Functional Alterations in β'-Actin from a KB Cell Mutant Resistant to Cytochalasin B

Sakuji Toyama and Sumi Toyama

Institute for Virus Research, Kyoto University, Kyoto 606, Japan

Abstract. We recently described the isolation of mutant KB cells (Cyt 1 cells) resistant to the cytotoxic effect of cytochalasin B (CB). This mutant carried an altered β-actin; i.e., β'-actin (Toyama, S., and S. Toyama. 1984. *Cell.* 37:609–614). In the present study, we have examined the functional properties of actin in Cyt 1 cells. Our results showed that increased resistance of Cyt 1 cells to CB was reflected in altered properties of β'-actin itself. This was shown directly by two findings. First, the polymerization of β'-actin was more resistant than that of β- or γ-actin to the multiple effects of CB. Second, β'-actin bound less CB than β- or γ-actin. The functional alteration of β'-actin in Cyt 1 cells was further supported by the observation that, although treatment of KB cells with CB increased the pool of unpolymerized actin, the same treatment did not affect the pool of unpolymerized actin in Cyt 1 cells, and that microfilaments of Cyt 1 cells were more resistant to the disrupting action of CB than those of KB cells. These results strongly suggest that the primary site of action of CB on cell motility processes is actin.

C YTOCHALASINS, a class of fungal metabolites, have widely been used as a powerful tool for the analysis of cellular motility processes (for review, see Tannenbaum, 1978). Recently, in vitro studies using purified actin have clearly indicated that cytochalasins inhibit actin polymerization (Brenner and Korn, 1979; Brown and Spudich, 1979; Flanagan and Lin, 1980; Lin et al., 1980; MacLean-Fletcher and Pollard, 1980) by reducing the rate of monomer addition to the barbed end of growing filaments (MacLean-Fletcher and Pollard, 1980; Lin et al., 1980; Brown and Spudich, 1981; Pollard and Mooseker, 1981; Mabuchi, 1983; Bonder and Mooseker, 1986). Several reports have also demonstrated that the actin-related gelation of cytoplasmic extracts can be inhibited by low concentration of cytochalasin B (CB)[1] (Hartwig and Stossel, 1976; Pollard, 1976; Weihing, 1976; Ishiura and Okada, 1979; MacLean-Fletcher and Pollard, 1980). Although these results suggest that cytochalasins affect cellular motility processes by interacting with cytoplasmic actin, it is still uncertain that all of the effects of CB on cellular motility processes are mediated through inhibition of actin, especially in view of its multiple classes of binding sites.

We feel that a genetic approach is useful for analyzing the relationship between the in vitro effect of CB on actin and its in vivo effect on cellular motility processes. To this end, we have isolated a mutant of KB cells (Cyt 1 cells) resistant to CB under the assumption that, if CB interacts with cytoplasmic actin, then some of the drug-resistant mutants should contain alterations in cytoplasmic actin (Toyama and Toyama, 1984). The Cyt 1 cells carry a structural alteration

in β-actin (β'-actin), and the alteration is not the result of a posttranslational modification. However, it remains uncertain whether the β'-actin is functionally altered. The present study was undertaken to determine whether the β'-actin itself shows increased resistance to the action of CB.

Materials and Methods

Cell Culture

KB 100 and KB 100 Cyt 1 cell lines used in this study have been described (Toyama et al., 1977; Toyama and Toyama, 1984). Cells were routinely grown in monolayers in MEM supplemented with 10% calf serum and 60 μg/ml kanamycin.

Purification of Actin

Cells were grown in suspension culture to a density of ∼5 × 10⁵ cells/ml in MEM (spinner modification) supplemented with 10% calf serum and 60 μg/ml kanamycin. The cells were harvested by low speed centrifugation, and washed twice with PBS and twice with 0.1 M Hepes, 0.1 mM MgSO₄, pH 7.5. The cells were then suspended in buffer G (3 mM imidazole, 0.1 mM CaCl₂, 0.5 mM ATP, 0.4 mM dithiothreitol [DTT], pH 7.5), 2 ml/g of cells, and homogenized using a Potter-Elvehjem homogenizer. Actin was purified by chromatography on DEAE-cellulose, followed by a cycle of polymerization and depolymerization and gel filtration on Sephadex G-150 as described by Gordon et al. (1976). β-, β'-, and γ-actin were isolated by chromatography on hydroxyapatite according to Segura and Lindberg (1984) with slight modification. Purified actin (∼10 mg) was passed through a PD-10 column (Pharmacia Japan Inc., Tokyo) equilibrated with 5 mM potassium phosphate, pH 7.6, 0.5 mM DTT, 0.5 mM ATP, 0.1 mM CaCl₂, and applied to a column (1 × 14 cm) of hydroxyapatite (Bio-Gel HTP; Bio-Rad Laboratories Japan, Tokyo) previously equilibrated with 5 mM potassium phosphate, pH 7.6, 1 mM DTT, 0.1 mM CaCl₂. The column was then eluted with 80 ml of a linear gradient from 5 mM potassium phosphate, pH 7.6, 0.5 mM DTT, 0.1 mM CaCl₂ to 40 mM potassium phosphate, pH 7.6,

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1. *Abbreviation used in this paper:* CB, cytochalasin B.

1.5 M glycine, 0.5 mM DTT, 0.1 mM CaCl₂. The flow rate was 1.0 ml/10 min and 1-ml fractions were collected into tubes containing 0.5 μmol or 50 μl of 10 mM ATP. Peak fractions were pooled, concentrated by ultra-filtration on an Amicon YM-10 membrane (W. R. Grace & Co. Japan, Tokyo), and dialyzed extensively against buffer G containing 0.02% NaN₃. Actin was kept on ice and used within 2 weeks. Concentration of actin was determined spectrophotometrically using an extinction coefficient of 0.63 ml·mg⁻¹.

Actin Polymerization Studies

Actin labeled with N-(1-pyrenyl)iodoacetamide was prepared by the procedure of Kouyama and Mihashi (1981) with the modification of Northrop et al. (1986).

The polymerization of actin was followed continuously by measuring the increase in fluorescence of the pyrenyl actin. Fluorescence measurements were made at 25°C using a spectrofluorometer (model 1850; Hitachi Ltd., Tokyo). The excitation and emission wavelengths were 366 nm and 388 nm, respectively.

Cytochalasin Binding Assay

The isoelectric precipitation assay was performed as previously described (Lin and Lin, 1980). Briefly, polymerized actin (20 μg) was diluted to 400 μl with buffer G containing 1 mM MgCl₂ and [³H]cytochalasin B (Amersham Japan Corp., Tokyo). After incubation at room temperature for 10 min, 32 μl of 2 M NaH₂PO₄ solution was added to the assay mixture. After 10 min, the assay mixture was centrifuged at 7,000 g at 4°C for 10 min. The supernatant was carefully removed, and the radioactivity of the pellet and of the supernatant was determined by scintillation counting. Specific binding of CB is defined as total binding minus nonspecific binding that cannot be eliminated by the presence of a 200-fold excess of unlabeled CB, as determined in a parallel assay.

Assay for Actin

Each plate was rinsed three times with PBS. Cells were lysed by pipetting with 500 μl of MT-buffer (100 mM Pipes, pH 6.75, 1 mM MgCl₂, 1 mM GTP, 1 mM EGTA, 0.2 mM DTT, 1 M sucrose, 0.5% Triton X-100). All subsequent operations were carried out at 0–4°C. For the assay of total actin, 100 μl of the lysate was mixed with 100 μl of MT-buffer and 200 μl of actin-dissociating buffer (Blikstad et al., 1978). The mixture was sheared by squirting through a 26-gauge needle to reduce viscosity. To determine the concentration of unpolymerized actin, the lysates were centrifuged at 1,500 g for 5 min to remove unlysed nuclei, and supernatants were used directly for the assay. The use of MT-buffer was necessary to stabilize the pool of filamentous actin in KB and Cyt 1 cells as observed in HeLa cells (Blikstad and Carlsson, 1982).

The amount of actin in the extracts was determined by the DNase-inhibition assay described by Blikstad et al. (1978).

Immunofluorescence Microscopy

For staining with rhodamine–phalloidin, cells grown on coverslips were fixed in 2.5% glutaraldehyde in PBS for 10 min, and then in 2.5% glutaraldehyde and 0.5% Triton X-100 in PBS for 30 min at room temperature. Fixed coverslips were rinsed in PBS, treated with 1% NaBH₄ in PBS (Weber et al., 1978), and washed again in PBS. The cells were treated with rhodamine–phalloidin (Molecular Probes Inc., Junction City, OR) for 45 min at room temperature, washed several times with PBS, and mounted in a 1:1 mixture of PBS and glycerol.

The coverslips were viewed in a Nikon Optiphot microscope (Nippon Kogaku K. K., Tokyo) equipped for epifluorescence. Fluorescence micrographs were photographed with Neopan 400 film (Fuji Photo Film Co. Ltd., Tokyo).

Isoelectric Focusing

IEF was done in vertical slab gels as described by Neefjes et al. (1986).

Results

Purification of Actin

KB and Cyt 1 actin were purified according to the proce-

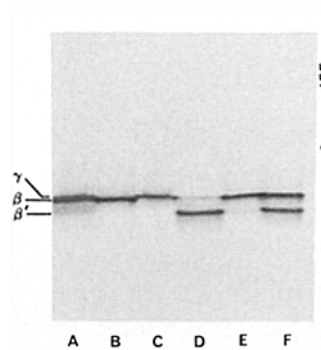Figure 1. IEF gel of purified actins. A, KB actin; B, β-actin; C, γ-actin from KB cells; D, β'-actin; E, γ-actin from Cyt 1 cells; F, Cyt 1 actin.

dure previously described for *Acanthamoeba* actin (Gordon et al., 1976). The purity of KB and Cyt 1 actin was >98% pure as judged by SDS-PAGE, and the final yield of actins from KB and Cyt 1 cells was ~2–3 mg for 10 g of packed cells. The KB actin was a mixture of β- and γ-species, and the Cyt 1 actin was a mixture of β'- and γ-species. Typically, both KB and Cyt 1 actin contained ~35% γ-actin as determined by densitometric scanning of the Coomassie Blue-stained IEF gels. However, the proportion of γ-actin in Cyt 1 actin was somewhat variable, since there were instances where the yield of β'-actin was low. The β- and γ-actin, and the β'- and γ-actin could be isolated by chromatography on hydroxyapatite from KB and Cyt 1 actin, respectively. Fig. 1 shows the IEF gel pattern of purified isoactins. The cross-contaminations between β- and γ-actin and between β'- and γ-actin were <10%.

Effect of CB on the Polymerization of Purified Actin

Since our preliminary experiments showed that β-, β'- and γ-actin could copolymerize randomly, and that pyrenyl actin
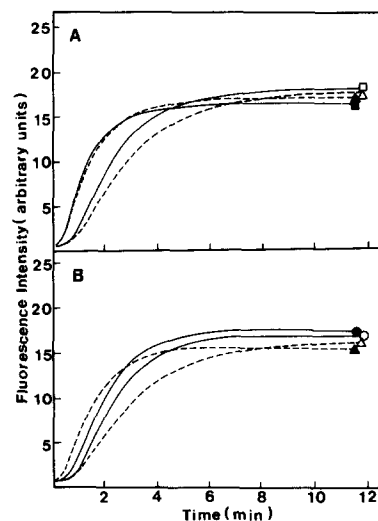

Figure 2. Effect of CB on the spontaneous polymerization of purified isoactins. Actin (12 μM containing 5% pyrenyl β-actin) was polymerized at 25°C by the addition of 100 mM KCl, 2 mM MgCl₂ in the absence (□, ○, and △) and the presence (■, ●, and ▲) of 2 μM CB. A, β- and γ-actin isolated from KB cells; B, β'- and γ-actin isolated from Cyt 1 cells. β-actin (□ and ■); β'-actin (○ and ●); γ-actin (△ and ▲).
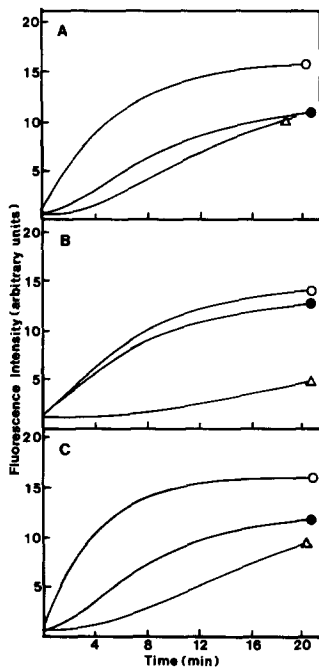
Figure 3. Effect of CB on the nucleated polymerization of purified actin. Actin (12 μM containing 5% pyrenyl β-actin) was polymerized in buffer G containing 1 mM MgCl₂ at 25°C. Actin alone (△); actin plus 0.5 μM corresponding actin fully polymerized in 2 mM MgCl₂ and sonicated immediately before use (O); actin in 2 μM CB plus 0.5 μM sonicated actin nuclei (●). *A*, β-actin; *B*, β'-actin; *C*, γ-actin from Cyt 1 cells.

Figure 4. Scatchard plot analysis of the binding of CB to polymerized actin. The lines drawn through the data points were obtained by the least squares method. β-actin (O); β'-actin (●); γ-actin from KB cells (△); γ-actin from Cyt 1 cells (▲).

displayed greatly reduced affinity for CB, the experiments described below were performed with actin solution containing 5% pyrenyl β-actin. To show that the resistance of Cyt 1 cells to CB is due specifically to an alteration in β'-actin, we first examined the effect of CB on the spontaneous polymerization of purified actin isoforms. In 100 mM KCl/2 mM MgCl₂, 2 μM CB accelerated the polymerization of β-actin, γ-actin from KB cells, and γ-actin from Cyt 1 cells but decreased slightly the final extent of polymerization of these actins (Fig. 2). In contrast, CB accelerated only slightly the polymerization of β'-actin and did not affect significantly the final extent of polymerization (Fig. 2 *B*). Next, we examined the effect of CB on the nucleated polymerization of purified actin isoforms in 1 mM MgCl₂, where nucleation was slow during the time of interest. Addition of polymerized actin nuclei to actin monomers resulted in rapid polymerization of actin. CB at 2 μM reduced the maximum elongation rate of β-actin and γ-actin from Cyt 1 cells by 60–70% (Fig. 3, *A* and *C*). Essentially identical results were obtained when γ-actin from KB cells was used instead of γ-actin from Cyt 1 cells (results not shown). As expected, β'-actin was less sensitive than β- or γ-actin to the inhibitory action of CB (Fig. 3 *B*). Interestingly, in 1 mM MgCl₂, the lag time for polymerization of β'-actin was longer than that of β-actin, and the elongation rate of β'-actin was lower than that of β-actin (Fig. 3, *A* and *B*).

CB Binding to Purified Actin

We have previously reported that Cyt 1 cells bind less CB than parental KB cells, and that the reduced binding of CB to Cyt 1 cells is not due to the decreased drug permeability of plasma membrane (Toyama and Toyama, 1984). These results suggest that the mechanism of CB resistance in Cyt 1 cells resulted from a reduced CB-binding affinity in their β'-actin. To test this hypothesis more directly, we analyzed
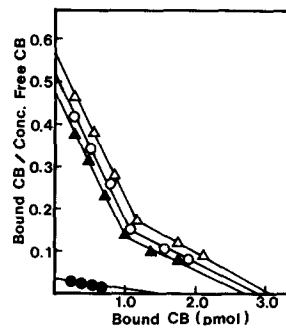
the binding of CB to purified isoactins. The dissociation constants of high affinity binding sites were 3.1, 3.0, and 3.2 × 10⁻⁹ M, respectively, for β-actin, γ-actin from KB cells, and γ-actin from Cyt 1 cells; for β'-actin the dissociation constant was 2.8 × 10⁻⁸ M (Fig. 4). The lower affinity sites of β-actin, γ-actin from KB cells, and γ-actin from Cyt 1 cells exhibited dissociation constants of ∼1 × 10⁻⁹ M. This value was much higher than the dissociation constant of β'-actin, suggesting that the low affinity binding site is structurally correlated with the high affinity binding site. However, the exact nature of the low affinity binding site remains to be elucidated.

Effect of CB on the Pools of Unpolymerized and Filamentous Actin

To determine whether the Cyt 1 mutation affects the organization of actin in vivo, we first measured the pools of unpolymerized and filamentous actin in extracts of KB and Cyt 1 cells by DNase-inhibition assay. Table I shows the results. Cyt 1 cells showed increases in the total actin content as compared with KB cells ($P < 0.001$). The fraction of unpolymerized actin in Cyt 1 cells was 36% and almost identical with that in KB cells. Next, we have investigated whether treatment of cells with CB affected the distribution between unpolymerized and filamentous actin. Treatment of KB cells with 6 μM CB increased the pool of unpolymerized actin from 34 to 49% ($P < 0.001$), whereas the same treatment did not affect the pool of unpolymerized actin in Cyt 1 cells (Table I). Control experiments showed that the DNase-inhibitory activity of β'-actin was the same as that of β- and γ-actin (data not shown).

Effect of CB on the Organization of Microfilaments

The distribution of actin filaments was examined by means of fluorescence using rhodamine–phalloidin, which bound only to filamentous actin. In normal interphase KB cells, the polymerized actin was concentrically arranged in the cell periphery, and formed cables (stress fibers) that run parallel to each other from one end of the cell to the other (Fig. 5 *A*). Cyt 1 cells did not form long actin fibers in the cytoplasm. Rather, most of the actin bundles were organized in arrays of short fibers which were located mainly in regions in contact with neighboring cells and on the bottom of the cells (Fig. 5 *B*). Exposure of KB cells to CB had a dramatic effect on actin cables. The effect was directly proportional to the drug concentration. At 6 μM CB, polymerized actin aggregated into small patches in the cytoplasm and into patches

Table I. *Effect of CB on Actin Pools*

Cell line	Cytochalasin B	Total actin*	Unpolymerized actin*	
	μM	*μg/100 μg protein*	*μg/100 μg protein*	%
KB	0	3.48 ± 0.11 (11)	1.18 ± 0.09 (11)	33.9
KB	6	3.60 ± 0.08 (8)	1.76 ± 0.05 (8)	48.8
Cyt 1	0	4.41 ± 0.13 (11)	1.60 ± 0.09 (11)	36.3
Cyt 1	6	4.43 ± 0.17 (8)	1.73 ± 0.10 (8)	39.1

Cells were plated into plastic tissue culture dishes (60 mm in diameter) at 1×10^6 cells/dish, and incubated at 37°C in a humidified incubator containing 5% CO_2 for 48 h. The medium was then replaced with fresh MEM containing no drug or containing 6 μM CB, and the cells were incubated at 37°C for 60 min. Actin pool measurements were performed as described in Materials and Methods.
* Results are shown as the mean ± SEM and the number of separate extract preparations is indicated in parentheses.

closely associated with the bases of zeiotic knobs (Fig. 5 *C*). However, some actin cables were still preserved. At 20 μM CB, actin cables disappeared completely and most of the polymerized actin was gathered into more or less discrete aggregates of varying size (Fig. 5 *E*). In contrast, treatment of Cyt 1 cells with CB at a concentration of 6 μM or 20 μM resulted in little alteration in the distribution of actin filaments (Fig. 5, *D* and *F*). Although some short fibers appeared to collapse, redistribution of actin filaments into aggregates did not occur even in the presence of 20 μM CB (Fig. 5 *F*).

Discussion

We have demonstrated that the increased resistance of Cyt 1 cells to CB in vivo is reflected in altered properties of β'-actin in vitro. This is most directly shown by the finding that the polymerization of β'-actin is more resistant than that of β- or γ-actin to the multiple effects of CB. In addition to being CB resistant the β'-actin exhibits unique polymerization characteristics. At relatively low salt conditions, such as 1 mM $MgCl_2$, the nucleation of β'-actin is slower than that of β-actin and the elongation rate of β'-actin is lower than that of β-actin (Fig. 3, *A* and *B*). However, the differences in polymerization properties between β'- and β-actin are minimal under favorable conditions for polymerization, such as 100 mM KCl, 2 mM $MgCl_2$ (Fig. 2, *A* and *B*).

The functional alteration of actin in Cyt 1 cells is further supported by the finding that, although treatment of KB cells with CB increases the pool of unpolymerized actin, the same treatment does not affect the pool of unpolymerized actin in Cyt 1 cells, and that microfilaments of Cyt 1 cells are more resistant to the disrupting action of CB than those of KB cells. The observation that the organization of microfilaments in Cyt 1 cells is different from that in KB cells provides further evidence for the functional alteration of β'-actin in Cyt 1 cells. Although Cyt 1 cells grow normally, they exhibit some different phenotypes (such as different cell morphology and weak adhesiveness to substratum) from KB cells. These phenotypes may result from functional alterations in microfilaments.

CB accelerates the initial rate of polymerization of β- and γ-actin while reducing slightly the final extent of polymerization (Fig. 2). This acceleration of the polymerization is a result of enhanced nucleation, since CB inhibits the elongation of β- and γ-actin filaments (Fig. 3). Similar effects of cytochalasins have been reported for α-actin (Löw and Dancker, 1976; Tellam and Frieden, 1982). Based on the observations

that cytochalasin D binds to monomeric actin and induces dimer formation (Goddette and Frieden, 1985, 1986), Goddette and Frieden have suggested that cytochalasin D–induced dimers serve as nuclei to enhance the polymerization of actin. If this is the case for CB, the finding that β'-actin has a reduced ability to respond to the stimulatory effect of CB (Fig. 2 *B*) would suggest that the CB-binding site on monomeric actin is structurally correlated with the high affinity binding site on filamentous actin. In contrast to cytochalasin D, CB decreases only slightly the final extent of polymerization (Fig. 2). This may be correlated with the previous observation that CB-induced ATPase activity is inhibited under conditions which favor actin polymerization (Löw and Dancker, 1976).

The data presented here show that Cyt 1 mutant cells possess β'-actin with increased resistance to the multiple effects of CB. This increased resistance is reflected in reduced affinity for the binding of CB by β'-actin. However, since γ-actin is still present in the Cyt 1 cells, it is difficult to reconcile the reduced CB-binding affinity of β'-actin as the mechanism of resistance with the general belief that CB inhibits actin polymerization (and/or interaction with actin-binding proteins) by capping the barbed end of actin filaments (Lin et al., 1980; MacLean-Fletcher and Pollard, 1980; Brown and Spudich, 1981; Pollard and Mooseker, 1981; Mabuchi, 1983; Bonder and Mooseker, 1986); i.e., if β'-actin at the barbed end of actin filament has reduced ability to bind CB, the further addition of γ-actin to this terminal β'-actin should restore the ability of the actin filament to bind the drug. There are two possible explanations to resolve this conflict. First, β- and γ-actin are segregated into functionally distinct microfilaments. The logical consequence of this model is that γ-actin is dispensable for cell motility processes, at least, for cytokinesis which is the selective marker for the isolation of Cyt 1 mutant. Second, β- and γ-actin are functionally interchangeable and, consequently, the sensitivity of cells to CB is determined by the relative amount of β'-actin to γ-actin; since β'-actin is a major isoform in Cyt 1 cells, the cells could escape from the inhibitory action of CB. We favor the second hypothesis, since our preliminary experiments indicate that the polymerization in vitro of unfractionated Cyt 1 actin (or reconstituted Cyt 1 actin composed of 65% purified β'-actin and 35% purified γ-actin) is more resistant than that of unfractionated KB actin (or purified β- or γ-actin) to the multiple effects of CB (Toyama, S., and S. Toyama, unpublished results). This hypothesis is also compatible with recent reports. McKenna et al. (1985) have demonstrated that microinjection of a mixture of muscle and nonmuscle isoac-
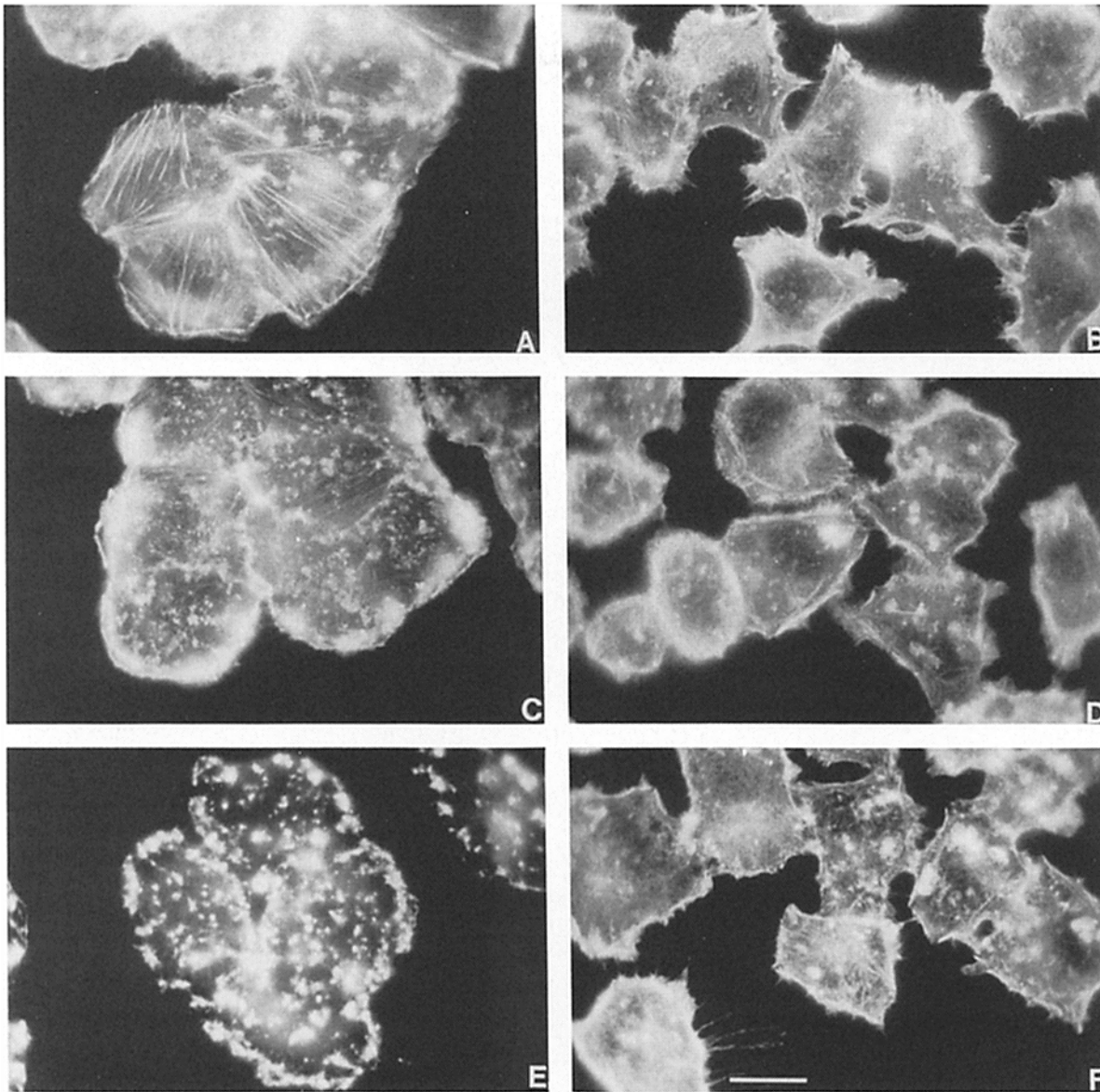
Figure 5. Fluorescence micrographs of actin filaments in KB and Cyt 1 cells. Cells were treated for 60 min at 37°C with CB before fixation. *A*, untreated KB cells; *B*, untreated Cyt 1 cells; *C*, KB cells treated with 6 µM CB; *D*, Cyt 1 cells treated with 6 µM CB; *E*, KB cells treated with 20 µM CB; *F*, Cyt 1 cells treated with 20 µM CB. Bar, 20 µm.

tins into fibroblasts results in an identical distribution of the isoactins introduced. Similarly, Otey et al. (1986), using γ-actin–specific antibody to localize γ-actin in a variety of cultured cells, have detected the γ-actin in all microfilament-containing structures, such as stress fibers, ruffled edges, and contractile rings, and have observed no subcellular sorting of the nonmuscle actin isoforms.

To our knowledge, this is the first time that a β-actin mutation resulting in alteration of polymerization properties in vitro has been proved. Further biochemical studies with this mutant should provide valuable information regarding the structure/function relationship of β-actin.

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