Essential Role of Caldesmon in the Actin Filament Reorganization Induced by Glucocorticoids

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Abstract. Glucocorticoids induce the remodeling of the actin cytoskeleton and the formation of numerous stress fibers in a protein synthesis-dependent fashion in a variety of cell types (Castellino, F., J. Heuser, S. Marchetti, B. Bruno, and A. Luini. 1992. *Proc. Natl. Acad. Sci. USA.* 89:3775–3779). These cells can thus be used as models to investigate the mechanisms controlling the organization of actin filaments. Caldesmon is an almost ubiquitous actin- and calmodulin-binding protein that synergizes with tropomyosin to stabilize microfilaments in vitro (Matsumura, F., and Yamashiro, S. 1993. *Current Opin. Cell Biol.* 5:70–76). We now report that glucocorticoids (but not other steroids) enhanced the levels of caldesmon (both protein and mRNA) and induced the reorganization of microfilaments with similar time courses and potencies in A549 cells. A caldesmon antisense oligodeoxynucleotide targeted to the most abundant caldesmon isoform in A549 cells dramatically inhibited glucocorticoid-induced caldesmon synthesis and actin reorganization with similar potencies. Several control oligonucleotides were inactive. These results demonstrate that caldesmon has a crucial role in vivo in the organization of the actin cytoskeleton and suggest that hormone-induced changes in caldesmon levels mediate microfilament remodeling.

CALDESMON is an almost ubiquitous intracellular protein that binds actin filaments in a calcium- and calmodulin-regulated fashion. Two main types of caldesmon isoforms have been discerned based on their mobility in SDS-PAGE: a high molecular mass form estimated at 130–150 kD (h-caldesmon), found in smooth muscle, and a lower molecular mass form of 70–80 kD (l-caldesmon), in nonmuscle cells. Based on examination of the primary structure and on the properties of the purified protein, caldesmon has been proposed to possess binding domains for actin, myosin, calmodulin and tropomyosin as well as several phosphorylation sites. A wealth of data (mostly in vitro) implicates caldesmon in diverse cellular functions, including the regulation of smooth muscle and nonmuscle contraction, secretion, and, more recently, the dynamics of the actin cytoskeleton (see Sobue and Sellers, 1991 and Matsumura and Yamashiro, 1993 for reviews).

Several lines of evidence suggest a role of l-caldesmon (hereafter referred to as caldesmon for simplicity) in the organization of actin filaments. Immunohistochemical studies have shown that caldesmon colocalizes with actin filaments with a distribution coincident with that of tropomyosin (see Sobue and Sellers, 1991 and references therein), and that microinjected caldesmon is quickly incorporated into microfilaments in fibroblasts (Yamakita et al., 1990). In vitro work has shown that caldesmon potentiates the ability of tropomyosin to inhibit the severing action of gelsolin (Ishikawa et al., 1989*a*) and enhances the reannealing effect of tropomyosin on actin filaments after severing by gelsolin (Ishikawa et al., 1989*b*). Caldesmon also regulates the interaction in vitro between actin filaments and the actin cross-linking protein filamin (see Sobue et al., 1988 and references therein).

While these in vitro data point to a "stabilizing" role of caldesmon on actin filaments, the complementary results in live cells are much less direct and abundant. A piece of correlative evidence in favor of caldesmon's stabilizing role is that actin filaments become disorganized during mitosis in parallel with the dissociation of caldesmon from them, probably as a consequence of phosphorylation of caldesmon by the cdc2 kinase (Yamashiro et al., 1990, 1991). Another evidence is that caldesmon levels are decreased in transformed cells, where stress fibers are scarce or partially replaced with a diffuse meshwork of short microfilaments (Novy et al., 1991); however, the concentrations of several actin-binding proteins also change upon transformation (see Novy et al., 1991 and references therein),

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1. *Abbreviations used in this paper*: AO, antisense oligonucleotide; GC, glucocorticoid; RT-PCR, reverse transcription-PCR.

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making it difficult to discern the role of caldesmon from that of other cytoskeletal components.

In the course of our studies on the role of the actin cytoskeleton in the glucocorticoid (GC)-induced inhibition of ACTH release from pituitary cells we found that GCs induce the reorganization and stabilization of the actin cytoskeleton in pituitary cells as well as in other cell types (Castellino et al., 1992), and that this effect contributes to the GC suppression of ACTH release. A particularly marked effect of GCs was observed in the human lung adenocarcinoma cell line A549. Interestingly, caldesmon levels were increased in GC-treated cells. Here we report that the striking actin-reorganizing effect of GCs in A549 cells correlates both in time and degree with a large increase in the cellular levels of caldesmon (both at the protein and mRNA level); and, more important, that the selective inhibition of caldesmon expression by a caldesmon antisense oligonucleotide (AO) inhibits the reorganization of the actin cytoskeleton induced by GCs. These results demonstrate the essential in vivo role of caldesmon in the actin cytoskeleton organization and suggests that hormone-induced changes in caldesmon levels can mediate microfilament remodeling.

Materials and Methods

Cell Culture

A549 cells were cultured in Minimum Essential Medium Eagle's Modified with 10% fetal calf serum, in a humidified atmosphere with 5% CO_2. Cells were always used at 50–70% confluence.

Antibodies, Oligonucleotides and Other Reagents

Polyclonal and monoclonal antibodies against nonmuscle caldesmon and tropomyosin were as described by Yamashiro-Matsumura and Matsumura (1988), Lin et al. (1984), and Matsumura et al. (1983*a*,*b*). Purified caldesmon from bovine liver was prepared as described by Yamakita et al. (1990). Anti-cytokeratin antibodies were a gift of Dr. L. Masuelli (University of Chieti). Anti-actin, anti-gelsolin, and anti-tubulin antibodies were purchased from Amersham Corp. (Arlington Heights, IL). Oligodeoxynucleotides were synthesized using a Beckman System 200A DNA synthesizer and purified by methanol precipitation. HPLC-purified oligodeoxynucleotides were purchased from Duotech (Milan).

Gel Electrophoresis and Immunoblotting Analysis

For two-dimensional electrophoresis cells were sonicated in a PBS buffer containing 0.05% Triton X-100 and a cocktail of protease inhibitors. Proteins were then separated by IEF-SDS/PAGE performed according to the Bravo's procedure (Bravo, 1984). The pH gradient in the first dimension ranged from 4.5 to 7 after electrophoresis. The majority of cellular and cytoskeletal proteins falls within this range. The second dimension was run on 10% SDS-PAGE.

For immunoblotting experiments, after cell disruption by sonication, proteins were separated by 10% SDS-PAGE, electrophoretically transferred to nitrocellulose membrane, and then probed with antibodies to gelsolin, tropomyosin, actin, tubulin, and cytokeratin. For the semiquantitative assay of caldesmon, sonicated cells were heated for 10 min at 100°C, cooled on ice for 30 min and centrifuged. The supernatants were analyzed by urea/SDS-PAGE, using 8% polyacrylamide gels containing 4 M urea, then transferred to nitrocellulose and stained with the monoclonal antibody SM12 (Yamashiro-Matsumura and Matsumura, 1988) to caldesmon. This antibody reacts with the NH_2 terminus of a variety of muscle and nonmuscle caldesmons from *Xenopus* to human. Judging from the conservation of the amino acid sequence in this region of caldesmon from several species, the antibody is most likely to react equally with bovine and human caldesmons (Yamashiro-Matsumura and Matsumura, 1988). The samples, prepared as described above, were processed in parallel with known amounts of purified bovine liver caldesmon (see Yamakita et al., 1992), used to construct a calibration curve. Electrotransfer onto nitrocellulose was nearly complete for both purified caldesmon and caldesmon from cell extracts. Immunostained bands were then analyzed by densitometry with LKB Ultroscan XL. The amount of caldesmon in cells was calculated by interpolation in the calibration curve.

Northern Blotting

Total cell RNA was isolated by the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987). Northern blotting was performed as described by Sambrook et al. (1987). Equal amounts of total cell RNA was loaded on a 1% formaldehyde/agarose gel. After electrophoresis the RNA was transferred to a nitrocellulose membrane (Millipore) by the capillary method. DNA probes of rat nonmuscle caldesmon (clone D3; see Yamashiro et al., 1995) were labeled according to a random prime labeling protocol (Feinberg and Vogelstein, 1983). Hybridization was performed in 50% formamide 5 × SSC, 5× Denhardt's solution at 37°C. The membrane was washed in 0.1 × SSC, 0.5% SDS at 37°C.

Reverse Transcription-PCR

There are two types of isoforms of human nonmuscle caldesmon, one cloned from HeLa cells and the other from WI-38 cells; they differ by the first 24 amino-terminal residues, while the rest of the sequences is identical. Of each isoform there are two subtypes (I and II), differing only by the insertion of a stretch of 26 amino acids in type I (positions 202–227 of the HeLa type I isoform). To reveal these isoforms reverse transcription-PCR (RT-PCR) experiments were performed. RT-PCR of WI-38 isoforms was carried out as follows: the first strand cDNA was synthesized from total RNA of A549 cells using oligo (dT)12-18 as a primer. The cDNA of WI-38 isoforms was then amplified with the same sense and antisense oligonucleotides as those used by Hayashi et al. (1992). Briefly, the primers used were: Pm, antisense primer complementary to the common sequence in all caldesmon isoforms, nucleotide positions 832–859 in WI-38 caldesmon type II; Pn2, sense primer specific to the short amino-terminal sequence of the WI-38 caldesmon type I and type II isoforms; Pi, antisense primer complementary to the insertion sequence present in all type I caldesmon. The following pairs of primers were used; Pn2-Pm, to amplify both the WI-38 isoforms type I and II; Pn2-Pi, to amplify the WI-38 isoform type I. For the HeLa isoforms, because they are very minor in A549 cells, RT-PCR was modified as follows: The first strand cDNA was synthesized using the antisense primer (Pm) common to all the caldesmon isoforms instead of oligo (dT)12-18, and the cDNAs for HeLa isoforms were amplified by two rounds of PCR. In the first round, the cDNA was amplified with the sense primer Pn (specific for the short amino-terminal sequence of the HeLa caldesmon type I and II isoforms) and the antisense primers Pi or Pm. The products were used as templates for a second round of PCR primed with a sense primer (Pnn, 5′-ATCCGGATCGCATGGAAGACGCAG) nested to Pn, and the same antisense primers as in the first round. Thus, Pnn-Pi will amplify the HeLa isoform type I and Pnn-Pm will amplify both the HeLa isoforms types I and II. The PCR products were separated by electrophoresis in 1.5% agarose gel.

Fluorescence Microscopy

Cells were plated in 24 well Falcon dishes at a density of 1×10^4 cells/dish on coverslips coated with gelatin or poly-D-lysine (0.1 mg/ml) for 1 h. Microfilaments were labeled by fixing the cells for 15 min in 2% paraformaldehyde, permeabilizing with Triton 0.02% in phosphate-buffered saline and incubating for 30 min in rhodamine-phalloidine (33 ng/ml in phosphate-buffered saline). Intermediate filaments were labeled with anti-cytokeratin antibodies by fixation in 4% paraformaldehyde, permeabilization with 0.05% saponin, then incubation with the primary antibody followed by the FITC-conjugated secondary antibody. Caldesmon was immunostained as described by Hosoya et al. (1993). Microtubules were labeled with anti-tubulin antibodies following exposure to methanol at −20°C for 5 min.

Results

Characterization of the Glucocorticoid-induced Reorganization of the Actin Cytoskeleton

A549 cells cultured in the presence of dexamethasone for 24 h became more polygonal, flattened and extended in

size. Staining of actin filaments with rhodamine-phalloidine (Cooper, 1987), revealed marked differences in actin organization between control and dexamethasone-treated cells. Control cells stained diffusely, with scarce and disorganized microfilaments, rare stress fibers and frequent ruffle-like protuberances ("flowers") previously shown to contain actin in a short filamentous form just beneath the plasma membrane (Boschek et al., 1981). Dexamethasone induced the appearance of well developed stress fibers and the disappearance of flowers (Fig. 1). No significant alteration in the organization of microtubules and intermediate filaments (stained with anti-tubulin and anti-cytokeratin antibodies, respectively, not shown) was observed in steroid-treated cells.

The effects of dexamethasone were maximal or near-maximal at 10^{-7} M and just detectable at 10^{-9} M (EC_{50}: $\sim 5 \times 10^{-9}$ M). Other GCs such as betamethasone and hydrocortisone were active but less potent, with EC_{50}s of $\sim 10^{-8}$ and $\sim 10^{-7}$ M, respectively, while non-GC steroids (estradiol, progesterone, and testosterone) were inactive at concentrations up to 10^{-6} M. The effects of dexamethasone on actin filaments became detectable within 12 h of treatment (Fig. 1) and were near-maximal after 24 h, then remained stable for up to three days. Although 10^{-7} M dexamethasone had near-maximal effects, a concentration of 10^{-6} M was used in this and many other experiments to abolish the variability sometimes noticed between different dexamethasone batches. To obtain a semiquantitative estimation of the effects of GCs (and of agents interfering with their action, see below) on microfilaments we set up two simple criteria: (*a*) the disappearance of flowers and (*2*) the increase in number, apparent thickness and parallel orientation of stress fibers (see Table I for details).

Based on these parameters, the GC effects were largely prevented by the protein synthesis inhibitors actinomycin D (at 0.2 μg/ml) (not shown) and cycloheximide (Fig. 1 *D* and Table I). Cycloheximide alone did not seem to detectably modify the actin cytoskeleton in control cultures and its only visible effect was a slight rounding of the cells (not shown). The inhibitory effect of cycloheximide is consistent with the well known mechanism of GC action via intracellular receptors controlling the synthesis of specific proteins (Ringold, 1985).

The Glucocorticoid-induced Reorganization of Actin Filaments Is Associated with an Increase in the Cellular Levels of Caldesmon

Changes in the actin cytoskeleton might be due to altered cellular levels of proteins involved in microfilament stability such as caldesmon or tropomyosin (Matsumura and Yamashiro, 1993). Under conditions that maximized the GC effects (10^{-6} M dexamethasone for 24 h), the cellular concentrations of caldesmon were markedly increased over control levels (Fig. 2 *B*). In well-resolved urea gels two closely adjacent caldesmon bands were visible, possibly reflecting mitotic (cdc2 kinase-phosphorylated) and nonphosphorylated protein (Yamashiro et al., 1991). The relative intensity of the two bands did not seem to be affected by dexamethasone, suggesting that this steroid does not alter the phosphorylative regulation of caldesmon. The levels of a few other actin-binding proteins such as the four tropomyosin isoforms shown in Fig. 2 *C* and gelsolin (Fig. 2 *A*) as well as the levels of actin itself (Fig. 2 *D*) were not significantly altered by dexamethasone. The seeming discrepancy between the lack of GC effect on gelsolin and the marked increase in the levels of this protein by dexamethasone observed in L929 cells by Lanks and Kasambalides (1983) is likely to be due to the well known cell type specificity of the GC actions (Ringold, 1985). The general cell protein pattern of metabolically labeled cells was examined in two-dimensional gels. Dexamethasone detectably increased 6 spots and decreased one, confirming the relative specificity of the steroid's action (Fig. 2, *F* and *G*). The increase in caldesmon, like the GC-induced microfilament remodeling, was time-dependent, was in-

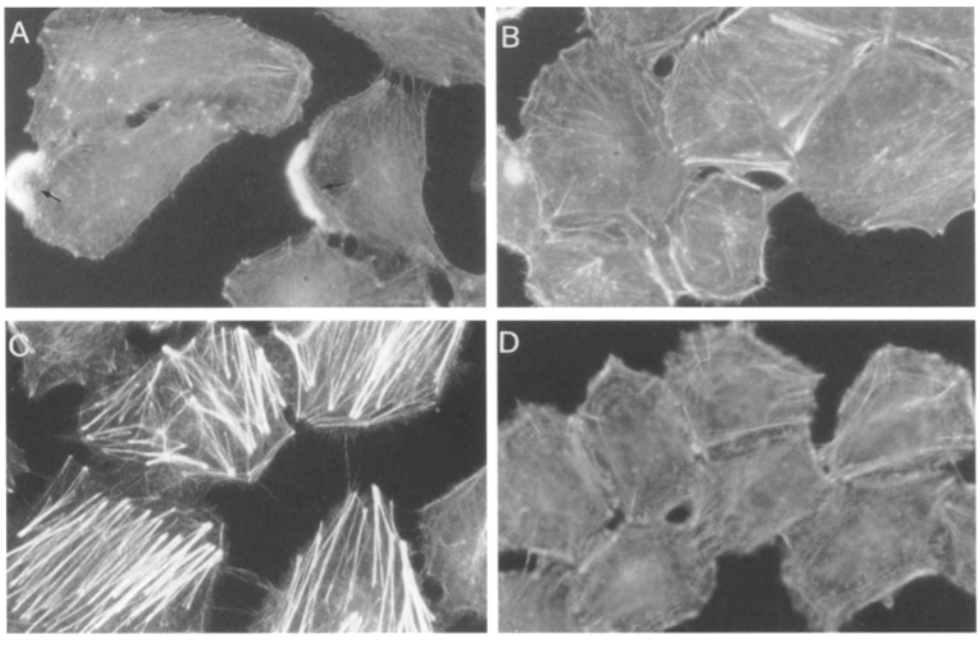

Figure 1. Reorganization of the actin cytoskeleton by dexamethasone. A549 cells were plated and grown for 3 d to ~60% confluency. Dexamethasone (10^{-6} M) and/or cycloheximide (0.1 μg/ml) were applied for the times specified below via addition in 10 μl of vehicle to the medium during the last period of growth. *A*, control cells; *B*, dexamethasone for 12 h; *C*, dexamethasone for 24 h; *D*, dexamethasone and cycloheximide for 24 h. Cycloheximide alone had no apparent effect on actin filaments in control cells. Arrowhead, flower; see text for details. This experiment was repeated at least five times with similar results. Bar, 5 μm.

Table I. Effects of Dexamethasone, Oligonucleotides, and Cycloheximide on Actin Filament Organization in A549 Cells

Treatment		AO		SO		CAO		
				μm				
		30	100	30	100	30	100	Cyclo
	0.8 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	1.3 ± 0.2	0.9 ± 0.1
DEX (12 h)	2.2 ± 0.1	ND		ND		ND		ND
DEX (24 h)	3.5 ± 0.2	1.6 ± 0.2*	1.5 ± 0.1*	3.6 ± 0.3	3.4 ± 0.1	3.5 ± 0.2	3.4 ± 0.3	1.2 ± 0.2

10^{-6} M Dexamethasone (*DEX*) and 0.1 mg/ml cycloheximide (*CYCLO*) were used as described in the legend to Fig. 1, antisense oligonucleotide (*AO*), sense oligonucleotide (*SO*), and chicken antisense oligonucleotide (*CAO*) as described in the legend to Fig. 5. The effects of the treatments were quantified by a trained technician in a blind fashion according to the following procedure: 30 randomly chosen cells from each treatment (15 cells from each duplicate) were examined at a magnification of 400× under a fluorescence microscope and given individual scores from 0 to 4, using the criteria defined in the text. For instance, a cell with a flower and very scarce and thin stress fibers received a score of 0; a cell without flowers and scarce fibers a score of 1; a cell without flowers and abundant, parallel, and bright stress fibers a score of 4. Flowers were absent in cells with developed fibers. Values represent means ± SE of 30 individual scores.

*Different from controls (DEX for 24 h) by the Duncan's test ($p < 0.01$). Similar results were obtained in two experiments performed on different days.

hibited by cycloheximide (0.1 μg/ml), and could be elicited by betamethasone and hydrocortisone (EC_{50}s: dexamethasone, ~3×10^{-9} M; betamethasone, ~10^{-8} M; hydrocortisone, ~7×10^{-8} M), but not by estradiol, progesterone and testosterone. Môreover, the time course (Fig. 2 *E*) of the caldesmon increase and that of microfilament reorganization were roughly parallel, suggesting a causal link between the two phenomena. GC did not seem to change on the cellular distribution of caldesmon in that the protein was found associated with microfilaments (see Yamakita et al., 1990) both in control and dexamethasone-treated cells, although the scarcity and disorganization of actin filaments in the former often made such association difficult to demonstrate (Fig. 3).

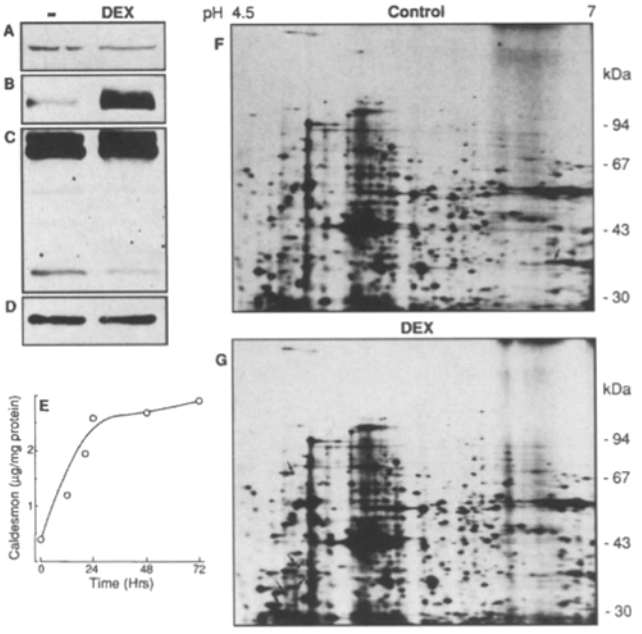

Figure 2. Selective effects of dexamethasone on the cellular levels of caldesmon. Cells were treated with dexamethasone (10^{-6} M for 24 h) (*DEX*) as described in the legend to Fig. 1, then sonicated and used for immunoblotting analysis with antibodies directed against gelsolin (*A*), caldesmon (*B*), tropomyosin (*C*), actin (*D*). The anti-tropomyosin antibody reveals both low and high MW isoforms of the protein. Human tropomyosin is expressed as a family of isoforms (Lin et al., 1984). Although we haven't investigated which isoforms are present in A549 cells, we can state, based on the extensive characterization of the antibody used (see Methods) that the four bands in *C* are indeed tropomyosin isoforms. (*E*) Caldesmon levels as a function of time of exposure to dexamethasone; (*F* and *G*) Total protein pattern in two-dimensional gels of control A549 cells and of cells treated with dexamethasone, respectively. Arrows indicate spots detectably modified by the steroid. Caldesmon focuses between pH 7 and 8 and is not seen in these gels. The experiment was repeated on three different occasions with similar results.

The Caldesmon Increase Is Accompanied by a Similar Increase in Caldesmon mRNA Levels

To investigate the mechanism of caldesmon induction by GC, we sought to determine whether the protein increase was associated with a corresponding elevation of caldesmon mRNA. Northern blot analysis was performed on total RNA isolated from A549 cells. Fig. 4 *A* shows that the levels of caldesmon mRNA prepared from cells that had been treated with 10^{-6} M dexamethasone for different periods of time were markedly elevated and that the increase was similar in extent to that of the caldesmon protein. The increase in caldesmon mRNA peaked at or before the 7th hour of dexamethasone treatment, a few hours before the peak of the caldesmon protein (compare Figs. 4 *A* with 2). This lag might be due to the slow turnover of the protein (caldesmon, when microinjected, is stably incorporated in stress fibers for over three days; see Yamakita et al., 1990).

Caldesmon Isoforms in A549 Cells

The rat caldesmon cDNA probe is likely to reveal all nonmuscle caldesmon isoforms, of which there are four known types (HeLa I and II, and WI-38 I and II; see Materials and Methods). To determine which of them are present in A549 cells, we performed RT-PCR experiments. The results in Fig. 4 suggest that all the isoforms are present and that the WI-38 isoforms are by far the more abundant, as might be expected given the similar tissue derivation (human lung alveoli) of WI-38 and A549 cells. Indeed, two rounds of PCR were needed to detect clearly the HeLa isoforms (Fig. 4 *B*, lanes *a* and *b*). It should also be noted that type I isoforms are less abundant than isoforms type II, as shown by the observation that although the primer pairs Pn2-Pm or Pnn-Pm should amplify both types I and II (Fig. 4 *B*, lanes *a* and *c*; see Materials and Methods), only type II isoforms were visible, and only after amplification by a second round of PCR with the primer pair Pn2-Pm did a faint band with a slower mobility corresponding to the WI-38 type I isoform become detectable (Fig. 4 *C*).

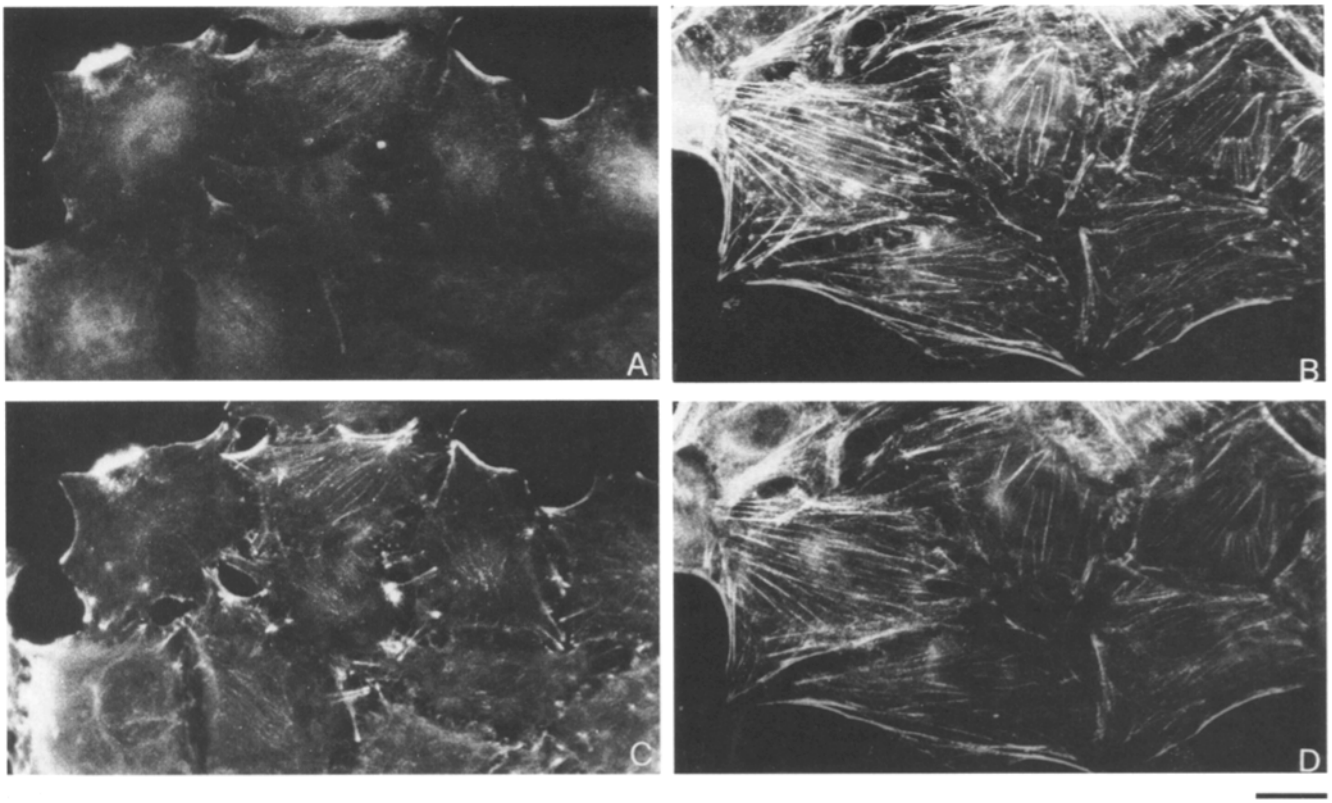

Figure 3. Caldesmon and actin filament localization in dexamethasone-treated cells. Cells were treated with dexamethasone (10^{-6} M for 24 h) as described in the legend to Fig. 1, then fixed and double stained for actin filaments (*A* and *B*) and caldesmon (*C* and *D*). Bar, 4 μm.

A treatment with GC resulted in a stimulatory trend of all caldesmon isoforms; thus, although RT-PCR is not quantitative, these results appear to agree with the Northern blotting experiments.

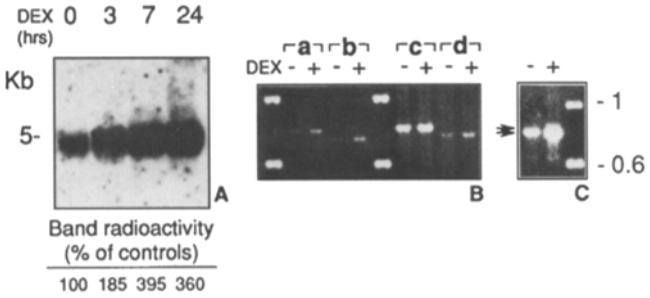

Figure 4. Effects of dexamethasone on caldesmon mRNA and characterization of the caldesmon isoforms in A549. Cells were treated with 10^{-6} M dexamethasone for the specified times as described in the legend to Fig. 1. (*A*) Northern blot analysis. Rat caldesmon cDNA was used to probe total RNA samples isolated from A549 cells. 10 μg of total RNA were loaded into each lane. Band radioactivity was quantified by a PhosphorImager (Molecular Dynamics). (*B*) RT-PCR analysis. RT-PCR was performed using first strand cDNA from control A549 cells or from cells treated with 10^{-6} M dexamethasone for 7 h (the time of peak caldesmon mRNA expression). The following pairs of primers were used: Pnn-Pm (HeLa isoforms types I and II) (*a*); Pnn-Pi (HeLa isoform type I) (*b*); Pn2-Pm (WI-38 isoforms types I and II (*c*); Pn2-Pi (WI-38 isoform type I) (*d*). The expected sizes were 0.8 kb for Pnn-Pm and Pn2-Pm and 0.7 kb for Pnn-Pi and Pn2-Pi. (*C*) amplification of the PCR products obtained with the primer pair Pn2-Pm by a second round of PCR with the same primer pair in order to show the presence of the expected doublet corresponding to the two WI-38 isoforms: the minor upper band in lane (+) corresponds to the less abundant type I isoform. The experiments were repeated twice with similar results.

A Caldesmon Antisense Oligonucleotide Inhibits the Effect of Dexamethasone on Both Caldesmon Cellular Levels and Microfilament Reorganization

To examine whether a causal link might exist between caldesmon expression and cytoskeletal reorganization we sought to selectively inhibit the GC-induced increase in caldesmon and then determine whether the steroids could still cause microfilament reorganization. To this end, we constructed an oligodeoxynucleotide complementary to the first 18 nucleotides of the abundant WI-38 isoforms (Novy et al., 1991; Hayashi et al., 1992) beginning from the AUG translation initiation codon. The first 18 translated nucleotides are a commonly used and often effective target site of antisense oligonucleotides (Chiang et al., 1991). Indeed, the AO inhibited the caldesmon increase induced by dexamethasone (Fig. 5) in a dose-dependent fashion, with a marked effect at 30 μM. These concentrations are similar to those found effective in other antisense studies aimed at blocking protein expression, although it should be noted that such concentrations may vary widely depending on experimental conditions (Jaskulsky et al., 1988; Chiang et al., 1991; Kirsch et al., 1993). The AO had only a slight effect on caldesmon levels in control cells (see Discussion) and did not appear to be toxic, as judged by its

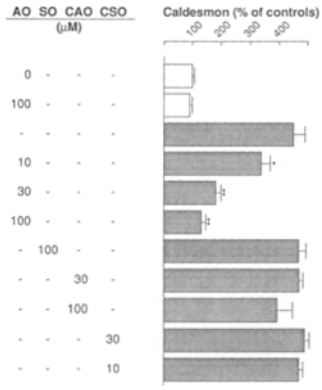

Figure 5. Effect of the antisense oligonucleotide on the cellular levels of caldesmon. Cells were grown in 2% serum (pretreated by heating at 56°C for 1 h to minimize serum nuclease activity). This did not affect cell morphology and moderately reduced cell growth. The treatment with dexamethasone (10^{-6} M for 24 h) was as described in the legend to Fig. 1. Oligonucleotides were applied in 20 μl of saline to 2 ml of growth medium for the 24 h preceding, as well as during, the dexamethasone treatment. The addition was repeated every 12 h (namely, 24 and 12 h before, simultaneously with, and 12 h after, the application of dexamethasone) to compensate for oligonucleotide degradation and uptake into cells, for a total of four additions. The real oligonucleotide concentrations throughout the treatment are therefore unknown. We assume that oligonucleotides are gradually degraded and/or taken up during the 12 h following each addition. The concentrations specified in the figure are those produced by the first addition. Cells were then sonicated and the caldesmon content assayed by quantitative immunoblotting. (*Empty bars*) Controls; (*gray bars*) dexamethasone; *AO*, antisense oligonucleotide; *SO*, sense oligonucleotide; *CAO*, chicken antisense oligonucleotide; *CSO*, chicken sense oligonucleotide. Values are means ± SD of three different experiments. * Different from controls (10^{-6} M dexamethasone) by the Duncan's test at $p < 0.05$; ** at $p < 0.01$. Oligonucleotides were prepared as described in Materials and Methods; commercial HPLC-purified oligonucleotides yielded the same effects.

lack of effect on cell growth rate, general cell protein pattern, levels of other cytoskeletal proteins and Trypan Blue tests (not shown). Three other oligodeoxynucleotides, namely, the human sense oligonucleotide and two other oligonucleotides complementary to the first 18 bases (starting from the translation initiation site) of the sense and antisense strand of chicken smooth muscle caldesmon mRNA (Bryan et al., 1989) were used as controls. In the chicken mRNA three out of the first 18 bases are different from those found in caldesmon WI-38 mRNA. Since three mismatches are usually sufficient to abolish AO activity (Wahlestedt, 1994), the chicken AO provides a sensitive control of the specificity of the effect of the human WI-38 AO designed to inhibit caldesmon expression in A549 cells. All control oligonucleotides, including chicken AO (at 30 mM), had no effects on GC-induced caldesmon expression. The slight effect of 100 mM of the chicken AO (Fig. 5) is statistically non significant. Thus, only the human AO inhibited GC-induced caldesmon expression.

The effects of the AO on GC-dependent actin remodeling were then examined. Strikingly, the AO at 30 mM largely prevented the cytoskeleletal changes induced by dexamethasone (Fig. 6 and Table I). By contrast, the GC-induced actin reorganization was not affected by control oligonucleotides, including chicken AO, at concentrations up to 100 mM. To better examine the relationship between GC-dependent caldesmon expression and degree of microfilament organization, the effects of GC and oligonucleotides on the actin cytoskeleton were quantified and summarized in Table I. Indeed, a good agreement seems to exist between the effects of oligonucleotides on caldesmon levels and those on the actin cytoskeleton, indicating the presence of a causal link between these two cellular actions of the GCs (compare Table I and Fig. 6 with Figs. 2 and 5). Unexpectedly, both the antisense and sense oligonucleotides slightly modified (in the absence of GCs) the actin organization in control cells with a similar potency (Fig. 6 and Table I), in that they all induced a more polygonal cell shape and a modest apparent enhancement in microfilament organization. The nature of such effects is unclear but it is nonspecific insofar as it is unrelated to the oligonucleotide sequence. The only oligonucleotide able to inhibit the cytoskeletal effects of GC was the human AO, further confirming the specificity of its action.

Discussion

The finding here reported that caldesmon plays an essential role in the rearrangement of filamentous actin induced by GCs in A549 cells is based on two lines of evidence. One is the correlation between GC-induced microfilament reorganization and caldesmon increase with respect to the time course of the two effects, the inducing potency of various steroids, and the suppressing effect of inhibitors of proteins synthesis. The second and more decisive proof is that both GC-dependent caldesmon increase and actin filament reorganization are dramatically and specifically inhibited by an AO targeted to the most abundant caldesmon isoform in A549 cells (see below).

The potent induction of caldesmon by GCs is likely to be due to an accelerated rate of the protein's synthesis, as indicated by the large elevation in caldesmon mRNA caused by the steroids. Together with the sensitivity of the caldesmon increase to protein synthesis inhibitors, this evidence is consistent with the accepted model of GC action by which these hormones act by binding to the GC-responsive elements in the promoters of a specific set of genes in target cells, thereby inducing the synthesis of the corresponding proteins (Ringold, 1985). Canonical GC-responsive elements, however, are absent in the promoter regions of caldesmon in chicken genes (Yano et al., 1994). It is possible that the human caldesmon promoter might differ from its chicken counterpart (and contain GC-responsive elements); another possibility is that GCs may act indirectly, by inducing the expression of proteins that, in turn, promote caldesmon synthesis. Several examples of this second mechanism of action of GCs are known (Ringold, 1985).

The human AO, in contrast with its high efficacy in inhibiting the GC-dependent caldesmon increase, failed to alter control caldesmon levels. This discrepancy might be in part explained by the existence in A549 cells of both of the known types of human caldesmon isoforms (cloned from HeLa and WI-38 cells; see Hayashi et al., 1992 and Fig. 4) one of which (the HeLa type), is insensitive to the AO. However, since the HeLa form is quantitatively minor, at least at the mRNA level, a more likely explanation may be based on the fact that caldesmon is a long-lived protein (when microinjected, it is stably incorporated in microfilaments for over three days, see Yamakita et al.,

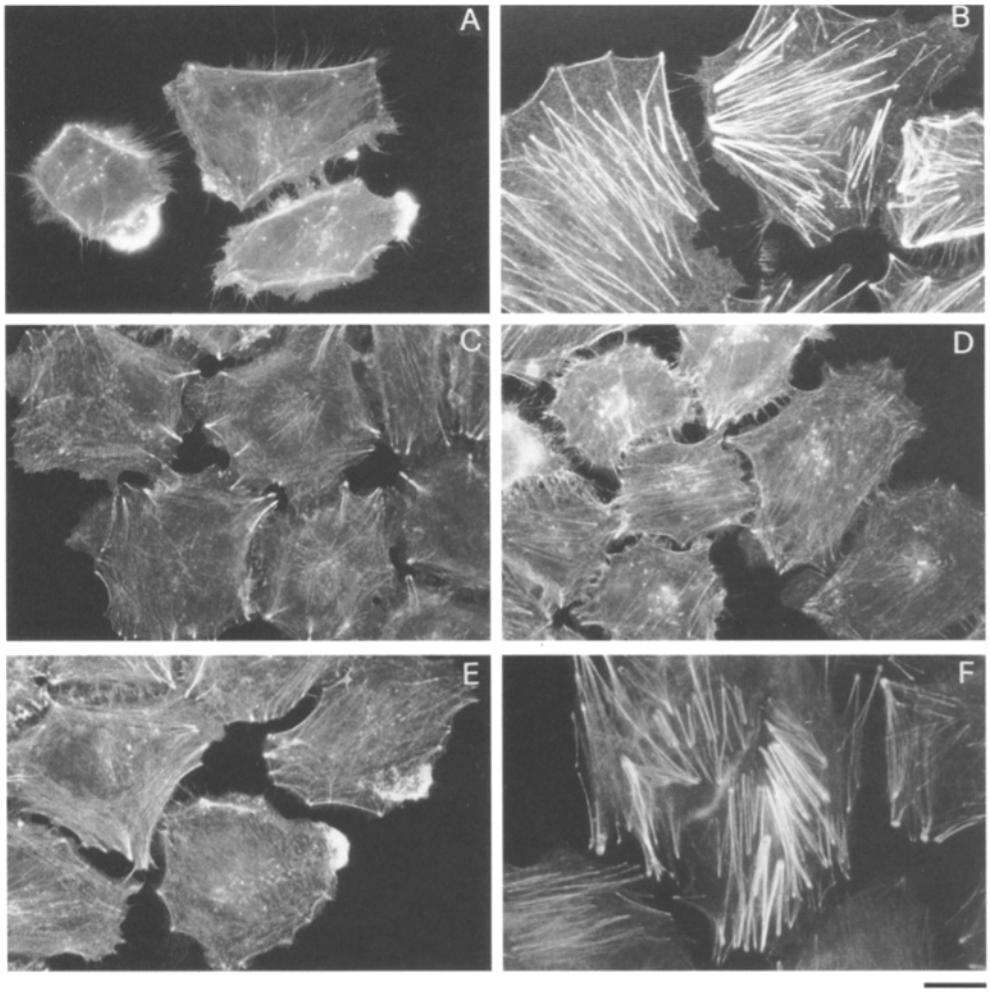

Figure 6. Effects of oligonucleotides on the glucocorticoid-induced reorganization of the actin cytoskeleton. Cells were grown and treated with dexamethasone (10^{-6} M for 24 h) and oligonucleotides exactly as described in the legend to Fig. 5. (*A*) control cells; (*B*) dexamethasone; (*C*) 30 mM AO; (*D*) dexamethasone and 30 mM AO; (*E*) 100 mM sense oligonucleotide; (*F*) dexamethasone and 100 mM sense oligonucleotide. Both sense and antisense chicken caldesmon oligonucleotides at 30 mM were without effect. The experiment was repeated four times with similar results. Bar, 5 μm.

1990). Thus, caldesmon would not be appreciably degraded during the treatment with AO (two days), whereas new synthesis of the protein (activated by GCs) would be efficiently prevented.

GCs are known to modify the expression of several proteins in target cells (Ringold, 1985); indeed, at least 6 proteins were increased and one decreased by dexamethasone in A549 cells (see Fig. 2, *F* and *G*). It is therefore interesting to ask whether caldesmon functions alone or in synergy with other GC-induced proteins to produce the observed cytoskeletal rearrangements. The transition from the background meshwork of scarce microfilaments in control cells to the numerous typical stress fibers in GC-treated cells is likely to require the annealing of preexisting short filaments into longer units and the bundling of these structures into stress fibers. Indeed, the in vitro properties of caldesmon to enhance the ability of tropomyosins to inhibit microfilament severing by gelsolin, to stimulate filament reannealing and lengthening (Ishikawa et al., 1989*a*,*b*), and, possibly, to induce cross-linking of actin filament (Sobue et al., 1985, 1988; Cross et al., 1987), appear to be well suited to account for the observed reorganization of the actin cytoskeleton. For this to occur in vivo, the intracellular concentrations of this protein must fall in the appropriate range. The in vitro effects of caldesmon are maximal at a caldesmon to actin ratio of ~1:18 (Ishikawa et al., 1989*b*). This ratio in live untreated A549 cells appears to be ~1:100, since caldesmon accounts for ~0.05% of total cellular proteins (see Fig. 2, in good agreement with Yamakita et al., 1990), and actin for ~5% (a value commonly presumed correct for most cultured cells). As the five- to sevenfold stimulation by GC would change this ratio to ~1:15–20 (within the range of optimal caldesmon activity), our data appear to be in line with the idea that an increase in caldesmon levels alone may be sufficient to reorganize the actin cytoskeleton. Support for this hypothesis comes from recent results demonstrating that overexpression of caldesmon through cDNA transfection techniques enhances the stability and the degree of organization of microfilaments into stress fibers (our unpublished results; Sugurcheva and Bryan, 1995). Warren et al. (1994) have reported that the overexpression of a large COOH-terminal fragment of caldesmon in CHO cells resulted in enhanced resistance to cytochalasin B of actin filaments (similar to our previous results, see Castellino et al., 1992), but not in the reorganization of the actin cytoskeleton of the type described in this paper. This difference might be due either to the fact that the whole caldesmon molecule has different properties than its COOH-terminal fragment used by Warren et al. (1994) or to the fact that different cellular backgrounds have been used in the two studies. The CHO cells used by these authors exhibit a well organized actin cytoskeleton even before overexpression of the caldesmon COOH-terminal domain whereas

A549 cells, where actin fibers are scarce, provide a suitable background to study microfilament remodeling.

Altogether, the above results and considerations demonstrate that a marked increase in caldesmon is absolutely required for reorganization of the actin cytoskeleton induced by GC. They also strongly suggest that such increase might be sufficient alone to support these cytoskeletal changes, but do not formally exclude the possibility that other proteins might play a role in the GC effects. A physiologically important corollary of these results is that variations in the cellular levels of caldesmon induced by hormonal stimuli may play a crucial role in the adaptive changes of the actin-based cytoskeleton that are known to accompany, and in part determine, a number of variations in the functional state of the cell. For example, the inhibition of ACTH secretion from pituitary cells (Castellino et al., 1992), the GC induction of a more differentiated phenotype in certain transformed cells (Furcht et al., 1978; Matin et al., 1990), the growth arresting effect of GCs observed in several cell lines (Jones et al., 1978), the inhibition of macrophage and fibroblast motility by GCs (Mantovani, 1985) might be in part due to caldesmon-mediated stabilization and organization of actin filaments.

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