Localization of Caldesmon and Its Dephosphorylation during Cell Division

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Abstract. Mitosis-specific phosphorylation by cdc2 kinase causes nonmuscle caldesmon to dissociate from microfilaments during prometaphase. (Yamashiro, S., Y. Yamakita, R. Ishikawa, and F. Matsumura. 1990. *Nature (Lond.).* 344:675–678; Yamashiro, S., Y. Yamakita, H. Hosoya, and F. Matsumura. 1991. *Nature (Lond.)* 349:169–172). To explore the functions of caldesmon phosphorylation during cytokinesis, we have examined the relationship between the phosphorylation level, actin-binding, and in vivo localization of caldesmon in cultured cells after their release of metaphase arrest. Immunofluorescence studies have revealed that caldesmon is localized diffusely throughout cytoplasm in metaphase. During early stages of cytokinesis, caldesmon is still diffusely present and not concentrated in contractile rings, in contrast to the accumulation of actin in cleavage furrows during cytokinesis. In later stages of cytokinesis, most caldesmon is observed to be yet diffusely localized although some concentration of caldesmon is observed in cortexes as well as in cleavage furrows. When daughter cells begin to spread, caldesmon shows complete colocalization with F-actin–containing structures. These observations are consistent with changes in the levels of microfilament-associated caldesmon during synchronized cell division. Caldesmon is missing from microfilaments in prometaphase cells arrested by nocodazole treatment, as shown previously (Yamashiro, S., Y. Yamakita, R. Iskikawa, and F. Matsumura. 1990. *Nature (Lond.).* 344:675–678). The level of microfilament-associated caldesmon stays low (12% of that of interphase cells) when some cells start cytokinesis at 40 min after the release of metaphase arrest. When 60% of cells finish cytokinesis at 60 min, the level of microfilament-associated caldesmon is recovered to 50% of that of interphase cells. The level of microfilament-associated caldesmon is then gradually increased to 80% when cells show spreading at 120 min. Dephosphorylation appears to occur during cytokinesis. It starts when cells begin to show cytokinesis at 40 min and completes when most cells finish cytokinesis at 60 min. These results suggest that caldesmon is not associated with microfilaments of cleavage furrows at least in initial stages of cytokinesis and that dephosphorylation of caldesmon appears to couple with its reassociation with microfilaments. Because caldesmon is known to inhibit actomyosin ATPase and/or regulate actin assembly, its continued dissociation from microfilaments may be required for the assembly and/or activation of contractile rings.

MICROFILAMENTS undergo profound changes in their cytoplasmic organization during mitosis. In cultured cells, microfilament bundles present in interphase cells are disassembled when cells enter prophase. Subsequently, contractile rings are transiently formed for cytoplasmic division. After cytokinesis, microfilaments reassemble into stress fibers of two daughter cells (Sanger and Sanger, 1976; Schroeder, 1976). While a variety of actin-binding proteins are believed to be involved in these processes, little is known regarding their functions in these processes.

We have shown previously that nonmuscle caldesmon, one of the actin-binding proteins, is specifically dissociated from microfilaments during prometaphase as a result of phosphorylation by $p34^{cdc2}$ kinase (Yamashiro et al., 1990 and 1991). Cdc2 kinase is a catalytic subunit of maturation or mitosis promoting factor (MPF)[1] which contains cyclin as the other subunit (Arion et al., 1988; Dunphy et al., 1988; Gautier et al., 1988; Labbe et al., 1988). MPF is both sufficient and necessary to induce all mitotic events including nuclear envelope breakdown, chromosome condensation, and spindle formation (for reviews see Hunt, 1989; Lohka, 1989; Norbury and Nurse, 1989). Although it has been reported that cdc2 kinase phosphorylates a variety of substrates (for reviews see Moreno and Nurse, 1990) including histone H1 (Arion et al., 1988), Large T (McVey et al., 1989), RNA

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1. *Abbreviations used in this paper*: MPF, maturation or mitosis promoting factor; NCS, newborn calf serum.

polymerase II (Cisek and Corden, 1989), nuclear lamins (Peter et al., 1990), pp60$^{c\text{-}src}$ (Morgan et al., 1989; Shenoy et al., 1989), c-Abl (Kipreos and Wang, 1990), intermediate filament protein (Chou et al., 1990), and caldesmon (Mak et al., 1991; Yamashiro et al., 1991), it is an open question how cdc2 kinase regulates these complex changes in cell structures. Our finding of cell cycle–dependent phosphorylation of caldesmon by cdc2 kinase suggests that cdc2 kinase directly affects microfilament reorganization during mitosis.

Caldesmon is a unique actin-binding protein, whose binding to actin is regulated by Ca^{2+}/calmodulin (Sobue et al., 1981). While caldesmon was first identified in smooth muscle, it is widely distributed in nonmuscle cells as well. These nonmuscle caldesmons have lower molecular masses on SDS gels (ranging from 70–80 kD) than that of smooth muscle caldesmon (140 kD) (Bretscher, 1984; Bretscher and Lynch, 1985; Koji-Owada et al., 1984; Yamashiro-Matsumura and Matsumura, 1988), yet it shows similar properties to that of muscle caldesmon, such as Ca^{2+}/calmodulin regulation of actin binding, regulation of actin–myosin interaction, cross-reactivity to antibodies, and incorporation of microinjected caldesmon into stress fibers (Dingus et al., 1986; Sobue et al., 1985*b*; Yamashiro-Matsumura and Matsumura, 1988; Yamakita et al., 1990). Nonmuscle caldesmon is localized both in stress fibers and in membrane ruffles (Bretscher and Lynch, 1985; Koji-Owada et al., 1984; Yamashiro-Matsumura et al., 1988). It is distributed along stress fibers in a periodic fashion that is coincident with the distribution of tropomyosin and complementary to the distribution of alpha-actinin.

In vitro biochemical studies have suggested two different regulatory roles for caldesmon in the microfilament organization. First, caldesmon inhibits actin-activated ATPase activity of myosin (Dabrowska et al., 1985; Hemric and Chalovich, 1988; Horiuchi et al., 1986; Ngai and Walsh, 1984; Smith et al., 1987; Sobue et al., 1985; for reviews see Bretscher, 1986; Sobue and Sellers, 1991). Second, caldesmon coupled with tropomyosin inhibits both severing and capping activities of gelsolin (Ishikawa et al., 1989*a*,*b*). In the former case, the dissociation of nonmuscle caldesmon from microfilaments may release caldesmon's inhibition on actomyosin ATPase, leading to the contraction of actomyosin. In the latter case, the dissociation of caldesmon may release the inhibition of gelsolin activities by caldesmon–tropomyosin complexes, thereby resulting in the fragmentation of microfilaments into short filaments. In either case, the dissociation of caldesmon could cause large changes in microfilament organization. To further explore the functions of caldesmon, we asked two questions in this study: (*a*) Where does caldesmon localize in the cell during cytokinesis?, and (*b*) How long does caldesmon stay phosphorylated after metaphase? To this end, we examined the relationships among phosphorylation states, association with microfilaments, and localization of caldesmon after release of metaphase arrest. We have found that caldesmon shows diffuse localization during metaphase as well as during initial stages of cytokinesis. This is in contrast to the accumulation of actin and other actin binding proteins in cleavage furrows including myosin, tropomyosin, and α-actinin (Fujiwara and Pollard, 1978; Fujiwara et al., 1976; Ishimoda-Takagi, 1979). Caldesmon is found to be fully colocalized with F-actin structures as daughter cells begin to spread. Biochemical analyses with synchronized cells support the notion that caldesmon stays dissociated from F-actin during early stages of cytokinesis. Reassociation of caldesmon with F-actin appears to be coupled with dephosphorylation of caldesmon. Our results suggest that continued dissociation of caldesmon from microfilaments may be required for the reorganization of microfilaments seen during cell division, in particular, the disassembly of stress fibers during cell rounding at prophase and the formation and/or activation of contractile rings at anaphase.

Materials and Methods

Cell Culture Preparation of Mitotic Cells

SV-40 transformed rat embryo cells (REF-4A) were used in the present study. REF-4A cells were maintained in DME containing 10% newborn calf serum (NCS) in an atmosphere of 5% CO_2 and 95% air at 37°C. For culture of REF-4A cells in large scale, cells were grown in large square plates (245 × 245 × 20 mm, Nunc, Denmark) in the DME containing 6% NCS.

Cells at metaphase and later stages during cell division were prepared as follows. Cells were first treated for 3 h with 0.25 μg/ml nocodazole, and mitotic cells were collected as described previously (Yamashiro et al., 1990). After washing with DME at 0°C to remove nocodazole, cells were plated in fresh culture dishes or on glass coverslips, and incubated at 37°C in DME containing 10% NCS to allow cell cycle progression.

Indirect Immunofluorescence

Cells on glass coverslips were washed with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.3) and fixed with 3.7% formaldehyde solution in PBS for 5 min except for the staining with anti-tubulin antibody. After being washed in PBS, the cells were made permeable with absolute acetone (−20°C) for 10 min. The cells were then washed in PBS and reacted for 30 min at room temperature with mouse monoclonal anti-caldesmon antibody (Yamashiro-Matsumura and Matsumura, 1988) together with FITC-conjugated phalloidin (Sigma Immunochemicals, St. Louis, MO). Cells were then washed in PBS for 30 min and reacted with rhodamine-conjugated goat anti-mouse IgG (1:20 dilution; Cooper Biochemical Inc., Malvern, PA). After a further washing, the coverslips were mounted in Gelvatol (Monsanto brand of polyvinyl alcohol; Monsanto, St. Louis, MO) containing 100 μg/ml 1,4-diazabicyclo[2,2,2]-octane (Sigma Immunochemicals) to prevent bleaching.

For the staining with anti-tubulin antibody, cells on coverslips were fixed with absolute methanol for 15 min at −20°C. After rehydration in PBS, the coverslips were stained with a rabbit polyclonal anti-tubulin antibody (1:50 dilution; Polyscience Inc., Warrington, PA) followed by the staining with FITC-conjugated goat anti-rabbit IgG (1:20 dilution; Cooper Biochemical Inc.).

Isolation of Microfilaments from Cultured Cells

The method of microfilament isolation was described in detail in our previous paper (Matsumura et al., 1983). Briefly, cells were harvested and extracted for 2 min with Triton–glycerol solution (0.1 M Pipes–NaOH, 5 mM $MgCl_2$, 0.2 mM EGTA, 4 M glycerol, and 0.05% Triton X-100) to stabilize the cytoskeleton. After washing with PBS containing 0.2 mM EGTA and 5 mM $MgCl_2$, cell residues were collected and homogenized in the presence of 5 mM ATP, 0.5 mM DTT, and 5 mM PMSF. After centrifugation, 0.02 volumes of ascites fluid of a mAb against tropomyosin, IV15, were added to the supernatant to induce aggregation of microfilaments into bundles. The microfilament bundles were then collected by low speed centrifugation (12,000 *g*) for 8 min at 4°C and washed twice with PBS. The final pellet was suspended in 50 μl PBS and subjected to SDS-PAGE.

Urea/SDS-PAGE of Heat-stable Fractions from Cultured Cells

Urea/SDS-PAGE of heat-stable fractions were performed as described (Yamashiro et al., 1990) as mitotically phosphorylated caldesmon shows retarded mobility in this system. Briefly, cells were first washed three times

with PBS, and homogenized in PBS containing 5 mM ATP, 5 mM PMSF, and 0.5 mM DTT using a motor-driven Teflon homogenizer. In some experiments, 5 mM of NaF was further added to the buffer. Cell homogenates were then centrifuged at 12,000 *g* for 5 min at 4°C. Resulting supernatants were boiled for 8 min and chilled on ice for 30 min, followed by the centrifugation at 12,000 *g* for 5 min. The heat-stable supernatants were then subjected to SDS-PAGE containing 6 M urea in both stacking and separating gels.

In vivo Phosphorylation of Caldesmon

Cultured cells were labeled with ^{32}P-orthophosphoric acid as described previously (Yamashiro et al., 1990). Briefly, cells were incubated for 3 h in phosphate-free DME containing 10% dialyzed NCS, 0.25 μg/ml nocodazole and 0.125 mCi/ml ^{32}P-orthophosphoric acid. Mitotic cells were selected, washed quickly with phosphate-free DME to remove nocodazole, and incubated at 37°C in phosphate-free DME containing the same radioactivity of ^{32}P. Cells at different stages after release of mitotic arrest were quickly washed with PBS, and lysed in SDS sample buffer. These lysates were homogenized by several passages through a 25-gauge needle, heated for 3 min at 100°C, and immediately used for immunoprecipitation using anti-caldesmon mAb (Yamashiro-Matsumura et al., 1988). The immunoprecipitates were then analyzed on urea/SDS-gel, followed by autoradiography.

Analyses of Caldesmon and Histone H1 Kinase Activities

Caldesmon kinase and histone H1 kinase activities in REF-4A cell lysates were assayed using purified caldesmon and histone H1 (Boehringer Mannheim Corp., Indianapolis, IN) as substrates, respectively. Nonmuscle caldesmon was purified from REF-4A cells as described previously (Yamashiro-Matsumura et al., 1988). For the preparation of cell lysates, REF-4A cells at different stages of cell division were treated with Triton/glycerol solution for 2 min, washed three times with PBS containing 5 mM $MgCl_2$ and 0.2 mM EGTA, and homogenized in the same saline in the presence of 5 mM PMSF and 1 mM DTT. After centrifugation (100,000 *g* for 10 min), the supernatants were used as kinase fractions. Either purified caldesmon (55 μg/ml) or histone H1 (38 μg/ml) in 60 μl of PBS was mixed with kinase fractions (1 mg/ml) in the presence of 5 mM $MgCl_2$, 0.2 mM EGTA, 500 nM A-kinase inhibitor peptide (Sigma Immunochemicals), 0.125 mCi/ml of [gamma-^{32}P]ATP and 0.72 mM ATP. The mixtures were incubated at room temperature for 20 min, the reactions were stopped by heating at 100°C for 10 min, and the samples were subjected to SDS-PAGE followed by autoradiography. In some experiments, caldesmon and histone H1 bands were excised from the gel and the radioactivities of those bands were counted by a scintillation counter using the Cerenkov method.

Other Procedures

SDS-PAGE was performed as described by Blatter et al. (1972) using 12.5% polyacrylamide except that the buffer system of Laemmli (1970) was used. Urea/SDS-PAGE was carried out using 12.5% polyacrylamide gel containing 6 M urea as described previously (Yamashiro et al., 1990). The intensities of each protein band on SDS gels were analyzed with a densitometer (Chromoscan 3, Joyce-Loebl) as described (Ishikawa et al., 1989*a*).

For immunoblotting, the proteins were electrophoretically transferred (Towbin et al., 1979) from gels to PVDF membranes (Millipore Corp., Bedford, MA) at 100 V for 2 h. The membranes were reacted with anti-caldesmon mAb, SM12 (Yamashiro-Matsumura and Matsumura, 1988). Immunoreactive bands were visualized by using peroxidase-conjugated anti-mouse IgG.

Protein concentrations were determined by the method of Bradford (1976) using BSA as a standard.

Results

Cell Synchronization

Cell division was allowed to proceed in mitotic-arrested cells by removing nocodazole from media, and then cell cycle progression was monitored by both phase contrast and immunofluorescent microscopy to examine cell synchronization. As Fig. 1, *a* and *b*, shows, >85% of the cells were found

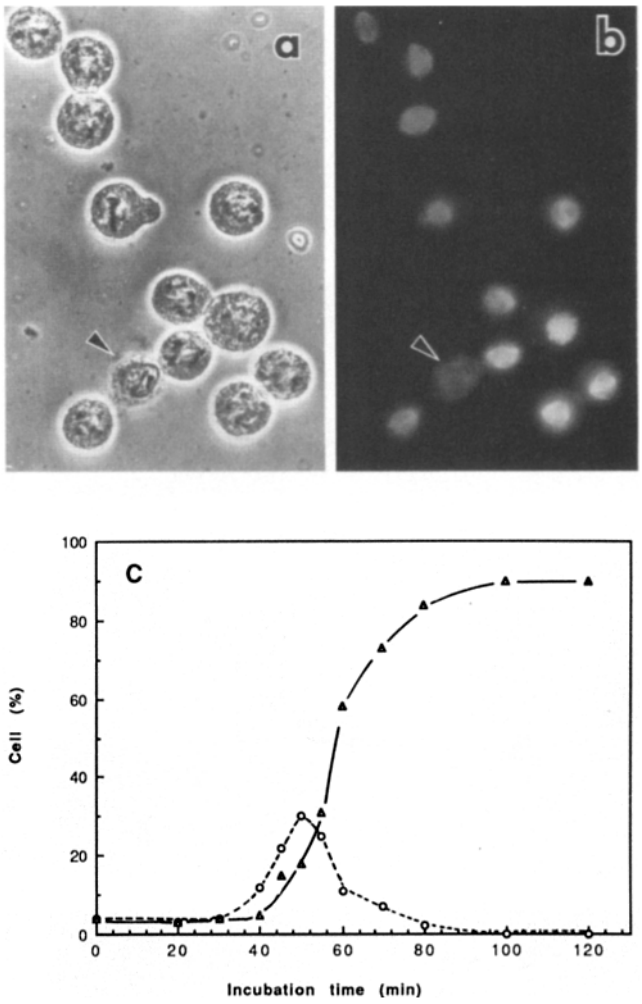
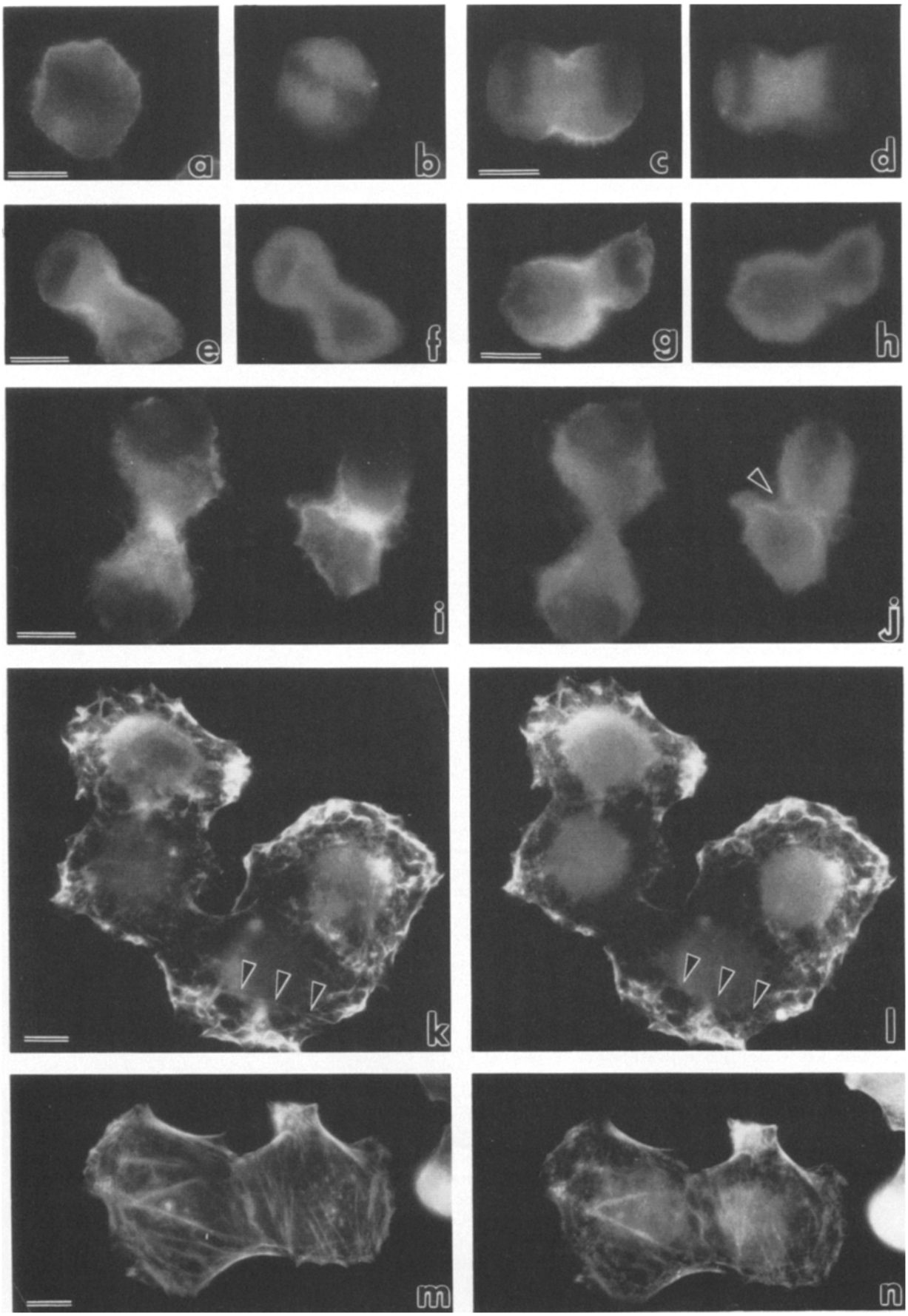


Figure 1. Analyses of cell synchronization after the release from mitotic arrest. Mitotic cells were collected, washed to remove nocodazole, and incubated at 37°C to start cell cycle progression. Cells were fixed at certain time, stained with anti-tubulin antibody, and observed by phase-contrast and fluorescent microscopy. (*a*) Phase-contrast micrograph of cells at 20 min incubation; (*b*) fluorescent micrograph of the same cells. All cells except one (*arrowhead*) have recovered spindles. (*c*) Percentages of cells with cleavage furrows (○) or cells which have finished cytokinesis (△) are plotted against the incubation time after the removal of nocodazole.

to recover metaphase spindles at 20 min incubation after removal of nocodazole.

Most cells finished cell division in a short time between 40 and 60 min. Fig. 1 *c* shows the changes in the fractions of two morphologically different cells; i.e., cells having finished cell division, and cells with cleavage furrows. At 40 min, cells with cleavage furrows were first observed but almost no cells were found to finish cytokinesis. While the fraction of cells with cleavage furrows reached to the maximum (30%) at 50 min, the fraction of cells having finished cytokinesis was still low (<20%). In the next 10 min (at 60 min), the fraction of divided cells was rapidly increased to 60%, and the fraction of cells with cleavage furrows was decreased to ~10%. Afterward, the fraction of divided cells was gradually increased to 85–90% over the next 40 min.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Localization of 83-kD Caldesmon during Cell Division

We first examined, by immunofluorescence, changes in the localization of caldesmon during cytokinesis after release of mitotic arrest. F-actin localization was examined simultaneously by double labeling with rhodamine-phalloidin.

Immunofluorescent observation has shown that caldesmon is not concentrated in cleavage furrows during early stages of cytokinesis. Before the formation of cleavage furrows (up to 40 min), diffuse caldesmon staining was observed throughout the cytoplasm (Fig. 2 *b*), while rhodamine-phalloidin stained cell cortex (Fig. 2 *a*). This observation is consistent with our previous biochemical analyses that caldesmon is dissociated from microfilaments during mitosis (Yamashiro et al., 1990). At the initial stage of cytokinesis, F-actin fluorescence was brighter in cleavage furrows (Fig. 2 *c*). Double-label immunofluorescence, however, revealed that caldesmon was still diffusely present and not accumulated at these cleavage furrows (Fig. 2 *d*). As cytokinesis proceeded, phalloidin strongly stained cleavage furrows (Fig. 2, *e* and *g*). Caldesmon fluorescence still had not accumulated in the cleavage furrows (Fig. 2, *f* and *h*) though some staining in cortexes was observable (Fig. 2 *h*). At late stages of cytokinesis, caldesmon showed some accumulation in the furrow (Fig. 2 *j*, *arrowhead*). Its concentration in the furrow is, however, much less than that of F-actin (Fig. 2, *i* and *j*). When two daughter cells began spreading at 80 min, caldesmon was found to be localized in cell periphery including membrane ruffles and surface projections where strong phalloidin staining was also found (Fig. 2, *k* and *l*). As the daughter cells spread further, stress fibers reassembled (Fig. 2, *m* and *n*). At this stage, caldesmon was found to be fully colocalized with actin.

Analysis of Microfilament-associated Caldesmon

The above results suggested that caldesmon stays dissociated from microfilaments at least during early stages of cytokinesis. To examine this further, we determined the levels of microfilament-associated caldesmon at different stages of cell division after the release of mitotic arrest. After removal of nocodazole, equal numbers of mitotic cells were plated on dishes to allow cell cycle progression and microfilaments were prepared as described previously. We found that similar amounts of microfilaments were obtained from equal numbers of mitotic cells at different stages. The association of caldesmon is thus normalized by loading equal amounts of microfilaments.

As Fig. 3 shows, caldesmon was absent from the microfilaments in mitotic prometaphase cells (0 min), confirming our previous data (Yamashiro et al., 1990). When some cells started to show cytokinesis at 40 min after the removal of nocodazole, the level of microfilament-associated caldesmon was only 12% of that of caldesmon associated with interphase microfilaments. At 60 min when most cells (60%) finished cytokinesis (see Fig. 1 *c*), its level was increased to 50%, and at 120 min, the level of microfilament-associated caldesmon was recovered to 80% of the interphase level. Thus the reassociation of caldesmon to microfilaments and the changes in the fraction of divided cells (Fig. 1 *c*) appears to show similar time course. This suggests that caldesmon is mostly disassociated from microfilaments at least during early stages of cytokinesis.

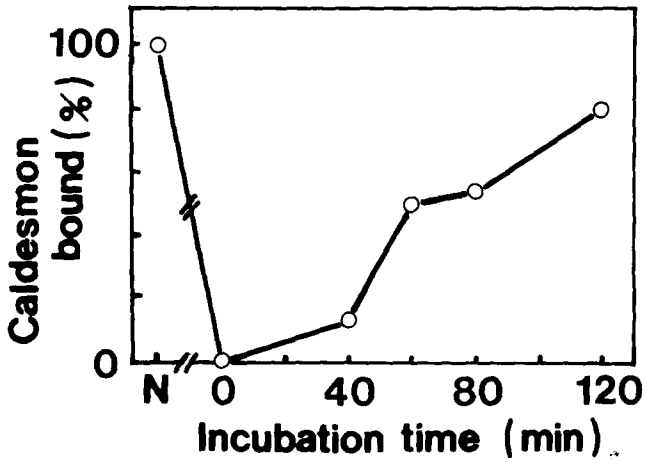

Figure 3. Changes in the levels of microfilament-associated caldesmon during mitosis. Microfilaments were isolated from cells at 0, 40, 60, 80, and 120 min of incubation at 37°C after the removal of nocodazole (see the legend to Fig. 1) and the levels of microfilament-associated caldesmon were determined by densitometry. The levels of microfilament-associated caldesmon are expressed as percentage of the level of caldesmon in microfilaments isolated from nonmitotic cells (*N*).

Analysis of the Phosphorylation State of Caldesmon during Cell Division

We examined the phosphorylation state of caldesmon during the cell cycle progression after the release of mitotic arrest. To this end, we have analyzed total caldesmon of heat-stable fractions by urea/SDS-PAGE because a phosphorylated form of caldesmon shows a slower mobility compared with an unphosphorylated one on urea/SDS gels (Yamashiro et al., 1990). As Fig. 4 *A* shows, caldesmon at 0 min (Fig. 4 *A*, lane 2) showed a lower mobility as expected for a fully phosphorylated form of caldesmon. At the beginning of the formation of cleavage furrows (40 min; Fig. 4 *A*, lane 3), most of the caldesmon still showed the lower mobility while some caldesmon appeared to show faster mobility, suggesting that

Figure 2. Intracellular localization of F-actin and caldesmon in REF 4A cells during mitosis. Cells at a variety of mitotic stages were prepared by the release of mitotic arrest as described in the legend to Fig. 1. Cells were double stained with phalloidin (*a*, *c*, *e*, *g*, *i*, *k*, and *m*) and anti-caldesmon antibody (*b*, *d*, *f*, *h*, *j*, *l*, and *m*) at 30 (*a* and *b*), 40 (*c* and *d*), 50 (*e*, *f*, *g*, and *h*), 60 (*i* and *j*), 80 (*k* and *l*), and 120 (*m* and *n*) min of the incubation at 37°C after the removal of nocodazole. Note that caldesmon is not concentrated in the cleavage furrows at early stages of cytokinesis (*d*, *f*, and *h*) whereas F-actin shows the accumulation in these cleavage furrows. At later stages, some concentration of caldesmon in cortexes as well as in cleavage furrows (shown by *arrowhead* in *j*) was observable. When cells begin to spread, both F-actin and caldesmon show colocalization. Arrowheads in *k* and *l* indicate the assembly of stress fibers at an early stage. Bar, 10 μm.

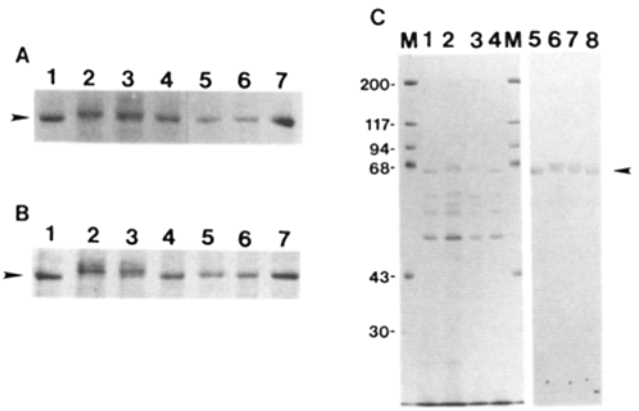

Figure 4. Analyses of the phosphorylation level of caldesmon during mitosis by urea/SDS-PAGE. Cells at different stages of mitosis were prepared as described in the legend to Fig. 1. Heat-stable fractions were prepared from mitotic cells at 0 (lane *2*), 40 (lane *3*), 60 (lane *4*), 80 (lane *5*), and 120 (lane *6*) min of incubation at 37°C after the removal of nocodazole. As a control, heat-stable fractions were also prepared from nonmitotic cells (lanes *1* and *7*) (*A*) Heat-stable fractions prepared in the absence of 5 mM NaF; (*B*) heat-stable fractions prepared in the presence of 5 mM NaF. The position of caldesmon is indicated by arrowheads. (*C*) Heat-stable fractions prepared from 0 min cells in the presence (lanes *2* and *6*) or the absence (lanes *3* and *7*) of 5 mM NaF were subjected to urea/SDS-PAGE as well as heat-stable fractions from nonmitotic cells (lanes *1*, *4*, *5*, and *8*). Proteins were transferred to a nitrocellulose membrane and stained with anti-caldesmon antibody (lanes *5–8*). Molecular mass markers (lane *M*) are indicated in kilodaltons.

partial dephosphorylation started. The dephosphorylated form of caldesmon with a higher mobility became predominant at 60 min (Fig. 4 *A*, lane *4*) when most cells (60%) completed cytokinesis. At both 80 and 120 min when many cells began to spread and form stress fibers (Fig. 4 *A*, lanes *5* and *6*), almost all caldesmon migrated at the unphosphorylated position, which was the same position shown by interphase caldesmon (Fig. 4 *A*, lanes *1* and *7*).

To minimize possible effect of phosphatase(s) during preparation of heat-stable fractions, the same experiments were performed in the presence of a potent phosphatase inhibitor, NaF. As Fig. 4 *B* shows, dephosphorylation began at 40 min and was almost complete at 60 min, yielding essentially the same results as observed in the absence of NaF. In the presence of NaF, however, we observed minor bands which migrated more slowly than those observed in the absence of NaF. These minor bands were identified as caldesmon by Western blotting using an anti-caldesmon mAb (Fig. 4 *C*). This might suggest that caldesmon could be partially dephosphorylated in the absence of NaF or hyperphosphorylated in the presence of NaF during preparation.

The in vivo phosphorylation state of caldesmon was also examined by immunoprecipitation of caldesmon from total cell lysates which had been labeled with ^{32}P-orthophosphate in vivo. Fig. 5 shows urea/SDS gel analyses of caldesmons immunoprecipitated from the cells at 0 or 40 min, where they again showed retarded mobility on this gel system (Fig. 5, lanes 2 and *3*). Autoradiography revealed that both caldesmons were heavily phosphorylated (Fig. 5, lanes *6* and *7*). Quantitative analyses by radioactive counting showed that the level of caldesmon phosphorylation at 40 min was ~30% less than that at 0 min, suggesting partial dephosphorylation

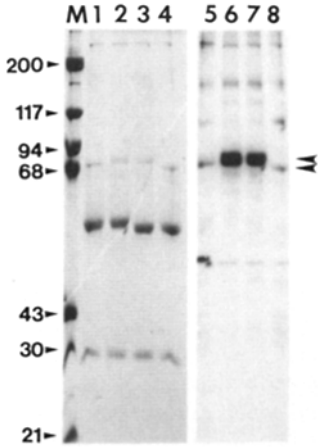

Figure 5. In vivo phosphorylation of caldesmon during mitosis. Cells were in vivo labeled with ^{32}P-orthophosphoric acid as described in Materials and Methods, and harvested at different stages of mitosis as described in the legend of Fig. 1. Caldesmon was immunoprecipitated from ^{32}P-labeled lysates of nonmitotic cells (lanes *1* and *5*) or mitotic cells at 0- (lanes *2* and *6*), 40- (lanes *3* and *7*), and 120- (lanes *4* and *8*) min incubation after removal of nocodazole. The immunoprecipitates were subjected to urea/SDS-PAGE (lanes *1–4*) followed by autoradiography (lanes *5–8*). The position of caldesmon is indicated by arrowheads. Molecular mass markers (lane *M*) are indicated in kilodaltons.

of caldesmon at 40 min. On the other hand, caldesmon isolated from cells at 120 min (Fig. 5, lanes *4* and *8*) was almost completely dephosphorylated; the level of phosphorylation was even lower than that of nonmitotic cells (Fig. 5, lanes *1* and *5*). These observations are consistent with the results obtained by the urea/SDS gel analyses of isolated caldesmons (Fig. 4).

Analysis of Caldesmon Kinase Activities during Cell Division

The state of caldesmon phosphorylation can be affected by changes in the activities of either kinase or phosphatase. We thus examined whether caldesmon kinase activities change during cell division. As cdc2 kinase appears responsible for the mitosis-specific phosphorylation of caldesmon, both caldesmon and histone H1 were used as substrates. Kinase activities were assayed with total cell extracts prepared at different stages after release of mitotic arrest. As Fig. 6 shows, caldesmon kinase activity was very low in interphase cells (Fig. 6, lanes *1* and *5*). The activity increased 3.5-fold in mitotically arrested cells (at 0 min; Fig. 6, lanes *2* and *6*). At 40 min (Fig. 6, lanes *3* and *7*), the activity was still high although it was decreased to 60% of that of 0 min cells. The activity could hardly be detected in 120 min cells (Fig. 6, lanes *4* and *8*). The changes in histone H1 kinase activity, namely cdc2 kinase activity, were well coupled with those of the caldesmon kinase activity (data not shown).

Discussion

Dissociation of Caldesmon from Microfilaments during Cell Division

We have previously demonstrated that caldesmon is disassociated from microfilaments during prometaphase, as a consequence of mitosis-specific phosphorylation of caldesmon by p34^{cdc2} kinase (Yamashiro et al., 1990, 1991). Our present results strongly suggest that caldesmon remains dissociated from microfilaments during cytokinesis at least in its early stages. Immunofluorescence (Fig. 2) has revealed the following: (*a*) caldesmon is not detected in cleavage furrows in early stages of cytokinesis; (*b*) there seems to be

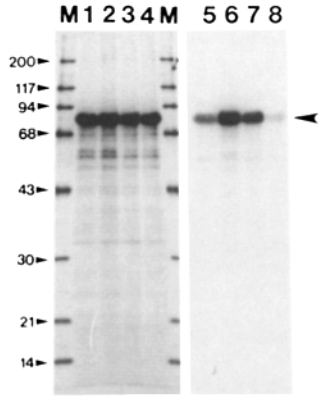

Figure 6. Changes in caldesmon kinase activity of cell lysates during mitosis. Cells at different stages of mitosis were prepared as described in the legend of Fig. 1. Cell lysates (crude kinase fractions) were prepared as described in Materials and Methods. Purified caldesmon (55 μg/ml) was mixed with the kinase fractions (0.2 mg/ml) of nonmitotic (lanes *1* and *5*), 0 (lanes *2* and *6*), 40 (lanes *3* and *7*), and 120 (lanes *4* and *8*) min cells, and incubated for 20 min at 37°C. The reactions were stopped by heat treatment and the samples were subjected to urea/SDS-PAGE (lanes *1–4*) followed by autoradiography (lanes *5–8*). The position of caldesmon is indicated by an arrowhead. Molecular mass markers (lane *M*) are indicated in kilodaltons.

some accumulation of caldesmon in the furrow as well as the cortex during late cytokinesis, however, the concentration of caldesmon in the furrow is much less than that of F-actin; and (*c*) actin and caldesmon become fully colocalized in membrane ruffles and microspikes of spreading cells after cell division. Kinetic analyses of the caldesmon–actin association using synchronized cells (Fig. 3) support these immunofluorescence observations. The level of microfilament-associated caldesmon is only 12% of that of interphase cells during early stages of cytokinesis (40 min), and is increased to 50% when most (60%) cells complete cytokinesis at 60 min. During the next 60 min, the level of microfilament-associated caldesmon is gradually increased to 80% of that of interphase cells. It is worthy of note that the time course of the extent of reassociation of caldesmon to actin is similar to that of the increase in the fraction of divided cells. Taken together, these data lead to the conclusion that caldesmon stays disassociated from microfilaments at least during early stages of cytokinesis. At later stages of cytokinesis, caldesmon may be partly associated with microfilaments.

The analyses of phosphorylation states of caldesmon have revealed that dephosphorylation of caldesmon occurs between 40 to 60 min. Because 60% of the cells finished cytokinesis at 60 min, this suggests that dephosphorylation is completed during cytokinesis. The data may also suggest that dephosphorylation slightly precedes reassociation of caldesmon to microfilaments. In any case, these results strongly suggest that reassociation of caldesmon to microfilaments appears to couple with dephosphorylation of caldesmon.

The dephosphorylation of caldesmon appears to be caused by the decrease in the caldesmon kinase activity, or cdc2 kinase. The apparent activities of caldesmon kinase present in crude cell extracts are proportional to the level of caldesmon phosphorylation at different mitotic stages (Fig. 6). This observation is consistent with the reported activities of cdc2 kinase during mitosis. It has been reported that cdc2 kinase activity or MPF drops rapidly during metaphase to anaphase transition by the destruction of cyclin (Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Murray and Kirschner, 1989; for review see Hunt, 1989; Lohka, 1989; Lewin, 1990).

Possible Functions of Nonmuscle Caldesmon in Cytokinesis

Our results have shown that caldesmon is unique in that it is not concentrated in cleavage furrows during cytokinesis. In contrast, other actin binding proteins including α-actinin, myosin, and tropomyosin have been reported to be concentrated in cleavage furrows (Fujiwara and Pollard, 1976; Fujiwara et al., 1978; Sanger et al., 1989; Ishimoda-Takagi, 1979). What is the physiological significance of the absence of caldesmon in cleavage furrows?

The first possibility is that the dissociation of caldesmon from microfilaments is required to allow contraction of the actin–myosin system in cleavage furrows during mitosis as caldesmon inhibits actin-activated myosin ATPase activity (for review see Bretscher, 1986; Sobue and Sellers, 1991). The continued dissociation of caldesmon may thus be required for the activation of contractile rings during cytokinesis. It is worthy of note that the dissociation of caldesmon from microfilaments at an earlier stage (during prophase) may induce contraction of stress fibers, resulting in rounding-up of the cell shape. Indeed, Sanger and co-workers have observed that stress fibers contract during prophase (Sanger et al., 1989).

The second possibility is that the dissociation of caldesmon may allow gelsolin-like activities to sever microfilaments into short filaments as caldesmon coupled with tropomyosin inhibits both severing and capping activities of gelsolin (Ishikawa et al., 1989*a*,*b*). This severing may first lead to the disassembly of stress fibers and the concomitant morphological alterations during prophase. During cytokinesis, gelsolin activity may also be required for the disassembly of the microfilament structure of contractile rings because the contraction of contractile rings should be accompanied with their simultaneous disassembly.

Our results have revealed that caldesmon stays dissociated from microfilaments from prometaphase to at least early stages of cytokinesis. This may suggest that caldesmon does not play an active role in cytokinesis. Rather, the continued dissociation of caldesmon may be required for the mitosis-specific organization of microfilaments including disassembly of stress fibers, and formation and activation of contractile rings. The reassociation of caldesmon to microfilaments in spreading daughter cells may suggest that caldesmon is specifically needed for the assembly of microfilaments into stress fibers in interphase cells.

This work is supported by grant R37 CA42742 from the National Cancer Institute, and grant CD-40B from the American Cancer Society to F. Matsumura and S. Yamashiro, and by grants-in-aid from the Ministry of Education of Japan to N. Hosoya. F. Matsumura is a recipient of Research Career Development Award K04 CA01304 from the National Cancer Institute. N. Hosoya is a Postdoctoral Fellow of Japan Society for Promoting Science and was supported by Katsuma and Jean Dan Fellowing in 1989.

Received for publication 26 May 1992 and in revised form 9 February 1993.

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