

Overexpression of Human Fibroblast Caldesmon Fragment Containing Actin-, Ca⁺⁺/Calmodulin-, and Tropomyosin-Binding Domains Stabilizes Endogenous Tropomyosin and Microfilaments

Kerri S. Warren, Jenny L.-C. Lin, Dawn D. Wamboldt, and Jim J.-C. Lin

Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242-1324

Abstract. Fibroblast caldesmon is a protein postulated to participate in the modulation of the actin cytoskeleton and the regulation of actin-based motility. The cDNAs encoding the NH₂-terminal (aa.1-243, CaD40) and COOH-terminal (aa.244-538, CaD39) fragments of human caldesmon were subcloned into expression vectors and we previously reported that bacterially produced CaD39 protein retains its actin-binding properties as well as its ability to enhance low *M_r* tropomyosin (TM) binding to actin and to inhibit TM-actin-activated HMM ATPase activity in vitro (Novy, R. E., J. R. Sellers, L.-F. Liu, and J. J.-C. Lin. 1993. *Cell Motil. Cytoskeleton*. 26:248-261). Bacterially produced CaD40 does not bind actin. To study the in vivo effects of CaD39 expression on the stability of actin filaments in CHO cells, we isolated and characterized stable CHO transfectants which express varying amounts of CaD39. We found that expression of CaD39 in CHO cells stabilized microfilament bundles

as well as endogenous TM. CaD39-expressing clones displayed an increased resistance to cytochalasin B and Triton X-100 treatments and yielded increased amounts of TM-containing actin filaments in microfilament isolation procedures. In addition, analysis of these clones with immunoblotting and indirect immunofluorescence microscopy with anti-TM antibody revealed that stabilized endogenous TM and enhanced TM-containing microfilament bundles parallel increased amounts of CaD39 expression. The increased TM observed corresponded to a decrease in TM turnover rate and did not appear to be due to increased synthesis of endogenous TM. Additionally, the phenomenon of stabilized TM did not occur in stable CHO clones expressing CaD40. Therefore, it is likely that CaD39 can enhance TM's binding to F-actin in vivo, thus reducing TM's rate of turnover and stabilizing actin microfilament bundles.

CALDESMON is an actin-, myosin-, tropomyosin-, and Ca⁺⁺/Calmodulin-binding protein found on actin-based filaments of smooth muscle and nonmuscle cells (for reviews see Sobue and Sellers, 1991; Marston and Redwood, 1991; Matsumura and Yamashiro, 1993). Though the smooth muscle and nonmuscle forms differ in molecular weight due to the presence of a central repeat region in smooth muscle caldesmon (Hayashi et al., 1991; Sobue et al., 1988), they exhibit similar properties in vitro. These properties include the ability to bind myosin (Marston et al., 1992), to bind actin, and enhance tropomyosin (TM)¹ binding to actin, and to inhibit the ATPase activity of actomyosin (Sobue et al., 1988; Yamashiro-Matsumura and Matsumura, 1988; Sobue and Sellers, 1991; Marston and Redwood, 1991; Matsumura and Yamashiro, 1993), all in a manner that

is reversibly regulated by Ca⁺⁺/Calmodulin (Hayashi et al., 1991) and caldesmon's phosphorylation state (Yamashiro et al., 1990; Yamashiro and Matsumura, 1991; Mak et al., 1991). In addition, nonmuscle caldesmon has been shown to improve TM's ability to protect actin from gelsolin severing and capping (Ishikawa et al., 1989a,b). Even though these interactions have been well characterized in vitro, the functions of caldesmon in the living cell have yet to be assigned.

There are several reports, consistent with in vitro observations, which suggest in vivo roles for nonmuscle caldesmon in the modulation/stabilization of actin microfilaments, and in the regulation of intracellular granular movement and secretion. For example, Hegmann et al. (1991) demonstrated that microinjection of a monoclonal anti-caldesmon antibody, which interferes with caldesmon's ability to bind actin, drastically reduced intracellular granular movement in chick embryo fibroblasts. In pituitary gonadotrophs, the injection of an anti-caldesmon antibody, which disrupts caldesmon-actin interactions, led to a decrease in detectable F-actin bundles and an increase in hormone-stimulated release of gonadotropin (Janovick et al., 1991). Additionally, the glu-

Address all correspondence to Dr. Jim J.-C. Lin, Department of Biological Sciences, University of Iowa, Iowa City, IA 52242-1324.

1. *Abbreviation used in this paper:* TM, tropomyosin.

corticoid-stimulated increase of caldesmon expression in pituitary cells accompanied the actin filament stabilization responsible for the inhibition of leutenizing hormone release (Castellino et al., 1992). More evidence to suggest that non-muscle caldesmon affects microfilament stability and organization comes from reports that document mitosis-specific phosphorylation of caldesmon, and caldesmon's subsequent release from microfilaments during mitotic cytoskeletal remodeling (Yamashiro et al., 1990; Hosoya et al., 1993). Many transformed lines also show a reorganized, diminished actin cytoskeleton, and such transformed lines also demonstrate a reduction in caldesmon expression (Novy et al., 1991; Owada et al., 1984).

To directly study the function of human nonmuscle caldesmon, we have subcloned cDNAs encoding the NH₂-terminal fragment (CaD40, aa.1-243) and the COOH-terminal fragment (CaD39, aa.244-538) of human nonmuscle caldesmon into prokaryotic and eukaryotic expression vectors. As previously reported (Novy et al., 1993b), bacterially produced CaD39 protein, which contains actin, TM, and Ca⁺⁺/Calmodulin-binding domains (see Fig. 1), retained the abilities to bind actin, potentiate TM binding to actin, and to inhibit actin-TM-activated myosin ATPase activity. Bacterially made CaD40 protein displayed no actin binding. The difference in actin-binding ability between the two CaD fragments was also seen in *in vivo* experiments with DNA transfections, and, in this paper, we report that expression of CaD39 in CHO cells, via stable transfection, stabilizes endogenous TMs and actin microfilament bundles.

Materials and Methods

Plasmid DNA Constructs

cDNAs encoding the NH₂-terminal fragment (CaD40, aa.1-243) and COOH-terminal fragment (CaD39, aa. 244-538) of human fibroblast caldesmon (see Fig. 1, Novy et al., 1991) from pETCaD40 and pETCaD39, respectively (Novy et al., 1993b), were subcloned into the XbaI site of the eukaryotic expression vector pCB6hx, a modified version of pCB6 (kindly provided by Dr. M. Stinski, The University of Iowa). The modification removed a DNA sequence from the polylinker region that contained an ATG predicted to cause unwanted translation initiation. The pCB6hx expression vector contains the neo^r gene to allow for selection in the presence of G418, a synthetic neomycin. Recombinant CaD fragment-containing plasmids are referred to as pCBCaD39 and pCBCaD40.

Cell Culture and Transfection

CHO cells were grown in DMEM plus 10% FCS and were kept in a 37°C humidified incubator with 5% CO₂. DNA transfection was performed via calcium phosphate coprecipitation, as described (Lourim and Lin, 1992). Plasmid DNA was prepared with Qiagen columns according to manufacturer's direction (Qiagen Inc., Chatsworth, CA).

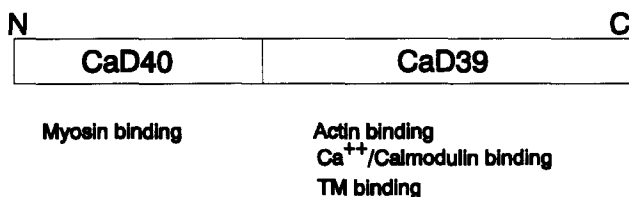


Figure 1. Schematic cartoon of human fibroblast caldesmon fragments CaD40 and CaD39, including the proposed binding sites residing in each fragment. (for detail see Matsumura and Yamashiro, 1993.)

For transient transfections, cells were assayed by immunofluorescence at 18–24 h after transfection. For the establishment of stably expressing CaD39 and CaD40 lines, cells at one day after transfection, were selected for in DMEM plus 10% FCS with 500 µg/ml G418 (GIBCO BRL Gaithersburg, MD). After 2 wk of selection, single cells were cloned, expanded, and tested for CaD39 or CaD40 expression. Stable lines 39L12, 39C11, 39C15 and 40C11, 40C22, 40C7, and 40C17, expressing low to high amounts of CaD39 or CaD40, respectively, were obtained for use in this study. Nonexpressing, G418-resistant lines 39C5 and 40N2 were isolated for use as controls.

Monoclonal Antibodies

Anti-TM5 tropomyosin antibody CG3 and anti-caldesmon antibody C21 were prepared and characterized previously (Lin et al., 1985, 1988, 1991). C21 recognizes an epitope in the COOH-terminal half of caldesmon that is highly conserved between human, rat, and chicken caldesmon (Novy et al., 1991). C21 cross-reacts with both CaD39 and endogenous CHO caldesmon, although the naturally low levels of endogenous CHO caldesmon allowed detection of CaD39 alone when 1,000-fold dilutions of C21 ascites fluid were used for immunofluorescence microscopy of transfected cells. For detection of endogenous caldesmon, C21 was used at 250-fold dilution. CaD40-specific monoclonal antibody C57 was generated for use in this study by immunizing mice with purified bacterially produced CaD40 protein. Generation of C57 followed procedures described (Lin et al., 1985). C57 antibody was tested for CaD40-immunospecificity by ELISA, Western blotting, and immunofluorescence. C57 does not cross-react with CHO caldesmon. C21 and C57 are IgG1 class; CG3 is an IgM class antibody and its epitope has been mapped to aa #29-44 of human TM5 (Novy et al., 1993b). Broad species-specific polyclonal anti-caldesmon was a generous gift of Dr. A. Bretscher, Cornell University (see Bretscher and Lynch, 1985).

Immunofluorescence Microscopy

Cells were prepared for indirect immunofluorescence as described (Warren and Lin, 1993). Primary antibodies CG3, C21, C57, and polyclonal anti-caldesmon were described above. The secondary antibodies used included FITC-conjugated goat anti-mouse IgG (γ chain specific, Sigma Chem. Co., St. Louis, MO), TRITC-conjugated goat anti-mouse IgM (μ chain specific, Cappel, Durham, NC), Lissamine-rhodamine-conjugated goat anti-mouse IgG (heavy and light chain, Boehringer Mannheim, Indianapolis, IN), and rhodamine-conjugated goat-anti-rabbit IgG (heavy and light chain, Cappel, Durham, NC). Cells were observed and photographed using a Zeiss epifluorescence photomicroscope III.

Cytochalasin B Treatment

Cells were grown to ~80% confluency on glass coverslips. Treatment with cytochalasin B involved exposure to 1.0, 1.25, or 1.5 µg/ml cytochalasin B for 30 min at 37°C. Cytochalasin B was solubilized in DMSO before addition to the culture dishes, so a DMSO-treated control group of cells were also prepared. Cells were then fixed, acetone permeabilized, and stained with CG3 to visualize the remaining TM-containing microfilament bundles.

Western Blot and SDS-Polyacrylamide Gel Electrophoresis

Cells at 90% confluence were rinsed three times in wash solution (PBS, containing 5 mM MgCl₂ and 0.1 mM EGTA), and then harvested in a small volume of 2× gel sample buffer (0.1 M DTT, 2% SDS, 0.08 M Tris pH 6.8, 15% glycerol, 0.006% bromophenol blue) and heated for 3 min at 100°C. Western immunoblotting, after protein resolution by 12.5% SDS-PAGE, was performed as described (Lin et al., 1985). The antibodies used C21, C57, and CG3 were described above. Amido black staining was used to determine relative protein loading.

Pulse Labeling and Two-Dimensional Gel Electrophoresis

Cells cultured to 85–90% confluency were labeled for 4 h with [³⁵S]methionine (100 µCi, 1100 Ci/mmol, Amersham Corp., Arlington Heights, IL) in methionine-free DMEM and 2.5% FCS, and then harvested in 2× gel sample buffer. Samples were subjected to two-dimensional gel electrophoresis as described (Lin et al., 1984). Gels were dried and exposed to

Table I. Level of CaD39 Fragment Expression in Selected CaD39 Cell Lines

Cell lines	% of total cell extract		Molar Ratio
	CHOCaD	CaD39	CaD39:CHOCaD
39C5	0.0080	0.0000	0.0
39L12	0.0080	0.0640	16.3
39C11	0.0086	0.3100	73.4
39C15	0.0080	0.3100	78.9

Autoradiographs from C21 immunoblots of purified CaD39 protein standards and known quantities of CaD39 cell extracts were analyzed using Image-1/AT software. Data are from a representative experiment, one of two. The M_r s of 33.4 Kd and 68 Kd for CaD39 and CHOCaD, respectively, were used to calculate molar ratios.

X-OMAT autoradiography film (Eastman Kodak Co., Rochester, NY) for 3 d.

Pulse-Chase Experiments

39C5 and 39C15 cells were pulse-labeled for 25 min with 100 μ Ci of [35 S]methionine in methionine-free DMEM with 2.5% FCS. To begin the chase, the labeling media was quickly replaced with DMEM media containing excess methionine (3 mg/ml) and 10% FCS. Samples were harvested in 2 \times sample buffer as described above at 0, 2, 6, 10, 14, and 22 h after starting the chase. 2D gel electrophoresis and fluorography were performed as described in Lin et al., 1984, and gels were then dried on paper and exposed to X-OMAT autoradiography film (Eastman Kodak Co., Rochester, NY).

Intensity Analysis with Image-1/AT Software

Images were obtained with a Hamamatsu CCD camera, model XC77, with camera controller C2400 (Hamamatsu, Hamamatsu-city, Japan) and analyzed using Image-1/AT image processing and analysis system, version 4.13 (Universal Imaging Corporation, West Chester, PA). After autoradiograph images were acquired, hand-traced regions representing 1D gel bands or 2D gel spots were measured for area enclosed and average gray value. The product (area \times average gray value) minus the background gray value gave the relative intensity value.

For the C21 immunoblot data shown in Table I, autoradiograph bands from known amounts of purified, bacterially produced CaD39 (Novy et al.,

1993) and their corresponding band intensity values were used to construct a standard curve against which the CaD39 cell extract caldesmon and CaD39 autoradiograph bands were measured. For the pulse-chase experiments graphed in Fig. 8, the gel spots corresponding to TM4, TM5, and a control protein were traced and analyzed for intensity values. The values for TM4 and TM5 were plotted against chase time.

Other Procedures

The TM-enriched microfilaments were isolated from various CaD39-expressing cells (39L12, 39C11, and 39C15) as well as the non-expressing cells (39C5), as previously described (Lin et al., 1984). The yield of isolated microfilaments was estimated from [35 S]methionine-labeled cells, according to the previously established method (Lin et al., 1984), and expressed as the actin content. The probes for various tropomyosin isoforms, and the procedure used for Northern blot analysis were described previously (Novy et al., 1993a).

Results

Force-expressed CaD39 Localizes to Stress Fibers and Membrane Ruffle Regions in CHO Cells

To determine if human caldesmon fragments CaD39 and CaD40 could display, *in vivo*, the same binding properties seen with bacterially produced fragments *in vitro*, we transiently transfected CHO cells with recombinant plasmids pCBCaD39 or pCBCaD40. At 24 h after transfection, the force-expressed CaD39 and CaD40 proteins were visualized in the cells with monoclonal antibodies C21, specific to an epitope in the COOH-terminal half of caldesmon, and C57, NH₂-terminal specific antibody. Consistent with *in vitro*-based predictions, immunofluorescence microscopy revealed that CHO-expressed CaD39 protein is found on stress fibers and ruffle regions. This localization is seen irrespective of the level of expression. Fig. 2 shows the staining pattern of CaD39 in transfected cells from two different fields. Note that cytoplasmic staining is also seen in cells expressing high amounts of CaD39 (Fig. 2 C), perhaps due to a saturation

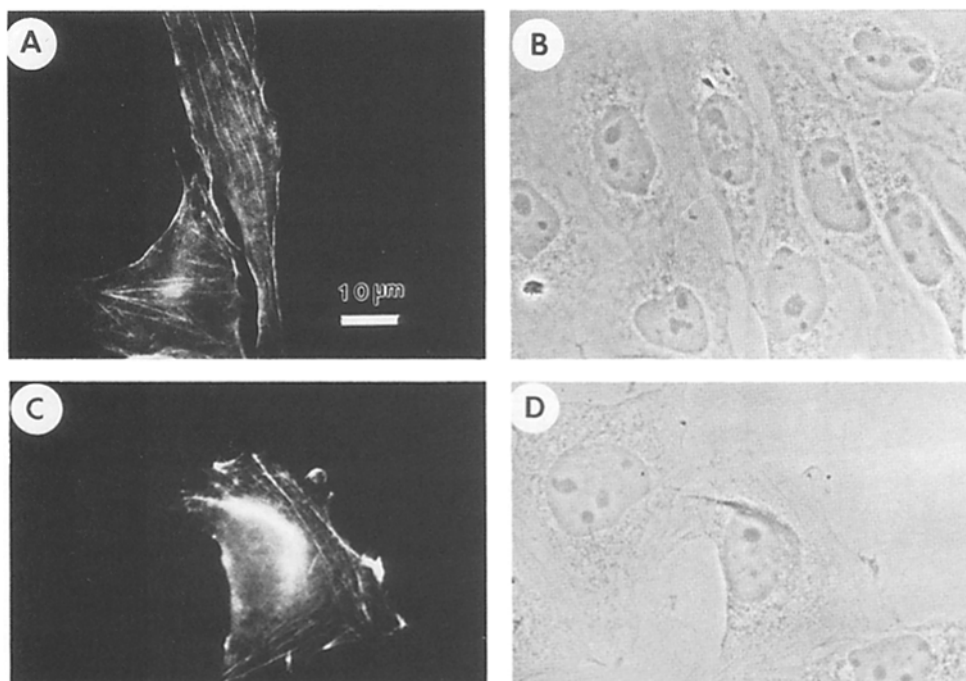


Figure 2. Transient transfection of CHO cells with pCBCaD39. Immunofluorescence micrographs of CHO cells, taken 24 h after transient transfection with pCBCaD39 (A and C). The corresponding phase micrographs appear in B and D. Force-expressed CaD39 localizes to stress fibers, edges, and ruffle regions, and, when expressed in high amounts, also distributes within the cytoplasm (C). C21 antibody, which cross-reacts strongly with CaD39 protein and weakly with endogenous caldesmon, was used at a high dilution to ensure detection of CaD39 alone. The non-reactive cells in each field serve as a C21 specificity control. Bar equals 10 μ m.

of available microfilament-binding sites. The untransfected cells in the micrographs serve as controls to show that C21 antibody, although it recognizes both CaD39 and CHO caldesmon at low dilution, can be suitably used at 1,000 \times dilution to detect CaD39 expression alone.

Also in agreement with predictions, CaD40 expression in CHO cells did not result in a stress fiber staining pattern. Fig. 3 immunofluorescence micrographs show transiently transfected CHO cells which express CaD40 in low (Fig. 3 A), medium, or high (Fig. 3 C) amounts. CaD40, which does not possess actin or TM-binding sites, distributed throughout the cytoplasm and appeared to concentrate in the nucleus. The nuclear staining may be a nonspecific consequence of CaD40's small size, but, interestingly, CaD40 sequence does contain several stretches of positively charged amino acid residues, which may serve as a nuclear localization signal (for sequence detail, see Novy et al., 1991). Western blot analysis of total cell extracts, harvested after transient transfection, revealed C21-positive (CaD39) and C57-positive (CaD40) bands at the expected positions after gel electrophoresis (data not shown), indicating that CaD39 and CaD40 are being expressed in their entirety in these transfection experiments.

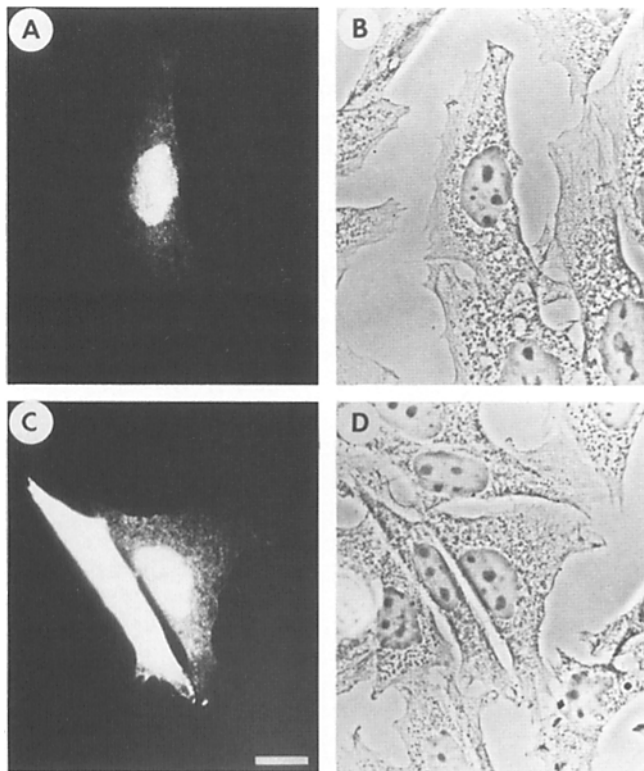


Figure 3. Transient transfection of CHO cells with pCBCaD40 resulted in a nuclear and cytoplasmic distribution of CaD40 protein, as detected by C57 antibody. A and C show immunofluorescence micrographs of CaD40 expression 24 h after transfection. The corresponding phase micrographs are B and D, respectively. Note that there is no apparent association with microfilament bundles in the transfected cells expressing low (A) medium or high (C) levels of CaD40. Bar equals 10 μ m.

High Amounts of CaD39 and CaD40 Are Expressed in Stable CHO Cell Lines

To better examine the consequences of CaD39 and CaD40 expression in CHO cells, we generated stable lines of clones expressing either fragment. After drug selection, single cell isolation, and expansion, we tested the cell lines for expression level by Western blot analysis, and for localization via immunofluorescence microscopy. The immunofluorescence distribution of expressed CaD39 or CaD40 in the cells from stable clones was the same as described for the transient transfection experiments (see Fig. 2 for CaD39 and Fig. 3 for CaD40). Both sets of stable lines have morphologies indistinguishable from parent CHO cells. Of the stable CaD39 and CaD40 lines examined, there were no apparent expression-related changes in cell shape, size, or overall microfilament organization (see Fig. 9 for CaD39 lines).

A quantitative analysis of CaD39 line expression levels, relative to endogenous caldesmon and to each other, is shown in Table I. Known quantities of CaD39 line total cell extracts and purified CaD39 protein standards were subjected to Western blot analysis with C21 antibody. Autoradiograph band intensities were obtained using Image-1/AT image analysis software, and protein amounts were quantified using a standard curve constructed from the purified CaD39 band intensities. The Table I results show that all stable lines express very low levels of CHO caldesmon, that clones 39L12, 39C11, and 39C15 express 16.3, 73.4, and 78.9 times more CaD39 than endogenous caldesmon, respectively, and that 39C5, our non-expressing, G418-resistant control expresses no CaD39. Monoclonal antibody C21 was used in this analysis because it recognizes an epitope, aa. 480-529 of human fibroblast caldesmon, that is conserved with 96% identity between human, rat and chicken caldesmon (Novy et al., 1991), and C21 does recognize CHO caldesmon to the same extent that it recognizes CaD39. To confirm that the differences seen between endogenous caldesmon levels and expressed CaD39 were not due to properties peculiar to C21, we used polyclonal anti-caldesmon antibodies (Bretscher and Lynch, 1985) to stain control and CaD39-expressing cells (data not shown). A Western blot demonstrating the negative, low, medium-high, and high levels of CaD39 expression in the stable lines is shown in Fig. 4 B: 39C5 (lane 1), 39L12 (lane 2), 39C11 (lane 3), and 39C15 (lane 4). The relative levels of CaD40 expression in the selected CaD40 stable clones were also analyzed by immunoblotting. Antibody C57 (Fig. 5 A) was used to detect the CaD40, and clones 40N2 (lane 5), 40C11 (lane 4), 40C22 (lane 2), 40C7 (lane 3), and 40C17 (lane 1) were chosen to represent negative control, low, medium, medium-high, and high CaD40 levels. The purified, bacterially produced CaD40 fragment, included to demonstrate CaD40 size and C57 immunospecificity, is shown in Fig. 5 A (lane pETCaD40).

Low M, Tropomyosin Levels Increase with Increasing Levels of CaD39 Expression

When immunoblots of stable CaD39 lines (Fig. 4 C) and CaD40 lines (Fig. 5 B) were probed with CG3 for endogenous TM5, an interesting phenomenon was observed. Fig. 4 demonstrates that CaD39 lines contain an increased amount of detectable TM that roughly parallels the amount

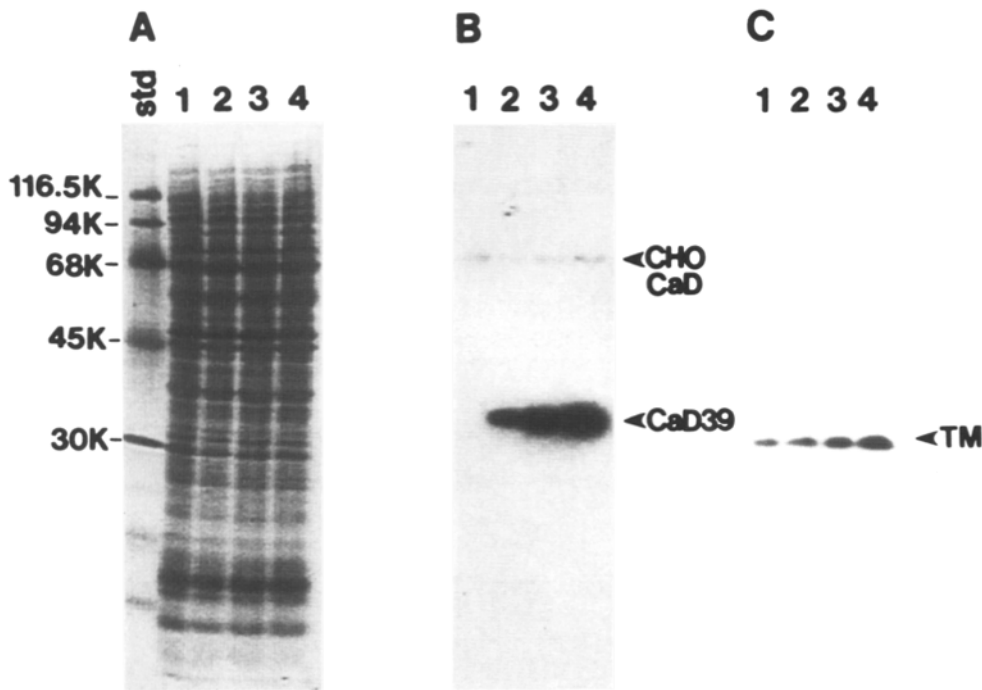


Figure 4. Western blot analysis of CaD39 lines 39C5 (lane 1), 39L12 (lane 2), 39C11 (lane 3), and 39C15 (lane 4) was done in duplicate to reveal the steady state levels of CaD39 and endogenous CHO caldesmon with C21 antibody (B) and the level of endogenous low M_r TMs with CG3 antibody (C). There is no expression of CaD39 in 39C5 cells (B, lane 1) and increasing levels of CaD39 expression in 39L12 (lane 2), 39C11 (lane 3), and 39C15 (lane 4). Notice that the amount of TM detected is increased in the CaD39 lines, roughly parallel to CaD39 expression levels (compare to lanes 1-4 of panel C). The amido-black-stained panel A shows the total protein loadings for the corresponding Western blots. C21 recognition of endogenous caldesmon in panel B reveals that there is no apparent change in caldesmon levels (panel B, lanes 1-4). CHO CaD; CHO endogenous caldesmon.

of CaD39 expressed (compare the lanes in panel 4 B with the corresponding lanes in panel 4 C). This is not seen in CaD40-expressing lines (Fig. 5) where steady-state levels of TM5 are unchanged across all four CaD40 lines and the negative control (compare the anti-CaD40 blot in Fig. 5 A

to the anti-TM panel in Fig. 5 B). The increase in endogenous TM5 levels, therefore, appears to be specific to CaD39 expression. Additionally, CaD39 or CaD40 overexpression does not appear to have an effect on CHO caldesmon as levels of endogenous caldesmon are unaltered in stable lines ex-

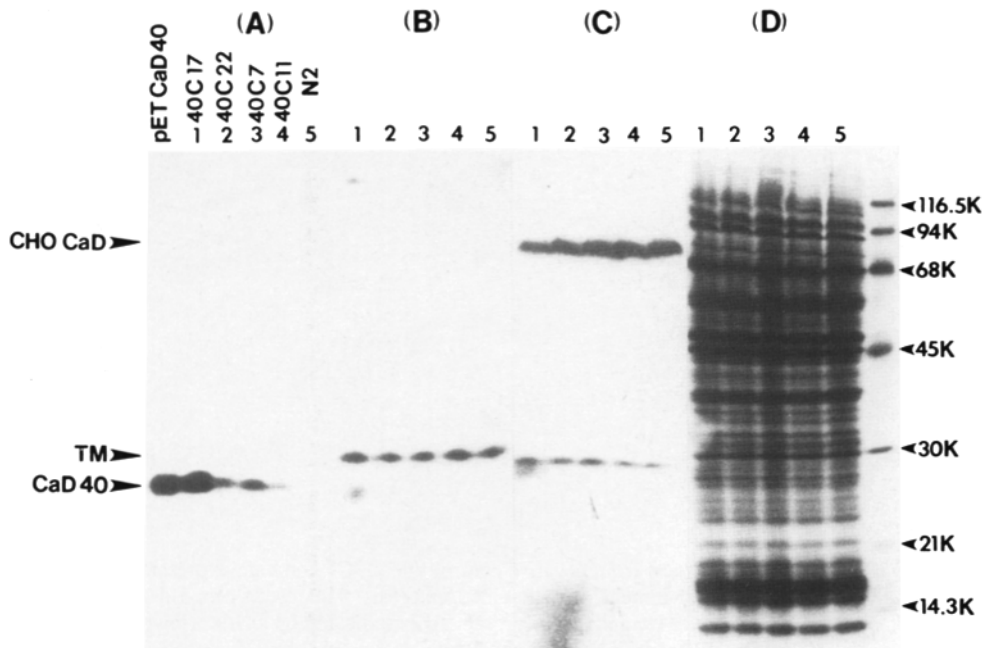


Figure 5. Western blot analysis of CaD40-expressing lines 40C17, 40C22, 40C7, and 40C11 as well as negative control line 40N2 and purified, bacterially expressed CaD40 fragment, pETCaD40 is shown. The blots were probed with C57 antibody for CaD40 (A), CG3 antibody for endogenous low M_r TM (B), and C21 (250X dilution) for endogenous caldesmon (C). The amido black-stained panel shows the relative total protein loaded. Note that endogenous TM and caldesmon levels do not change relative to CaD40 expression in the CaD40 lines (compare lanes in panels B and C to the same lanes in panel A). The fast migrating band recognized by C21 in panel C is a degradation product of caldesmon. CHO CaD; endogenous CHO caldesmon.

pressing CaD39 (Table I and Fig. 4 B) or CaD40 (Fig. 5 C). The fast-migrating species recognized by C21 in Fig. 5 C is a degradation product of CHO caldesmon often detectable in long exposures.

CaD39-Specific Increase in TM Steady-State Levels Is Not Due to Increased TM Synthesis

A comparison of the pulse-labeled protein profiles for all four CaD39 lines revealed no differences in the amount of low M_r TM protein synthesized during the 4 h label incorporation. Fig. 6 shows the result of two-dimensional gel electrophoresis on total extracts from [35 S]methionine-labeled negative control cell line, 39C5 (Fig. 6 A), and high-expressing line 39C15 (Fig. 6 B). The two small arrowheads indicate the positions of endogenous low M_r TM isoforms, TM4 and TM5. The identification of TM4 and TM5 was based on two-dimensional gel immunoblotting and two-dimensional gel analysis of heat-resistant microfilament fractions (data not shown). This data suggests that increased steady-state levels of TM detected by immunoblotting (Fig. 4 C) are due to accumulation of protein rather than an increase in TM production. We also examined the message levels of TM4 and TM5 in CaD39-expressing lines by Northern blot analysis. Again, no differences in TM mRNA levels were detected (data not shown), adding to the suggestion that TM protein is stabilized and accumulates in CHO cells which express CaD39.

CaD39 Expression Decreases the Rate of Low M_r TM Protein Turnover

To more directly address the question of whether TM protein is stabilized in CaD39-expressing cells, we performed pulse-chase experiments on stable CaD39 cell lines. Cells

from non-expressing 39C5 and high-CaD39-expressing 39C15 lines were pulse-labeled 25 min, and then chased for 0, 2, 6, 10, 14, and 22 h before harvesting. The [35 S]methionine-labeled total cell extracts were separated by two-dimensional gel electrophoresis, processed for fluorography, and the corresponding autoradiographs were analyzed for gel spot intensity (see Materials and Methods). Fig. 7 shows the result of plotting gel spot intensity values against the chase time for TM4 (A) and TM5 (B). A comparison of the plots from 39C5 and 39C15 reveals a significant decrease in the protein turnover rates for both TM4 and TM5 in the CaD39-expressing cells. The estimated 39C5 half lives for TM4 (10 h) and TM5 (5 h) are significantly different from TM half lives seen in 39C15 cells (22 h for TM4 and 17 h for TM5). Similar results were obtained from multiple two-dimensional gel runnings for a single pulse-chase experiment, as well as with a separate pulse chase experiment which used a shorter pulse-labeling period. The turnover rate for a randomly selected control protein was not different in 39C5 and 39C15 lines (data not shown).

CaD39 Expression Stabilizes Microfilaments

To investigate the consequence of overexpression of CaD39 and elevation of endogenous TM levels on the fate of microfilaments, TM-enriched microfilament fractions were isolated from CHO cells (Fig. 8 A) and the CaD39 clones (Fig. 8 B) by a previously developed method (Lin et al., 1984). The results of a microfilament isolation from CHO cells are shown in Fig. 8 A, a Coomassie blue-stained gel of the microfilament fraction (lane 1), the heat-treated supernatant (lane 2), and the heat-treated pellet (lane 3). Note that CHO cells contain low M_r TM isoforms, TM4 and TM5, but not high M_r isoforms, TM2 or TM3. They do possess a

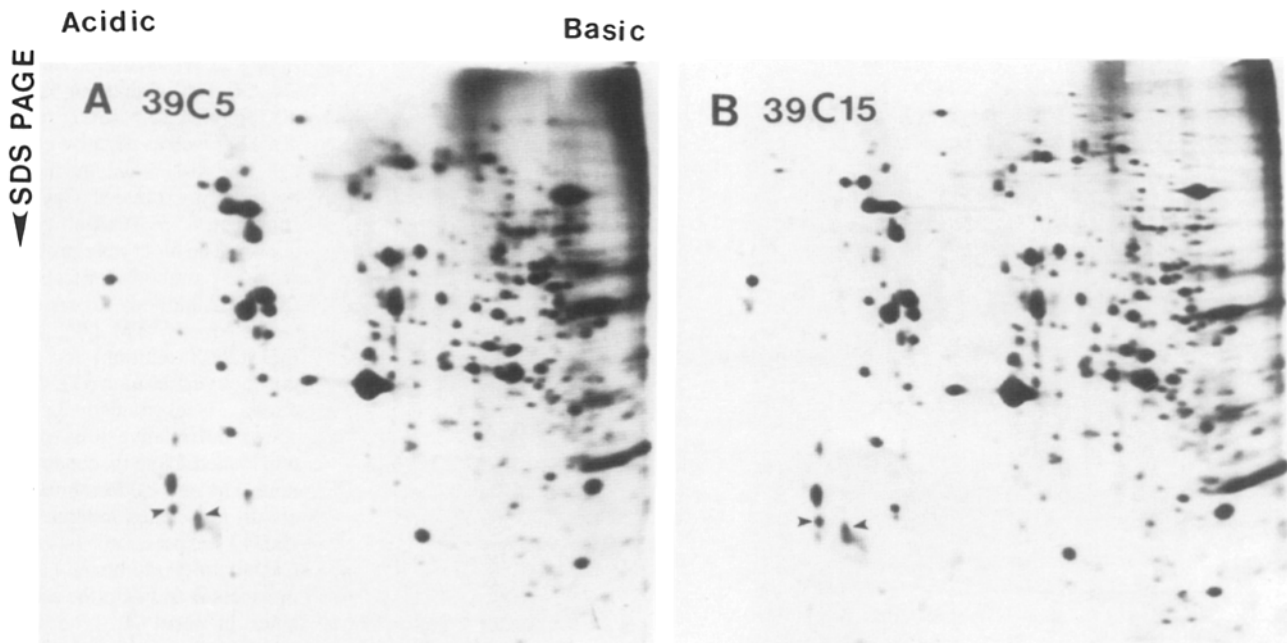


Figure 6. Two dimensional gel analysis of 35 S-labeled total cell extracts from negative control line, 39C5 and high expression line, 39C15. Cells were labeled for 4 h, and then harvested and subjected to two-dimensional gel electrophoresis. The results of a 3-d autoradiograph exposure for each are shown. The amount of low M_r TMs, CHO TM4, and TM5 (protein spots indicated by small arrowheads), synthesized during incubation with 35 S-label, does not appear to be different in the two cell lines.

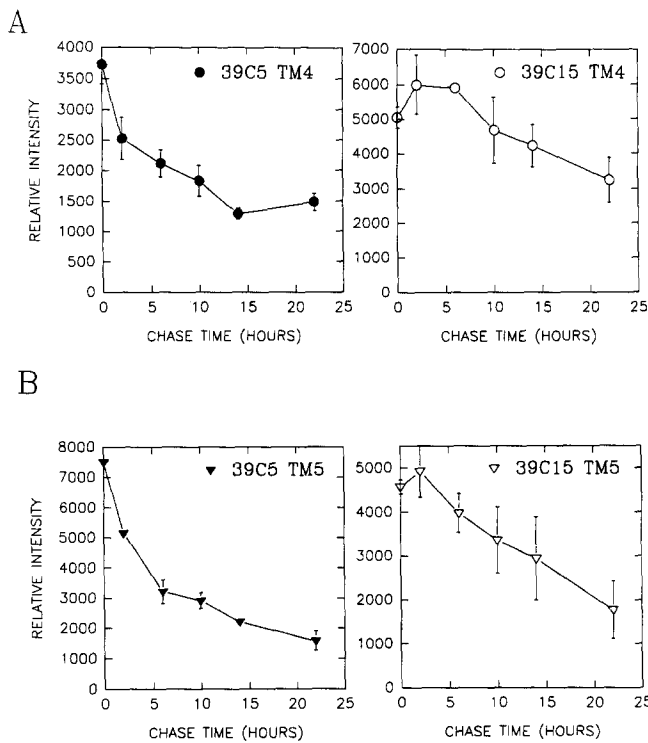


Figure 7. Pulse-chase analysis of low M_r TM turnover rates in 39C5 and 39C15 cell lines. Cells from non-expressing 39C5 and high-CaD39-expressing 39C15 lines were pulse-labeled 25 min with [35 S] methionine, and then incubated in chase media before harvest. Cell extracts from six chase time points were subjected to two-dimensional gel electrophoresis and the autoradiographs were analyzed for relative gel spot intensity. The graphs demonstrate the TM4 (A) and TM5 (B) spot intensity values over chase time for a representative experiment, one of two. Note the decreased turnover rate for both low M_r TMs in the CaD39-expressing 39C15 cells.

protein that has the apparent size (indicated with an asterisk) of a TM1, but this protein does not show heat-stability, a characteristic property of TM (compare Fig. 8 A; lanes 1 and 2). To quantify the microfilaments from the CaD39 lines, cells were labeled in vivo for 16 h with [35 S]methionine before isolation of TM-enriched microfilaments. Aliquots of samples from each step of the isolation procedure were analyzed on SDS-PAGE. The actin bands of each fraction were cut out and their radioactivity quantified by a liquid scintillation counter. The yield of isolated microfilaments was calculated and expressed as percent of total actin. As can be seen in Table II, high-expressing cells, 39C11 and 39C15, appeared to have significantly higher amounts of TM-enriched microfilaments. This increase is also apparent in the Fig. 8 B autoradiograph from SDS-PAGE resolution of microfilament fractions from 39C5 (lane 1), 39L12 (lane 2), 39C11 (lane 3), and 39C15 (lane 4). These data suggest that the stabilized, endogenous TM, together with overexpressed CaD39 protein, can further stabilize microfilaments inside the cell. On the other hand, the CaD39-stabilized microfilament may protect endogenous TM from turnover, thereby resulting in CaD39-expressing clones which contain more endogenous TM.

Table II. Amounts of TM-enriched Microfilaments Isolated from Various CaD39 Expressing Cell lines

Cell lines	% of total actin in TM-enriched microfilaments
39C5	7.60
39L12	7.92
39C11	11.17
39C15	11.30

Amounts of [35 S]methionine incorporation into the actin band of each fraction were used for calculation of percent. The data are from a representative experiment, one of two.

Resistance to Cytochalasin B Disruption Parallels CaD39 Expression

Cytochalasins are fungal toxins which can shift the overall microfilament organization in treated cells from a stress fiber network, to short fragments in focal accumulations. Cytochalasins act by binding actin filament barbed ends, thus inhibiting filament growth and also, possibly, filament inter-

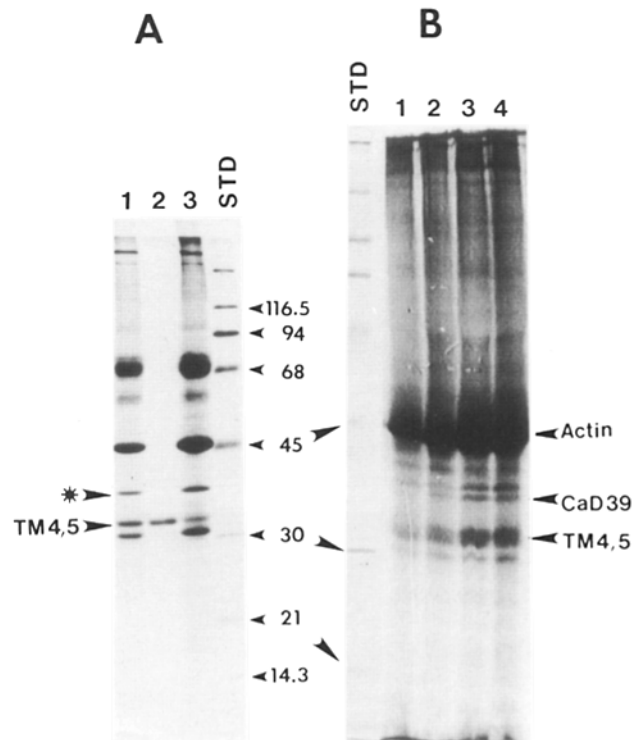


Figure 8. TM-enriched microfilaments were prepared from CHO cells (A) and CaD39 lines (B). The Coomassie blue-stained gel from the CHO microfilament fraction (A, lane 1), the heat-treated supernatant (lane 2) and the heat-treated pellet (lane 3) indicates the presence of microfilament-associated low M_r TM isoforms, TM4 and TM5, as well as a protein (*) which has the apparent M_r of a TM1, but is not heat-resistant (compare lanes 1 and 2). CHO cells do not have high M_r TM isoforms, TM2 or TM3. The autoradiograph shown in B is from [35 S]methionine-labeled microfilament fractions from 39C5 (B, lane 1), 39L12 (lane 2), 39C11 (lane 3), and 39C15 (lane 4) cells. Note the increase in actin, TM4 and TM5, that roughly parallels the amount of CaD39 present in these microfilament fractions.

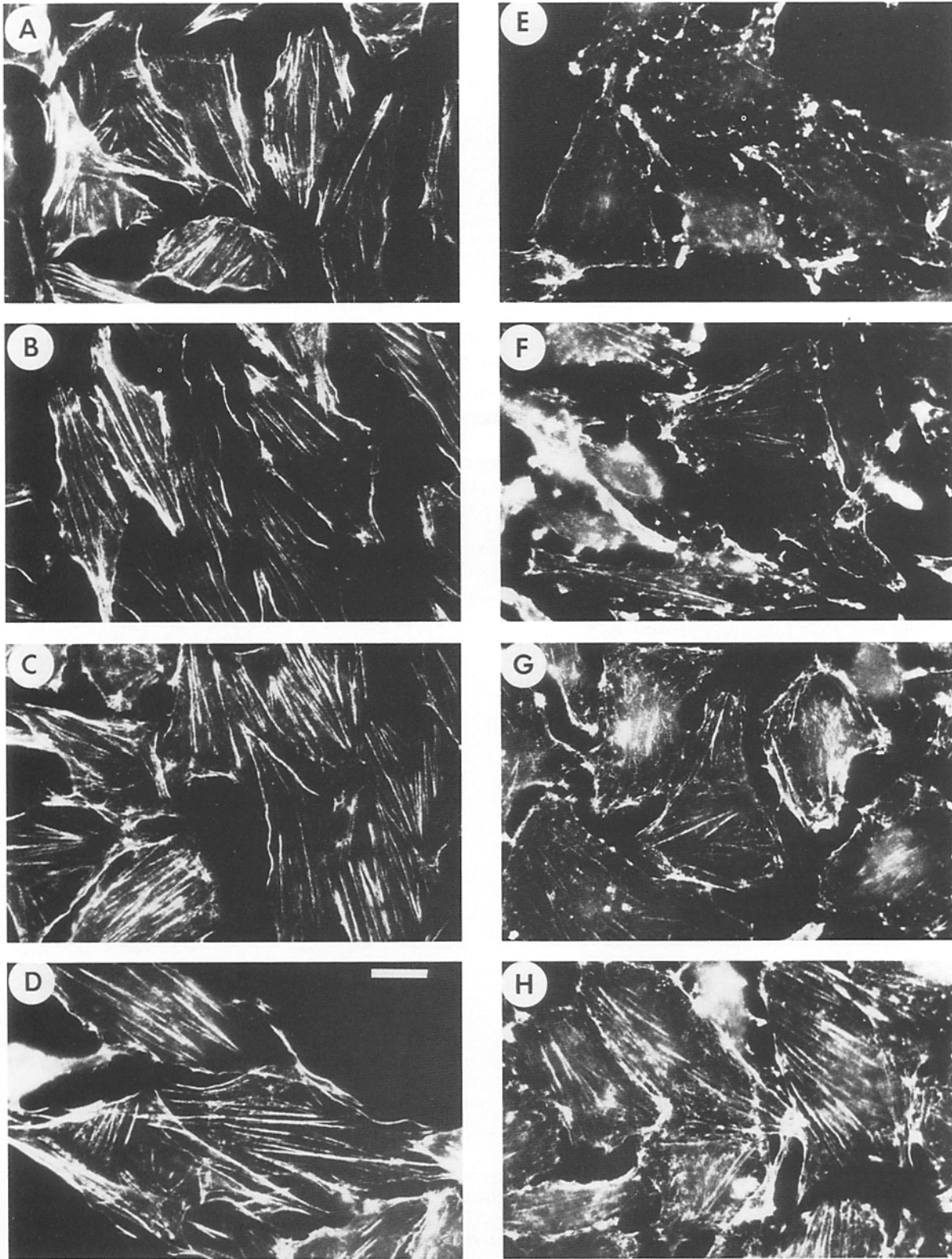


Figure 9. CaD39-expressing lines display resistance to a 30-min cytochalasin B treatment of 1.25 $\mu\text{g}/\text{ml}$. Immunofluorescence microscopy with CG3 identifies the TM-containing microfilament bundles in CaD39 lines exposed to DMSO alone (*A*, *B*, *C*, and *D*) or to cytochalasin B dissolved in DMSO (*E*, *F*, *G*, and *H*). In the absence of cytochalasin B, there are no obvious differences in the cell morphology, size, shape, and microfilament organization of the CaD39 cell lines 39C5 (*A*), 39L12 (*B*), 39C11 (*C*), and 39C15 (*D*). After exposure to cytochalasin B, non-expressing 39C5 (*E*) cells have virtually no intact TM-containing filament bundles after exposure to cytochalasin B, whereas 39L12 (*F*), 39C11 (*G*), and 39C15 (*H*) cells increasingly show more resistant microfilament bundles and fewer focal aggregations. Bar equals 10 μm .

actions with the cell's own capping and anchoring proteins (Cooper, 1987). If CaD39 expression in CHO cells does stabilize TM-containing microfilament bundles, we hypothesized that CaD39 expression might confer resistance to cytochalasin B. Cells from each CaD39 line were grown on glass coverslips, treated to 1.0, 1.25, or 1.5 $\mu\text{g/ml}$ cytochalasin B, or DMSO alone, and stained with CG3 to visualize the remaining TM-containing microfilament bundles in the cells. We discovered that CaD39 expression did render the CaD39 lines less sensitive to the effects of cytochalasin B. Under the control treatment (DMSO alone) 39C5 (Fig. 9 A), 39L12 (Fig. 9 B), 39C11 (Fig. 9 C), and 39C15 (Fig. 9 D) cells display a microfilament organization characteristic of CHO cells. An automatic exposure meter was used to take the micrographs, therefore no TM staining intensity differences are seen. Fig. 9 micrographs E-H show the results from cells exposed 30 min to 1.25 $\mu\text{g/ml}$ cytochalasin B, the concentration which best demonstrates the graded response to cytochalasin. The non-expressing line 39C5 (Fig. 9 E), displays an almost complete filament rearrangement into focal aggregations. Low and medium-high expressors, 39L12 (Fig. 9 F) and 39C11 (Fig. 9 G), show intermediate effects, and cells from high expressing line 39C15 (Fig. 9 H) demonstrate resistant, partially intact stress fiber networks. Treated cells, examined with rhodamine-phalloidin for F-actin staining, had identical filament staining patterns (data not shown). CaD39 expression also conferred increased resistance to Triton X-100 (data not shown), suggesting that the cortical cytoskeleton is also stabilized.

Discussion

The proposed role of caldesmon in the regulation of fibroblast microfilament stability is supported by several reports describing *in vitro* observations. *In vitro* analysis of caldesmon's interactions with actin and TM revealed that caldesmon can stimulate actin polymerization (Galazkiewicz et al., 1989) and greatly enhance TM's binding to actin (Yamashiro-Matsumura and Matsumura, 1988; Novy et al., 1993b). Caldesmon was also found to protect actin filaments from gelsolin severing and to augment reannealing of fragmented actin filaments (Ishikawa et al., 1989a,b).

The *in vivo* functions of nonmuscle caldesmon have not been well characterized, but several lines of correlative *in vivo* evidence have also suggested that caldesmon might stabilize microfilaments. In the fibroblast cell, caldesmon does localize to actin and TM-rich stress fibers and ruffle regions (Lin et al., 1988; Dingus et al., 1986; Bretscher and Lynch, 1985; Owada et al., 1984). In cells which have stabilized and thickened microfilament bundles after dexamethasone treatment, steady-state levels, of caldesmon (but not actin, TM, or gelsolin) was shown to be increased (Castellino et al., 1992). In addition, caldesmon is found to be reduced or dissociated from microfilaments in cells that do not display a hearty cytoskeleton. For example, caldesmon is phosphorylated and subsequently released from microfilaments in mitotic cells actively dissolving their actin filament bundles (Yamashiro et al., 1990; Hosoya et al., 1993). Also, in transformed cells which exhibit a diminished and diffuse actin cytoskeleton, caldesmon expression is downregulated (Novy et al., 1991; Owada et al., 1984). We are seeking to further understand the stabilizing role of nonmuscle caldes-

mon and, in this paper, provide direct evidence for the first time, that the actin-, TM-, and Ca^{++} /Calmodulin-binding COOH-terminal fragment of human fibroblast caldesmon can stabilize TMs and microfilament bundles *in vivo*.

In the stably transfected CaD39 lines, we see an increase in the steady-state level of endogenous TM5. The fact that the TM5 increase is not due to increased TM synthesis, suggests that this TM is somehow protected from normal protein turnover. Pulse-chase experiments have confirmed that TM5 as well as TM4 turn over more slowly in CaD39-expressing 39C15 cells. This protection of both low M_r TM isoforms likely involves CaD39-enhanced TM binding to F-actin. We have previously characterized bacterially expressed CaD39's ability to enhance low M_r TM binding to F-actin (Novy et al., 1993b), and have shown in this report that CaD39 expressed in CHO cells localizes to stress fibers and ruffles, and is a component of isolated, TM-enriched microfilaments. The amount of actin isolated in our microfilament preparations is also increased, suggesting further that interactions between TM and actin filaments are stabilized. The enhanced accumulation of TM appears to be a phenomenon specific to CaD39 stable clones, as levels of endogenous caldesmon are not altered in CaD39 lines, and levels of TM are not altered in CaD40-expressing lines. We have also examined endogenous caldesmon steady-state levels in stable CHO lines which express mouse skeletal βTM . No changes in caldesmon levels were seen (data not shown), even though force-expressed βTM readily assembles onto CHO microfilaments (Warren and Lin, 1993). A hierarchy of interactions is suggested here, in that overexpression of CaD39 leads to an accumulation of TM, but overexpression of TM does not affect steady-state levels of caldesmon.

In support of an actual stabilization of microfilament bundles, we also found that CaD39 expression confers, upon expressing cells, enhanced resistance to cytochalasin B disruption and Triton extraction. Consistent with our observations that CaD39-expressing lines possessed more intact microfilament bundles after exposure to cytochalasin B, Castellino et al. (1992) reported that cells with dexamethasone induced caldesmon overexpression, were also less susceptible to cytochalasin disruption. Cytochalasins are thought to possibly act by destabilizing actin filament interactions with capping proteins important for holding filaments in place. Stress fibers have also been seen to contract upon cytochalasin treatment, as if released from their attachments (Cooper, 1987). CaD39-associated protection may be a combined response to stabilized filaments, stabilized attachments, and an inhibited contraction response. That these CaD39-expressing cells are also resistant to a Triton X-100 treatment strong enough to completely collapse the cytoskeleton of non-expressing cells, supports the notion that microfilament and microfilament-membrane attachments are stabilized. In addition, preliminary analysis of these CaD39 lines has indicated that their ability to attach to substrate is also enhanced. Further studies on the effect of CaD39 overexpression on cell attachment and spreading on different substrata are currently underway.

We did not observe expression-related alterations in the size, shape, or microfilament organization of the CaD39 cell lines (see Fig. 9), even though the estimated levels of CaD39 reached up to 79 times that of endogenous caldesmon. One could argue that CaD39, a fragment of caldesmon, might regulate, and/or be regulated, differently than intact caldesmon. Unfortunately, using the pCB6hx expression vector,

we failed to obtain stable lines expressing intact caldesmon. We are currently working towards an inducible expression of full-length caldesmon to address this important issue. It is important to note, however, the findings of Yamakita et al. (1990), that microinjection of intact gizzard caldesmon into fibroblasts did not alter cell morphology or the actin cytoskeleton, even though the gizzard caldesmon remained incorporated into stress fibers and ruffle regions for days after injection. Additionally, microinjected COOH-terminal caldesmon fragment behaved indistinguishably from injected full-length gizzard caldesmon (Yamakita et al., 1990).

The approach of artificially expressing a given protein in cultured cells, and observing the resultant phenotype, has been used to examine the normal roles for proteins such as villin (Friederich et al., 1989), gelsolin (Cunningham et al., 1991), and vinculin (Rodriguez Fernandez et al., 1992), to name a few. In this paper, we have used transfection mediated expression of caldesmon fragments to study nonmuscle caldesmon function. As mentioned earlier, the expression effects of the CaD39 fragment may not fully represent the consequences of overexpressing full length caldesmon. However, since caldesmon itself has been implicated in microfilament stability, CHO-expressed CaD39 and microinjected COOH-terminal gizzard caldesmon fragment (Yamakita et al., 1990) are able to localize to the same structures as endogenous caldesmon, and COOH-terminal gizzard caldesmon fragment (Marston and Redwood, 1993) and bacterially produced CaD39 (Novy et al., 1993b) were shown to retain the properties, in vitro, of whole caldesmon, we feel that the CaD39-mediated stabilization of the actin cytoskeleton is a valid indication of one function of caldesmon in nonmuscle cells. We are currently exploring other caldesmon functions in our CaD39 stable lines with experiments analyzing cell motility.

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