Special type of morphological reorganization induced by phorbol ester: Reversible partition of cell into motile and stable domains

(morphogenesis/tumor promoter/microfilaments/microtubules)

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ABSTRACT The phorbol ester phorbol 12-myristate 13-acetate (PMA) induced reversible alteration of the shape of fibroblastic cells of certain transformed lines—namely, partition of the cells into two types of domains: motile body actively extending large lamellas and stable narrow cytoplasmic processes. Dynamic observations have shown that stable processes are formed from partially retracted lamellas and from contracted tail parts of cell bodies. Immunofluorescence microscopy and electron microscopy of platinum replicas of cytoskeleton have shown that PMA-induced narrow processes are rich in microtubules and intermediate filaments but relatively poor in actin microfilaments; in contrast, lamellas and cell bodies contained numerous microfilaments. Colcemid-induced depolymerization of microtubules led to contraction of PMA-induced processes; cytochalasin B prevented this contraction. It is suggested that PMA-induced separation of cell into motile and stable parts is due to directional movement of actin structures along the microtubular framework. Similar movements may play an important role in various normal morphogenetic processes.

Reversible morphological reorganizations of fibroblasts and other cultured cells are actively studied as prototypes of normal morphogenetic processes; alterations of cytoskeleton regulated by the membrane-generated signals seem to play central roles in these reorganizations (for reviews, see refs. 1–3). In this paper we describe a special type of reversible alteration of cell shape and cytoskeleton induced in certain fibroblastic lines by the tumor promoter phorbol 12-myristate 13-acetate (PMA). This agent caused reversible cell partition into a motile pseudopod-forming body and nonmotile elongated processes rich in microtubules and intermediate filaments. Analysis of this reorganization suggests that alterations of interactions between the actin cortex and microtubules are of key importance in its development. It is possible that reorganizations of a similar type are involved in normal morphogenetic processes—e.g., in the formation of neurites by neural cells, in forward translocation of polarized fibroblasts, etc.

MATERIALS AND METHODS

Several types of cultured fibroblastic cells were used: secondary cultures of mouse embryo fibroblasts, minimally transformed mouse BALB/3T3 line, rat NRK (normal rat kidney) line, transformed PS-103 and PS-104 lines obtained in this laboratory from mouse sarcomas induced by implanted plastic films, and cloned subline 152/15 of the spontaneously transformed CAK-7 line of mouse fibroblasts (4). The cells were grown at 37°C in Eagle's medium with 10% fetal calf serum. The cells were seeded on the coverslips placed into Petri dishes at a concentration of 10^4 cells per ml. The experiments were started at 48 hr after seeding.

Colcemid, PMA, and its analogues were from Serva (Heidelberg), and cytochalasin B was from Aldrich. Rho-damine-labeled phalloidin was a gift of T. Wieland (Max Planck Institute for Medical Research, Heidelberg). Polyclonal antibodies to tubulin and myosin were obtained from V. I. Gelfand, F. K. Gioeva, and A. D. Bershadsky (Moscow State University), and monoclonal antibodies to vimentin were from O. S. Rochlin (Cardiological Center, Moscow). The methods of indirect immunofluorescent examination of cytoskeleton (5) and electron microscopic examination of the platinum replicas (6) were performed as described in previous publications (5, 6). To perform dynamic-phase contrast microscopy of living cells, the cultures were grown in special glass chambers at 37°C and photographed at various time intervals, usually each 5–10 min.

RESULTS

Alterations of Cell Shape. Treatment of several types of mouse tumorigenic lines (PS-103, PS-104, 152/15) with PMA (10–20 ng/ml) leads to similar morphological alterations: contraction of cell body and lamellas, accompanied by the formation of narrow cytoplasmic processes. These changes were observed in 70–90% of cells after 2–4 hr of incubation. Similar changes were induced in the minimally transformed BALB/3T3 and NRK lines, but here the effective concentrations of PMA were higher (25–50 ng/ml) and percentages of the altered cells were lower (20–40%).

The cultures of mouse embryo fibroblasts were the least sensitive to PMA: a high concentration of this agent (100 ng/ml) induced only partial contraction of lamellas in 10–20% of the cells. The 152/15 line had the highest sensitivity to PMA. Most cells of this line underwent drastic morphological changes after the 1- to 2-hr incubation with low concentrations of the drug (2–5 ng/ml). Therefore, these cells treated with a standard concentration of PMA (5 ng/ml) were selected for further detailed study of the effects of the tumor promoter. Control cells of this line, spread on the substratum, had typical fibroblast-like shape with small leading lamellas and short tail processes; formation of small ruffles at the leading edges and contraction of the tail accompanied their translocation. Dynamic observations of individual cells (Fig. 1) had shown that the first morphological changes were observed at about 20–30 min after the addition of PMA. The large fan-like lamellas were formed at the cell edge; simultaneously, some contraction of the cell body took place. Later, at 30–60 min, the newly formed lamellas contracted into the narrow cylindrical processes; these processes occasionally had thick parts ("bulbs") and small lamellar zones. New lamellas were extended from other sites of the edge simultaneously with the retraction of the old ones. These extensions and retractions went on for many hours. Often they were not accompanied by any considerable displacements of the cell body; this body became surrounded by several narrow processes formed from the retracted lamellas.

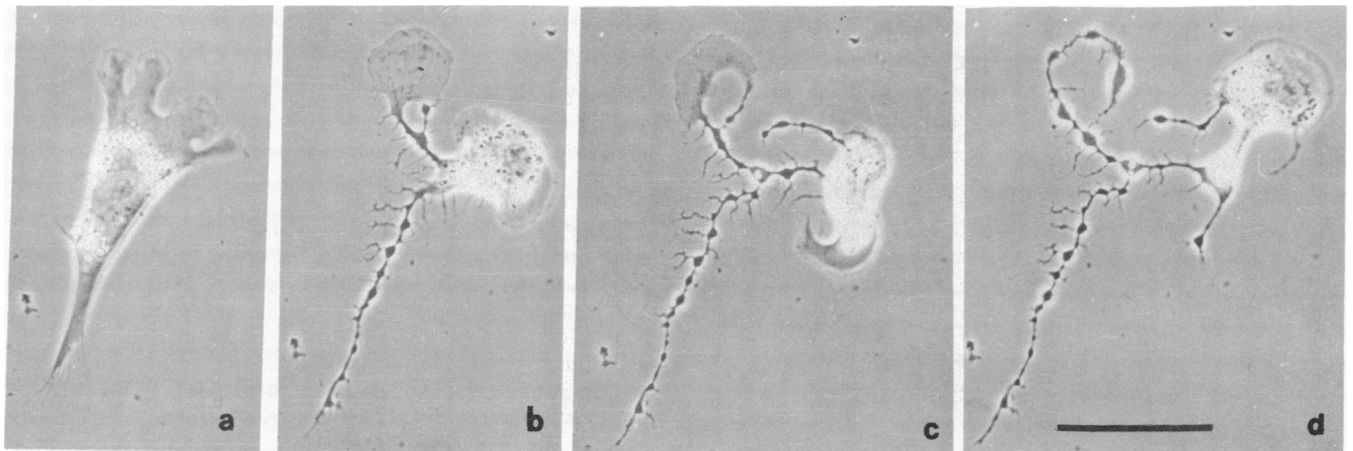Abbreviation: PMA, phorbol 12-myristate 13-acetate.

FIG. 1. Phase-contrast micrographs of the cell of the 152/15 line immediately before the addition of PMA to the medium (*a*) and 25 min (*b*), 70 min (*c*), and 90 min (*d*) after the addition. In *b* and *c*, two large lamellas were formed at the leading edge, and the cell body moved into the right lamella; the tail part of the body and a part of the left lamella contracted into narrow processes. In *b–d*, the left lamella was completely transformed into the narrow process. The cell body formed new lamellas and moved toward the right upper corner. The tail narrow process was elongated at its proximal end but otherwise retained the stable shape. (Bar = 50 μm.)

In other cases translocations of the cell body were observed; the body was displaced from time to time toward the newly extended lamellas, while the tail part of the body contracted. These contractions lead to elongation of the proximal parts of narrow processes. These processes, once formed by contraction of lamellas and cell bodies, usually did not change their shape during the next several hours, except for occasional formation and disappearance of bulbs.

Morphological alterations induced by PMA were not accompanied by any significant changes of the substratum area occupied by one cell. At 16–20 hr, formation of narrow processes was gradually reversed in spite of continued presence of PMA in the medium; these processes became wider and shorter. At 24–30 hr, the morphology of PMA-treated cultures became indistinguishable from that of control cultures. When, after 4–6 hr of incubation, the PMA-containing medium was substituted by the control medium, the original morphology was restored 2–3 hr later. Two analogues of PMA devoid of tumor-promoting activity, 4-oxymethyl-PMA (100 ng/ml) and phorbol (100 ng/ml), did not induce any morphological changes in the cultures of the 152/15 line. In contrast, the PMA-related tumor promotor,

mezerein (1 μg/ml), induced formation of narrow processes in these cultures.

Alterations of Cytoskeleton. Immunofluorescence examination (Fig. 2) revealed thin actin bundles in the cytoplasm of control 152/15 cells. These cells had well-developed systems of microtubules and intermediate filaments. The cells incubated with PMA for 2 hr had newly formed lamellas with bands of intensely stained actin near their outer edges; proximal parts of lamellas and bodies also contained large intensely stained areas, whereas discrete bundles were usually absent. The intensity of actin staining of the narrow processes was similar to or weaker than that of the cell body; the bulbs often were stained more intensely than other parts of the processes. Staining for myosin was more intense in the central part of the body than in lamellas and narrow processes. Microtubules were numerous in all parts of the PMA-treated cell; they formed a dense network in lamellas and compact cables in the narrow processes. The distribution of intermediate filaments was similar to that of microtubules except that these filaments were absent from the distal parts of lamellas.

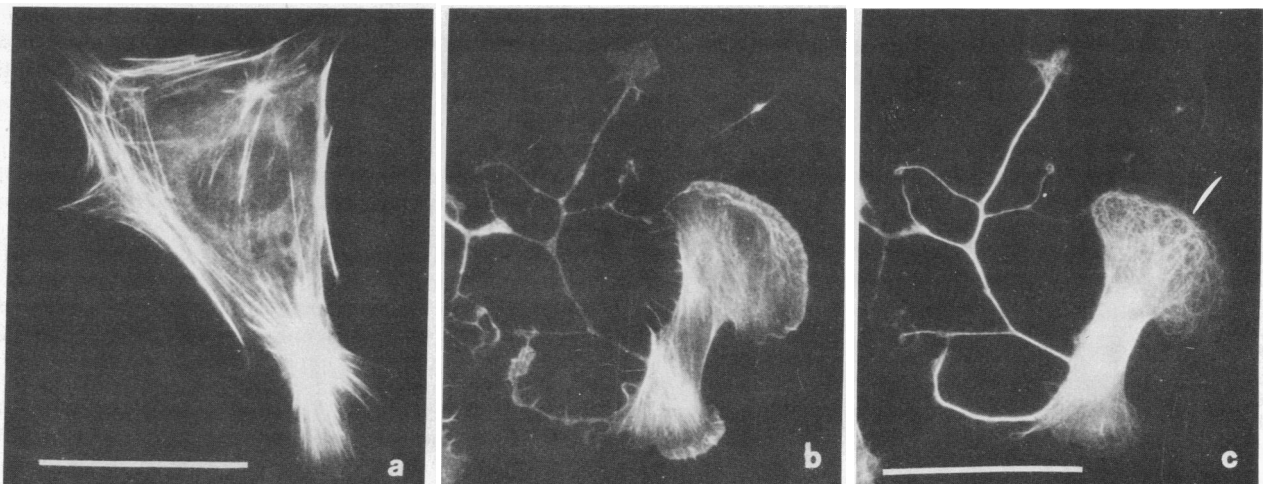Examination of platinum replicas (Fig. 3) of the control

FIG. 2. Fluorescence microscopy after rhodamine-phalloidin treatment (*a* and *b*) or after tubulin antibody revealed by fluorescein isothiocyanate-labeled second antibody (*c*). (*a*) Actin bundles in the control 152/15 cell. (*b* and *c*) PMA-treated cell. This cell has an actin-rich body and lamellas; narrow processes contain less actin (*b*). Numerous microtubules are present in all the parts of the same cell (*c*). (Bars = 50 μm.)
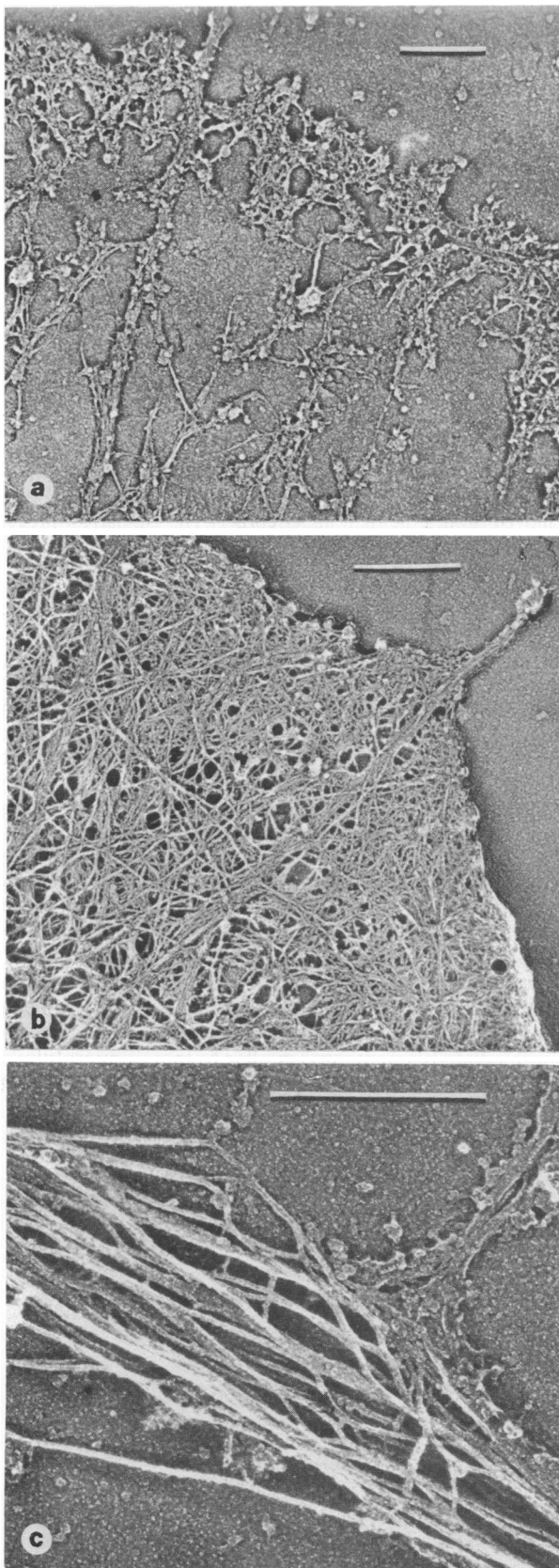
FIG. 3. Electron microscopy of platinum replicas of the cytoskeleton. (*a*) Microfilament meshwork near the leading edge of a control cell. (*b*) More dense and wide network in the lamella of a PMA-treated cell. (*c*) Microtubules and intermediate filaments in the narrow process of a PMA-treated cell. (Bars = 0.5 μm.)

cells revealed a dense network of microfilaments at the active edge of lamella; the zone of sparsely spaced microfilaments was adjacent to this marginal zone. The marginal microfilamental meshwork at the active edges of lamellas of PMA-treated cells was more wide than in the control cells and consisted of longer fragments of microfilaments. The adjacent sparse zone usually disappeared. The narrow processes of PMA-treated cells contained numerous longitudinally oriented microtubules and intermediate filaments. Microfilaments were present mostly in the form of meshwork-like patches at the periphery of processes and in the bulbs.

Effects of Inhibitors. The 152/15 cells incubated with colcemid (1 μg/ml) for 1 hr lost their elongated shape and acquired irregular polygonal contour with short thick processes extended from all parts of the edge (1). These cells did not contain microtubules; intermediate filaments collapsed around the nucleus. Short and thin actin bundles were randomly distributed in the cytoplasm. Addition of PMA to the colcemid-containing medium did not induce any additional morphological changes of these cells.

In other experiments the cells were first incubated with PMA for 2–3 hr and then colcemid was added to the same medium. In this case colcemid caused gradual disappearance of large lamellas and of narrow processes (Fig. 4), so that the cells incubated for 1–2 hr in the medium containing both PMA and colcemid became morphologically indistinguishable from those treated with colcemid alone. Cytochalasin B (10 μg/ml) caused characteristic arborization of the cells. Subsequent addition of PMA to the medium did not induce further changes of shape. When the cells were pretreated with PMA for 2 hr and then cytochalasin B was added to the medium for 1 hr, arborization of lamellas was observed, but the narrow processes were not considerably changed. When colcemid was added to the medium of these cells, it did not induce any additional morphological alterations.

DISCUSSION

Numerous effects of PMA on various cultured cells have been described (see reviews in refs. 7–9). The list of these effects includes some morphological alterations, such as disappearance of actin bundles (9), induction of ruffles (9), and contraction of the cell body (10). In our experiments these changes were associated with another previously unknown effect—namely, with the reversible division of cell into several parts: motile cell body and stable elongated processes. These processes were formed from the retracted lamellas; later they often were elongated at their proximal ends because of contraction of the body, which moved away from these ends. The immunomorphological and electron microscopic examination had shown that lamellas and the cell body of PMA-treated cells contain extensive actin networks. The relationship of the number of actin microfilaments to that of microtubules was much lower in the narrow processes than in the lamellas. We have to conclude that, when the processes are formed from lamellas and tail parts of the body, polymerized or depolymerized actin moves away from them along the microtubular framework. PMA did not cause any shape alterations in the cells pretreated either with colcemid or with cytochalasin B. Thus, both microtubules and microfilaments are essential for the development of PMA-induced alterations of shape. One possible suggestion is that PMA somehow detaches actin microfilaments from the microtubules and/or intermediate filaments.

The network of actin microfilaments in the lamellas of untreated fibroblasts develops centripetal tension (1–3). Actin cortex of PMA-treated fibroblasts may be reversibly detached from the other components of cytoskeleton; when this cortex contracts, microtubules and intermediate filaments are left behind. Stable narrow processes are, possibly,
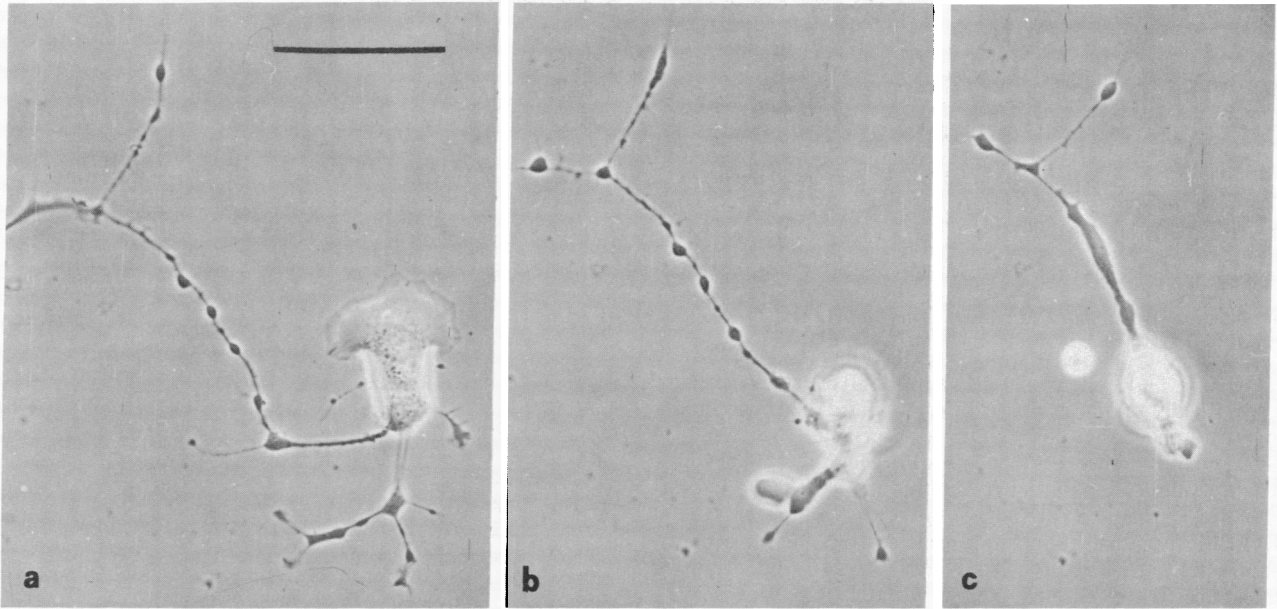
FIG. 4. Phase-contrast micrographs of the cell of 152/15 line preincubated for 1 hr with PMA before the addition of colcemid (1 μg/ml) (*a*) and 15 min and 45 min after the addition (*b* and *c*, respectively). Note the progressing shortening of the long process and translocation of the cell body toward the end of the process. (Bar = 50 μm.)

formed in this way. Actin-rich "bulbs" are, possibly, the fragments of contracted cortex remaining on these processes. Actin transported from the old lamellas may be used for the formation of microfilamental network within the newly extended lamellas.

Microtubules seem to be essential not only for the transport of actin from lamellas but also for the prevention of its return into the stable processes. Destruction of microtubules by colcemid removes the ban for the restoration of actin network and leads to contraction of the processes; the inhibitory effect of cytochalasin B confirms the essential role of microfilaments in this contraction (see also ref. 11).

The molecular mechanisms of the effects of PMA on the cytoskeleton remain completely obscure. PMA is a specific activator of protein kinase C participating in the transduction of membrane-generated signals (12). The long list of proteins phosphorylated by this enzyme contains, among other substrates, some cytoskeleton proteins such as vinculin, certain microtubule-associated proteins, etc. (13). Possibly, phosphorylation of one of these substrates modifies in some way the interactions of microtubules with actin cortex.

Factors determining the relative sensitivity of various types of fibroblasts to the induction of shape changes by PMA remain unknown. The alterations of cell shape, like many other effects of PMA, are spontaneously reversed after prolonged incubation. Possibly, this reversion is due to the down-regulation of the main cellular target of PMA, protein kinase C (14).

We suppose that movements of actin cortex along the microtubular framework take place not only in PMA-treated fibroblasts of certain lines but also in many cell types during their normal morphogenesis. For instance, the growing processes of the neuron consist of stable parts rich in microtubules and of motile lamellar growth cones rich in microfilaments. When the growth cone moves forward, its proximal part retracts and is transformed into the more narrow stable processes. The moving untreated fibroblast continually forms actin-rich lamellas and narrow tails; pos-

sibly, actin moves continually toward the leading edge. Dissociation of actin from the other components of cytoskeleton may be less complete in these untreated cells than in PMA-treated ones because their tails remain contractile and repeatedly disappear during movement. In other words, PMA possibly exaggerates normal reorganizations essential for locomotion. Thus, experiments described in this paper suggest that the cells have a membrane-activated mechanism that reversibly separates large or small motile parts from stable cell parts. This mechanism possibly operates by moving actin along microtubules.

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