Correction. In the article "Occurrence of caldesmon (a calmodulin-binding protein) in cultured cells: Comparison of normal and transformed cells" by M. Koji Owada, Akira Hakura, Kazuko Iida, Ichiro Yahara, Kenji Sobue, and Shiro Kakiuchi, which appeared in number 10, May 1984, of Proc. Natl. Acad. Sci. USA (81, 3133–3137), pages 3135 and 3136 were misplaced relative to each other: the material on page 3135 should have appeared on page 3136 and vice versa.
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. KOI OWADA*, AKIRA HAKURA*, KAZUKO IIDA†, ICHIRO YAHARA‡, KENJI SOBUE‡, AND SHIRO KAKIUCHI†§

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

Communicated by Kenichi Fukui, February 7, 1984

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 Ca2+- and calmodulin-dependent "flip-flop" fashion, thereby regulating the function of actin filaments. Here we report that various lines of cultured cells contain a Mr 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 [35S]methionine, the Mr 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 cells. 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 Mr 77,000 protein and actin filaments. We propose to refer to this Mr 77,000 protein as caldesmon77. The cellular level of caldesmon77 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 caldesmon77 observed at a permissive temperature (35°C) was about one-third of that at a nonpermissive temperature (38.5°C). These changes of caldesmon77 in transformed cells may correlate with the loss of Ca2+ regulation in the transformed state.

Caldesmon, a protein composed of Mr's 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 Ca2+-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), Ca2+-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 Ca2+ ions, 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 NaDodSO4/polyacrylamide gel electrophoretic separation of a caldesmon-binding protein fraction from fibroblast cells, could not detect polypeptide bands at a molecular weight (Mr ≈150,000) region similar to that of caldesmon. Instead, a band of an estimated Mr of about 77,000 was strongly stained by the immunoblotting technique. Therefore, the possibility was not excluded that the detection of the Mr 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 35S-pulse-labeled proteins of fibroblast cells, the Mr 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 Mr 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 NaDodSO4/polyacrylamide gel electrophoresis, showing only a doublet of Mr's 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 NaDodSO4 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

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

§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-[35S]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% CO2/90% air and 100% humidity. It was confirmed that the 68 N2 clone is temperature sensitive for the transformed morphology and the pp60src kinase activity: at the nonpermissive temperature (38.5°C), the latter activity was one-fourth that at the permissive temperature (33°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/NaCl) consisting of 150 mM NaCl, 5 mM KCl, 20 mM KH₂PO₄/Na₂HPO₄ (pH 7.2) and fixed with 3.5% (wt/vol) formaldehyde in P/NaCl for 20 min. The cells were then washed three times with P/NaCl and then treated with 0.2% Triton X-100 in P/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/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/NaCl several times and mounted on glass slides in 50% glycerol/50% P/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 NaDodSO₄/polyacrylamide gel (6% polyacrylamide). A doublet band of Mr 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 Mr 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/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 [35S]methionine and lysed, the extracts being immunoprecipitated and electrophoresed by a modification of the procedure described in ref. 15 as follows: semi-
fected 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 M₇,77,000 protein from the M₇,77,000 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 M₇,77,000 protein band in the autoradiography picture decreased. Note that, among the protein bands appearing in the autoradiogram, only the M₇,77,000 band was subject to such change. Anticaldesmon antibody was used to localize the M₇,77,000 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. 3A, B, and H; and Fig. 3D–F and I). In addition, anticaldesmon immunofluorescence decorated leading edges of the cell (Fig. 3A) in a manner similar to antiaxin. Although the fluorescence patterns seen with antiaxin 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. 3D). 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 antiaxin fluorescence.

In Fig. 4, the cellular levels of the M₇,77,000 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 M₇,77,000 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 M₇,77,000 protein band was cut from the gel and its radioactivity was determined. The radioactivities contained in the M₇,77,000 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-h pulse-labeling time, uptakes of [³⁵S] into the trichloroacetic acid sediments were about the same between the transformed and untransformed cells.

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

FIG. 4. Comparison of the cellular levels of M₇,77,000 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 [³⁵S]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 [³⁵S]methionine. Other conditions (cell labeling, cell lysis, and subsequent NaDodSO₄ gel electrophoretic analysis of labeled proteins) were essentially the same as that of the experiment in Fig. 1, except that the duration of [³⁵S]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 NaDodSO₄/polyacrylamide gel electrophoresis. Fig. 1 demonstrates the protein bands separated on the gel and visualized by autoradiography. A protein band having an estimated Mr of 77,000 was predominant in the experimental gel (lane a), which was lacking in the control (lane b). The presence of the M₇,77,000 protein band was also seen in Fig. 2, lane a, where the affinity-purified anticaldesmon IgG was used. We next examined the ef-
confluent cell cultures (2 × 10⁶ cells on a 60-mm dish) were washed two times with 3 ml each of P₄/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 P₄/NaCl, the cells were incubated with 1 ml per dish of the same medium containing 125 μCi of [³⁵S]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 P₄/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% NaDodSO₄/10 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 dithiothreitol, 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% NaDodSO₄/10% 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 NaDodSO₄/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 [³⁵S]methionine. The lysates

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 anticaldesmon (affinity-purified protein) for 2 hr at 38.5°C, washed six times with 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% NaDodSO₄/10% 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 NaDodSO₄/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 [³⁵S]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. 5B), 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 Mr 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 (Mr 150,000/147,000) was excised from the NaDodSO₄ gel, on which chicken gizzard caldesmon had been electrophoresed, and transferred to nitrocellulose paper, the latter being used to affinity purify anticaldesmon. The Mr 77,000 protein was specifically immunoprecipitated with the affinity-purified anticaldesmon. Moreover, the binding of the Mr 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 [³⁵S]methionine, the Mr 77,000 protein was the only protein band on the NaDodSO₄ 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 Mr 150,000 on the NaDodSO₄ gel, although minor variations were observed among them (9). Gizzard and platelet caldesmons resolved into a doublet of Mr 150,000 and 147,000, whereas aorta and uterus caldesmons gave a single band of Mr 150,000 on the NaDodSO₄ gel. The possibility that the Mr 77,000 protein is a proteolytic fragment of the Mr 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 Mr 150,000/147,000 caldesmon and presence of Mr 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 Mr 150,000/147,000 caldesmon and Mr 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 Mr 77,000 protein as caldesmon77.

The intracellular distribution of the caldesmon77 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 caldesmon77 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 substrate (20-22). Therefore, it is attractive to assume that the calmodulin-caldesmon77 system plays a regulatory role in some of the functions of actin filaments in nonmuscle tissues. In support of this notion, we have found recently caldesmon77 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 Ca²⁺ concentration (unpublished results).

The cellular level of caldesmon77 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 caldesmon77 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 caldesmon77 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 Ca²⁺ 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.

We thank Miss Tomoko Nagasaka for typing the manuscript. This work was supported in part by grants from the Scientific Research Fund of Ministry of Education, Science and Culture, Japan, and Mitsubishi Science Foundation.