contained: (a) microtubule protein + 1.1×10^{-5} M free Ca^{2+}; (b) microtubule protein + 0.75 mg of troponin C per ml + 1.1×10^{-5} M free Ca^{2+}; and (c) microtubule protein + 3.0 mg of CDR per ml + 2.2×10^{-5} M free Ca^{2+}.

Table 1. Physical properties and effects on microtubule assembly of several calcium-binding proteins

Calcium-binding protein	M_r	pI	Metal binding sites/molecule Ca^{2+}	Metal binding sites/molecule Ca^{2+}-Mg^{2+}	Concentration, mg/ml	Inhibition of microtubule assembly,* %
CDR†	17,000	3.9	4	0	2.5	94.0
					0.6	20.0
Troponin C‡	18,000	4.4	2	2	0.6	89.0
					0.2	41.0
Parvalbumin§	11,000	4.5	0	2	5.0	12.0

* Percent inhibition values were calculated from the difference in maximum ΔOD_{320} measurements between samples in the absence and presence of Ca^{2+}-binding protein at the same free Ca^{2+} concentration of 1.1×10^{-5} M.

† The physical characteristics of rat testis CDR have been described (1, 2).

‡ The molecular weight of rabbit skeletal muscle troponin C was approximated from the sequence of the protein (27). Other physical properties have been described (24).

§ The sequence of carp muscle parvalbumin has been determined (28) and the metal binding properties have been described (4).

Ca^{2+}-binding proteins did vary significantly in their effectiveness as inhibitors. Under the conditions of these assays, troponin C was the most potent inhibitor, CDR was only slightly less effective, while parvalbumin was a poor inhibitor of microtubule assembly. The molecular basis for this variation in potency as well as the mechanism(s) of inhibition remain to be elucidated. However, the data of Table 1 suggest that the inhibition is stoichiometric rather than catalytic. CDR inhibited microtubule assembly 94% at 2.5 mg/ml, which is an approximate 8-fold molar ratio of CDR to tubulin dimers. When the concentration of CDR was reduced to 0.6 mg/ml (~2-fold molar ratio), a proportional reduction in inhibition was noted. Troponin C yielded 89% inhibition of an approximate 2-fold molar ratio to tubulin dimers (0.6 mg/ml), and a lower concentration of this protein resulted in less inhibition. Parvalbumin proved to be a poor inhibitor of microtubule assembly, yielding only 12% inhibition at a concentration of 5.0 mg/ml, which is approximately a 25-fold molar ratio of parvalbumin to tubulin dimers.

DISCUSSION

The development of monospecific antibodies directed against rat testis CDR has allowed the use of indirect immunofluorescence techniques for the subcellular localization of this regulatory molecule. In interphase cells CDR-specific immunofluorescence is concentrated in fibrous networks which have been identified by phase contrast and Nomarski microscopy as cellular stress fibers (8). Potential interactions between CDR and microfilaments are not unanticipated, since CDR is homologous to skeletal muscle troponin C (2, 3). In contrast, the immunofluorescent observations of Welsh *et al.* (8), confirmed in this report (Fig. 1*a*), that CDR is localized in the spindle of mitotic cells were initially surprising. A comparison of the anti-CDR and anti-tubulin immunofluorescence staining patterns showed that during anaphase CDR was restricted to the chromosome-to-pole region of the mitotic apparatus, whereas anti-tubulin decorated the entire spindle complex. The localization of CDR in that region of the mitotic spindle where overall microtubule depolymerization occurs during anaphase chromosome movement (26) suggested that CDR might facilitate the Ca^{2+}-dependent disassembly of microtubules. The data presented in this report have directly demonstrated that rat testis CDR can inhibit *in vitro* microtubule polymerization at a high physiological Ca^{2+} concentration (1.1×10^{-5} M) while having little or no inhibitory properties at lower physiological Ca^{2+} concentrations ($\sim 5 \times 10^{-7}$ M). In addition, CDR was shown to cause essentially total disassembly of intact microtubules in response to an increase in the free Ca^{2+} concentration which, in the absence of any Ca^{2+}-binding proteins, resulted in slight microtubule depolymerization (Fig. 4). Thus, a well-characterized, ubiquitous cellular protein which binds Ca^{2+} with high affinity and has been shown to regulate a number of Ca^{2+}-dependent enzymatic activities, such as cyclic nucleotide phosphodiesterase (29–31), brain adenylate cyclase (32, 33), and erythrocyte membrane Ca^{2+}-Mg^{2+}-ATPase (34–36), has now been demonstrated to control the Ca^{2+}-dependent inhibition and reversal of microtubule assembly *in vitro*. Furthermore, the correlation of the immunofluorescent localization of CDR in the mitotic half-spindle with the biochemical demonstration of CDR-dependent microtubule depolymerization offers strong circumstantial evidence for Ca^{2+} as an endogenous regulator of microtubule disassembly. These observations also provide a potential link (CDR) in a mechanistic chain from Ca^{2+} release to subsequent microtubule disassembly and anaphase chromosome movement.

The mechanism by which CDR and the other Ca^{2+}-binding proteins act to depolymerize microtubules remains to be elucidated. However, the biphasic nature of the CDR- and troponin C-induced disassembly of microtubules indicates that there may be multiple disassembly pathways. The initial precipitous decrease in turbidity or viscosity suggests that at high concentrations the Ca^{2+}-binding proteins may act along the entire surface lattice of the microtubule to cause disassembly, perhaps by binding to exposed sites on the tubulin subunits. The long period of slow decrease in turbidity is more consistent with events occurring at the ends of the microtubules. The recent demonstration of opposite end assembly-disassembly for microtubule polymerization *in vitro* by Margolis and Wilson (37) provides a conceptual framework for considering events at microtubule ends. The Ca^{2+}-binding proteins might act at the assembly end to form a cap structure in analogy to the mechanism of substoichiometric colchicine inhibition of microtubule assembly (38). Otherwise, the Ca^{2+}-binding proteins might act at the disassembly end by binding to subunits as they are released and preventing their subsequent reincorporation at an assembly site.

The preparation of CDR antibodies used in this study was shown to be monospecific by a number of criteria, and troponin C was specifically shown not to crossreact.* Additionally, attempts to isolate and purify troponin C from nonmuscle sources have resulted in the purification of either S-100 protein and CDR in the case of brain tissue (39) or CDR alone from a variety of tissues (40–42). Thus, it is apparently CDR or an antigenically crossreactive homologue and not troponin C which is localized in the anaphase mitotic half-spindle and may function in the Ca^{2+}-dependent disassembly of microtubules during chromosome movement. However, in the absence of experimental evidence concerning the possible presence of troponin C in the spindle, it is premature to rule out an involvement for troponin C in Ca^{2+}-mediated microtubule disassembly.

Although the molecular mechanism of Ca^{2+}-binding protein-dependent inhibition of microtubule assembly remains to be determined, the data of Table 1 indicate that this inhibition

is stoichiometric rather than catalytic. However, the variation in efficacy of the Ca^{2+}-binding proteins as inhibitors (Table 1) is somewhat perplexing, particularly the apparent 4-fold potency difference between troponin C and CDR. It should be emphasized that the biochemical results presented here were obtained in a reconstituted *in vitro* system. Therefore, it is preliminary to assume that the high molar ratios of CDR to tubulin necessary *in vitro* are required for effective inhibition *in vivo*. The *in vitro* assay conditions used may be suboptimal, and other factors may be required for efficient CDR inhibition of microtubule assembly. It is possible, in analogy to the troponin system in muscle, that CDR is part of a multiprotein complex. Such complexes might include proteins that would act to bind and localize CDR in the mitotic half-spindle or the cytoplasmic microfilament bundles, thus providing the cell with another level of specificity and control over these organelle systems.

The observation that Ca^{2+}-binding proteins can dramatically alter the Ca^{2+} sensitivity of microtubule polymerization *in vitro* suggests a possible resolution of the Ca^{2+} controversy. CDR is abundant in brain tissue (micromolar amounts); thus the amount of exogenous Ca^{2+} required for inhibition of microtubule polymerization in brain extracts will depend on how much free Ca^{2+} is liberated during processing of the tissue. Although 1 mM EGTA is a universal component in microtubule homogenization buffers, methods of tissue preparation and disruption vary widely. The reported differences in the Ca^{2+} sensitivity of microtubule polymerization in purified preparations may reflect differences in the protein compositions of the microtubule protein solutions. We have observed that CDR is inefficiently removed during the assembly-disassembly cycle purification of microtubule protein, especially from bovine brain tissue. Therefore, the presence of variable amounts of CDR or other Ca^{2+}-binding proteins in purified preparations of microtubule protein might account for the micromolar Ca^{2+} sensitivity reported by Rosenfeld *et al.* (13) and others (43), and the millimolar Ca^{2+} sensitivity reported by Olmsted and Borisy (12). There is insufficient data available to determine if the factor described by Nishida and Sakai (17), which is present in porcine brain extracts and can increase the Ca^{2+} sensitivity of polymerization of purified microtubule protein, is CDR, another Ca^{2+}-binding protein, or an uncharacterized Ca^{2+}-modulator of microtubule assembly.

In conclusion, we have demonstrated by indirect immunofluorescence that a ubiquitous, low molecular weight, Ca^{2+}-binding regulatory protein, CDR, is localized in the half-spindle of mitotic cells. Rat testis CDR and a homologous Ca^{2+}-binding protein, skeletal muscle troponin C, were shown to both inhibit and reverse *in vitro* microtubule assembly in a Ca^{2+}-dependent manner. These results provide evidence for Ca^{2+} as an endogenous regulator of microtubule disassembly during mitosis by binding to and activating CDR.

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1. Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. & Means, A. R. (1977) *J. Biol. Chem.* **252,** 8415–8422.
2. Dedman, J. R., Jackson, R. L., Schreiber, W. E. & Means, A. R. (1978) *J. Biol. Chem.* **253,** 343–346.
3. Dedman, J. R., Potter, J. D. & Means, A. R. (1977) *J. Biol. Chem.* **252,** 2437–2440.
4. Potter, J. D., Johnson, J. D., Dedman, J. R., Schreiber, W. E., Mandel, F., Jackson, R. L. & Means, A. R. (1977) in *Calcium-Binding Proteins and Calcium Function*, eds. Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H. & Siegel, F. L. (Elsevier North-Holland, New York), pp. 239–250.
5. Dabrowska, R., Aromatorio, D. K., Sherry, J. M. F. & Hartshorne, D. J. (1977) *Biochem. Biophys. Res. Commun.* **78,** 1263–1272.
6. Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K. & Hartshorne, D. J. (1978) *Biochemistry* **17,** 253–258.
7. Blomberg, F., Cohen, R. & Siekevitz, P. (1977) *J. Cell Biol.* **74,** 204–225.
8. Welsh, M. J., Dedman, J. R., Brinkley, B. R. & Means, A. R. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1867–1871.
9. Weisenberg, R. C. (1972) *Science* **177,** 1104–1106.
10. Borisy, G. G. & Olmsted, J. B. (1972) *Science* **177,** 1196–1197.
11. Olmsted, J. B. & Borisy, G. G. (1973) *Biochemistry* **12,** 4282–4289.
12. Olmsted, J. B. & Borisy, G. G. (1975) *Biochemistry* **14,** 2996–3005.
13. Rosenfeld, A. C., Zackroff, R. V. & Weisenberg, R. C. (1976) *FEBS Lett.* **65,** 144–147.
14. Keihart, D. P. & Inoué, S. (1976) *J. Cell Biol.* **70,** 230a.
15. Fuller, G. M. & Brinkley, B. R. (1976) *J. Supramol. Struct.* **5,** 497–514.
16. Salmon, E. D. & Jenkins, R. (1977) *J. Cell Biol.* **75,** 295a.
17. Nishida, E. & Sakai, H. (1977) *J. Biochem.* **82,** 303–306.
18. Fuller, G. M., Brinkley, B. R. & Boughter, J. M. (1975) *Science* **187,** 948–950.
19. Brinkley, B. R., Fuller, G. M. & Highfield, D. P. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4981–4985.
20. Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B. & Johnson, K. A. (1975) *Ann. N. Y. Acad. Sci.* **253,** 107–132.
21. Schacterle, G. R. & Pollak, R. L. (1973) *Anal. Biochem.* **51,** 654–655.
22. Kretsinger, R. H. & Nockolds, C. E. (1973) *J. Biol. Chem.* **248,** 3313–3326.
23. Perrin, D. D. & Sayce, I. G. (1967) *Talanta* **14,** 833–842.
24. Potter, J. D. & Gergely, J. (1975) *J. Biol. Chem.* **250,** 4628–4633.
25. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
26. Inoué, S. & Ritter, H. (1975) in *Molecules and Cell Movement*, eds. Inoué, S. & Stephens, R. E. (Raven, New York), pp. 3–29.
27. Collins, J. H., Greaser, M. L., Potter, J. D. & Horn, M. J. (1977) *J. Biol. Chem.* **252,** 6356–6362.
28. Coffee, C. J. & Bradshaw, R. A. (1973) *J. Biol. Chem.* **248,** 3305–3312.
29. Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun.* **33,** 533–538.
30. Cheung, W. Y. (1971) *J. Biol. Chem.* **246,** 2859–2869.
31. Kakiuchi, S., Yamazaki, R. & Nakajima, H. (1970) *Proc. Jap. Acad.* **46,** 587–592.
32. Brostrom, C. O., Huang, Y.-C., Breckenridge, B. M. & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 64–68.
33. Lynch, T. J., Tallant, E. A. & Cheung, W. Y. (1976) *Biochem. Biophys. Res. Commun.* **68,** 616–624.
34. Gopinath, R. M. & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun.* **77,** 1203–1209.
35. Jarrett, H. W. & Penniston, J. T. (1977) *Biochem. Biophys. Res. Commun.* **77,** 1210–1216.
36. Luthra, M. G., Au, K. S. & Hanahan, D. J. (1977) *Biochem. Biophys. Res. Commun.* **77,** 678–688.
37. Margolis, R. L. & Wilson, L. (1978) *Cell* **13,** 1–8.
38. Margolis, R. L. & Wilson, L. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3466–3470.
39. Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharef, F. & Vanaman, T. C. (1976) *J. Biol. Chem.* **251,** 4501–4513.
40. Brooks, J. C. & Siegel, F. L. (1973) *Biochem. Biophys. Res. Commun.* **55,** 710–716.
41. Teo, T. S. & Wang, T. H. (1973) *J. Biol. Chem.* **248,** 588–595.
42. Kuo, I. C. Y. & Coffee, C. J. (1975) *J. Biol. Chem.* **251,** 1603–1609.
43. Haga, T., Abe, T. & Kurokawa, M. (1974) *FEBS Lett.* **39,** 291–295.