Morphological Evidence for Cyclic AMP-Induced Reverse Transformation in Vole Cells Infected with Avian Sarcoma Virus†

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Normal fibroblasts of the vole displayed moderately spread or flattened, spindle-shaped, or polygonal morphologies and attached firmly to a substrate. Topographic features of these cells included sparse microvilli, ruffles, and filopodia. Microfilament bundles, intermediate filaments, and long microtubules generally parallel to each other, and the long axis of the cell or its extensions were present in the cytoplasm. Fibronectin was abundant, and fibronectin fibrils often formed junctions at the cell membrane with microfilament bundles. Transformation with avian sarcoma virus converted 90% of the cells to spheres 5 to 10 μm in diameter. In contrast to the normal vole cells, microfilament bundles were absent, microtubules were short and randomly arranged, and fibronectin was no longer visible. Exposure to dibutyl cyclic AMP and testololactone caused a majority of the spherical cells to stretch and flatten, a process referred to as reverse transformation. Microtubules radiated out to the cell periphery and became parallel in cell extensions, while long microfilament bundles appeared in the cytoplasm. Parallel intermediate filaments were arranged throughout the cell. This ultrastructural analysis of reverse transformation in avian sarcoma virus-transformed vole cells detailed the status of the cytoskeletal system and showed agreement with earlier findings (Puck et al., *J. Cell. Physiol.* 107:399-412, 1981) using indirect immunofluorescence.

The reverse transformation (RT) response in CHO cells includes changes in cell topography and shape (14, 16, 31, 34). Electron microscopy shows a rearrangement of microtubules and microfilaments, collectively referred to as the microtubule-microfilament or microfibrillar system (32), as being responsible for these morphological changes. During RT, microtubules increase in number and become parallel to both the long axis of the cell and 6-nm microfilaments (actin-containing). These microfilaments become oriented into thick subplasmalemmal bundles (25, 31). In addition, the blebbing or knob activity caused by a breakdown in the structural rigidity of microtubules and a disordered microfilament contraction and relaxation ceases (25). In further support of the observation that the microfibrillar system plays a key role in the shape changes, the RT response can be prevented or reversed by agents that depolymerize microtubules and mi-

crofilaments (16, 34). The RT agents responsible for such changes increase the intracellular level of cyclic AMP (cAMP) (15, 40).

A CHO $\times$ CH brain hybrid undergoes a different kind of shape change than the CHO cell when treated with dibutyl cAMP (dbcAMP) (24). This hybrid becomes stellate shaped, forming processes that are contiguous with neighboring cells and imparting a network-like appearance to cells of a colony. Such phenotypic changes are characteristic of the brain cell rather than the parent CHO cell. Cytoskeletal elements appear to play an important role in this shape change: microtubules, intermediate filaments, and microfilaments are visualized within the nerverlike processes. These events are also inhibited by the cytoskeletal disrupting drugs, colcemid and cytochalasin B. This unique response of the hybrid may be affected by nerve cell microtubule organizing centers that respond to a specific threshold level of cAMP imparting the nerve cell phenotype. Microtubules then assemble and increase in length to form the backbone of the radiating processes. Microfilaments and intermediate filaments also are impor-

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tant in forming and stabilizing the new cell shape.

The status of microtubules in cultured, transformed cells is a subject of intense interest, and contradictory reports appear (1, 5, 7, 28). Studies concerning the status of microfilaments in cultured, transformed cells are more generally in agreement. A shape change occurs in several different cell types infected with sarcoma virus (3, 8, 42, 43, 45), simian virus 40 (30, 41), or adenovirus (12) that causes these cells to become more rounded as the viral gene is expressed, an event that can be temperature sensitive. Microfilament bundles are lost in most cases (2, 8, 23, 27, 30, 41, 42).

In transformation studies, most work has been done with microtubules and microfilaments. The use of indirect immunofluorescence and antibodies to tubulin and actin allows for the easy analysis of large numbers of cells. The morphological role and chemical composition of another filamentous system distinct from microtubules and microfilaments, known as the intermediate filaments (10-nm diameter), are becoming better understood. Their role in cell transformation and regulation of cell shape is suggested by the observation that sarcoma virus transformation of a hamster cell line alters the organization of intermediate filaments (17).

It is believed that abnormalities occur in the microfibrils of transformed cells that in turn are phenotypically expressed as topographical changes, cell rounding, loss of contact inhibition of growth, changes in distribution of cell surface receptors, anchorage-independent growth, impaired active transport, and abnormal enzyme inductions (32). The sum of these changes is a cell that becomes transformed and exhibits a range of transformation stigmata. Puck postulates that changes in the microtubule-microfilament system may further affect growth regulation and chromosomal stability by direct interactions between cytoskeletal and nuclear elements (32).

The *src* gene product from cells infected with avian sarcoma virus (ASV) is a cAMP-independent protein kinase with a molecular weight of 60,000 called pp60^{src}. pp60^{src} produces morphological changes in transformed cells that are reversed by cAMP (6, 9, 35). With this information available, it seemed important to determine by what common mechanisms viral transformation, cAMP, and earlier work with the spontaneous transformant CHO were related. Attempts to infect the CHO cell with ASV were unsuccessful. In preliminary studies, the vole cell (*Microtus agrestis*) proves to be the proper model for our study because it contains the *src* gene product and its response to cAMP is similar to that of CHO cells (26).

An introductory paper describes growth characteristics, pp60^{src} activity, and tubulin and actin immunofluorescence of transformed vole cells in the absence and presence of dbcAMP (33). The present paper concentrated more on the ultrastructural changes that result from dbcAMP treatment. Additional work with antiactin indirect immunofluorescence, antifibronectin indirect immunofluorescence, and the fluorescent derivative of phalloidin (46) were discussed. The results of this study further substantiate the proposal that viral transformation with the *src* gene and spontaneous transformation of the CHO cell may have similar mechanisms and that cAMP works through the cytoskeletal system to restore normal cell morphology (33). Results were discussed with particular emphasis on the ultrastructural data, the effect of the *src* gene on microtubules and microfilaments, and the ability of dbcAMP and synergists to restore the cytoskeletal system to a status comparable with the normal cell.

MATERIALS AND METHODS

The origin and properties of the vole (*Microtus agrestis*) cells used and details of their transformation with *src*-containing ASV are described elsewhere (47). Normal and transformed cells were routinely cultured in plastic dishes in F12 medium supplemented with 5 to 8% fetal calf serum or the macromolecular fraction thereof and 100 U each of streptomycin and penicillin per ml. Cultures were incubated at 37°C in an atmosphere of high humidity and 5% CO₂ in air. For some experiments, the cells were grown on a glass substrate which was previously washed with sterile 0.1% aqueous polylysine hydrobromide (Polysciences, Inc., Warrington, Pa.). The cover slip or slide was then dried and washed three times with sterile, phosphate-buffered saline (PBS) or sterile, distilled water. These were dried overnight, before seeding of the cells.

For scanning electron microscopy (SEM), normal vole and transformed vole cells (1×10^3 to 2×10^3 cells each) were seeded into 35-mm plastic petri dishes (Lux Scientific Corp., Newbury Park, Calif.) containing carbon-coated or uncoated glass cover slips (11 by 22 mm). The cells were seeded into medium with or without 1 to 2 mM 6-*N*,2-*O*-dibutyryl adenosine 3',5'-cyclic monophosphoric acid (the monosodium salt) (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10^{-5} M testolactone (E. R. Squibb & Sons, Princeton, N. J.). After 48 to 120 h, cells were fixed for both SEM and transmission electron microscopy (TEM) in one of the following ways: (i) for enhanced microfilament bundle visualization, 2.3% glutaraldehyde in PBS with 0.2% tannic acid (11) at pH 7.4 and 37°C for 40 to 60 min; (ii) for enhanced microtubule visualization, 2.0% glutaraldehyde in PBS with microtubule stabilizers (22) at pH 7.4 and 37°C for 40 to 60 min; and (iii) as a standard or usual type of fixation, 2.5% glutaraldehyde in 0.1 M cacodylate buffer in saline G at pH 7.4 and 37°C for 40 to 60 min. Cells were next washed three times with the respective buffer and then postfixed, dehydrated, critical point dried, mounted, and observed as previously described (24).

For TEM, the same 35-mm petri dishes used in SEM were processed for TEM. The cells that grew on the dish to the side of the glass cover slip were fixed simultaneously with those cells to be observed by SEM. These cells were washed in the proper buffer and dehydrated in a graded ethanol series (5- to 10-min changes in 30, 50, 70, and 90% ethanol and three such changes in 100% ethanol), in a 1:1 Epon 812-ethanol mixture for 1 h, and in a 2:1 Epon 812-ethanol mixture overnight. This mixture was decanted, the dishes were drained for 2 h, Epon 812 was added, and the dishes were cured for 18 h at 60°C. Normal vole (10^3) and transformed vole (1.5×10^4) cells were also seeded into each chamber of four-chamber glass slides (Lab-Tek Products, Westmont, Ill.). Medium was added to each chamber, and cells were grown under proper conditions. Fixation was done by the same methods as described in SEM. Dehydration, embedment, curing, and sectioning were done as previously described (24). Sections were observed and photographed with a Jeol JEM-100C operated at 60 kV.

For visualization of actin, cells were prepared by two methods. (i) The cells were grown on glass cover slips and immediately fixed in 2.7% formaldehyde in PBS at pH 7.2 and 37°C for 10 min, washed two to three times in PBS at room temperature, treated with fluorescent phalloidin (1.5 µg/ml) in 1% dimethyl sulfoxide for 40 min, and washed two to three times in PBS, and the cover slips were mounted with PBS and glycerol. (ii) Antiactin serum was used as described previously (12, 21).

The indirect immunofluorescence technique with antibody to human cold insoluble globulin or fibronectin was detailed previously (26).

The procedure used for time lapse microcinematography was reported previously (25).

RESULTS

Normal vole fibroblast. SEM revealed the normal vole fibroblast as moderately flat to totally flat with only a few microvilli on interphase cells, while microvilli were abundant on more spherical or mitotic cells (Fig. 1). Filopodia were present along the borders of spread interphase cells and resembled long guy wires that appeared to anchor mitotic cells to the substrate. Ruffles or lamellipodia were a frequent topographic feature of normal cells (Fig. 1). Knobs or blebs were usually seen only on dividing cells.

With fluorescent phalloidin, numerous microfilament bundles were observed, usually parallel to one another and to the long axis of the normal vole cell (Fig. 2). The same results were obtained with antiactin.

TEM confirmed these results of indirect immunofluorescence and others with antitubulin (33). Microtubules were parallel to both the long axis of the cell and microfilament bundles (Fig. 3). Fibronectin was seen as a fibrillar material along the cell surface (Fig. 3 and 4). The fibronectin was very thick in the normal vole cultures with long strands extending from cell to cell and

short bundles appearing on some cells (Fig. 5). When normal cells were prepared for TEM, the fibronectin fibrils appeared as in Fig. 3 and 4. Junctions between the fibronectin and intracellular microfilament bundles at the cell surface were observed (Fig. 4). These are similar to those described for hamster and human fibroblasts by Singer (38).

Transformed vole fibroblasts. SEM of the ASV-transformed cells showed the spherical cells covered with microvilli (Fig. 6). Filopodia like those seen on mitotic normal cells radiated from the cell body. Time lapse cinematography revealed (results not shown) that a majority of the cells remained spherical throughout their life cycle. Many cells became detached from the plastic or glass substrate and floated in the medium. Cells also clumped together in large clusters apparently held together by some type of surface material (Fig. 6). Few knobs or blebs were seen on these cells. The spherical cells are represented by 80 to 96% of the population, while 2 to 12% are partially flat, 2 to 5% are flat, and 1 to 6% are spindle shaped (33).

With fluorescent phalloidin, a rim of actin was noted below the cell membrane of the spherical cells (Fig. 7). However, no microfilament cables were present in these cells, and short, disorganized microtubules were seen beneath the cell membrane and in the deeper cytoplasm (Fig. 8).

Transformed vole cells in the presence of RT agents. With an increasing concentration or exposure time of the RT agent, more cells became flattened and resembled the normal fibroblast (Fig. 9). Coating the substrate with polylysine did not affect the transformed cell shape when grown in normal medium, but the coated substrate increased the RT response. This increased response was also seen after treating the substrate with collagen and cold insoluble globulin. This latter treatment causes a much quicker RT reaction (26), suggesting that substrate adhesion played a key role in the changes. Time lapse cinematography showed that the cells reached a fully spread shape within 2 h if plated on a collagen substrate. Cells continued to multiply, round up for mitosis, and spread just after division. The ruffling that was so common to the normal cells occurred in some RT cells, but most cells had rigid, smooth borders. Time lapse cinematography revealed that the response was readily reversible; most of the cells reverted to the spherical shape when the cAMP derivatives were washed out.

Earlier results with antiactin fail to show a restoration of actin bundles in the RT vole cell (33). Current results with fluorescein-tagged phalloidin and further studies with actin antibody showed that microfilament bundles were restored and localized to filopodia, directly be-

FIG. 1. Normal vole fibroblasts in various phases of the cell cycle. These are flat or spindle shaped. A few microvilli are present on the rounder cells. Filopodia (Fil) extend from the cell edges. Ruffles or lamellipodia (arrowheads) occur along some cell borders. 30° tilt. 875 $\times$.

FIG. 2. Normal vole fibroblasts stained with fluorescent phalloidin. They show many stress fibers. Some actin bundles follow closely the curved cell membranes. 3,388 $\times$.

FIG. 3. Normal vole cell, with microtubules (arrowheads), in addition to microfilament bundles (MfB) and intermediate filaments (IF), traversing the cell and parallel to the long axis of the cell. Fibronectin (Fn) is attached to the cell surface. This cell is representative of one of the spindle-shaped cells in Fig. 1. 12,450 $\times$.

FIG. 4. High magnification of a normal vole cell showing microfilament bundles (MfB) in the cytoplasm forming a junction or fibronexus (arrowheads) with the fibronectin fibrils (Fn). The function of these junctions is not understood, but a close relationship between microfilaments and fibronectin is implied. 19,500 $\times$.

FIG. 5. Fibronectin in normal vole cells. Fibronectin is the bright fluorescent material in this culture of normal vole cells. Some long fibrils can be seen running from cell to cell (arrows), and other strands are apparent on the surface of cells. Since the cell in the upper center was in focus, other parts of the picture appear out of focus. 2,970 $\times$.

FIG. 6. SEM micrograph of the ASV-infected vole cells showing the dramatic change in shape. Spherical cells are prevalent with microvilli covering their surface. Some cells are attached to one another by surface material or cytoplasmic extensions (arrowheads). Footlike processes (arrow) link some cells to the glass substrate. 0 $^{\circ}$ tilt. 1,680 $\times$.

FIG. 7. Transformed vole cells stained with fluorescent phalloidin. They have actin deposited around the cell membrane and aggregated in some areas. Actin bundles could not be seen in these cells in any plane of focus. 2,364 $\times$.

neath the straight or smoothly curved cell membrane, over the nucleus, and parallel to the long axis of the cell (Fig. 10 and 11). The reason for the discrepancy in the two techniques might be some artifactual damage resulting from the preparation of the cells for actin antibody labeling and indirect immunofluorescence. When using the fluorescent phalloidin, it is unnecessary to permeabilize the cell with acetone or detergent since the phalloidin is such a low-molecular-weight protein, and furthermore, it has a high affinity for actin.

TEM confirmed the results seen with antitubulin indirect immunofluorescence and the present results with fluorescent phalloidin and antiactin: long microtubules ran parallel to the subplasmalemmal microfilament bundles, and intermediate filaments were parallel to the other microfibrils and prevalent in slender processes (Fig. 12). Vertical sections (perpendicular to the substrate) showed that most microtubules, intermediate filaments, and microfilaments were parallel and arranged in a single plane (not pictured). The microfilaments were closely related

to the cell membrane, in agreement with TEM pictures of horizontal sections.

Fibronectin was absent on the surface of the transformed vole cell (Fig. 13). The long strands, which extended from cell to cell in normal cultures, were also absent. However, in the RT vole cell, short fibronectin fibrils appeared along the cell periphery (Fig. 14). Earlier work also shows fibronectin deposition between contiguous cells after cAMP treatment (26).

DISCUSSION

This study presented the ultrastructural characteristics of ASV-transformed vole cells as they reverted to a shape similar to that of the normal cell following treatment with dbcAMP. This process, named reverse transformation, was accompanied by changes in the orientation of and a possible increase in the number or length of microtubules, microfilaments, and intermediate filaments. These cytoskeletal elements appeared to be responsible for the shape changes. The status of microfilament bundles and fibronectin was also analyzed in greater detail with the use of the new actin labeling treatment, fluorescent phalloidin, and antifibronectin (cold insoluble globulin).

Earlier work with CHO cells shows similar ultrastructural results (31). dbcAMP also causes different, but notable, morphological changes in a hybrid made from fusing CHO and human brain cells (24). Similar results are found in cultured human glioma cells exposed to dbcAMP (19). Microtubules and microfilaments are prevalent structures in the nervelike processes of such cells, and they are obvious in 3T3 and L929 cells subjected to dbcAMP treatment (44). Microtubules are parallel to subplasmalemmal microfilament bundles in the 3T3 and L929 cells, further suggesting that dbcAMP acts near the level of the cell membrane to induce microtubule and microfilament polymerization and organization. The rigid, straight borders of the RT vole cells were maintained by an underlying backbone of 6-nm microfilaments and long microtubules. This finding adds credibility to our model of knob formation (25) in which cAMP causes a cessation of the random microfilament contractions and relaxations by increasing bundle organization; the cell membrane is further strengthened upon polymerization of the microtubules. dbcAMP appears to induce polymerization of G-actin which is commonly observed in ruffles.

Fibronectin is lost in both the CHO (26) and the transformed vole cell, and its reappearance is dependent on at least one agent, dbcAMP. The short, linear arrangements of fibronectin on the RT vole cell surface resembled short actin bundles. This orientation of fibronectin was pos-

FIG. 8. TEM micrograph of a transformed vole cell in which short, random microtubules (arrowheads) are present below the cell membrane and within the deeper cytoplasm. Microtubules are seen in both cross- and longitudinal-section profiles indicative of their random arrangement. 25,600 $\times$.

sibly due to the return of subplasmalemmal microfilament bundles since it is believed that fibronectin is linked to actin filaments by a transmembrane protein (18, 39). This is also supported by studies in which the CHO is treated with a RT agent to induce fibronectin deposition and then treated with cytochalasin B. The result is a complete removal of the fibronectin (26).

In further TEM studies of the cell surface protein fibronectin and microfilament bundles, a junctional complex was observed between these two elements at the cell membrane. The junction of an intracellular microfilament bundle and extracellular fibronectin fibrils is referred to as a fibronexus (38). This junction is described as an electron-dense zone located just under the plasma membrane at the insertion point of stress fibers. Electron microscopic immunolabeling experiments to insure that the bundles were indeed actin microfilaments within the cell or that the bundles outside the cell were fibronectin were not performed, since their distinction was clear enough from the study of low-magnifica-

tion normal vole cell micrographs. This same type of morphological relationship has also been observed in human skin fibroblasts. The function of the fibronexus was not determined although it has been hypothesized that such areas could be nucleation sites for actin bundle formation (29). Further study of this junctional complex and the significance of its absence in the transformed vole cell is necessary. The fibronexus was not observed in the reverse-transformed vole cells. Its return might become evident in cultures treated for longer periods with RT agents.

The *src* gene product may have induced changes at or near the cell membrane which either broke down microfilament bundles or in some way disturbed the assembly mechanism of bundle formation. The *src* gene product (pp60^{src}) of the Schmidt-Ruppin strain of ASV is localized at the inner surface of the plasma membrane (43). Using the same technique of electron microscopy immunocytochemistry, the intracellular location of the *src* protein (p21) in cells transformed by the Harvey strain of murine

FIG. 9. SEM picture of transformed vole cells treated with dbcAMP and testololactone. It shows that their shape and surface features are similar to the normal cells seen in Fig. 1. The cells have sparse, short microvilli and no ruffling edges. Filopodia (Fil) extend from some cells. The large cell at left center is so flat that some organelles can be seen. 30° tilt. 1,400 $\times$.

FIG. 10. Long, parallel microfilament bundles stained with fluorescent phalloidin in this well-spread RT vole cell. 2,285 $\times$.

FIG. 11. Two RT vole cells labeled with actin antibody for indirect immunofluorescence. Actin is localized in the filopodia, long cytoplasmic extensions, and some fluorescence is seen directly beneath the cell membrane in the cell on the left. 2,364 $\times$.

FIG. 12. Another transformed vole cell after treatment with dbcAMP and testololactone showing the typical arrangement of subplasmalemmal microfilament bundles (MfB) and generally parallel microtubules (arrowheads). Some intermediate filaments (IF) in a parallel arrangement are also seen. 19,500 $\times$.

FIG. 13. Transformed vole cells stained with antifibronectin. The lack of fibronectin is apparent in this group of transformed vole cells stained with antifibronectin. Compare with Fig. 5 to note the total absence of fibronectin. 1,350 $\times$.

FIG. 14. Short strands of fibronectin appearing along the cell border of this RT vole cell. The technique used for visualizing the fibronectin was the same as that used for Fig. 5 and 13. 2,362 $\times$.

sarcoma virus is also at the inner surface of the plasma membrane (45).

It was also likely that the *src* gene product could phosphorylate one of the many accessory proteins associated with actin, such as α -actinin, myosin, tropomyosin, troponin, calmodulin, filamin, villin, or vinculin. Villin, isolated from the microvillus core of intestinal epithelial cells, cross-links with F-actin and functions as an actin-bundling or gelation factor (4). It was unlikely that this occurred in vole cells since villin is not widely distributed in nonmuscle cells and is not detectable in tissue culture cells by immunofluorescence or by the immune replica method. However, α -actinin does have actin-bundling capabilities, and its presence in tissue culture cells is clear (21). Another cytoskeletal protein called vinculin (molecular weight, 130,000) is found in intracellular plaques at substrate attachment sites near the bottom of various chicken embryo cells, in some points of cell-cell contact, and at the termini of stress fibers (10). If the relationship between an actin-binding protein like α -actinin or vinculin and microfilament bundles were disturbed, the attachment site and structural rigidity of the cell would be affected and the cell would become detached or assume a rounded morphology. In support of this, the *src* gene product of the Rous sarcoma virus is present at adhesion plaques or sites of cell substrate attachment in transformed rat kidney cells (36). Once bundle formation or stress fiber attachment sites are affected, it is possible that fibronectin release from the cell surface could occur by a simultaneous effect on proteins that cross-link actin bundles and fibronectin.

Since microfilament-disrupting agents such as cytochalasin B caused cells to round up and arborize, resembling the virally transformed cell, the microfilament network, whether disturbed at its attachment to the cell membrane or along its length, seems to be directly or indirectly a target site for the viral gene product. Microtubule-disrupting drugs such as colcemid do not cause the arborization in fibroblasts as seen with cytochalasin B, but surface changes are noticeable (25). Therefore, microtubules would be secondarily involved in the morphological changes. However, evidence that microtubules are affected is presented by Sen and Todaro (37) who isolated a low-molecular-weight protein phosphokinase from murine sarcoma virus virions which binds to both microtubules and microfilaments and inhibits the *in vitro* polymerization of microtubules.

dbcAMP acted to undo the transformative effects of the viral gene product in the vole fibroblast. Since pp60^{src} uniquely phosphorylates tyrosine residues while cAMP phosphory-

lates only threonine and serine, the mechanism by which transformation is accomplished could be through phosphorylation of tyrosine residues (20) on either tubulin and its associated proteins, actin and its associated proteins, or intermediate filament proteins. All three of these cytoskeletal proteins in the filamentous form have a role in the spreading of fibroblasts and muscle cells.

This morphological study served to support the conclusion that a common mechanism is involved in the spontaneous transformation of CHO cells and the viral transformation of vole fibroblasts and that this mechanism could be overridden by dbcAMP. It would prove interesting to determine if the same mechanism exists in chemically transformed cells, since morphological changes do occur in such cells with loss of microfilament bundles and reduction in microtubule number (13).

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