Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 1884–1888, March 1990
Cell Biology

Cytoskeletal reorganizations responsible for the phorbol ester-induced formation of cytoplasmic processes: Possible involvement of intermediate filaments

(actin microfilaments/microtubules/protein kinase C)

A. D. BERSHADSKY*, O. Y. IVANOVA†, L. A. LYASS†, O. Y. PLETYUSHKINA†, J. M. VASILIEV*†, AND I. M. GELFAND†

*All-Union Cancer Research Center of the U.S.S.R. Academy of Medical Sciences, Moscow, U.S.S.R.; and †Laboratory of Molecular Biology and Bioorganic Chemistry of the Moscow State University, Moscow, U.S.S.R.

Contributed by I. M. Gelfand, December 13, 1989

ABSTRACT **The tumor promoter phorbol 12-myristate 13-acetate (PMA) induces characteristic reversible changes of cell shape in certain fibroblastic lines: motile lamellas are transformed into noncontractile narrow processes; simultaneously, the actin microfilament network of lamellas is locally disorganized. This reaction to PMA may be regarded as a prototype of reorganizations involving formation of stable cytoplasmic processes. Specific drugs, Taxol and Colcemid, were used to study the role of microtubules and vimentin-containing intermediate filaments (IF) in the development of PMA-induced reorganizations. PMA readily induced formation of noncontractile processes in Taxol-treated fibroblasts; these cells had a profoundly altered microtubular system but noncollapsed IF. A short (1 hr) exposure to PMA induced formation of processes in control cells but not in the Colcemid-treated cells, which had depolymerized microtubules and IF that collapsed around the nucleus. Longer (3–4 hr) exposure of the Colcemid-treated cells to PMA induced partial reversal of the IF collapse; those parts of the peripheral lamellas that contained IF were transformed into narrow noncontractile processes. It is suggested that the local interaction of IF with the actin system is an essential step in the formation of processes from lamellas. The microtubular system controls distribution of IF in the cytoplasm and thus plays an indirect role in the reorganization of the actin cortex.**

Alterations of the degree of cell segregation into two types of cytoplasmic domains, motile lamellas and nonmotile narrow processes, are characteristic of many morphological differentiations (1). Reversible enhancement of this segregation accompanied by the pronounced formation of nonmotile long processes from motile lamellas is induced in certain fibroblastic and epithelial cultures by phorbol 12-myristate 13-acetate (PMA). This phenomenon is convenient for the experimental analysis of mechanisms controlling segregation (1–5, ‡). PMA-induced transformation of lamellas into stable processes was found to be accompanied by profound reorganization of the cytoskeleton, especially by the disappearance of the network of actin microfilaments filling lamellas and by the loss of contractility of the actin cortex (3–5). These alterations of the microfilament system were shown to be microtubule dependent (4, ‡): their development after short-term PMA treatment was prevented by preincubation of cells with Colcemid, which caused complete depolymerization of microtubules and was accompanied by perinuclear collapse of vimentin-containing intermediate filaments (IF). These data suggest that the cell has a special microtubule-dependent mechanism controlling the actin microfilament system (4).

Experiments presented in this paper show that when the time of exposure of Colcemid-treated cells to PMA is increased to 3–4 hr, microtubules remain depolymerized, but IF collapse is reversed; simultaneously, formation of narrow processes from lamellas takes place. The main cytoskeletal elements present in these newly formed processes are IF. PMA also induced characteristic shape changes in the presence of Taxol, a drug that caused profound disorganization of the microtubular system but did not produce the collapse of IF. Analysis of these data suggests that IF interactions with the actin system play a central role in the development of PMA-induced reorganization of cell shape.

MATERIALS AND METHODS

Cloned subline 152/15 (6) of the spontaneously transformed CAK-7 line (7) of mouse fibroblasts was used in all the experiments. Cultivation, preparation of cell models, and assessment of ATP-induced contraction of these models were performed as described (3, 4). PMA (Sigma) was used at a final concentration of 10 ng/ml, Colcemid (demecolcine, Sigma) was used at a final concentration of 0.5 μg/ml, and Taxol (a gift of M. Suffness, National Cancer Institute) was used at a final concentration of 12 μM. Immunofluorescence staining with antibodies to vimentin and tubulin and staining of polymerized actin with rhodamine-conjugated phalloidin were performed as described (8).

RESULTS

Control cells had fan-like, fusiform, or polygonal shapes. During the first hour of incubation with PMA, >90% of these cells acquired long narrow cytoplasmic processes. Simultaneously, new lamellas were extended from the cell bodies; these lamellas later collapsed into new processes. These reorganizations were gradually reduced after 8–12 hr. Their dynamics have been described in more detail elsewhere (1, 3).

Control cells had numerous microfilament bundles; numerous microtubules and IF radiated from the central part of their bodies into the peripheral cytoplasm. The processes of the PMA-treated cells were stained less intensely for actin than other parts of the cytoplasm. Lamellas and bodies of PMA-treated cells had numerous radiating microtubules and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; IF, vimentin-containing intermediate filament(s).
‡Danowsky, B. A. (1988) International Congress of Cell Biology, Aug. 14–19, 1988, Montreal, abstr. 2.19.24.

IF; the processes were also filled with uninterrupted microtubules and IF.

The cells incubated with Taxol for 20 hr had a polygonal shape (Fig. 1*A*); they were less elongated than control ones. When PMA was added to the medium of these cells for 1 hr, 90–95% of them acquired narrow cytoplasmic processes (Fig. 1*B*); these processes had more bends and thickenings than those of cells treated with PMA alone. The normal system of microtubules radiating from the center to the periphery was partially or completely absent in the cells treated with Taxol alone or with Taxol and PMA; instead, these cells had numerous bundles of microtubules not connected with any centers and scattered in all parts of the cytoplasm (Fig. 1*C*). This disintegration of the microtubular system by Taxol has been described in various cell types; it is regarded to be a result of the promotion of microtubule assembly and stabilization of microtubules in the cytoplasm (9–11). The cells treated with Taxol alone or in combination with PMA contained dense networks of IF in the peripheral parts of the cytoplasm (Fig. 1*D*).

The cells preincubated with Colcemid for 20 hr had polygonal or star-like shapes. During the first hour after addition of PMA to the medium of Colcemid-treated cells, many cells extended large lamellas, but no narrow processes were formed (Figs. 2 *A* and *B* and 3*E*). However, after 3–4 hr of incubation with PMA, many cells (50–70%) developed processes that had more bends and local thickenings than those induced by PMA in the absence of Colcemid (Fig. 2*C*). The shapes of these PMA-induced processes were clearly different from those of the straight and short processes occasionally seen in cells treated with Colcemid alone. Dynamic observations had shown that the formation of processes in Colcemid-treated cells was a result of narrowing and incomplete retraction of previously formed lamellas (Fig. 2 *A–C*). Extension of new lamellas and their transformation into the processes went on for the next 4–6 hr; later the processes gradually disappeared, and the morphology of the cell became similar to that in the cultures incubated with Colcemid alone. The cells incubated with Colcemid alone or in combination with PMA for any period of time had no cytoplasmic microtubules. The cells incubated with Colcemid alone for 20 hr had a completely collapsed IF system: IF were absent from the cell periphery and accumulated near the nuclei, forming compact ring-like or spherical aggregates (Fig. 2 *D* and *E*). This distribution of IF in Colcemid-treated cells was not changed 1 hr after the addition of PMA to their medium. However, when Colcemid-treated cells were fixed 3–4 hr after the addition of PMA, anti-vimentin staining revealed partial reversal of the IF collapse: most cells had more loose perinuclear aggregates with one or several IF bundles radiating from aggregates to the cell periphery (Fig. 2 *F* and *G*). These radiating bundles were closely associated with the newly formed processes; usually each narrow process contained the peripheral part of the IF bundle. Conversely, most bundles detached from perinuclear aggregates radiated into the PMA-induced process and not into the other parts of cell periphery. The staining for polymerized actin was less intense in the PMA-induced processes than in the cell bodies.

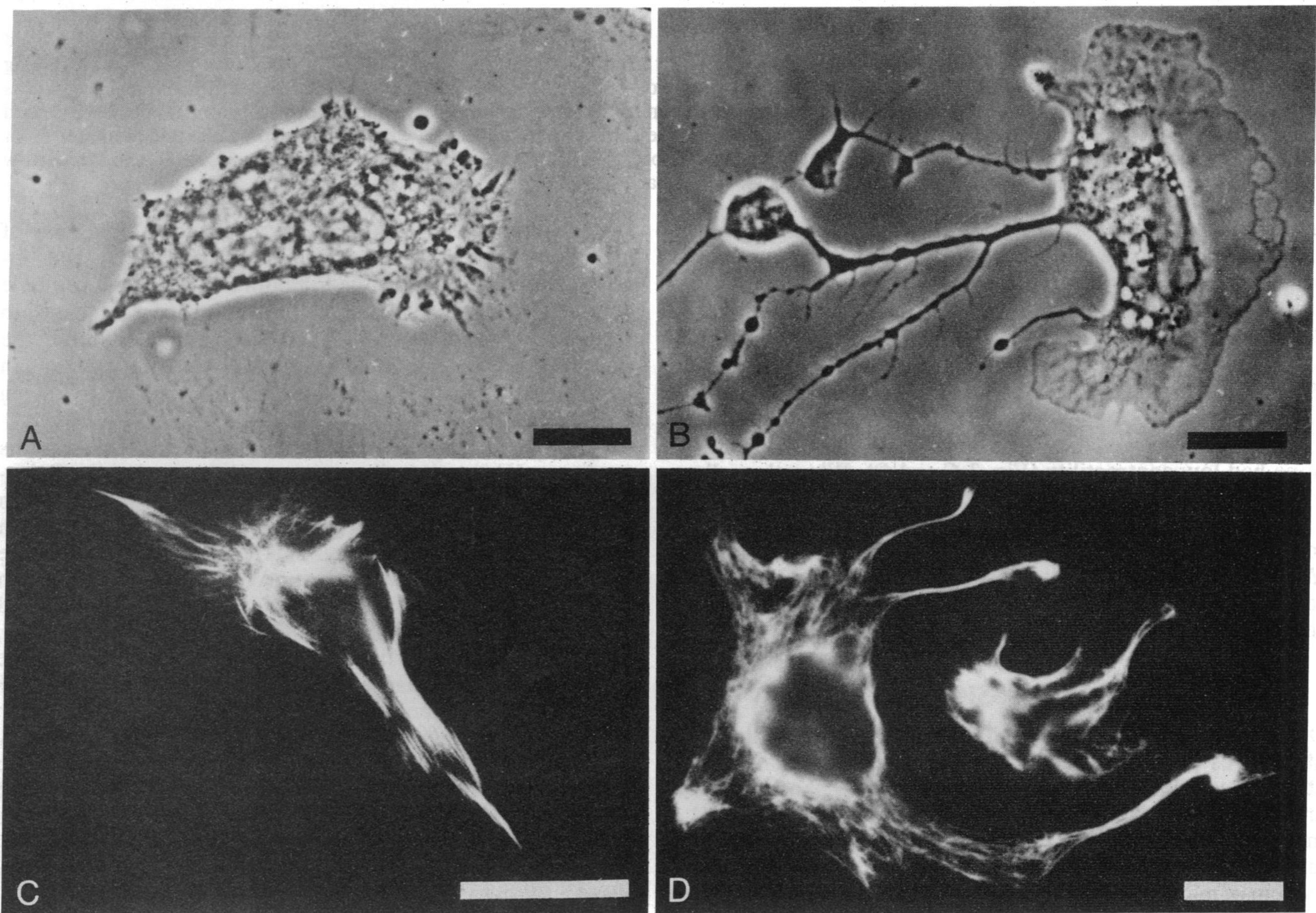

FIG. 1. Effects of PMA on Taxol-treated 152/15 cells. (*A*) Phase-contrast photograph of cells incubated with Taxol (12 μM) alone for 20 hr. (*B–D*) Cells were incubated with Taxol alone for 19 hr and then with Taxol and PMA (10 ng/ml) for 1 hr. (*B*) Phase-contrast photograph of lamella and narrow stable processes induced with PMA. (*C*) Tubulin antibody staining detected by fluorescent microscopy. Bundles of microtubules are scattered in the cytoplasm of the cell body and the processes. (*D*) Vimentin antibody staining detected by fluorescent microscopy. Numerous IF can be seen in the cytoplasm of cell body and the processes. (Bars = 20 μm.)

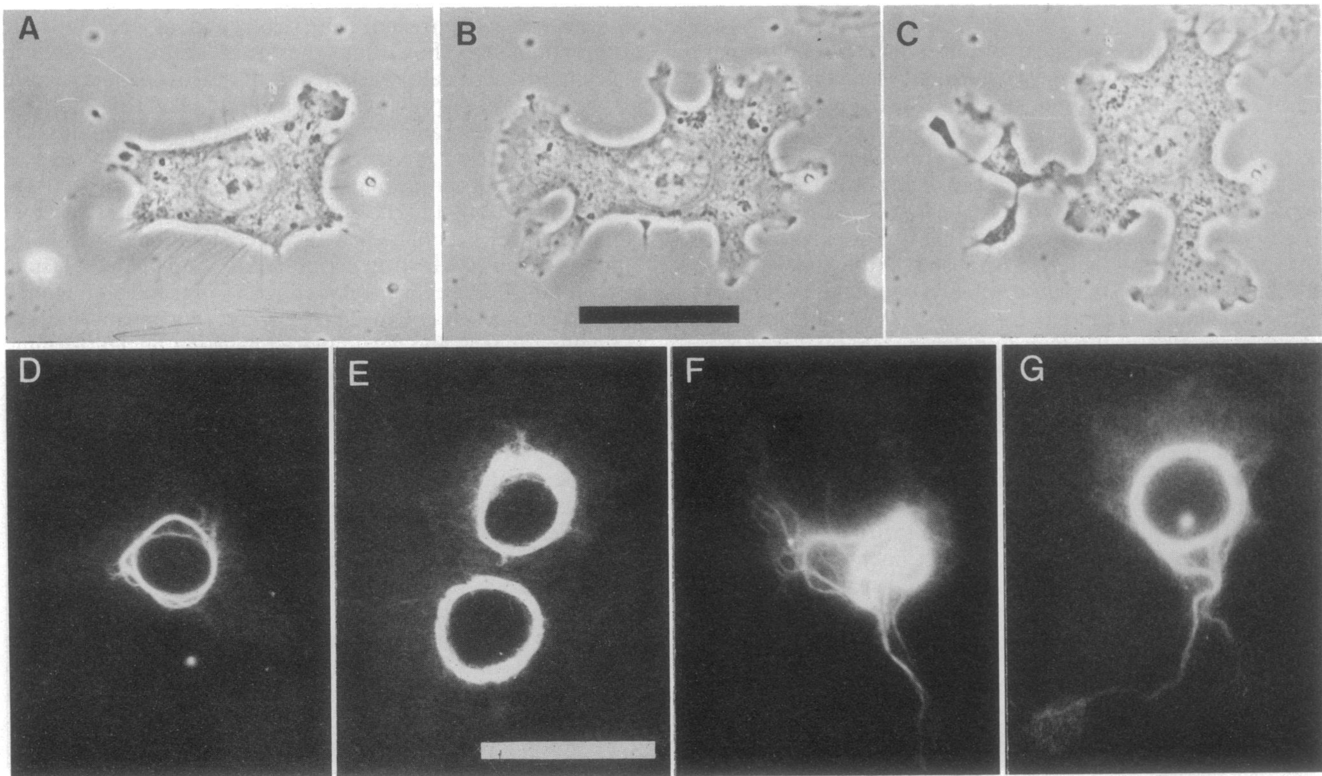

FIG. 2. Effects of PMA (10 ng/ml) on Colcemid-treated cells. (*A–C*) The same cell photographed after 20 hr of incubation with Colcemid (0.5 μg/ml) immediately before the addition of PMA (*A*) as well as 1 hr (*B*) and 3 hr (*C*) after the addition of PMA into Colcemid-containing medium. Notice that PMA induced the formation of lamella (*B*, left side of the cell); then this lamella was transformed into a branched process with thickenings (*C*). (*A–C*, bar = 20 μm.) (*D–G*) Vimentin antibody staining detected by fluorescent microscopy. (*D* and *E*) Cells were treated with Colcemid alone for 20 hr. The perinuclear rings of collapsed IF are shown. (*F* and *G*) Cells were treated with Colcemid alone for 20 hr and then with Colcemid and PMA for 4 hr. Partial reversal of the collapse of IF: bundles of IF go from perinuclear aggregates to the narrow processes induced by PMA (lower parts of both photographs). (*D–G*, bar = 20 μm.)

Permeabilized preparations of control cells (also called "cell models") incubated with ATP underwent rapid contraction: in a few minutes almost their entire cytoplasm acquired a nearly spherical shape (Fig. 3 *A* and *B*). As described earlier (4), similar high contractility was also characteristic of the models of cells treated with Colcemid alone and of the models of Colcemid-treated cells incubated for 1 hr with PMA (Fig. 3 *E* and *F*).

In contrast, the models of cells incubated for 1 hr with PMA alone had low contractility (Fig. 3 *C* and *D*). When the cells were incubated with Colcemid in combination with PMA for a longer period (4 hr), their newly formed processes did not contract after addition of ATP to cell models (Fig. 3 *G* and *H*). The models of cells treated with Taxol alone had high contractility, whereas the models of cells treated with Taxol and PMA had low contractility.

DISCUSSION

Our previous experiments (4) with short-term PMA treatment of cultures preincubated with Colcemid showed that alterations of cell shape and contractility induced by phorbol ester are microtubule dependent. Further experiments presented in this paper show that the role of microtubules in these alterations is indirect. In particular, the presence of a unified system of microtubules radiating from the single center is not essential for the formation of stable noncontractile processes, as these processes had been induced by PMA in Taxol-treated cells filled with the groups of isolated microtubules not connected with any center.

What is more, under certain conditions, formation of the processes could be observed even in cells with depolymerized microtubules. This was the case when the time of incubation of Colcemid-treated cells with PMA was increased to 3–4 hr. Thus, the inhibitory effect of microtubule depolymerization on the induction of processes seems to require some mediator, which remains inactivated during the first hour of PMA action on Colcemid-treated cells but, in contrast to microtubules, is restored during the following hours.

IF are the most likely candidates for the role of this mediator. IF remain collapsed around the nucleus in Colcemid-treated cells during the first hour of incubation with PMA; later this collapse is gradually reversed, and IF begin to radiate to the cell periphery. This reversal coincides in time and place with the formation of narrow processes from lamellas: the newly formed processes always contain IF, and most radiating IF bundles go into these processes. Thus, transformation of a certain zone of peripheral lamella into the narrow process is associated with the appearance of IF bundles in this zone. Results of other experiments are in agreement with this conclusion: stable processes induced by PMA in control and in Taxol-treated cells always contained numerous IF at the cell periphery. Thus, noncontractile processes induced by PMA in these various experiments contain IF.

What is the mechanism of the reversal of IF collapse by PMA? Earlier studies (12) showed that this collapse (that is, perinuclear aggregation of IF in the cells with depolymerized microtubules) is actin dependent: disorganization of the actin microfilament system by cytochalasins prevents or reverses IF collapse. It was suggested (12) that IF are kept in the central part of the cell by contractile action of the associated actin cortex. On the other hand, immunomorphological data

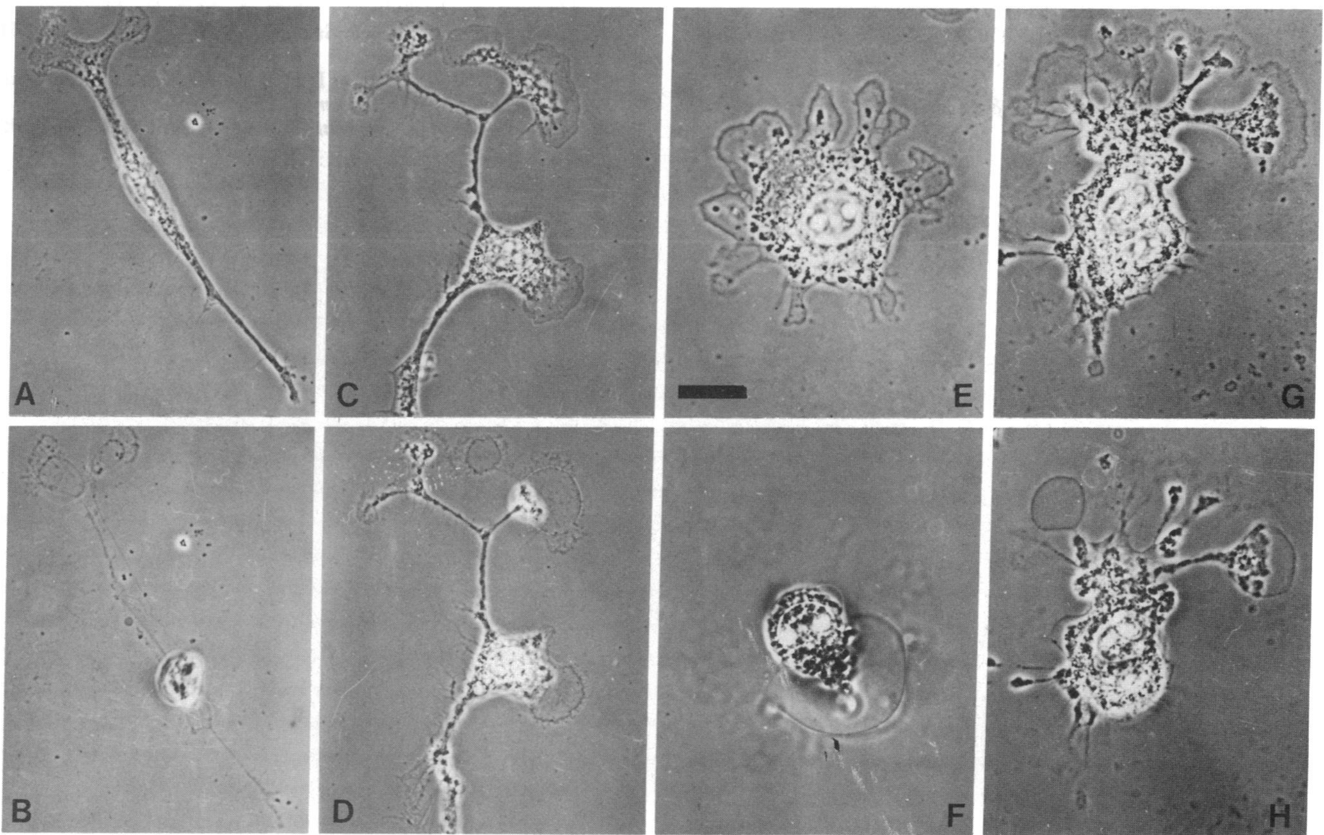

FIG. 3. Contractility of the models permeabilized with saponin after incubation with PMA (10 ng/ml) and Colcemid (0.5 μg/ml). Pairs of phase-contrast photographs of the same cell models immediately before (*A*, *C*, *E*, and *G*) and 2 min after (*B*, *D*, *F*, and *H*) the addition of ATP (2 mM) to the incubation medium. (*A* and *B*) Control cell. High contractility: the whole cell contracted, acquiring almost a spherical shape. (*C* and *D*) The cell was incubated with PMA for 1 hr. Low contractility: the processes did not contract; local contractions of the body and of the lamellas (upper right parts of photographs) located at the ends of the processes. (*E* and *F*) The cell was incubated with Colcemid alone for 20 hr and then with Colcemid and PMA for 1 hr. High contractility: notice the absence of PMA-induced narrow processes (compare *E* and *C*). (*G* and *H*) The cell was incubated with Colcemid alone for 20 hr and then with Colcemid and PMA for 4 hr. Low contractility: the processes (upper part of the model) did not contract; some contraction is seen in the lower part of the body. (Bar = 20 μm.)

(1, 3) and examination of cell model contractility (see ref. 4 and the data in this paper) indicate that PMA-induced transformation of motile lamellas into stable narrow processes is always accompanied by local morphological disorganization and functional inactivation of the actin cortex. These two groups of facts suggest that PMA-induced reversal of IF collapse is a result of changes in the IF–actin interactions. More specifically, inactivation of the actin cortex, which contracts the IF aggregate, may lead to uncoiling of IF.

Alterations of the IF–actin cortex interactions may also play an essential role in the formation of stable processes from lamellas. It is possible, for instance, that PMA-induced activation of protein kinase C may modulate some molecules, linking IF with the actin cortex. The microtubule-associated protein MAP-2 is one of the possible candidates for the role of this molecule: it binds not only to microtubules but also to IF and actin microfilaments (13, 14), and it is phosphorylated by many kinases including protein kinase C (15). IF themselves can be altered by protein kinase C, as this enzyme phosphorylates vimentin (16). It is also possible that some still unknown cell components controlling the organization of the actin cortex are associated with IF and are activated by protein kinase C, so that alteration of the surrounding zone of the actin cytoskeleton is then induced. Thus, the existing data suggest that IF participate in the organization of noncontractile processes, although their exact function remains to be determined.

Many facts show that microtubules are colocalized with vimentin-containing IF and in many situations determine the distribution of IF in fibroblasts (17). Therefore, microtubules may be involved in the formation of processes and alterations of the actin cortex indirectly, by controlling the positions of IF.

PMA-induced segregation of motile and stable domains in the cytoplasm may be regarded as a prototype of many important morphogenetic reorganizations, such as extension of neurites by neurons, formation of polarized motile mesenchymal cells from nonpolarized epithelial cells, etc. (1, 2). Our experiments suggest that IF have an essential function in reorganizations of this type.

1. Vasiliev, J. M. (1978) *J. Cell Sci. Suppl.* **8,** 1–18.
2. Vasiliev, J. M. & Gelfand, I. M. (1988) *Cell Differ.* **24,** 75–82.
3. Dugina, V. B., Svitkina, T. M., Vasiliev, J. M. & Gelfand, I. M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4122–4126.
4. Lyass, L. A., Bershadsky, A. D., Vasiliev, I. M. & Gelfand, J. M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9538–9541.
5. Danowsky, B. A. & Harris, A. K. (1988) *Exp. Cell Res.* **177,** 47–59.
6. Bershadsky, A. D., Brodskaya, R. M., Mansurov, P. G., Stavrovskaya, A. A. & Stromskaya, T. B. (1984) *Exp. Onkol.* **6,** 27–32.
7. Farber, R. A. & Liskay, R. M. (1974) *Cytogenet. Cell Genet.* **13,** 384–396.
8. Bershadsky, A. D., Tint, I. S. & Svitkina, T. M. (1987) *Cell Motil. Cytoskeleton* **8,** 274–283.
9. Green, K. J. & Goldman, R. D. (1983) *Cell Motil.* **3,** 283–305.
10. Geuns, G., DeBrabander, M., Nuydens, R. & DeMay, J. (1983) *Cell Biol. Int. Rep.* **7,** 35–47.
11. Forry-Schaudies, S., Murray, I. M., Toyama, I. & Holtzer, H. (1986) *Cell Motil. Cytoskeleton* **6,** 324–338.

12. Hollenbeck, P. J., Bershadsky, A. D., Pletjushkina, O. Y., Tint, I. S. & Vasiliev, J. M. (1989) *J. Cell Sci.* **92,** 621–631.
13. Hirokawa, N., Hisanaga, S.-I. & Shiomura, Y. (1988) *J. Neurosci.* **8,** 2769–2779.
14. Pollard, T. D., Selden, S. C. & Maupin, P. (1984) *J. Cell Biol.* **99,** 33s–37s.
15. Akiyama, T., Nishida, E., Ishida, J., Sagi, N., Ogawara, H., Hoshi, M., Miyata, Y. & Sakai, H. (1986) *J. Biol. Chem.* **261,** 15648–15651.
16. Huang, C.-K., Devanney, I. F. & Kennedy, S. P. (1988) *Biochem. Biophys. Res. Commun.* **150,** 1006–1011.
17. Traub, P. (1985) *Intermediate Filaments* (Springer, New York).