Direct Proof That the Primary Site of Action of Cytochalasin on Cell Motility Processes Is Actin

Haruo Ohmori, Sumi Toyama, and Sakuji Toyama

Institute for Virus Research, Kyoto University, Kyoto 606-01, Japan

Abstract. We have previously described the isolation of a mutant KB cell (Cyt 1 mutant) resistant to the cytotoxic effect of cytochalasin B (CB). The Cyt 1 mutant carries an altered form of β-actin (β′-actin) and lacks normal β-actin (Toyama, S., and S. Toyama. 1984. *Cell.* 37:609–614). Increased resistance of the Cyt 1 mutant to CB in vivo is reflected in altered properties of β′-actin in vitro (Toyama, S., and S. Toyama. 1988. *J. Cell Biol.* 107:1499–1504). Here, we show that the mutation in β-actin is solely responsible for the cytochalasin-resistant phenotype of the Cyt mutant. We have isolated a cDNA clone encoding β′-actin from Cyt 1 cells. Sequence analysis reveals two mutations in the coding region that substitute two amino acid residues ($Val^{139} \rightarrow$ Met and $Ala^{295} \rightarrow$ Asp). Expression of the β′-actin cDNA confers cytochalasin resistance upon transformed cytochalasin-sensitive KB cells. Levels of resistance to CB in the transformed cell clones correlate well with amounts of β′-actin polypeptide. Both of the two mutations in β′-actin are necessary for the high level expression of cytochalasin resistance. Overall, we conclude that the primary site of action of cytochalasin on cell motility processes in vivo is actin.

CARTER (1967) first reported the effect of cytochalasins on cell motility processes, such as inhibition of cell movement, prevention of cytoplasmic cleavage, and extrusion of nucleus. Since then, cytochalasins have been widely used as a tool to study motility-related processes in a variety of cell types (for review see Tannenbaum, 1978), and cytochalasin sensitivity has often been taken as an indication of the involvement of an actin-based microfilament system in the absence of proof. In vitro studies using purified actin have clearly indicated that cytochalasins bind one end of actin filaments (Brown and Spudich, 1979; Flanagan and Lin, 1980; Lin et al., 1980) and inhibit its 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). Recently, Goddette and Frieden (1985, 1986*a*,*b*) examined the interaction of monomeric actin with cytochalasin D to explain the finding that cytochalasin B (CB)[1] accelerates the initial rate of polymerization while reducing the final extent (Löw and Dancker, 1976). They found that cytochalasin D binds actin monomer and induces dimer formation, and proposed a mechanism which explains both the enhanced rate of polymerization and the increased critical concentration. The mechanism depends on the cytochalasin-induced formation of dimer (to enhance the rate) and on the cytochalasin-enhanced rate of ATP hydrolysis in the dimer to yield higher concentrations of monomeric actin containing ADP (to increase the critical concentration). Although these results suggest that cytochalasins affect cell motility processes by interacting with cytoplasmic actin, the possibility that cytochalasins have target(s) other than actin cannot be totally excluded (for review see Cooper, 1987).

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 an altered form of β-actin (β′-actin) and lack normal β-actin. The alteration in β′-actin is not the result of a posttranslational modification. Furthermore, increased resistance of Cyt 1 cells to CB is reflected in altered properties of β′-actin itself (Toyama and Toyama, 1988). These results strongly suggest that the mutation of β′-actin is responsible for the cytochalasin resistance of Cyt 1 cells, but left unanswered the question of whether Cyt 1 cells might have mutation(s) in molecules other than β-actin which could account for the cytochalasin resistance. Here, we present evidence that the expression of β′-actin cDNA in cytochalasin-sensitive parental KB cells confers resistance to CB, and conclude therefore that the primary site of action of cytochalasin on cell motility processes is actin.

1. *Abbreviations used in this paper*: CB, cytochalasin B; tk, thymidine kinase.

Materials and Methods

Cell Line and Cell Culture

The KB100 and KB100 Cyt 1 cell lines used in this study have been described (Toyama et al., 1977; Toyama and Toyama, 1984). Cells were routinely maintained in monolayers at 37°C in MEM supplemented with 10% newborn calf serum and 50 μg/ml kanamycin. The thymidine kinase (tk)-deficient KB cell line (KB100 TK^-) was isolated from KB100 cells by the stepwise treatment of 5-bromodeoxyuridine (Toyama, S., unpublished results).

Cloning and Sequencing of β'- and γ-Actin cDNAs

cDNA was synthesized with oligo-dT primer from 5 μg/ml of poly(A)$^+$ mRNA isolated from Cyt 1 cells using the cDNA synthesis kit from Pharmacia Fine Chemicals, (Pharmacia Japan Inc., Tokyo). After adding EcoRI adaptors, cDNA was ligated into λgt10 and packaged in vitro (Gigapack II Plus; Strategene Cloning Systems, Inc., La Jolla, CA). cDNA clones containing β'- and γ-actin sequences were identified by plaque hybridization using digoxigenin-labeled human cDNA clone previously shown to encode β-actin (Hanukoglu et al., 1983). The digoxigenin labeling and detection of digoxigenin-labeled probe were carried out by using the nonradioactive DNA labeling and detection kit (Boehringer Mannheim GmbH, Mannheim, Germany). cDNAs were then subcloned into Bluescript II plasmid (Stratagene Cloning Systems, Inc.). Nested sets of deletions were made by exonuclease III digestion, and the cDNAs were sequenced by the dideoxy chain termination method (Sanger et al., 1977).

Construction of Expression Vector

Standard techniques were used for construction of the expression vector (Maniatis et al., 1982). The plasmid pTKSVβ'-act 1 is shown in Fig. 2. This plasmid contains the following elements: (*a*), PvuII-NcoI fragment of tk gene from herpes simplex virus (BclI-ClaI portion of pTKSVβ'-act 1); (*b*), PstI-BamHI fragment of pSV2neo (ClaI-BamHI portion of the plasmid); (*c*), BamHI-BglII fragment of pSV2hph (BamHI-BglII portion of the plasmid); (*d*), MstI-EcoRI fragment of β'-actin cDNA (BglII-HindIII portion of the plasmid); (*e*), HindIII-AccI fragment of pSV2neo (HindIII-MluI portion of the plasmid); (*f*), AccI-ApaLI fragment of pSV2neo (MluI-ApaLI portion of the plasmid); (*g*), ApaLI-ApaLI fragment of pUC19 (ApaLI-ApaLI portion of the plasmid); and (*h*), ApaLI-EcoRI fragment of pSV2neo (ApaLI-BclI portion of the plasmid).

To construct truncated tk gene of herpes simplex virus, the segment flanked by two MluI sites was removed through digesting the DNA with MluI, blunting the ends by the Klenow fragment of *E. coli* DNA polymerase I, and ligating the blunted ends by T4 DNA ligase. This deletion leaves intact the enhancer/promoter region but removes all of the wild type-nontranslated leader and the first 10 codons for tk polypeptide.

DNA Transformation

Plasmids were transfected as supercoiled circular DNA using the calcium phosphate precipitation procedure (Graham and van der Eb, 1973). The final amount of DNA added to a 25-cm^2 flask was 10 μg. The medium was changed 4 h before transfection. The precipitates in a final volume of 0.5 ml were added to 5 ml medium. After incubation for 16 h, the cells were treated with 15% glycerol in Hepes-buffered saline (20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, pH 7.5) for 3 min, and were then incubated for 20 h in MEM. The cells were harvested by trypsin-EDTA treatment, counted, and replated at ~2 × 10^6 cells per 100-mm dish in HAT medium (MEM containing 100 μM hypoxanthine, 4 μM aminopterine, and 16 μM thymidine). The medium was changed every 4 d. On the 16th day after the addition of HAT medium, the medium was replaced by HT medium (MEM containing 100 μM hypoxanthine and 16 μM thymidine) containing 2 μg/ml of CB. After a further 5 d, the cells were fed again with MEM containing CB at 2 μg/ml, and continued to incubate for 5 d. Individual colonies were isolated with cloning cylinders and propagated into mass culture.

Assay for Cytochalasin Resistance

The resistance of cells to the growth-inhibitory effect of cytochalasin was determined by plating 150 cells at various drug concentrations in 6-well multiwell plate (Falcon 3046; Becton Dickinson and Co., Ltd., Tokyo) containing 4 ml MEM. After 12 d of incubation at 37°C in a CO_2 incubator, the cells were stained with 1% methylene blue in 50% ethanol. The resistance is expressed by D_{10} value: the drug concentration that reduces cell survival to 10%.

Southern Blot Analysis

Genomic DNA was digested with 6 U restriction endonuclease per μg DNA at 37°C for 16 h, and electrophoresed in 0.7% agarose (10 μg DNA per slot). The electrophoresed DNA fragments were transferred to nylon membranes (Nytran-N; Schleicher & Schuell, Inc., Keene, NH). The membranes were hybridized with digoxigenin-labeled probes. Probes were prepared using nonradioactive DNA labeling kit (Boehringer Mannheim GmbH). Hybridization and washing were done according to the protocol in the kit. The hybridized probes were detected with antidigoxigenin antibody: alkaline phosphatase conjugate (Boehringer Mannheim GmbH) and by soaking in the luminescent substrate (Lumiphos 530; Lumigen, Inc., Detroit, MI). The filters were wrapped in Saranwrap and exposed to Fuji RX X-ray film (Fuji Photo Film Co. Ltd., Tokyo).

IEF in Vertical Slab Gel

IEF in 4.5% acrylamide vertical slab gel was carried out as described previously (Toyama and Toyama, 1988), except that cathode electrode solution was 20 mM NaOH and anode electrode solution was 10 mM H_3PO_4. Gels were stained using silver stain kit Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Lanes were scanned and the peaks were integrated with a scanning densitometer (Biomed Instruments Inc., Fullerton, CA).

Results

Defining the Mutation on β'-Actin by DNA Sequencing

We have previously reported that the isoelectric point of

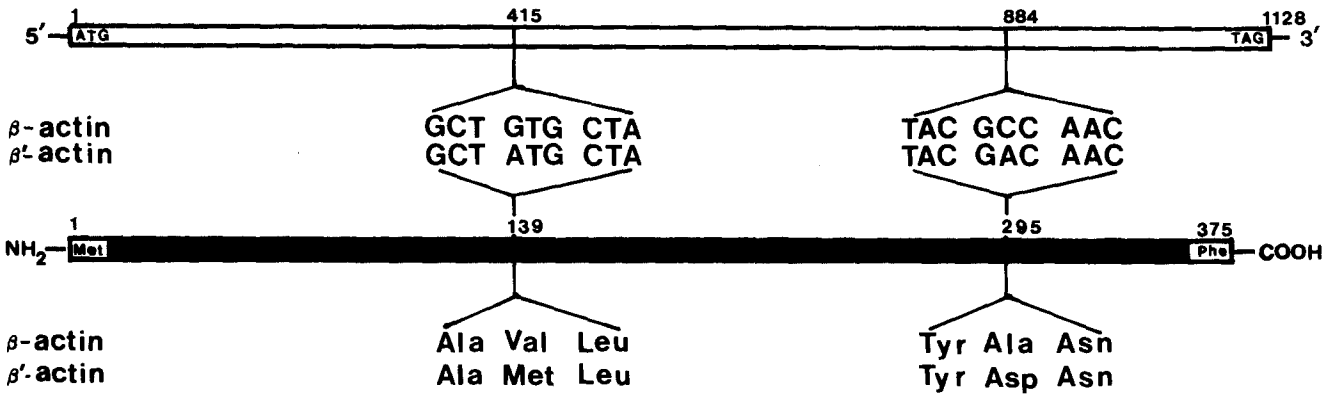

Figure 1. Locating and sequencing the Cyt 1 mutation. Schema of cDNA and polypeptide of β'-actin are presented, and the nucleotide sequence and predicted amino acid sequence surrounding mutation sites are presented. These sequence data are available from EMBL/GenBank/DDBJ under accession number X63432.

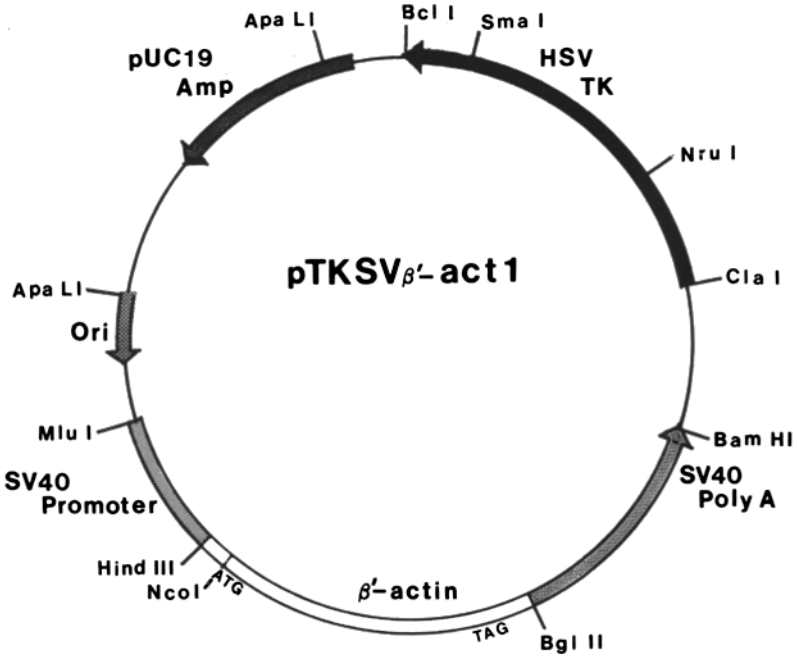

Figure 2. Structure of pTKSVβ'-act 1. pTKSV β'-act 1 carries a 1.27-kb EcoRI-MstI fragment from β'-actin cDNA containing the entire coding region plus 87 nucleotides of 5'-noncoding sequence and 52 nucleotides of 3'-noncoding sequence. The β'-actin cDNA is under the control of SV40 enhancer/promoter combination and followed by the SV40 splice and polyadenylation signal. Arrows indicate the direction of transcription. Some restriction sites used for the construction and the further analysis of the vector are indicated.

β'-actin is more acidic than that of β-actin, by approximately one electrical charge, and that this alteration is not the result of a posttranslational modification (Toyama and Toyama, 1984). These findings suggest that β'-actin has a substitution of amino acid involving a charged one. To prove this directly, we isolated cDNA clones encoding β'- and γ-actins from Cyt 1 cells, and sequenced these cDNA clones. The coding region of β'-actin cDNA had nucleotide sequence identical to that of a reported β-actin cDNA (Ponte et al., 1984), except for two base substitutions that alter the predicted amino acid sequence (Fig. 1). A first and unexpected mutation was found in codon 139, and this was a GC to AT transition (GTG → ATG) to give an amino acid substitution of Val^{139} → Met. A second mutation had a CG to AT transversion (GCC → GAC) at codon 295. This would replace Ala^{295} → Asp; the predicted gain of a positive charge accounts for the altered isoelectric point of β'-actin.

The nucleotide sequence of coding region of γ-actin cDNA was identical to that of the γ-actin gene from HeLa cells (Erba et al., 1988).

Strategy for the Construction of Expression Vector

To exclude the possibility that mutations in molecules other than β-actin may account for the CB resistance of Cyt 1 cells, it is necessary to express the β'-actin cDNA in cytochalasin-sensitive parental KB cells. Since in vitro polymerization studies have indicated that the resistance to CB is determined by the relative amount of β'-actin to β-actin or of β'-actin to γ-actin (Toyama, S., unpublished results), and since actin is the most abundant protein in KB cells, we reasoned that high level expression of the transfected β'-actin cDNA is required for transformed cells to express cytochalasin-resistant phenotype. Therefore, we designed an expression vector for the direct screening of transformed cells with high level expression. As a selectable gene, we chose truncated tk gene of herpes simplex virus (see Materials and Methods). This is based on the property of Δ1 deletion in tk gene. This deletion removes the first 27 codons of tk polypeptide, and consequently, translation initiates from the second AUG codon (Halpern and Smiley, 1984). The Δ1 produces ~40% of the tk protein synthesized by wild-type virus (Irmiere et al., 1989). After transfection of the expression vector carrying the truncated tk gene into TK^- cells, it is expected that only such transformed cells that express high levels of tk polypeptide will survive HAT selection. Because there is some relation between expression level and copy number of gene, high copy number of the integrated expression vector could compensate the reduced expression level of tk protein. Consequently, these cells are expected to exhibit high expression levels for the linked nonselected genes. Since the level of expression is also determined by the strength of enhancer/promoter activity, we tested various enhancer/promoter combinations from different sources (such as SV40, cytomegalovirus, and β-actin). Among the tested ones, SV40 enhancer/promoter combination was the most efficient to express β'-actin cDNA on KB cells. Based on these considerations, we have constructed the expression vector depicted in Fig. 2.

Expression of β'-Actin cDNA Confers Cytochalasin Resistance upon Transformed Cells

KB100 TK^- cells were transfected with β'-actin expression vector pTKSVβ'-act 1. TK^+ transformants were first selected in HAT medium, and they were then incubated in medium containing 2 μg/ml of CB. ~35% of the TK^+ transformants survived the CB treatment. Eight individual clones were picked and grown to mass culture (Tfβ'-11–Tfβ'-18). These clones were tested for their ability to grow in the presence of varying concentrations of CB, and the amount of β'-actin in these clones was quantitated from densitometric scan of IEF gels. Fig. 3 shows an IEF pattern of proteins from these transformant clones. All of the transformant clones displayed a protein band which had the same isoelectric point as β'-actin of Cyt 1 cells, but the intensity of the bands varied among

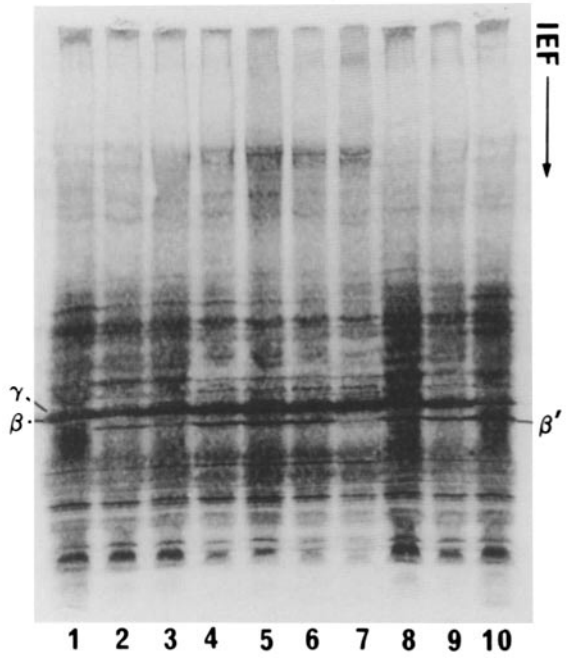

Figure 3. IEF analysis of extracts from KB cells transformed with pTKSVβ'-act 1. Monolayers of cells were washed two times with PBS. The cells were harvested by scraping with a rubber policeman and washed two times with PBS. The final pellets were dissolved directly in O'Farrel's lysis buffer (O'Farrel, 1975). The gels were run as described in Materials and Methods. (Lane *1*) KB100 TK⁻; (lane *2*) Tfβ'-11; (lane *3*) Tfβ'-12; (lane *4*) Tfβ'-13; (lane *5*) Tfβ'-14; (lane *6*) Tfβ'-15; (lane *7*) Tfβ'-16; (lane *8*) Tfβ'-17; (lane *9*) Tfβ'-18; and (lane *10*) KB100 Cyt 1.

different clones. As expected, expression of the β'-actin cDNA conferred cytochalasin resistance upon transformed cells, and expression levels of β'-actin correlated well with levels of resistance to CB (Table I). Essentially identical results were obtained, when TK⁺ transformants were selected in HAT medium and these clones were individually analyzed without CB selection, except that ~65% of the TK⁺ transformants expressed relatively low amounts of β'-actin (<5% of the total actin) and did not exhibit any increased resistance to CB. No cytochalasin-resistant colonies appeared in control groups that were mock transfected, or transfected with the expression vector containing either γ-actin cDNA or chimeric β-actin cDNA (Fig. 4). Transformant clones also exhibited increased resistance to cytochalasins D and E (results not shown).

To confirm further the relationship between the amount of β'-actin and the level of resistance to CB, we selected one relatively unstable transformant clone (Tfβ'-17) and serially passaged it in the stepwise increasing concentration of CB (3, 5, and 9 μg/ml). The cells termed Tfβ'-17(9), were then passaged for 40 times in the absence of CB [Tfβ'-17(40')]. Expression levels of β'-actin and levels of CB resistance in these cells were determined as described above. The results are shown in Table I. The passage in CB led to the significant increase in the amount of β'-actin, which comprised ~40% of the total actin in Tfβ'-17(9) cells. Concomitantly, Tfβ'-17(9) cells exhibited a D_{10} for CB of 9.7 μg/ml, which is only slightly lower than that of Cyt 1 cells (12 μg/ml), when Tfβ'-17(9) cells had been passaged in the absence of CB [Tfβ'-17(40')], the amount of β'-actin was reduced to 10% of the total actin. This decrease in the expression level of β'-actin paralleled decrease in the resistance level to CB (D_{10}, 1.9 μg/ml).

Mutation of Both Val^{139} → Met and Ala^{295} → Asp Is Required to Produce Cytochalasin Resistance

Since the β'-actin cDNA carries two mutations that lead two amino acid substitutions, we ask whether both mutations are required to produce cytochalasin resistance. To this end, we constructed hybrid cDNAs by exchange of restriction fragments between β'- and γ-actin cDNA (Fig. 4). Plasmid containing these chimeric cDNA (pTKSVβ'-act 2 and pTKSV β'-act 3) were transfected into KB100 TK⁻ cells, and trans-

Table I. Relationship between Expression Levels of β'-Actin and Levels of CB Resistance

Transfected actin cDNA	Strain	Mutation	Fraction of β'-actin (% of total actin)	D_{10}
				μg/ml
None	TK⁻	None	0	1.4
None	Cyt 1	Val^{139}→Met, Ala^{295}→Asp	50	12.0
β'-Actin 1	Tfβ'-12	Val^{139}→Met, Ala^{295}→Asp	7	1.5
β'-Actin 1	Tfβ'-16	Val^{139}→Met, Ala^{295}→Asp	8	1.6
β'-Actin 1	Tfβ'-14	Val^{139}→Met, Ala^{295}→Asp	24	2.0
β'-Actin 1	Tfβ'-15	Val^{139}→Met, Ala^{295}→Asp	25	2.2
β'-Actin 1	Tfβ'-11	Val^{139}→Met, Ala^{295}→Asp	27	2.2
β'-Actin 1	Tfβ'-13	Val^{139}→Met, Ala^{295}→Asp	28	2.5
β'-Actin 1	Tfβ'-18	Val^{139}→Met, Ala^{295}→Asp	28	2.6
β'-Actin 1	Tfβ'-17	Val^{139}→Met, Ala^{295}→Asp	32	3.2
β'-Actin 1	Tfβ'-17(9)	Val^{139}→Met, Ala^{295}→Asp	41	9.7
β'-Actin 1	Tfβ'-17(40')	Val^{139}→Met, Ala^{295}→Asp	10	1.9
β'-Actin 2	Tfβ'-21	Val^{139}→Met	30*	1.8
β'-Actin 2	Tfβ'-22	Val^{139}→Met	24*	1.7
β'-Actin 3	Tfβ'-31	Ala^{295}→Asp	20	1.6
β'-Actin 3	Tfβ'-32	Ala^{295}→Asp	34	1.8

* Values were calculated from the β-/γ-actin ratios of the transformed and wild-type TK⁻ cells.

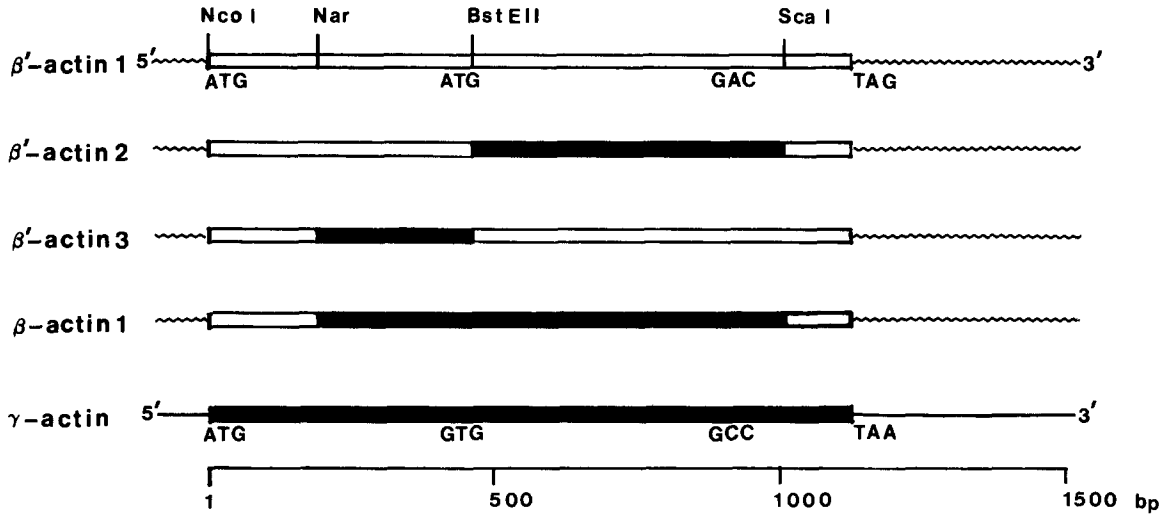

Figure 4. Construction of restriction fragment chimeras. Restriction fragment chimeras were constructed by exchange of restriction fragment between β′-actin and γ-actin cDNAs. To create the unique NcoI site at the same position as in the β′-actin cDNA, guanine at 1 nucleotide upstream from the initiator ATG codon of γ-actin cDNA was replaced by cytosine (by site-directed mutagenesis). The restriction sites that can be used for the construction of chimeras are indicated.

formants were selected as described in the preceding section. The transformants thus obtained were tested for their ability to grow in the presence of varying amounts of CB and the amount of mutant actin in these transformants was determined by scanning IEF gels. The expression of cDNA carrying mutation $Ala^{295} \rightarrow Asp$ (β′-actin 3) produced a polypeptide that migrated to the same position as β′-actin, whereas the expression of cDNA carrying mutation $Val^{139} \rightarrow Met$ (β′-actin 2) produced a polypeptide that could not be separated from β-actin (Fig. 5). This provides direct evidence that mutation $Ala^{295} \rightarrow Asp$ is responsible for the altered isoelectric point of β′-actin. The expression of cDNA carrying either of two single substitutions ($Ala^{295} \rightarrow Asp$ or $Val^{139} \rightarrow Met$) conferred a low level of resistance to CB (Table I). The expression levels of mutant actin in transformants containing $Ala^{295} \rightarrow Asp$ substitution were comparable to those containing both $Ala^{295} \rightarrow Asp$ and $Val^{139} \rightarrow Met$ substitutions (Table I). The transformants expressing $Val^{139} \rightarrow Met$ mutation did exhibit an increase in the β-/γ-actin ratio. Based on the comparison of β-/γ-actin ratio between the transformed and wild-type KB cells, we infer that the expression levels of mutant actin in these transformants are comparable to those in transformants containing $Ala^{295} \rightarrow Asp$ substitution (Table I).

These results indicate that mutation of both $Val^{139} \rightarrow Met$ and $Ala^{295} \rightarrow Asp$ is required to produce a CB-resistant phenotype of Cyt 1 cells. Substitution of either one of these residues results in a low level of CB resistance.

Southern Blot Analysis of Integrated Plasmid Sequences

To exclude the little possibility that the cytochalasin-resistant phenotype of transformants is due to the integration of vector DNA into specific site of TK^- cell genome (i.e., insertional mutation of a special gene that determines sensitivity to cytochalasin), chromosomal DNAs from six transformant clones were digested with the enzyme BamHI, which cuts the pTKSVβ′-act 1 sequence at a unique site, transferred to a nylon membrane after electrophoresis on agarose gel, and probed for a tk gene sequence (Fig. 6 *A*). DNA from five clones [Tfβ′-19, Tfβ′-110, Tfβ′-111, Tfβ′-112, and Tfβ′-17(9)] yielded a prominent 6.58-kb fragment that was the size of intact linear pTKSVβ′-act 1 plasmid. Minor bands were also visible on the blot. These bands most probably correspond to DNA fragments containing the joining site between plasmid and cellular DNA. Each cell clone yielded a specific pattern of joining fragments. Similar results were obtained when a β′-actin probe was used instead of the tk probe, except that several additional weak signals which derived from endogenous actin-related genes were observed (Fig. 6 *B*). Restriction enzymes which do not cut out the pTKSVβ′-act 1 sequence produced fragments of more than 23 kb in size (data not shown). An exception is clone Tfβ′-113. This clone, which did not reveal any hybridization signal, no longer exhibited cytochalasin resistance and failed to show the β′-actin polypeptide on IEF gels (data not shown), suggesting that the transfected plasmid sequence had been lost during the process of cloning.

These results clearly show that the plasmid pTKSVβ′-act 1 sequence is integrated in various sites of the chromosomal DNA as tandemly arranged intact units. As expected, these results also suggest that the high level expression of β′-actin is correlated with high copy number of the integrated expression vector.

CB-induced Cellular Responses in Transformed Cells

To show further that the effect of CB on cell motility processes is through actin, we have analyzed some CB-induced cellular responses in transformed Tfβ′-17(9) cells.

Untreated KB100 TK^- cells displayed prominent array of stress fiber (Fig. 7 *A*). The expression of β′-actin in these cells caused reorganization of actin filaments. In Tfβ′-17(9) cells, the filaments became thinner in width and shorter in length. They intersected with each other (Fig. 7 *C*). This or-

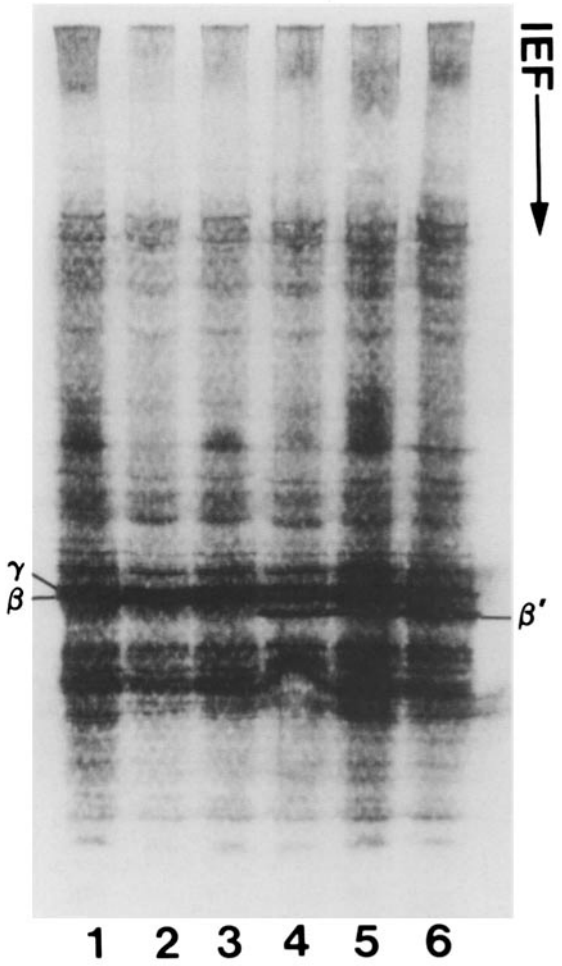

Figure 5. IEF analysis of extracts from KB cells transformed with pTKSVβ′-act 2 and pTKSVβ′-act 3. Lysates were prepared from transformants expressing Val^{139} → Met mutation (Tfβ′-21 and Tf β′-22) and Ala^{295} → Asp mutation (Tfβ′-31 and Tfβ′-32) as described in the legend to Fig. 3. (Lane *1*) KB100 TK^-; (lane *2*) Tf β′-21; (lane *3*) Tfβ′-22; (lane *4*) Tfβ′-31; (lane *5*) Tfβ′-32; and (lane *6*) KB100 Cyt 1.

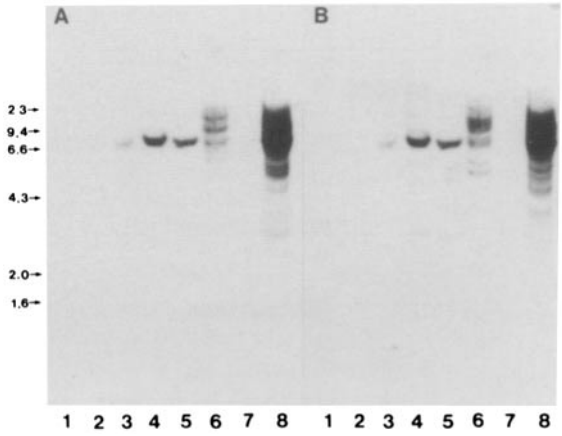

Figure 6. Southern blot analysis of independent pTKSVβ′-act 1 transformants. High molecular weight DNA from the transformant cells were digested with BamHI, submitted to electrophoresis in 0.7% agarose, transferred to a nylon membrane, and hybridized to a 1.57-kb ClaI-BclI fragment (tk probe) from pTKSVβ′-act 1 (*A*). The membrane was stripped off the hybridized tk probe and reprobed with a 1.18-kb NcoI-BglII fragment (β′-actin probe) from pTKSVβ′-act 1 (*B*). The size of standard DNA is indicated in kilobase pairs. (Lanes *1*) pTKSVβ′-act 1 cleaved with BamHI (10.4 pg: approximating single-copy gene present in the amount of DNA loaded to gel); (lane *2*) KB100 TK^-; (lanes *3*) Tfβ′-19; (lanes *4*) Tfβ′-110; (lanes *5*) Tfβ′-111; (lane *6*) Tfβ′-112; (lanes *7*) Tfβ′-113; and (lanes *8*) Tfβ′-17(9).

ganization of actin filaments was close to that of Cyt 1 cells (Fig. 7 *E*). Exposure of KB100 TK^- cells with CB resulted in a disruption of actin filament bundles and most of the polymerized actin was gathered into aggregates of varying size (Fig. 7 *B*). In contrast, treatment of Tfβ′-17(9) and Cyt 1 cells with CB resulted in little alteration in the distribution of actin filaments (Fig. 7, *D* and *F*).

The projection of knobby protuberance at cell surface (zeiosis) is a general cellular response to CB. When KB100 cells were treated with 20 μM CB, the cell surface was beset by the cluster of zeiotic knobs within 10 min (Fig. 8, *A* and *B*). In contrast, the cell surface of Tfβ′-17(9) and Cyt 1 cells appeared to remain unchanged in the presence of CB (Fig. 8, *C–F*).

Discussion

Our goal was to prove that the mutation in β-actin is solely responsible for the cytochalasin-resistant phenotype of Cyt 1 cells. We have isolated and sequenced the cDNA encoding cytochalasin-resistant β′-actin. The β′-actin cDNA was subcloned into expression vector and expressed in cytochalasin-sensitive parental KB cells. Sequence analysis of the β′-actin cDNA reveals the presence of two amino acid substitutions (Val^{139} → Met and Ala^{295} → Asp). Expression of the β′-actin cDNA in wild-type KB cells results in the synthesis of β′-actin and confers cytochalasin resistance. Levels of resistance to CB in transformed cells correlate well with amounts of β′-actin polypeptides. Based on these results together with our previous finding that β′-actin is more resistant than β- or γ-actin to the multiple effects of CB (Toyama and Toyama, 1988), we conclude that the primary site of action of CB on cell motility processes is actin.

Interestingly, our results obtained by transfection experiments with chimeric actin cDNA clearly demonstrate that mutation of both Val^{139} → Met and Ala^{295} → Asp is required for high level expression of cytochalasin resistance in transformed cells. This suggests that residues Val^{139} and Ala^{295} are located in or close to a region which is involved in the binding of cytochalasins, since our previous work has shown that purified β′-actin displays a decreased binding affinity for CB (Toyama and Toyama, 1988). Very recently, the structure of the actin–DNase I complex has been solved at 2.8 Å resolution by X-ray crystallography and an atomic model for F actin has also been proposed (Kabsch et al., 1990; Holmes et al., 1990). According to the atomic model of actin, Val^{139} is located in the α-helix (residues 137–144) of subdomain 1 and Ala^{295} is located adjacent to the short helix (residues 288–294) in the subdomain 3. Residues Val^{139} and Ala^{295} stand at opposite sides of the cleft between subdomains 1 and 3. The logical consequence of these considerations is that cy-

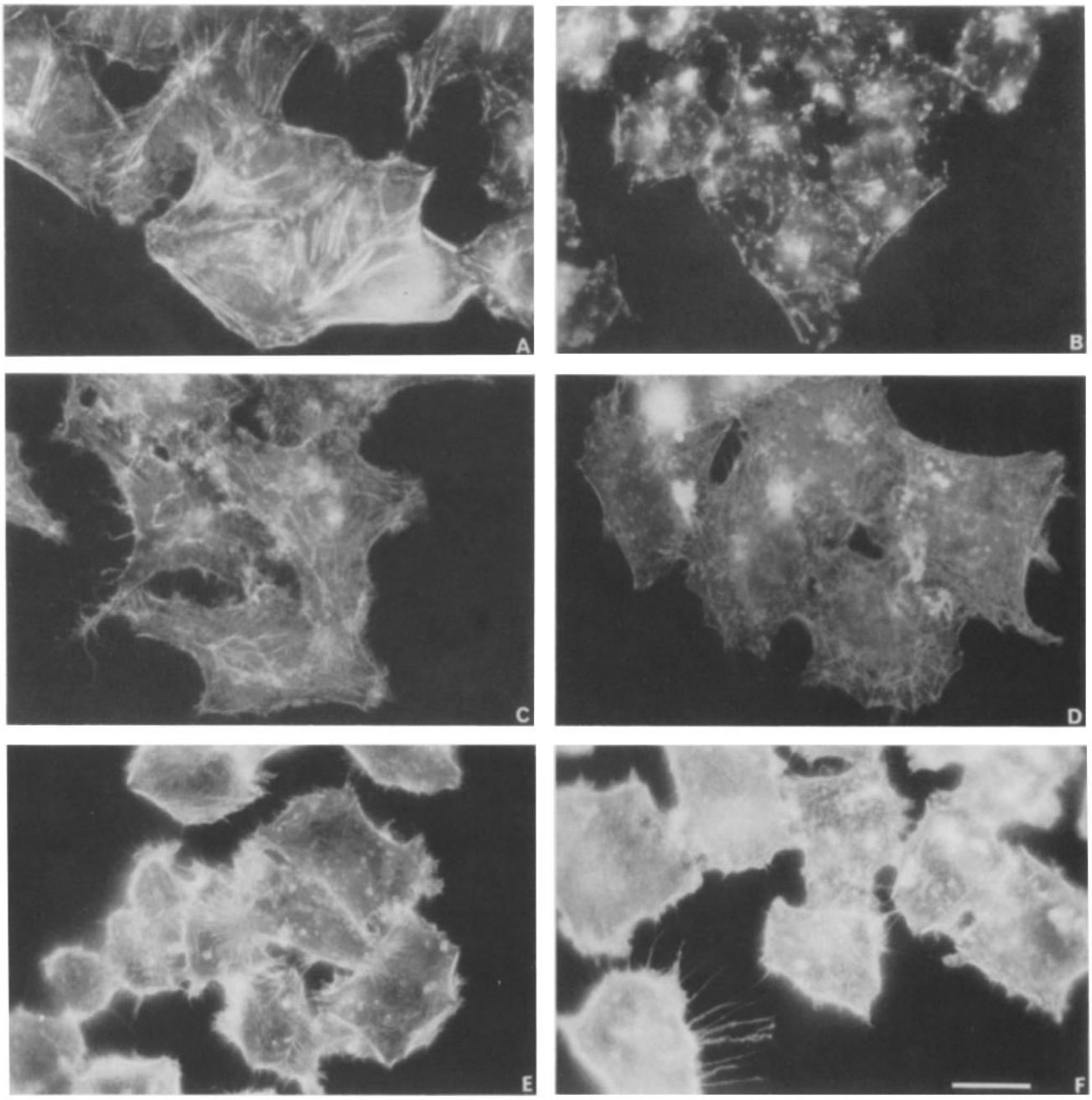

Figure 7. Fluorescence micrographs of actin filaments in CB-treated cells. Cells were treated with 20 μM CB for 60 min at 37°C. They were then fixed and stained with rhodamine-phalloidin as described previously (Toyama and Toyama, 1988). (*A*) Untreated KB100 TK^- cells; (*B*) CB-treated KB100 TK^- cells; (*C*) untreated Tfβ'-17(9) cells; (*D*) CB-treated Tfβ'-17(9) cells; (*E*) untreated KB100 Cyt 1 cells; and (*F*) CB-treated KB100 Cyt 1 cells. Bar, 20 μm.

tochalasin is bound near the base of the cleft between subdomains 1 and 3. Based on this interpretation, together with our previous finding that the CB binding site on monomeric actin is structurally correlated with the high affinity binding site on filamentous actin (Toyama and Toyama, 1988), we propose that the cytochalasin-binding site on monomeric and filamentous actin is the same but its binding affinity for cytochalasin is determined by the conformation about the cleft. Polymerization of monomeric actin to the barbed end of filamentous actin results in a conformational change about the cleft to a state where cytochalasin is bound more tightly. The predicted cytochalasin binding site is located near the hinge which links the small and large domain at the base of the cleft. The binding of cytochalasins could result in a conformational change about the hinge, leading to a change in the relative orientation of the two domains. This model provides the structural basis to explain multiple effects of cytochalasins to actin. The present study represents the first step to investigate the molecular dynamics of actin–cytochalasin interaction.

Our previous studies have shown that the increased resistance to CB of Cyt 1 cells is reflected in reduced affinity for the binding of CB by β'-actin (Toyama and Toyama, 1988).

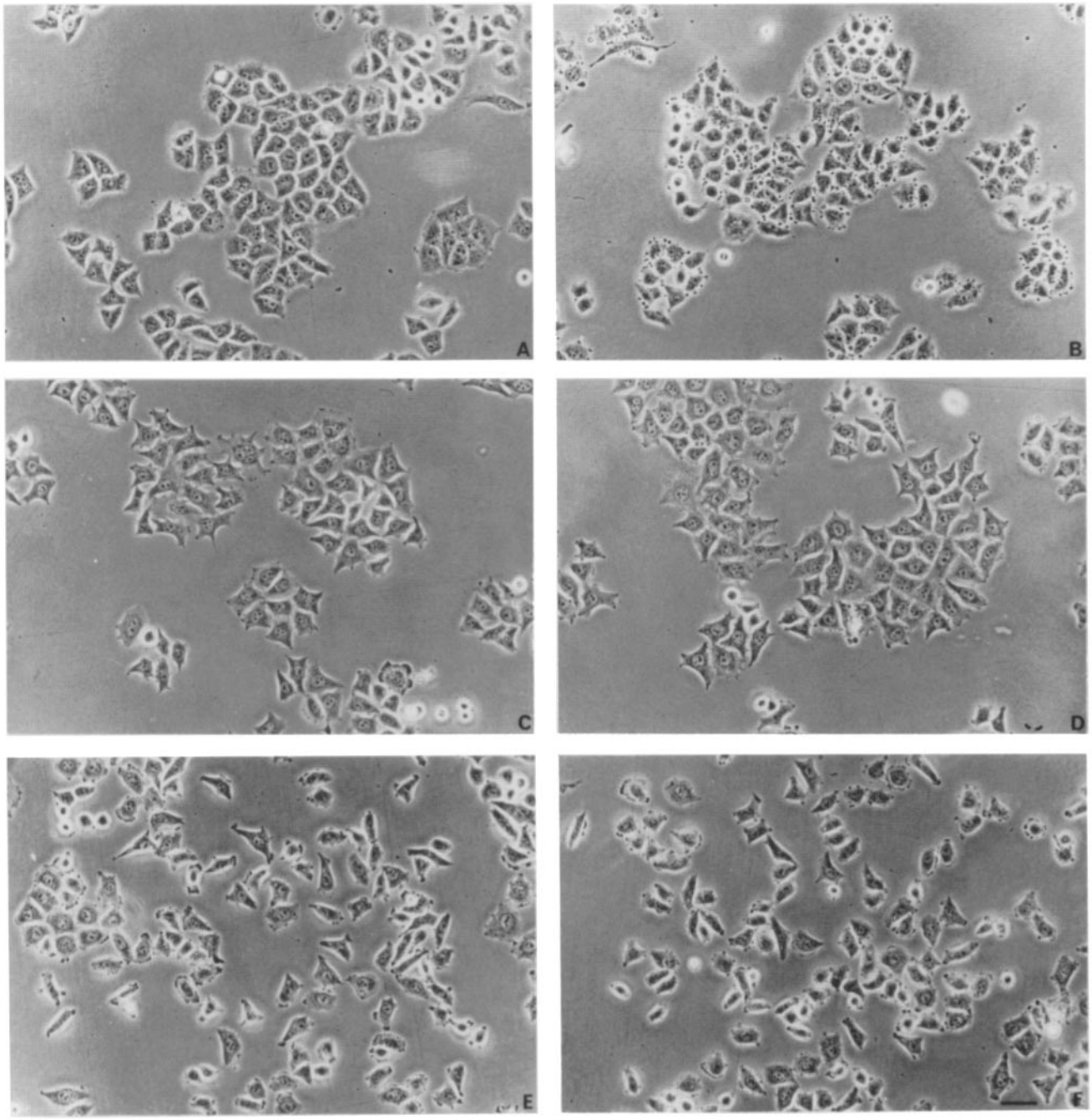

Figure 8. Effect of CB on cell morphology. Cells were incubated at 37°C for 7 min in the presence of 20 μM CB and then examined by phase-contrast microscopy. (*A*) Untreated KB100 TK^- cells; (*B*) CB-treated KB100 TK^- cells; (*C*) untreated Tfβ′-17(9) cells; (*D*) CB-treated TFβ′-17(9) cells; (*E*) untreated KB100 Cyt 1 cells; and (*F*) CB-treated KB100 Cyt 1 cells. Bar, 50 μm.

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 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. To solve this conflict, we proposed that 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. This explanation is substantiated by our present finding that the level of cytochalasin resistance in cells transformed with β′-actin cDNA is determined by the relative amount of exogenous β′-actin to endogenous β- and γ-actins (i.e., incomplete dominance).

Further support for the functional interchangeability between β- and γ-actin is provided by the observation that the γ-actin cDNA carrying two mutations (Val[139] → Met and Ala[295] → Asp) confers cytochalasin resistance upon transformed KB cells (Ohmori, H., and S. Toyama, unpublished results).

It has heretofore not been possible to unambiguously demonstrate differences in the function associated with given actin isoforms in vivo. This is largely because of the lack of direct methods to examine the functional capacity of different actin isoforms. Gene transfer method with actin isoform cDNA carrying cytochalasin-resistant mutation can be used to solve this difficulty; if more cytochalasin is required to inhibit a process in the transformed cells that express cytochalasin-resistant actin isoform than is required in control cells, one may be reasonably confident that the newly introduced actin isoform could be involved in the process. Gene transfer experiments described above with γ-actin cDNA carrying two mutations present a model example of this kind of analysis.

We thank Drs. Elaine Fuchs, Margaret E. Buckingham, and Kozo Makino for generously providing actin cDNA clones and cloned β-actin promoter.

This work was supported in part by a grant from the Ministry of Education, Science, and Culture of Japan.

Received for publication 15 August 1991 and in revised form 5 November 1991.

References

Bonder, E. M., and M. S. Mooseker. 1986. Cytochalasin B slows but does not prevent monomer addition at the barbed end of the actin filament. *J. Cell Biol.* 102:282–288.

Brenner, S. L., and E. D. Korn. 1979. Substoichiometric concentrations of cytochalasin D inhibit actin polymerization. *J. Biol. Chem.* 254:9982–9985.

Brown, S. S., and J. A. Spudich. 1979. Cytochalasin inhibits the rate of elongation of actin filament fragments. *J. Cell Biol.* 83:657–662.

Brown, S. S., and J. A. Spudich. 1981. Mechanism of action of cytochalasin: evidence that it binds to actin filament ends. *J. Cell Biol.* 88:487–491.

Carter, S. B. 1967. Effects of cytochalasins on mammalian cells. *Nature (Lond.).* 213:261–264.

Cooper, J. A. 1987. Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* 105:1473–1478.

Erba, H. P., R. Eddy, T. Shows, L. Kedes, and P. Gunning. 1988. Structure, chromosomal location, and expression of the human γ-actin gene: differential evolution, location, and expression of the cytoskeletal β- and γ-actin genes. *Mol. Cell. Biol.* 8:1775–1789.

Flanagan, M. D., and S. Lin. 1980. Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J. Biol. Chem.* 255:835–838.

Goddette, D. W., and C. Frieden. 1985. The binding of cytochalasin D to monomeric actin. *Biochem. Biophys. Res. Commun.* 128:1087–1092.

Goddette, D. W., and C. Frieden. 1986*a*. The kinetics of cytochalasin D binding to monomeric actin. *J. Biol. Chem.* 261:15970–15973.

Goddette, D. W., and C. Frieden. 1986*b*. Actin polymerization. The mechanism of action of cytochalasin D. *J. Biol. Chem.* 261:15974–15980.

Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456–467.

Halpern, M. E., and J. R. Smiley. 1984. Effects of deletion on expression of the herpes simplex virus thymidine kinase gene from the intact viral genome: the amino terminus of the enzyme is dispensable for catalytic activity. *J. Virol.* 50:733–738.

Hanukoglu, I., N. Tanese, and E. Fuchs. 1983. Complementary DNA sequence of a human cytoplasmic actin. Interspecies divergence of 3′ noncoding regions. *J. Mol. Biol.* 163:673–678.

Holmes, K. C., D. Popp, W. Gerhard, and W. Kabsch. 1990. Atomic model of the actin filament. *Nature (Lond.).* 347:44–49.

Irmiere, A., M. M. Manos, J. G. Jacobson, J. S. Gibbs, and D. M. Coen. 1989. Effect of an amber mutation in the herpes simplex virus thymidine kinase gene on polypeptide synthesis and stability. *Virology.* 168:210–220.

Kabsch, W., H. G. Mannherz, D. Suck, E. F. Pai, and K. C. Holmes. 1990. Atomic structure of the actin: DNase I complex. *Nature (Lond.).* 347:37–44.

Lin, D. C., K. D. Tobin, M. Grumet, and S. Lin. 1980. Cytochalasins inhibit nuclei-induced actin polymerization by blocking filament elongation. *J. Cell Biol.* 84:455–460.

Löw, I., and D. Dancker. 1976. Effect of cytochalasin B on formation and properties of muscle actin. *Biochim. Biophys. Acta.* 430:366–374.

Mabuchi, I. 1983. Electron microscopic determination of the actin filament end at which cytochalasin B blocks monomer addition using the acrosomal actin bundle from horseshoe crab sperm. *J. Biochem. (Tokyo).* 94:1349–1352.

MacLean-Fletcher, S., and T. D. Pollard. 1980. Mechanism of action of cytochalasin B on actin. *Cell.* 20:329–341.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

O'Farrel, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.

Pollard, T. D., and M. S. Mooseker. 1981. Direct measurement of actin polymerization rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores. *J. Cell Biol.* 88:654–659.

Ponte, P., S.-Y. Ng, J. Engel, P. Gunning, and L. Kedes. 1984. Evolutionary conservation in the untranslated region of actin mRNA; DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res.* 12:1687–1696.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 78:3824–3828.

Tannenbaum, S. F. 1978. Cytochalasins: Biochemical and Biological Aspects. North-Holland Publishing Co., Amsterdam. 561pp.

Toyama, S., and S. Toyama. 1984. A variant form of β-actin in a mutant of KB cells resistant to cytochalasin B. *Cell.* 37:609–614.

Toyama, S., and S. Toyama. 1988. Functional alterations in β′-actin from a KB cell mutant resistant to cytochalasin B. *J. Cell Biol.* 107:1499–1504.

Toyama, S., S. Toyama, and H. Uetake. 1977. Altered cell fusion capacity of KB cells resistant to Sendai virus-induced cytolysis. *Virology.* 76:503–515.