# Identification and Localization of Immunoreactive Forms of Caldesmon in Smooth and Nonmuscle Cells: A Comparison with the Distributions of Tropomyosin and α-Actinin

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ABSTRACT Caldesmon is an F-actin cross-linking protein of chicken gizzard smooth muscle whose F-actin binding activity can be regulated in vitro by $Ca^{2+}$-calmodulin (Sobue, K., Y. Muramoto, M. Fujita, and S. Kakiuchi, 1981, *Proc. Natl. Acad. Sci. USA*, 78:5652–5655). It is a rod-shaped, heat-stable, F-actin bundling protein and is the most abundant F-actin cross-linking protein of chicken gizzard smooth muscle presently known (Bretscher, A., 1984, *J. Biol. Chem.*, 259:12873–12880). We report the use of polyclonal antibodies to caldesmon to investigate its distribution and localization in other cells. Using immune blotting procedures, we have detected immunoreactive, heat-stable forms of caldesmon in cultured cells having either approximately the same apparent polypeptide molecular weight as gizzard caldesmon (120,000–140,000) or a substantially lower molecular weight (71,000–77,000). Through use of affinity-purified antibodies in indirect immunofluorescence microscopy, we have localized the immunoreactive forms to the terminal web of the brush border of intestinal epithelial cells and to the stress fibers and ruffling membranes of cultured cells. At the light microscope level caldesmon is distributed in a periodic fashion along stress fibers that is coincident with the distribution of tropomyosin and complementary to the distribution of α-actinin.

Of the three major filament systems that make up the cytoskeleton of eucaryotic cells, the microfilaments have been most clearly implicated in the determination of cellular shape and in many cellular motile events. Associated with the F-actin of microfilaments are proteins responsible for organizing the filaments into functional arrays (for review see references 1–3). In addition to myosin, its regulatory proteins, and tropomyosin, mammalian cells contain a number of distinct classes of these microfilament-associated proteins. One class of these proteins are those that gel F-actin, such as filamin (actin binding protein), that are capable of cross-linking random networks of actin filaments (4, 5). A second class is the F-actin bundling proteins, such as α-actinin, fimbrin, and villin, that, under appropriate conditions, cross-link actin filaments into parallel bundles of filaments (6–9). A third class is the F-actin capping and/or severing proteins, such as gelsolin, villin, and related proteins that can regulate the lengths of actin filaments (8–15).

The activity of various members of these classes of microfilament-associated proteins can be regulated, at least in vitro, by physiological concentrations of $Ca^{2+}$ ions. The mechanism of $Ca^{2+}$ regulation has been found to be either direct or indirect. Indirect regulation has been shown to involve the ubiquitous $Ca^{2+}$-binding protein calmodulin (for review see reference 16). An example is the activation of myosin light chain kinase activity by $Ca^{2+}$-calmodulin, which in turn regulates the activity of myosin (17). A number of examples of direct $Ca^{2+}$-regulation exist: for example, the cross-linking activity of nonmuscle α-actinin is abolished by the presence of $>10^{-6}$ M free $Ca^{2+}$ (18), and the activation of the severing activity of gelsolin is mediated by $Ca^{2+}$ (10). The level of $Ca^{2+}$ can also switch a protein from one functional class to another, such as the conversion of villin at low levels of $Ca^{2+}$ from an F-actin bundling protein to an F-actin severing protein at $>10^{-6}$ M free $Ca^{2+}$ (8).

Sobue et al. (19) recently reported that chicken gizzard smooth muscle contains a protein whose F-actin binding and cross-linking activity can be inhibited by $Ca^{2+}$-calmodulin. This protein, which has an apparent polypeptide molecular weight of about 140,000, binds calmodulin in a $Ca^{2+}$-depend-



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ent manner and was named "caldesmon" to reflect its $Ca^{2+}$-calmodulin regulated activity. We independently found that smooth muscle contains polypeptides with apparent molecular weights of 135,000 and 140,000 that remain soluble after heat treatment at 90°C. Making use of this unusual property, we developed a rapid, high-yield method for the purification of these polypeptides (20). Characterization of the purified material showed that it was caldesmon, with no apparent loss of activity due to the heat treatment. Our characterization has shown that caldesmon is a rod-shaped protein and belongs to the F-actin bundling class of proteins. In addition, estimates based on gel scans of the total protein profile of chicken gizzard smooth muscle indicated that caldesmon is the most abundant cross-linking protein presently known in this tissue (20). It is therefore important to establish the tissue distribution of caldesmon and its subcellular localization to help elucidate its role in contractile and other microfilamentous structures. A survey of contractile tissues revealed caldesmon cross-reacting proteins in bovine aorta and uterus, and in human platelets (21). Additionally, a preliminary report describing the presence and localization of caldesmon immunoreactive species in cultured cells has recently appeared (22).

In this report, we identify immunoreactive forms of caldesmon in smooth muscle and nonmuscle cells and show that, like smooth muscle caldesmon, they are heat stable. We find considerable heterogeneity in the caldesmon species in cultured cells, a situation more complex than that indicated by Owada et al. (22). We also localize the immunoreactive species in several cell types and explore the relationship between the distribution of caldesmon immunoreactive species and other microfilament-associated proteins of higher cells. These results indicate that caldesmon may be a component of a contractile unit composed of at least actin, myosin, caldesmon, tropomyosin, and myosin light chain kinase.

## MATERIALS AND METHODS

*Preparation of Antibodies:* Caldesmon was prepared as described (20). The purified protein was subjected to preparative SDS gel electrophoresis, the bands were excised and recovered by electrophoretic elution (24). Antibodies were elicited in rabbits and the caldesmon-specific antibodies purified by affinity chromatography on immobilized caldesmon (24). Preparations of affinity-purified antibodies from two different rabbit sera were prepared. Affinity-purified antibodies to bovine brain tropomyosin and chicken gizzard $\alpha$-actinin have been described (25).

*Immune Blotting and Indirect Immunofluorescence Microscopy:* The following cultured cells were used in this study: mouse fibroblasts (3T3 cells), rat mammary cells (RMC cells), normal rat kidney cells (NRK cells), chicken embryo fibroblasts, a smooth muscle-like cell line from mouse brain ($BC_3H1$ cells) and a rat aorta smooth muscle cell line (A7R5 cells). Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 2.5 $\mu$g/ml fungizone, except for $BC_3H1$ cells that were grown in medium supplemented with 20% fetal calf serum.

For immune blotting experiments, cells were grown to confluence, unless otherwise described, and 1–2 drops of the protease inhibitor diisopropylfluorophosphate was added per plate. After 5 min, the cells were washed twice with culture medium without serum, then twice with phosphate-buffered saline (PBS) and scraped into a high salt extraction buffer (0.3 M KCl, 0.5 mM $MgCl_2$, 1.0 mM EDTA, 50 mM Imidazole-HCl [pH 6.9]) and either precipitated with 10% trichloroacetic acid and prepared for gel electrophoresis (for total proteins) or boiled for 5 min. Boiled samples were centrifuged at 50,000 *g* for 30 min and the pelleted and supernatant fractions prepared for gel electrophoresis. In some cases, protease inhibitors (4 $\mu$g/ml leupeptin, 5 mM iodoacetate, 5 $\mu$g/ml pepstatin, 5 $\mu$g/ml aprotinin, 100 $\mu$g/ml phenylmethylsulfonylfluoride, 130 $\mu$g/ml benzamidine, 1 mM *o*-phenanthroline, 20 $\mu$g/ml soybean trypsin inhibitor, and 5 $\mu$g/ml diethyl pyrocarbonate) were included in the preparation of samples to examine the effects of inhibiting proteases.

Samples of proteins from cultured cells were subjected to gel electrophoresis and immune blotting, avoiding the use of the non-ionic detergent Nonidet P-40, essentially as described (26).

For immunofluorescence microscopy, cells were grown on coverslips, fixed for 5 min in 3.7% formaldehyde in PBS, extracted in −20°C methanol for 5 min and rehydrated in PBS. Primary antibodies were used at 20–50 $\mu$g/ml and incubated with the extracted cells for 30 min at room temperature in a humid chamber. The coverslips were then washed in PBS, then washed in PBS adjusted to pH 9.0 with 1 M Tris base, and then again in PBS. After these washes, the coverslips were incubated with a 1:20 dilution of a commercial (Miles-Yeda, Israel) fluorescein-labeled goat anti-rabbit IgG, washed as before and mounted on glass slides. Frozen sections were prepared and processed as described (27). Cells were viewed in a Zeiss Universal microscope fitted with epifluorescence optics and appropriate filters for fluorescein fluorescence and photographed on Kodak Tri-X film.

## RESULTS

### *Antibodies to Smooth Muscle Caldesmon*

Chicken gizzard smooth muscle caldesmon purified by our new procedure (20) appears homogeneous as determined by SDS PAGE (Fig. 1 *B*). However, to ensure the purity of the

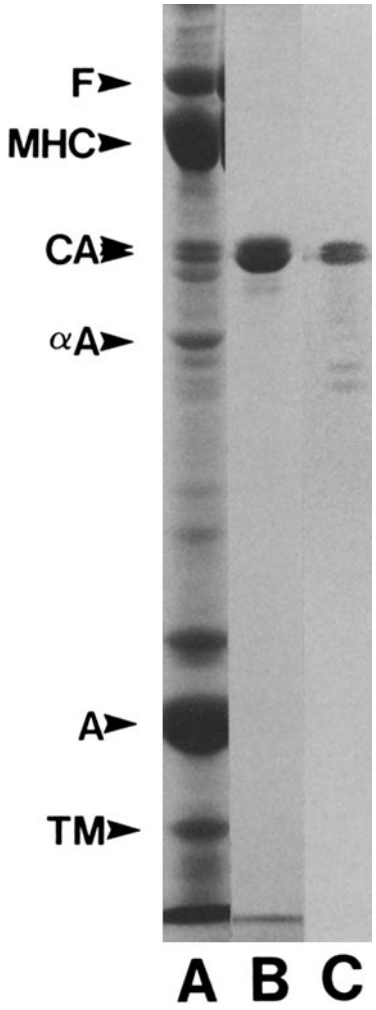


FIGURE 1 Specificity of the caldesmon antibody. 7.5% SDS PAGE of (*A*) the total proteins of chicken gizzard smooth muscle and (*B*) purified caldesmon. To test the specificity of the caldesmon antibody, we transferred the proteins shown in *A* to nitrocellulose and processed them to detect caldesmon immunoreactive species with an affinity-purified antibody (*C*). Some major smooth muscle polypeptides are indicated. *F*, filamin; *MHC*, myosin heavy chain; *CA*, caldesmon polypeptides; *αA*, α-actinin; *A*, actin; *TM*, the lower molecular weight subunit of tropomyosin.

protein used for immunization, the purified protein was subjected to preparative SDS PAGE, the 135,000- and 140,000-mol-wt caldesmon polypeptides were excised, and the protein was eluted and prepared for immunization. Two rabbits were immunized with different preparations of caldesmon and both responded by producing antibodies to caldesmon. Double diffusion analysis with purified caldesmon gave a single precipitin line (Fig. 2). Immune blots of total gizzard proteins probed with either antiserum or antigen/affinity-purified antibodies, detected the caldesmon polypeptides (Fig. 1*C*). In addition, a few minor immunoreactive species were seen that are probably degradation products, as caldesmon is very susceptible to proteolysis in tissue homogenates (20); these immunoreactive species co-migrate with characteristic degradation products that appear in preparations of purified caldesmon after storage for a week or more at 4°C.

Affinity-purified antibodies to caldesmon were used to explore whether immunologically related polypeptides are present in other cells. Fig. 3 shows the immunoreactive forms detected in immune blots of the total proteins from cultured cells. Two major classes of caldesmon immunoreactive species were found for each sample tested: one or two polypeptides in the molecular weight region of 130,000 (ranging from 118,000 to 140,000) and one or more polypeptides in the region of 74,000 (ranging from 71,000 to 77,000). Similar results were obtained with either of the affinity-purified antibody preparations or the crude sera. Immunoreactive forms of both molecular weight classes were seen in all cultured cells, although the apparent ratio between them varied from line to line and somewhat between preparations. This variability led us to explore whether the lower molecular weight species were derived by proteolysis from the higher molecular weight polypeptides during sample preparation. We do not believe this to be the case: extensive experiments to reduce or demonstrate proteolysis of the samples failed to alter the pattern of immunoreactive forms for a given preparation of cells. For example, to examine protocols for reducing proteolysis, samples were either (*a*) scraped into PBS, washed, and dissolved in sample buffer, or (*b*) dissolved directly in hot sample buffer in the culture dish; or (*c*) cell proteins were immediately precipitated after removal of the medium with 10% trichloroacetic acid before solubilization for gel electrophoresis. All these methods gave the same pattern of immunoreactive forms. To test for proteases in the samples, we added purified smooth muscle caldesmon to a homogenate of cultured cells, and withdrew samples for gel electrophoresis after incubation for various periods of time. Immune blots of these samples showed no evidence for caldesmon proteolysis in the samples. It should also be noted that immune blots show no evidence for immunoreactive material of low molecular weight that would run at the dye front. This again suggests that there has been little proteolysis of the samples. Our results suggest that if the lower molecular weight immunoreactive species are derived from the 130,000 class species, then this

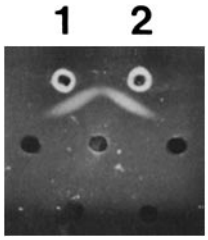


FIGURE 2 Double diffusion analysis of caldesmon antisera. The two different rabbit antisera were placed in wells 1 and 2 and pure caldesmon in the center well. A single precipitin line was observed.

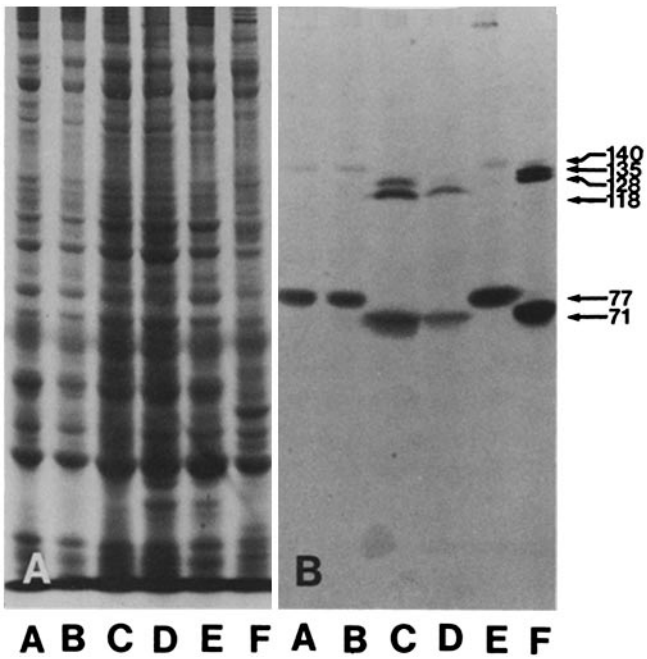


FIGURE 3 Immunoreactive forms of caldesmon in cultured cells. Duplicate samples of the total protein mixture from each cell line were separated on 7.5% SDS PAGE and one sample of each stained with Coomassie Blue (Panel *A*). The other sample was electrophoretically transferred to nitrocellulose and processed to detect immunoreactive forms of caldesmon (Panel *B*). The samples applied were (*A*) NRK cells, (*B*) rat mammary cells, (C) 3T3 cells, (*D*) $BC_3H1$ cells, (*E*) A7R5 cells, and (*F*) chicken embryo fibroblasts. Apparent molecular weights ($\times 10^{-3}$), shown on the right, were estimated from standard proteins run on the same gel.

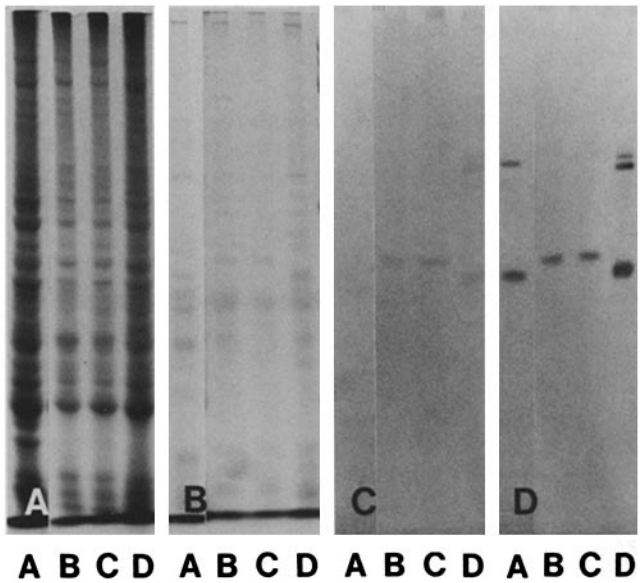


FIGURE 4 Caldesmon immunoreactive forms are heat soluble. Cultured cells were suspended in a high salt buffer and heated to 90°C for 5 min. The heat-precipitated and heat-soluble material were prepared and subjected to gel electrophoresis on 7.5% gels. Panel *A* shows the heat-precipitated material, panel *B* the heat-soluble material, panel C the caldesmon immunoreactive forms in the heat-precipitated material, and panel *D* the immunoreactive forms in the heat-soluble material. The cell lines analyzed were (*lanes A*) $BC_3H1$ cells, (*lanes B*) NRK cells, (*lanes C*) rat mammary cells, and (*lanes D*) 3T3 cells.

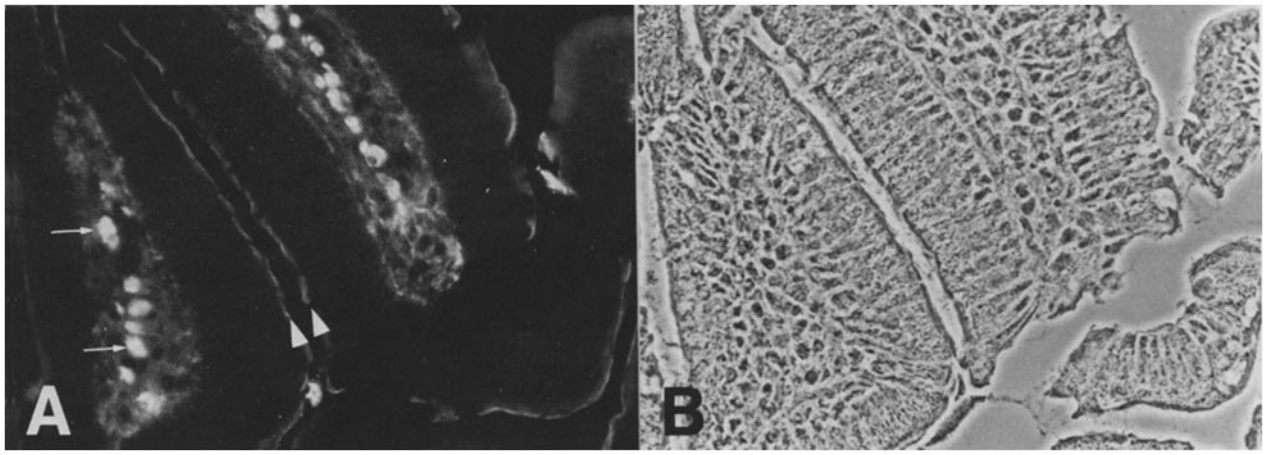


FIGURE 5 Fluorescence (*A*) and phase-contrast (*B*) micrographs of a frozen section of mouse intestine stained with antibodies to caldesmon. Arrows indicate smoooth muscle cells, arrowheads the brush border of intestinal epithelial cells. × 320.

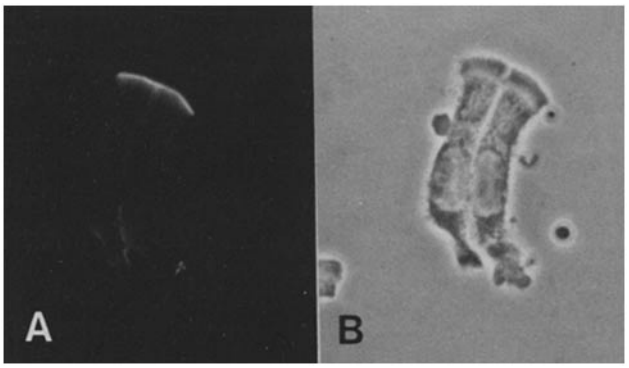


FIGURE 6 Fluorescence (*A*) and phase-contrast (*B*) micrographs of mouse intestinal epithelial cells stained with caldesmon antibody. × 950.

probably happened in vivo before the preparation of the samples.

An important question is whether the immunoreactive forms are structurally and functionally related to smooth muscle caldesmon. Caldesmon from smooth muscle is stable after heat treatment at 90°C for at least several minutes (20), a property shared by a very limited number of cytoplasmic proteins. Heat treatment of total cell protein in high salt showed that the caldesmon immunoreactive forms were also heat stable, whereas the vast majority of cellular proteins were precipitated by this treatment (Fig. 4).

### *Localization of Immunoreactive Forms of Caldesmon*

Initial experiments using indirect immunofluorescence microscopy with affinity-purified antibodies to caldesmon suggested that it might show a similar distribution to tropomyosin. We therefore compared the distribution of these two proteins with the distribution of actin in various tissues and cell lines.

One of the best understood microfilament arrangements is the cyoskeleton of the brush border of intestinal epithelial cells. We therefore examined the localization of caldesmon in frozen tissue sections of mouse intestine (Fig. 5). It was detected in the smooth muscle cells of the villi and in the brush border of the intestinal epithelial cells. Since it is extremely difficult to determine from frozen sections which part of the brush border was stained, we also examined the distribution of caldesmon in isolated intestinal epithelial cells (Fig. 6). In these preparations, caldesmon was found to be localized in the terminal web, with no fluorescence detectable in the microvilli.

Examination of the distribution of actin, caldesmon, and tropomyosin in preparations of three different cultured cells—chicken embryo fibroblasts, rat aorta smooth muscle A7R5 cells, and mouse fibroblast 3T3 cells—indicated a very similar distribution for caldesmon and tropomyosin (Fig. 7). Careful examination of the localization in these cells, revealed that tropomyosin and caldesmon (a) are both associated with all detectable stress fibers in these cells, and (b) both appear to show regions of periodicity down the length of stress fibers.

The periodic distribution along stress fibers of caldesmon and tropomyosin was examined in some detail. The periodicity for tropomyosin has previously been reported (23) and shown to be complementary to the periodic distribution of $\alpha$-actinin along stress fibers (28). We therefore examined how the periodicity of caldesmon related to that seen for tropomyosin and $\alpha$-actinin. Fig. 8 shows selected areas of cultured cells stained either with antibodies to caldesmon, $\alpha$-actinin, or tropomyosin alone, or simultaneously with pairwise combinations of these antibodies. The periodicity seen for caldesmon in A7R5 cells (*A*) or in 3T3 cells (*B*) is evident, but is less distinct than the periodic distribution of $\alpha$-actinin (*C*) or tropomyosin (*D*) in 3T3 cells. Simultaneous staining for tropomyosin and caldesmon (*E*), caldesmon and $\alpha$-actinin (*F*), or tropomyosin and $\alpha$-actinin (*G*) revealed periodicities when tropomyosin and caldesmon were localized simultaneously, but a continuous distribution when either of these were localized together with $\alpha$-actinin. The distribution of caldesmon along stress fibers is therefore at least largely coincident with tropomyosin and complementary to $\alpha$-actinin. In addition, caldesmon is found in the leading edge of motile cultured cells (Fig. 9).

## DISCUSSION

In this report we have shown that immunoreactive, heat-stable forms of smooth muscle caldesmon are present in a wide variety of cells. However, there appears to be considerable heterogeneity in the apparent polypeptide molecular weights of these molecules, with some being in the range of gizzard smooth muscle (~120,000–140,000), the '130,000

class,' and with some at about half this (71,000–77,000), the '74,000 class.' Despite attempting many different preparative procedures to reduce proteolysis, we have so far been unable to prepare samples of cell lines that do not contain a representative from the 74,000-mol-wt class. We therefore conclude that it represents a major form of caldesmon in these cells. However, it is still quite likely that the 74,000 class peptides are processed in vivo from the 130,00 class; such a

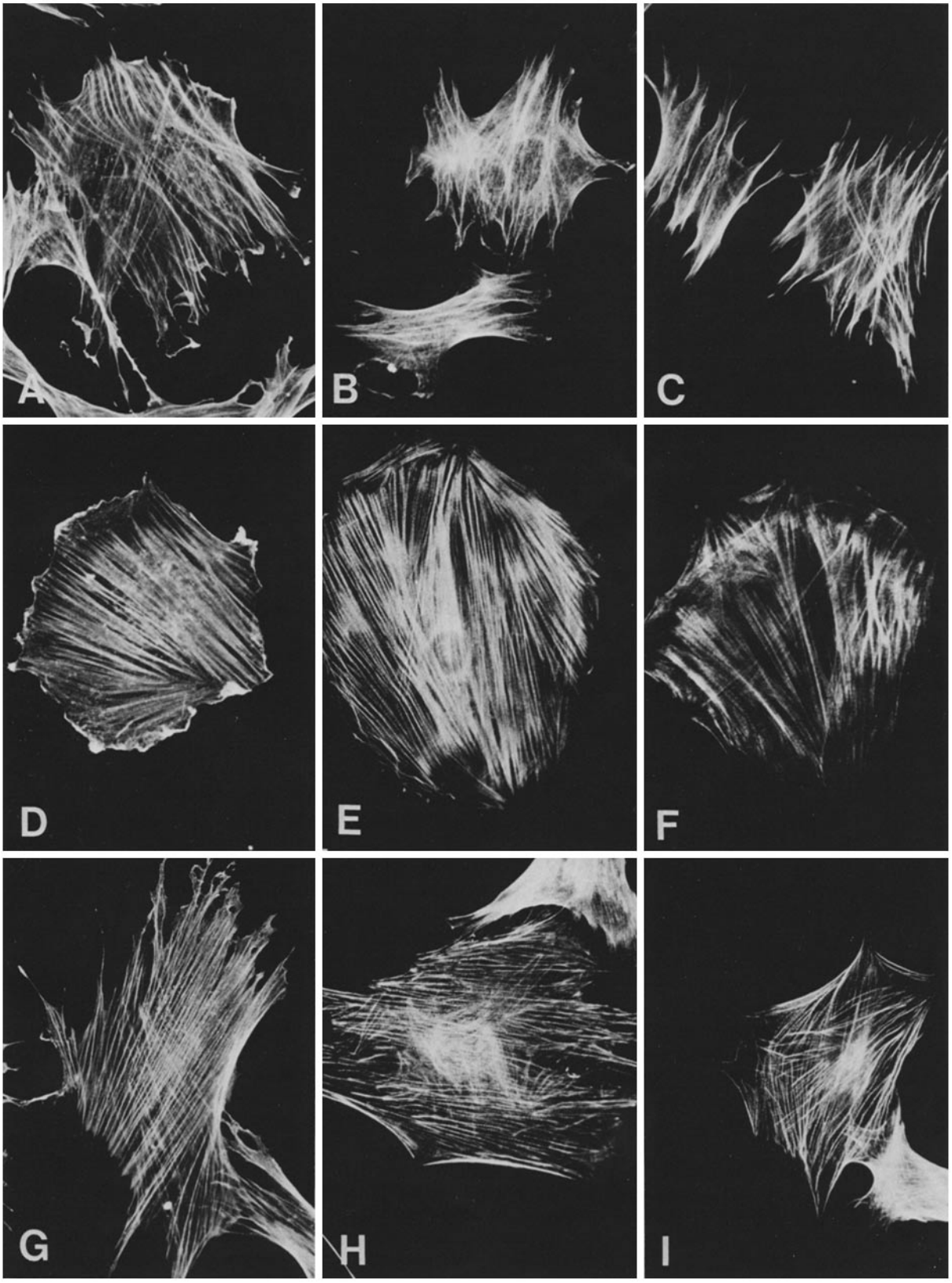


FIGURE 7 Survey fluorescence micrographs of the localization of actin (*A*, *D*, and *G*), caldesmon (*B*, *E*, and *H*), and tropomyosin (*C*, *F*, and *I*) in chicken embryo fibroblasts (*A–C*), A7R5 smooth muscle cells (*D–F*), and mouse 3T3 fibroblasts (*G–I*). × 300.

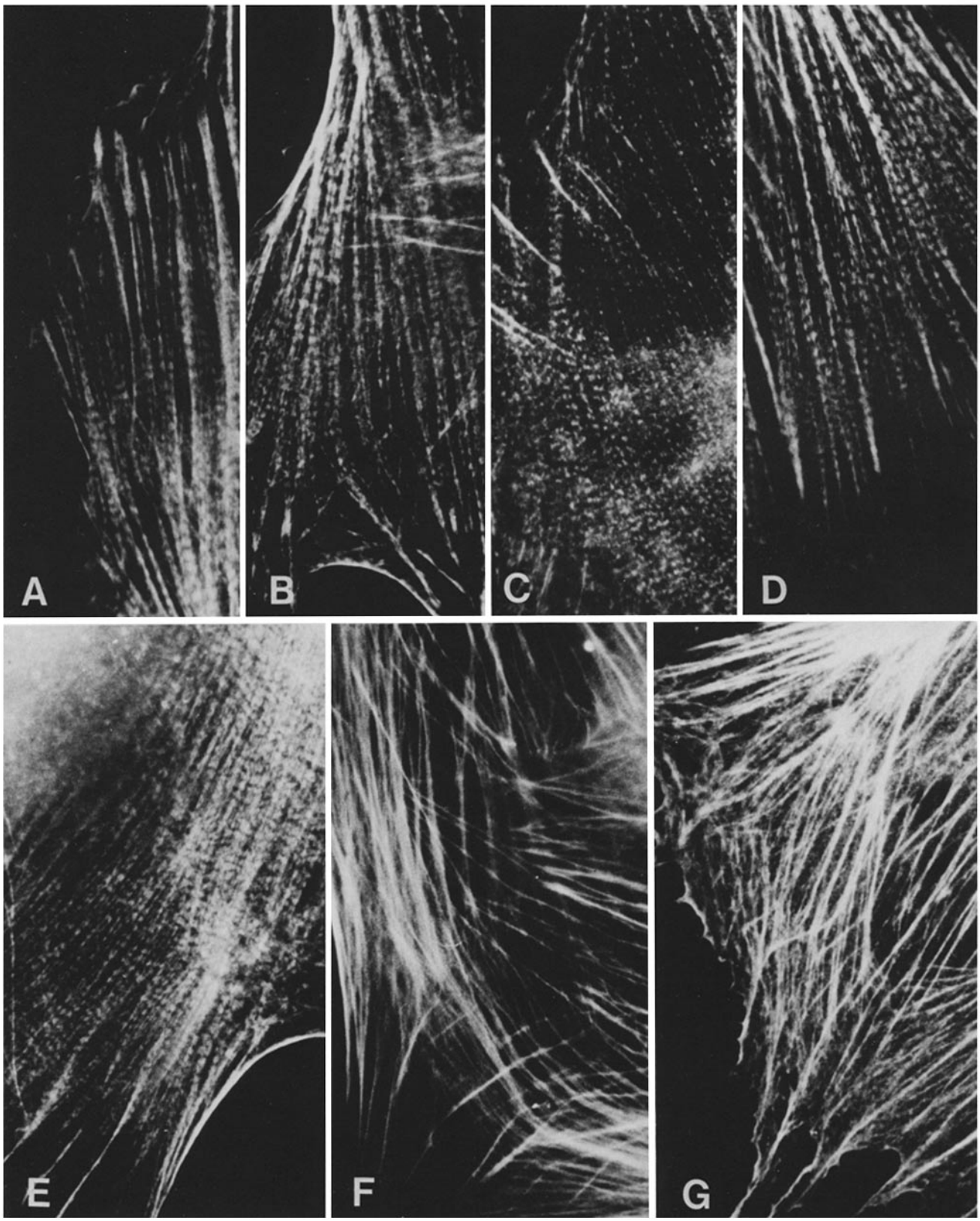


FIGURE 8 Characterization of the periodic distribution of caldesmon along stress fibers of cultured cells. Selected areas of cells are shown stained as follows: (*A*) A7R5 cell stained for caldesmon, (*B*) 3T3 cell stained for caldesmon, (*C*) 3T3 cell stained for α-actinin, (*D*) 3T3 cell stained for tropomyosin, (*E*) 3T3 cell stained simultaneously for caldesmon and tropomyosin, (*F*) 3T3 cell stained simultaneously for caldesmon and α-actinin, and (*G*) 3T3 cell stained simultaneously for tropomyosin and α-actinin. × 1,350.

possibility is presently being investigated. The 130,000 and 74,000 species found in the cultured cells are heat stable, an unusual property that is found for smooth muscle caldesmon (20). This indicates that the immunoreactive forms of the 130,000- and 74,000-mol-wt classes may be structurally similar. Preliminary data indicate that all the heat-stable, immunoreactive species in 3T3 cells bind F-actin in vitro and, like smooth muscle caldesmon, the binding is inhibited by the presence of calcium and calmodulin. However, since these studies were done with a crude heat-stable supernatant, proof that the 74,000 class proteins have similar properties to smooth muscle caldesmon will have to await their purification and in vitro characterization.

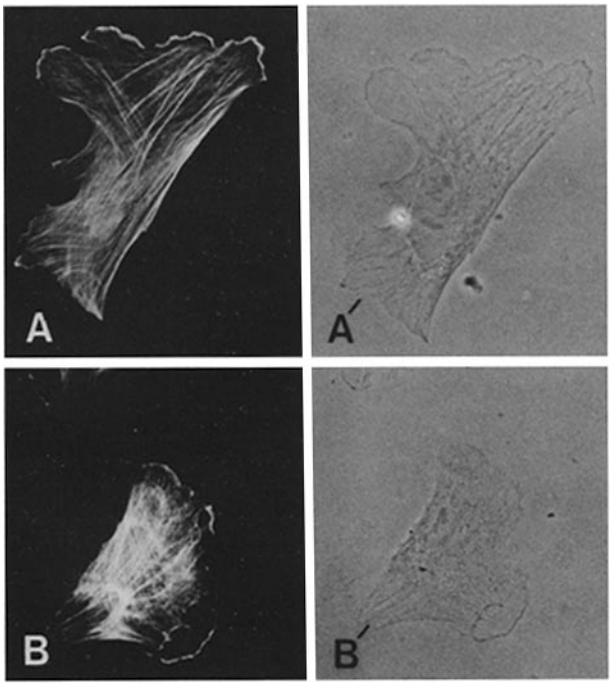


FIGURE 9 Fluorescence (*A* and *B*) and phase-contrast (*A'* and *B'*) micrographs of chicken embryo fibroblasts stained for actin (*A* and *A'*) and caldesmon (*B* and *B'*). Note that the membrane ruffles are stained by both antibodies. × 360.

Very recently, Owada et al. (22) reported that cultured Fischer rat fibroblasts, normal rat kidney cells, and chicken embryo fibroblasts contain a 77,000-mol-wt polypeptide that could be immunoprecipitated with caldesmon antibody. Our results indicate a more complex situation. For example we have shown that cultured cells, including chicken embryo fibroblasts, contain immunoreactive species in both the 130,000 and 74,000 classes. Moreover, the apparent molecular weights of species of both classes vary between cell lines.

We have so far not been able to find any firm correlation between the origin of cells, or their state of growth, and the caldesmon immunoreactive species they contain. For example, we initially thought that smooth muscle cells might contain only the 130,000 class polypeptides, and nonmuscle cells the 74,000 class polypeptides. This is clearly wrong, as platelets contain the 130,000 class polypeptides (21) and cultured smooth muscle cells (A7R5 cells) contain the 74,000 class polypeptides in addition to the 130,000 class. Another possibility was that dividing cells contain both the 130,000 and 74,000 classes, whereas terminally differentiated, or possibly just resting, cells contained the 130,000 class only. In preliminary experiments, we have found caldesmon immunoreactive species of both classes in rapidly dividing cultured cells and in arrested contact-inhibited cells. It is of interest to note, however, that cultured chicken gizzard embryo cells, as well as 12-d embryonic gizzard, contains species of both molecular weight classes, whereas adult chicken gizzard smooth muscle contains exclusively the 130,000 class polypeptides (not shown). Further experiments aimed at understanding the heterogeneity of the caldesmon immunoreactive forms are in progress.

We have used the affinity-purified antibodies to caldesmon in indirect immunofluorescence microscopy to localize the immunoreactive forms in intestinal epithelial cells and in several cultured cell lines. Caldesmon is localized in the terminal web of the brush border of intestinal epithelial cells and along the stress fibers and in the ruffling membrane of

TABLE I

*Localization of Known Microfilament-associated Proteins in Cultured Cells and in the Brush Border of Intestinal Epithelial Cells*

| | Cultured cells | | | Intestinal brush border | | |
|---|---|---|---|---|---|---|
| Protein | Stress fibers | Ruffling membrane | Adhesion plaques | Microvillus | Terminal web | Reference |
| Actin | Continuous | + | + | + | + | 25, 28 |
| Myosin | Periodic (pattern A) | − | − | − | + | 25, 30 |
| Myosin light chain kinase | Periodic (pattern A) | ND | ND | ND | ND | 31 |
| Tropomyosin | Periodic (pattern A) | −* | − | − | + | 23, 25, 28 |
| α-Actinin | Periodic (pattern B) | + | + | − | + | 25, 28, 32 |
| Filamin (actin binding protein) | Continuous‡ | + | + | − | + | 25, 33, 44 |
| Caldesmon | Periodic (pattern A) | + | ND | − | + | This work |
| Fimbrin | Absent | + | + | + | ND | 35 |
| Villin§ | Absent | + | ND | + | ND | 27, 36 |
| Gelsolin | Absent | + | ND | − | + | 37 |
| Vinculin | Absent | − | + | − | + | 38, 39 |
| Talin | Absent (generally) | + | + | ND | ND | 40 |
| Spectrin/fodrin family | Absent | + | − | − | + | 41–43 |

* However, see reference 45 for evidence that tropomyosin is present in the ruffling membrane.
‡ However, see reference 34 for evidence that filamin localization might be striated in cultured cells.
§ Villin is not detectable in most cultured cells (27). The data given for cultured cells reflects the distribution found after microinjection of native villin in living cells (27).
*ND*, Not determined.
+, present; −, not detectable.

cultured cells. In addition, we have shown that caldesmon is distributed in a periodic fashion along stress fiber bundles of cultured cells. Using simultaneous localization of caldesmon with tropomyosin or α-actinin, we have found that the caldesmon periodicity is similar to the distribution of tropomyosin and complementary to the distribution of α-actinin along stress fibers.

What might the role of caldesmon in smooth muscle and nonmuscle cells be? A comparison of the localization of caldesmon with the other known microfilament-associated proteins of higher cells (Table I) shows that its distribution is unique. It is the only F-actin cross-linking protein known that is present along stress fibers with the same periodocity as tropomyosin and also present in the ruffling membrane. So far it has not been possible to determine whether caldesmon is present in adhesion plaques. However, of particular significance is the finding that caldesmon is found along stress fibers in a periodic fashion that, at least at the light microscope level, appears to be similar to the distribution of tropomyosin, myosin, and myosin light chain kinase ('pattern A' in Table I) and complementary to the distribution of α-actinin ('pattern B' in Table I). This finding, together with caldesmon's localization with known contractile proteins in the terminal web of the brush border (see reference 25 and Table I), may suggest a functional contractile unit that contains at least the five proteins actin, myosin, tropomyosin, myosin light chain kinase, and caldesmon. Previous work has shown that thick filament regulation of contraction involves phosphorylation of a specific myosin light chain (17). The finding that caldesmon, a $Ca^{2+}$-calmodulin–regulated F-actin bundling protein, is co-localized with tropomyosin together with myosin might indicate that they form part of a thin filament $Ca^{2+}$-regulatory system, as already suggested (29). The finding that tropomyosin and caldesmon do not compete in their binding for F-actin in vitro, and in fact might show some cooperativity (20), is not inconsistent with a regulatory role for these proteins in smooth muscle and in stress fibers. However, this is clearly not the only function of caldesmon as it is also a component of the ruffling membrane of cultured cells, a region in which myosin cannot be detected (see Table I). Therefore, to understand in more detail the role of caldesmon and its 130,000 and 74,000 class immunoreactive polypeptides, the effects of these purified proteins on reconstituted microfilament systems will have to be carefully examined.

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*Note Added in Proof:* We have recently purified to homogeneity the 71K caldesmon species from 3T3 cells. Like smooth muscle caldesmon, it binds immobilized calmodulin in a $Ca^{2+}$-dependent manner and it binds F-actin in a $Ca^{2+}$-calmodulin inhibitable manner (Lynch, W., and A. Bretscher, manuscript in preparation).

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