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Expression of high and low molecular weight caldesmons during phenotypic modulation of smooth muscle cells

(actin/myosin/calmodulin/mRNA/BC_3H1 cells)

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ABSTRACT **We investigated the expression of two molecular weight forms of caldesmon in a wide range of tissues and cells. The distribution of high molecular weight caldesmon (*h*-caldesmon, M_r 120,000–150,000) was restricted to smooth muscles where it was found in large quantity. The low molecular weight protein (*l*-caldesmon, M_r 70,000–80,000) was widely distributed in nonmuscle tissues and cells. Therefore, the expression of *h*-caldesmon might be much more specific to smooth muscles. We then examined the expressional changes of two caldesmons during phenotypic modulation of smooth muscle cells (SMCs). In developing gizzards, the expression of caldesmons switched from the *l*- to the *h*-form. Contrarily, the expression turned from *h*- to *l*-caldesmon in association with dedifferentiation of aortic SMCs in primary culture. In agreement with these observations, the levels of those mRNAs that direct the synthesis of both caldesmons were apparently in proportion to the quantities of protein, as determined by use of an *in vitro* translation system. In addition, *h*-caldesmon in smooth muscle-like BC_3H1 cells increased in its amount with a concomitant reduction of *l*-caldesmon following serum-depleted and contact-inhibited cytodifferentiation. These results suggest that the expressional changes of two caldesmons are closely correlated with the phenotypic modulation of SMCs.**

Caldesmon, which is composed of a doublet with M_r 150,000 and 147,000 subunits, was originally purified from chicken gizzard smooth muscle (1). Subsequently, a similar molecular weight form of caldesmon (M_r 120,000–150,000) was purified from or identified in smooth muscles (2–7). We have called this high molecular weight form of protein *h*-caldesmon (8, 9). It has an alternative binding ability to either calmodulin or actin filaments depending upon the concentration of Ca^{2+} (flip-flop binding) (1, 10). Accumulating evidence suggests that caldesmon is involved in smooth muscle contraction as an actin-linked regulatory protein (11–16). In addition, we have obtained evidence that it also regulates the interaction between the high molecular weight actin-binding protein (ABP or filamin) and actin in a Ca^{2+}- and calmodulin-dependent manner (17, 18). Recent studies have indicated the presence of low molecular weight caldesmon (*l*-caldesmon, M_r 70,000–80,000) in nonmuscle tissues and cells (8, 19–23). It possesses some features in common with *h*-caldesmon, including the flip-flop binding ability to either calmodulin or actin filaments (20, 24), the inhibitory action on the actin–myosin interaction (20, 24), and the immunological cross-reactivity (19–24).

In this report, we surveyed the distribution of both caldesmons. The distribution of *h*-caldesmon was much more specific for smooth muscles than nonmuscle tissues and cells. On the contrary, *l*-caldesmon was widely distributed in tissues and cells with the exception of smooth muscles. Using three different smooth muscle systems we found that the enhanced expression of *h*-caldesmon with a concomitant reduction of *l*-caldesmon expression is critical in the differentiation of smooth muscle cells (SMCs).

MATERIALS AND METHODS

Materials. ^{125}I-labeled protein A (30 mCi/mg of total protein A; 1 Ci = 37 GBq), [^{35}S]methionine (>1000 Ci/mmol), [^{3}H]thymidine (40–60 Ci/mmol) and ^{125}I-labeled α-bungarotoxin (>200 Ci/mmol) were purchased from Amersham. Oligo(dT)-cellulose and angiotensin II were obtained from Collaborative Research (Waltham, MA) and Peninsula Laboratories (San Carlos, CA), respectively.

Measurement of Caldesmons in Tissues and Cells. The purification of *h*- and *l*-caldesmons was carried out as described in refs. 1 and 20. The preparation of anti-*h*-caldesmon IgG from antiserum was performed as described (20). Gizzards were taken from embryos on days 7, 10, 13, 16, and 19, from chicks on day 3 after hatching, and from adult chickens. Gizzards and cultured cells were homogenized with 20 mM Tris·HCl, pH 7.5/mM dithiothreitol/1 mM EGTA/2 mM EDTA/0.1 M KCl/5 μg of leupeptin per ml/20 μg of trypsin inhibitor per ml. Each homogenate was treated with 2% $NaDodSO_4$ under reducing conditions and heated for 3 min in a boiling water bath. After centrifugation, the proteins in the supernatant were fractionated with $NaDodSO_4$/polyacrylamide gel electrophoresis and then transferred to nitrocellulose paper by the method of Towbin *et al.* (25). After overnight incubation in buffer A (phosphate-buffered saline containing 1% bovine serum albumin) the nitrocellulose blot was incubated with the antibodies (2.5 μg/ml) in buffer A for 1 hr, then washed three times with buffer A, and incubated with ^{125}I-labeled protein A (0.5 μCi/ml) in buffer A for 30 min at room temperature. After thorough washing in buffer A, the radioactive bands corresponding to the two caldesmons were cut out, and their radioactivities were assayed. In some experiments, the blots were exposed to Kodak X-Omat x-ray film with an intensifying screen for autoradiography.

Cell Culture. SMCs isolated from the aortic media of adult Sprague–Dawley rats were primarily cultured by the method of Chamley *et al.* (26). The cells were plated (2×10^4/cm^2) on 60-mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and were cultured at 37°C in humidified 5% CO_2/95% air. The responsiveness of cells to angiotensin II (10 μM) was monitored with sequential microphotographs.

BC_3H1 cells, BALB/c 3T3 cells, NRK (normal rat kidney) cells, BHK (baby hamster kidney) cells, MDCK (Madin–

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Abbreviations: *h*-caldesmon, high molecular weight (120,000–150,000) caldesmon; *l*-caldesmon, low molecular weight (70,000–80,000) caldesmon; SMCs, smooth muscle cells.

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Darby canine kidney) cells, and CHO-K1 (Chinese hamster ovary) cells were purchased from the American Type Culture Collection. BC_3H1 cells were grown in DMEM supplemented with 20% fetal calf serum. When necessary, transfer of the growing cells to a medium containing 1% fetal calf serum resulted in the arrest of cell division within 24 hr. The other cell lines were grown in DMEM supplemented with 10% fetal calf serum.

Isolation of RNA. Total cellular RNAs were prepared from gizzards (12-day-old chicken embryo gizzards and 3-day-old posthatched chick gizzards) and from aortic SMCs in culture by the guanidinium/cesium chloride method (27). The RNAs thus obtained were chromatographed on an oligo(dT)-cellulose column to prepare the poly(A)$^+$ RNA fraction.

Cell-Free Translation. The poly(A)$^+$ RNA fraction was translated in a micrococcal nuclease-treated rabbit reticulocyte lysate (28). The reaction mixture (final volume, 0.1 ml) consisting of 120 μCi of [^{35}S]methionine (>1100 Ci/mmol) and 8 μg of poly(A)$^+$ RNA (heated at 65°C for 5 min prior to addition) was incubated at 30°C. After 60 min of incubation, 5 mM unlabeled methionine and 10 vol of RIPA buffer [50 mM Tris·HCl, pH 8.2/0.15 M NaCl/1% Triton X-100/1% deoxycholate/0.1% $NaDodSO_4$/10 mM EDTA/50 μg of phenylmethylsulfonyl fluoride per ml/5 μg of leupeptin per ml/0.1 mM diisopropyl fluorophosphate and 1% (vol/vol) Trasylol] were added to stop the reaction. The mixture was then centrifuged at 6000 × *g* for 20 min at 4°C. The resulting supernatant was subjected to the immunoprecipitation assay as described (19). After $NaDodSO_4$/polyacrylamide gel electrophoresis, the radioactive protein bands were detected by fluorography with Kodak X-Gmatic R film.

Other Methods. Electrophoresis was performed on 8% polyacrylamide gels in the presence of 0.1% $NaDodSO_4$ (29).

RESULTS

Caldesmons in Tissues and Cells. Since the affinities of *h*- and *l*-caldesmon for the antibodies were different, we developed a method for the simultaneous measurement of both proteins. Fig. 1 shows the dose dependence of respective caldesmon binding to the antibodies with a technique of immunoblotting using ^{125}I-labeled protein A. The affinity of

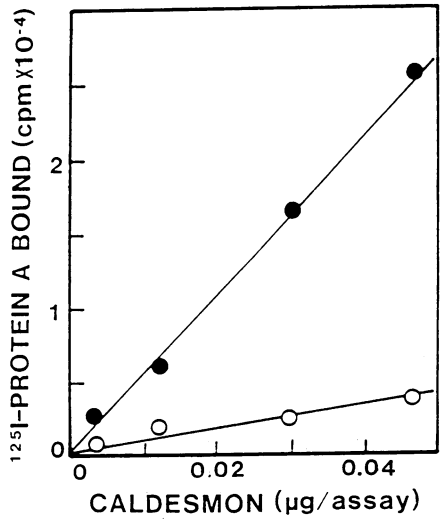

FIG. 1. Comparison of the affinities of *h*-caldesmon (●) and *l*-caldesmon (○) for the caldesmon antibodies. After $NaDodSO_4$/polyacrylamide gel electrophoresis of the known amounts of caldesmons, the bindings were performed by immunoblotting with the caldesmon antibodies and ^{125}I-labeled protein A (^{125}I-protein A). The details of assay procedure are as described.

h-caldesmon for the antibodies was 6-fold higher than that of *l*-caldesmon. Therefore, the amounts of the two caldesmons were corrected from the data of Fig. 1. Table 1 shows the distribution of the two proteins in tissues and cells as determined by this method. In the smooth muscles, *h*-caldesmon was detected in large quantities. *l*-Caldesmon was not detected in these smooth muscles. The content of *l*-caldesmon in nonmuscle tissues was much higher than that of *h*-caldesmon. In agreement with this result, vascular smooth muscles contained in visceral tissues were intensely stained with the caldesmon antibodies (30). The parenchymal cells of these visceral tissues, on the other hand, were only faintly stained. In cultured cells examined, *l*-caldesmon was predominantly present with a trace amount of *h*-caldesmon. These results suggest that the expression of *h*-caldesmon might be much more specific to smooth muscles than to nonmuscle tissues and cells.

Developmental Changes of Caldesmons and Other Cytoskeletal Proteins in the Gizzards. Morphological studies indicated that, during embryogenesis, the massive proliferation of mesenchymal cells causes an enlargement in the bulk of chicken embryo gizzards following differentiation of mesenchymal cells into SMCs (31). We compared the expression of caldesmons and other cytoskeletal proteins in developing chicken gizzards (Fig. 2). Each protein was identified by comigration of the purified protein with the $NaDodSO_4$ gels or by means of an immunoblotting technique using corresponding antibodies. *l*-Caldesmon, in addition to *h*-caldesmon, was simultaneously detected by immunoblotting (Fig. 2*B*). The quantitative analyses of cytoskeletal proteins in developing gizzard are summarized in Table 2.

Patterns of protein accumulation can be classified roughly into two groups. Actin and desmin already appeared in early embryos <7-day-old and increased gradually. In contrast, myosin and β-tropomyosin became detectable in 10-day-old embryo gizzards following abrupt increases. The amount of *h*-caldesmon increased in a manner similar to that of myosin and β-tropomyosin, with a concomitant decrease in the amount of *l*-caldesmon during development of gizzards. These developmental changes in cytoskeletal proteins including caldesmons are in good agreement with morphological differentiation of gizzard SMCs (31).

Expression of Caldesmons During Dedifferentiation of Aortic SMCs in Culture. The change from the contractile to

Table 1. Contents of caldesmons

Tissue or cells	*h*-Caldesmon, μM	*l*-Caldesmon, μM
Chicken gizzard	9.2	<0.06
Bovine		
Aorta (media)	3.0	<0.06
Adrenal medulla	0.04	0.82
Adrenal cortex	0.05	0.68
Anterior pituitary	0.03	0.44
Posterior pituitary	0.03	0.64
Rat		
Spleen	0.05	0.43
Brain	<0.01	0.25
Liver	0.03	0.78
Kidney	0.03	0.66
Cultured cells		
BALB/c 3T3	0.03	0.62
NRK	<0.01	0.35
BHK	<0.01	0.53
MDCK	<0.01	0.40
CHO-K1	<0.01	0.48

The contents of caldesmons were determined by immunoblotting with the caldesmon antibody and ^{125}I-labeled protein A. The details of the assay procedure are as described.

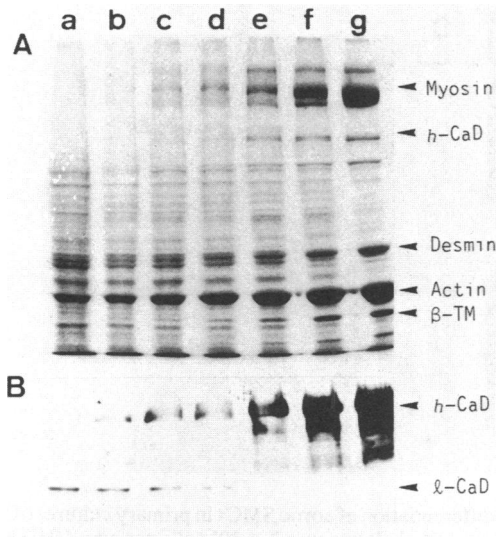

FIG. 2. (*A*) Accumulation of cytoskeletal proteins in developing gizzards. The whole homogenates (corresponding to 1 mg wet weight of gizzards) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue and destained. Lanes: a–e, embryonic chicken gizzards [7 days (lane a), 10 days (lane b), 13 days (lane c), 16 days (lane d), and 19 days (lane e)]; f and j, 3-day posthatched gizzard and adult gizzard, respectively. (*B*) Detection of caldesmons in developing gizzards. Autoradiograms of immunoreplica corresponding to developing gizzards in *A*. The positions of each cytoskeletal protein are indicated. *h*-CaD, *h*-caldesmon; β-TM, β-tropomyosin; ℓ-CaD, ℓ-caldesmon.

synthetic state of vascular SMCs is a characteristic of dedifferentiation in primary culture (26, 32). For example, angiotensin II (10 μM) induced a slow contraction of 2-day-cultured aortic SMCs. The maximum contraction was attained at 5 min after addition of angiotensin II (Fig. 3*C*). When cultured over 3 days, active DNA synthesis and cellular proliferation were initiated as determined by [^{3}H]-thymidine uptake and by cell count (Fig. 3*A*). The cells became confluent by 7–10 days and grew in multilayers, forming the typical hills-and-valleys pattern (26). However, these proliferative and confluent cells did not show any contractile response to angiotensin II and morphologically resembled fibroblasts.

We investigated the expression of caldesmons during dedifferentiation of aortic SMCs (Fig. 3*D*). In the contractile states, >90% of all caldesmons was detected as the *h*-form. In contrast, a dramatic decrease in the amount of *h*-caldesmon with a concomitant increase in that of ℓ-caldesmon was observed in the synthetic state.

Detection of Caldesmons Synthesized *in Vitro*. Poly(A)$^+$ RNAs from the gizzards of 12-day-old embryos and of 3-day-old chicks and from 2- and 7-day cultures of aortic SMCs were translated in cell-free mRNA-dependent reticulocyte lysates. The translational products were analyzed by immunoprecipitation with the caldesmon antibodies. When poly(A)$^+$ RNA from 12-day-old embryo gizzard was translated, both caldesmons were synthesized (Fig. 4, lane a). The ratio of ℓ-caldesmon RNA to *h*-caldesmon RNA reflects the protein levels of two caldesmons in the embryonic gizzards. When the RNA from 3-day-old posthatched gizzards was translated, *h*-caldesmon, but not ℓ-caldesmon, was synthesized (Fig. 4, lane b). These results are consistent with those in Fig. 2 and Table 1, showing that *h*-caldesmon is the sole protein that is immunologically detected by the caldesmon antibodies in the gizzards at this developing stage.

The RNA from a 2-day culture of aortic SMCs synthesized *h*-caldesmon in addition to a small amount of ℓ-caldesmon (Fig. 4, lane c). In contrast, the RNA from 7-day cultured cells programmed mainly the synthesis of ℓ-caldesmon (Fig. 4, lane d). Moreover, the RNA-directed synthesis of *h*-caldesmon was dramatically decreased. These results also indicate that the expression of the RNA levels in cultured aortic SMCs during phenotypic modulation corresponded to those of the protein levels.

Expression of Caldesmons in BC_3H1 Cells. The BC_3H1 mouse cell line has been demonstrated to possess many properties characteristic of SMCs. The smooth muscle-like properties are induced by serum-depleted growth arrest or contact inhibition (34–38). In our experiments, the growing cells supplemented with 20% fetal calf serum did not show the significant amount of acetylcholine receptor expression (0.056 pg per 10^2 cells) as was determined by the ^{125}I-labeled α-bungarotoxin-binding assay (34). Transfer of growing cells to medium supplemented with 1% fetal calf serum resulted in arrest of cell division following changes in cell shape, and enhanced expression of the acetylcholine receptor could be observed (1.33 pg per 10^2 cells).

We then examined the expression of caldesmons during differentiation of BC_3H1 cells. In growing cells, ℓ-caldesmon was mainly detected with a small amount of *h*-caldesmon (Fig. 5, lane a). After differentiation under conditions of serum depletion (Fig. 5, lanes c and d) and contact inhibition (Fig. 5, lane b), the amount of *h*-caldesmon expressed was increased 3- to 4-fold with a concomitant reduction of ℓ-caldesmon.

Table 2. Changes of cytoskeletal proteins in developing gizzards

Developing chicken	Proteins, μM					
	Myosin	*h*-Caldesmon	ℓ-Caldesmon	Desmin	Actin	β-Tropomyosin
Embryo						
7 days	ND	ND	0.9	31	219	ND
10 days	0.4	0.1	0.6	32	224	1.1
13 days	3.1	0.9	0.3	38	329	1.9
16 days	5.7	1.9	0.2	54	433	4.3
19 days	12.1	2.8	<0.06	84	609	12.5
Posthatched						
3-day chick	28.1	5.7	ND	123	731	39
Adult chicken	48	9.2	ND	193	970	76

The contents of cytoskeletal proteins with the exception of caldesmons were estimated from densitometric scans of the $NaDodSO_4$ gels in Fig. 1. Because our electrophoretic conditions did not entirely separate α-tropomyosin from actin, the actin content was expressed as a difference between the two scanning values corresponding to the area of actin and to that of β-tropomyosin. The concentrations of caldesmons were determined by immunoblotting with the antibody using ^{125}I-labeled protein A. ND, not detectable. The molecular weight values used were M_r 500,000 for myosin, M_r 300,000 for *h*-caldesmon dimer, M_r 150,000 for ℓ-caldesmon dimer, M_r 52,000 for desmin monomer, M_r 43,000 for actin monomer, and M_r 35,000 for β-tropomyosin.

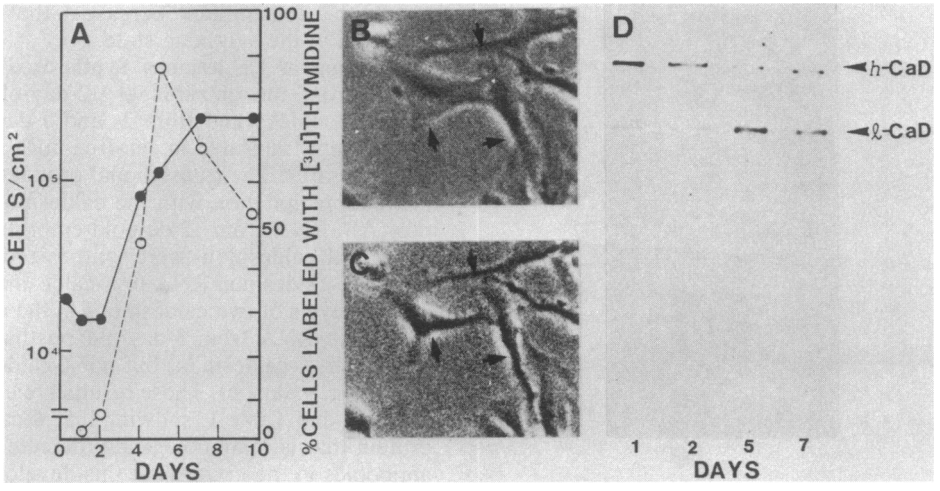

FIG. 3. Changes of *h*- and *ℓ*-caldesmon (*h*-CaD and *ℓ*-CaD) expression during dedifferentiation of aortic SMCs in primary culture. (*A*) Growth curve (●) and [^{3}H]thymidine uptake (○) of aortic SMCs in culture. The cell density for initial plating was 2×10^4 cells per cm^2. [^{3}H]Thymidine uptake into the cultured cells was determined by the method described in ref. 33. (*B* and *C*) Phase-contrast microphotographs of contractile response of 2-day cultured aortic SMCs before (*B*) and after (*C*) addition of angiotensin II (10 μM). (×290.) (*D*) Conversion of *h*-caldesmon to *ℓ*-caldesmon during dedifferentiation of aortic SMCs in culture. Autoradiograms are shown of the "immunoreplica" corresponding to aortic SMCs at the indicated culture days.

DISCUSSION

Several approaches were used to study the biochemical events following phenotypic modulation of SMCs, resulting in the following observations: (*i*) in differentiating smooth muscle, cytoskeletal proteins (myosin, actin, tropomyosin, and desmin) accumulate sequentially and in specific quantities (39); (*ii*) specific expression of isoactin depends on their phenotypic modulation at the protein and gene levels (39, 40); and (*iii*) cell surface acetylcholine receptors are increasingly synthesized following differentiation (34, 41). In spite of these laborious studies, the expression of smooth muscle-specific proteins are still not well understood.

Recent biochemical and immunocytochemical studies have led to the conclusion that caldesmon is distributed over a wide range of tissues and cells and is involved in contractile processes as an actin-linked regulatory protein (see the Introduction). Further studies have indicated the presence of two molecular weight forms of caldesmon that possess some features in common, resulting in a complication of the picture. Using three different systems, we first investigated the expression of caldesmons at the protein level following phenotypic modulation of SMCs. Based on our present results, it appears that the specific enhancement of *h*-caldesmon expression is closely related to differentiation of SMCs. In developing gizzards, aortic SMCs in culture, and smooth muscle-like BC$_3$H1 cells, the increase in quantity of *h*-caldesmon during differentiation was the counterpart of the increase in the expression of *ℓ*-caldesmon in association with dedifferentiation and vice versa.

In the cell-free translation experiments, it was demonstrat-

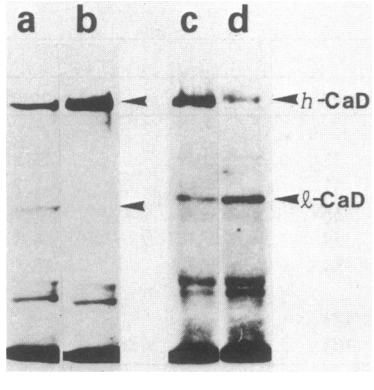

FIG. 4. *In vitro* translation of RNAs from gizzards and aortic SMCs in cell-free reticulocyte lysates. The details of the assay condition are as described. Lanes: a, 12-day-old embryo chicken gizzards; b, 3-day-old posthatched chick gizzards; c, 2-day cultured aortic SMCs; d, 7-day cultured aortic SMCs. *h*-CaD, *h*-caldesmon; *ℓ*-CaD, *ℓ*-caldesmon.

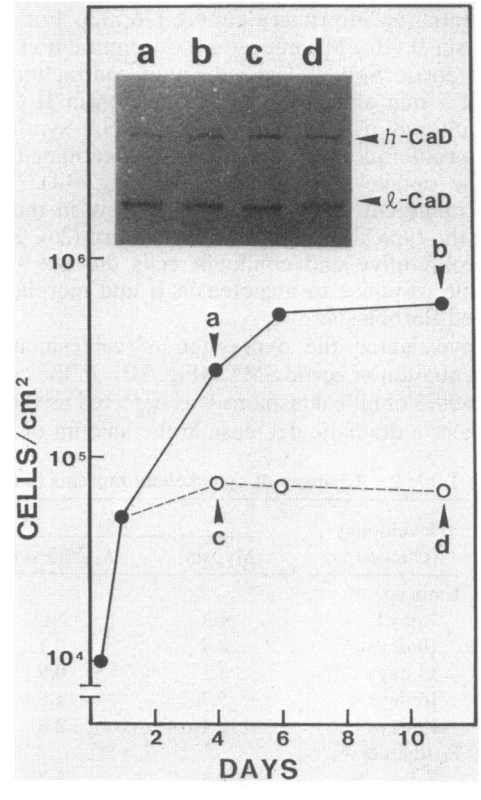

FIG. 5. Expression of caldesmons during phenotypic modulation of BC$_3$H1 cells. The cells were cultured in DMEM containing 20% fetal calf serum. On day 1, half of the cultures were refed with DMEM containing 1% fetal calf serum (○), and the remaining cultures were maintained with DMEM containing 20% fetal calf serum (●). (*Inset*) Autoradiograms are shown of the "immunoreplica" corresponding to the cells at the indicated culture days (arrowheads). *h*-CaD, *h*-caldesmon; *ℓ*-CaD, *ℓ*-caldesmon.

ed that the level of caldesmon RNAs in developing gizzards and aortic SMCs in culture directly reflected the level of caldesmons. These results suggest that the expression of two caldesmons is regulated at the level of RNA. However, we cannot completely exclude the possibility of a precursor–product relationship between *h*- and *l*-caldesmon or some other type of posttranslational modification. For example, specific proteases that cleave *h*-caldesmon into *l*-caldesmon and other products with modified activities might be present in an active form in the cell-free translation system. Nevertheless, such a situation is unlikely because, in our preliminary experiments, we identified two mRNAs of different molecular weights (5000- and 3000-nucleotide RNA-size classes) that direct the synthesis of *h*- and *l*-caldesmon, by RNA blot analysis using the hybridization of caldesmon cDNA probe (unpublished data). This observation supports an assumption that the expression of caldesmons during phenotypic modulation may be regulated at the level of mRNA. It is apparent that the enhanced expression of *h*-caldesmon results in the suppression of *l*-caldesmon expression and vice versa. Therefore, the syntheses of both caldesmons seem to be controlled under "a coordinate regulation."

As shown in this report, a trace amount of *h*-caldesmon was detected in some cultured cells examined. Bretscher and Lynch also have reported the presence of *h*-caldesmon in addition to *l*-caldesmon in several cultured cells (21). However, according to our results, any changes of caldesmon expression could not be observed in several cell lines in the growing and resting states with the exception of BC_3H1 cells (data not shown). Although the biological significance of *l*-caldesmon expression in nonmuscle cells is still unknown, at least the enhanced expression of *h*-caldesmon might be closely associated with differentiation of SMCs. As a result, *h*-caldesmon is a promising marker protein for studies on smooth muscle differentiation.

The functional differences between the two caldesmons are still unknown. In our separate experiments, we found that the two caldesmons had a common calmodulin- and actin-binding domain with similar molecular weights (M_r 32,000–35,000). If there are functional differences between the two caldesmons, the remaining domains in both proteins may be responsible for the different properties. The possible function of *h*-caldesmon in SMCs may be the involvement of the "latch" mechanism (42). In the future, it is important to determine whether or not the remaining domain of *h*-caldesmon has such a function.

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