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Role of the Microfibrillar System in Knob Action of Transformed Cells

William D. Meek and Theodore T. Puck

Eleanor Roosevelt Institute for Cancer Research and the Florence R. Sabin Laboratories for Genetic and Developmental Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

Transformed cells often display knobs (or blebs) distributed over their surface throughout most of interphase. Scanning electron microscopy (SEM) and time-lapse cinematography on CHO-K1 cells reveal roughly spherical knobs of 0.5–4 μm in diameter distributed densely around the cell periphery but sparsely over the central, nuclear hillock and oscillating in and out of the membrane with a period of 15–60 sec. Cyclic AMP derivatives cause the phenomenon of reverse transformation, in which the cell is converted to a fibroblastic morphology with disappearance of the knobs. A model was proposed attributing knob formation to the disorganization of the jointly operating microtubular and microfilamentous structure of the normal fibroblast. Evidence for this model includes the following: 1) Either colcemid or cytochalasin B (CB) prevents the knob disappearance normally produced by cAMP, and can elicit similar knobs from smooth-surfaced cells; 2) knob removal by cAMP is specific, with little effect on microvilli and lamellipodia; 3) immunofluorescence with antiactin sera reveals condensed, amorphous masses directly beneath the membrane of CB-treated cells instead of smooth, parallel fibrous patterns of reverse-transformed cells or normal fibroblasts; 4) transmission electron microscopy (TEM) of sections show dense, elongated microfilament bundles and microtubules parallel to the long axis of the reverse-transformed CHO cell, but sparse, random microtubules throughout the transformed cell and an apparent disordered network of 6-nm microfilaments beneath the knobs; 5) cell membranes at the end of telophase, when the spindle disappears and cleavage is complete, display typical knob activity as expected by this picture.

Key words: **knobs or blebs, transformed cells, reverse transformation, time-lapse cinematography, scanning EM, transmission EM, microtubules and microfilaments or microfibrillar system, colcemid, cytochalasin B, dibutyryl cyclic AMP, indirect immunofluorescence, antiactin, antitubulin**

Knobs or blebs are a characteristic topographic feature of many transformed cells in culture [1–3], although time-lapse cinematography of other cells growing in vivo also occasionally display similar features [4]. Rounded membrane protuberances or the pro-

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cesses which form them have been described by a variety of names including potocytosis [5], balloon-like structures [6], hyaline bulges or protrusions [7], blisters [8], zeiosis (boiling over) [9], bubbling [10], anaphase bubbling or blebbing [11], bosses [12], and membranous whorls [13]. It is not known to what extent these represent similar or different phenomena. Because of this large variety of observed cell excrescences we adopted the term "knob" for the rounded, oscillating protrusions which characterize the transformed cells studied by us and which display the specific morphologic property of disappearing in response to the addition of agents which increase the level of cyclic AMP in the cell [1,2,14].

Knobs are sparse on normal cells [1–3, 15, 16], but are commonly seen on mammalian cells transformed by viruses [17, 18], chemicals [19], and radiation [15, 20, 21]. This paper describes comparative studies carried out on the transformed Chinese hamster ovary cell (CHO-K1) and two other permanent Chinese hamster cell lines from the ovary (CHO-III) and from the lung (CHL) that display reasonably normal fibroblastic morphology in culture. These studies amplify our previous demonstration that cyclic AMP plays an important role in organizing the microfibrillar structure of particular transformed cells into the pattern resembling that of a normal fibroblast and that knob activity is intimately associated with the microfibrillar structure.

In its native state in normal growth medium CHO-K1 exhibits the following transformation characteristics: 1) The cell is compact, pleomorphic and studded with knobs; 2) single cells grow with virtually 100% plating efficiency, either in agar suspension or on solid surfaces; 3) cells deposited on surfaces produce colonies displaying heavy, three-dimensional growth in the central region and a roughly circular outline, indicating that growth proceeds randomly in all directions. Addition of cyclic AMP derivatives to such a culture causes the following changes: 1) Knobs disappear and the cell becomes elongated, assuming a typical fibroblastic morphology; 2) the capacity to grow in suspension in ordinary serum concentrations disappears; 3) colonial growth on surfaces becomes strongly monolayered, and growth in such colonies displays the typical fibroblastic pattern associated with density-dependent growth inhibition, in which the cells pack closely together in parallel to their long dimension and exhibit loops and whorls resembling stacked sheaves of wheat; 4) cell adhesion to plastic surfaces and to each other is markedly increased so that the satellite colonies which characterize plates grown in normal growth medium virtually disappear [1, 2, 14]. We have named the processes caused by cyclic AMP reverse transformation. Reverse-transformed CHO cells also differ from their native counterparts in resisting the cell-rounding action of antisera against cell surface antigens and the killing action of such antisera in the presence of complement [22]. The reverse-transformed cells are also more resistant to the capping phenomenon which is readily exhibited by the native cells when challenged with lectins [23]. In this paper we present experimental data and interpretations dealing with the knobbing phenomenon that support a picture of this cell surface activity of knobbing as a manifestation of alterations in the microfibrillar structure.

MATERIALS AND METHODS

CHO-K1, CHO-III, and CHL were grown in the standard F12 medium [24] supplemented with 5–20% fetal calf serum (FCS) and an equivalent amount of the macromolecular portion (FCM) of fetal calf serum. All cultures were provided with 100 units/ml of streptomycin and penicillin, and were incubated at 37°C in an atmosphere of high humidity and 5% CO_2 in air. The chemicals used were as follows: N^6,O^2-dibutyryl adenosine 3′-5′-cyclic monophosphoric acid, monosodium salt (Sigma Chemical Co., St. Louis) testololactone

(E. R. Squibb and Sons, Inc., Princeton), colcemid (Sigma Chemical Co.), and cytochalasin B (Sigma Chemical Co.). Several different concentrations of the above reagents were used which included: 0.5–7;5 mM dbcAMP + 10^{-5} M testololactone, 1.4–14 μM colcemid, 1.0–10 μM cytochalasin B. After several experiments with concentrations within these ranges it was found that 1 mM dbcAMP + 10^{-5} M testololactone, 1.4 μM colcemid, and 1.0 μM cytochalasin B yielded optimal results for our particular analysis, as discussed below.

Time-Lapse Microcinematography

For time-lapse cinematography, 3×10^3 cells are inoculated into a growth chamber made by placing a Teflon ring on the bottom of a 35 × 10-mm plastic Petri dish (Lux Scientific Corp., Newbury Park, California) with sterile, Dowex silicone grease as an adhesive. The ring is completely filled with medium. A plastic coverslip (Lux Plastics, No. 5407) is attached to the top of the ring with silicone grease, care being taken to exclude air bubbles. The growth chamber fits snugly into a depression drilled into a brass block, fitted with heater and thermostat so that the temperature is maintained at 37.5 ± 0.5°. After the growth chamber is filled, the top of the Petri dish is fitted over the bottom, and 5% CO_2 warmed to 37° is injected continuously into the Petri dish through a fine plastic tube passing through a port in the brass block and through a small hole in the side of the Petri dish. Test solutions are added to the chamber by lifting the coverslip and quickly aspirating the old medium and pipetting the fresh medium containing added agents. It is possible, by this means, to observe the effect of the agent immediately after administration. Agents to be tested are added in growth medium containing the macromolecular fetal calf serum component. It is found that the chamber supports clonal growth of single cells with high efficiency. The entire assembly is placed on the stage of a Wild M40 inverted microscope, with quartz halogen light source, fitted with phase optics and Bolex-Wild variotimer. Exposures are made for 0.5 sec at 30-sec intervals. A Bolex H16SBM 16-mm camera and Kodak Plus-X reversal film are used. Films are studied at low speed (1–4 frames per second). Negatives for prints are made from the 16-mm movie film using Kodak Plus-X film. Magnification in all pictures is 260.

Scanning Electron Microscopy

Into 35 × 10-mm plastic tissue culture dishes containing uncoated coverslips (11 × 22 mm) 3×10^4 cells are inoculated and then incubated for 48–72 h in growth medium supplemented with FCM. Agents like 1 mM dbcAMP plus 10^{-5} M testololactone are added and incubation is continued for 3 h. The cultures are washed briefly with saline G (pH = 7.4; 37°C) [25]. Cells are then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.4; 37°C) for 10 min. The preparations are dehydrated in a graded acetone series (one 5-min change in 1%, 15%, 30%, 50%, 70%, 90%; two 5-min changes in 100%). The cell-attached coverslips are dried in a Sorvall critical-point CO_2 drying apparatus (Ivan Sorvall, Inc., Newton, Connecticut) [26]. Fragments of the coverslips are placed on a stud or specimen holder. These cell-containing coverslip fragments are coated with a thin layer of gold and studied with a Kent Cambridge Stereoscan scanning electron microscope [S4] mounted with a LaB_6 gun. Photographs are taken on a Polaroid P/N Type 55 film at tilt angles of 30–40°.

Transmission Electron Microscopy

CHO-K1 [27, 28] is inoculated at a density of 3×10^3 cells per chamber into four-chamber tissue culture slides (Lab-Tek Products, Westmont, Illinois). Following a 48- to 72-h growth period, the cells are treated for 6 h with 1 mM dbcAMP + 10^{-5} M testololactone

in medium either with the macromolecular portion of fetal calf serum, in a concentration equivalent to 5% of that in whole serum, or without any serum supplementation. Cells are next washed and fixed by the same method as used in SEM. Dehydration is performed by a graded ethanol series (one 10-min change in 30%, 50%, 70%, 95%; and two 10-min changes in 100%) followed by two 15-min changes in propylene oxide; followed by a 10-min change in 1:1 propylene oxide : Epon 812 mixture;followed by a 10-min change in Epon 812. The slides are then flat-embedded in Epon 812. Slides are later removed from the plastic by immersion in boiling water for 10–20 sec. Areas of cells, visible with the light microscope, are cut from the plastic and mounted upon Epon bullets for thin-sectioning. Sections are cut with a Porter-Blum MT-2 ultramicrotome and stained with uranyl acetate and lead citrate. The resulting specimens are examined and photographed with a Jeol 100C transmission electron microscope operated at 60 kV.

Immunofluorescence

On the day prior to the experiment, 2.5×10^5 cells (CHO-K1, CHO-III, or CHL) are plated onto 11 × 22-mm sterile glass coverslips in 35 × 10-mm Petri dishes and allowed to attach and grow overnight. After overnight incubation the cells are treated with 0.5–7.5 mM dbcAMP + 10^{-5} M testololactone, 8 μM colcemid, or 2 μM cytochalasin B in growth medium for periods of 1–4 h. Initial gifts of antisera against tubulin and actin, respectively, were kindly furnished to us by B. R. Brinkley and E. Lazarides. The immunofluorescence procedures follow those described by these investigators [29–31].

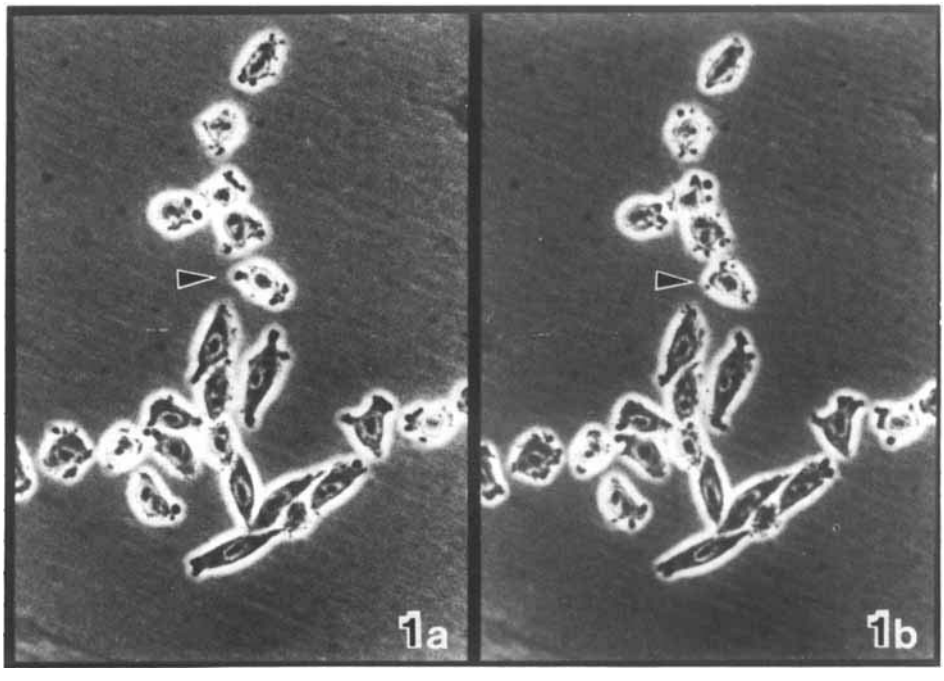


Fig. 1. Time-lapse pictures taken at 30-sec intervals during the growth of CHO-K1 in standard growth medium (F12FCM10). A single cell (arrowhead) is shown in two frames to demonstrate the frequency at which the round protuberances or knobs extrude from and then return to the cell body. Each knob can be seen to form and disappear during a 30-sec period. × 260.

RESULTS

Time-Lapse Microcinematography

The knobs on the CHO-K1 cell extend from the main body of the cell membrane to a distance of approximately 2 μ and move in and out with a period of approximately 30 sec (Fig. 1). This rapid oscillatory activity is confined to distinct positions and so is clearly different from ruffling that appears in time-lapse movies as an extended waving or undulating motion of a large portion of the membrane. Knobs are exhibited by CHO-K1 cells throughout the entire life cycle except during the first part of mitosis, when the cells are almost completely spherical. At the end of telophase or early G1, knob activity is resumed and is more intense than that which occurs throughout the rest of interphase. The knobs characteristic of the cells just completing cytokinesis are frequently seen in the polar region, but are not necessarily restricted to these areas (Fig. 2).

The addition of cyclic AMP derivatives to CHO-K1 cultures produces virtually complete disappearance of the knobs in all parts of the cell life cycle (Fig. 3 and Table I). Concomitantly the membrane loses its oscillating foci and becomes much more tranquilized. Knob formation is also virtually completely suppressed in the telophase/early G1 cells under conditions of high cyclic AMP concentration (> 1 mM), although transient knob action may occasionally be visible at this particular period. By the use of 0.5–1 mM dibutyryl cyclic AMP plus 10^{-5} M testololactone knobbing can be suppressed without interference with normal cell division of the CHO-K1 cell (Table I).

Even smooth-surfaced cells can exhibit knob action at the telophase/G1 boundary of the life cycle in normal growth medium (Table II). Addition of cyclic AMP to CHO-III

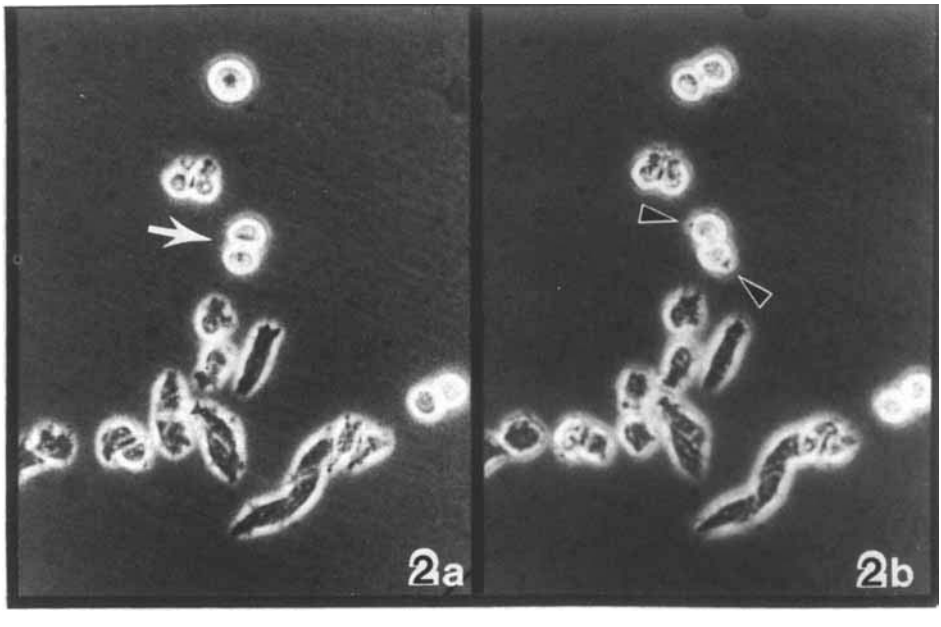


Fig. 2. These time-lapse pictures show the knob activity of a late telophase/early G1 CHO-K1 cell (arrow in a) in standard medium (F12FCM10). The first frame (2a) is 6.5 min after anaphase and 2b is 6.5 min later. Knobs (arrowheads) begin to form in the polar regions 13.0 min after anaphase (2b). Compare with the same colony at a later time in Figure 3. × 260.

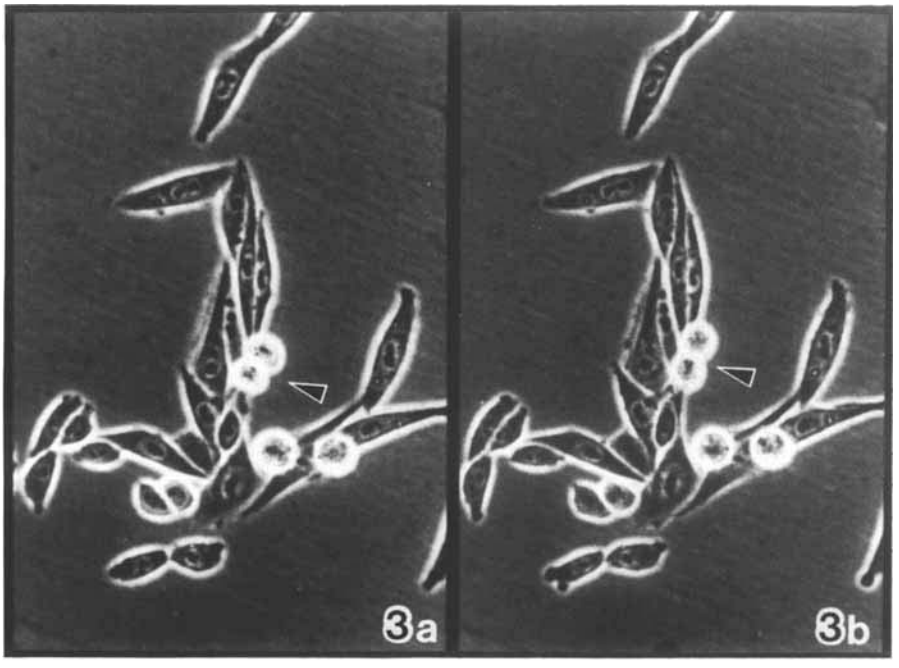


Fig. 3. These time-lapse pictures show the same field as in Figure 2 after the cells have been treated with 1 mM dibutyryl cyclic AMP + 10^{-5}M testololactone in F12FCM10. The first frame (a) is 4.3 h after the addition of the agent and 6.5 min after anaphase; frame b is 6.5 min later. The cells are elongated and possess no knobs. The knob formation as seen in Figure 2 also fails to occur in the late telophase/early G1 cell (arrow). × 260.

TABLE I. Demonstration of Knob Activity in CHO-K1 After Various Concentrations of dbcAMP, Colcemid, and Cytochalasin B Treatment

	Knobbed cells (%)	Smooth-surfaced elongated cells (%)
Normal medium (F12FCMS)	81	19
1 mM dbcAMP + 10^{-5}M testololactone	15	85
3 mM dbcAMP + 10^{-5}M testololactone	8	92
10^{-2}M dbcAMP + 10^{-5}M testololactone	3	97
2.7 μM Colcemid	96	4
2.7 μM Cytochalasin B	99	1

Elongated = one dimension at least three times greater than the other.

or CHL fibroblasts produces little, if any, change in their overall morphology as determined by viewing with time-lapse cinematography (Table II).

Addition of colcemid at a concentration 1.4–14 μM to smooth-surfaced CHO-III or CHL fibroblasts or to CHO-K1 cells that have been reverse-transformed by the presence of cyclic AMP derivatives causes an outbreak of typical knob activity in most points of the

TABLE II. Knob Activity by Culture Condition as Viewed by Time-Lapse Microcinematography

	Normal medium		1 mM dbcAMP +10^{-5} M testololactone		Colcemid (1.4–14 μM)		Cytochalasin B (1.0–10 μM)	
Cell type	Inter-phase	Telo-phase-G1	Inter-phase	Telo-phase-G1	Inter-phase	Telo-phase-G1	Inter-phase	Telo-phase-G1
CHO-K1	+	+	–	±	+	X	+	+
CHO-III	–	±	–	±	±	X	+	+
CML	–	±	–	±	+	X	+	+

Symbols: + = knob presence or formation; – = no knob presence or formation; X = no cell observed in that portion of the cell cycle because of the mitotic arrest produced by colcemid.

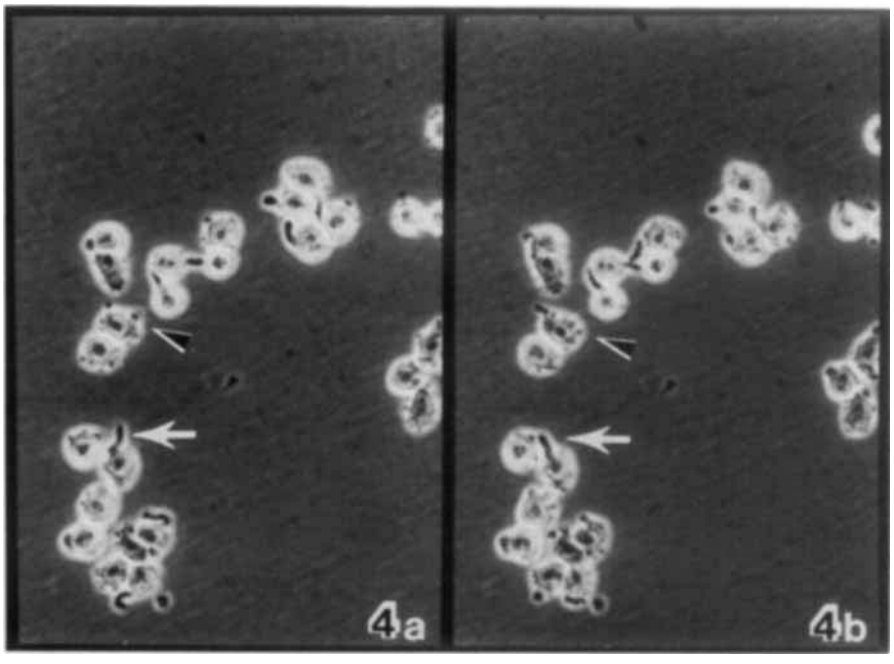


Fig. 4. In the time-lapse picutres a and b, changes are depicted that occur when 1.4 μM colcemid is added to the growth medium (F12FCM10) of CHO-K1. The first frame is 2.2 h after addition of colcemid; frame b is 1 min later. Elongated knobs (arrows), as well as the more spherical type (arrowheads), are seen emanating from the cells. The former wave around in the medium over the period pictured. × 260.

life cycle. CHO-III and CHL cells become almost indistinguishable from the appearance of CHO-K1 in the presence of ordinary growth medium (Table II), and an increase in knob formation is seen in colcemid-treated K1 cells (Table I). Colcemid can also cause extrusion of elongated sausage-shaped knobs from the cell surface (Fig. 4a,b), which undergo waving movements in the medium. When cultures of CHO-K1 are treated with a combination of colcemid and dbcAMP + testololactone, the two sets of reagents antagonize each other's action [1, 2, 14]. In appropriate concentrations the dbcAMP + testololactone can prevent both the knob activity that occurs normally and that which is stimulated by colcemid. Under these circumstances, ruffling is commonly seen along the cell borders (Fig. 5a,b). The addition of cAMP derivatives to cells in colcemid also produces some cell elongation, although

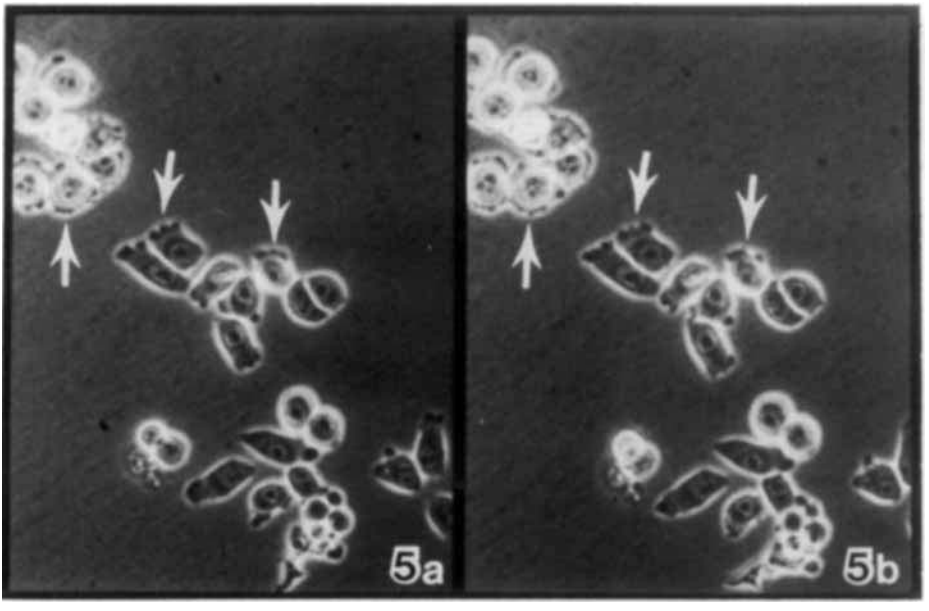


Fig. 5. In these two pictures (a and b) taken at 1-min intervals, another CHO-K1 culture is seen 1.5 h after treatment with both 1.4 μM colcemid and 1 mM dbcAMP + 10^{-5}M testololactone in growth medium (F12FCM10). Knob formation does not occur but lamellipodia or ruffles (arrows) are present along cell borders, and these structures persist over the 1-min period. Some cells have assumed a more flattened appearance. × 260.

not as much as that produced by dbcAMP + testololactone alone. Lamellipodia or ruffles are distinguished from knobs by their restriction to the cell periphery, by their undulating or wavy appearance, and by their continuous existence in contrast to the knobs, whose pulsations cause them to appear and disapper.

Cytochalasin B administered to cell cultures also increases the number of knobs on CHO-K1 (Table I), causes knobs to form on CHO-III and CHL (Table II and Fig. 6a,b), and appears to increase the frequency of the oscillatory knob action on K1 as viewed by time-lapse cinematography. The knobs are somewhat smaller than those normally present in CHO-K1 (compare with Fig. 1) and form so quickly that the cell topography changes appreciably in periods smaller than 30 sec. Cytochalasin B treatment at low concentrations may leave the general shape of the cell unchanged, so that one may obtain a typical spindle-shaped or epithelioid cell studded with knobs. At higher concentrations of CB, cells with a stellate appearance can result. DbcAMP plus testololactone added with the CB erases the knobs and some ruffling is observed (Fig. 7a,b).

These experiments demonstrate that knob activity exists throughout most of the life cycle for the transformed CHO-K1 cell, but only at the telophase/G1 boundary in smooth-surfaced fibroblastic cells like CHO-III or CHL; it can be diminished or removed from cells in which it is present by reagents which increase the concentration of cyclic AMP; it can be induced in some smooth-surfaced cells in which it is absent by either colcemid or cytochalasin B. Knob activity is associated with production of a hyperactive membrane. The substrate attachment of CHO-K1 cells exhibiting such activity is diminished considerably [32, 33]. The oscillatory knob activity may explain at least in part why cells possessing knobs grow in random fashion rather than in specifically oriented patterns in colonies developing on solid surfaces.

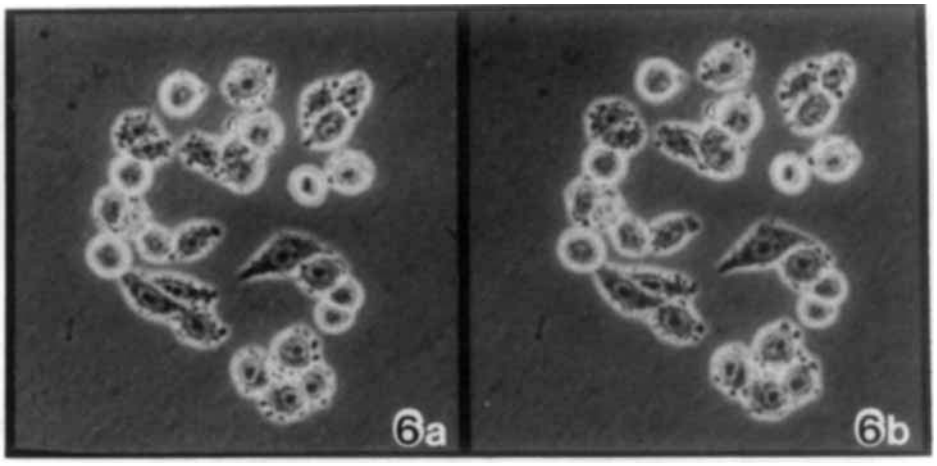


Fig. 6. In these time-lapse pictures (a and b), a culture of CHO-K1 is seen after addition of 1.0 μM cytochalasin B to the growth medium (F12FCM10). Knobs are prevalent over most cells including the elongated ones, and these cells still remain in the shape characteristic of CHO-K1. Frame a is 2.2 h after addition of CB; frame b is 1 min later. Most knobs form and disappear over the 1-min interval. × 260.

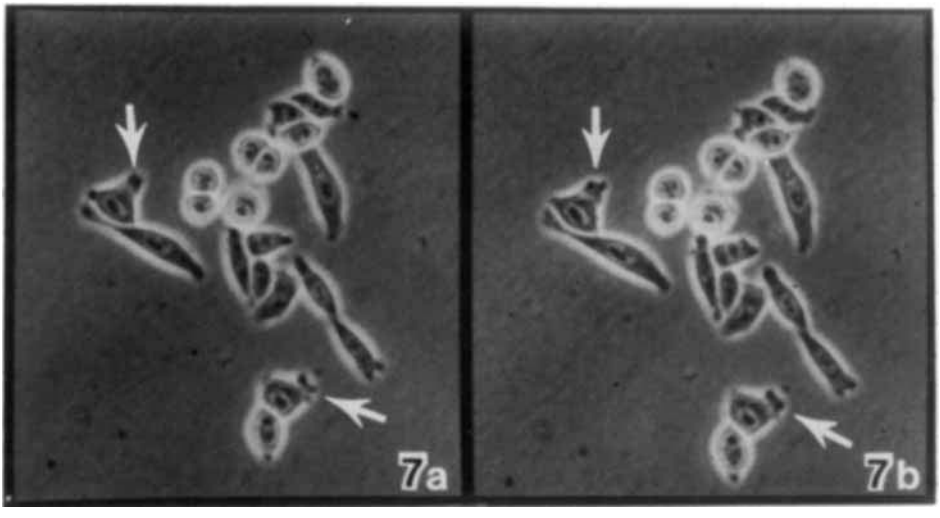


Fig. 7. In these time-lapse pictures (a and b) taken at 1-min intervals, a culture of CHO-K1 is seen 1.5 h after treatment with both 1 μM cytochalasin B and 1 mM dbcAMP + 10^{-5}M testololactone in growth medium (F12FCM10). Knobs are not seen and some cells have elongated. Ruffles (arrows) replace the knobs as surface features. × 260.

Scanning Electron Microscopy

The production of knobs in the CHO-K1 cell by the action of microtubule disorganizing agents like colcemid and vinblastine – or by cytochalasin B, which disorganizes or disrupts the 6-nm microfilaments – indicates that the integrity of both sets of fibrils is required in order to maintain the smooth, knob-free surface of the fibroblastic cell. SEM studies of native CHO-K1 cells reveal that the shape of the knob is roughly spherical, with occasional indentations (Fig. 8). Continuity between knobs and the cell body is

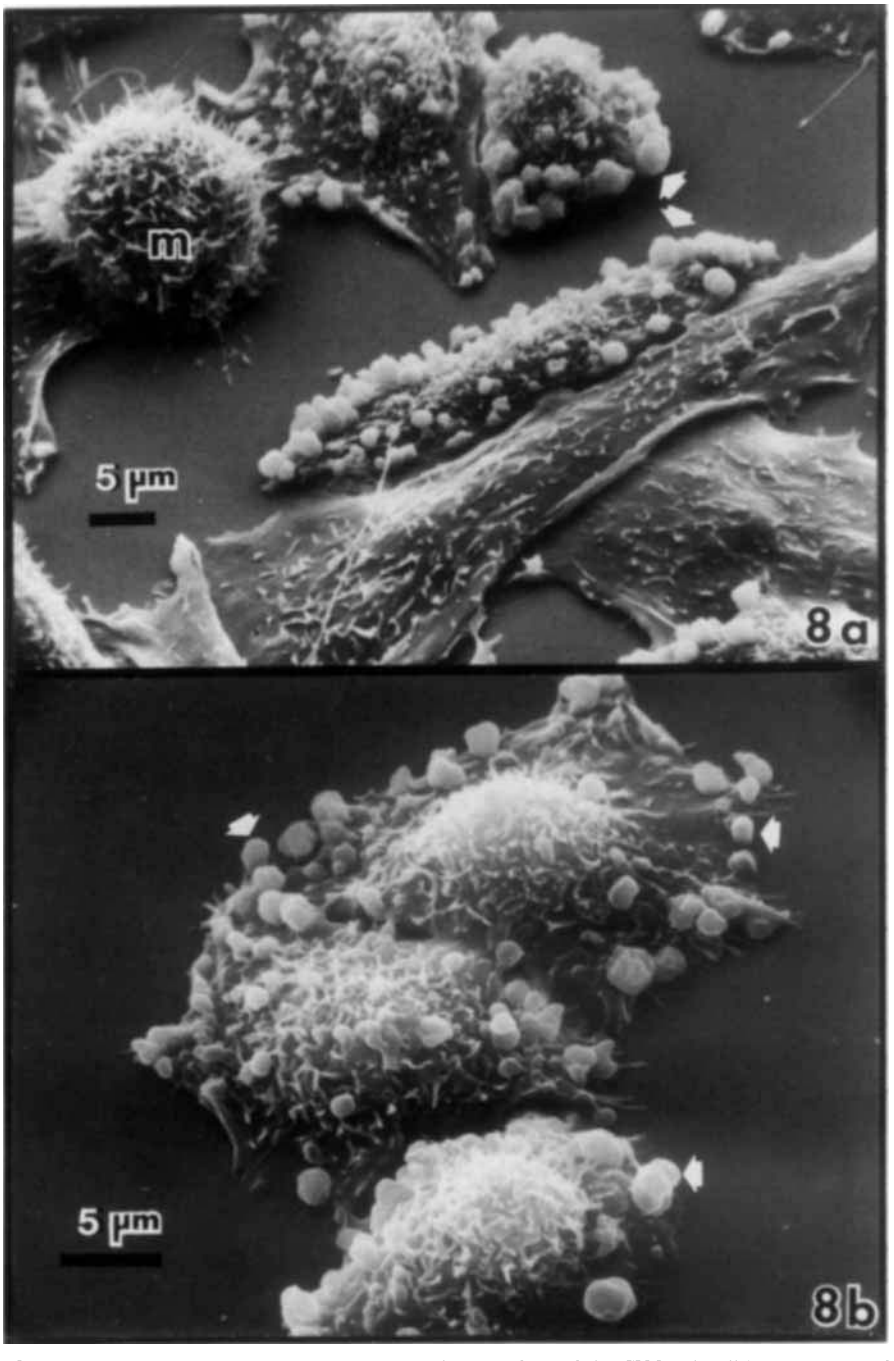


Fig. 8. a: Knobs (arrows) are present over most of the surface of the CHO-K1 cell in the center of the micrograph and along the base of one rounded cell. Other surface features include microvilli, characteristic of the rounded, mitotic cells (m). Some cells are either elongated or flat and noticeably devoid of knobs. F12FCM5 medium; 40° tilt. × 2,000. b: Knobs (arrows) are arranged around the periphery of these cells, while the central, nuclear hillock is covered mainly with microvilli. The two upper cells appear to be in early G1, still connected by the cytoplasmic bridge. F12FCM5 medium; 30° tilt. × 2,625.

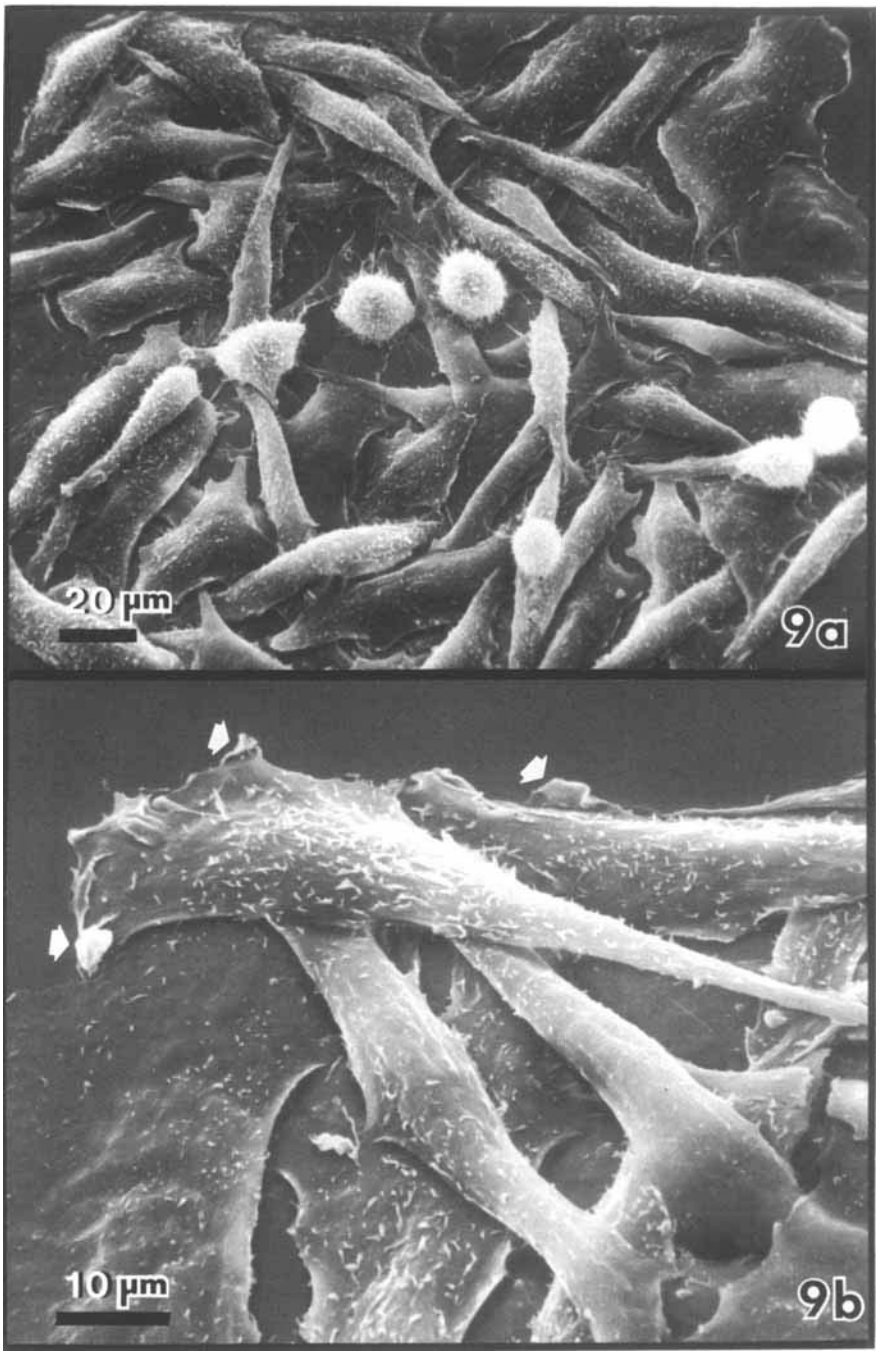


Fig. 9. a: In this low-magnification view of CHO-K1 treated with 0.5 mM dbcAMP + 10^{-5}M testololactone for 3 h, the absence of knobs is noted. Several mitotic cells are present in this colony and most of the other cells are spindle-shaped. F12FCM5 medium; 40° tilt. × 600. b: Lamellipodia or ruffles (arrows) are present in CHO-K1 cells following treatment as in (a). Such higher-magnification micrographs show the detail of the surface as smooth, with scattered microvilli. F12FCM5 medium; 40° tilt. × 1,729.

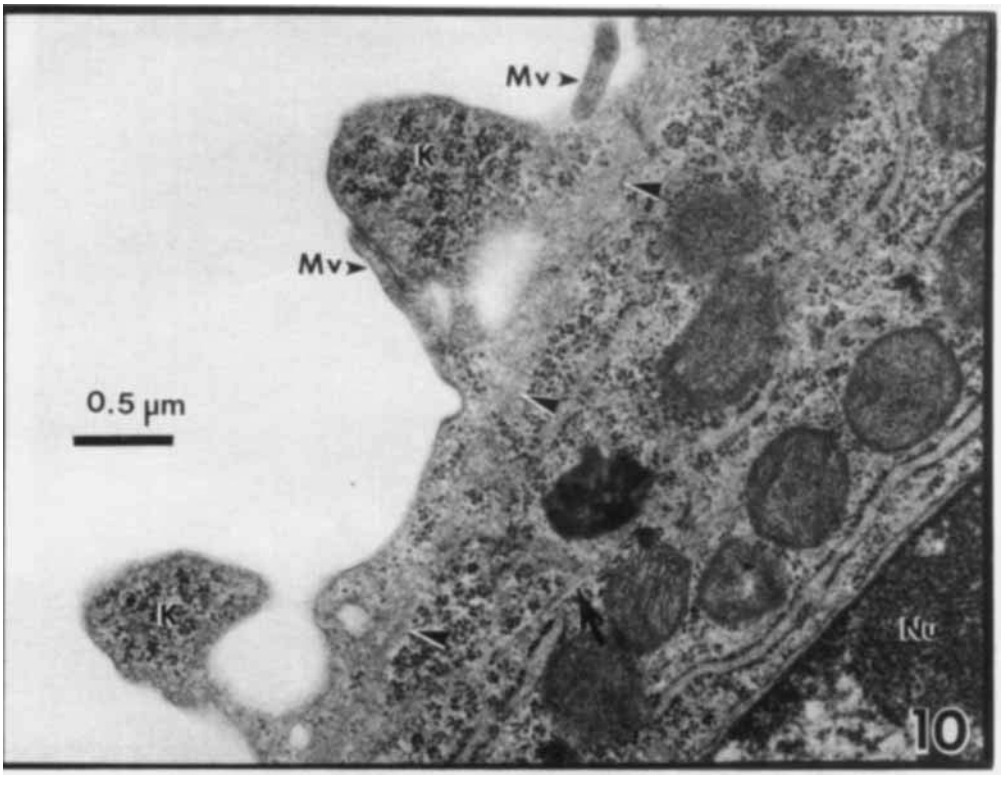


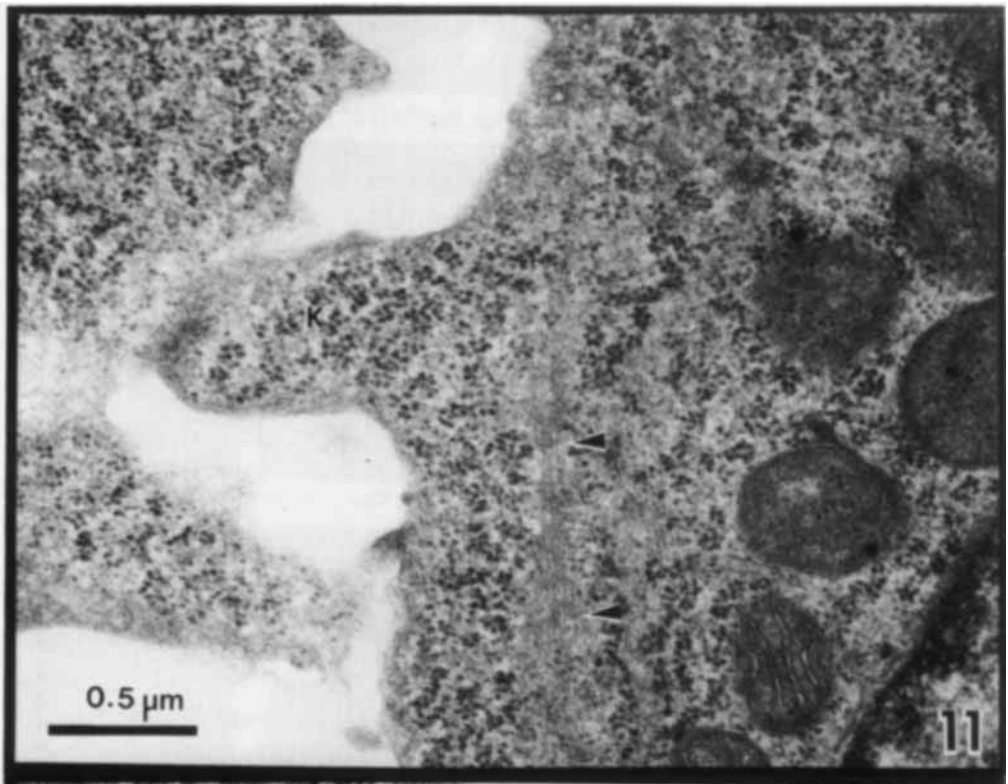
Fig. 10. A high-magnification transmission electron micrograph of CHO-K1 shows knobs (K) and microvilli (Mv) along the surface. Beneath the ribosome-filled knobs and in the cortical cytoplasm, a microfilament bundle (arrowheads) is seen. The filaments are closely related to the cell membrane, and ribosomes are present on both sides of the bundle. A microtubule is seen at the arrow. Nu, Nucleus. F12 medium. × 26,000.

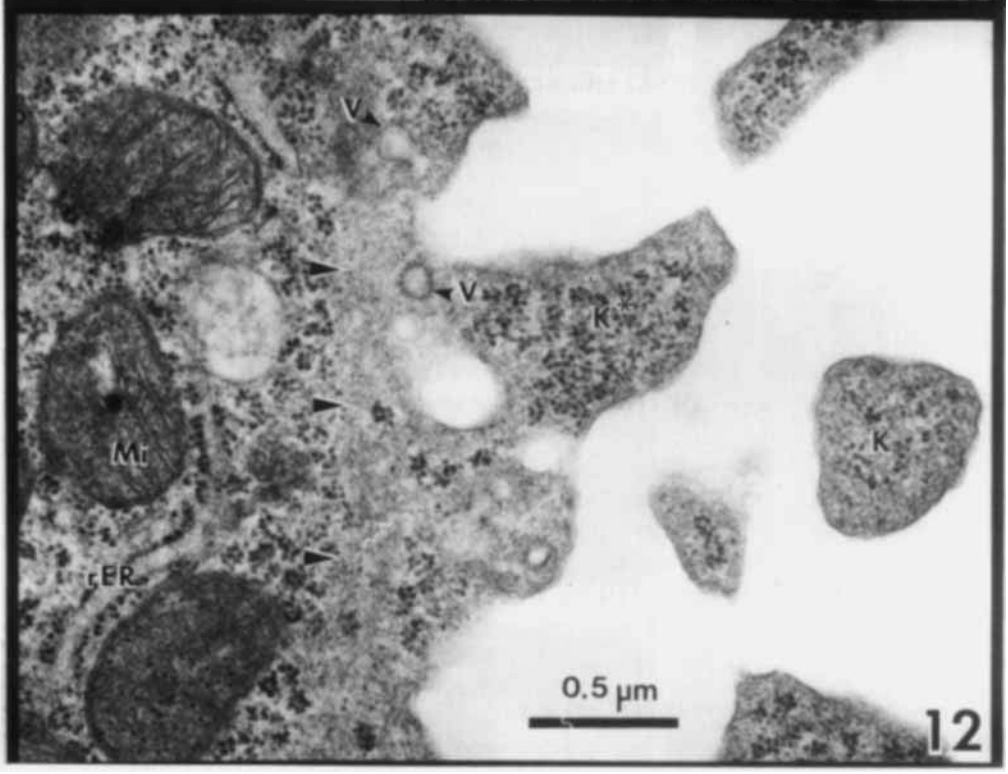
evident. There are always a few CHO-K1 cells visible which lack knobs, particularly in densely packed cultures. These include elongated cells, and some broad, flattened cells, as well as mitotic cells (Fig. 8a). The knobs in the transformed CHO-K1 cells are commonly distributed around the cell periphery while the central, nuclear hillock region contains a dense distribution of microvilli, with a somewhat lower knob density than that found in peripheral areas (Fig. 8b).

The effect of the addition of dbcAMP plus testololactone is shown in Figure 9. The knobs are removed with the appearance of ruffles. Microvilli seem to be little affected (Fig. 9). At the reagent concentrations pictured, most of the cells are elongated within 3h. More rapid and complete response can be achieved with higher drug concentrations, resulting in elongated, spindle-shaped cells (Table I). The change in surface activity observed in

Fig. 11. In this transmission electron micrograph of a CHO-K1 cell, a bundle of microfilaments (arrowheads) is seen near the base of a knob (k) and courses at an oblique angle to the cell membrane. F12 medium. × 39,000.

Fig. 12. This section of CHO-K1 passes through one knob (K*) at its attachment site. Subplasmalemmal filaments (arrowheads) have a felt-like pattern seemingly in a disarranged state. Vesicles (V) are also present in this area. Ribosomes are separated by the filamentous mass, and it can be seen that other organelles such as mitochondria (Mi) and rough endoplasmic reticulum (rER) are not present in the knobs. F12 medium. × 39,866.


K
0.5 µm
11


V
V
K
K
Mi
ER
0.5 µm
12

the time-lapse microcinematography appears to be a direct result of the elimination of the knobs which form the foci of oscillatory movement in the cell membrane.

Transmission Electron Microscopy

The knobs of the CHO-K1 cell contain large numbers of free ribosomes (Figs. 10, 11, 12). A zone of material containing either intact filaments or a fuzzy, poorly organized meshwork of filaments often separates the knobs from the body of the cytoplasm (Figs. 10, 11, 12). This meshwork resembles the amorphous material commonly seen in the subplasmalemmal region after cytochalasin B treatment [34, 35]. Dense accumulations of ribosomes appear on both sides of the filamentous region and pinocytotic vesicles are sometimes present in the cortical regions near the base of knobs (Fig. 12).

After a 6-h exposure of cells to 1 mM dbcAMP plus 10^{-5} M testololactone, the knobs have disappeared (Fig. 13a), and there appears a new, highly ordered arrangement of microfilament bundles, arranged in parallel to the cell membrane together with a parallel array of microtubules adjacent to the filament bundles (Fig. 13b). Some microtubules run at oblique angles to the others but the overall effect is of a much more dense and orderly pattern of microfibrils than that of the transformed cell. The pattern of the reverse-transformed cell resembles that of the fibroblastic cell. The microfibrils tend to be more closely packed and more nearly paralleled to each other and to the long axis of the cell; the more this is so, the longer, more narrow, and more spindle-shaped is the resulting fibroblast.

Indirect Immunofluorescence

Use of indirect immunofluorescence with antibodies against tubulin and actin provides a means of studying microtubules and 6-nm-diameter microfilaments; this serves as an independent check of the electron micrograph data and also furnishes a different level of magnification for examination of these structures.

Treatment of native CHO-K1 cells with antibodies against tubulin reveals a cytoplasmic microtubular network. Cells that are compact or rounded usually display diffuse fluorescence, although most cells exhibit at least some recognizable microtubule patterns, which in the transformed cell frequently involve tubules randomly oriented with respect to each other (Fig. 14a; also see Porter et al [36]). Treatment of these cells with dbcAMP plus testololactone greatly increases the number of stretched cells which display dense strands of tubules parallel to each other and to the long axis of the cell (Fig. 14b).

Treatment of such cells with colcemid destroys the tubular patterns and produces only a diffuse fluorescence throughout the cell cytoplasm (Fig. 14c). The knobs that become more prominent under these circumstances may contain tubulin because of their faint fluorescence (Fig. 14c). Wherever parallel studies were carried out, our data agree with the results described by Brinkley and his co-workers [29].

Immunofluorescence examination of CHO-III and CHL cells that display a reasonably normal fibroblastic shape demonstrates their microtubular patterns to resemble that obtained in CHO-K1 cells treated with dbcAMP plus testololactone. Using antibodies to actin, parallel stress fibers are seen throughout the cytoplasm of the normal CHO-III cell (Fig. 14e). Treatment of the CHO-III cell with CB prior to staining with antiactin reveals localized patches of fluorescence often concentrated through the cell in a fashion similar to that of the knobs that these cells display under such circumstances (Fig. 14f and Table II). Control experiments were performed in identical fashion except for use of normal rabbit

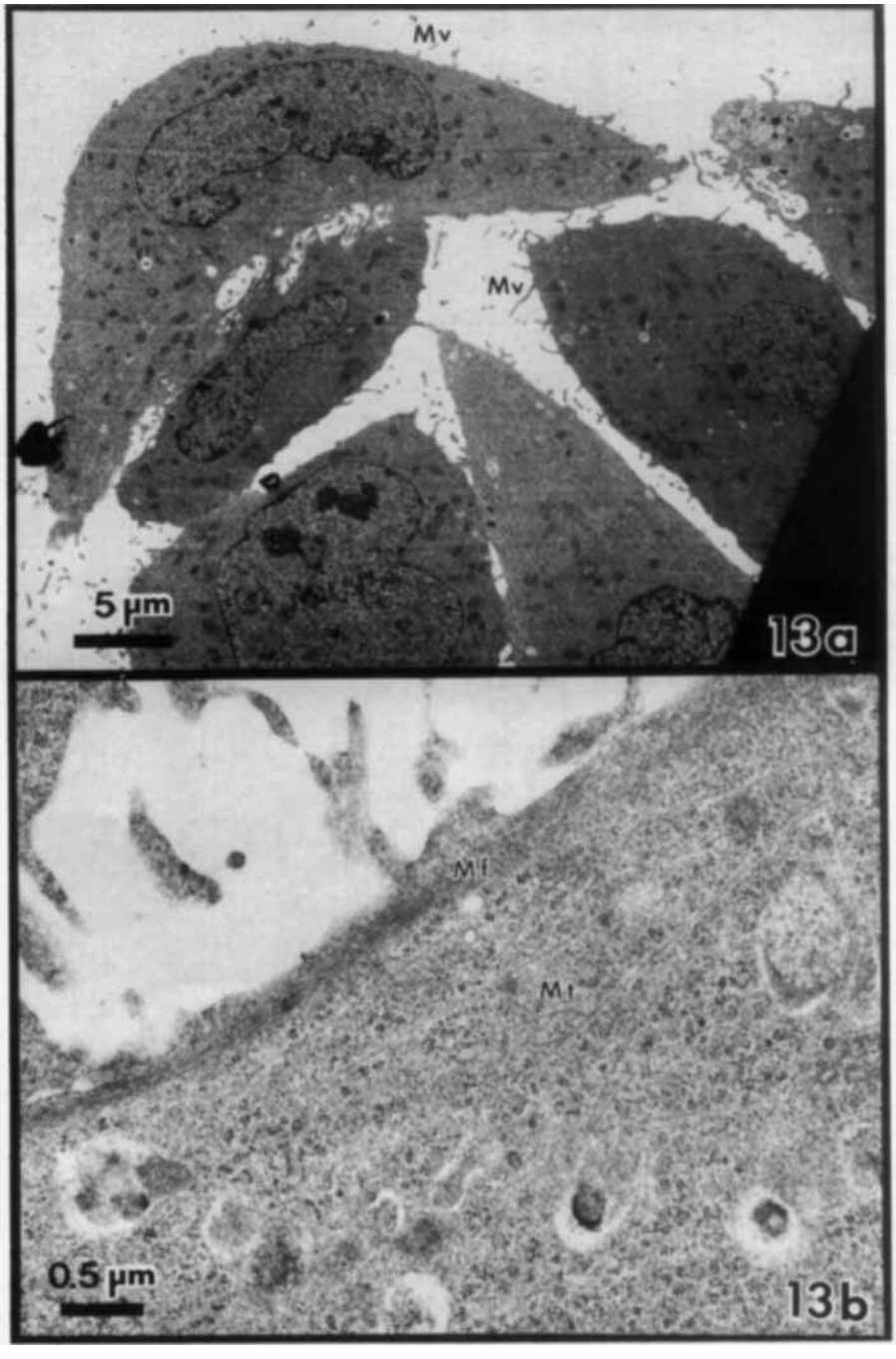


Fig. 13. a: CHO-K1 after treatment with 1 mM dbcAMP + 10^{-5}M testololactone for 6 h displays microvilli (Mv) as the only surface feature. F12 medium. × 3,000. b: A higher magnification of a CHO-K1 cell as treated in panel a shows a compact bundle of microfilaments (Mf) directly beneath the cell membrane. Microtubules (Mt) run generally parallel to the filament layer in the nearby cytoplasm. F12 medium. × 24,000.

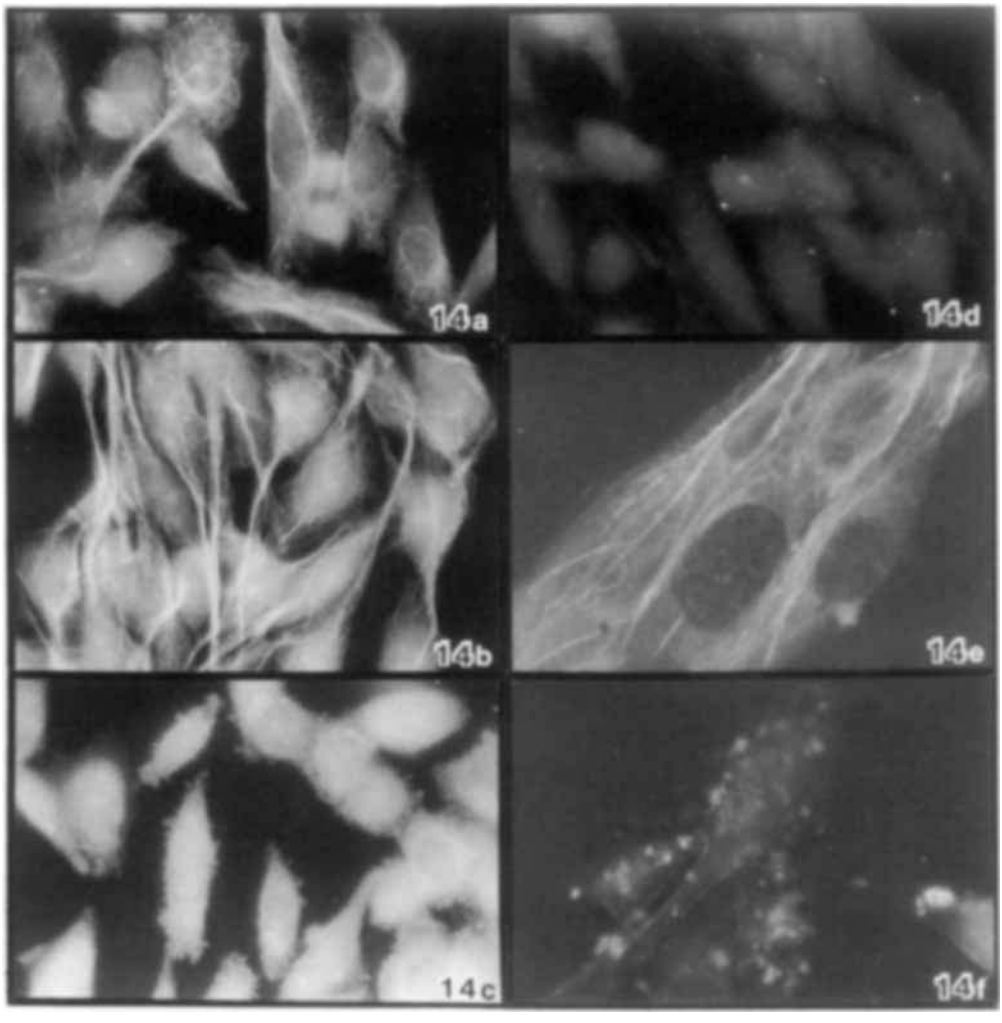


Fig. 14. a: The network of microtubules in CHO-K1 is apparent when cells are labeled with antitubulin sera. × 1,800. b: Parallel bundles of microtubules are seen in many CHO-K1 cells treated with 7.5 mM dbcAMP + 10^{-5}M testololactone for 1 h. × 1,800. C; CHO-K1 cells show a diffuse fluorescence when exposed to 8 μM colcemid for 1 h prior to treatment with antitubulin sera. Knobs are visible along the periphery of the cells. × 1,800. e: CHO-III cells, which display morphology of normal fibroblasts, reveal bundles of stress fibers when treated with serum prepared against the contractile protein actin. Branching bundles are seen in the cytoplasm of these cells. × 2,400. f: Foci of fluorescence appear when CHO-III cells are treated with 2 μM cytochalasin B for 1 h prior to exposure to the antiactin sera. The actin cables are not present as in panel e × 2,400. d: No specific staining was observed in cells treated with normal sera × 1,800.

serum instead of antiserum to actin, and they are presented in Figure 14d. Similar results are obtained in such control experiments carried out with CHO-III and CHL.

Use of Other Fibroblastic Cells

While each cell type has its unique features, the general pattern of findings that we have reported for the fibroblastic CHO-III cell is exhibited by other long-term, cultured fibroblastic cells such as the CHL cell and the V79 cell described earlier [2]. In normal

medium these exhibit smooth, knob-free cell membranes except for a brief interval at the telophase/G1 boundary. Addition of dbcAMP plus testololactone produces no effect except for occasional slight cell elongation. Either colcemid or cytochalasin B causes knob extrusion although the concentrations and the times required may vary somewhat with the different cells employed.

DISCUSSION

This paper constitutes part of a continuing study designed to illuminate the relationship between cyclic AMP metabolism and microfibrillar structure in mammalian cells and the relevance of these to cell transformation.

It has been demonstrated by us that in CHO-K1, cyclic AMP is necessary for organization of microtubules into a parallel network, with concomitant effects on the overall shape of the cell. The pattern assumed under these circumstances appears to be genetically or epigenetically predetermined [1, 37].

The fact that the addition of cytochalasin B, colcemid, or other microfibril-disrupting compounds to a stretched, fibroblastic cell [2] can cause it to assume the condensed, pleomorphic structure characteristic of transformed cells implies that the fibroblastic structure is dependent on the integrity of cellular microfilaments and microtubules. This conclusion is strengthened by the fact that in the fibroblastic morphology, which is achieved by cells like CHO-K1 on the addition of cyclic AMP derivatives, microtubules are arranged in parallel to each other and to the long dimension of the stretched cell [36]. A similar dependence of cellular morphology on microtubular integrity in brain cell hybrids that send out nerve-like processes on treatment with dbcAMP supports this general thesis. Ultrastructural studies described elsewhere demonstrate these processes to contain bundles of microtubules parallel to the long dimension of the process [38].

The role of knob formation, which occurs when either microtubules or microfilaments are disrupted, requires more intimate elucidation. We have proposed the working hypothesis that the relatively smooth membranes of normal fibroblasts are not a reflection of the absence of forces operating at the membrane but rather of the fact that such forces are in balance so as to produce a smooth, stretched membranous configuration. The contractile tendency of the actin-containing filaments is balanced by the opposition of the rigid microtubules, the possible opposition of 10-nm filaments, and elastic constraints of the membrane. Disruption of the microtubules or of cytochalasin B-sensitive microfilaments upsets this balance of forces and permits random shortening and relaxation of the remaining contractile elements. Contraction initiated by elements parallel to the membrane would cause the membrane to bulge out to form the typical knob-like structure, which would return into its unstretched position when the contraction is succeeded by relaxation. The tentative model shown in Figure 15 demonstrates how this motion could occur.

The role of the cellular filaments appears to be more complex than that of the microtubules. Apparently treatment with cytochalasin B does not eliminate all activity of contractile elements associated with the cell membrane, since cytochalasin B treatment can cause the appearance of oscillating knob activity. It is possible that cytochalasin B in the concentrations used here, affects only part of the organized contractile activity associated with the cell membrane and permits the remainder to act in unbalanced fashion, which results in the knobbed oscillations. The cytochalasins may also cause a hypercontraction of the actin filaments, an action which might underlie the amorphous deposits of filamentous material in the cortical regions [39]. Other, more complex actions cannot yet be ruled out.

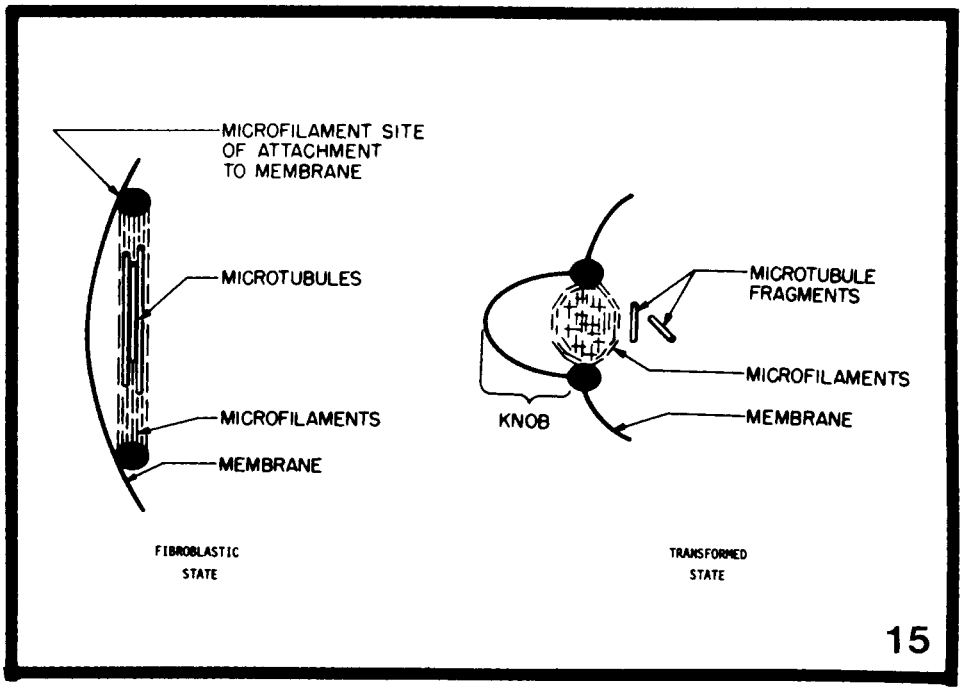


Fig. 15. A highly simplified model of how the knob activity on the cell membrane might occur. In the left diagram microfilaments are pictured as attached to specific membranous sites. The tendency of longitudinal microfilaments to contract is opposed by other forces, such as the rigidity of the associated microtubules, and possible antagonistic action of other contractile elements. In the right diagram, the balance of forces is upset by disorganization of the microtubule or of counteracting contractile 6-nm microfilaments. Random contraction and relaxation of longitudinal filaments may occur, causing the membrane to bulge at a particular point

We have pointed out how these considerations afford a natural explanation of the knob activity that is observed even in normal cells during the brief period between the end of telophase and the initiation of G1 [40]. In mitosis all or most of the cellular tubulin is mobilized into the spindle, and the cell becomes spherical. At the end of telophase, the spindle dissociates and its component tubulin moieties begin to reform the microtubules of the interphase cytoskeleton. The cellular microfilaments also play important roles in the complex events associated with mitosis [41, 42]. Until the interphase cytoskeleton structure is completed the microtubular and microfilamentous elements are partially disorganized in a state similar to that of the interphase CHO-K1 cell. Consequently knob activity is to be expected during this brief interval.

That both microtubules and microfilaments are involved in knob activity is strengthened by the newly discovered microtrabeculae [43]. This network resembles the lattice-like architecture of spongy bone and connects or joins various elements of the cytoplasm, including microfilaments and microtubules with the cell membrane. Hence, it becomes apparent that when one of the cytoskeletal elements is influenced by destabilizing agents, the remainder may also experience organizational change that will be transferred to the cell membrane. The involvement of the microtrabeculae in cell topography has not been described, but its presence in a large number of cultured cells investigated by Wolosewick and Porter [43], and its intimate relationship with the microfibrillar system, seem to make this network a candidate for a role by which knob activity is expressed.

Of the various transformation characteristics that are reversed by the action of cAMP derivatives, the removal of the knob action appears to be the one that is most rapid and that promises to lend itself readily to precise kinetic experiments.

These considerations imply that every transformed cell that displays knob activity throughout its interphase period suffers from a defective cytoskeletal structure. We have elsewhere suggested as a working hypothesis that this structure plays a role in cellular regulation of growth [1, 14] and in the evolutionary development of a malignant cell from a premalignant form [44].

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