Caldesmon Inhibits Arp2/3-mediated Actin Nucleation*

Yoshihiko Yamakita‡, Fumio Oosawa§, Shigeko Yamashiro‡, and Fumio Matsumura¶

From ‡Rutgers University, Department of Molecular Biology and Biochemistry, Nelson Laboratories, Busch Campus, Piscataway, New Jersey 08854 and the §Aichi Institute of Technology, Yakausa-cho, Toyota 470-03, Japan

Received for publication, August 26, 2002
Published, JBC Papers in Press, March 11, 2003, DOI 10.1074/jbc.M208739200

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

17937

The Arp2/3 complex greatly accelerates actin polymerization, which is thought to play a major role in cell motility by inducing membrane protrusions including ruffling movements. Membrane ruffles contain a variety of actin-binding proteins, which would modulate Arp2/3-dependent actin polymerization. However, their exact roles in actin polymerization remain to be established. Because caldesmon is present in membrane ruffles, as well as in stress fibers, it may alter Arp2/3-mediated actin polymerization. We have found that caldesmon greatly retards Arp2/3-induced actin polymerization. Kinetic analyses have revealed that caldesmon inhibits the nucleation process, whereas it does not largely reduce elongation. Caldesmon is found to inhibit binding of Arp2/3 to F-actin, which apparently reduces the ability of F-actin as a secondary activator of Arp2/3-mediated nucleation. We also have found that the inhibition of the binding between actin and caldesmon either by Ca2+/calmodulin or by phosphorylation with cdc2 kinase reverses the inhibitory effect of caldesmon on Arp2/3-induced actin polymerization. Our results suggest that caldesmon may be a key protein that modulates membrane ruffling and that this may involve changes in caldesmon phosphorylation and/or intracellular calcium concentrations during signal transduction.

Arp2/3-dependent actin polymerization plays a critical role in controlling motile structures including membrane ruffling (1–7). Two small G-proteins, Rac and Cdc42, regulate actin polymerization and are responsible for the assembly of membrane ruffles and filopodia, respectively (8). Recent work has elucidated the essential role of the Arp2/3 complex in the Rac- or Cdc42-mediated actin polymerization (6). Actin alone polymerizes rather slowly because of the rate-limiting process of nucleation. The Arp2/3 complex greatly accelerates the nucleation process in a Rac- or Cdc42-dependent way; Rac or Cdc42 activates the effector proteins of WASP family proteins including N-WASP and WAVE (SCAR). Together they activate the nucleation activity of the Arp2/3 complex and induce rapid actin polymerization. The Arp2/3 complex also binds to the side of actin filaments and initiates branched actin polymerization, resulting in the formation of a dendritic actin network.

The appearance and extent of membrane ruffling vary widely depending on cell types, as well as cellular conditions and external circumstances. For example, fibroblasts generally show more vigorous ruffling than do epithelial cells. Membrane ruffling is regulated by a variety of external signals including serum or growth factors, extracellular matrix, and cell-cell contacts (9–12). On the other hand, intrinsic signals appear to control constitutive membrane ruffling of motile cells. It is thus conceivable that, in addition to the small G-proteins, other proteins may modulate Arp2/3-dependent actin polymerization. Candidates with such functions are actin-binding proteins present in membrane ruffles. For example, cortactin activates Arp2/3-dependent actin polymerization and stabilizes branched actin polymerization in vitro (13–15). Tropomyosin, on the other hand, has been shown to reduce Arp2/3-dependent branching and branched nucleation (16). Most recently, coronin has been reported to bind to the Arp2/3/VCA complex and inhibit the de novo nucleation activity of Arp2/3 (17).

Caldesmon is an actin-binding protein that is localized in stress fibers, as well as in membrane ruffles (18, 19). In vitro, caldesmon inhibits actin-activated myosin ATPase (20), and caldesmon together with tropomyosin protects actin filaments from severing activities of gelsolin and annelis gelsolin-severed actin filaments (21, 22). These two activities are likely to be involved in regulating the actomyosin contractility and stability of stress fibers, respectively (23–28). It is not clear, however, what functions caldesmon plays in membrane ruffles. We have examined the effects of caldesmon on Arp2/3-dependent actin polymerization and found that caldesmon inhibits the Arp2/3-dependent actin nucleation process.

MATERIALS AND METHODS

Protein Purification—Skeletal muscle G-actin was prepared as described (29), and pyrene dye was conjugated as described (30). The Arp2/3 complex was purified from HeLa cells using the method described in Ref. 31, except that the incubation at 37 °C was omitted. The GST-tagged C-terminal region of N-WASP (GST-VCA) was cloned by PCR using the N-WASP-expressing baculovirus (a kind gift from Drs. Takenawa and Miki, Tokyo University) as the template, expressed in bacteria, and purified as described (31). Rat nonmuscle caldesmon was expressed in bacteria and purified as described (32). Smooth muscle caldesmon was purified from chicken gizzard by the method described in Ref. 33 with slight modification as described (34). Cdc2 kinase was prepared as described previously (35). Calmodulin and λ-phosphatase were purchased from Sigma and New England BioLabs (Beverly, MA), respectively.

Polymerization Assay—Actin polymerization was measured by pyrene fluorescence using a Perkin Elmer spectrofluorometer, LS-50B. Mg-ATP G-actin (2 μM 5% pyrene-labeled actin) was mixed with various proteins in polymerization buffer (final condition: 10 mM imidazole buffer, pH 7, 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 10 mM DTT) at time 0. When we examined the effects of Ca2+/calmodulin, 0.4 mM CaCl2 was added instead of EGTA. The high concentration of DTT was included to prevent the dimerization of caldesmon (36).

Phosphorylation of Caldesmon with cdc2 Kinase—Smooth muscle caldesmon was phosphorylated by cdc2 kinase as described (35). The abbreviations used are: GST, glutathione S-transferase; DTT, dithiothreitol; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor.
level of phosphorylation (determined by $^{32}$P incorporation) was found to be 4.5 ± 0.5 mol/mol. In some experiments, phosphorylated caldesmon was dephosphorylated by the incubation with λ-phosphatase at 30 °C for 30 min, which resulted in the decrease of the incorporation to 0.8 ± 0.4 mol/mol. λ-Phosphatase was denatured by heat treatment, and dephosphorylated caldesmon was recovered in a heat-stable fraction.

Actin Binding Assay—F-actin was polymerized from Mg-ATP G-actin (final concentration, 2 μM) in the polymerization buffer (10 mM imidazole buffer, pH 7, 50 mM KCl, 1 mM MgCl$_2$, 1 mM EGTA, 10 mM DTT, 4 μM phalloidin) for 40 min. After polymerization, caldesmon (final concentration, 0.5 μM) was added and incubated for 10 min, and then varying concentrations (5–20 nM) of Arp2/3 and GST-VCA (50 nM) were added. The samples were incubated for 20 min at room temperature and centrifuged in a Beckman Airfuge (26 p.s.i. × 20 min). Both pellets and supernatants were suspended in SDS sample buffer and analyzed by the Western blot method using mouse monoclonal antibody against Arp3 (BD Biosciences Pharmingen, San Diego, CA). A standard curve was made by immunoblotting known concentrations of Arp2/3 complex with the same antibody and used to quantitatively determine the levels of Arp3 using Kodak one-dimensional Image Analysis Software (Eastman Kodak Co., Rochester, NY).

Electron Microscopy—The average length of actin filaments at the equilibrium state (after pyrene fluorescence reached a plateau) was measured by electron microscopy using negative staining technique (36). More than 1000 actin filaments were measured for each polymerization experiment.

RESULTS

Caldesmon Effectively Inhibits Arp2/3-dependent Actin Polymerization—We first examined effects of caldesmon on Arp2/3-mediated actin polymerization. Actin (2 μM), Arp2/3 (15 nM), and VCA (50 nM) were mixed with varying concentrations (0–2 μM) of either smooth muscle or nonmuscle caldesmon, and polymerization was initiated by the addition of salt at time 0. As Fig. 1 (A and B) shows, the higher the concentration of caldesmon, the longer the delay in actin polymerization. To quantitate the effects of caldesmon, we measured the delay times required to reach one-fifth of the final fluorescence, as well as slopes at the half-maximal fluorescence (proportional to the number of barbed ends, assuming that caldesmon did not change the elongation rate). As Fig. 2 shows, the delay time is increased from 100 to 200 s for smooth muscle caldesmon and to 210 s for nonmuscle caldesmon. The delay is saturated at 0.25 μM for smooth muscle caldesmon and at 0.5–1 μM for nonmuscle caldesmon. The inhibition of the polymerization rate is saturated at approximately 1 μM of either type of caldesmon. It is noteworthy that caldesmon at a very low concentration (50 nM) has more effect on the delay time than on the slope of polymerization. The inhibition by caldesmon is at least as effective as that reported for tropomyosin (16).

Without Arp2/3, the effect of caldesmon on actin polymerization was minimal. The delay time was changed from 165 to 180 s when 0.5 μM of caldesmon was added in the absence of Arp2/3 (Fig. 1C). The difference was only 9%. In contrast, the same concentration (0.5 μM) of caldesmon delayed Arp2/3-dependent polymerization to a much greater extent (from 99.4 to 193.2 s, corresponding to a 94% increase; see Fig. 2B). The polymerization rate (slopes at the half-maximal fluorescence) also showed little change in the absence of Arp2/3; caldesmon decreased the rate by merely 5%. In contrast, the same concentration of caldesmon decreased the rate by 62% in the presence of Arp2/3 (Fig. 2B). These results suggest that caldesmon affects Arp2/3-dependent processes of actin polymerization.

The C terminus of caldesmon is indispensable for the inhibitory effect. Although the N terminus of caldesmon contains a major myosin-binding site, the C terminus has the actin-binding domains in addition to the domains that bind to tropomyosin or calmodulin (37). We found that the C-terminal half (Glu335–Val531 of rat nonmuscle caldesmon) was able to show inhibitory effects on Arp2/3-dependent actin polymerization similar to the full-length molecule, whereas the N terminus showed no effects on actin polymerization (results not shown). This result suggests that actin binding ability is important for the inhibition.

Caldesmon Does Not Largely Alter Elongation Rates—The
retardation of Arp2/3-dependent actin polymerization could be caused by the reduction of the elongation rate from barbed ends, by the inhibition of actin nucleation, or by both. We first examined whether caldesmon slows the elongation from barbed ends of Arp2/3-capped actin seeds. To make seeds, actin (2 mM, without pyrene label) was polymerized in the presence of the Arp2/3 complex (20 nM) and VCA (50 nM). The seeds were then diluted 10-fold and incubated with 0.3 mM pyrene-labeled G-actin with or without caldesmon to monitor barbed end polymerization. We conclude from the following reasons that this assay reasonably tests the effects of caldesmon on elongation from barbed ends. First, the concentration of G-actin is below the critical concentration of pointed ends so that barbed end polymerization was monitored (final concentrations: 0.2 mM F-actin seeds, 0.3 mM G-actin, with or without 0.5 mM caldesmon). Second, caldesmon did not increase the annealing of Arp2/3-capped actin filaments in this condition, and thus the number of barbed ends was not changed with or without caldesmon binding is faster than actin elongation at the barbed ends. Because the binding of caldesmon to F-actin is rather a fast reaction ($k = 1 \times 10^7 M^{-1} s^{-1}$) (38), most actin filaments would be saturated with caldesmon before any significant actin polymerization could occur at the free barbed ends; half of caldesmon binds to actin in 0.1 s in this condition, whereas only 0.2% of actin seems to polymerize at the ends at the same time.

As Fig. 3B shows, caldesmon inhibits the elongation rates to...
a small extent. The rates were decreased 20% by smooth muscle caldesmon and 10% by nonmuscle caldesmon. This small extent of inhibition, however, does not account for the large decreases in the rates of actin polymerization by caldesmon as shown in Figs. 1 and 2 in which the slope at half-maximum fluorescence was decreased by 62% by 0.5 μM caldesmon.

Caldesmon Reduces Nucleation Rates—We then examined how caldesmon affects the apparent nucleation rate, $k^*$, and the apparent elongation rate, $k^+$. According to the theory of actin polymerization devised by Oosawa and Asakura (39), the nucleation rate $k^*$ is proportional to the inverse of $<i>_t$, where $<i>$ is the average length of actin filaments at the equilibrium state, and $t$ is the time when a certain amount of monomers has polymerized. On the other hand, the elongation rate $k^+$ is proportional to $<i>_t$.

Fig. 4 shows the time courses of actin polymerization in the absence (panel A) or presence (panel B) of caldesmon when the concentrations of the Arp2/3 complex were changed from 0 to 20 nM. At the equilibrium, the lengths of more than 1000 actin filaments were measured by electron microscopy, and the length distribution is shown as histograms in Fig. 5. It is clear that filament length becomes longer when caldesmon is present. Table I shows the average lengths, $<i>_t$, of actin filaments, as well as times ($t_{1/5}$) at which 20% of F-actin was polymerized relative to the final level of F-actin. Both average lengths and $t_{1/5}$ become longer in the presence of caldesmon than in its absence. We have then calculated relative changes in $k^*$ and $k^+$ by normalizing the rates, $k^*$ and $k^+$, of polymerization of actin alone to 1.0. Fig. 6 shows the plots of relative values of $k^*$ and $k^+$ versus Arp2/3 concentrations.

These plots indicate that caldesmon greatly decreases the nucleation rate, $k^*$ (Fig. 6A), but not the elongation rate, $k^+$ (Fig. 6B). As expected, $k^*$ increased as the concentration of Arp2/3 increases (Fig. 6A). Importantly, $k^+$ in the presence of caldesmon (open squares) is decreased to one-third of that in the absence of caldesmon (filled squares). On the other hand, $k^+$ (Fig. 6B) seems to be the same whether caldesmon is present (open squares) or absent (filled squares), although $k^+$ decreases when the Arp2/3 concentration becomes high. The reason for the decrease is not clear at this time. It is interesting to note that the Arp2/3-dependent increase in $k^+$ is proportional to the second power of Arp2/3 concentrations (Fig. 6A, inset). This relationship may be reflected by the fact that Arp2/3 nucleation activity is greatly increased when the Arp2/3 complex binds to the side of an actin filament (29, 40).
Inhibition of Arp2/3-mediated Actin Nucleation by Caldesmon

### Table I

| Caldesmon (μM) | Arp2/3 (nM) | Average length of actin filaments (μM) | Delayed time (t_1/5) |
|---------------|-------------|----------------------------------------|----------------------|
| 0             | 0           | 4.72                                   | 168                  |
| 5             | 0           | 2.64                                   | 92                   |
| 10            | 0           | 1.33                                   | 61                   |
| 20            | 0           | 0.597                                  | 46                   |
| 1             | 0           | 5.30                                   | 185                  |
| 5             | 0           | 3.49                                   | 135                  |
| 10            | 0           | 2.27                                   | 112                  |
| 20            | 0           | 1.07                                   | 76                   |

*Average lengths of actin filaments were measured by electron microscopy when polymerization reached equilibrium.

**Delayed times were measured when actin polymerization reached 20% of the final level in Fig. 4.**

---

**Fig. 6. Caldesmon reduces nucleation rates of Arp2/3-dependent actin polymerization but not the elongation rates. A, the relative nucleation rate constants (k*) are calculated from Table I according to the theory of Oosawa and Asakura on protein polymerization (39) and plotted versus Arp2/3 concentrations. The rate constants are normalized as actin alone to 1. Inset, the Arp2/3-dependent increases in the nucleation rates (k* − 1) are proportional to the square of Arp2/3 concentrations. Closed squares, without caldesmon; open squares, with caldesmon. B, the relative elongation rate constants (k−) calculated from Table I are plotted versus Arp2/3 concentrations. The constants are normalized as in A. Closed squares, without caldesmon; open squares, with caldesmon.**

**Caldesmon Does Not Interact with the Arp2/3 Complex.** The kinetic analyses shown above (Figs. 4–6) strongly suggest that caldesmon inhibits the nucleation activity of Arp2/3 complexes. One possibility was that caldesmon might directly associate with Arp2/3 complex or VCA and interfere the nucleation activity. However, we were unable to detect the binding of caldesmon to the Arp2/3 complex, VCA, or a VCA-Arp2/3 complex when the association was examined by a pull-down assay using affinity column chromatography or by the surface plasmon resonance method (data not shown). This suggests that caldesmon may indirectly inhibit the nucleation activity of Arp2/3 complex.

**Caldesmon Inhibits Branched Nucleation of Arp2/3 by Reducing Actin Binding of Arp2/3**—How does caldesmon directly reduce the nucleation activity of Arp2/3? Arp2/3 is able to nucleate actin in two ways. One is de novo nucleation (without polymerized actin), and the other is branched nucleation via binding of Arp2/3 to the side of F-actin filaments. Branched nucleation has been reported to be far more effective than de novo nucleation (39, 40). Because caldesmon binds to the side of actin filaments, it is possible that caldesmon inhibits the latter nucleation by inhibiting Arp2/3-F-actin binding.

To test this possibility, we performed the following two experiments. We first asked whether caldesmon reduces the ability of F-actin seeds as the secondary activator of Arp2/3 nucleation. F-actin seeds (final concentration, 0.3 μM) were first mixed with or without 0.5 μM caldesmon, and then Arp2/3, VCA, and pyrene-labeled G-actin were added to initiate polymerization. As Fig. 7 shows, actin polymerization in the presence of Arp2/3 occurred very rapidly with almost no lag time when F-actin seeds without caldesmon were added (Fig. 7, filled squares; compare the actin polymerization without F-actin seeds shown in Fig. 1). The addition of caldesmon (Fig. 7A, nonmuscle caldesmon; Fig. 7B, smooth muscle caldesmon) to F-actin seeds, however, greatly reduces the ability of F-actin seeds (compare open squares with filled squares). The effect is specific to Arp2/3-dependent actin polymerization because, in the absence of Arp2/3, the addition of either nonmuscle (Fig. 7A) or smooth muscle (Fig. 7B) caldesmon showed only small (for nonmuscle caldesmon) or no (for smooth muscle caldesmon) effects on actin polymerization (compare filled and open circles). These results suggest that caldesmon inhibits branched nucleation mediated by the binding of Arp2/3 to F-actin seeds. However, because there was an excess amount of caldesmon in these assays, it is possible that free caldesmon may also inhibit de novo nucleation of Arp2/3.

We next examined whether caldesmon inhibits binding of Arp2/3 to F-actin, because actin binding of Arp2/3 is required for branched nucleation. Using co-sedimentation assays, we examined whether caldesmon affects actin binding of Arp2/3. F-actin (2 μM) with or without caldesmon (0.5 μM) was incubated with varying concentrations of Arp2/3 (5–20 nM), and the levels of Arp2/3 in F-actin pellets were determined by quantitative Western blot method using the monoclonal antibody against Arp3. As Fig. 8 shows, the levels of bound Arp2/3 are reduced to 30–60% of the controls, when actin was preincubated with either nonmuscle or smooth muscle caldesmon. These results indicate that caldesmon inhibits Arp2/3 binding to F-actin.

**Reversal of the Inhibition of Caldesmon by Ca2+/Calmodulin or by Phosphorylation with cdc2 Kinase**—The interaction of caldesmon with actin is regulated by Ca2+/calmodulin or by phosphorylation of caldesmon with a variety of kinases including cdc2 kinase (35, 41). Because actin binding of caldesmon is important for its effects on Arp2/3, we examined whether these agents regulate the effects of caldesmon on Arp2/3 nucleation. As Fig. 9 shows, Ca2+/calmodulin negated the effects of the inhibition of caldesmon. Although caldesmon alone (filled triangles) retarded Arp2/3-dependent actin polymerization greatly (compare with filled squares), the addition of Ca2+/calmodulin (open triangles) to caldesmon almost completely reversed the inhibitory effect of caldesmon. As an additional
Inhibition of Arp2/3-mediated Actin Nucleation by Caldesmon

**DISCUSSION**

Our data indicate that caldesmon reduces the nucleation rate of the Arp2/3-dependent actin polymerization by inhibiting actin binding of Arp2/3. Three lines of evidence support this conclusion. First, kinetic analyses showed that caldesmon reduced the nucleation rate, whereas it did not alter the elongation rate largely (Fig. 6). Second, Arp2/3 seeds bound with caldesmon were much less effective in activating nucleation by Arp2/3 than were F-actin seeds without caldesmon (Fig. 7). Finally, caldesmon inhibited actin binding of Arp2/3 (Fig. 8).

We also found that phosphorylation of caldesmon with cdc2 kinase completely negates the inhibition of caldesmon (Fig. 10). Although unphosphorylated caldesmon (Fig. 10, X) greatly inhibited Arp2/3-dependent actin polymerization, caldesmon phosphorylated by cdc2 kinase to 4–5 mol/mol showed no inhibition on Arp2/3-dependent actin polymerization. The time course without caldesmon (filled squares) was identical to that with phosphorylated caldesmon (open circles). The release of inhibition was phosphorylation-specific; when caldesmon was dephosphorylated by X-phosphatase, caldesmon regained its inhibitory effect on Arp2/3-dependent actin polymerization (filled triangles). Dephosphorylation was not complete (phosphorylation level was 0.8 ± 0.4 mol/mol), which appears to explain the fact that dephosphorylated caldesmon showed less inhibition than unphosphorylated caldesmon.

Although unphosphorylated caldesmon (Fig. 10, X) greatly inhibited Arp2/3-dependent actin polymerization, caldesmon phosphorylated by cdc2 kinase to 4–5 mol/mol showed no inhibition on Arp2/3-dependent actin polymerization. The time course without caldesmon (filled squares) was identical to that with phosphorylated caldesmon (open circles). The release of inhibition was phosphorylation-specific; when caldesmon was dephosphorylated by X-phosphatase, caldesmon regained its inhibitory effect on Arp2/3-dependent actin polymerization (filled triangles). Dephosphorylation was not complete (phosphorylation level was 0.8 ± 0.4 mol/mol), which appears to explain the fact that dephosphorylated caldesmon showed less inhibition than unphosphorylated caldesmon.

**A**

**B**

**FIG. 7.** Actin filaments with caldesmon are less effective activator of Arp2/3. Actin polymerization using F-actin seeds with or without nonmuscle (A) or smooth muscle (B) caldesmon is shown. F-actin seeds were made by polymerizing 6 μM Mg-ATP G-actin (without pyrene) in the same buffer conditions as described in Fig. 1 with 12 μM phalloidin for 30 min and used for seeds. For F-actin seeds with caldesmon, 10 μM caldesmon was added after actin polymerization and incubated for 10 min. F-actin seeds (final concentration, 0.3 μM) with (open symbols) or without (filled symbols) caldesmon (final concentration, 0.5 μM) were mixed with pyrene labeled G-actin (final concentration, 2 μM G-actin, 5% pyrene-labeled) to start actin polymerization. Squares, with Arp2/3 and GST-VCA; circles, without Arp2/3 and GST-VCA. Filled squares, F-actin seeds without caldesmon, with 15 nM Arp2/3 and 50 nM GST-VCA. Open squares, F-actin seeds with caldesmon, with 15 nM Arp2/3, and 50 nM GST-VCA. Filled circles, F-actin seeds without caldesmon, without Arp2/3 or GST-VCA. Open circles, F-actin seeds with caldesmon, without Arp2/3 or GST-VCA.

**FIG. 8.** Caldesmon reduces the affinity of Arp2/3 to F-actin. A, a representative Western blot showing the levels of F-actin-bound Arp3 in the presence or absence of caldesmon. F-actin (2 μM) with or without caldesmon was incubated with 5–20 nM of Arp2/3 and GST-VCA (50 nM) for 20 min. Then the mixtures were centrifuged to pellet F-actin. The levels of Arp3 in F-actin pellets were detected by immunoblotting with monoclonal antibody against Arp3. Control, without caldesmon; +NM CaD, with 0.5 μM nonmuscle caldesmon; +SM CaD, with 0.5 μM smooth muscle caldesmon. The total amounts of Arp2/3 added to F-actin are indicated below each lane. B, the levels of F-actin-bound Arp2/3 were determined by scanning the Western blot shown in A. A standard curve was made by blotting the series of known concentrations of Arp2/3 on the same membranes and used to calculate the levels of Arp2/3. The total amounts of Arp2/3 added to F-actin were indicated below each lane. The error bars represent the variations of three independent experiments.
squares filled squares muscle caldesmon.

The molar ratio of caldesmon to actin could be between 1:100 and 1:12, in which range caldesmon was shown to affect actin polymerization with caldesmon and Ca^{2+}. The conditions were: 2 μM actin (5% pyrene labeled actin); 50 nM VCA; 15 nM Arp2/3 complex in 10 mM imidazole buffer, pH 7, 50 mM KCl, 1 mM MgCl_2, 0.4 mM CaCl_2, 10 mM DTT, and 0.5 μM caldesmon. A, nonmuscle caldesmon; B, smooth muscle caldesmon. Filled squares, Arp2/3-induced polymerization; open squares, with Arp2/3 and calmodulin (2 μM) alone; open triangles, Arp2/3-induced actin polymerization with caldesmon and Ca^{2+}/calmodulin (2 μM); filled triangles, Arp2/3-induced polymerization with caldesmon but without calmodulin.

Caldesmon concentrations appear to alter the motile activity of the peripheral membranes. For example, caldesmon has been reported to be down-regulated in many transformed cells (19, 26). This down-regulation is well correlated to the increases of membrane ruffling upon cell transformation. Furthermore, in our preliminary experiments, we found that microinjection of caldesmon into normal rat kidney cells reduced membrane ruffling movement considerably (data not shown).

Caldesmon may be a key molecule that could confer phosphorylation-dependent or Ca^{2+}/calmodulin-dependent regulation of Arp2/3-mediated actin polymerization. We have demonstrated that the inhibition of caldesmon of Arp2/3-dependent actin polymerization is released by phosphorylation with cdc2 kinase (Fig. 10) and by Ca^{2+}/calmodulin (Fig. 9). This release is likely to be caused by the inhibition of actin binding of caldesmon by these agents. Although tropomyosin has been reported to inhibit Arp2/3-dependent actin polymerization (16), it is mainly localized in stress fibers, and the actin binding of tropomyosin is not regulated like caldesmon. Because other kinases including MAPK (43) and protein kinase C (44) were also reported to regulate the actin binding of caldesmon, caldesmon may play a role in motile phenomena in a variety of signal transduction pathways.

For example, growth factors like PDGF are known to induce membrane ruffles. PDGF also causes caldesmon phosphorylation by MAPK. Because the phosphorylation sites of caldesmon are different from those by cdc2 kinase, phosphorylation by MAPK is likely to reverse the inhibition of caldesmon on Arp2/3 nucleation. Indeed, our preliminary data showed that caldesmon phosphorylated by MAPK lost its inhibition of Arp2/3-dependent actin polymerization (data not shown). Thus, although growth factors like PDGF activate small G-proteins such as Cdc42 and Rac, which in turn initiate membrane ruffles, phosphorylation of caldesmon by MAPK would enhance Arp2/3-induced actin polymerization, leading to more vigorous membrane ruffling and cell movement during growth factor treatment (45, 46). This notion appeared to be supported by a report showing that caldesmon phosphorylation...
by MAPK is involved in PDGF-stimulated cell migration of smooth muscle cells (46).

Caldesmon phosphorylation by cdc2 kinase may also have physiological significance. It has been recently reported that Arp2/3 plays an important role in the completion of cytokinesis in yeast (47) as well as Drosophila (42). We have shown here that phosphorylation of caldesmon by cdc2 kinase completely abolished the inhibition of caldesmon on Arp2/3 (Fig. 10). Because our previous study has shown that caldesmon is phosphorylated by cdc2 kinase during mitosis (35, 41), it is possible that the release of the inhibition of caldesmon may be involved in Arp2/3-mediated assembly of contractile rings. Future studies should be conducted to define how the regulation of actin-caldesmon binding controls actin polymerization and cell motility during signal transduction.

Acknowledgment—We thank Dr. F. Deis (Rutgers) for critical reading of this manuscript.

REFERENCES

1. Condeelis, J. (2001) Trends Cell Biol. 11, 288–293
2. Condeelis, J. S., Wyckoff, J. B., Bailly, M., Pestell, R., Lawrence, D., Backer, J., and Segall, J. E. (2001) Semin. Cancer Biol. 11, 119–128
3. Higgs, H. N., and Pollard, T. D. (1999) J. Biol. Chem. 274, 32531–32534
4. Higgs, H. N., and Pollard, T. D. (2001) Annu. Rev. Biochem. 70, 649–676
5. Mullins, R. D., and Pollard, T. D. (1999) Curr. Opin. Struct. Biol. 9, 244–249
6. Pollard, T. D., Blanchard, L., and Mullins, R. D. (2000) Annu. Rev. Biophys. Biomol. Struct. 29, 545–576
7. Weed, S. A., and Parsons, J. T. (2001) Oncogene 20, 6418–6434
8. Hall, A., Paterson, H. F., Adamson, P., and Ridley, A. J. (1993) Philos. Trans. R. Soc. Lond. B Biol. Sci. 340, 267–271
9. Ridley, A. J., Allen, W. E., Pappelenbosch, M., and Jones, G. E. (1999) Biochem. Soc. Symp. 65, 111–123
10. Lim, L., Manser, E., Leung, T., and Hall, C. (1996) Eur. J. Biochem. 242, 171–185
11. Bonnastre, J., Rijksen, B., Hummel, B., Cremers, F., Verkleij, A., and van Bergen en Henegouwen, P. (1995) Cell Biol. Int. 19, 413–430
12. Smith, C. E. (1998) Crit. Rev. Oral Biol. Med. 9, 128–161
13. Uruno, T., Liu, J., Zhang, P., Fun, Y., Egie, C., Li, R., Mueller, S. C., and Zhan, X. (2001) Nat. Cell Biol. 3, 259–266
14. Weaver, A. M., Karginov, A. V., Kinley, A. W., Weed, S. A., Li, Y., Parsons, J. T., and Cooper, J. A. (2001) Curr. Biol. 11, 370–374
15. Weed, S. A., Karginov, A. V., Schafer, D. A., Weaver, A. M., Kinley, A. W., Cooper, J. A., and Parsons, J. T. (2000) J. Cell Biol. 151, 29–40
16. Blanchon, L., Pollard, T. D., and Hitchcock-DeGregori, S. E. (2001) Curr. Biol. 11, 1300–1304
17. Humphries, C. L., Balcer, H. I., D’Agostino, J. L., Winsor, B., Drubin, D. G., Barnes, G., Andrews, B. J., and Goode, B. L. (2002) J. Cell Biol. 159, 993–1004
18. Yamakita, Y., Yamashiro, S., and Matsumura, F. (1990) J. Cell Biol. 111, 2487–2498
19. Koji-Owada, M. K., Hakura, A., Iida, K., Yahara, I., Sobue, K., and Kakiuchi, S. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3133–3137
20. Lim, M. S., and Walsh, M. P. (1986) Biochem. J. 238, 523–530
21. Ishikawa, R., Yamashiro, S., and Matsumura, F. (1989) J. Biol. Chem. 264, 7490–7497
22. Ishikawa, R., Yamashiro, S., and Matsumura, F. (1989) J. Biol. Chem. 264, 16764–16770
23. Lenaerts, W., Craig, R., Lu, J., and Moody, C. (1989) J. Muscle Res. Cell Motil. 10, 101–112
24. Matsumura, F., and Yamashiro, S. (1993) Curr. Opin. Cell Biol. 5, 70–76
25. Helfman, D. M., Levy, E. T., Berthier, C., Shuttman, M., Rivelino, D., Grushka, I., Lachish-Zalait, A., Elbaum, M., and Bershady, A. D. (1999) Mol. Biol. Cell 10, 3097–3112
26. Yamashiro, S., Yoshida, K., Yamakita, Y., and Matsumura, F. (1994) in International Conference on the Biophysics, Biochemistry, and Cell Biology of Actin (Estes, J. E., and Higgins, P. J., eds) pp. 113–122, Plenum Publishing Corp., Troy, NY
27. Lamb, N. J., Fernandez, A., Menguelo, M., Labbe, J. P., Kassab, R., and Fattoum, A. (1996) Eur. J. Cell Biol. 69, 36–44
28. Castellino, F., Ono, S., Matsumura, F., and Laini, A. (1995) J. Cell Biol. 131, 1223–1230
29. Higgs, H. N., Blanchin, L., and Pollard, T. D. (1999) Biochemistry 38, 15212–15222
30. Koyama, T., and Mihashi, K. (1981) Eur. J. Biochem. 114, 33–38
31. Egie, C., Leisel, T. P., Laurent, V., Li, R., Pantaloni, D., Sansonetti, P. J., and Carrier, M. F. (1999) J. Cell Biol. 146, 1319–1332
32. Yamashiro, S., Chern, H., Yamakita, Y., and Matsumura, F. (2001) Mol. Biol. Cell 12, 239–250
33. Bretscher, A. (1984) J. Biol. Chem. 259, 12873–12880
34. Yamashiro-Matsumura, S., Ishikawa, R., and Matsumura, F. (1988) Protol. Genes (Suppl. 2) 9–21
35. Yamakita, Y., Yamashiro, S., and Matsumura, F. (1992) J. Biol. Chem. 267, 12022–12029
36. Yamashiro-Matsumura, S., and Matsumura, F. (1988) J. Cell Biol. 106, 1573–1583
37. Hayashi, K., Yamada, S., Kanda, K., Kimizuka, F., Kato, I., and Sobue, K. (1989) Biochem. Biophys. Res. Commun. 161, 38–45
38. Cronich, J. M., Chen, Y. D., Dudke, R., and Luo, H. (1995) J. Biol. Chem. 270, 9911–9916
39. Osawa, F., and Asakura, S. (1975) Thermodynamics of the Polymerization of Proteins, Academic Press, Orlando, FL
40. Marchand, J. R., Kaiser, D. A., Pollard, T. D., and Higgs, H. N. (2001) Nat. Cell Biol. 3, 76–82
41. Yamashiro, S., Yamakita, Y., Hosoya, H., and Matsumura, F. (1991) Nature 349, 169–172
42. Stevens, V., Hudson, A., Cooley, L., and Themurkou, W. E. (2002) Curr. Biol. 12, 765–711
43. Hedges, J. C., Yamboliev, I. A., Ngo, M., Hovorka, B., Adam, L. P., and Gerthoffer, W. T. (1998) Am. J. Physiol. 275, C527–C534
44. Vorotnikov, A. V., Gusev, N. B., Hua, S., Collins, J. H., Redwood, C. S., and Marston, S. B. (1994) J. Muscle Res. Cell Motil. 15, 37–48
45. Gerasimova, E. A., Vorotnikov, A. V., Gracheva, E. O., Albert, W. C., and Carlier, M. F. (1999) J. Cell Biol. 145, 111–123
46. Gerasimova, E. A., Vorotnikov, A. V., Carlier, M. F., and Higgs, H. N. (2001) J. Biol. Chem. 276, 12301–12308
47. Pelham, R. H. C., and Chang, F. (2002) Nature 419, 82–86