Microinjection of Nonmuscle and Smooth Muscle Caldesmon into Fibroblasts and Muscle Cells

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Abstract. Caldesmon is present in a high molecular mass form in smooth muscle and predominantly in a low molecular mass form in nonmuscle cells. Their biochemical properties are very similar. To examine whether these two forms of caldesmon behave differently in cultured cells, we microinjected fluorescently labeled smooth muscle and nonmuscle caldesmons into fibroblasts. Simultaneous injection of both caldesmons into the same cells has revealed that both high and low relative molecular mass caldesmons are quickly (within 10 min) and stably (over 3 d) incorporated into the same structures of microfilaments including stress fibers and membrane ruffles, suggesting that nonmuscle cells do not distinguish nonmuscle caldesmon from smooth muscle caldesmon. The effect of calmodulin on the incorporation of caldesmon has been examined by coinjection of caldesmon with calmodulin. We have found that calmodulin retards the incorporation of caldesmon into stress fibers for a short period (10 min) but not for a longer incubation (30 min). The behavior of caldesmon in developing muscle cells was also examined because we previously observed that caldesmon disappears during myogenesis (Yamashiro, S., R. Ishikawa, and F. Matsumura. 1988. *Protoplasma Suppl.* 2: 9–21). We have found that, in contrast to its stable incorporation into stress fibers of fibroblasts, caldesmon is unable to be incorporated into thin filament structure (I-band) of differentiated muscle.

CALDESMON, first identified in smooth muscle, is a unique actin binding protein whose binding to actin is regulated by Ca^{2+}/calmodulin (24). A wide variety of nonmuscle cells have been reported to contain caldesmon, which shows lower molecular masses on SDS gels than does smooth muscle caldesmon (ranging from 70,000 to 80,000 vs. 140,000 D of smooth muscle caldesmon) (3, 8, 16, 29). In cultured cells, nonmuscle caldesmons are found to be colocalized with actin, suggesting their involvement in microfilament organization and the regulation of cell motility. Both muscle and nonmuscle caldesmon have similar properties including a rodlike molecular shape, heat stability, calmodulin regulation of actin binding, self-association through disulfide bonds, periodic localization along actin filaments, and stimulation of actin binding of tropomyosin (1–3, 8, 16, 24, 28, 29). Bryan et al. have recently revealed a structural difference between these two types of chicken caldesmon through cDNA cloning (4; Bryan, J., R. Lee, S. J. Chang, and W. G. Lim. 1989. *J. Cell Biol.* 109[No. 4, Pt. 2]:189a [Abstr.]). While NH_2-terminal (200 amino acids) and COOH-terminal (324 amino acids) regions are exactly the same in smooth muscle and nonmuscle caldesmons, nonmuscle caldesmon is missing 232 amino acids from the central region of smooth muscle caldesmon, including a characteristic domain with an eightfold repeat of a 13 amino acid sequence. The sequence data together with the studies of domain mapping by others (11, 15, 22, 25, 30) have placed the actin- and calmodulin-binding domain at the COOH-terminal end of the molecule and the myosin binding domain at the NH_2 terminus. These studies have also revealed that caldesmons have much lower molecular masses than predicted by SDS-PAGE. The calculated molecular masses for chicken smooth muscle and nonmuscle caldesmons are 87,000 and 60,000 D, respectively.

The differences in biological functions of these caldesmons caused by the structural difference are not clear. The wide occurrence of the nonmuscle type of caldesmon in nonmuscle tissue and culture cells suggests that nonmuscle caldesmon may function in a way different from smooth muscle caldesmon. In this study, we have microinjected fluorescently labeled muscle and nonmuscle caldesmons into fibroblasts in order to see whether these two types of caldesmons behave differently in vivo. We have found that both high and low relative molecular mass caldesmons are incorporated into the same structures of microfilaments despite the structural difference. In addition, because we previously observed that caldesmon disappears during myogenesis (28), we have examined whether caldesmon is stably incorporated into actin structures of muscle cells. We have observed that, unlike fibroblasts, muscle cells actively exclude caldesmon from thin filament structure.

Materials and Methods

Protein Purification

Smooth muscle caldesmon was purified from frozen bovine aorta and chicken gizzard by the method of Bretscher (1) with slight modification as

described (28). Nonmuscle caldesmon was purified from fresh bovine liver by the method described previously (28) except that the last hydroxylapatite column was omitted. COOH-terminal 40-kD fragments of chick gizzard smooth muscle caldesmon were prepared according to Fujii et al. (11). Skeletal muscle actin was purified as described previously (18). Alpha-actinin was purified from chicken gizzard as described by Feramisco and Burridge (9), and conjugated with FITC as described (23).

Conjugation of Caldesmon with Fluorescent Dyes

Caldesmons from smooth muscle and nonmuscle cells were conjugated with tetramethylrhodamine-5-(and-6)-iodoacetamide (TMRIA)[1] or 5-iodoacetamidofluorescein (IAF) (Molecular Probes Inc., Eugene, OR). Caldesmons are known to form oligomers through disulfide bonds when reducing agents are removed by dialysis. Because this will eliminate free cysteine residues from caldesmon for conjugation with the dyes, we have conjugated caldesmons with excess amounts of the dyes over the concentration of reducing agents present in caldesmon preparation. In fact we found that caldesmon was conjugated very poorly with the dyes when reducing agents were removed by dialysis.

Caldesmons (1–5 mg/ml) in 20 mM Tris-acetate buffer containing 0.1 M KCl and 0.2 mM DTT were incubated with 0.5–1 mM of either TMRIA or IAF on ice for 4 h. The reaction was terminated by the addition of 2-mercaptoethanol to a final concentration of 50 mM. The free dye was removed by using Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) as described (19). For purification of caldesmon with actin binding ability, dye-conjugated caldesmon (final concentration, 0.5 mg/ml) was incubated at room temperature for 1 h with 1 mg/ml of actin in 20 mM imidazole buffer of pH 7.0 containing 0.1 M KCl and 1 mM DTT. After centrifugation with an Airfuge (26 psi × 20 min; Beckman Instruments, Inc., Palo Alto, CA), the pellets were suspended in 50 μl of 20 mM potassium phosphate or 20 mM imidazole buffer of pH 7.0 containing 0.1 M KCl and 1 mM DTT. After heat treatment at 100°C for 10 min, dye-conjugated caldesmons (1 mg/ml) were recovered in the supernatants by centrifugation with an Airfuge, and used for microinjection or actin binding assay.

The dye/protein ratios of TMRIA-conjugated caldesmons were estimated based on a molar extinction coefficient of 47,000 at 552 nm (7), and assuming that molecular masses of nonmuscle and smooth muscle caldesmon were 60,000 and 87,000 D, respectively (4; Bryan, J., R. Lee, S. J. Chang, and W. G. Lim. 1989. *J. Cell Biol.* 109[No. 6, Pt. 2]:189a[Abstr.]).

Cell Culture

REF-52 cells (an established rat embryo cell line) and gerbil fibroma cells (CCL-146; American Type Culture Collection, Rockville, MD) were maintained in DME containing 10% newborn calf serum in an atmosphere of 5% CO_2 and 95% air at 37°C. Chick embryo myoblasts were prepared from the thigh muscle of an 11-d chick and cultured in DME supplemented with 15% horse serum and 2% of chick embryo extracts. They began to fuse at 2 d and differentiated into myotubes with sarcomere structures at ~10 d. Chick embryo fibroblasts were also maintained in DME supplemented with 15% horse serum and 2% of chick embryo extracts.

Microinjection

The technique of Graessmann and Graessmann (12) was used for microinjection of the fluorescently labeled proteins into cells. Needles were made with a Narishige needle puller and injection was performed with a Narishige micromanipulator (Narishige, Tokyo, Japan) attached to an Olympus inverted microscope (IMT-2). After injection, cells were incubated in an CO_2 incubator, fixed at a certain time (usually at 16 h) with 3.7% formaldehyde in PBS, washed with PBS, and mounted on slides with gelvatol. Fluorescence was observed with an Olympus BH-2 microscope equipped for epifluorescence and phase contrast. Fluorescent photographs were taken using Kodak T-Max P3200 films and developed with T-Max developer for an ASA rating of 3,200. Phase-contrast photographs were taken with Kodak Technical Pan films and developed with Rodinal developer. When the incorporation of fluorescent caldesmon in living cells was examined, or when fluorescence was at a low level, images were taken with a SIT video camera (Dage-MTI, Inc.), processed with an Argus 10 image processor (Phototonic, Hamamatsu City, Japan), and subsequently photographed from the monitor with 35 mm Technical Pan film (developed with Technidol liquid developer).

Binding of Dye-conjugated Caldesmon on Permeabilized Cell Models

REF-52 cells were treated on ice for 90 s with 0.03% Triton X-100 containing 0.1 M Pipes, 5 mM $MgCl_2$, 1 mM EGTA. After gentle washing with PBS containing 5 mM $MgCl_2$, permeabilized cells were incubated at 37°C for 15 min with fluorescently labeled caldesmons (0.3–0.5 mg/ml) in 20 mM imidazole buffer of pH 7.0, 0.1 M KCl, and 1 mM DTT, then washed gently with PBS containing 5 mM $MgCl_2$, fixed with 3.7% formaldehyde in PBS, and observed by an epifluorescent microscope. In some experiments, 0.4 mg/ml of calmodulin with either 0.2 mM $CaCl_2$ or 1 mM EGTA were added to fluorescently labeled caldesmons to see effects of Ca^{2+}/calmodulin on the binding of caldesmon to cell models.

Other Procedures

Immunofluorescence was performed as described (27). Protein concentrations were determined by the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. Protein concentrations of dye-conjugated caldesmons were determined as follows. Dye-conjugated caldesmon, together with intact caldesmons with known concentrations were run on SDS gels, stained with Coomassie blue, and quantified by densitometry as described (26). SDS-PAGE was performed as described (29). Actin binding activity of caldesmon was assayed as described before (29).

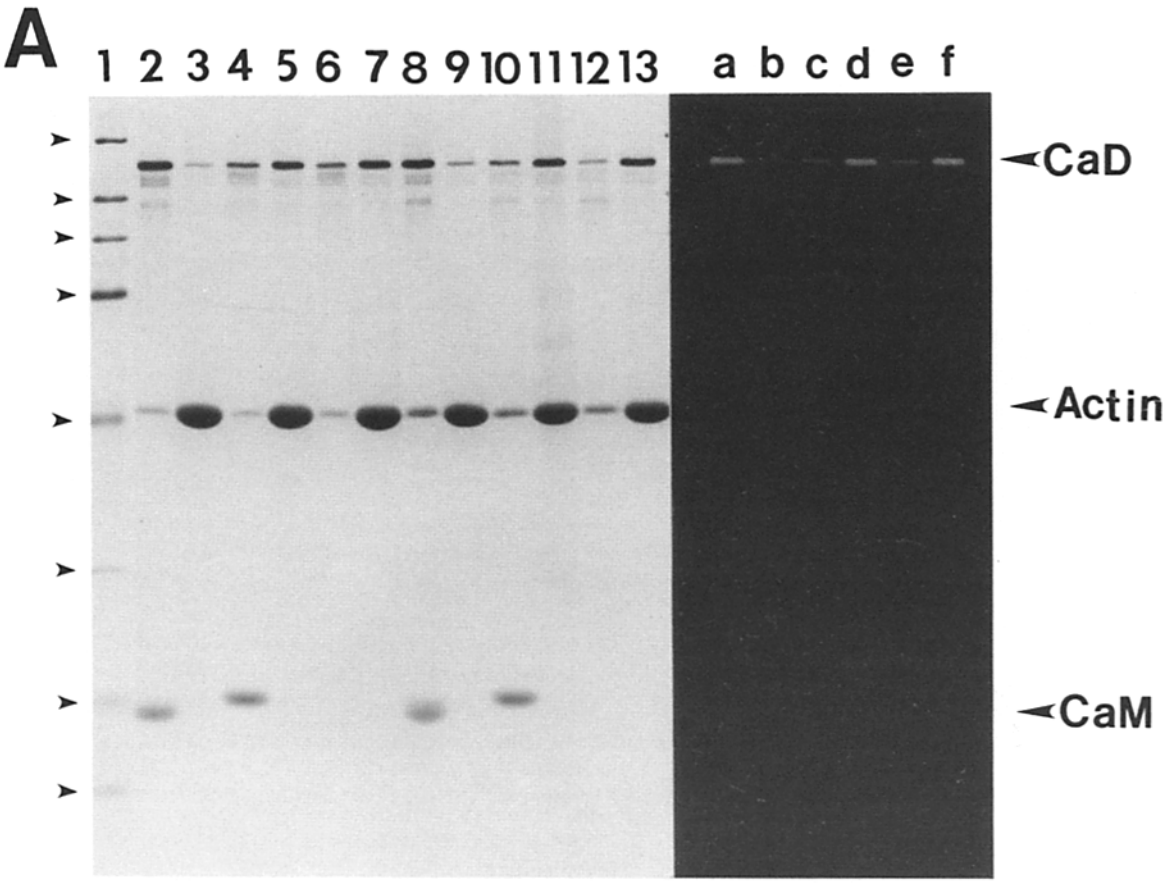
Results

Actin Binding Properties of Dye-conjugated Smooth Muscle and Nonmuscle Caldesmons

For the conjugation of smooth muscle and nonmuscle caldesmons, we initially tested two kinds of fluorescent dyes that are different in the reactivities. One is iodoacetamides, which reacts with cysteine residues of protein. The other is isothiocyanates, which reacts with ε-amino residues. Although caldesmons conjugated with either dye were observed to be incorporated into stress fibers when injected, caldesmon conjugated with the latter dyes showed much higher background and more diffuse staining than did caldesmon conjugated with the former dyes. We have thus chosen fluorescein or rhodamine iodoacetamide for this study. Dye-conjugated caldesmons were further purified by using

1. *Abbreviations used in this paper*: IAF, 5-iodoacetamidofluorescein; TMRIA, tetramethylrhodamine-5-(and-6)-iodoacetamide.

Figure 1. Actin binding of TMRIA-conjugated smooth muscle (*A*) and TMRIA-conjugated nonmuscle (*B*) caldesmons. Actin (0.5 mg/ml) was incubated for 1 h at room temperature with either 0.2 mg/ml of bovine aorta caldesmon (*A*) or 0.1 mg/ml of bovine liver caldesmon (*B*) in 20 mM imidazole buffer, 0.1 M KCl, and 0.5 mM DTT in the presence or absence of calmodulin (0.4 mg/ml). After centrifugation with an Airfuge (28 psi × 20 min), both supernatants and pellets are suspended in equal amounts of SDS sample buffer and analyzed by SDS-PAGE. Lane *1*, molecular weight markers (from *top* to *bottom*, 200,000, 116,000, 97,000, 66,000, 43,000, 31,000, 21,000, and 14,000); lanes *2–7*, actin binding of unconjugated caldesmon control; lanes *8–13* and *a–f*, actin binding of dye-conjugated caldesmon; lanes *1–13*, Coomassie blue staining; lanes *a–f*, the same gel as in lanes 8-13 photographed with ultraviolet light. Lanes *3*, *5*, *7*, *9*, *11*, *13*, *b*, *d*, and *f* are pellets; lanes *2*, *4*, *6*, *8*, *10*, *12*, *a*, *c*, and *e* are supernatants. Lanes *2*, *3*, *8*, *9*, *a*, and *b*, are in the presence of calmodulin and 0.1 mM $CaCl_2$; lanes *4*, *5*, *10*, *11*, *c* and *d*, are in the presence of calmodulin and 1 mM EGTA; lanes *5*, *6*, *12*, *13*, *e*, and *f*, are in the absence of both calmodulin and $CaCl_2$.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 a b c d e f
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Actin
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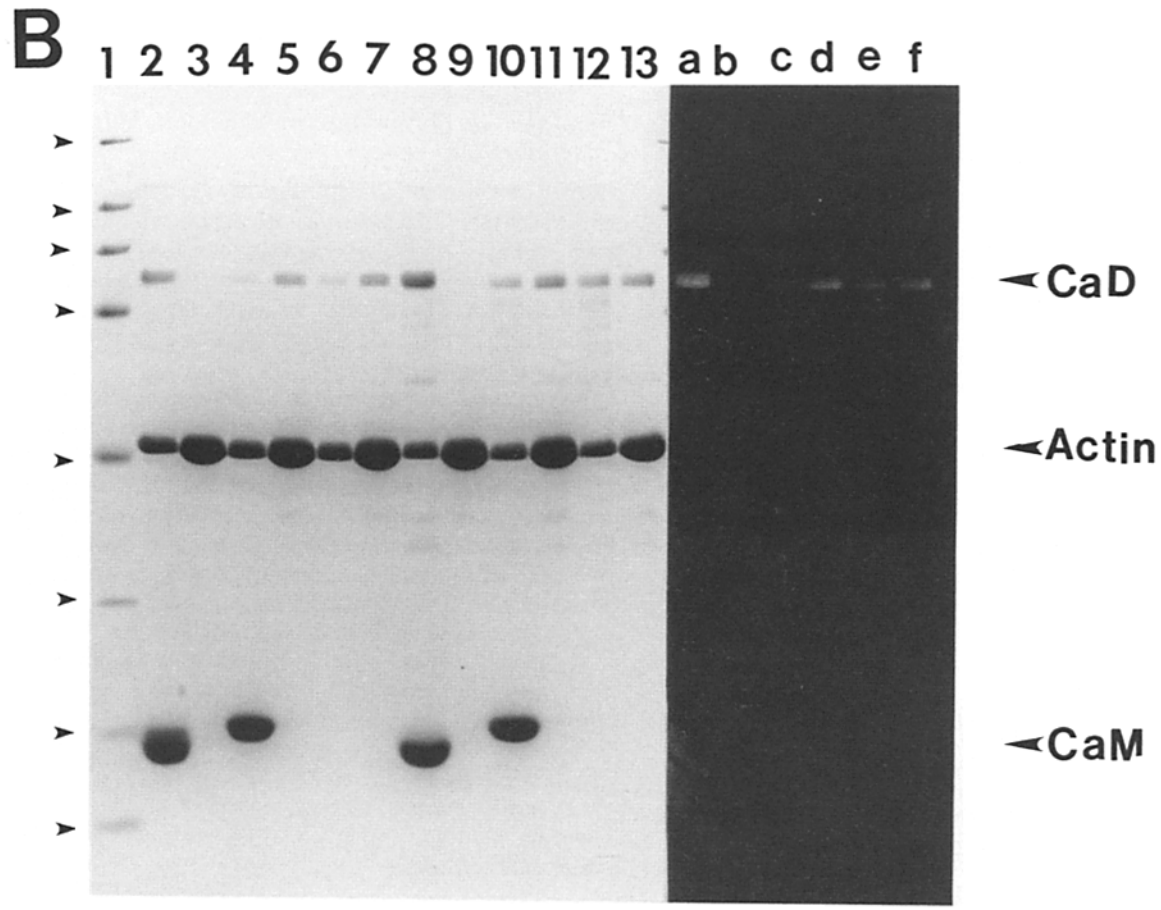
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CaM

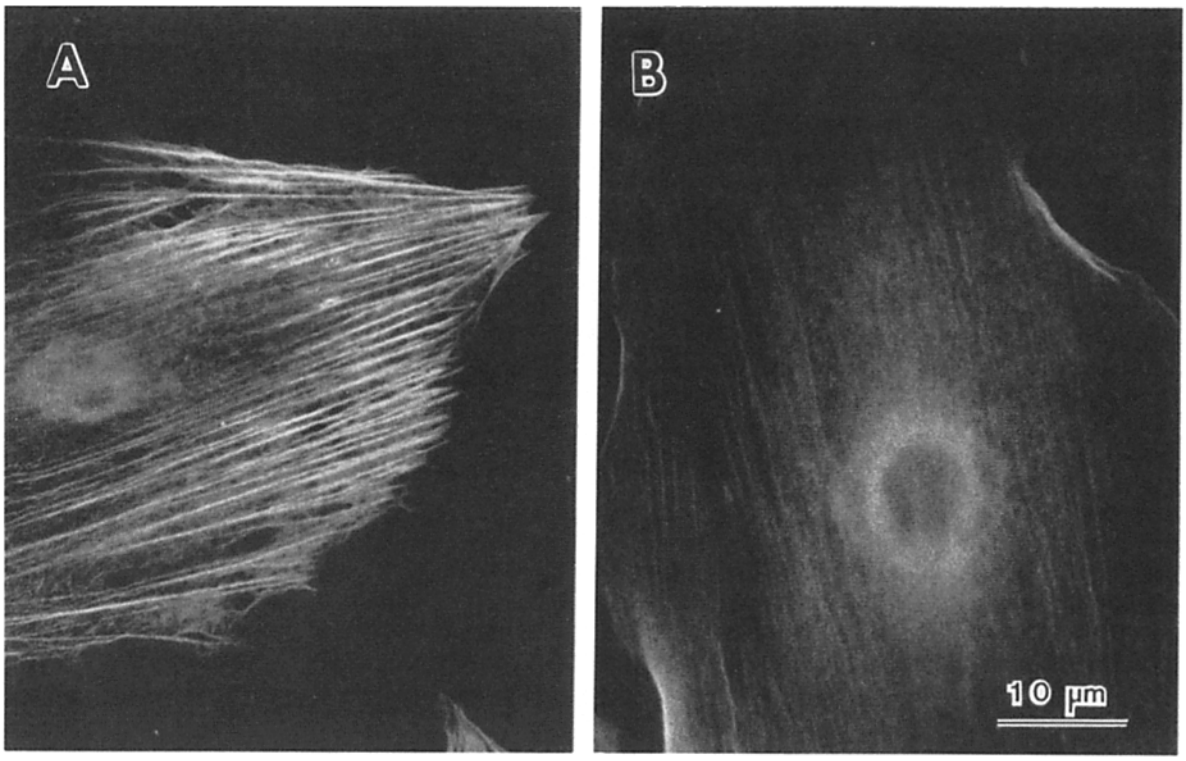


Figure 2. Binding of TMRIA-conjugated bovine liver caldesmon to cell models (REF-52 cells permeabilized with Triton X-100). (*A*) In the absence of calmodulin; (*B*) in the presence of Ca^{2+}/calmodulin.

F-actin binding ability of caldesmon as described in Materials and Methods. The molar ratios of dye (TMRIA) to the caldesmons were estimated as 0.75 for bovine aorta smooth muscle caldesmon, 0.5 for chicken gizzard caldesmon, and 0.35 for bovine liver nonmuscle caldesmon.

We have examined actin binding properties of purified dye-conjugated bovine smooth muscle and nonmuscle caldesmons. As Fig. 1, *A* and *B* show, dye-conjugated caldesmons appear to show actin binding ability similar to that of intact proteins. Furthermore, actin binding of dye-conjugated smooth muscle and nonmuscle caldesmon is regulated by Ca^{2+}/calmodulin in the same way as that of intact molecule. Chick gizzard caldesmon labeled with the same dye also shows actin binding properties similar to those of intact proteins (data not shown).

To further examine the actin binding property of dye-conjugated caldesmons, we have examined their binding to a permeabilized cell model with or without calmodulin. When calmodulin is absent, TMRIA-conjugated liver caldesmons bind to stress fibers either in the presence or absence of Ca^{2+} (Fig. 2 *A*). When calmodulin is present, the binding is regulated by Ca^{2+}/calmodulin as is the binding of dye-conjugated caldesmons to actin (Fig. 1). In the presence of Ca^{2+}, caldesmon shows very weak binding to stress fibers (Fig. 2 *B*), while caldesmon binds well to a cell model in the absence of Ca^{2+}. Similar results were obtained with dye-conjugated smooth muscle caldesmon (data not shown).

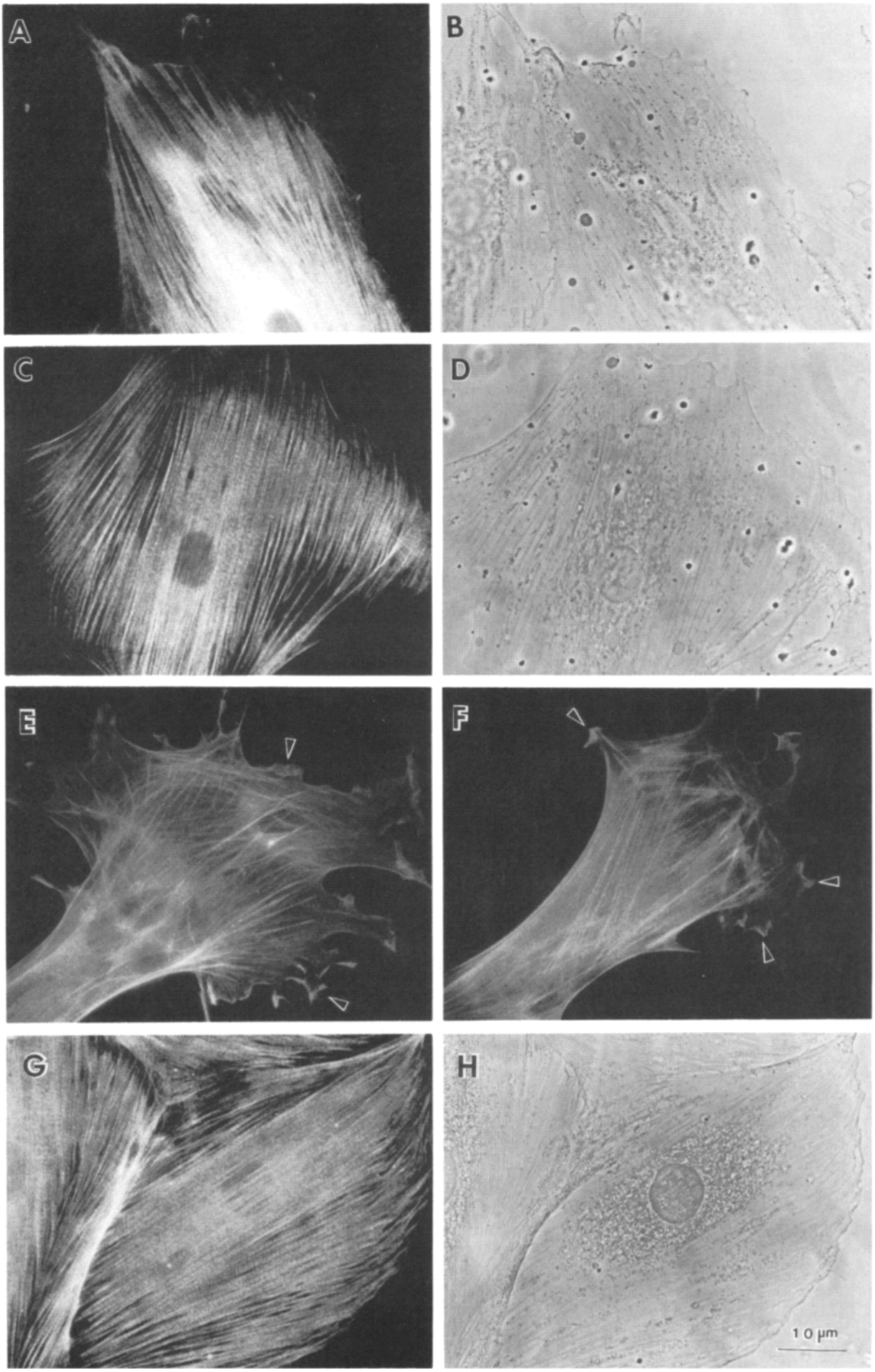
Microinjection of Nonmuscle and Smooth Muscle Caldesmons into Fibroblasts

We then microinjected them into rat fibroblasts (REF 52 cells) to see their incorporation into the microfilament structures. As Fig. 3 shows, both bovine muscle and nonmuscle caldesmons are incorporated into stress fibers periodically (*A* and *C*). These patterns of the incorporation correspond to the localization of endogenous caldesmon revealed by immunofluorescence (Fig. 3 *G*).

The injected caldesmons, regardless whether they come from nonmuscle or smooth muscle cells, are quickly and stably incorporated into stress fiber structures. These caldesmons are observed in stress fibers within 10 min after injection and are stably present for over 3 d although the intensity of fluorescence is decreased by dilution due to cell division.

We have also examined incorporation of caldesmon into membrane ruffles because caldesmon is known to be localized in membrane ruffles as well. To this end, we have microinjected caldesmon into chick embryo fibroblasts, which have more membrane ruffle structures than REF 52 cells. As Fig. 3, *E* and *F* show, both bovine smooth muscle

Figure 3. Distribution of dye-conjugated caldesmon in REF-52 cells (*A–D*) or in chick embryo fibroblasts (*E* and *F*). Injection was performed with 1 mg/ml of TMRIA-conjugated bovine aorta caldesmon (*A*, *B*, and *E*), or 1 mg/ml of TMRIA-conjugated bovine liver caldesmon (*C*, *D*, and *F*). Arrows show incorporation of caldesmon into membrane ruffles. (*G* and *H*) Immunofluorescence of REF-52 cells stained with anti-caldesmon monoclonal antibody (SM12). (*A*, *C*, *E*, *F*, and *G*) Fluorescent images; (*B*, *D*, and *H*) phase-contrast images.


A
B
C
D
E
F
G
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10 μm

and nonmuscle caldesmons are incorporated into membrane ruffles as well as stress fibers. Similar incorporation to microfilament structures is also seen with injection of dye-conjugated chick gizzard caldesmon (data not shown).

We have observed distribution of injected caldesmon in living cells because Cooper et al. (6) have shown that fixation changes the distribution of microinjected gelsolin from diffuse to fibrillar patterns. As Fig. 4 shows, injected caldesmon was observed to be incorporated into stress fibers of living REF 52 cells (Fig. 4 *A*), as well as membrane ruffles of living gerbil fibroma cells (Fig. 4 *B*). Although the background is somewhat higher than that of fixed images, the incorporation of caldesmon in living cells appears very similar to the pattern of distribution shown by fixed cells.

Simultaneous Injection of Smooth and Nonmuscle Caldesmons into the Same Cells

Because the incorporation of smooth muscle caldesmon appears to be similar to that of nonmuscle caldesmon, we asked whether these two types of caldesmons are incorporated into the same structures of microfilaments. To distinguish smooth and nonmuscle caldesmon in the same cells, these two caldesmons were separately labeled with two different dyes (TMRIA and IAF), mixed to make a equal molar concentration (6 μM of smooth muscle and 6 μM of nonmuscle caldesmon), and injected into REF 52 or gerbil fibroma cells. As Fig. 5, *A*, *B*, and *C* show, coinjected smooth muscle and nonmuscle caldesmons show indistinguishable incorporation into stress fibers in the same REF 52 cells. The injection into gerbil fibroma cells (Fig. 5, *D*, *E*, and *F*) has clearly demonstrated that two species of caldesmon are equally distributed into two different microfilament structures, i.e., stress fibers and membrane ruffles. The fluorescent structures derived from smooth muscle caldesmon are found to correspond exactly to those derived from nonmuscle caldesmon.

To examine whether these two species of caldesmon compete each other in the incorporation into stress fibers, we microinjected 5 μM (final concentration) of dye-conjugated bovine liver or chick smooth muscle caldesmon that had been mixed with varying concentrations (0–80 μM) of either smooth muscle or liver unconjugated caldesmon. If, for example, smooth muscle caldesmon bind to stress fibers better than nonmuscle species, smooth muscle unconjugated caldesmon would compete fluorescent nonmuscle caldesmon out of stress fibers more effectively than nonmuscle unconjugated caldesmon does. We have found, however, that both species of caldesmon give the same effects; fluorescently conjugated liver caldesmon on stress fibers was equally diluted out as the concentrations of unconjugated caldes-

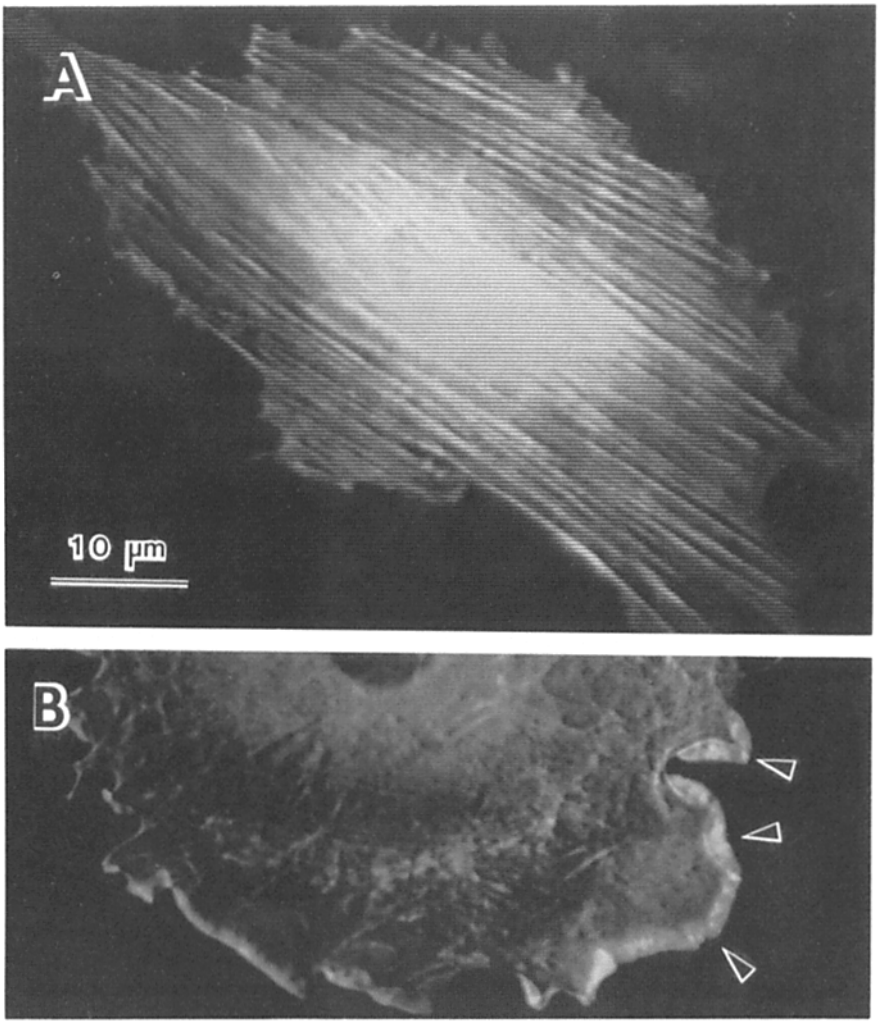

Figure 4. Distribution of TMRIA-conjugated caldesmon (1 mg/ml) in living REF-52 cells (*A*) and gerbil fibroma cells (*B*). The incorporation into stress fibers (*A*) and into membrane ruffles (*B*, *arrows*) is similar to those observed in fixed cells (Figs. 3 and 5).

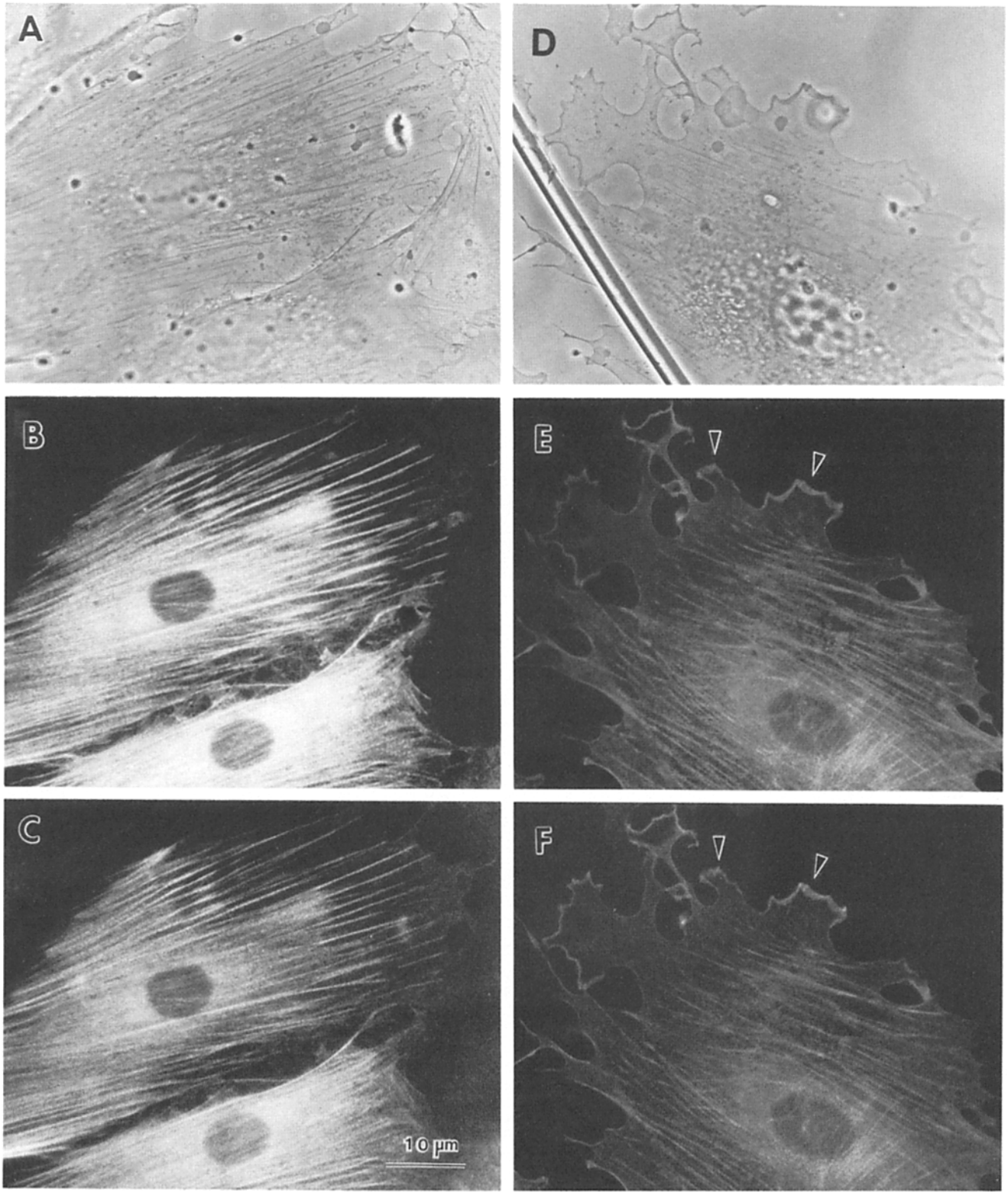

Figure 5. Simultaneous injection of smooth muscle and nonmuscle caldesmon into the same cells. REF-52 cells (*A–C*) or gerbil fibroma cells (*D–F*) were injected with a mixture of TMRIA-conjugated bovine aorta caldesmon (*B* and *E*) and IAF-conjugated bovine liver caldesmon (*C* and *F*). (*A* and *D*) Phase contrast; (*B, C, E,* and *F*) fluorescent image. Arrows show membrane ruffles. Note that two species of caldesmon are incorporated into the same structures of microfilaments. Injection with a mixture of IAF-conjugated smooth muscle and TMRIA-conjugated nonmuscle caldesmons gave similar results.

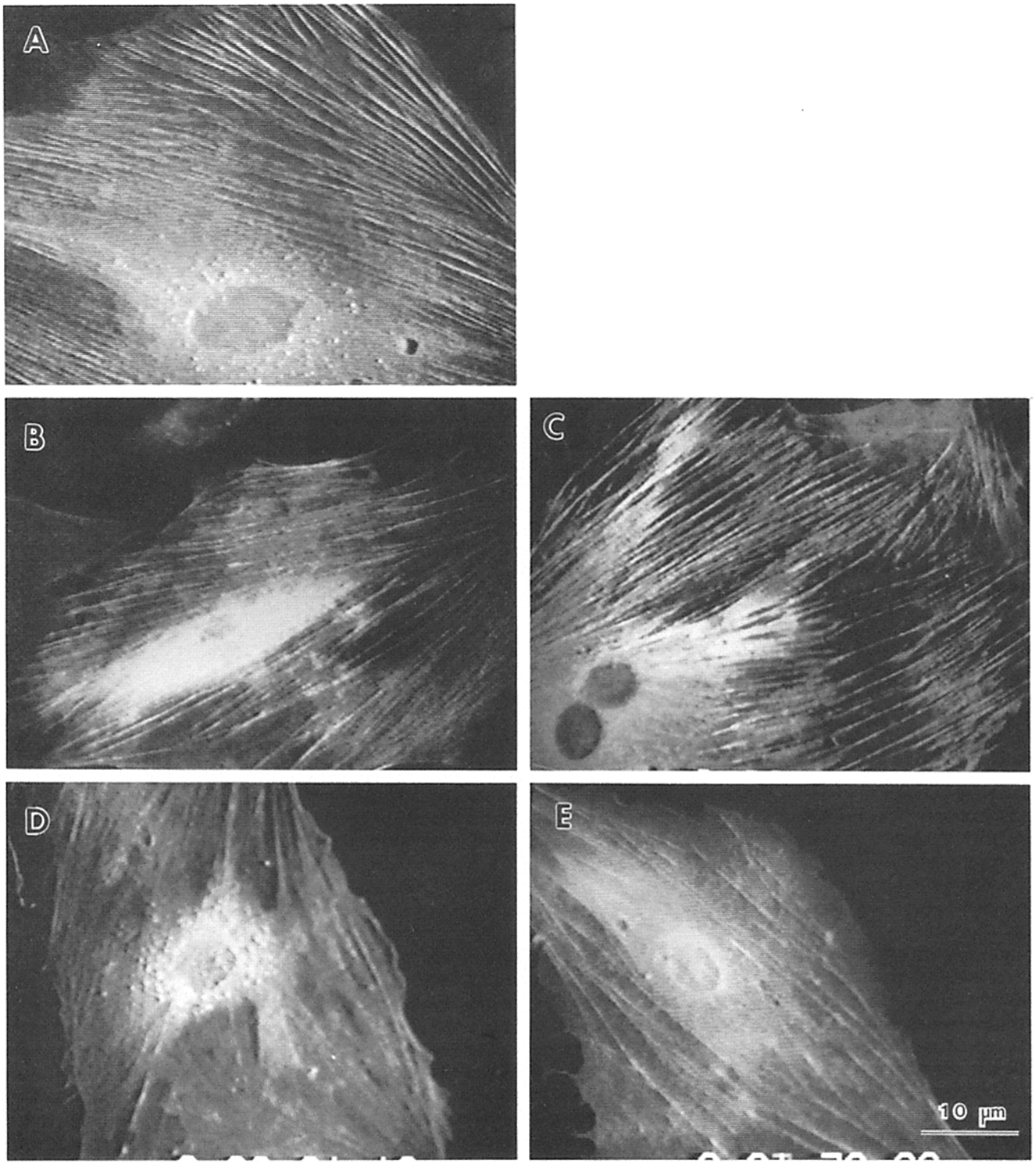

Figure 6. Smooth muscle and nonmuscle caldesmons do not compete each other in the incorporation into stress fibers. TMRIA-conjugated bovine liver caldesmon (final concentration, 5 μM) was first mixed with buffer alone (*A*), or varying concentrations of unconjugated smooth muscle (*B*, 40 μM; *D*, 80 μM) or nonmuscle (*C*, 40 μM; *E*, 80 μM) caldesmon, and injected into REF-52 cells. Fluorescent images were taken with a SIT video camera as described in Materials and Methods. Note that both species of caldesmon equally decrease the fluorescence of bovine liver caldesmon incorporated into stress fibers, suggesting that cells do not distinguish between these two species of caldesmon.

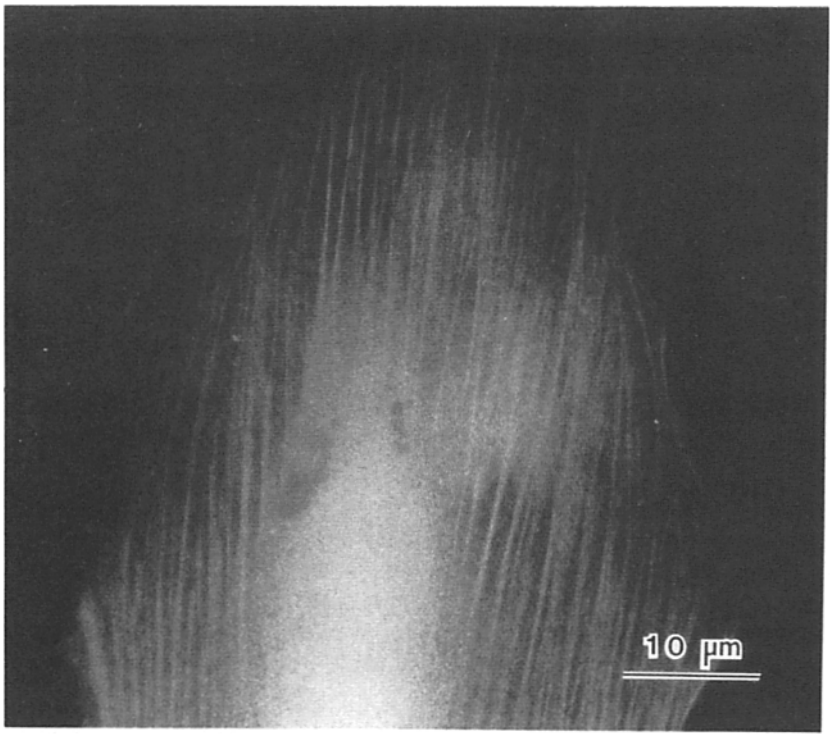

Figure 7. Injection of COOH-terminal 40-kD chymotryptic fragments (0.3 mg/ml) into REF-52 cells.

mon, regardless of species of caldesmon, were increased (Fig. 6). These results suggest that nonmuscle and smooth muscle caldesmons appear to have an indistinguishable ability of the incorporation into stress fibers.

Injection of COOH-Terminal Fragments into Fibroblasts

Because both high and low relative molecular mass caldesmons are incorporated into the same structures of stress fibers despite the structural differences, we asked whether even small fragments of caldesmon with the functional actin binding domain are incorporated into stress fibers. Studies on domain structure of gizzard caldesmon have revealed that the actin binding and calmodulin binding domain is localized in COOH-terminal 40-kD fragments (11, 15, 22, 25, 30). The protein sequence derived from cDNA cloning has revealed that one of the two cysteine residues of chick gizzard caldesmon are localized in the middle of the COOH-terminal fragments, allowing us to conjugate COOH-terminal fragments with TMRIA to see their incorporation. Dye-conjugated COOH-terminal fragments was also purified by cosedimentation with F-actin and injected into REF-52 cells. As Fig. 7 shows, COOH-terminal fragments are incorporated into stress fibers. Their incorporation in some places appears periodic as seen in the case of the injection of intact molecule.

Effects of Coinjection with Calmodulin

Effects of coinjection of caldesmon with Ca^{2+}/calmodulin have been examined because actin binding of caldesmon is inhibited by Ca^{2+}/calmodulin in vitro. To ensure the complex formation, dye-conjugated caldesmon (1 mg/ml) is incubated with 20 times molar excess (final concentration, 4 mg/ml) of calmodulin over caldesmon in the presence of 0.1 mM of $CaCl_2$ and then injected into fibroblasts. As a control, caldesmon in 0.1 mM $CaCl_2$ but without calmodulin was injected. As Fig. 8, *A* and *B* show, coinjection with calmodulin retards the incorporation of caldesmon for a short time after injection. While control caldesmon without calmodulin (Fig. 8 *B*) is quickly incorporated into stress fibers within 10 min, most of caldesmon complexed with calmodulin (*A*) is diffusely present at the same time. The effect of calmodulin, however, disappears as the cells are incubated for longer than 30 min. As Fig. 8, *C* and *D* show, the incorporation of caldesmon coinjected with calmodulin (*C*) appears indistinguishable from that (*D*) of control.

Microinjection of Dye-conjugated Caldesmon into Muscle Cells

Our results indicate that caldesmon is a stable protein that remains bound to stress fibers over 3 d. On the other hand, we previously observed that nonmuscle caldesmon present in myoblasts disappears during fusion of myoblasts into myotubes (28). We have thus examined whether smooth muscle or nonmuscle caldesmon is stably incorporated into actin microfilaments of developing muscle cells. Chick myoblasts were used for this purpose because these cells are easily differentiated into myotubes with sarcomere structures.

As Fig. 9, *A* and *B* show, caldesmon is unexpectedly found in A-bands but not in I-bands when it is injected into myotubes with sarcomere structures. This A-band incorporation (Fig. 9 *C*) is quite unstable and quickly disappears after a 16-h incubation (Fig. 9 *E*). As a control, we have coinjected FITC-labeled α-actinin into myotubes. As Fig. 9, *D* and *F* show, α-actinin remains stably incorporated into Z-bands after 16 h. Similar results were obtained with the injection of smooth muscle caldesmon.

We have also injected caldesmon into myotubes that have

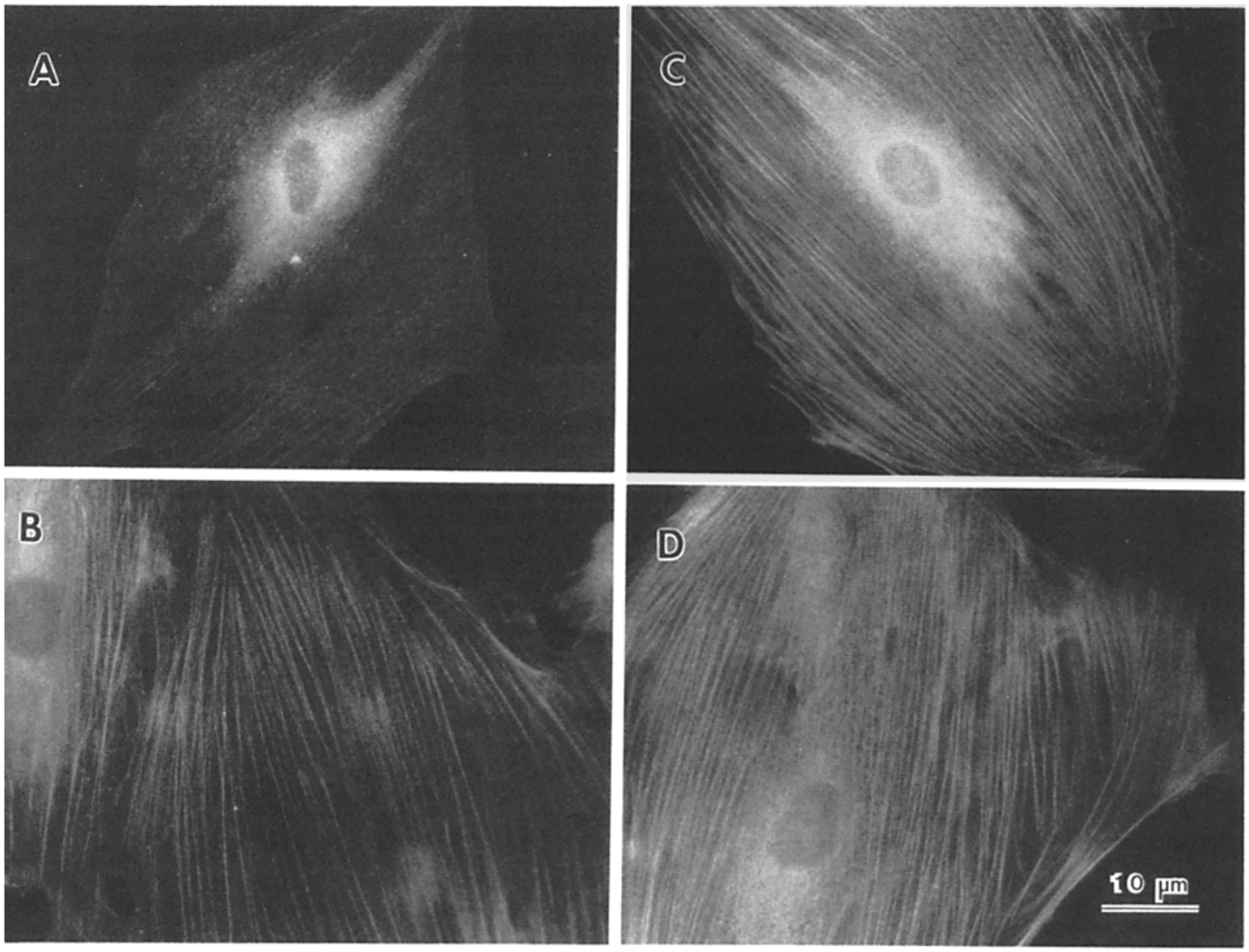

Figure 8. Effects of coinjection with calmodulin on the incorporation of caldesmon. *A* and *C* are fluorescent images at 10 and 30 min, respectively, after coinjection of TMRIA-conjugated caldesmon with calmodulin in the presence of 0.1 mM $CaCl_2$. *B* and *D* are fluorescent images at 10 and 30 min, respectively, after injection with TMRIA-conjugated caldesmon in 0.1 mM $CaCl_2$ without calmodulin.

not developed sarcomere structures. While caldesmon is found to be incorporated into microfilament bundles of myotubes, the incorporation is again very unstable and disappears after a 16-h incubation (data not shown). These results suggest that, unlike fibroblasts, muscle cells actively exclude caldesmon from their actomyosin system.

Discussion

Nonmuscle Cells Do Not Distinguish Smooth Muscle and Nonmuscle Caldesmons

Smooth muscle caldesmon has additional central regions (27 kD) including a repeated domain while both nonmuscle and smooth muscle share NH_2-terminal and COOH-terminal portions (4; Bryan, J., R. Lee, S. J. Chang, and W. G. Lim. 1989. *J. Cell Biol.* 109[No. 4, Pt. 2]:189a[Abstr.]). Our results indicate that the central domain does not inhibit the incorporation of caldesmon into actin structures of nonmuscle cells. Both smooth muscle and nonmuscle caldesmons are quickly and stably incorporated into stress fibers as well as membrane ruffles (Fig. 5). The incorporation is apparently normal because it is indistinguishable from the localization of endogenous caldesmon (Fig. 3). Furthermore, one species of caldesmon does not appear to compete the other out from stress fibers (Fig. 6). These results indicate that nonmuscle cells do not distinguish smooth muscle and nonmuscle caldesmons in terms of the incorporation into microfilaments. This notion is extended by the observation that a 40-kD actin and calmodulin binding proteolytic fragment is incorporated into stress fibers (Fig. 7), suggesting that caldesmon incorporation needs only the actin-binding domain.

The introduction of smooth muscle caldesmon at a concentration comparable to the endogenous nonmuscle caldesmon does not appear to affect the organization of the microfilament system in fibroblasts. The highest concentration of smooth muscle caldesmon used for microinjection was 7 mg/ml. Microinjection usually introduces $\sim 10^{-13}$ liter of solution into most fibroblasts (10), which allows for the introduction of 4×10^6 molecules per cell. Because ~0.03–0.08% of the cellular protein is nonmuscle caldesmon (Ishikawa, R., and F. Matsumura, unpublished results), each cell contains $\sim 2\text{–}6 \times 10^6$ molecules of caldesmon per

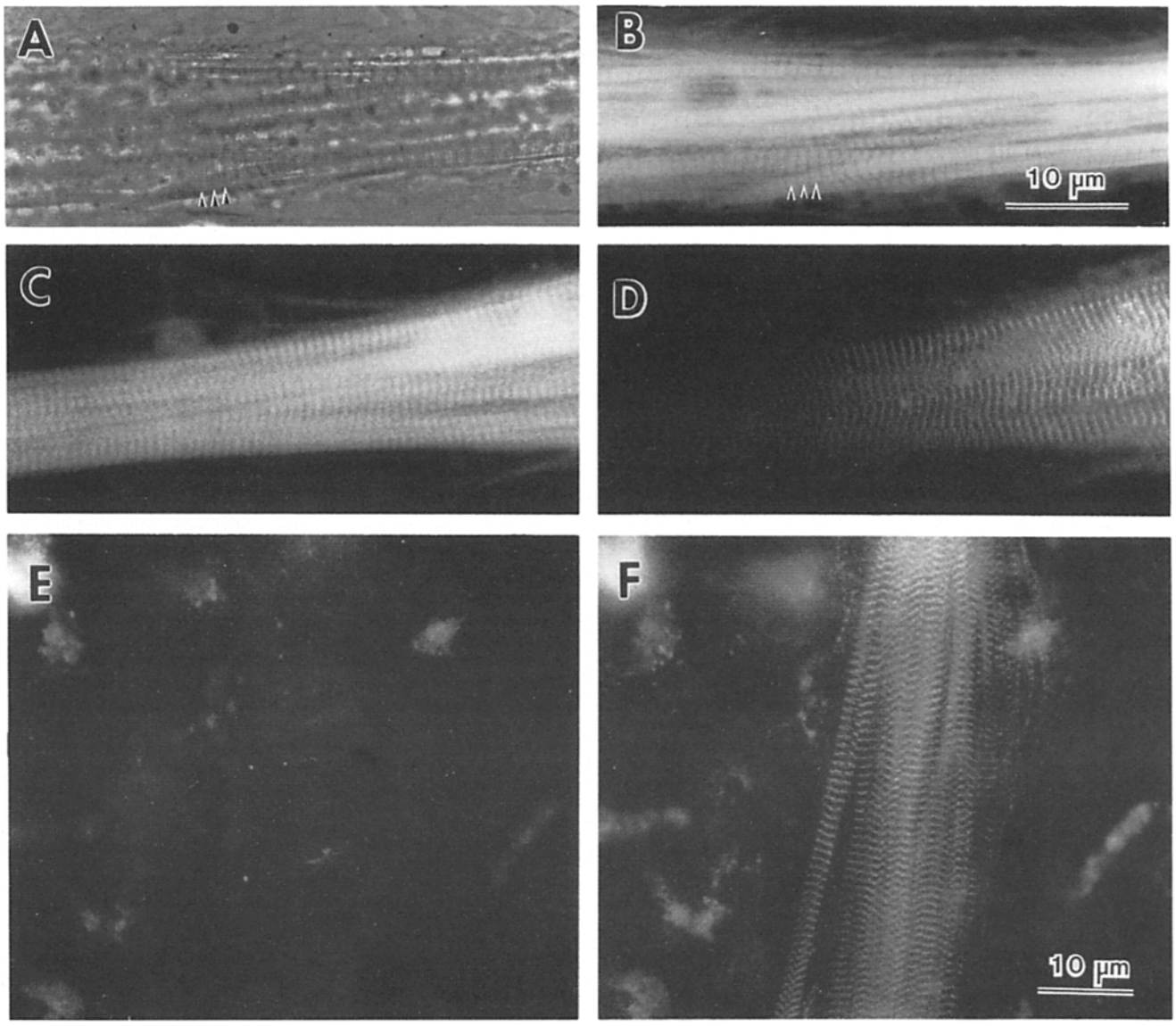

Figure 9. Injection of TMRIA-conjugated bovine liver caldesmon (1.0 mg/ml) into chick myotubes. (*A*) Phase contrast; (*B*) fluorescent image. Arrowheads indicate A-bands. In *C–F*, FITC-conjugated α-actinin (2 mg/ml) was coinjected as a control. (*C* and *D*) 2 h after injection; (*E* and *F*) 16 h after injection; (*C* and *E*) fluorescent images of TMRIA-conjugated caldesmon; (*D* and *F*) images of FITC-conjugated α-actinin.

cell assuming that a fibroblast contains ~1 ng of protein. The injected smooth muscle caldesmon thus roughly corresponds to the concentration of the endogenous caldesmon, but the injected caldesmon does not have any observable effects on the organization of microfilaments.

The incorporation of caldesmon into developing muscle cells is very different from its incorporation into fibroblasts. When caldesmon is injected into myotubes with sarcomere structures, it is unexpectedly incorporated into the A-band, rather than the I-band, just for a short time. On overnight incubation, caldesmon is excluded from muscle cells. This A-band incorporation is probably due to the direct binding of caldesmon to myosin (13, 14). These results are consistent with our previous observation that caldesmon disappears during fusion of myoblasts into myotubes but inconsistent with the report describing the presence of caldesmon in muscle cells (21). Further, these results suggest that thin filaments of developing muscle cells have some structure that inhibits the binding of caldesmon. At present, we do not know what inhibits the actin binding of caldesmon. One candidate for such structure may be troponin/tropomyosin system. However, we could not observe competitive inhibition of actin binding of caldesmon by tropomyosin/troponin in vitro (data not shown).

Effects of the Modification of Cysteine Residues on the Actin Binding

The modification of thiol residues of chicken smooth muscle caldesmon or its 40-kD chymotryptic fragments with either fluorescein or rhodamin iodoacetamides does not appear to alter actin-binding properties of these proteins, nor to

change regulation of their actin binding by Ca^{2+}/calmodulin. This is consistent with the domain structure of the 40-kD fragments. According to the domain mapping studies (4, 22), a cysteine residue is located approximately at the middle of the 40-kD fragments, and COOH-terminal 25-kD fragments generated by chemical cleavage at the cysteine residue still retain actin and calmodulin binding properties.

Effects of Calmodulin

The coinjection of Ca^{2+}/calmodulin with caldesmon inhibits initial incorporation of caldesmon for 10 min. This retardation may represent the time period for the dissociation of a ternary complex of Ca^{2+}/calmodulin/caldesmon inside cells. The level of endogenous calmodulin in CHO cells is reported as 150 ng/10^6 cells (20). Assuming that REF 52 cells have the same level of calmodulin, the amount of calmodulin injected would be three to six times higher than the concentration of endogenous calmodulin, and this level of calmodulin would be high enough for the dissociation of both endogenous and exogenous caldesmons from actin filaments if calcium is present. This estimation may thus suggest that calcium concentration inside cells may be quickly dropped below the micromolar level. Other possibilities for this short-term effect by calmodulin are that calmodulin may be consumed for other cellular functions, or calmodulin may not function in the dissociation of caldesmon in vivo. Lehman, for example, questioned the regulatory role of calmodulin for the dissociation of caldesmon because he has observed that content of smooth muscle caldesmon is not changed when native thin filaments are prepared in either Ca^{2+}-free or Ca^{2+}-containing medium in the presence of endogenous calmodulin (17). Further studies are needed for the elucidation of the biological significance of calmodulin regulation of actin–caldesmon interactions.

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