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Cell Biology

Occurrence of caldesmon (a calmodulin-binding protein) in cultured cells: Comparison of normal and transformed cells

(actin-binding protein/transformation/Rous sarcoma virus/temperature-sensitive changes/flip-flop regulation)

M. KOJI OWADA*, AKIRA HAKURA*, KAZUKO IIDA†, ICHIRO YAHARA†, KENJI SOBUE‡, AND SHIRO KAKIUCHI‡§

*Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita, Osaka 565; †The Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113; and ‡Department of Neurochemistry and Neuropharmacology, Institute of Higher Nervous Activity, Osaka University Medical School, Nakanoshima, Kita-ku, Osaka 530, Japan

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ABSTRACT **Caldesmon is a calmodulin-binding and F-actin-binding protein originally purified from chicken gizzard smooth muscle. This protein binds to F-actin filaments in a Ca^{2+}- and calmodulin-dependent "flip-flop" fashion, thereby regulating the function of actin filaments. Here we report that various lines of cultured cells contain a M_r 77,000 protein that specifically reacts with the affinity-purified caldesmon antibody raised against chicken gizzard caldesmon. Among the fibroblast proteins that had been pulse-labeled with [^{35}S]methionine, the M_r 77,000 protein was the only protein band detected on the NaDodSO$_4$ gel that reacted with the anticaldesmon. The subcellular distribution of the M_r 77,000 protein was investigated by the indirect immunofluorescence technique using the anticaldesmon. In all fibroblast cell lines examined, the immunofluorescence localized along the cellular stress fibers and in leading edges of the cell. In Rous sarcoma virus-transformed cells (S7-1), however, the distribution of the fluorescence changed to a diffuse and blurred appearance. These staining patterns of anticaldesmon obtained with the normal and transformed cells coincided with those of antiactin in the corresponding states, strongly suggesting the functional linkage between the M_r 77,000 protein and actin filaments. We propose to refer to this M_r 77,000 protein as caldesmon$_{77}$. The cellular level of caldesmon$_{77}$ in transformed S7-1 cells decreased to about one-third of that in their normal counterparts (cell line no. 7). Essentially the same result was obtained with normal rat kidney cells infected with the temperature-sensitive transformation mutant Schmidt–Ruppin strain of Rous sarcoma virus (68 N2 clone). The cellular level of caldesmon$_{77}$ observed at a permissive temperature (35°C) was about one-third of that at a nonpermissive temperature (38.5°C). These changes of caldesmon$_{77}$ in transformed cells may correlate with the loss of Ca^{2+} regulation in the transformed state.**

Caldesmon, a protein composed of M_rs 150,000 and 147,000 subunit polypeptides, was discovered in chicken gizzard as a major calmodulin-binding protein also able to bind to F-actin (1, 2). It is now regarded as a key protein through which the Ca^{2+}-dependent regulatory action of calmodulin is transmitted to F-actin filaments with the following properties: while binding of caldesmon to F-actin causes modulation of the function of F-actin filaments (see below), Ca^{2+}-dependent binding of calmodulin to F-actin eliminates the interaction between caldesmon and F-actin. Since two types of interactions, calmodulin–caldesmon and caldesmon–F-actin, occur alternately, depending upon the presence or absence, respectively, of Ca^{2+}, we have called this mode of action "flip-flop" regulation (1, 2).

This flip-flop regulation represents at least one type of mechanism by which calmodulin regulates the function of F-actin. Thus, using contractile proteins isolated from gizzard smooth muscle, we were able to reconstitute systems in which filamin-induced gelation of F-actin filaments (3) or myosin–F-actin interaction measured by superprecipitation or ATPase activity (4) was controlled by the caldesmon-linked flip-flop mechanism. Caldesmon by itself did not produce gel formation or shortening of actin filaments nor did it influence reannealing of fragmented actin filaments (3). In this respect, it differs from other F-actin interacting proteins, such as gelsolin (5), actinogelin (6), fragmin (7), and villin (8).

Subsequently, caldesmon was shown to be present in bovine aorta and uterus and in human platelets (9). Caldesmon samples from these tissues fulfilled all criteria for caldesmon (9), including the flip-flop-regulated binding to F-actin filaments. During the search for caldesmon in tissues other than chicken gizzard, we observed that cultured fibroblast cells also contained a protein that reacted with caldesmon antiserum raised against chicken gizzard caldesmon (9). By use of the indirect immunofluorescence technique, the caldesmon antibody was found to be localized in the cellular stress fibers and leading edges. However, the immunoblotting method, following NaDodSO$_4$/polyacrylamide gel electrophoretic separation of a caldesmon-binding protein fraction from fibroblast cells, could not detect polypeptide bands at a molecular weight ($M_r \approx 150{,}000$) region similar to that of caldesmon. Instead, a band of an estimated M_r of about 77,000 was strongly stained by the immunoblotting technique. Therefore, the possibility was not excluded that the detection of the M_r 77,000 protein may have been due to the presence of contaminating antibodies. In the present investigation such the possibility was ruled out by using the affinity-purified caldesmon antibody. Thus, among the ^{35}S-pulse-labeled proteins of fibroblast cells, the M_r 77,000 protein was the only protein species that specifically reacted with the affinity-purified caldesmon antibody. The present paper also reports that the level of the M_r 77,000 protein decreased in Rous sarcoma virus-transformed cells.

MATERIALS AND METHODS

Materials. Caldesmon was prepared from chicken gizzard smooth muscle as in ref. 2. The preparation was homogeneous on NaDodSO$_4$/polyacrylamide gel electrophoresis, showing only a doublet of M_rs 150,000 and 147,000 polypeptide bands. To prepare a homogeneous preparation of actin to use for the immunization of rabbits, chicken gizzard proteins were electrophoresed on a NaDodSO$_4$ gel and the actin band on the gel was cut out as described in ref. 10. The sources of commercial materials used in this work were as follows: phenylmethylsulfonyl fluoride and diisopropyl

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§To whom reprint requests should be addressed.

fluorophosphate, Sigma; DE-52, Whatman Ltd.; protein A-coupled Sepharose 4B, Pharmacia Japan, Tokyo; dimethyl sulfoxide, Wako Pure Chemical Industries Ltd., Osaka, Japan; leupeptin, Peptide Institute Inc., Osaka; Trasylol, Bayer, Leverkusen, West Germany; fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, Cappel Laboratories, Cochranville, PA; and fetal calf serum, M. A. Bioproducts, Walkersville, MD. L-[^{35}S]Methionine (>100 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. X-Omatic R film is a product of Eastman Kodak.

Cell Culture. Normal Fischer rat cell line no. 7 (11), which showed typical properties of the untransformed cell line, and the Rous sarcoma virus-transformed cell line no. 7 (S7-1) were mainly used. Normal rat kidney cells infected with the temperature-sensitive mutant Schmidt–Ruppin strain of Rous sarcoma virus (subgroup A) (68 N2 clone) (12) was a generous gift of R. Hirai (The Tokyo Metropolitan Institute of Medical Science). Chicken embryo fibroblasts were prepared from 10-day embryos. C3H-2K cells that originated from the kidney tissue of a newborn C3H/He mouse are epithelial-like cells (13). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, penicillin G (20 units/ml), and streptomycin (50 μg/ml). Cells were kept at a temperature of 37°C, with the exception of the 68 N2 clone (see below), in an atmosphere of 10% CO_2/90% air and 100% humidity. It was confirmed that the 68 N2 clone is temperature sensitive for the transformed morphology and the pp60$^{v\text{-}src}$ kinase activity: at the nonpermissive temperature (38.5°C), the latter activity was one-fourth that at the permissive temperature (35°C) (unpublished observation).

Immunofluorescence Microscopy. All procedures were carried out at room temperature. Cells grown on glass coverslips were washed with phosphate-buffered saline (P_i/NaCl) consisting of 150 mM NaCl, 5 mM KCl, 20 mM KH_2PO_4/Na_2HPO_4 (pH 7.2) and fixed with 3.5% (wt/vol) formaldehyde in P_i/NaCl for 20 min. The cells were then washed three times with P_i/NaCl and then treated with 0.2% Triton X-100 in P_i/NaCl for 5 min. The Triton-treated cells were incubated with rabbit anticaldesmon IgG (100 μg of protein per ml) or antiactin (100 μg of protein per ml) for 30 min. After washing with P_i/NaCl four times, the cells were stained with fluorescein-conjugated goat anti-rabbit IgG at a 1:10 dilution for 60 min. The cells were again washed with P_i/NaCl several times and mounted on glass slides in 50% glycerol/50% P_i/NaCl (vol/vol). Microscopic examination of fluorescence was carried with an Olympus fluorescence microscope model BHF. Photographs were taken with Kodak Tri-X film with an exposure time of 10 sec.

Preparation of Antibody. Antiserum to chicken gizzard caldesmon was produced in rabbits as in ref. 9. The anticaldesmon IgG was prepared from the serum by ammonium sulfate precipitation (48% saturation) and then by DEAE-cellulose (DE-52) column chromatography (9). The anticaldesmon IgG was retained on the column with 20 mM phosphate buffer (pH 7.2) and then was eluted from the column with 20 mM phosphate buffer (pH 7.2) plus 50 mM KCl. Antiactin antibody to chicken gizzard actin was produced in rabbit and was partially purified as in anticaldesmon described above. The affinity-purified anticaldesmon IgG was prepared in the manner described in ref. 14: chicken gizzard caldesmon was electrophoresed on the NaDodSO$_4$/polyacrylamide gel (6% polyacrylamide). A doublet band of M_rs 150,000 and 147,000, which was located by Coomassie brilliant blue staining, was excised from the gel and then transferred to nitrocellulose paper electrophoretically. The nitrocellulose paper was incubated with the partially purified anticaldesmon IgG overnight and then was washed with 10 mM Tris·HCl buffer (pH 7.5) containing 0.9% NaCl and 0.05% Tween 20. Antibody that bound to the M_r 150,000/147,000 caldesmon was eluted with 0.05 M acetic acid, neutralized immediately with 2 M Tris, and then dialyzed overnight against P_i/NaCl.

Immunoprecipitation. For the experiments shown in Figs. 1, 2, and 4, cultured cells (cell line nos. 7, S7-1, and 68 N2) were labeled with [^{35}S]methionine and lysed, the extracts being immunoprecipitated and electrophoresed by a modification of the procedure described in ref. 15 as follows: semi-

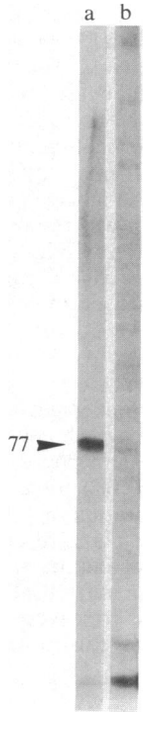

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled fibroblast proteins coprecipitated with antibody to chicken gizzard caldesmon. Cultures of the no. 7 cell line grown at 37°C for 1 day were pulse-labeled with [^{35}S]methionine for 30 min at 37°C and then lysed. The cell lysates were incubated with either anticaldesmon IgG (5 μg of protein) (lane a) or preimmune rabbit serum (50 μg of protein) (lane b). To the incubation mixture, protein A-Sepharose 4B was added to precipitate the proteins adsorbed to protein A. These proteins were then dissolved with a NaDodSO$_4$ solution, boiled, and electrophoresed on the NaDodSO$_4$/polyacrylamide gel. The proteins resolved on the gel were visualized by autoradiography. Protein bands other than the M_r 77,000 band (shown as $M_r \times 10^{-3}$) are seen in both the experimental (lane a) and control (lane b) gels, suggesting that these were nonspecifically adsorbed to protein A.

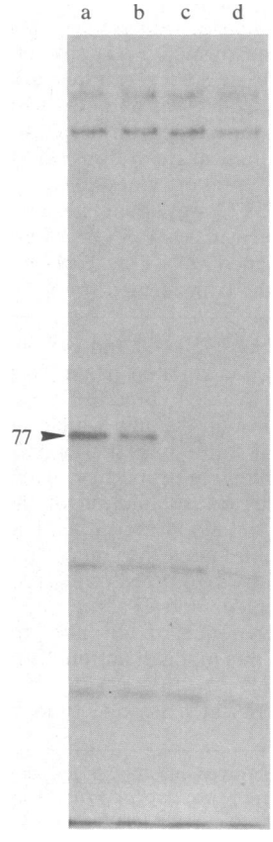

FIG. 2. Demonstration of the competitive displacement of the M_r 77,000 protein (shown as $M_r \times 10^{-3}$) from the immune complex by addition of chicken gizzard caldesmon. The procedure was essentially the same as that of the experiment in Fig. 1, except that affinity-purified anticaldesmon IgG, instead of partially purified anticaldesmon IgG, was used and, during the incubation of cell lysates with anticaldesmon, highly purified chicken gizzard caldesmon was added to the incubation mixtures as follows: lane a, no addition (control); lane b, 0.12 μg; lane c, 1.2 μg; lane d, 12 μg.

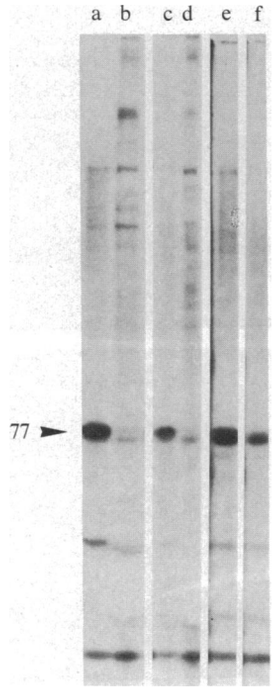

FIG. 4. Comparison of the cellular levels of M_r 77,000 protein in the transformed and untransformed states. Lanes a and c, a cell line no. 7 derived from Fischer rat fibroblasts (lane a) and its Rous sarcoma virus-transformed derivative S7-1 clone (lane c). Cells were grown at 37°C for 1 day before the [^{35}S]methionine labeling. Lanes b and d, control experiments for no. 7 and S7-1, respectively, in which the cell lysates were incubated with preimmune rabbit serum instead of anticaldesmon. Lanes e and f, a clone derived from normal rat kidney cells that were infected with the temperature-sensitive transformation mutant Schmidt–Ruppin strain of Rous sarcoma virus was grown either at 38.5°C (lane e) or 35°C (lane f) for 1 day before labeling. These temperatures were also maintained during the incubation with methionine-free medium and the subsequent incubation with [^{35}S]methionine. Other conditions (cell labeling, cell lysis, and subsequent NaDodSO$_4$ gel electrophoretic analysis of labeled proteins) were essentially the same as that of the experiment in Fig. 1, except that the duration of [^{35}S]methionine labeling was 3 hr.

were incubated with anticaldesmon, and protein complexes formed were separated and collected by protein A-Sepharose 4B and then subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. Fig. 1 demonstrates the protein bands separated on the gel and visualized by autoradiography. A protein band having an estimated M_r of 77,000 was predominant in the experimental gel (lane a), which was lacking in the control (lane b). The presence of the M_r 77,000 protein band was also seen in Fig. 2, lane a, where the affinity-purified anticaldesmon IgG was used. We next examined the effect of adding increasing concentrations of the highly purified chicken gizzard caldesmon to incubation mixtures containing cellular lysates and anticaldesmon to see whether such additions cause increasing degrees of competitive displacement of the M_r 77,000 protein from the M_r 77,000 protein–anticaldesmon complex. The results shown in Fig. 2, lanes b–d, indicate that this is the case. As the concentration of added caldesmon increased, the intensity of the M_r 77,000 protein band in the autoradiography picture decreased. Note that, among the protein bands appearing in the autoradiogram, only the M_r 77,000 band was subject to such change.

Anticaldesmon antibody was used to localize the M_r 77,000 protein in cultured fibroblast cells (chicken embryo fibroblasts and C3H-2K) by means of indirect immunofluorescence. As shown in Fig. 3, anticaldesmon immunofluorescence coincided with the cellular stress fibers that are known to be composed mainly of actin filament bundles (compare Fig. 3 *A*, *B*, and *H*; and Fig. 3 *D–F* and *I*). In addition, anticaldesmon immunofluorescence decorated leading edges of the cell (Fig. 3*A*) in a manner similar to antiactin. Although the fluorescence patterns seen with antiactin and anticaldesmon antibodies are similar, one difference was observed: anticaldesmon produced some periodicities in its staining pattern of the stress fibers in C3H-2K cells (Fig. 3*D*). Similar periodicities along the stress fibers were observed when cells were stained with the α-actinin (17), tropomyosin (18), and myosin light chain kinase (19) antibodies. No periodicities have been reported for antiactin fluorescence.

In Fig. 4, the cellular levels of the M_r 77,000 protein were compared between a Rous sarcoma virus-transformed clone (S7-1) and its normal counterpart cell line (no. 7). A comparison was also made, using a clone (68 N2) isolated after infection of normal rat kidney cells with the temperature-sensitive mutant Schmidt–Ruppin strain of Rous sarcoma virus, between permissive (35°C) and nonpermissive (38.5°C) temperatures. With autoradiography, the decreased intensity of the M_r 77,000 protein band was seen in transformed cells (Fig. 4, lane c) or infected cells at the permissive temperature (Fig. 4, lane f) when compared with their untransformed (Fig. 4, lane a) or nonpermissive temperature (Fig. 4, lane e) counterparts, respectively. In a separate experiment, the M_r 77,000 protein band was cut from the gel and its radioactivity was determined. The radioactivities contained in the M_r 77,000 protein bands of Fig. 4, lanes c and f, were about one-third the radioactivities in their untransformed (Fig. 4, lane a) or nonpermissive temperature (Fig. 4, lane e) counterparts. During the 3-hr pulse-labeling time, uptakes of ^{35}S into the trichloroacetic acid sediments were about the same between the transformed and untransformed cells.

Fig. 5 *A* and *B* compare cell morphologies between the normal (no. 7) and transformed (S7-1) cells with respect to anticaldesmon immunofluorescence. As described (see

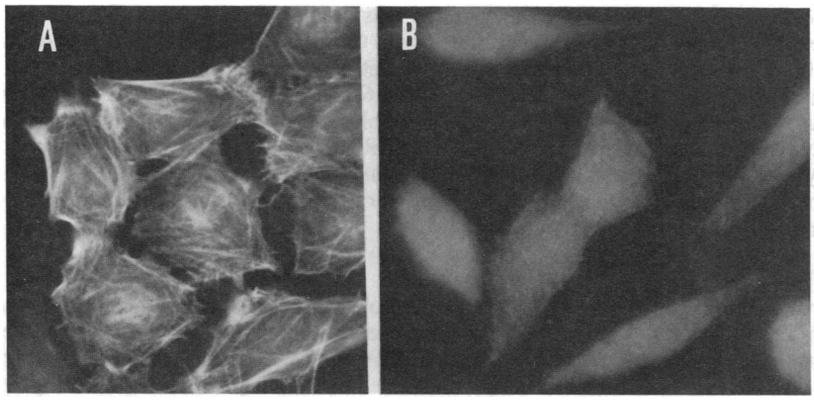

FIG. 5. Fluorescence micrographs of no. 7 (*A*) and S7-1 (*B*) cells stained with anticaldesmon. Fluorescein-labeled goat anti-rabbit IgG was used to visualize the primary antibody. Note the shape change and reduction of the size of transformed cells (*B*) when compared with their normal counterparts (*A*). (×400.)

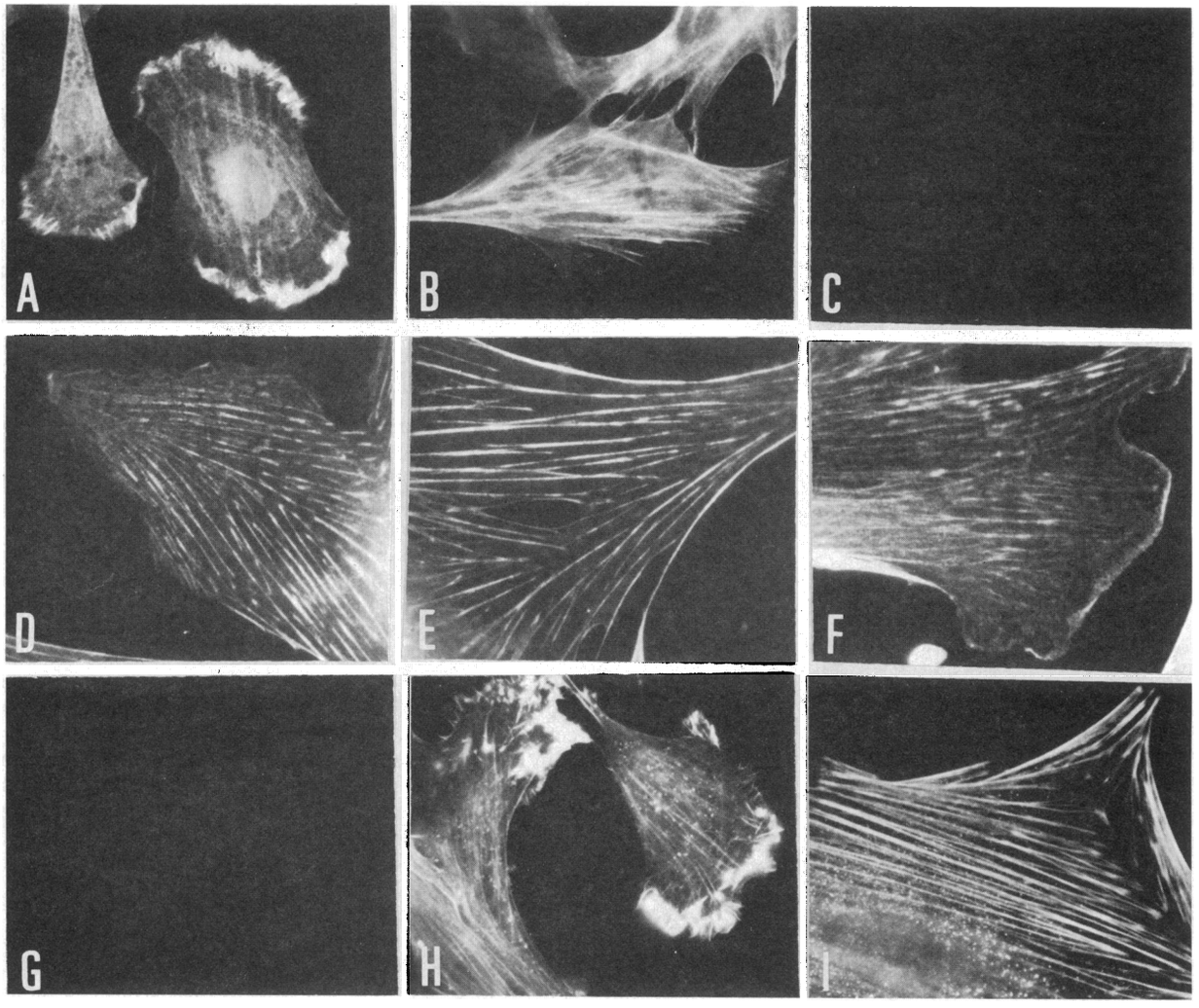

FIG. 3. Immunofluorescence localization of anticaldesmon in cultured cells. (*A* and *B*) Chicken embryo fibroblast cells; (*D–F*) C3H-2K cells. (*H* and *I*) For comparison, chicken embryo fibroblast and C3H-2K cells, respectively, were stained with antiactin instead of anticaldesmon. (*C* and *G*) Controls (chicken embryo fibroblast and C3H-2K cells, respectively) in which anticaldesmon was omitted and cells were treated with only fluorescein-labeled goat anti-rabbit IgG. (×700.)

confluent cell cultures (2×10^6 cells on a 60-mm dish) were washed two times with 3 ml each of P_i/NaCl and incubated for 2 hr with 1 ml per dish of methionine-free medium containing 5% dialyzed calf serum. After washing the cells two times with P_i/NaCl, the cells were incubated with 1 ml per dish of the same medium containing 125 μCi of [^{35}S]methionine for the appropriate time indicated in the legend to each figure. Incubation temperature was either 37°C (for no. 7 and S7-1 cells), 35°C (permissive temperature for 68 N2 clone), or 38.5°C (nonpermissive temperature for 68 N2 clone). The labeled cells were washed two times with P_i/NaCl and lysed with 1.5 ml per dish of a modified RIPA buffer (15) (50 mM Tris·HCl, pH 8.2/0.15 M NaCl/1% Triton X-100/1% deoxycholate/0.1% $NaDodSO_4$/10 mM EDTA/20 mM NaF) containing a protease inhibitor mixture consisting of final concentrations of 50 μg of phenylmethylsulfonyl fluoride per ml, 5 μg of leupeptin per ml, 0.1 mM diisopropyl fluorophosphate, and 1% (vol/vol) Trasylol. The lysate was centrifuged at 6000 × *g* for 30 min at 4°C. From the resulting clear supernatant, a 100-μl sample was taken, to which either 5 μg of protein (anticaldesmon IgG) (Figs. 1 and 4) or 2.5 μg of protein (affinity-purified anticaldesmon) (Fig. 2) was added. In control experiments, anticaldesmon was replaced by rabbit preimmune serum (50 μg of protein). The mixture was incubated for 1 hr at 4°C. The incubation mixture was mixed with 28 μg of protein A-Sepharose 4B and then further incubated for 30 min at 4°C. The mixture was centrifuged at 6000 × *g* for 1 min. The pellet was washed six times with the modified RIPA buffer/protease inhibitor mixture and the proteins adsorbed to the protein A-Sepharose 4B were dissolved with 50 μl of 0.1 M Tris·HCl, pH 6.8/25% glycerol/5% $NaDodSO_4$/10% 2-mercaptoethanol/0.01% bromophenol blue. The solution was boiled for 2 min and centrifuged at 6000 × *g* for 2 min. The supernatant was subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis as in ref. 3. The concentration of acrylamide was 7.5%. After the electrophoresis the gels were stained with Coomassie brilliant blue. Radioactive protein bands were detected by fluorography using sodium salicylate as a fluor (16). In short, the gels were soaked in 1 M sodium salicylate, blotted, dried under vacuum, and then exposed to X-Omatic R film at −80°C. In some experiments, the radioactive gel bands were cut out and solubilized, and the radioactivities were determined in a liquid scintillation counter.

RESULTS

To search for the protein that reacts with anticaldesmon, cellular lysates were prepared from cultured fibroblast cells [clone no. 7 derived from Fischer rat fibroblasts (11)] that had been pulse-labeled with [^{35}S]methionine. The lysates

above), anticaldesmon immunofluorescence distributed along the cellular stress fibers and cell leading edges in the untransformed normal cells. In contrast, in the transformed cells (Fig. 5*B*), these patterns were disordered and were replaced by a diffuse or blurred appearance of immunofluorescence. Leading edges were no longer decorated with the fluorescence in the transformed cells.

DISCUSSION

The present results demonstrate that various types of cultured fibroblast cells contain the M_r 77,000 protein that reacts with the antibody raised against chicken gizzard caldesmon. To exclude the possibility that the detection of this protein was due to the presence of contaminating antibodies, we used the affinity-purified caldesmon antibody. In this experiment, only the caldesmon doublet (M_r 150,000/147,000) was excised from the NaDodSO$_4$ gel, on which chicken gizzard caldesmon had been electrophoresed, and transferred to nitrocellulose paper, the latter being used to affinity purify anticaldesmon. The M_r 77,000 protein was specifically immunoprecipitated with this affinity-purified anticaldesmon. Moreover, the binding of the M_r 77,000 protein to the affinity-purified anticaldesmon IgG was competitively inhibited by adding increasing concentrations of highly purified gizzard caldesmon.

Among the fibroblast proteins that were pulse-labeled with [^{35}S]methionine, the M_r 77,000 protein was the only protein band on the NaDodSO$_4$ gel that cross-reacted with the caldesmon antibody specifically. This was somewhat unexpected because caldesmons from smooth muscles and human platelets showed values close to M_r 150,000 on the NaDodSO$_4$ gel, although minor variations were observed among them (9). Gizzard and platelet caldesmons resolved into a doublet of M_r 150,000 and 147,000, whereas aorta and uterus caldesmons gave a single band of M_r 150,000 on the NaDodSO$_4$ gel. The possibility that the M_r 77,000 protein is a proteolytic fragment of the M_r 150,000/147,000 caldesmon is quite unlikely because a mixture of protease inhibitors was included in the ruptured cell preparations at all times. In addition, absence of M_r 150,000/147,000 caldesmon and presence of M_r 77,000 protein were consistent observations in repeated experiments using the same and different cell lines. Although the exact basis for the antigenic relationship between M_r 150,000/147,000 caldesmon and M_r 77,000 protein is unclear at present, the result strongly suggests that both proteins share some degree of homology in their molecular structures. We propose to refer to the M_r 77,000 protein as caldesmon$_{77}$.

The intracellular distribution of the caldesmon$_{77}$ in fibroblast cells was investigated by the indirect immunofluorescence technique using anticaldesmon. In all cell lines examined (chicken embryo fibroblasts, C3H-2K, and no. 7) the anticaldesmon immunofluorescence coincided with the antiactin immunofluorescence—i.e., the cellular stress fibers and leading edges were strongly stained. The results suggest the functional linkage of the caldesmon$_{77}$ with actin filaments. In nonmuscle cells, actin-containing microfilaments have been thought to be implicated in a number of cellular functions, such as maintenance of cell morphology, cell locomotion, membrane ruffling, and cell adhesion to a substratum (20–22). Therefore, it is attractive to assume that the calmodulin–caldesmon$_{77}$ system plays a regulatory role in some of the above functions of actin filaments in nonmuscle tissues. In support of this notion, we have found recently caldesmon$_{77}$ in a variety of nonmuscle tissues of rat and have purified it from bovine adrenal medulla (unpublished results). The purified protein was found to bind to both calmodulin and F-actin. In a preliminary experiment, these bindings were regulated in a flip-flop fashion depending upon the Ca^{2+} concentration (unpublished results).

The cellular level of caldesmon$_{77}$ decreased in transformed cells to about one-third of that in the untransformed cells. To confirm that this change is associated with the transformation of the cell, we next used the 68 N2 clone, which is a transformed clone isolated after infection of normal rat kidney cells with the temperature-sensitive mutant Schmidt–Ruppin strain of Rous sarcoma virus. At a permissive temperature (35°C), but not at a restrictive temperature (38.5°C), such a change occurred. The intracellular distribution of caldesmon$_{77}$ also changed to a diffuse and blurred appearance in transformed cells. This is very much the same pattern as the antiactin pattern in the transformed state, in which the ordered stress fibers consistently seen in the normal cells are replaced by a diffuse network of actin-containing matrix (23, 24). These changes of caldesmon$_{77}$ occurring in transformed cells—i.e., decrease in its level and distortion of its normal distribution pattern in the cell—may correlate with the loss of Ca^{2+} regulation in transformed cells.

It has been thought that phenotypic changes characteristic of transformed cells, such as rounded cell shape and loss of cell substrate anchorage, are related to the morphological and functional alterations of actin filaments in the cell (23–25). In this connection, the caldesmon–calmodulin system with its possible regulatory role on actin filaments would provide a clue to the mechanisms underlying the phenotypic expression of the transformed state.

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