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Altered distributions of the cytoskeletal proteins vinculin and α-actinin in cultured fibroblasts transformed by Rous sarcoma virus

(cell adhesion/immunofluorescence/interference reflection microscopy/oncogenic viruses)

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ABSTRACT It was recently shown by combined immunofluorescence and interference reflection microscopy that a protein named vinculin, along with α-actinin, is concentrated at focal adhesion plaques inside cultured normal fibroblasts [Geiger, B. (1979) *Cell* 18, 193–205]. These plaques are the discrete, isolated sites of strong adhesions formed between the ventral surfaces of the cells and the substrata on which they are grown. We show that after transformation of fibroblasts by Rous sarcoma virus a majority of the cells have many fewer focal adhesion plaques and now exhibit a cluster of small patches that are immunolabeled for both vinculin and α-actinin. Such a cluster (rosette) is located near the ventral surface of the cell, usually partly under the nucleus. The significance that these altered distributions of vinculin and α-actinin may have for the rounding up and loss of adherence of transformed cells is discussed.

Viral transformation of cultured fibroblasts is often associated with a rounding-up of the originally well-spread cells and a concomitant decrease in their tendency to form strong cell–cell and cell–substratum adhesions (for review, see ref. 1). Normal cultured fibroblasts are thought to adhere to the substratum at two types of specialized sites, focal adhesions and close contacts (2). Focal adhesions are small, discrete areas of intimate approach (10–15 nm) of the cell surface to the substratum and are probably the sites of strong cell–substratum adhesion (3, 4). Inside the cell, the focal adhesion plaques are the sites at which bundles of microfilaments appear to terminate (1, 5). Close contacts are broader areas with a larger separation (≈30 nm) of cell surface and substratum and appear also to be implicated in cell adhesiveness (5). The two types of sites can be distinguished in light microscopy of cell cultures by suitable interference reflection optics (2, 6); focal adhesions appear as black images, whereas close contacts appear gray. Upon transformation of fibroblasts by oncogenic viruses, the intracellular bundles of microfilaments are disrupted and the filaments become disorganized (1, 7–12), but what happens to the focal adhesion plaques and close contacts is not clear (1, 13).

It has recently been shown (14) that a protein of molecular weight 130,000 named vinculin, isolated from chicken gizzard smooth muscle, is in part concentrated at the sites of focal adhesions on the cytoplasmic side of the surface membrane of cultured normal chicken cells. This has been demonstrated by immunofluorescence labeling for vinculin combined with interference reflection microscopy and has been confirmed (15). The protein α-actinin is also found at these focal adhesion plaques (14, 16). In view of the morphological and structural changes that are observed with transformed fibroblasts, it was therefore of interest to examine whether changes occurred in the distributions of vinculin and α-actinin upon transformation. To this end, the initial system chosen for study was a temperature-sensitive mutant (LA23) of the Rous sarcoma virus (RSV) infecting a normal rat kidney (NRK) cell line (9, 17, 18). These infected cells exhibit the transformed phenotype when grown at the permissive temperature (33°C) and the normal phenotype at the nonpermissive temperature (39°C). At neither temperature do these infected cells produce infectious RSV. LA23-NRK cells grown at the two temperatures therefore allow a fairly direct comparison to be made between normal and transformed phenotypes. In addition, uninfected NRK cells were used to examine the normal phenotype, and NRK cells infected with the wild-type RSV, B77, were studied to provide another example of the transformed phenotype. These experiments have revealed that vinculin and α-actinin are distributed at the ventral surfaces of NRK cells in ways that are strikingly different for the normal and transformed phenotypes, closely correlated with structural changes in the cell–substratum adhesion sites.

MATERIALS AND METHODS

Normal and Virus-Infected Cell Cultures. For these experiments, stocks of uninfected NRK cells, LA23-infected NRK cells, and wild-type B77-infected NRK cells were the gifts of Peter K. Vogt. The cells were cultured on 22-mm^2 coverslips in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum and antibiotics under an atmosphere of 10% CO_2/90% air for at least 2 days at the indicated temperatures before immunolabeling experiments (19). At that time, the cell density was of the order of $2–5 \times 10^3$ cells per cm^2.

Immunochemical Reagents. Primary antibodies raised in guinea pigs to chicken gizzard vinculin and in rabbits to chicken gizzard α-actinin were used in these experiments because they gave sufficient crossreaction with the respective rat cell antigens for our purposes. For indirect immunofluorescent labeling, secondary goat antibodies to guinea pig IgG and to rabbit IgG were used; they were purified first on affinity columns of the homologous IgG and were then crossabsorbed on columns of the heterologous IgG (14). The goat antibodies to rabbit IgG were conjugated with fluorescein isothiocyanate; the antibodies to guinea pig IgG were conjugated with lissamine rhodamine β-sulfonylchloride. The methods and reagents used have been described (14).

Immunofluorescence Studies. Cells were first fixed for 20 min with 3% formaldehyde in phosphate-buffered saline (pH 7.6) containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The cells were permeabilized by treatment with 0.1% Triton X-100 for 4 min at room temperature, and double immunofluorescent labeling was carried out (14). Control experiments, substituting normal

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Abbreviations: RSV, Rous sarcoma virus; NRK, normal rat kidney.

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guinea pig IgG or rabbit IgG for the respective primary antibodies in the immunolabeling experiments, demonstrated that the labeling was specific for the appropriate antigen (not shown; see ref. 14).

Microscopy. Fluorescent microscopy was performed with a Zeiss photoscope III equipped with filter settings for fluorescein and rhodamine. The same microscope was also modified for recording interference reflection images according to Curtis (6). The lamp field was reduced in order to improve the image contrast, and an interference green filter ($\lambda = 546$ nm) and a polarizer were inserted into the optical path of the epifluorescence condenser RSIII. The observations were made through a ×63 oil objective. Photographs were taken on Kodak Tri-X film and developed in Diafine.

RESULTS

LA23-Infected NRK Cells Grown at the Nonpermissive Temperature (39°C). At the nonpermissive temperature, LA23-NRK cells examined by interference reflection microscopy (Fig. 1*C*) or by double immunofluorescence labeling for vinculin (Fig. 1*A*) and α-actinin (Fig. 1*B*) appear similar to normal uninfected fibroblasts (ref. 14, and Fig. 2 *A–F*). Part of the labeling for vinculin was diffusely distributed throughout the cytoplasm, but part was characteristically concentrated inside the cell at sites that largely coincided with the elongated focal adhesion plaques that were recognized by their black appearance in interference reflection (arrows, Fig. 1 *A* and *C*). Some of these elongated patches were at the periphery of the cell, but most were distributed over the entire inside ventral surface. Immunolabeling for α-actinin inside the same cells was in part superimposed on the same focal adhesion plaques (arrow, Fig. 1*B*) but was also seen in the punctate linear arrays known to be associated with stress fibers in normal fibroblasts (16). Gray areas in the interference reflection images, signifying regions of close, but not focal, contact between the ventral cell surface and substratum, did not appear to be immunolabeled for vinculin or α-actinin. This was especially apparent in occasional isolated gray areas (arrowhead in Fig. 1*C* and corresponding areas in Fig. 1 *A* and *B*).

LA23-Infected NRK Cells Grown at the Permissive Temperature (33°C). These cells showed a range of morphological and immunolabeling characteristics, but all of them were clearly distinguishable from the normal phenotype exhibited by the cells grown at 39°C. Cells grown at 33°C were less well spread than those at 39°C. In about 20–30% of the cells grown at 33°C, elongated focal adhesions were seen by interference reflection to be present at the cell periphery but, in contrast to the normal phenotype, there were only a few elsewhere on the ventral surface of the cell. These peripheral focal adhesions were the primary sites of concentrated vinculin and α-actinin immunolabeling (not shown).

The majority (70–80%) of the LA23-NRK cells grown at 33°C, however, were distinguishable from the minority just discussed. They exhibited only a few focal adhesion sites, which were mostly at the cell periphery (Fig. 1*F*). These few sites were immunolabeled for vinculin or α-actinin. Another portion of the vinculin and α-actinin was diffusely labeled in the cytoplasm, with much less of the punctate linear distribution of α-actinin than was seen at 39°C (Fig. 1*B*). The most remarkable feature of these cells, however, was an intracellular cluster of

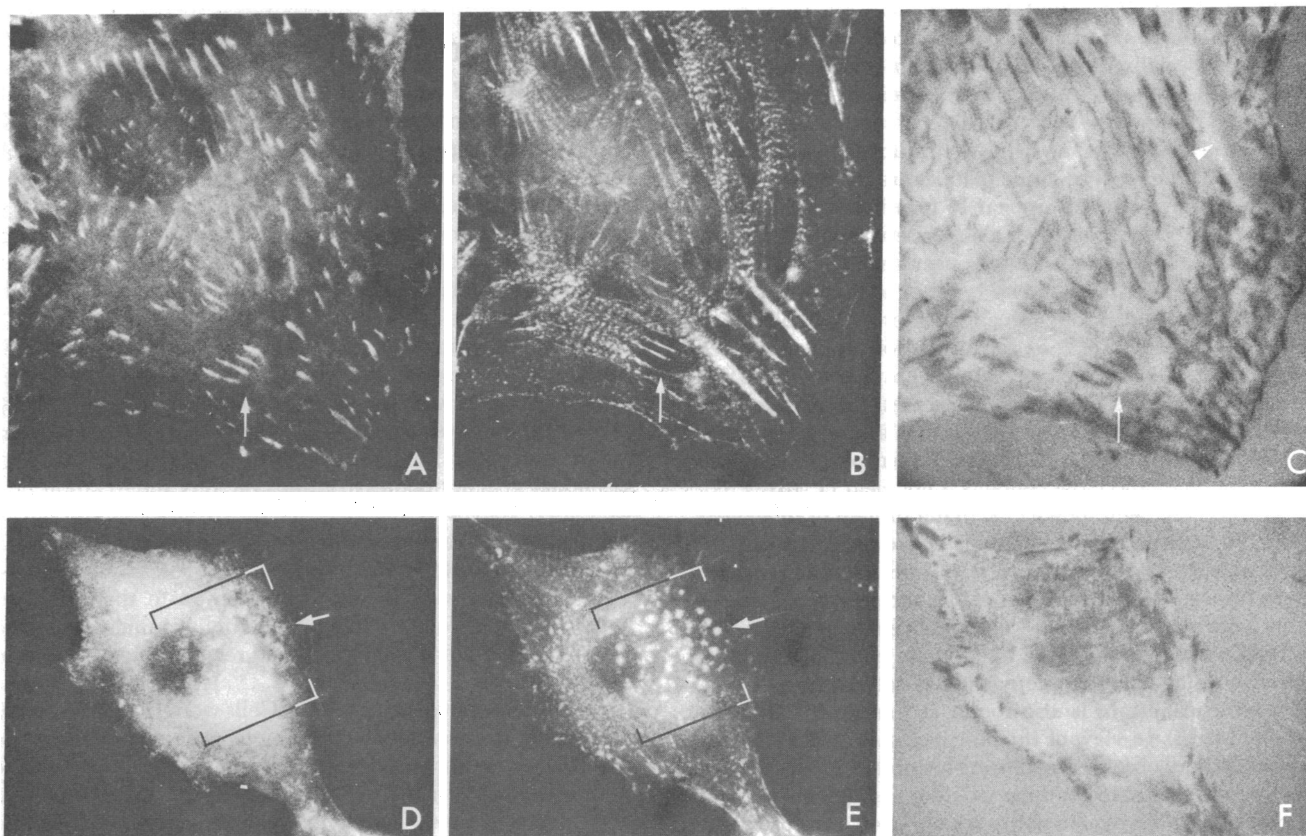

FIG. 1. LA23-infected NRK cells grown at the nonpermissive temperature (39°C) (*A–C*) and at the permissive temperature (33°C) (*D–F*). The cells were doubly immunofluorescent labeled for vinculin (*A* and *D*) and for α-actinin (*B* and *E*) and observed by interference reflection microscopy (*C* and *F*). Arrows in *A–C* correspond to the same focal adhesions. Brackets in *D* and *E* demarcate the area of the rosette of immunolabeled patches, and the arrows in *D* and *E* point out a single circular patch of vinculin and α-actinin labeling, respectively. Note the more peripheral staining for vinculin and the more central staining for α-actinin on this patch. (× 650.)

small round patches of immunolabeling for vinculin (Fig. 1*D*, brackets), and even more clearly, for α-actinin (Fig. 1*E*, brackets). It often appeared that the vinculin labeling of a single round patch was concentrated at the circumference of the patch (Fig. 1*D*, arrow) and the α-actinin labeling was more centrally located (Fig. 1*E*, arrow). Such a cluster of patches (which we refer to as a rosette) was observed only when the microscope was focused at the cell–substratum interface. In this focal plane, the area covered by a rosette corresponded to a gray mottled area in the interference reflection image of the same cell (Fig. 1*F*), the grayer portions closely coinciding with the immunolabeled patches. The total area covered by a rosette varied in location and in compactness in different cells in the population, but the total area of the gray mottled image in the interference reflection picure always corresponded with the area of the rosette (see also Fig. 2 *G–L*). These areas often lay partially beneath the cell nucleus.

Similar gray areas in the interference reflection image were also seen with these cells in the absence of any immunolabeling for vinculin or α-actinin (not shown) and, therefore, cannot be attributed to any optical effects due to the binding of the immunolabeling reagents themselves.

Such immunolabeled rosettes were never observed in LA23-NRK cells grown for longer than 48 hr at the nonpermissive temperature or in uninfected NRK cells (see below).

Uninfected NRK Cells. Normal cells were grown at both 39°C and 33°C to determine whether the growth temperature caused any large change in the normal phenotype that might be responsible for the gross differences seen with LA23-NRK cells grown at these two temperatures. Although there appeared to be some subtle differences between populations of NRK cells grown at the two temperatures, there was a strong overall similarity in the interference reflection patterns (Fig. 2 *C* and *F*) and also in the distribution of immunolabeling for vinculin (Fig. 2 *A* and *D*) and α-actinin (Fig. 2 *B* and *E*). In particular, NRK cells grown at 33°C never showed any structures resembling the clusters of circular patches (rosettes) of vinculin and α-actinin immunolabeling seen in the large majority of LA23-NRK cells grown at that temperature.

B77-Infected NRK Cells. NRK cells infected with a wild-type strain of RSV, B77, were grown at 39°C and 33°C. The objects of these experiments were to determine whether the results of immunolabeling and interference reflection obtained with LA23-NRK cells at 33°C were also observed with cells infected with wild-type virus and whether the growth temperature had any gross effects on these results. We found that over 95% of the B77-infected cells, whether grown at 33°C or 39°C, gave immunolabeling patterns for vinculin (Fig. 2 *G* and *J*) and α-actinin (Fig. 2 *H* and *K*), as well as interference reflection images (Fig. 2 *I* and *L*), that were closely similar to their counterparts obtained with the major fraction (70–80%) of LA23-NRK cells grown at 33°C. The most distinctive features of the immunolabeling patterns were the appearance at the ventral surface of the cell of rosettes of patches that were labeled both for vinculin and α-actinin and the marked reduction of labeled patches that corresponded to the focal adhesions characteristic of the normal phenotype. The area occupied by the rosette varied from cell to cell at both temperatures: an example of a compact rosette is shown in Fig. 2 *J–L* and an example of a more dispersed one is shown in Fig. 2 *G–I*. In the interference reflection images (Fig. 2 *I* and *L*) only a few black elongated patches were seen at the ventral surface; the main feature was a gray mottled area corresponding to the total area occupied by the rosette in that cell. The growth temperature had only subtle effects on the patterns observed with the B77-NRK cells. In particular, the patterns of B77-NRK cells grown at 39°C were totally different from those of LA23-NRK or uninfected NRK cells grown at the same temperature.

DISCUSSION

In view of the characteristically different adhesive properties of normal and transformed cells (1), it is probable that important insights into the mechanisms of cell transformation will come from a knowledge of the molecular ultrastructure of the focal adhesion plaques of the normal fibroblast and of the changes that are produced in this ultrastructure upon transformation. Vinculin (14) and α-actinin (14, 16) have previously been localized by immunofluorescence microscopy at the cytoplasmic surfaces of focal adhesion plaques inside cultured normal fibroblasts. In this paper, we show that, in conjunction with a decrease or disappearance of the focal adhesion plaques of NRK cells upon transformation by RSV, striking changes are observed in the distribution of immunolabeling for vinculin and α-actinin inside these cells. Parallel changes have been observed with NRK cells infected with a temperature-sensitive mutant of RSV when cells grown at the permissive or nonpermissive temperature were compared or uninfected NRK cells compared to those infected with a wild-type RSV grown at the same temperature. In the majority of cells exhibiting the transformed phenotype, vinculin and α-actinin were in part associated with the greatly decreased number of focal adhesion plaques that were situated at the cell periphery. However, in larger part they were associated with a cluster of small round patches (a rosette) that was localized near the inner ventral surface of the cells. The rosette was often located partially underneath the nucleus of the cell. The individual immunolabeled patches in the cluster appeared to coincide with local gray areas in the interference reflection images, suggesting that these were sites of close (but not focal) contact of the cell surface with the substratum (2, 20). The area covered by the entire cluster corresponded to a single, broad, gray mottled area where the cell surface was engaged in a cluster of such close contacts.

To offer an interpretation of these results, we first summarize some of the properties of vinculin. It has been proposed that vinculin is a structural protein that is generally involved in one type of linkage of the termini of microfilament bundles to normal cell membranes (14, 19). This suggestion is consistent with the results of immunofluorescence experiments with normal cultured fibroblasts (14), which show that vinculin is concentrated at the focal adhesion plaques formed between the cell surface and substratum, and is strongly supported by immunoelectron microscopic studies (19), which have shown that vinculin is specifically and closely apposed to several kinds of membrane sites at which microfilament bundles terminate—namely, the membrane-associated dense plaques in smooth muscle cells, the zonula adherens in the junctional complex in intestinal brush border, and the fascia adherens of the intercalated disk membrane of cardiac muscle cells. α-Actinin is also associated with all of these sites, except perhaps the intercalated disk membrane. By double immunoelectron microscopic labeling experiments (21), however, it was shown that vinculin is situated closer than is α-actinin to the membrane at these sites (unpublished results). For normal cultured fibroblasts, it has therefore been suggested that the vinculin that is immunolabeled at the sites of focal adhesion plaques may participate more directly than α-actinin in the intracellular linkage of the termini of bundles of microfilaments to these membrane sites.

The transformation of cells by RSV has been shown to require only a single gene (the *src* gene) in the viral genome, which apparently codes for the production of a single protein, designated $pp60^{src}$ (22, 23). This protein is a protein kinase, an enzyme that can use ATP to phosphorylate tyrosine residues (24)

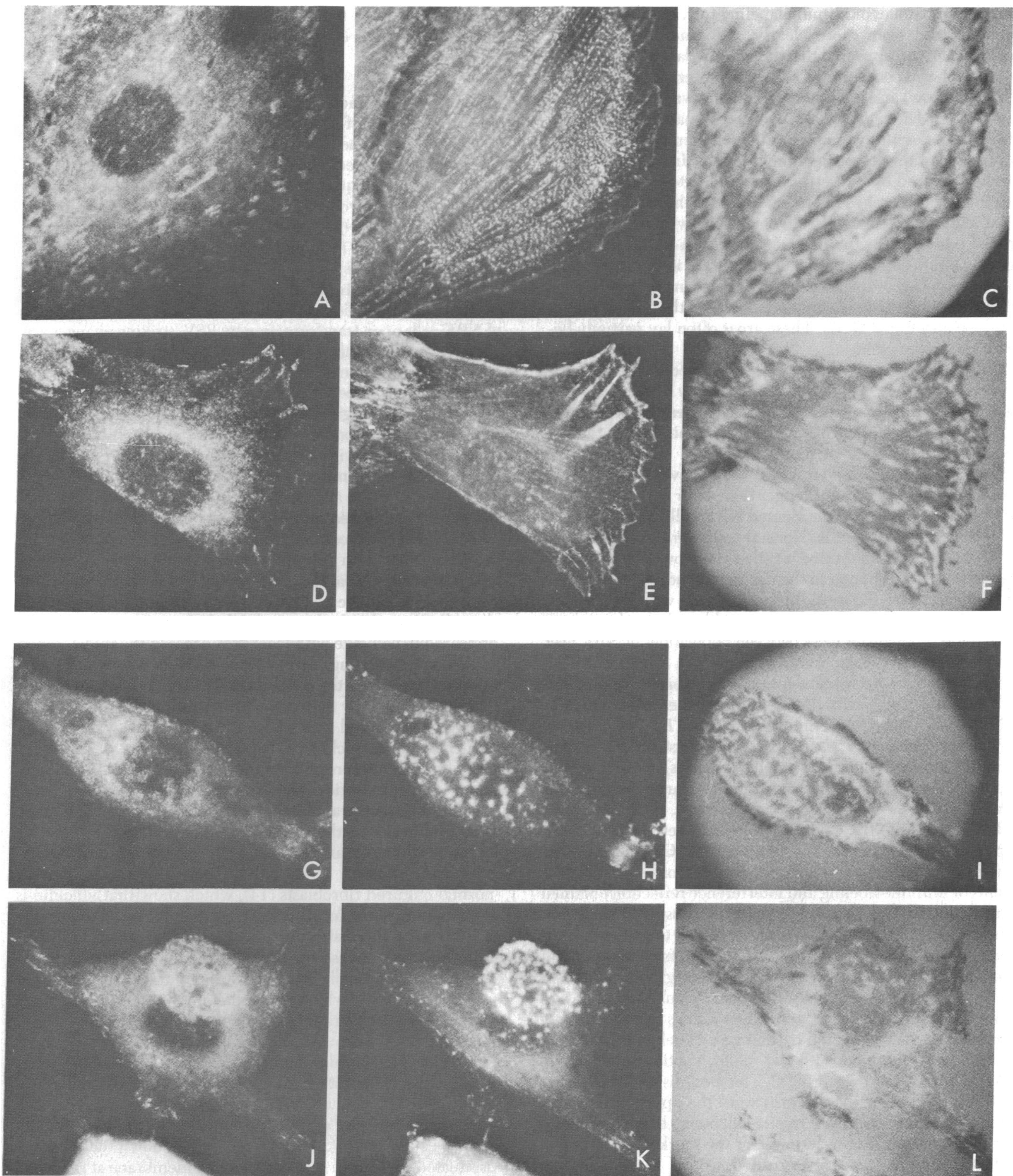

FIG. 2. NRK cells (*A–F*) and B77-infected cells (*G–L*). Cells shown in *A–C* and *G–I* were grown at 39°C; those shown in *D–F* and *J–L* were grown at 33°C. Each cell was stained simultaneously for vinculin (*A, D, G,* and *J*) and α-actinin (*B, E, H,* and *K*) and observed by interference reflection microscopy (*C, F, I,* and *L*). (× 650.)

on those protein molecules that are substrates for the enzyme. In the RSV-transformed cell, the level of phosphotyrosine is elevated about 10-fold over the level in the uninfected cell (24) but still accounts for only a small fraction of the total amount of phosphorylated protein residues (mostly phosphoserine and phosphothreonine) in the cell. It is therefore likely that there is only a limited number of protein substrates for the action of $pp60^{src}$ and that the phosphorylation of these proteins is responsible for a cascade of reactions that culminates in the transformation of the cell. It is therefore of considerable interest that it has recently been shown that vinculin is a specific substrate for $pp60^{src}$ inside RSV-transformed fibroblasts (B. M.

Sefton, E. H. Ball, T. Hunter, and S. J. Singer, unpublished results), its specific phosphotyrosine content increasing about 8-fold upon transformation. α-Actinin, however, is not phosphorylated under the same conditions. Such modification of vinculin may directly or indirectly alter its function and thereby affect the association of the microfilament bundles with the focal adhesion plaques. Such an effect could in turn play an important role in the disorganization of the microfilament bundles (9, 10), in the decrease in cell–substratum adhesion (25), and in the rounding-up of the cell that accompanies transformation of fibroblasts by RSV. It is, of course, possible that the modification by pp60^{src} action of other proteins in addition to vinculin is required to produce all of these changes.

Several reports have appeared concerning the immunolabeling of the pp60^{src} protein inside RSV-transformed cells (26–28) but they have not yielded a consistent pattern of localization of that protein. More recently, however, Rohrschneider (13) has presented evidence that pp60^{src} was in part concentrated at focal adhesion plaques inside RSV-transformed NRK cells. No description of rosettes was presented in any of these studies. It is not clear whether there was an absence of rosettes in these cells, the rosettes were present but were not immunolabeled for pp60^{src} and, hence, were not visible, or the rosettes were present and immunolabeled but went unrecognized against the diffuse background labeling. The facts that vinculin is a specific substrate *in vivo* for pp60^{src} and that pp60^{src} is concentrated at the focal adhesion plaques suggest the possibility that the vinculin molecules located at the focal adhesion plaques provide the sites of attachment of the pp60^{src} molecules.

Finally, we consider the nature and significance of the rosettes detected by immunolabeling of vinculin and α-actinin inside the majority of RSV-transformed NRK cells. They do not appear to be simply the detritus formed as a result of the disruption of microfilament bundles and of focal adhesion plaques in the rounded-up transformed cells. We have never observed such rosettes in normal fibroblasts that have been rounded-up during mitosis or shortly after replating, conditions which also result in the loss of the microfilament bundles and focal adhesions that are present in well-spread interphase cells. The individual patches in the rosettes appear to be associated with sites of close contact with the substratum at the ventral surface of the cell. This suggests a possible role for these structures in the residual adhesion of RSV-transformed NRK cells to their substrata. In other words, the redistributions of vinculin and α-actinin that we have observed upon transformation by RSV may directly reflect the intracellular molecular events that change the strongly adhesive focal contacts of the normal fibroblasts to the more weakly adhesive close contacts of the transformed cell.

It is possible that the individual patches seen in projection by immunofluorescence may be associated with, or correspond to, microvilli emanating from the ventral surface of the transformed cell and making close contact with the substratum. Microvilli have been observed by electron microscopy to be present at the ventral surfaces of transformed but not of normal fibroblasts (1). On the other hand, the patches appear to be too large, too circular in profile, and too regularly disposed to be attributed to such microvilli. Further work is required to determine if the rosettes represent defined ultrastructural entities that are observable in the electron microscope and if they are also present in fibroblasts transformed by a variety of different agents (29).

In dense or confluent cultures of normal fibroblasts, vinculin is found associated not only with focal adhesion plaques at the cell–substratum interface but also in patch-like distributions at regions of cell–cell contact (14). Upon transformation with RSV, these immunofluorescent-labeled patches at cell–cell contacts also undergo marked changes in appearance in the majority of the transformed cells, either becoming more fuzzy and rounded in outline or largely disappearing altogether (unpublished results). It is therefore likely that alterations of cell–cell contact regions upon transformation are correlated with the alterations in the cell–substratum contact regions that are discussed in this paper.

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