Calcium-Calmodulin Suppresses the Filamentous Actin-Binding Activity of a 135-Kilodalton Actin-Bundling Protein Isolated from Lily Pollen Tubes

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We have isolated a 135-kD actin-bundling protein (P-135-ABP) from lily (*Lilium longiflorum*) pollen tubes and have shown that this protein is responsible for bundling actin filaments in lily pollen tubes (E. Yokota, K. Takahara, T. Shimmen [1998] Plant Physiol 116: 1421–1429). However, only a few thin actin-filament bundles are present in random orientation in the tip region of pollen tubes, where high concentrations of Ca^{2+} have also been found. To elucidate the molecular mechanism for the temporal and spatial regulation of actin-filament organization in the tip region of pollen tubes, we explored the possible presence of factors modulating the filamentous actin (F-actin)-binding activity of P-135-ABP. The F-actin-binding activity of P-135-ABP in vitro was appreciably reduced by Ca^{2+} and calmodulin (CaM), although neither Ca^{2+} alone nor CaM in the presence of low concentrations of Ca^{2+} affects the activity of P-135-ABP. A micromolar order of Ca^{2+} and CaM were needed to induce the inhibition of the binding activity of P-135-ABP to F-actin. An antagonist for CaM, W-7, cancelled this inhibition. W-5 also alleviated the inhibition effect of Ca^{2+}-CaM, however, more weakly than W-7. These results suggest the specific interaction of P-135-ABP with Ca^{2+}-CaM. In the presence of both Ca^{2+} and CaM, P-135-ABP organized F-actin into thin bundles, instead of the thick bundles observed in the absence of CaM. These results suggest that the inhibition of the P-135-ABP activity by Ca^{2+}-CaM is an important regulatory mechanism for organizing actin filaments in the tip region of lily pollen tubes.

In pollen tubes, actin filament bundles with parallel orientation to the long axis of a tube are well developed, and are involved in cytoplasmic streaming and the transport of vegetative nuclei and generative cells to the growing tip (Pierson and Cresti, 1992; Mascarenhas, 1993; Li et al., 1997; Taylor and Hepler, 1997). In the tip region, however, only a few thin bundles and short individual actin filaments in random orientation have been observed by electron microscopy in samples prepared using the rapid freeze fixation technique (Lancelle et al., 1987; Tiwari and Polito, 1988; Lancelle and Hepler, 1992; Miller et al., 1996). At the tip of actively growing pollen tubes, a tip-focused Ca^{2+} gradient has been reported that is indispensable for the tip growth (Rathore et al., 1991; Miller et al., 1992; Pierson et al., 1994, 1996) and tube orientation (Malhó et al., 1994, 1995; Malhó and Trewavas, 1996). An influx of external Ca^{2+} at the tip supports the tip-focused gradient of Ca^{2+} (Kuhtreiber and Jaffe, 1990; Pierson et al., 1994, 1996; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). The disorganization of actin-filament bundles in the tip region of growing tubes is believed to be due to the tip-focused Ca^{2+} gradient. When growing pollen tubes are treated with caffeine that diminishes the tip-focused Ca^{2+} gradient (Pierson et al., 1994), tip growth ceases and extensive actin-filament bundles extend into the tip (Miller et al., 1996; Lancelle et al., 1997). This observation also suggests that higher concentrations of Ca^{2+} at the tip region suppress actin filament bundle formation. For further insights into the regulation of tip growth of pollen tubes, it is important to elucidate how the organization of actin filaments is modulated by Ca^{2+} in the tip region of growing tubes.

Architecture of the actin cytoskeleton and polymerization-depolymerization of actin filaments in the cell are mediated by and modulated temporally and spatially by actin-binding proteins (Stossel et al., 1985; Pollard and Cooper, 1986). Recently, we have isolated an actin-binding protein (P-135-ABP) from lily (*Lilium longiflorum*) pollen tubes, which arranges filamentous actin (F-actin) into bundles in vitro (Yokota et al., 1998). F-actin filaments in the bundle formed by P-135-ABP in vitro showed uniform polarity (Yokota and Shimmen, 1999). Immunofluorescence and immunoelectron microscopy using an antibody against P-135-ABP showed colocalization of P-135-ABP with actin-filament bundles in lily pollen tubes (Yokota et al., 1998; Vidali et al., 1999). These observations strongly suggest that P-135-ABP is a factor responsible for bundling actin filaments in pollen tubes and its activity may be suppressed at the tip region of tubes. The activities of several kinds of actin-bundling proteins, including villin (Bretscher

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and Weber, 1980; Mooseker et al., 1980) and plastin/fimbrin (Glenney et al., 1981; Namba et al., 1992; Lin et al., 1994; Prassler et al., 1997), are inhibited by Ca^{2+} at physiological concentrations. In contrast, the activity of purified P-135-ABP was independent of Ca^{2+} (Yokota et al., 1998). It was suggested that some unknown factor(s) gave Ca^{2+} sensitivity to P-135-ABP. In the present study, we show that the binding activity of P-135-ABP to F-actin is significantly suppressed by calmodulin (CaM) in the presence of Ca^{2+}.

RESULTS

Suppression of the Binding Activity of P-135-ABP to F-Actin by Ca^{2+}-CaM

CaM had no effect on the binding activity of P-135-ABP to F-actin in the presence of EGTA. Most of P-135-ABP was coprecipitated with F-actin in a cosedimentation assay in the presence of EGTA and CaM (Fig. 1C). In contrast, 70% to 80% of P-135-ABP did not bind to F-actin and remained in the supernatant in the presence of Ca^{2+}-CaM (Fig. 1D). As shown previously, Ca^{2+} alone did not affect the binding activity of P-135-ABP to F-actin in the cosedimentation assay (Fig. 1B; Yokota et al., 1998).

Dissociation of P-135-ABP from F-Actin by Ca^{2+}-CaM

Next, we confirmed whether Ca^{2+}-CaM possesses an ability to dissociate P-135-ABP from F-actin. At first P-135-ABP was mixed with F-actin in the presence of 0.2 mM EGTA for 20 min, and then with $CaCl_2$ (final concentration at 0.5 mM) alone or with both $CaCl_2$ and CaM (final concentration at 7.8 μM). In the case of the addition of $CaCl_2$ alone, most of P-135-ABP was coprecipitated with F-actin (Fig. 2C). However, 70% to 80% of P-135-ABP was detected in the supernatant (Fig. 2D) when both $CaCl_2$ and CaM were added to the mixture of F-actin and P-135-ABP. These results indicate that CaM caused dissociation of P-135-ABP from F-actin in the presence of Ca^{2+}.

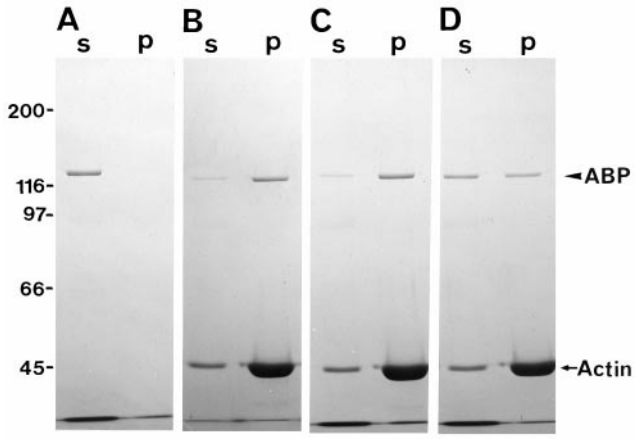

Figure 1. Suppression of binding activity of P-135-ABP to F-actin by Ca^{2+}-CaM. A mixture of 3.2 μg/mL P-135-ABP, 60 μg/mL F-actin, and 7.8 μM CaM was incubated in the absence (C) or the presence (D) of 0.5 mM $CaCl_2$ at 20°C for 20 min. After centrifugation, the resulting supernatant (s) and pellet (p) were analyzed by SDS-PAGE on a 7.5% (w/v) acrylamide gel. As control experiments, a mixture of P-135-ABP and CaM without F-actin (A) or P-135-ABP and F-actin without CaM (B) was analyzed in the presence of $CaCl_2$ as above. The arrowhead and the arrow indicate the 135-kD polypeptide of P-135-ABP (ABP) and actin, respectively. The molecular masses of standard proteins are indicated on the left in kD.

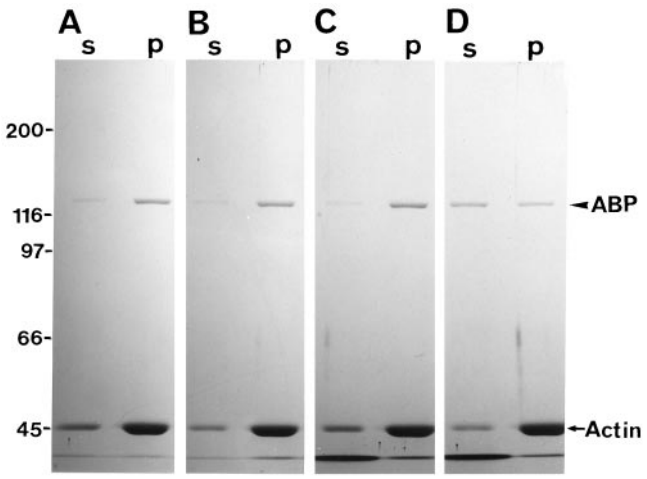

Figure 2. Dissociation of P-135 from F-actin by Ca^{2+}-CaM. P-135-ABP was mixed with F-actin. After a 20-min incubation at 20°C, samples were treated with no addition (A), with $CaCl_2$ alone (B), with CaM alone (C), and with both $CaCl_2$ and CaM (D). The mixtures were incubated for an additional 10 min at 20°C and then subjected to centrifugation. The resulting supernatant (s) and pellet (p) were analyzed by SDS-PAGE on a 7.5% (w/v) acrylamide gel. The final concentrations of P-135-ABP, F-actin, CaM, and $CaCl_2$ were 3.2 μg/mL, 60 μg/mL, 7.8 μM, and 0.5 mM, respectively. The arrowhead and the arrow indicate the 135-kD polypeptide of P-135-ABP (ABP) and actin, respectively. The molecular masses of standard proteins are indicated on the left in kD.

Suppression of Bundling Activity of P-135-ABP by Ca^{2+}-CaM

To examine the effect of Ca^{2+}-CaM on the formation of actin bundles by P-135-ABP, a mixture of rhodamine-phalloidin (RP)-labeled F-actin and P-135-ABP was observed under an epifluorescence microscope in the presence of Ca^{2+} alone or Ca^{2+}-CaM. In the presence of Ca^{2+} alone, RP-labeled actin filaments were arranged into thick bundles (2–12 μm in width) by P-135-ABP (Fig. 3B). This observation is consistent with the previous result showing the Ca^{2+}-insensitive bundling activity of P-135-ABP (Yokota et al., 1998). On the other hand, only thin bundles with widths below 2 μm were seen in the presence of Ca^{2+}-CaM (Fig. 3C). With these results, together with those obtained from the cosedimentation assay, it is concluded that the organization of F-actin into a bundle is sup-

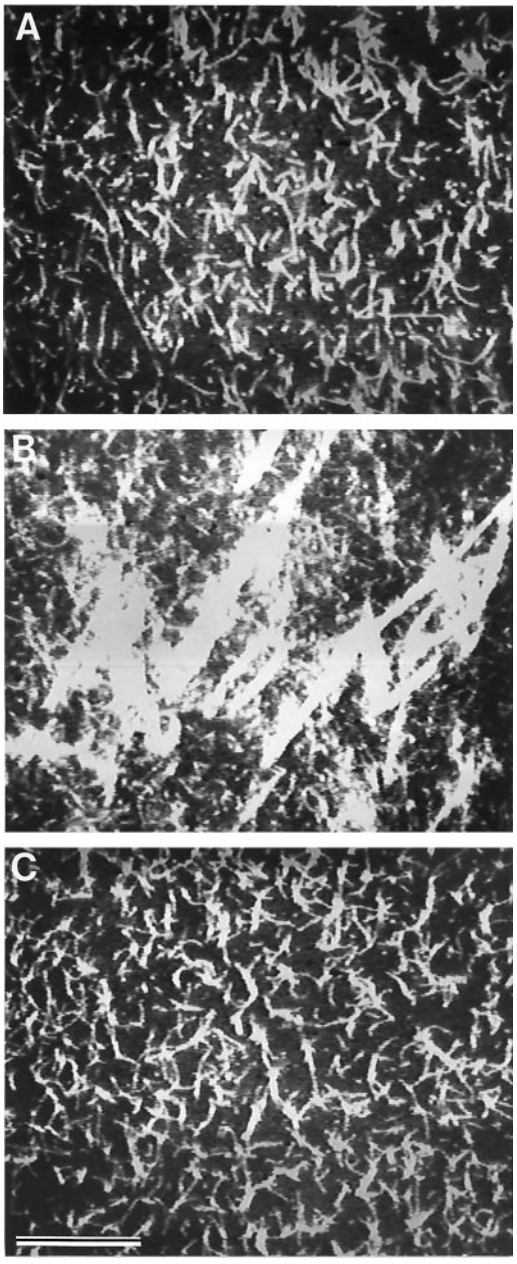

Figure 3. Fluorescence micrographs of mixtures of P-135-ABP and RP-labeled F-actin. P-135-ABP (4.6 μg/mL) was mixed with RP-labeled F-actin (4.2 μg/mL) in a solution containing 0.5 mM $CaCl_2$ with CaM (5.5 μM; C) or without it (B). The mixture was then observed under an epifluorescence microscope. A, RP-labeled F-actin alone in the presence of 0.5 mM $CaCl_2$. The bar represents 30 μm.

pressed by Ca^{2+}-CaM due to inhibition of P-135-ABP binding to F-actin.

Effect of Ca^{2+} and CaM Concentrations on the Binding Activity of P-135-ABP

The inhibitory effect of Ca^{2+}-CaM was intimately dependent on the concentration of both Ca^{2+} and CaM. Figure 4A shows the relation between CaM concentrations and binding of P-135-ABP to F-actin in the cosedimentation assay. The amount of P-135-ABP associated with F-actin decreased remarkably as CaM concentrations increased up to 1 μM. Since the native molecular mass of P-135-ABP is 260 kD (Yokota et al., 1998), the molecular ratio of 3.2 μg/mL P-135-ABP:1 μM CaM is calculated to be 1:83.

Figure 4B shows the relation between Ca^{2+} concentrations and binding of RP-labeled F-actin to the glass surface coated with P-135-ABP. EGTA contamination from the P-135-ABP fraction can be avoided in this experiment, because the medium in the flow chamber is completely replaced by the assay medium, whose Ca^{2+} concentrations are controlled (see "Materials and Methods"). In randomly selected microscope fields (2,000 $\mu m^2 \times 20$), only a few RP-labeled actin filaments were attached at their one end to the glass surface not treated with P-135-ABP (data not shown), indicating a low level of non-specific binding. An RP-labeled F-actin attached to the P-135-ABP-coated glass surface along its whole length was judged as a bound F-actin. The number of F-actin bound to the glass surface coated with P-135-ABP in the presence of CaM significantly decreased by elevating Ca^{2+} concentration up to 2.5 μM (pCa 5.6). Further increase in the Ca^{2+} concentration induced only a gradual decrease in bound RP-labeled F-actin. In the absence of CaM, the number of F-actin bound to the glass surface coated with P-135-ABP was not affected by Ca^{2+} (data not shown).

In both assays, 20% to 30% of P-135-ABP remained bound to F-actin in a Ca^{2+}-CaM-insensitive manner. For example, 28% of P-135-ABP was recovered in the F-actin pellet even in the presence of 0.5 mM Ca^{2+} and 7.8 μM CaM (Fig. 4A).

Ameliorating Effects of Antagonist for CaM on the Inhibitory Effect of Ca^{2+}-CaM

To confirm that the inhibitory effect of Ca^{2+}-CaM on the binding of P-135-ABP to F-actin is a specific phenomenon, we examined the influence of W-7 and W-5, antagonists for CaM, on the Ca^{2+}-CaM-induced inhibition of binding activity of P-135-ABP (Fig. 5). Figure 6 shows the relation between concentrations of W-7 or W-5 and cosedimentation of P-135-ABP with F-actin in the presence of Ca^{2+}-CaM. The amount of P-135-ABP associated with F-actin significantly increased when W-7 concentrations were elevated up to 10 μM. The addition of 30 μM W-7 increased the amount of P-135-ABP that coprecipitated with F-actin in the presence of Ca^{2+}-CaM to a level similar to that in the presence of Ca^{2+} alone (compare lane Ap with lane Cp in Fig. 5). Thus, W-7 cancels the inhibitory effect of Ca^{2+}-CaM on the binding activity of P-135-ABP to F-actin. W-5 also cancelled the inhibitory effect, but to a lesser extent (compare lane Dp with lane Cp in Figs. 5 and 6). The amount of P-135-ABP associated with F-actin was gradually increased

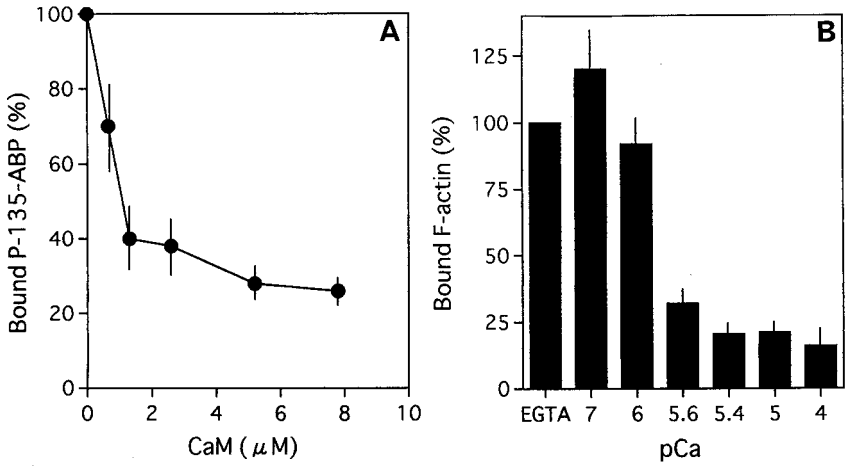

Figure 4. Dose-response relation of CaM and $CaCl_2$ on the binding activity of P-135-ABP to F-actin. A, Effect of CaM concentrations on P-135-ABP binding to F-actin analyzed by a coprecipitation assay. The mixture of P-135-ABP (3.2 μg/mL) and F-actin (60 μg/mL) containing 0.5 mM $CaCl_2$ in the presence of CaM at various concentrations was centrifuged and the supernatant and the pellet were electrophoresed on a 7.5% (w/v) acrylamide gel and subsequently analyzed by densitometry. The amount of P-135-ABP bound to F-actin was quantified and plotted using values relative to that for the mixture without CaM. The average values obtained from three separate preparations are shown. B, Effect of Ca^{2+} on the binding of RP-labeled F-actin on a glass surface coated with P-135-ABP. RP-labeled F-actin (0.5 μg/mL) and CaM (1.5 μM) in an assay medium containing Ca^{2+} of various concentrations were introduced into the flow chamber constructed using a P-135-ABP-coated coverslip. The amount of RP-labeled F-actin attached to the coverslip surface was quantified and plotted using values relative to that obtained in the presence of EGTA. The average values obtained from three separate preparations are shown.

with the elevation of the concentrations of W-5. However, only about 60% of P-135-ABP was coprecipitated with F-actin even in the presence of 30 μM W-5 (Fig. 6).

These CaM antagonists also alleviated and cancelled the inhibitory effect of Ca^{2+}-CaM on the arrangement of F-actin into bundles by P-135-ABP. Even in the presence of Ca^{2+}-CaM, F-actin filaments were arranged into thick bundles (Fig. 7, C and D) whose diameters appeared to be similar to those in the presence of Ca^{2+} alone (Fig. 7A) when W-7 at concentrations above 10 μM was supplied to the mixture. In contrast, images of mixture of F-actin, P-135-ABP, and Ca^{2+}-CaM in the presence of 10 μM W-5 (Fig. 7E) were identical with those in the absence of CaM antagonists (Fig. 7B). In the presence of 30 μM W-5, F-actin bundles became visible, (Fig. 7F) although the number and diameter of the bundles were significantly smaller than those observed in the presence of W-7.

DISCUSSION

In plant cells, it has been well known that CaM interacts with microtubules (Vantard et al., 1985; Wick et al., 1985; Fisher and Cyr, 1993) and that it is responsible for regulating the dynamics of cortical microtubules in a Ca^{2+}-dependent manner (Cyr, 1991; Fisher et al., 1996). It is thought that the interaction between CaM and microtubules is mediated by microtubule-associated proteins (MAPs). Elongation factor-1α is one of these MAPs, whose Ca^{2+}-CaM-dependent interaction with microtubules has been characterized in vitro: Ca^{2+}-CaM inhibits the bundling formation and stabilization of microtubules induced by the elongation factor-1α (Durso and Cyr, 1994; Moore et al., 1998). In addition to MAPs, it has been reported that the motile and binding activity in vitro of microtubule-based motor protein, kinesin-like CaM binding protein (Reddy et al., 1996), which identified in plant cells, is suppressed by Ca^{2+}-CaM through its binding to this protein (Song et al., 1997; Narasimhulu and Reddy, 1998).

In the present study, we have shown that the activity of an actin-binding protein from a higher plant is also modulated by CaM in a Ca^{2+}-sensitive manner in vitro. Binding of P-135-ABP to F-actin was inhibited by Ca^{2+}-CaM, but not by Ca^{2+} or CaM alone (Fig. 1). Moreover, Ca^{2+}-CaM caused the dissociation of P-135-ABP from F-actin (Fig. 2). In general, two high-affinity and two low-affinity binding sites for Ca^{2+} are present in each CaM molecule. The dissociation constants (K_d) of these binding sites are about 10^{-6} and 10^{-5} M, respectively (Klee, 1988). The saturable concentration of Ca^{2+} and CaM for the inhibition of F-actin-binding of P-135-ABP was above 2.5 and 1.0 μM, respectively (Fig. 4). This Ca^{2+} concentration is comparable to the K_ds for Ca^{2+} binding sites of the CaM molecule. Furthermore, W-7 cancelled the inhibitory effect of Ca^{2+}-CaM on binding (Figs. 5 and 6) and bundling activities of P-135-ABP (Fig. 7). W-5 also alleviated the inhibitory effect of Ca^{2+}-CaM, however, more weakly than W-7 (Figs. 5–7). It has been reported that W-5 interacted more weakly than W-7 with Ca^{2+}-CaM and inhibited to a lesser extent the activation of Ca^{2+}-CaM-dependent

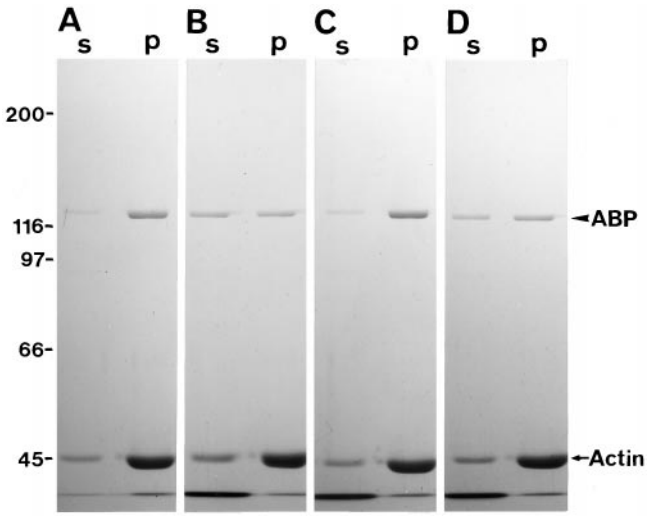

Figure 5. Ameliorating effect of W-7 and W-5 on the inhibitory effect of Ca^{2+}-CaM on P-135-ABP binding to F-actin in the presence of 0.5 mM $CaCl_2$. A mixture of P-135-ABP (4.6 μg/mL), F-actin (60 μg/mL), and CaM (7.8 μM) was centrifuged in the presence of 30 μM W-7 (C) or W-5 (D). Resulting supernatants (s) and pellets (p) were analyzed by SDS-PAGE on a 7.5% (w/v) acrylamide gel. As a control experiment, a mixture of P-135-ABP and F-actin without CaM (A) or with CaM (B) in the absence of antagonists was treated with the same manner as above. The arrowhead and the arrow indicate the 135-kD polypeptide of P-135-ABP (ABP) and actin, respectively. The molecular masses of standard proteins are indicated on the left in kD.

cyclic nucleotide phosphodiesterase in vitro (Tanaka et al., 1982). Together with the evidence showing that W-7 blocks the interaction of Ca^{2+}-CaM with its target enzymes (Hidaka et al., 1980; Tanaka et al., 1982), these results support the specific interaction of Ca^{2+}-CaM with P-135-ABP in the inhibition of P-135-ABP binding to F-actin.

Recently, it was revealed that P-135-ABP is a plant homolog of villin by cloning of a cDNA from a lily pollen library (Vidali et al., 1999). Villin is a well-characterized actin-bundling protein constructed from two domains: a gelsolin/severin domain and a head piece domain in COOH-terminal tail. At concentrations of Ca^{2+} below 10^{-6} M, villin bundles actin filaments through the head piece domain, whereas actin-filament severing and capping activities residing in the gelsolin/severin domain are expressed at Ca^{2+} concentrations above 10^{-6} M (Matsudaira and Janmey, 1988; Friederich et al., 1990; Otto, 1994). P-135-ABP also has these two domains (Vidali et al., 1999), however, the severing activity has not been demonstrated for isolated P-135-ABP even in the presence of Ca^{2+} at concentrations higher than 10^{-6} M (Yokota et al., 1998; this study). Furthermore, it has not been reported that non-plant villin thus far examined shows Ca^{2+}-CaM sensitivity in its binding and bundling activities in vitro. Additional biochemical and molecular biological studies are needed to elucidate and confirm whether Ca^{2+}-CaM sensitivity is characteristic for plant villin only and which regions or domains in the plant villin molecule provide this sensitivity.

Numerous actin-filament bundles are oriented parallel to the long axis of a pollen tube, from the grain into the subapical region in the growing tube. In contrast, only a few thin bundles are dispersed in random orientation at the tip, in which high concentrations of Ca^{2+} are present. In the case of lily pollen tubes, the Ca^{2+} concentration at the tip is estimated to be more than 3 μM (Pierson et al., 1994, 1996; Messerli and Robinson, 1997). This concentration is sufficient to induce inhibition of P-135-ABP binding to F-actin in the presence of 1.5 μM CaM (Fig. 4B). Furthermore, the tip focused distribution of CaM has been suggested in chemical fixed pollen tubes of tobacco and lily by using antibody against CaM (Tirlapur et al., 1994) and by fluphenazine that binds to CaM in a Ca^{2+}-dependent manner (Hauβer et al., 1984), respectively. However, it was recently reported that the distribution of fluorescein-conjugated CaM microinjected into pollen tubes of *Agapanthus umbellatus* was uniform and that no tip-focused gradient was observed (Moutinho et al., 1998). The concentration of CaM in plant cells has been estimated to be 1.3 μM in barley aleurone cells (Schuurink et al., 1996), 4 μM in carrot cell lines (Fisher et al., 1996), and 11 μM in the cytoplasm of stamen hair cells of *Tradescantia virginiana* (Vos and Hepler, 1998). Therefore, even if CaM is dispersed uniformly throughout pol-

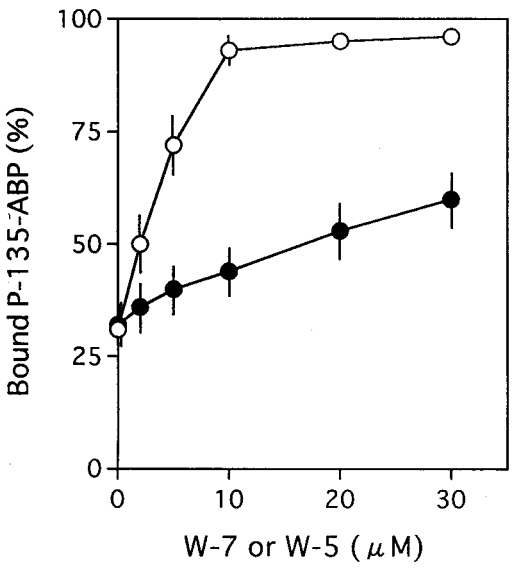

Figure 6. Dose-response relation of antagonists for CaM on the binding activity of P-135-ABP to F-actin in the presence of Ca^{2+}-CaM. The mixture of P-135-ABP (4.6 μg/mL), F-actin (60 μg/mL), and CaM (7.8 μM), containing 0.5 mM $CaCl_2$ in the presence of W-7 (○) or W-5 (●) at various concentrations, was centrifuged and the supernatant and the pellet were electrophoresed on a 7.5% (w/v) acrylamide gel and subsequently analyzed by densitometry. The amount of P-135-ABP bound to F-actin was quantified and plotted using values relative to that for the mixture without CaM and antagonists. The average values obtained from three analyses are shown.

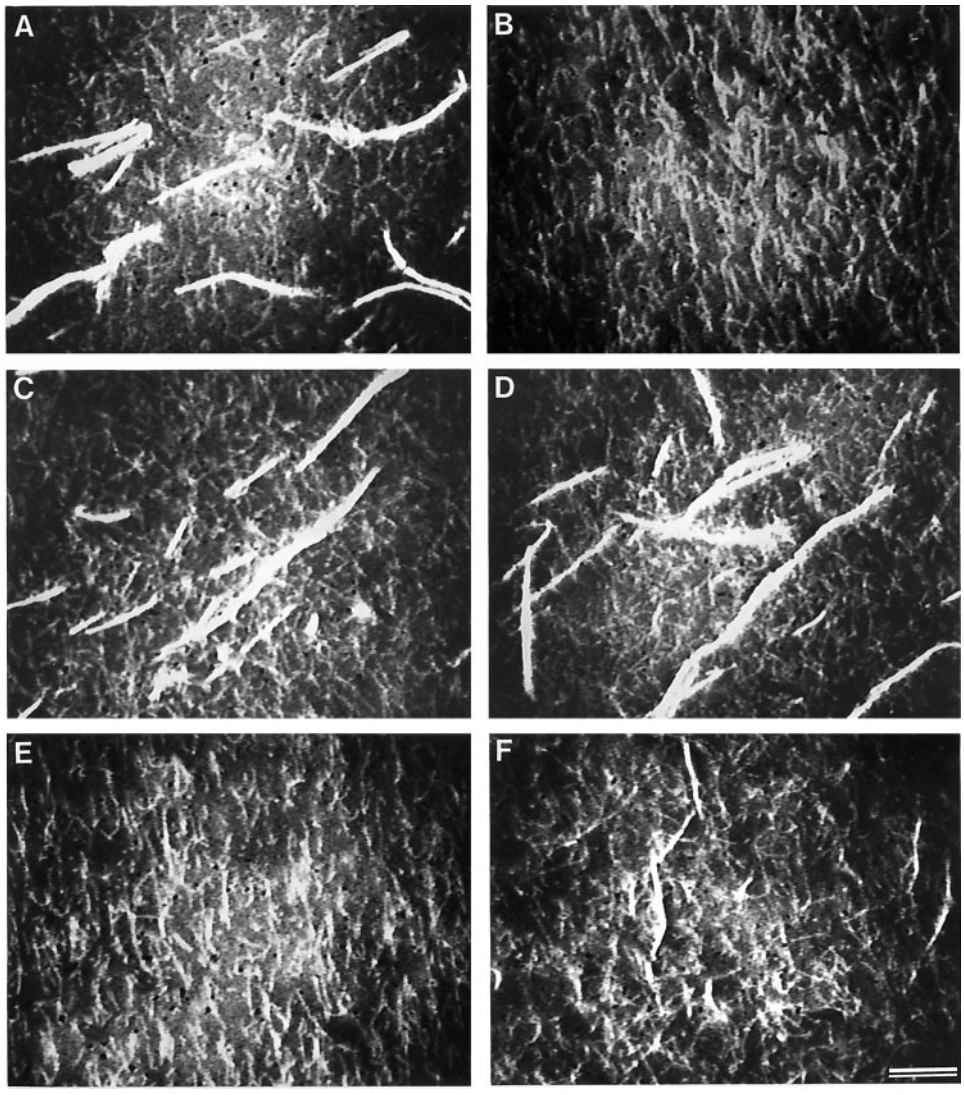

Figure 7. Ameliorating effect of W-7 and W-5 on the inhibitory effect of Ca^{2+}-CaM on bundling activity of P-135-ABP in the presence of 0.5 mM $CaCl_2$. P-135-ABP (4.6 μg/mL) was mixed with RP-labeled F-actin (4.2 μg/mL) in a solution containing 0.5% (v/v) DMSO (A), DMSO and 5.5 μM CaM (B), CaM and 10 μM W-7 (C), CaM and 30 μM W-7 (D), CaM and 10 μM W-5 (E), or CaM and 30 μM W-5 (F). The mixture was then observed under an epifluorescence microscope. The bar represents 30 μm.

len tubes, as reported by Moutinho et al. (1998), it will be reasonable to deduce that the concentration of CaM in the tip region is in the micromolar order, which is sufficient to induce the inhibition of P-135-ABP binding to F-actin (Fig. 4A). Consequently, it is speculated that a high Ca^{2+} concentration is a cause for the lack of large actin-filament bundles at the tip region.

In the cosedimentation assay (Fig. 4A) and the binding assay of RP-labeled F-actin to glass surface coated with P-135-ABP (Fig. 4B), 20% to 30% of P-135-ABP remained bound to F-actin even in the presence of Ca^{2+}-CaM. The thin bundles of RP-labeled F-actin in the presence of Ca^{2+}-CaM appeared to be formed by Ca^{2+}-CaM-insensitive P-135-ABP (Fig. 3C). The possibility is not excluded that a site or sites within a P-135-ABP molecule that interact with Ca^{2+}-CaM are denatured during purification steps, making the actin-binding protein insensitive to Ca^{2+}-CaM. However, it may also be that P-135-ABP that is insensitive to Ca^{2+}-CaM is inherently present in lily pollen tubes and works to form thin bundles of actin filaments in the tip region containing high concentrations of Ca^{2+}-CaM. This possibility remains unsolved.

In addition to P-135-ABP, other actin-binding proteins have been reported in pollen tubes such as a low M_r actin-sequestering and depolymerizing protein, profilin (Valenta et al., 1993; Mittermann et al., 1995; Huang et al., 1996; Vidali and Hepler, 1997; Gibbon et al., 1998), and an actin depolymerizing factor (ADF; Kim et al., 1993; Chung et al., 1995;

Lopez et al., 1996). Recently, we isolated an actin-binding protein that is composed of a 115-kD polypeptide (Nakayasu et al., 1998). In addition, the presence of some factors fragmenting actin filaments in the presence of Ca^{2+} is suggested in lily pollen tubes. This is based on the observation that actin filaments are fragmented by the elevation of the intracellular Ca^{2+} concentration in pollen tubes (Kohno and Shimmen, 1987). Hence, the possibility must be considered that various actin-binding proteins and factors are also involved in controlling the architecture of actin filaments together with P-135-ABP in the tip region of pollen tubes.

MATERIALS AND METHODS

Purification of P-135-ABP and CaM from Lily Pollen Tubes

P-135-ABP was purified from the germinating pollen of lily (*Lilium longiflorum*) according to the method described previously (Yokota et al., 1998). Purified P-135-ABP was dialyzed against a solution containing 90 mM KCl, 0.2 mM EGTA, 2 mM $MgCl_2$, 50 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 30 mM PIPES (1,4-piperazinediethanesulfonic acid)-KOH (pH 7.0). To remove any aggregate, dialysate was centrifuged at 300,000*g* for 20 min at 4°C. The resultant supernatant was used as P-135-ABP for various experiments described below.

CaM was also isolated from the germinating pollen of lily by the method described previously (Yokota et al., 1999). After dialysis against a solution containing 90 mM KCl, 50 μg/mL leupeptin, 0.5 mM PMSF, 1 mM DTT, and 30 mM PIPES-KOH (pH 7.0), CaM was stored at −80°C until use.

Cosedimentation Analysis of P-135-ABP with F-Actin

P-135-ABP was mixed with F-actin prepared from chicken skeletal muscle in an assay solution containing 90 mM KCl, 0.2 mM EGTA, 2 mM $MgCl_2$, 50 μg/mL leupeptin, 0.5 mM PMSF, 1 mM DTT, and 30 mM PIPES-KOH (pH 7.0) and left standing for 10 min at 20°C. To examine the effect of Ca^{2+}-CaM, $CaCl_2$ (final concentration at 0.5 mM) and various concentrations of CaM were added to the assay solution. As a control, P-135-ABP alone was treated in the same manner. The samples were centrifuged at 150,000*g* for 20 min, and supernatants and pellets were analyzed by SDS-PAGE on a 7.5% (w/v) polyacrylamide gel (following the method of Laemmli [1970]). The amount of P-135-ABP bound to F-actin was determined quantitatively according to the method described in the previous paper (Yokota et al., 1998).

To examine the effect of Ca^{2+}-CaM on the dissociation of P-135-ABP from F-actin, P-135-ABP was first mixed with F-actin. After a 20 min incubation at 20°C, $CaCl_2$ (final concentration at 0.5 mM) alone or both $CaCl_2$ and CaM were added to the mixture and left standing for 10 min at 20°C. The final concentrations of P-135-ABP, F-actin, and CaM were 3.2 μg/mL, 60 μg/mL, and 7.8 μM, respectively.

To examine the influence of W-7 and W-5, these chemicals (Sigma, St. Louis) dissolved in dimethylsulfoxide (DMSO) were added to a mixture of 4.6 μg/mL P-135-ABP, 60 μg/mL F-actin, and 7.8 μM CaM in the presence of 0.5 mM $CaCl_2$. As a control, the same volume of DMSO (0.5% [v/v]) was added to the mixture.

Binding Assay of F-Actin on the Glass Surface Coated with P-135-ABP

RP-labeled F-actin was prepared by incubating F-actin with RP (Molecular Probes, Eugene, OR) according to the method of Kohno et al. (1991). A washing solution contained 30 mM KCl, 5 mM EGTA, 6 mM $MgCl_2$, 5 mM DTT, 30 mM PIPES-KOH (pH 7.0), and various concentrations of $CaCl_2$. To calculate actual Ca^{2+} concentrations from the K_ds, a computer program was used (Kohno and Shimmen, 1988). Coverslips were coated with 0.2% (v/v) collodion dissolved in isopentyl acetate and then allowed to air dry. P-135-ABP was mixed with CaM and $CaCl_2$ in the same solution used for the cosedimentation procedure and left standing on ice. The final concentrations of P-135-ABP, CaM, and $CaCl_2$ were 4.1 μg/mL, 1.5 μM, and 0.5 mM, respectively. Fifty microliters of the mixture was placed on parafilm (American National Can, Neenah, WI) and a collodion-coated coverslip was laid on it. After 5 min at 25°C, a small amount of petroleum jelly was applied along the two opposite edges of the coverslip, and then this coverslip was placed on a glass slide to make a flow chamber with a volume of approximately 12 to 15 μL. The flow chamber was perfused with 100 μL of the washing solution and subsequently with 100 μL of the washing solution containing 0.5 μg/mL RP-labeled F-actin and 1.5 μM CaM. After 5 min, 100 μL of the washing solution was introduced into the chamber to remove unbound free RP-labeled F-actin.

To assess the binding activity of P-135-ABP, fluorescent images obtained by epifluorescence microscopy (BH2-RFC, Olympus, Tokyo) were recorded using a high-sensitivity television camera (C2400–08 SIT camera, Hamamatsu Photonics K. K., Hamamatsu, Japan) and a video tape recorder (model NV-FS65, National Co., Tokyo). For each condition, the number of RP-labeled F-actin bound to the glass surface coated with P-135-ABP was counted in 20 randomly selected areas of 2,000 μm^2.

Observation of the Mixture of RP-Labeled F-Actin and P-135-ABP

RP-labeled F-actin was mixed with P-135-ABP in the presence of both CaM and $CaCl_2$, or $CaCl_2$ alone, under the same conditions as that in the cosedimentation assay. The final concentrations of RP-labeled F-actin, P-135-ABP, CaM, and $CaCl_2$ were 4.2 μg/mL, 4.6 μg/mL, 5.5 μM, and 0.5 mM, respectively. To examine the influence of W-5 and W-7, these chemicals were added at final concentrations of 10 and 30 μM, respectively, to the mixture of RP-labeled F-actin, P-135-ABP, CaM, and $CaCl_2$. The mixture was

observed using the fluorescence microscope-video system described above.

Other Methods

SDS-PAGE was performed according to Laemmli (1970). Gels were stained with Coomassie Brilliant Blue. Protein concentrations were determined by the method of Bradford (1976). F-actin was prepared from acetone powder of chicken breast muscle according to Kohama (1981).

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