Reprinted from *Cell Motility*

Modification of Mammalian Cell Shape: Redistribution of Intracellular Actin by SV40 Virus, Proteases, Cytochalasin B and Dimethylsulfoxide

Robert Pollack
Microbiology Department
SUNY, Stony Brook, New York 11794

Daniel B. Rifkin
The Rockefeller University
New York, New York 10021

Variations in shape (Fig. 1) are the most obvious of all the many changes that occur in a mammalian cell as it proceeds through its replicative cycle. In order to cycle between the mitotic ball and the interphase disc, every proliferating cell must be able to carry out simultaneously many cyclic physiological changes involving the cell membrane and the cortical cytoplasm. For example, the cell must alter its adhesions to the substrate (Tobey, Anderson and Petersen 1967) by modifying its plasma membrane (Burger et al. 1972; Mannino and Burger 1975) so that the same substrate is "wet" by the membrane to a cyclically varying degree (Folkman and Greenspan 1976). At the same time it must assemble and dissemble intracytoplasmic structures containing polymers of actin (Lazarides 1975a) and tubulin (Fuller, Brinkley and Boughter 1975; Weber, Pollack and Bibring 1975).

Normal and malignant cells differ both in their ability to traverse the cell cycle under limiting conditions and in the manner in which they carry out these cyclic changes in shape. For a normal cell to complete successive cycles (i.e., for it to be able to form a colony), serum must be present in high amounts, cells must initially be far from each other so that cell–cell contact is minimal, and a wettable substrate such as glass or plastic must be provided for the cells to spread out in early G_1 (Fig. 1). Transformed cells have a diminished or absent requirement for these three environmental signals and therefore can grow well in low serum, high cell density or in the absence of an anchoring substrate (Risser and Pollack 1974).

Paralleling one of these variations in growth control, a major perturbation in cortical cytoplasmic structure has recently been found. Anchorage-independent cell lines have been shown to lack intracellular actin-containing cables (Pollack, Osborn and Weber 1975) and intracellular microtubules (Brinkley, Fuller and Highfield 1975) at all points in interphase. Modifications of cell structure that correlate specifically with changes in anchorage dependence are especially interesting insofar as anchorage-independence and tumorigenicity seem to be very well correlated (Shin et al. 1975; Freedman

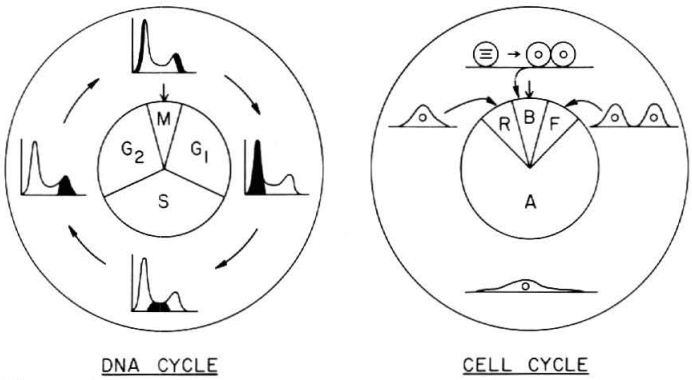

Figure 1

Shape changes in the cell cycle. On the left is the classic cycle, marked by the periods of DNA synthesis (S), mitosis (M) and the gaps between (G_1 and G_2). Histograms of DNA/cell show the shuttling of the population between 2C and 4C DNA content. On the right is an attempt to show the cell's shape changes as the cycle progresses. A indicates the adherent flattened cell, occurring in G_1, S and G_2; R, the rounding cell, occurring in late G_2 and early M; B, the ball or spherical cell, occurring in the middle of M; and F, the flattening cell, occurring in late M and early G_1. Actin-containing cables (see text) are absent throughout the cycle in anchorage-transformed cells and are present in normal cells only in A, disappearing in R and reappearing in F.

and Shin 1974; Evans and DiPaolo 1975). Clearly, experimental assembly and dissembly of the cortical actin-containing structures would contribute to our understanding both of the shape changes occurring in the cell cycle and of the mechanisms by which the oncogenic anchorage-independent phenotype is expressed.

With this in mind we attempted to convert the actin-containing cables within normal cells into the diffuse network characteristic of anchorage-transformed cells by use of a series of reagents known to alter cytoplasmic structure (Pollack and Rifkin 1975). Proteases were initially chosen because (1) one protease, plasmin, is a physiological concomitant of tumor growth in vivo (Reich, Rifkin and Shaw 1975), and (2) we had observed earlier that the amount of plasminogen activator produced by different SV40-transformed rat embryo fibroblast lines was well correlated with their ability to grow in the absence of anchorage (Pollack et al. 1974). More recently, the effects of cytochalasin B (CB) on the intracellular distribution of actin have been studied. In these studies we have found widely varying effects that are dependent on dosage (concentration × time of exposure).

METHODS

All studies reported here were carried out on an early passage population of cells isolated by trypsinization of 16-day-old rat embryos (Pollack and Rifkin 1975).

All experiments were performed at 36.5°C in an atmosphere of 10% CO_2, 90% air, with a relative humidity of 100%. Stock cultures were grown on plastic petri dishes (Falcon) in Dulbecco's modified Eagle's medium (DME; Gibco H21) with 10% fetal calf serum (Reheis) added. Transfers were made by trypsinization (0.25% trypsin, Gibco, in Ca^{++}- and Mg^{++}-free PBS), low-speed centrifugation, and resuspension in fresh DME plus 10% fetal calf serum. Cells were permitted to spread for 24 hours after transfer before any experimental protocol was initiated.

Proteases were made and used as described previously (Pollack and Rifkin 1975). Cytochalasin B was made as a stock of 1 mg/ml in DMSO. For all treatments of cells, cultures plated 24 hours earlier on 11-mm round glass cover slips at a density of $2 \times 10^4/cm^2$ were washed twice with prewarmed DME and held at 37°C for 1 hour. Proteases or other reagents were added

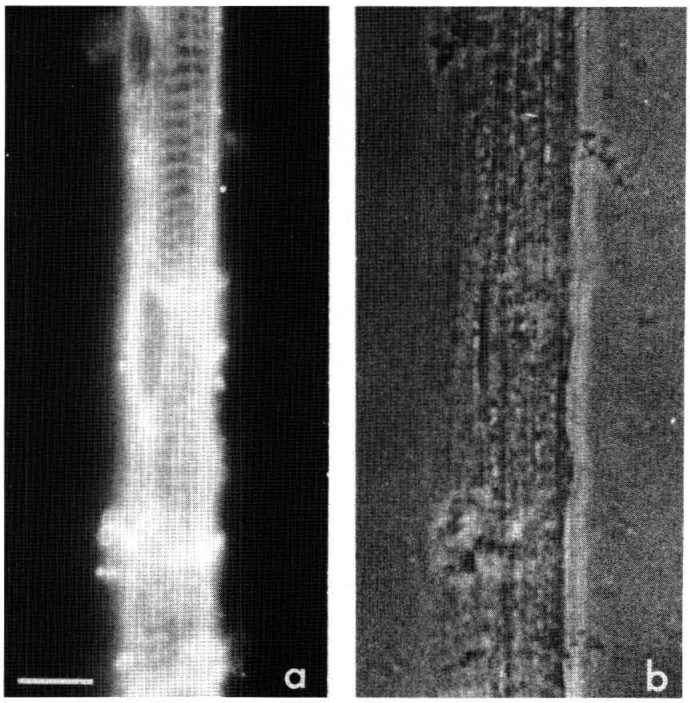

Figure 2

Immunofluorescence demonstration of specificity of antibody to actin. Antibody prepared against SDS-denatured actin (Lazarides 1975b) was layered on cover slips of cultured chick embryonic myotubes after formalin fixation-acetone postfixation (Pollack and Rifkin 1975). After washing in PBS, the cells were incubated with goat anti-rabbit IgG conjugated to fluorescein, washed, and mounted in Elvanol (Rodriguez and Deinhardt 1960). The same field is shown in *a* and *b*: (*a*) with UV-epi-illumination; (*b*) with phase contrast. Note the periodic variations in fluorescence and phase density characteristic of striated muscle. Presumably, bright fluorescent bands are the I-bands, and dense interbands are the A-bands. Note the absence of nuclear fluorescence and the presence of fine fluorescent fibers running perpendicular to the striations. Bar = 10 μ.

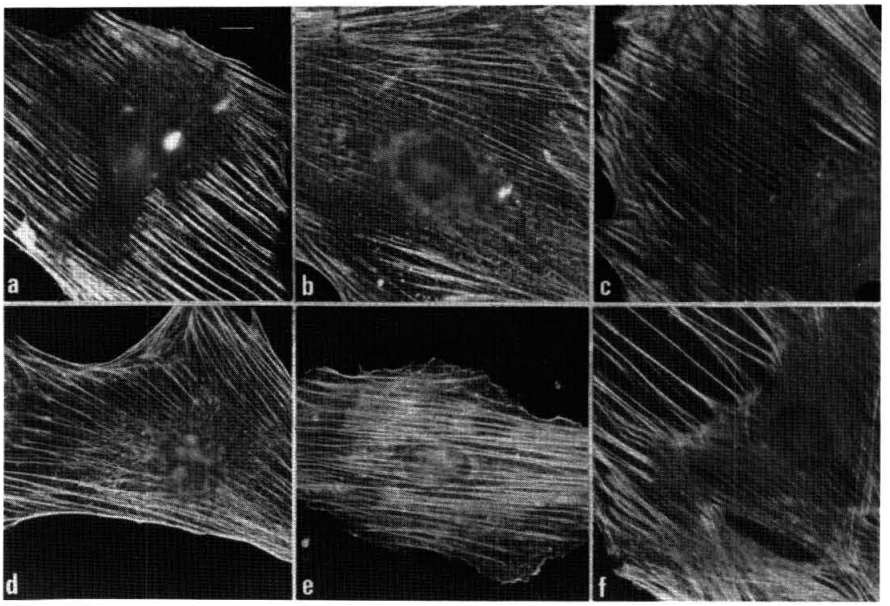

Figure 3
Actin cables remain in rat embryo cells in serum-free medium. Secondary rat embryo cells were trypsinized and permitted to spread for 24 hours on glass cover slips in DME plus 10% fetal calf serum. They were then washed twice with DME and examined for actin cables (as in Methods) after (*a*) 15 min, (*b*) 1 hr, (*c, d*) 2 hr, (*e*) 8 hr and (*f*) 24 hr in DME. These figures are typical of at least 90% of the rat embryo cells studied at any time. Bar = 10 μ. (Reprinted, with permission, from Pollack and Rifkin 1975.)

in DME to bring final concentrations to the desired level, and cultures were returned to 37°C until fixation.

Fixation and Staining

After exposure to reagents or control media, cover slips were fixed by immersing them directly in formaldehyde fixative (3.8% formaldehyde in PBS). Fixed cover slips were stored for periods of 1–5 days at 4°C before staining. To open cell membranes to antibody, fixed cover slips were washed in PBS and passaged for 5 minutes each in water:acetone (1:1), acetone, water: acetone (1:1) and PBS. The postfixed cover slips were then put in a moist chamber and incubated for 60 minutes at 37°C with 10 μl of a 1:40 PBS dilution of rabbit antibody to actin. The cover slips were then washed three times in PBS and incubated for 60 minutes with 10 μl of fluorescein isothiocynate-conjugated goat anti-rabbit IgG (Miles), diluted 1:10 in PBS.

After this counterstain, cover slips were washed three times in PBS and once in water and mounted cell-side down in Elvanol (Rodriguez and Deinhardt 1960). After drying for 24 hours, cells were examined at 830× with a Zeiss photomicroscope II, by epi-illumination, using an FITC narrow-pass exciter filter and a 500-nm barrier filter. Fields were photographed with Tri-X Kodak film, exposed at DIN 32 and developed with Diafine developer. (See Fig. 2.)

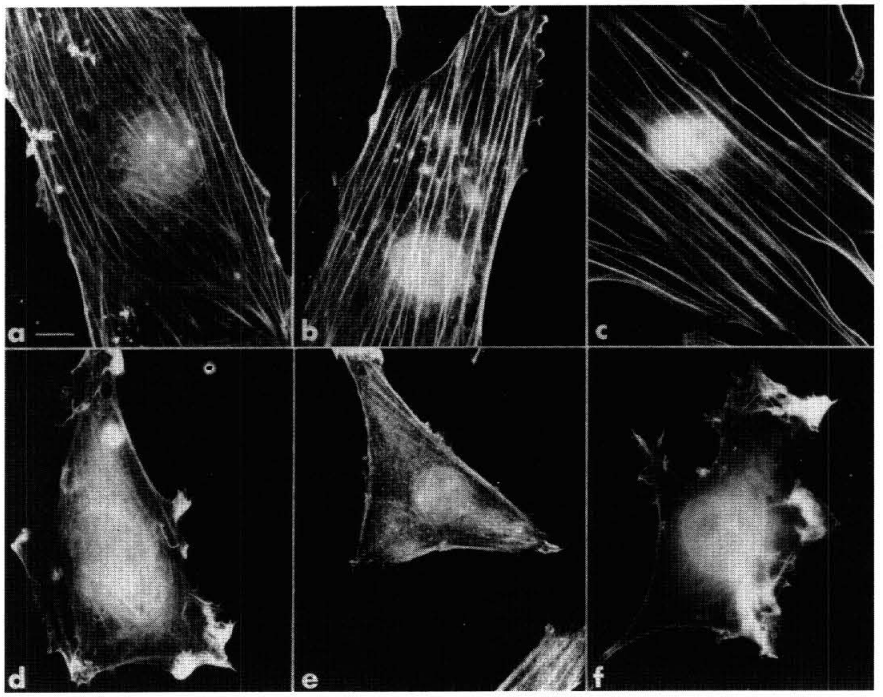

Figure 4
Effect of the plasmin system on actin cables in rat embryo cells. Cells were prepared as in Fig. 3. At 0 hr, DME was replaced by one of the following: (*a*) fresh DME; (*b*) DME plus 1.5 μg/ml plasminogen; (*c*) DME plus 25 μg/ml urokinase; (*d, e, f*) DME plus both 1.5 μg/ml plasminogen and 25 μg/ml urokinase. After 3 hr, cells were fixed and examined for actin cables as described in Methods. Bar = 10 μ. (Reprinted, with permission, from Pollack and Rifkin 1975.)

Scanning for Presence of Actin-containing Cables

All cover slips were labeled in an arbitrary fashion and scored by a person unable to identify the origin of the cover slip. At least 100 cells were scored on each cover slip. The microscope was focused on the edge of a cell and therefore on structures at the adherent side of the cell. If cables ran the length of the cell, the cell was scored as positive. Some cells had only finer structures, especially just behind ruffles. Such cells, and cells completely lacking cables, were scored as negative (Pollack and Rifkin 1975). (See Fig. 3.)

RESULTS AND DISCUSSION

Proteases

The results of protease treatment of REF cells are given in Table 1 (Pollack and Rifkin 1975). Trypsin and plasmin removed cables from within REF cells very efficiently, since 1–3 μg/ml of either protease converted more than

Table 1
Results of Protease Treatment of REF Cells

Protease[a]	*RE cells*[b] *with cables (%) after protease exposure for*			
	15 min	*1 hr*	*2 hr*	*4 hr*
None	97[b]	95	91	97
Plasmin				
plasminogen (1.5)	—	—	90	—
urokinase (25)	—	—	92	—
plasminogen (1.5) + urokinase (25)	—	—	23	—
plasminogen (7.5) + urokinase (50)	—	—	17	—
Trypsin				
(1.0)	83	64	—	—
(2.0)	100	35	13	0
(3.0)	56	30	0	—
(5.0)	67	16	0	—
Chymotrypsin				
(1.0)	—	79	—	47
(2.5)	—	—	51	—
(5.0)	76	—	65	26
(1.0)	—	—	37	14
Thrombin				
(5)	—	—	92	—
(10)	—	80	—	79
(50)	44	83	—	85
(100)	—	88	—	78

[a] Concentration in μg/ml given in parentheses.
[b] Average of at least two experiments for each point.

80% of the REF cells to a morphology resembling anchorage-transformed cells in 1–2 hours (Figs. 4, 5).

Not all proteases removed cables. For example, urokinase, whose proteolytic activity was necessary to activate the proenzyme plasminogen, was by itself unable to disrupt the cables (Fig. 5). Thrombin also had no effect on cables, even at very high doses (100 μg/ml for 4 hr). Chymotrypsin had an intermediate effect on cables: although no dose of chymotrypsin removed all cables, sufficiently high doses had some effect on them (Table 1). Although we have some evidence that proteases bound to beads also remove cables, we have not yet established with certainty whether the effects shown here are all the result of proteolysis from without or are instead the result of protease uptake (Hodges, Livingston and Franks 1973).

In these experiments, dosages of enzyme were low enough so that the cells were not dislodged from the cover slip as a result of exposure to the

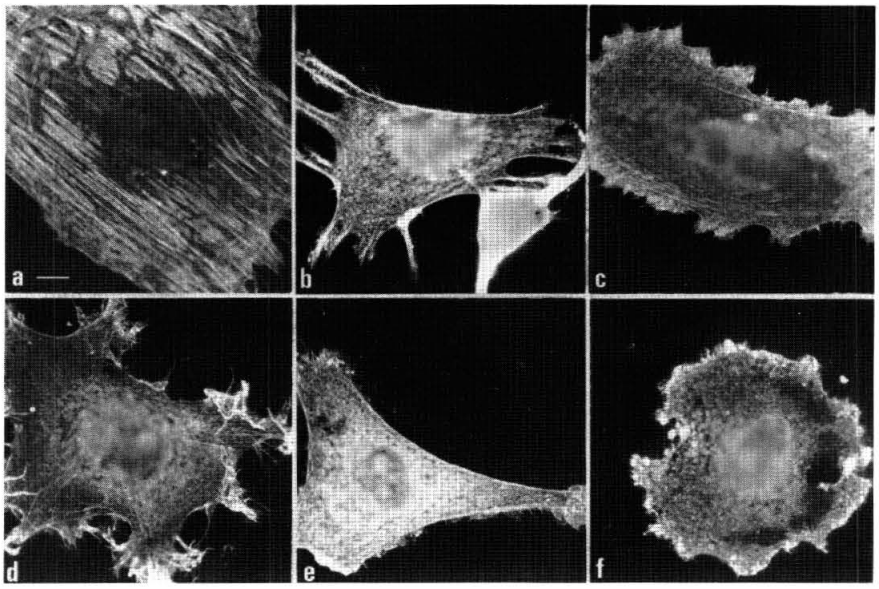

Figure 5
Time course of effect of trypsin on actin cables in rat embryo cells. Rat embryo cells were plated and examined for actin cables as described in Methods. Cells were fixed at various times after addition of DME plus 5 μg/ml trypsin: (*a*) 15 min; (*b*) 1 hr; (*c, d*) 2 hr; (*e*) 4 hr; (*f*) 8 hr. By 8 hr, approximately half of the rat embryo cells have detached from the glass. No cells detach after 2 hr in this concentration of trypsin. Bar = 10 μ. (Reprinted, with permission, from Pollack and Rifkin 1975.)

protease. Of course if a protease such as trypsin is presented to cells at high concentrations ($>$ 100 μg/ml), cell detachment will occur rapidly. These results suggest, therefore, that dispersion of intracellular actin cables is necessary, but not sufficient, for rounding and detachment.

Cytochalasin B and DMSO

Cytochalasin B (CB) has many interesting effects on cell morphology and motility. The direct site of CB's activity is unknown. It is clear, however, that both the plasma membrane and the actin-containing microfilaments are affected by the drug. For this reason we examined the distribution of actin-containing structures by immunofluorescence in RE cells after treatment with different doses of CB. Because of the complexity of CB's effects on RE cells, treated cells were classified as spread, retracted or detached (Table 2), as well as being scored for the fraction of spread cells retaining cables (Table 3).

Retraction and detachment were minimal after 15 minutes exposure to CB concentrations up to 3 μg/ml (Fig. 6a,b,c). At 10 μg/ml, a minority of spread cells showed a new distribution of actin: a disoriented, unraveled dispersion (Fig. 6d). At low CB concentration, there was some conversion of cables to this diffuse distribution among the spread cells (Table 3).

Table 2
Effects of Cytochalasin B in DMSO on Adherence of RE Cells

Treatment			*Distribution of cells (%)*		
CB (μg/ml)	*DMSO (%)*	*exposure (hr)*	*spread*[a]	*retracted*[b]	*detached*[c]
0	0	0.25	90[a]	0	10
		1	91	0	9
		24	80	0	20
3.0	0.3	0.25	94	1	5
		1	26	58	34
		24	0	0	100
10	1.0	0.25	37	57	5
		1	1	84	15
		24	0	90	10

[a] See Fig. 6a for actin distribution in typical spread cells.
[b] See Fig. 7c for actin distribution in typical retracted cells.
[c] See Fig. 8c for actin distribution in typical detached cell sites; shows dots remaining.

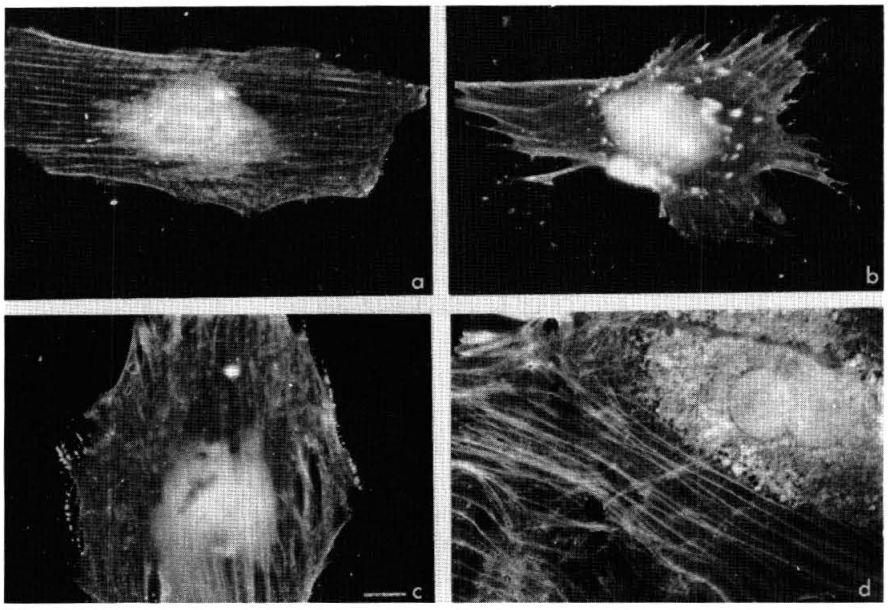

Figure 6
Intracellular distribution of actin in RE cells after 15 min exposure to cytochalasin B in serum-free medium. (*a*) Control, no CB; (*b*) 1 μg/ml CB, the cell has begun to retract, and actin-containing cables are less sharply defined; (*c*) μg/ml CB, retraction at the cell boundary has deposited pads of actin-containing material; (*d*) 10 μg/ml CB, the cytoplasm seems to have come apart, leaving unraveling, bent cables. All pictures at same magnification; bar = 10 μ.

Table 3
Effects of CB and DMSO on Intracellular Cables within Spread Cells

Treatment			*Spread cells retaining cables (%)*
CB (μg/ml)	*DMSO (%)*	*exposure (hr)*	
0	0	0.25	86
		1	90
		24	77
0	0.3	1	86
	1	1	71
	3	1	57
	10	1	43
1	0.1	0.25	60
		1	70
		24	89
3	0.3	0.25	80
		1	99
		24	NSC[a]
10	1.0	0.25	93
		1	NSC
		24	NSC

[a]NSC = no spread cells.

By 1 hour of exposure to concentrations of CB greater than 1 μg/ml (Fig. 7), cell retraction had proceeded extensively. The majority of cells exposed to 3 or 10 μg CB also retracted (Table 2). However, among the minority of spread cells, cables were still present (Table 3). The aberrant dispersion of cables was maintained at 6 and 10 μg/ml. While no fully spread cells remained after 1 hour in 6 or 10 μg of CB, retraction was less than in cells exposed to 3 μg/ml for 1 hour (Fig. 7, Tables 2 and 3).

After 24 hours of exposure to CB (Fig. 8), populations of RE cells became homogeneous, showing actin distributions that were dependent on drug dose (Table 3). In 3 μg/ml, all cells detached, leaving behind sets of cytoplasmic adherent pads that outlined the shapes of spread cells (Fig. 8c, Table 2). In 10 μg/ml CB, very few cells detached. Rather, almost all cells contained the same partially retracted dispersion of disoriented cables (Fig. 8d, Table 2). There were no fully spread cells after 1 hour in 10 μg/ml CB.

Since CB was given to RE cells as dilutions of a 1 mg/ml stock solution in 100% dimethylsulfoxide (DMSO), we tested the effect of DMSO itself. DMSO alone caused retraction of RE cells at concentrations of 3% and 10% (Table 4). These concentrations are three and ten times higher, respectively, than the concentration of DMSO present in a culture receiving 10 μg/ml of CB. These high concentrations of DMSO also disrupted actin cables in about half of the

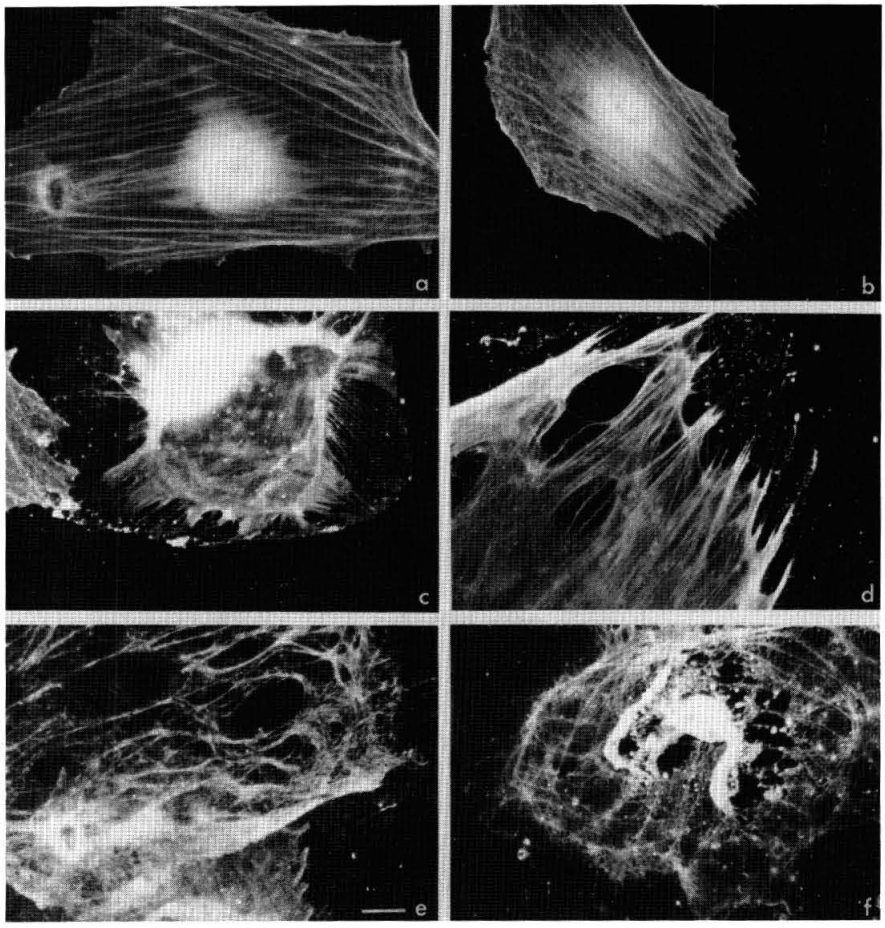

Figure 7
Intracellular distribution of actin in RE cells after 1 hr exposure to cytochalasin B in serum-free medium. (*a*) Control, no CB; (*b*) 0.3 μg/ml CB, slight retraction at edge of cell; (*c*) 1 μg/ml CB, extensive retraction; (*d*) 3 μg/ml CB, detail of large cell, retraction depositing pads; (*e*) 6 μg/ml CB, unraveling of cables as well as retraction; (*f*) 10 μg/ml CB, retraction, unraveling and disintegration of cytoplasm. All pictures at same magnification; bar = 10 μ.

cells (Table 5). However, 10% DMSO by itself for an hour did not cause appreciable cell detachment nor the appearance of unraveled cables. Thus these changes are apparently CB-specific.

The effects of CB and/or DMSO on the cables of spread and retracted RE cells were reversible within an hour after the cells were washed in fresh serum-free medium (Table 5). The material left behind by detached cells did not show any change in shape upon reversal, however.

CB-induced loss of actin cables within spread cells and detachment of cells from glass (Tables 3 and 4) did not proceed in a coordinated manner. Although cable loss in cells remaining attached during CB treatment was not markedly dosage-dependent, cell detachment was most effective at interme-

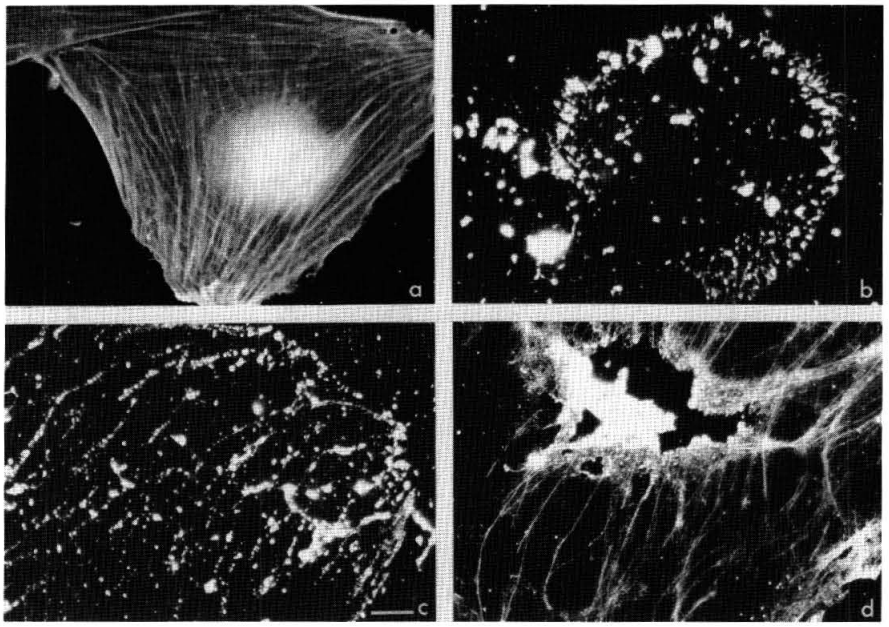

Figure 8
Intracellular distribution of actin in RE cells after 24 hr exposure to cytochalasin B in serum-free medium. (*a*) Control, no CB; (*b*) 1 μg/ml CB, complete detachment from glass, leaving pads outlining shape of spread cell; (*c*) 3 μg/ml CB, complete detachment of cell; (*d*) 10 μg/ml CB, unraveled cytoplasm does not detach. All pictures at same magnification; bar = 10 μ.

Table 4
Effect of DMSO Alone on Adherence of RE Cells

Treatment		*Adherence (% of cells)*		
DMSO (%)	*exposure (hr)*	*spread*	*retracted*	*detached*
0.3	0.25	90	8	2
	1	90	4	6
1.0	0.25	98	0	2
	1	87	8	5
3.0	0.25	46	32	12
10	0.25	16	73	11

diate concentrations of CB. At the optimal concentration of 3 μg/ml CB, cell loss exceeded 95%. After CB-induced detachment of the cell body, adherent bits of cytoplasm remained behind. These stained very brightly with anti-actin. Studies are underway to determine which other cell proteins are associated with these adherent bits of cell membrane and cytoplasm and to compare them with substrate-attached glycoproteins (Terry and Culp 1974; Culp 1974, 1975).

Table 5
Recovery from CB and DMSO

Treatment		*Time (hr)*		*Adherence (% cells)*			*Spread cells with cables*[a]
CB (μg/ml)	*DMSO (%)*	*exposure*	*recovery*	*spread*[a]	*retracted*	*detached*	*(%)*
0	0.3	1	—	90	8	2	86
		1	1	90	4	6	94
0	3	1	—	46	32	12	57
		1	1	70	16	14	91
0	10	1	—	16	73	11	43
		1	1	74	14	12	97
3	0.3	1	—	26	58	34	99
		1	1	67	10	23	88
10	1.0	1	—	1	84	15	NSC
		1	1	69	9	12	75

[a] Only the spread cells were scored for the presence of actin-containing cables.

Acknowledgments

This work was supported by grants from the Human Cell Program of the National Science Foundation; the National Cancer Institute, NIH; and the American Cancer Society. We thank Joel Gordon for chick embryo myotube cultures.

REFERENCES

Brinkley, B., G. Fuller and D. Highfield. 1975. Cytoplasmic microtubules in normal and transformed cells in culture. Analysis by tubulin antibody immunofluorescence. *Proc. Nat. Acad. Sci.* **72**:4981.

Burger, M. M., B. M. Bombik, B. M. Breckenridge and J. R. Sheppard. 1972. Growth control and cyclic alterations of cyclic AMP in the cell cycle. *Nature New Biol.* **239**:161.

Culp, L. A. 1974. Substrate-attached glycoproteins mediating adhesion of normal and virus-transformed mouse fibroblasts. *J. Cell Biol.* **63**:71.

Culp, L. A. 1975. Topography of substrate-attached glycoproteins from normal and virus-transformed cells. *Exp. Cell Res.* **92**:467.

Evans, C. and J. DiPaolo. 1975. Neoplastic transformation of guinea pig fetal cells in culture induced by chemical carcinogens. *Cancer Res.* **35**:1035.

Folkman, J. and H. P. Greenspan. 1976. Influence of geometry on control of cell growth. *Biochim. Biophys. Acta* **417**:211.

Freedman, V. H. and S. Shin. 1974. Cellular tumorigenicity in *nude* mice: Correlation with cell growth in semi-solid medium. *Cell* **3**:355.

Fuller, G. M., B. R. Brinkley and J. M. Boughter. 1975. Immunofluorescence of mitotic spindles by using monospecific antibody against bovine brain tubulin. *Science* **187**:948.

Hodges, G., D. Livingston and L. Franks. 1973. The localization of trypsin in cultured mammalian cells. *J. Cell Sci.* **12**:887.

Lazarides, E. 1975a. Immunofluorescence studies on the structure of actin filaments in tissue culture cells. *J. Histochem. Cytochem.* **23**:507.

———. 1975b. Tropomyosin antibody: The specific localization of tropomyosin in nonmuscle cells. *J. Cell Biol.* **65**:549.

Mannino, R. and M. M. Burger. 1975. Growth inhibition of animal cells by succinylated concanavalin A. *Nature* **256**:19.

Pollack, R. and D. Rifkin. 1975. Actin-containing cables within anchorage-dependent rat embryo cells are dissociated by plasmin and trypsin. *Cell* **6**:495.

Pollack, R., M. Osborn and K. Weber. 1975. Patterns of organization of actin and myosin in normal and transformed cultured cells. *Proc. Nat. Acad. Sci.* **72**:994.

Pollack, R., R. Risser, S. Conlon and D. Rifkin. 1974. Plasminogen activator production accompanies loss of anchorage regulation in transformation of primary rat embryo cells by simian virus 40. *Proc. Nat. Acad. Sci.* **71**:4792.

Reich, E., D. Rifkin and E. Shaw, eds. 1975. *Proteases and biological control. Cold Spring Harbor Conferences on Cell Proliferation,* vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Risser, R. and R. Pollack. 1974. A non-selective analysis of SV40 transformation of mouse 3T3 cells. *Virology* **59**:477.

Rodriguez, J. and F. Deinhardt. 1960. Preparation of a semipermanent mounting medium for fluorescent Ab studies. *Virology* **12**:316.

Shin, D., V. Freedman, R. Risser, and R. Pollack. 1975. Tumorigenicity of virus-transformed cells in *nude* mice is correlated specifically with anchorage-independent growth *in vitro*. *Proc. Nat. Acad. Sci.* **72**:4435.

Terry, A. H. and L. A. Culp. 1974. Substrate-attached glycoproteins from normal and virus-transformed cells. *Biochemistry* **13**:414.

Tobey, R. A., E. C. Anderson and D. F. Petersen. 1967. Properties of mitotic cells prepared by mechanically shaking monolayer cultures of Chinese hamster cells. *J. Cell. Physiol.* **70**:63.

Weber, K., R. Pollack and T. Bibring. 1975. Microtubular antibody: The specific visualization of cytoplasmic microtubules in tissue culture cells. *Proc. Nat. Acad. Sci.* **72**:459.