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Calcium-dependent regulator protein: Localization in mitotic apparatus of eukaryotic cells*

(calcium binding protein/mitosis/microfilaments/microtubules)

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ABSTRACT **Calcium-dependent regulator protein is a low molecular weight (17,000), thermostable, calcium binding protein which is structurally homologous to skeletal muscle troponin C. This protein is present in all nonmuscle cells and has been shown to decorate stress fibers in interphase cells by indirect immunofluorescence. Using this procedure we have investigated the distribution of the protein during mitosis of eukaryotic cells. As the cells enter prophase, the distinct cytoplasmic localization disappears commensurate with the dissolution of the cytoskeleton. The regulator protein seems to be randomly distributed throughout the prophase cell, including the region around the condensed chromosomes. However, at prometaphase, it is localized in association with the half-spindles of the mitotic apparatus. Through metaphase and most of anaphase, the protein remains localized between the chromosomes and the poles of the spindle. During late anaphase the protein is also found in the interzone region but rapidly condenses into two small regions, one on each side of the midbody that separates the daughter cells. The regulator protein is not localized in the cleavage furrow during telophase, whereas actin is demonstrable in this region. Indeed, placement of the protein during mitosis is distinct from both that of actin and that of tubulin. The localization of calcium-dependent regulator protein during mitosis suggests that it may mediate the calcium effects on the mitotic apparatus and thus play a role in chromosome movement.**

Cheung (1, 2) and Kakiuchi *et al.* (3) independently discovered that a low molecular weight, thermostable protein would activate brain cyclic nucleotide phosphodiesterase. Since these initial observations, similar activities have been demonstrated in virtually every tissue tested (4–6). Teo and Wang (7) further demonstrated that the activation required micromolar levels of calcium. Hence, the protein is referred to as the Ca^{2+}-dependent regulatory protein (CDR). Wang and coworkers (8, 9) first noted the structural similarity between CDR and troponin-C, the calcium-binding subunit of troponin that regulates skeletal muscle ATPase activity and muscle contraction. We have isolated CDR from rat testes and have shown that the primary amino acid sequence of CDR has approximately 50% direct homology with troponin-C (10). Although both proteins bind 4 mol of Ca^{2+} per mol of protein, the specificity and affinity of the metal-binding sites are quite distinct (11). CDR and troponin-C will, however, substitute for each other in their respective biological systems (12). We have made monospecific antibodies against native CDR and have used indirect immunofluorescence to localize CDR within interphase cells.† These observations suggest that CDR may provide a function in nonmuscle cells similar to that of tropinin-C in muscle. In this communication we demonstrate the specific localization of CDR in the mitotic spindle of a variety of vertebrate cells *in vitro*. In all cell types examined, anti-CDR fluorescence was associated with the mitotic apparatus in a pattern distinct from that of actin and tubulin.

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MATERIALS AND METHODS

Cells and Cell Culture. BALB/C 3T3 and PtK_1 cells are maintained routinely in our laboratories and were originally obtained from American Type Culture. The cells were cultured in Eagle's minimal essential medium (Gibco) supplemented with 10% fetal calf serum (Gibco) at 37° with 5% CO_2/95% air atmosphere. Both cell types were cultured in Corning plastic culture dishes (35 mm diameter) containing two 11 × 22-mm glass coverslips per dish.

Antibodies to CDR and Actin. CDR was purified from rat testis as described (11, 13). An adult male goat was used for production of antibodies against electrophoretically homogeneous CDR. The antibodies were purified by affinity chromatography on CDR–Sepharose.† Antibodies to rabbit muscle actin (IgG fraction) produced in rabbits were the generous gift of Janet Morgan and Brian Spooner (Kansas State University).

Indirect Immunofluorescence Procedure. The indirect immunofluorescence procedure was similar to that described by Fuller *et al.* (14). Coverslips on which the cells had been cultured were removed from the culture dishes. The upper left corner of each coverslip was broken off. In all subsequent manipulations the broken corner served to identify the side of the coverslip to which the cells were attached. Each coverslip was rinsed in Dulbecco's phosphate-buffered saline (P_i/NaCl), then fixed in 3% formalin/P_i/NaCl for 90 min at room temperature. The coverslips were rinsed in P_i/NaCl and subsequently fixed in absolute acetone at −20° for 10 min. After the preparations were rinsed in P_i/NaCl, they were inverted over a small drop (15 μl) of primary antibody for 5 hr at 34°. Actin IgG fraction was diluted 1:10 in P_i/NaCl before use and CDR antibody concentration was 100 μg of protein per ml. The preparations were washed twice for 30 min in P_i/NaCl, then incubated in second antibody for 45 min at 34°. When first antibody was anti-actin, second antibody was fluorescein-conjugated goat anti-rabbit IgG (IgG fraction, Cappel Laboratories), 1:50 in P_i/NaCl. When first antibody was anti-CDR, second antibody was fluorescein-conjugated rabbit anti-goat IgG (IgG fraction, Cappel Laboratories), 1:50 in P_i/NaCl. The coverslips were subjected to two 10-min in washes P_i/NaCl, rinsed briefly in distilled water, and mounted on glass microscope slides in a drop

Abbreviations: CDR, calcium-dependent regulator protein; P_i/NaCl, phosphate-buffered saline.

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† J. R. Dedman, M. J. Welsh, and A. R. Means, unpublished data.

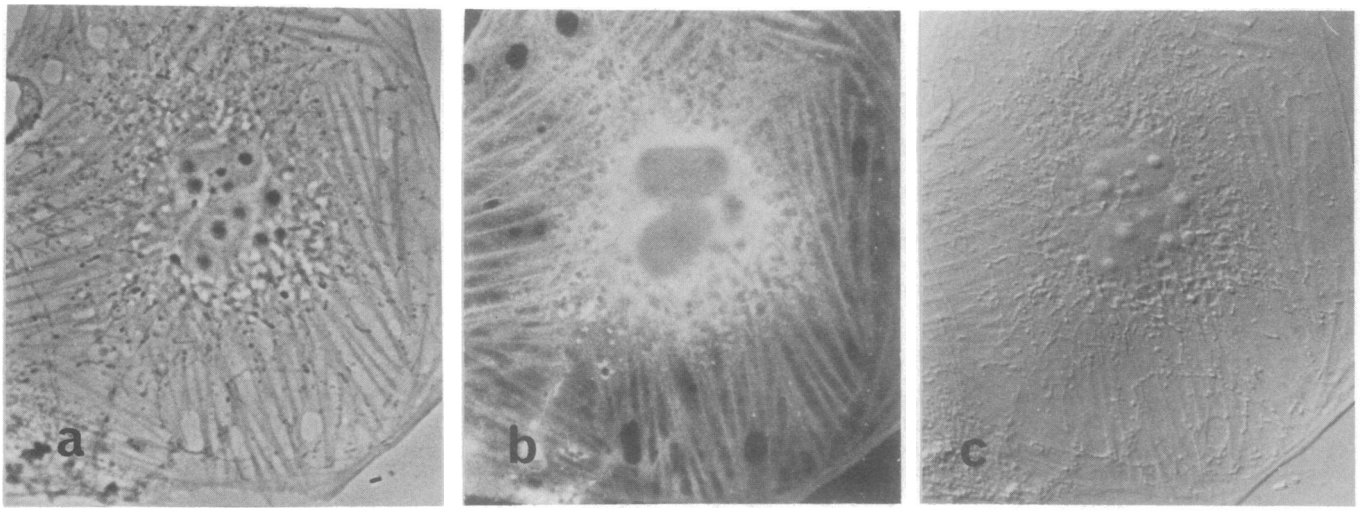

FIG. 1. CDR localization in interphase mouse 3T3 cell. Indirect immunofluorescence was performed as described in *Materials and Methods.* (*a*) Phase contrast microscopy. (*b*) Fluorescence microscopy. (*c*) Interference contrast microscopy. (All photographs ×570.)

of P_i/NaCl/glycerol (1:9), pH 8.5. Cells were viewed through a Leitz microscope with epi-illumination adapted for darkfield ultraviolet microscopy. Images were recorded on Kodak Tri-X film developed in either Kodak HC-110 developer (Tri-X rated at 400 ASA) or Acufine developer (Tri-X rated at 1000 ASA). Control preparations for CDR consisted of cells incubated in preimmune serum IgG fraction, in immune serum IgG fraction not retained on the CDR–Sepharose affinity column, or in fluorescein-conjugated second antibody only.

RESULTS

Interphase. CDR is localized in a pattern identical to the stress fibers seen when the cells are examined with a phase contrast or interference contrast microscope (Fig. 1).

Mitosis. During mitosis, CDR was specifically localized in the mitotic spindle (Figs. 2 and 3). Although the figures shown are representative for 3T3 and PtK_1 cells, identical localization was observed in all other cells examined (SV-3T3, HeLa, human testis fibroblasts, rat Sertoli cells, mouse mammary and mammary adenocarcinoma, mouse × human somatic cell hybrids, and chick liver fibroblasts). When cells were incubated with preimmune goat serum (Fig. 2*e*), with immune goat IgG serum fraction not retailed on the CDR affinity column (Fig. 2*f*), or with second antibody only (not shown), fluorescence was not seen in interphase or mitotic cells.

During all of prophase, diffuse staining of the cytoplasm was apparent, but no specific localization of CDR was evident on the early prophase spindle (Figs. 2*a* and 3*a*). CDR staining of the spindle first became evident at prometaphase (Fig. 2*b*) and became more intense at metaphase (Fig. 3*b*). Staining was most intense at the spindle poles and it projected toward the chromosomes in a distribution that gave the appearance of fibers. The pattern of CDR-specific fluorescence did not traverse the metaphase plate but terminated on or before reaching the chromosomes. In some cells at metaphase, especially in cells observed from a polar view or in flat metaphase cells, CDR appeared to be excluded from the region in the immediate vicinity of the centriole (Fig. 3*c*), suggesting that CDR may not be a component of the amorphous material surrounding the centrioles.

As the chromosome pairs separate during early anaphase, CDR was localized in the half-spindles but was absent from the interzonal region (Figs. 2*c* and 3*d*). This pattern is distinctly different from the immunofluorescence observed after antitubulin staining (14, 15). Tubulin localization is apparent in both the interzone (interpolar fibers) and the half-spindle. CDR-specific fluorescence was more intense near the poles and, as the chromosome-to-pole distance diminished, the fluorescence became more closely associated with the chromosomes. In late anaphase, patches of fluorescence appeared to be on or closely associated with the chromosomes (Figs. 2*c* and 3 *d* and *e*).

During late anaphase or early telophase, CDR appeared abruptly in the interzone region (Fig. 3*e*). The CDR is distributed as linear fibers parallel to the pole-to-pole axis of the dividing cell with a prominent discontinuity near the "Zwischenkorper." As late telophase approaches, polar fluorescence diminishes as the interzonal CDR condenses into two small, intensely fluorescent areas, one on each side of the midbody (Figs. 2*d* and 3 *f* and *g*). CDR was not localized in the cleavage furrow during cytokinesis (Fig. 3*h*) although actin was clearly present in this region (Fig. 3*i*) (see also ref. 16). Some CDR staining was apparent in the cytoplasm surrounding the spindle in all stages of mitosis, but the fluorescence was diffuse and not visibly organized into fibrous structures.

DISCUSSION

We have found that in many interphase cells, CDR was localized in fibrous elements of the cytoplasm, including the actin-containing cellular stress fibers (Fig. 1). Beginning in early prophase of mitosis, the cytoskeleton of cells became disorganized, as reflected by actin (17) and tubulin immunofluorescence (15). Likewise, the pattern of CDR in the cytoplasm became diffuse and was not visibly organized into fibrous structures. Cytoplasmic fluorescence in mitotic cells was often brighter than in interphase cells and may be attributed to the Beer–Lamberts law since mitotic cells tend to be more rounded and of greater thickness than cells in interphase. Increased cytoplasmic fluorescent intensity in mitotic cells may also be the result of disassembly of interphase cytoskeletal structures, freeing a larger pool of CDR which more readily binds with CDR antibody.

The present results suggest several possible roles for CDR in the mitotic process. As discussed in the introduction, CDR is structurally similar to troponin-C (10) and these proteins will crossreact in their respective biological systems (12). This sug-

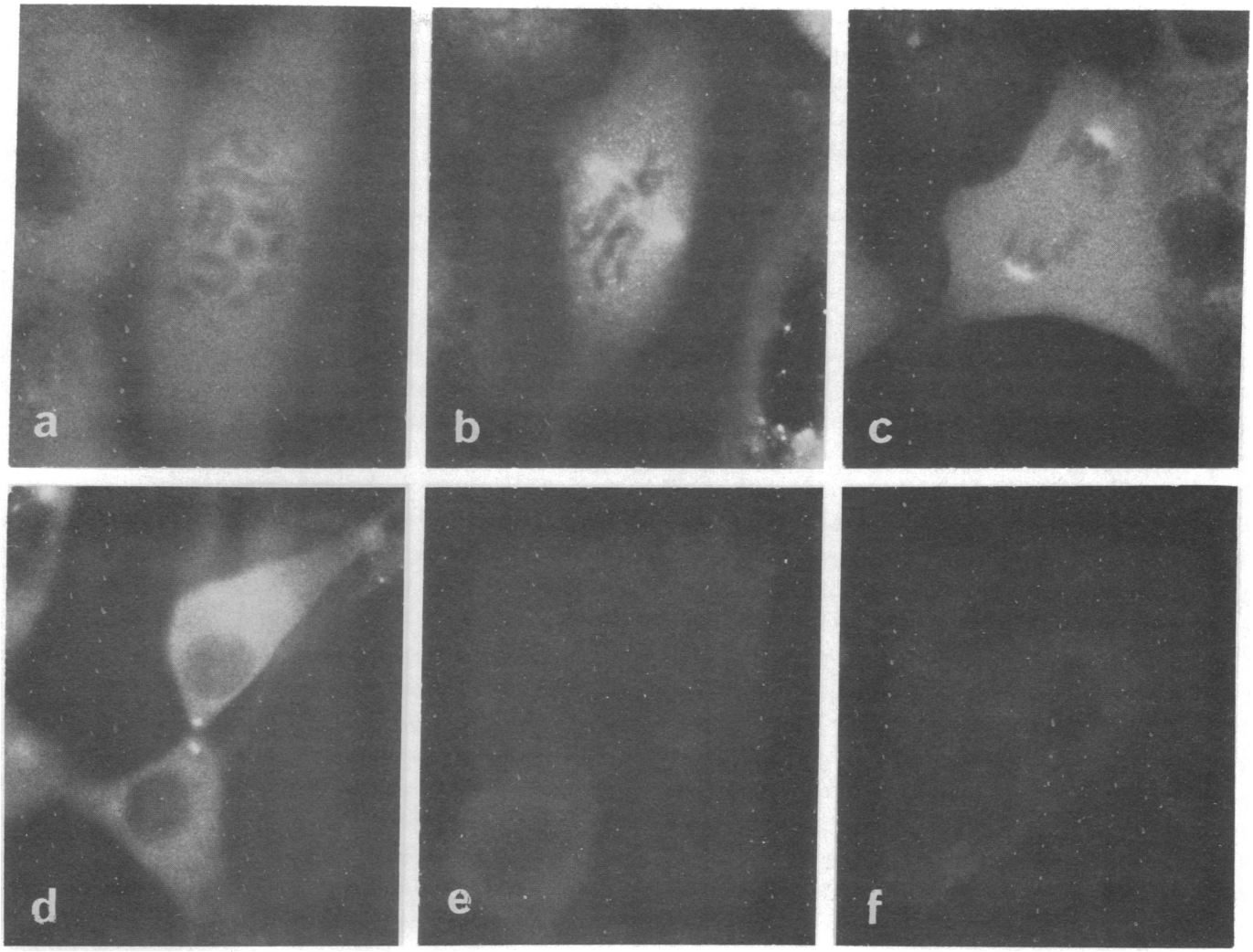

FIG. 2. CDR immunofluorescence in mitotic rat kangaroo PtK_1 cells. Indirect immunofluorescence was performed as described in *Materials and Methods*. (*a*) Prophase. (×800.) (*b*) Prometaphase. (×550.) (*c*) Anaphase. (×700.) (*d*) Telophase. (×550.) (*e*) Metaphase and anaphase, preimmune goat serum IgG used as primary antibody. (×525.) (*f*) Metaphase, immune goat IgG not retained on CDR affinity column used as primary antibody. (×525.)

gested the possibility that CDR might serve a troponin-C-like function during mitosis. There is considerable evidence for the localization and function of myosin (18) and actin (19–22) in the mitotic apparatus. In fact, the specific localization of actin in the half-spindles is similar to the pattern described for CDR in the present study. However, evidence for the presence of actin or myosin in the mitotic apparatus is controversial. Actin immunofluorescence (21) or heavy meromyosin decoration (19, 20, 22) of actin in the mitotic apparatus has been demonstrated only after lysis and extraction of cells with either glycerol (19, 20, 22) or Triton X-100 (21) prior to fixation. Extraction of the cells before fixing may allow artifactual redistribution of actin to the spindle. Fujiwara and Pollard (18) examined the localization of myosin in cultured cells using standard procedures of indirect immunofluorescence. When purified antimyosin was used, myosin-specific fluorescence was present in the cleavage furrow but not localized in the spindle. The results suggested that the major role of actin-myosin during mitosis might be in cytokinesis. Similar conclusions were reached by Kiehart *et al.* (23), who demonstrated that injection of antimyosin into marine oocytes did not affect chromosome movement but did inhibit cytokinesis. We also used standard procedures for indirect immunofluorescence and found actin to be specifically associated with the cleavage furrow during cytokinesis (Fig. 3*i*). At other stages of mitosis only diffuse cytoplasmic staining was observed. On the other hand, CDR, while present in the spindle, was not concentrated in the cleavage furrow (Fig. 3*h*).

The distribution of CDR-specific fluorescence in prominent parallel arrays in interphase cells is identical to the pattern of stress fibers seen in cells observed by phase contrast or interference contrast microscopy (Fig. 1), suggesting that CDR is a component of the microfilament bundles. The absence of CDR in the cleavage furrow (contractile ring) is interesting since this region also contains microfilament bundles. However, the microfilament bundles of the contractile ring may differ in molecular composition from those of the stress fibers in interphase cells. Alternatively, intracellular calcium concentrations may dictate binding specificity which results in differential affinity for the actin-myosin filaments. Indeed, Dabrowska *et al.* (24) have shown that CDR is required for the phosphorylation of smooth muscle myosin which is requisite for actin stimulation of myosin ATPase.

The similarity of CDR to troponin-C, the Ca^{2+}-binding regulatory protein of muscle, suggests an alternate role for this protein in mitosis. CDR will substitute for troponin-C in the

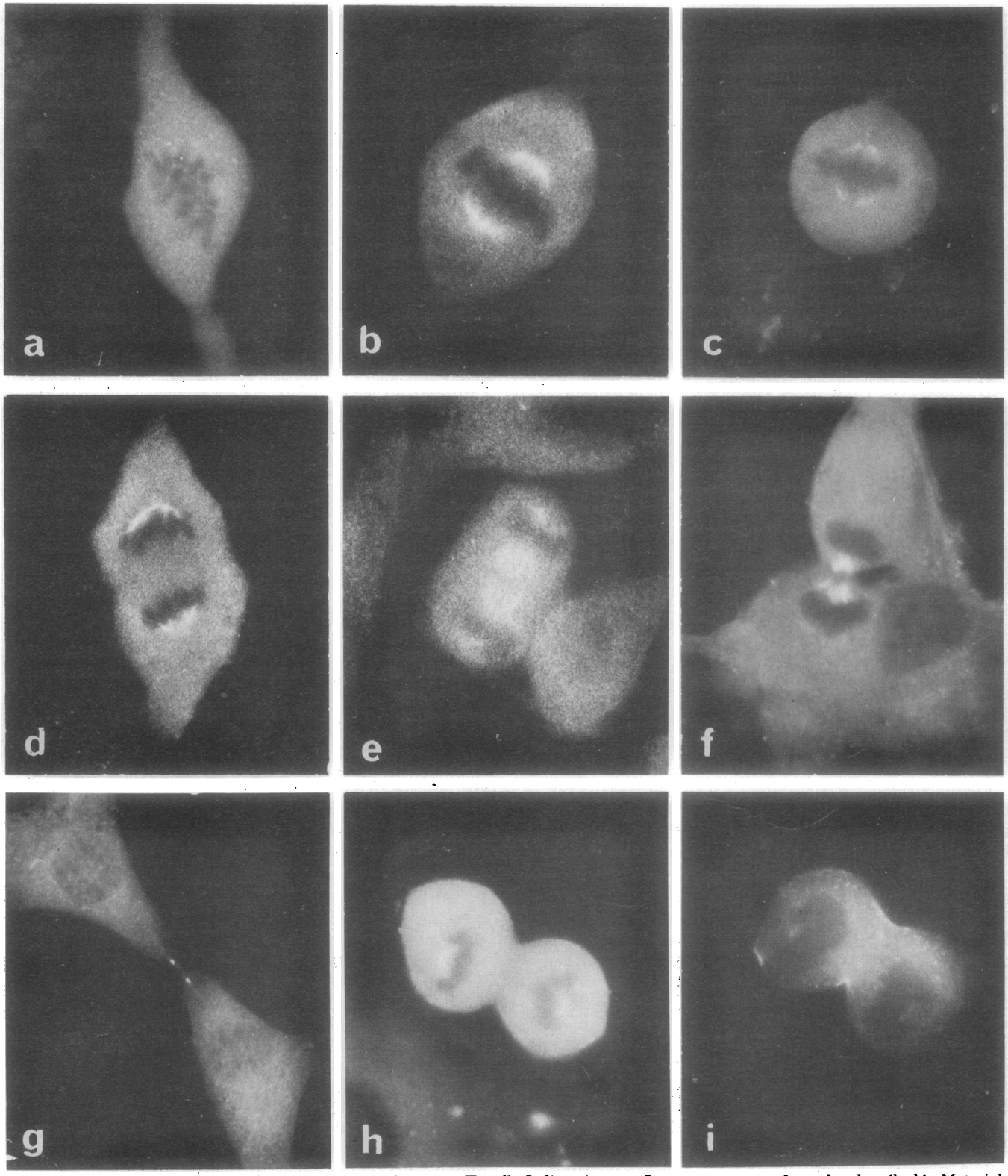

FIG. 3. CDR and actin immunofluorescence in mitotic mouse 3T3 cells. Indirect immunofluorescence was performed as described in *Materials and Methods*. (*a*) CDR in prophase. (×600.) (*b*) CDR in metaphase. (×650.) (*c*) CDR in metaphase. (×560.) (*d*) CDR in anaphase. (×600.) (*e*) CDR in late anaphase. (×700.) (*f*) CDR in telophase. (×560.) (*g*) CDR in daughter cells. (×480.) (*h*) CDR in telophase. (×700.) (*i*) Actin in telophase. (×700.)

regulation of actomyosin ATPase. In addition, CDR regulates the Ca^{2+}-Mg^{2+}-ATPase (calcium pump) of erythrocyte plasma membrane. A Ca^{2+}-sensitive ATPase has also been shown to be associated with the mitotic apparatus (25). The activity of this enzyme peaks during mitosis in many cell types (26, 27). Moreover, the Ca^{2+}-ATPase activity has been identified with the smooth endoplasmic reticulum of mitotic cells (28). Thus, it is possible that this vesicular system may serve a Ca^{2+} se-

questering function much like the sarcoplasmic reticulum (29, 30). Therefore, CDR, through its Ca^{2+}-binding properties, could play a functional role by regulating Ca^{2+} levels near the spindle.

Another possible role for CDR during mitosis involves microtubules. Microtubles are a major component of the spindle fibers (31). Chromosome movement to the poles requires the depolymerization of kineticore-associated microtubules (32, 33). Numerous investigators have shown that Ca^{2+} is a potent inhibitor of microtubule assembly. Although the localization of CDR and tubulin in the half-spindle is not identical in all stages of mitosis, CDR is found in the region where microtubule depolymerization is occurring. Thus, CDR could mediate microtubule depolymerization in a Ca^{2+}-dependent manner. Accordingly, CDR might also be expected to associate with cytoplasmic microtubules. The absence of CDR staining of cytoplasmic microtubules in our cells may be due, in part, to the relative density of microtubules in these two systems. Thus, spindle microtubules are packed into a small area of the cytoplasm whereas cytoplasmic microtubules are more dispersed. Of course, alternate mechanisms of Ca^{2+} regulation of spindle and cytoplasmic microtubules cannot be ruled out.

The association of CDR with the mitotic apparatus and its widespread distribution in a variety of eukaryotic cells suggest a fundamental role for this protein in motile systems in nonmuscle cells. Such a suggesion is strengthened by the multifunctional activities already demonstrated for CDR, including regulation of the calcium pump, actomyosin ATPase, and cyclic nucleotide metabolism. Additional biochemical and ultrastructural studies are needed to determine if CDR is directly associated with microfilaments and/or microtubules or with the smooth endoplasmic reticulum or if CDR may play multiple roles in coordinating the dynamics of the interphase cytoskeleton and the mitotic apparatus.

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1. Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun.* **33**, 533–538.
2. Cheung, W. Y. (1971) *J. Biol. Chem.* **246**, 2859–2869.
3. Kakiuchi, S., Yamazaki, R. & Nakajima, H. (1970) *Proc. Jpn. Acad.* **46**, 587–592.
4. Waisman, D., Stevens, F. C. & Wang, J. H. (1975) *Biochem. Biophys. Res. Commun.* **65**, 975–982.
5. Smoake, J. A., Song, S.-Y., & Cheung, W. Y. (1974) *Biochim. Biophys. Acta* **341**, 402–411.
6. Kakiuchi, S., Yamazaki, R., Teshima, Y., Venishi, K. & Miyamoto, E. (1975) *Biochem. J.* **146**, 109–120.
7. Teo, T. S. & Wang, J. H. (1973) *J. Biol. Chem.* **248**, 5950–5855.
8. Wang, J. H., Teo, T. S., Ho, H. C. & Stevens, F. C. (1974) *Adv. Cyclic Nucleotide Res.* **5**, 179–194.
9. Stevens, F. C., Walsh, M., Ho, H. C., Teo, T. S. & Wang, J. H. (1976) *J. Biol. Chem.* **251**, 4495–4500.
10. Dedman, J. R., Jackson, R. L., Schrieber, W. E. & Means, A. R. (1978) *J. Biol. Chem.* **253**, 343–346.
11. Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. & Means, A. R. (1977) *J. Biol. Chem.* **252**, 8415–8422.
12. Dedman, J. R., Potter, J. D. & Means, A. R. (1977) *J. Biol. Chem.* **252**, 2437–2440.
13. Dedman, J. R., Fakunding, J. L. & Means, A. R. (1977) in *Hormone Action and Molecular Endocrinology Workshop Syllabus*, eds. O'Malley, B. W. & Schrader, W. T. (The Endocrine Society, Bethesda, MD), pp. 901–946.
14. Fuller, G. M., Brinkley, B. R. & Boughter, J. M. (1975) *Science* **187**, 948–950.
15. Brinkley, B. R., Fuller, G. M. & Highfield, D. P. (1975) *Proc. Natl. Acad. Sci.* **72**, 4981–4985.
16. Schroeder, T. E. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1688–1692.
17. Lazarides, E. & Weber, K. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2268–2270.
18. Fujiwara, K. & Pollard, T. D. (1976) *J. Cell Biol.* **71**, 848–875.
19. Hinkley, R. & Telser, A. (1974) *Exp. Cell Res.* **86**, 161–164.
20. Sanger, J. W. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2451–2455.
21. Cande, W. Z., Lazarides, E. & McIntosh, J. R. (1977) *J. Cell. Biol.* **72**, 552–567.
22. Schloss, J. A., Milsted, A. & Goldman, A. D. (1977) *J. Cell Biol.* **74**, 794–815.
23. Kiehart, P., Inoúe, S. & Mabuchi, I. (1977) *J. Cell Biol.* **75**, 258a.
24. Dabrowska, R., Aromatorio, D., Sherry, J. M. F. & Hartshorne, D. J. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1263–1272.
25. Mazia, D., Petzelt, C., Williams, R. O. & Meza, I. (1972) *Exp. Cell Res.* **70**, 325–332.
26. Petzelt, C. & vonLedebur-Villiger, M. (1973) *Exp. Cell Res.* **81**, 87–94.
27. Petzelt, C. & Auel, D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1610–1613.
28. Harris, P. (1975) *Exp. Cell Res.* **94**, 409–425.
29. Hasselbach, W. & Makinose, M. (1963) *Biochem. Z.* **339**, 94–111.
30. Weber, A., Herz, R. & Reiss, I. (1966) *Biochem. Z.* **345**, 329–369.
31. Sato, H., Ellis, G. W. & Inoúe, S. (1975) *J. Cell. Biol.* **67**, 501–517.
32. Fuseler, J. (1975) *J. Cell Biol.* **67**, 789–800.
33. Inoúe, S. & Ritter, H. (1975) in *Molecules and Cell Movement*, eds. Inoúe, S. & Stephens, R. E. (Raven, New York), pp. 3–30.