A New Protein of Adhesion Plaques and Ruffling Membranes

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ABSTRACT A protein with a molecular weight on SDS polyacrylamide gels of 215,000 (referred to here as 215K) was purified from chicken gizzard smooth muscle. Antibodies against this protein localized it in fibroblasts to adhesion plaques (focal contacts), to regions underlying cell surface fibronectin, and to ruffling membranes. In the first two distributions it was similar to vinculin in cellular location, and this was confirmed by double-label immunofluorescence microscopy, but the concentration of 215K in membrane ruffles distinguished it from vinculin. There was no cross-reaction of the antibody against 215K with vinculin, and immunoprecipitation and antibody staining of SDS gels of whole cells revealed a single cross-reactive component with a molecular weight of 215,000. Immunoprecipitation from cultures labeled with [^{32}P]phosphate revealed 215K to be a phosphoprotein. Transformation of rat or chicken fibroblasts by Rous sarcoma virus resulted in a reorganization of 215K, in some cases into complex intracellular structures. The localization of 215K where microfilament bundles terminate as well as in close relation to cell surface fibronectin and in membrane ruffles suggests that the protein has some function in the organization of actin filaments at or close to regions of actin-membrane attachment.

Actin microfilament bundles (stress fibers) of cells such as cultured fibroblasts terminate at specialized regions of the plasma membrane where the cells adhere to the underlying substrate. The molecular details of these regions, known as adhesion plaques or focal contacts, are of considerable interest. Several cytoskeletal proteins have been identified in these regions where microfilament bundles terminate, including α-actinin (21), vinculin (6, 14), and fimbrin (3). The functions of these proteins either in the attachment of microfilaments to the membrane or in their organization have not been established. The properties of α-actinin and fimbrin in vitro (2, 7, 8, 15) suggest that these are probably involved in the "bundling" or packing of the actin filaments. Vinculin may also be involved in bundling (18) but it has also been shown to bind to the ends of actin filaments (8, 26), which could indicate an attachment role for this protein. Electron microscopic studies are consistent with this possibility, in that vinculin has been demonstrated to be closer to the plasma membrane than α-actinin in a number of locations (9, 25). An additional protein that has been localized in the adhesion plaque is pp60^{src}, the protein kinase coded for by the transforming gene of Rous sarcoma virus (RSV)[1] (22). This location of pp60^{src} could be responsible for the disruption of the actin cytoskeleton, which is a prominent feature of RSV-transformed cells.

In this paper we describe a new protein found in adhesion plaques, at the ends of microfilament bundles. This protein was found to have a molecular weight of 215,000 on SDS polyacrylamide gels; we refer to it here as 215K. Like vinculin it was localized in close relation to cell surface fibronectin. It differed from vinculin, however, in also being prominent in membrane ruffles. These cellular locations suggest that this protein may have a role either in the organization of actin filaments or in their attachment to membranes in specialized regions. A preliminary account of this work was recently presented (10).

MATERIALS AND METHODS

Purification of the 215K Protein: Frozen chicken gizzard smooth muscle was dissected from connective tissue, weighed, and blended in 7 vol of deionized water at 4°C, containing 0.5 mM phenylmethylsulfonyl fluoride. The muscle was blended at a high-speed setting in a Waring blender three times, each for 10 s. The homogenate was centrifuged for 10 min at 7,000 rpm in a Sorvall GSA rotor (E. I. Dupont de Nemours + Co., Inc., Sorvall Instruments Div., Newtown, CT). The supernatant was discarded and the pellet resuspended in 7 vol of cold deionized water containing 0.5 mM phenylmethylsulfonyl fluoride. The centrifugation was repeated at 10,000 rpm for 10 min, and again the supernatant was discarded. The pellet was resuspended in buffer A (2 mM

[1] *Abbreviations used in this paper:* NRK, normal rat kidney; and RSV, Rous sarcoma virus.

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Tris, 1 mM EGTA, and 0.5 mM phenylmethylsulfonyl fluoride, pH 9.0 at room temperature) and extracted for 30 min at 37°C with occasional stirring. The suspension was centrifuged at 12,000 rpm for 10 min. The supernatant was adjusted to pH 7.0 with acetic acid and then brought to a concentration of 13 mM $MgCl_2$. The suspension was stirred at room temperature for 15 min before centrifugation at 10,000 rpm for 10 min. The resulting supernatant was fractionated with solid ammonium sulfate at 4°C. Initially, 21.25 g of ammonium sulfate was added per 100 ml and stirred for at least 1 h. The pellet was collected by centrifugation and contained vinculin, α-actinin, and filamin. An additional 5.6 g of ammonium sulfate/100 ml was added to the supernatant. After being stirred for at least 1 h, the precipitated protein was collected by centrifugation. The resulting pellet, rich in the 215K protein, was dissolved in and dialyzed against buffer B (20 mM NaCl, 20 mM Tris-acetate, 0.1 mM EDTA, and 15 mM β-mercaptoethanol, pH 7.6). The dialyzed solution of protein was chromatographed on a 7.5 × 2 cm phosphocellulose column (Cellex-P, Bio-Rad Laboratories, Richmond, CA) equilibrated in buffer B. While initially loading the sample, a rapid flow rate (900 ml/h) was used, which prevented many of the contaminants from binding. The column was eluted with a linear gradient of buffer B containing 100 mM NaCl to buffer B containing 300 mM NaCl, with a flow rate of ~30 ml/h. Fractions were analyzed on SDS polyacrylamide gels. Fractions rich in the 215K protein were pooled, dialyzed against buffer B, and chromatographed on a column (5 × 2 cm) of DEAE-cellulose (Whatman DE52, Whatman Chemical Separation Inc., Clifton, NJ) equilibrated in buffer B, and eluted with a linear gradient of buffer B to buffer B plus 300 mM NaCl. Again, fractions were analyzed by SDS polyacrylamide gels and the fractions rich in 215K were pooled. These were concentrated by dialysis against 70% sucrose in buffer B, and then dialyzed against buffer B before loading on a column (90 × 1.2 cm) of Sepharose C1-4B (Pharmacia Fine Chemicals Div., Pharmacia Inc., Piscataway, NJ) equilibrated in buffer B. The fractions were analyzed by SDS polyacrylamide gels. Those fractions free from contaminating proteins were pooled and used for further work with the 215K protein.

Cell Cultures: Chick embryo fibroblasts from the skins of 11-d-old embryos were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The corresponding cells transformed by RSV were grown in the same medium as were RSV-transformed rat fibroblasts (RR1022). Transformed cells were a gift of Dr. P. Maness (University of North Carolina). Normal rat kidney cells (NRK) and gerbil fibroma cells (IMR-33) were cultured in the same medium but supplemented with 2% fetal calf serum. For labeling with [^{35}S]methionine, the cultures were grown in the same medium but with the methionine concentration reduced approximately fivefold. The medium of a 60-mm petri dish was supplemented with 50–100 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL). For labeling with [^{32}P]phosphate, the cells were cultured in the same medium with the phosphate concentration decreased about fivefold. The medium of a 60-mm dish was supplemented with ~2.5 mCi of [^{32}P]phosphate (New England Nuclear, Boston, MA).

Antibody Preparation: Antisera against the native 215K protein were made in New Zealand white rabbits by multiple subcutaneous injections of the protein (~0.2 mg) in an emulsion of Freund's complete adjuvant, followed twice at 3-wk intervals with subcutaneous injection of the protein with Freund's incomplete adjuvant.

Affinity-purified antibodies against 215K were prepared as follows: We ran preparative SDS polyacrylamide gels of preparations of 215K. The gels were stained in Coomassie Blue and the protein band was excised with a scalpel. The slices from several gels were destained and then equilibrated in buffer C (150 mM NaCl, 0.1% NaN_3, and 50 mM Tris-Cl, pH 7.5). The slices were finely ground either using a motor-driven homogenizer or by freezing with liquid nitrogen and grinding with a pestle and mortar. The gel fragments were poured into a column and washed with buffer C. Crude antiserum was passed slowly over the column, which was then washed until no protein could be detected by absorbance at 280 nm. The bound antibody was then eluted with 0.1 M glycine-HCl, pH 2.3. The fractions were neutralized with 1 M Tris base, concentrated, and dialyzed into buffer C.

Antibody Staining of SDS Gels: Immunoautoradiography was performed on SDS polyacrylamide gels essentially as has been described before (4, 5), except that the second antibody, affinity-purified goat anti-rabbit IgG, was iodinated by the chloramine T method (16). This iodinated second antibody was used with a specific activity of ~2 mCi/mg protein. Gel slices were overlaid with a solution containing 10^7 cpm/ml of antibody and ~5 mg/ml of carrier protein (hemoglobin).

Immunoprecipitation: Radioactively labeled cell cultures were dissolved in gel sample buffer (20), scraped from the culture dish, boiled, and passed several times through a narrow gauge needle to shear the DNA. The boiled sample was diluted with 20 vol of buffer C containing 1% Triton X-100 and 1% sodium deoxycholate (reducing the SDS concentration to 0.1%). The sample was then incubated with washed, fixed *Staphylococcus aureas* (19) (obtained from the Enzyme Center, Inc., Boston, MA). After 20 min the *S. aureus* were sedimented by centrifugation at 12,000 *g* for 2 min in a microfuge. The resulting supernatants were incubated overnight on ice after the addition of 2 μl of antiserum or nonimmune serum. Washed *S. aureus* were added in sufficient quantity to bind all the IgG, incubated for 20 min, and then centrifuged as described above. The pellet was washed four times with buffer C containing 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate. The pellets were then boiled in gel sample buffer, the *S. aureus* were sedimented, and the supernatants were analyzed on SDS polyacrylamide gels. Duplicate immunoprecipitations were performed on the ^{32}P-samples, and 100 μm $ZnCl_2$ was added to one sample throughout the experiment to inhibit protein phosphatases.

SDS Gel Electrophoresis: SDS PAGE was performed in slab gels using the buffer system of Laemmli (20). 10% polyacrylamide gels contained 0.13% bisacrylamide.

Indirect Immunofluorescence and Microscopy: Cells grown on glass coverslips were fixed in 3.7% formaldehyde in PBS for 5 min. After permeabilization in 0.1–1.0% Triton X-100 in buffer C, the cells were incubated with antibodies, either affinity-purified anti-215K or the anti-215K antiserum diluted to 1/50. For double-label studies the coverslips were simultaneously stained with either a mouse monoclonal antibody against fibronectin (generous gift of Dr. J. Lin, Cold Spring Harbor, NY) or guinea pig antivinculin. These were detected using fluorescein- or rhodamine-labeled second antibodies, either goat anti-rabbit, goat anti-mouse, or goat anti-guinea pig immunoglobulins (Cappel Laboratories, Inc., Cochranville, PA). Actin was visualized in cells with nitrobenzoxadiazol-phallacidin (Molecular Probes, Inc., Junction City, OR) (1). The coverslips were viewed in a Leitz Orthoplan or Ortholux microscope equipped for epifluorescence. Fluorescence micrographs were photographed with Kodak Tri-X film at 1600 ASA; phase micrographs were taken on Kodak Technical pan film at 100 ASA. Interference reflection microscopy was performed on a Zeiss microscope belonging to Dr. A. Harris (University of North Carolina).

RESULTS

Purification of 215K

The purification of 215K from chicken gizzard evolved from the purification scheme we use routinely for the purification of α-actinin and vinculin (13). Described in detail in

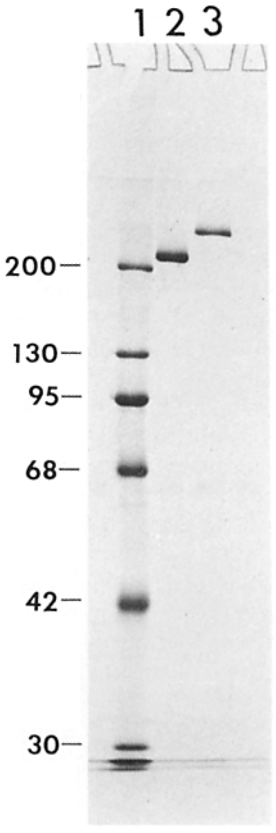

FIGURE 1 SDS polyacrylamide gel analysis of the purified 215K protein. A 10% polyacrylamide gel run in the presence of SDS and stained with Coomassie Blue is shown, on which were electrophoresed molecular weight markers (lane *1*), the purified chicken gizzard 215K protein (lane *2*), and purified chicken gizzard filamin (lane *3*). Molecular weight markers (× 10^3) included myosin (200), β-galactosidase (130), phosphorylase B (95), BSA (68), ovalbumin (42), and carbonic anhydrase (30).

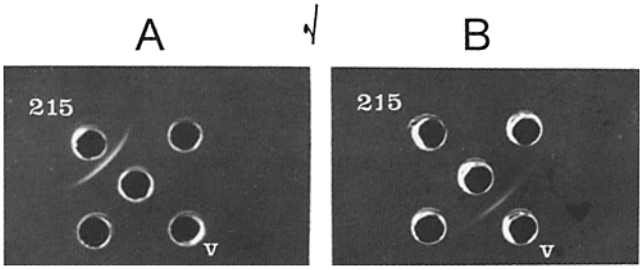

FIGURE 2 Ouchterlony double-diffusion analysis of anti-215K antiserum. In the center well of *A* was placed antiserum from a rabbit immunized with 215K, whereas in *B* the center well was filled with a rabbit antiserum prepared against vinculin. Purified chicken gizzard 215K and vinculin were placed in opposite wells as indicated. After 24 h, precipitin lines had formed between the anti-215K and 215K, but at no time was a cross-reaction seen with vinculin. Similarly no cross-reaction was observed between the antivinculin serum and the purified 215K protein.

Materials and Methods, the procedure entailed low ionic strength extraction of the smooth muscle, which was then fractionated first with $MgCl_2$ and secondly with ammonium sulfate, before chromatography successively on phosphocellulose and DEAE-cellulose, and finally gel filtration on Sepharose C1-4B. The resulting preparation of 215K was essentially free of contaminants as judged by SDS PAGE (Fig. 1). In Fig. 1, the purified protein is compared with filamin and a number of proteins used as molecular weight markers, including myosin. On such SDS gels the protein had an apparent molecular weight of 215,000. Upon storage, a band on SDS gels with a molecular weight of 190,000 was often seen to appear. The increase of this band with time suggests that it was probably a proteolytic degradation product.

Antisera against 215K

Antisera against 215K were raised in rabbits. These antisera were characterized by several techniques. In double-diffusion

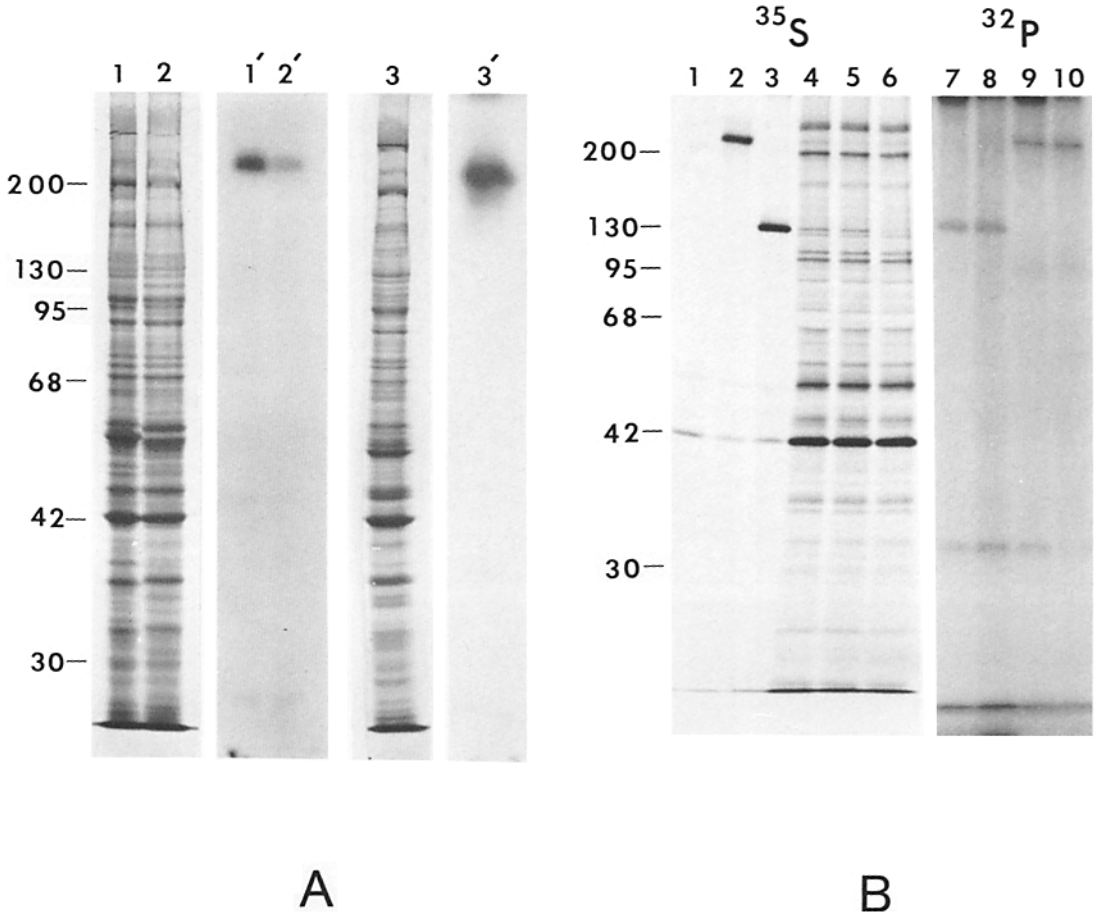

FIGURE 3 SDS polyacrylamide gel analysis of the cellular antigens cross-reacting with the anti-215K antiserum. In *A*, lanes *1*, *2*, and *3* show photographs of gel slices stained with Coomassie Blue on which were electrophoresed whole gerbil fibroma cells, NRK cells, or chick embryo fibroblasts, respectively. Lanes *1′*, *2′*, and *3′* show the autoradiographs of parallel gel slices which were reacted first with rabbit anti-215K protein and then with an ^{125}I-labeled goat anti-rabbit IgG. *1′* and *2′* were stained with whole rabbit serum against 215K, whereas *3′* was stained with affinity-purified anti-215K immunoglobulin. Note the prominent staining of a band at ~215,000 mol wt in the gels of all three cell lines. *B* shows gel analysis of immunoprecipitations of chicken embryo fibroblasts labeled either with [^{35}S]methionine or with [^{32}P]phosphate. Lanes *1–3* show the immunoprecipitates with nonimmunized rabbit serum (lane *1*), anti-215K antiserum (lane *2*), and antivinculin antiserum (lane *3*), and the corresponding supernatants are shown in lanes *4–6*. Note that in lane *5* a quite prominent band at 215,000 mol wt is decreased in intensity relative to the intensity of the same band in the other supernatants. Lanes *7–10* show the immunoprecipitates from cultures labeled with ^{32}P and precipitated either with antivinculin (lanes *7* and *8*) or anti-215K (lanes *9* and *10*). In lanes *7* and *9*, cells have been labeled in the presence of 1 μM $ZnCl_2$ and the immunoprecipitate buffers contained 100 μM $ZnCl_2$ to inhibit protein phosphatases.

analysis the antiserum against 215K formed a single precipitin line with the purified protein and did not cross-react with vinculin (Fig. 2). Immunoautoradiography on fixed SDS gels of whole cells labeled a single band at 215,000 mol wt, in gels of NRK cells and gerbil fibroma cells (Fig. 3*A*); but with gels of chick embryo fibroblasts, a second minor band at ~225,000 mol wt was labeled when this whole serum was used (data not shown). When, however, the affinity-purified antibodies were used, only a single band was labeled in gels of these cells (Fig. 3*A*). Immunoprecipitation of [^{35}S]methionine-labeled cultures with anti-215K antiserum resulted in a single band being precipitated specifically compared with a sample of nonimmune serum (Fig. 3*B*). Again, note that there was no cross-reaction with vinculin, as shown in Fig. 3*B*. In this figure, the supernatants from the three immunoprecipitations (nonimmune serum, anti-215K, and antivinculin) were also analyzed, and a decrease in the intensity of a band at 215,000 mol wt is visible and comparable to the decrease in intensity of the band at 130,000 mol wt. corresponding to vinculin in the supernatant from the vinculin immunoprecipitation.

When chick embryo fibroblast cultures were labeled with [^{32}P]phosphate, autoradiography of gels of immunoprecipitates indicated that 215K was a phosphoprotein (Fig. 3).

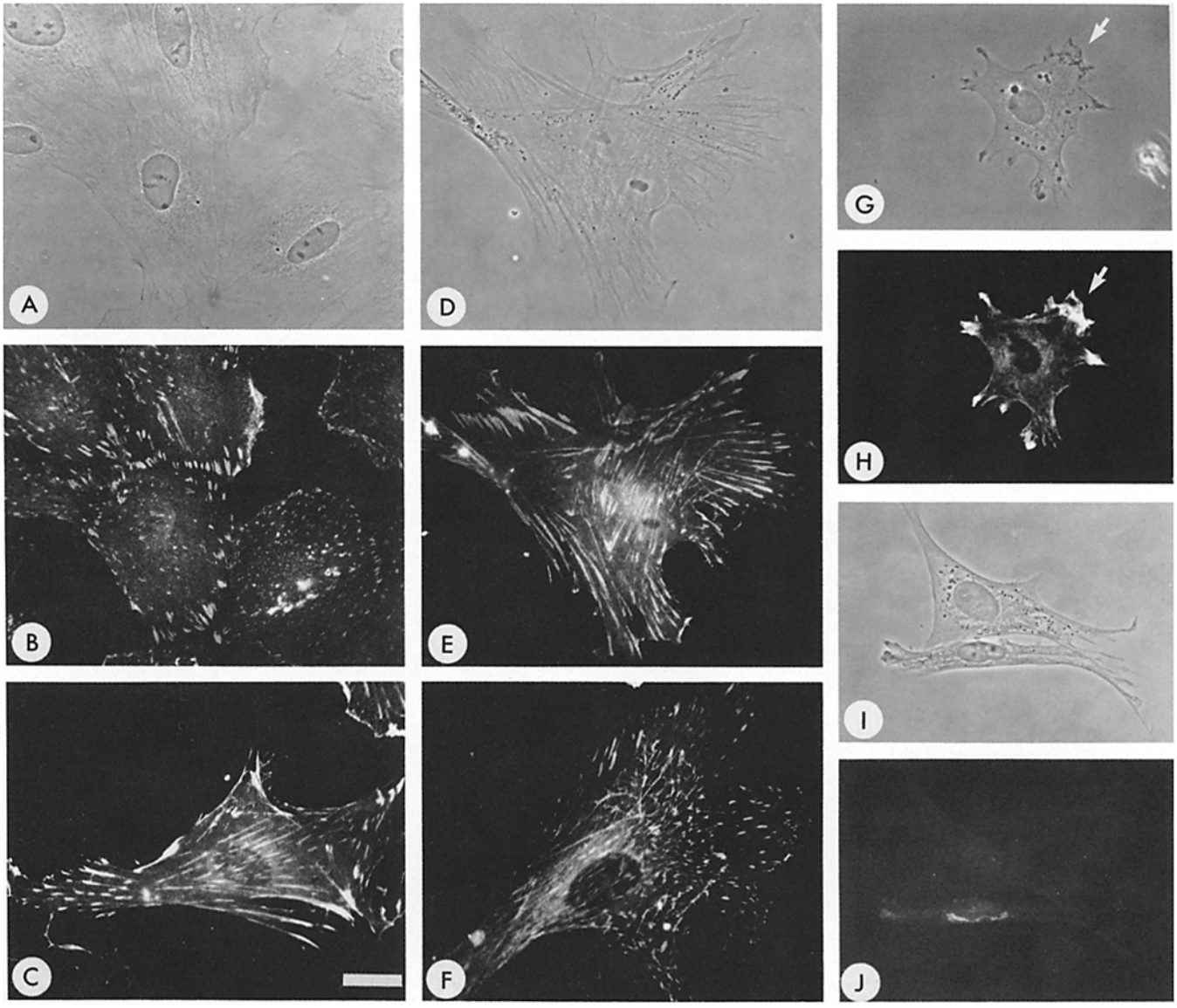

FIGURE 4 Phase and fluorescent micrographs of cells stained with anti-215K antibodies. *A* and *B* show the phase and fluorescent micrographs of NRK cells that were stained with affinity-purified anti-215K antibodies. Note that most of the staining is limited to the focal regions at the edge of the cells and that these regions correspond to the ends of phase dense stress fibers. *D* and *E* show phase and fluorescent micrographs of chick embryo fibroblasts stained with affinity-purified anti-215K antibodies. Note that focal staining is seen again at the ends of phase dense stress fibers but that frequently this also extends along the stress fibers for some of their length. *C* and *F* show chicken embryo fibroblasts stained with the whole antiserum against 215K and illustrate the different types of distribution revealed by this antibody. *G* and *H* show phase and fluorescent images of a chick embryo fibroblast that was respreading, with prominent ruffling membranes at its periphery (arrows) that are labeled by the antibody. *I* and *J* show phase and fluorescent micrographs of chick embryo fibroblasts that were stained with the anti-215K that had been absorbed first with the purified 215K protein. Note the relative absence of staining except for some faint perinuclear fluorescence. (*A–J*) Same magnification. Bar, 20 μm.

Again, the intensity of the autoradiographic band can be seen to be nearly equivalent to that of vinculin in the parallel vinculin immunoprecipitation (Fig. 3*B*).

Localization of 215K by Immunofluorescence

Several different cell types were stained with the antibodies against 215K, using either the diluted antiserum or the affinity purified anti-215K immunoglobulins. When NRK cells were examined (Fig. 4, *A* and *B*), we observed a staining pattern of bright fluorescent areas on the bottom of the cell close to the cell's margin. Frequently, these fluorescent areas could be seen at the ends of phase-dense stress fibers. There was a similar fluorescent pattern with chick embryo fibroblasts (Fig. 4, *C–F*), except that here the staining was more extensive, with the fluorescence extending back from the cell's margin for some distance along the phase-dense stress fibers. Streaky, fibrillar patterns were common (e.g., Fig. 4*F*) and reminiscent of the patterns generated when fluorescent vinculin is microinjected into cells (6). Ruffling membranes, often seen at the periphery of chick embryo fibroblasts, were also labeled by the anti-215K antibody (Fig. 4, *G* and *H*). These three distributions (i.e., the adhesion plaques, the fibrillar distribution, and the membrane ruffles) were revealed only when the staining was performed on permeabilized cells, indicating that 215K is not exposed on the cell surface. Moreover, the three staining patterns were abolished when the antiserum was absorbed with purified 215K protein (Fig. 4, *I* and *J*).

The prominent focal distribution of 215K coincided with the adhesion plaques as revealed both by interference reflection microscopy (Fig. 5, *A* and *B*) (11, 17) and by double-labeled immunofluorescence staining with antivinculin (Fig. 5, *C* and *D*). The areas of closest cell contact with the underlying substrate appeared darkest by interference reflection (Fig. 5*B*, arrows) and were brightly fluorescent when stained with anti-215K and viewed by fluorescence microscopy (Fig. 5*A*, arrow). Other fluorescent areas, however, were also evident which do not correspond to the adhesion plaques as defined by dark areas in interference reflection.

The bright fluorescent focal areas seen when cells were stained with anti-215K corresponded to the ends of the microfilament bundles as is revealed in Fig. 6, where the microfilament bundles have been visualized with nitrobenzoxadiazol-phallacidin (1).

We considered that the linear or fibrillar distribution, besides aligning with the stress fibers, might also relate to the distribution of fibronectin, since we have shown previously that vinculin can align with cell surface fibronectin (6). To study this possible co-alignment, we again used double-label fluorescence microscopy and compared the distribution of 215K revealed with a fluorescein-labeled second antibody with the distribution of fibronectin revealed with a mouse

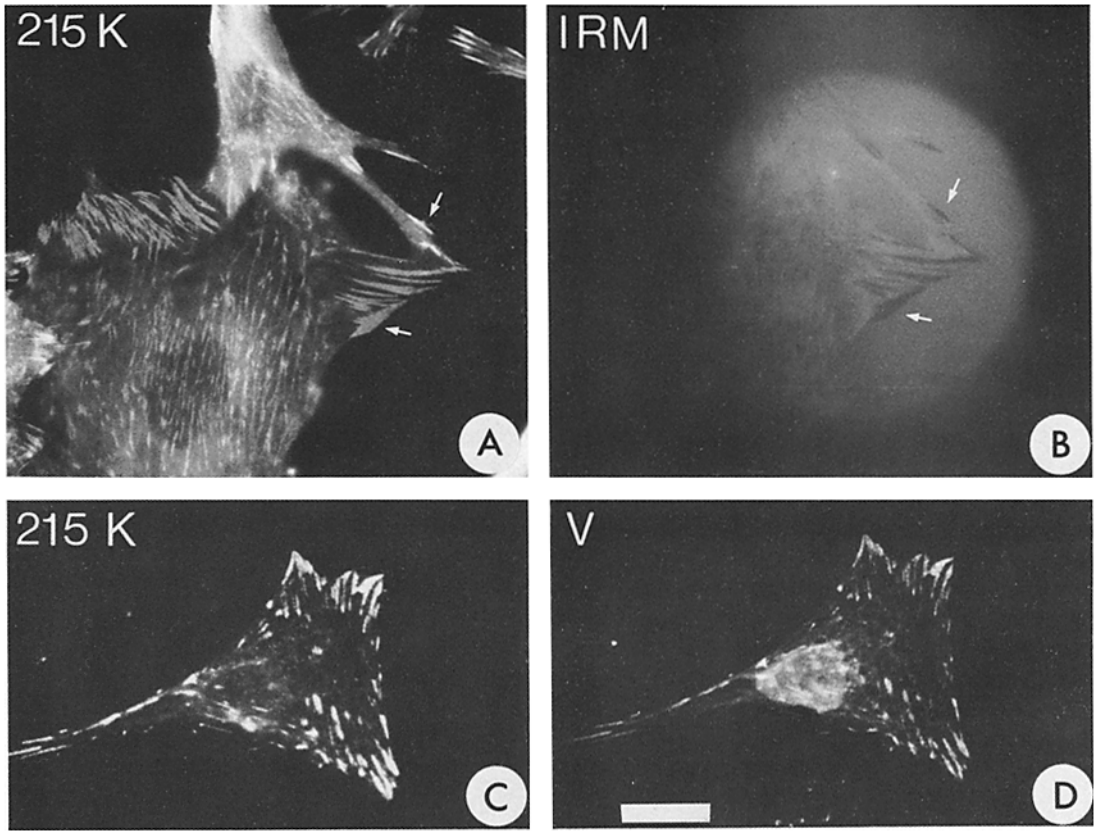

FIGURE 5 215K is concentrated in adhesion plaques. In *A* the distribution of 215K in chicken embryo fibroblasts is revealed by immunofluorescence microscopy. An area of the same field is viewed in *B* by interference reflection microscopy (*IRM*). Note that the dark areas (arrows) in *B* reveal the adhesion plaques (focal contacts), and that these same areas are brightly fluorescent in *A*, indicating the presence of 215K in these regions. *C* and *D* show the same NRK cell stained either with rabbit anti-215K (*C*) or guinea pig antivinculin (*D*) and visualized with fluorescein- or rhodamine-labeled second antibodies, respectively. Note the similar staining pattern with the two antibodies, except for the artifactual nuclear staining with the antivinculin antiserum that had not been affinity purified. Bar, 20 μm.

monoclonal antibody against fibronectin and a rhodamine-labeled second antibody (Fig. 7). We saw many examples of close correlation between the distribution of 215K and that of fibronectin. However, fibronectin could be seen to extend beyond the cell borders whereas 215K was confined strictly within the boundaries of the cell. With time, as fibronectin became more complex and elaborate on the cell surface, it became more difficult to relate the distribution of 215K to that of fibronectin.

The antibody used in these studies cross-reacted most strongly with chicken cells, but it was also used to stain NRK cells (Fig. 4) and gerbil fibroma cells (data not shown). No significant staining was detected with myofibrils prepared from chicken breast muscle. Staining was detected with primary cultures of chicken hepatocytes, but no staining was detected with two epithelial cell lines, Madin-Darby bovine kidney and Madin-Darby canine kidney. The lack of staining with these cell lines may, however, reflect poor antigenic cross-reactivity rather than lack of the 215K protein in these cells.

Effect of Transformation on the Distribution of 215K

In both rat and chick embryo fibroblasts transformed by RSV, the distribution of 215K was altered markedly (Fig. 8). Both transformed cell types showed a variety of distributions. In cells that are well spread and show less of the transformed phenotype, 215K continued to be seen in the adhesion plaque regions. In the more rounded transformed rat cells (Fig. 8*A*), the distribution appeared predominantly as fluorescent dots within the cells, reminiscent of the distribution of vinculin in similar RSV-transformed cells (12). With many of the RSV-transformed chick embryo fibroblasts, the cells were very rounded and appeared brightly fluorescent with little internal structure discernable. A few fluorescent adhesion plaque regions were usually visible in those cells that had spread, but the more extended fibrillar pattern was rarely, if ever, seen. Ruffling membranes were often pronounced on these cells and stained brightly with the antibody (Fig. 8*B*). A particularly well-spread RSV-transformed chick cell, in Fig. 8, *C* and *D*, continued to show the bright focal staining typical of adhesion plaques, but also revealed other fluorescent cytoplasmic structures. These were seen in many cells. The structures ranged in size and the large ones were often "C"- or "S"-shaped and appeared to be aggregates of the smaller structures. The individual structures had a dark, nonfluorescent core. These fluorescent structures, which may exceed 20 μm diam, did not appear to align with any recognizable structure in the corresponding phase micrographs. We noticed similar structures staining both with antiactin and with antivinculin (data not shown).

DISCUSSION

We have described here a new cytoskeletal protein with a molecular weight of 215,000 on SDS polyacrylamide gels.

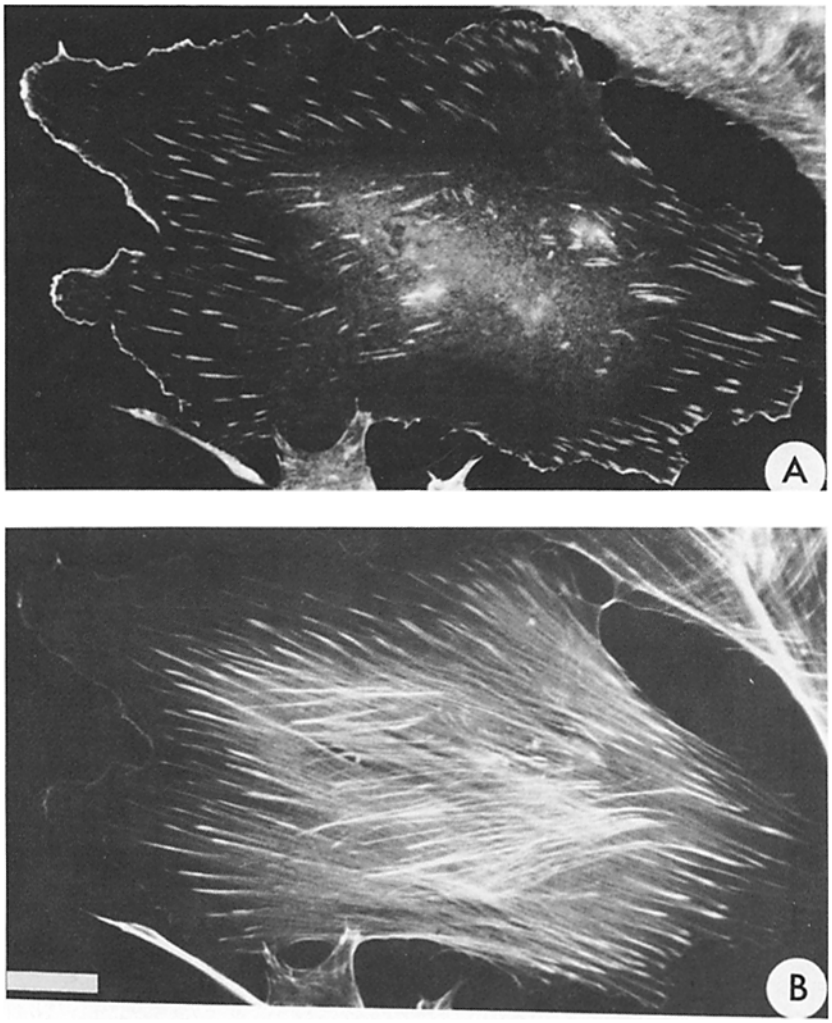

FIGURE 6 Comparison of the distributions of 215K (*A*) and actin (*B*) in the same cell. Chick embryo fibroblasts were stained with both nitrobenzoxadiazol-phallacidin and rabbit anti-215K followed by a rhodamine-labeled second antibody. Note the bright focal distribution of 215K that corresponds with the ends of the actin microfilament bundles. Note also that much of the margin of the cell is labeled both by nitrobenzoxadiazol-phallacidin and by anti-215K. Bar, 20 μm.

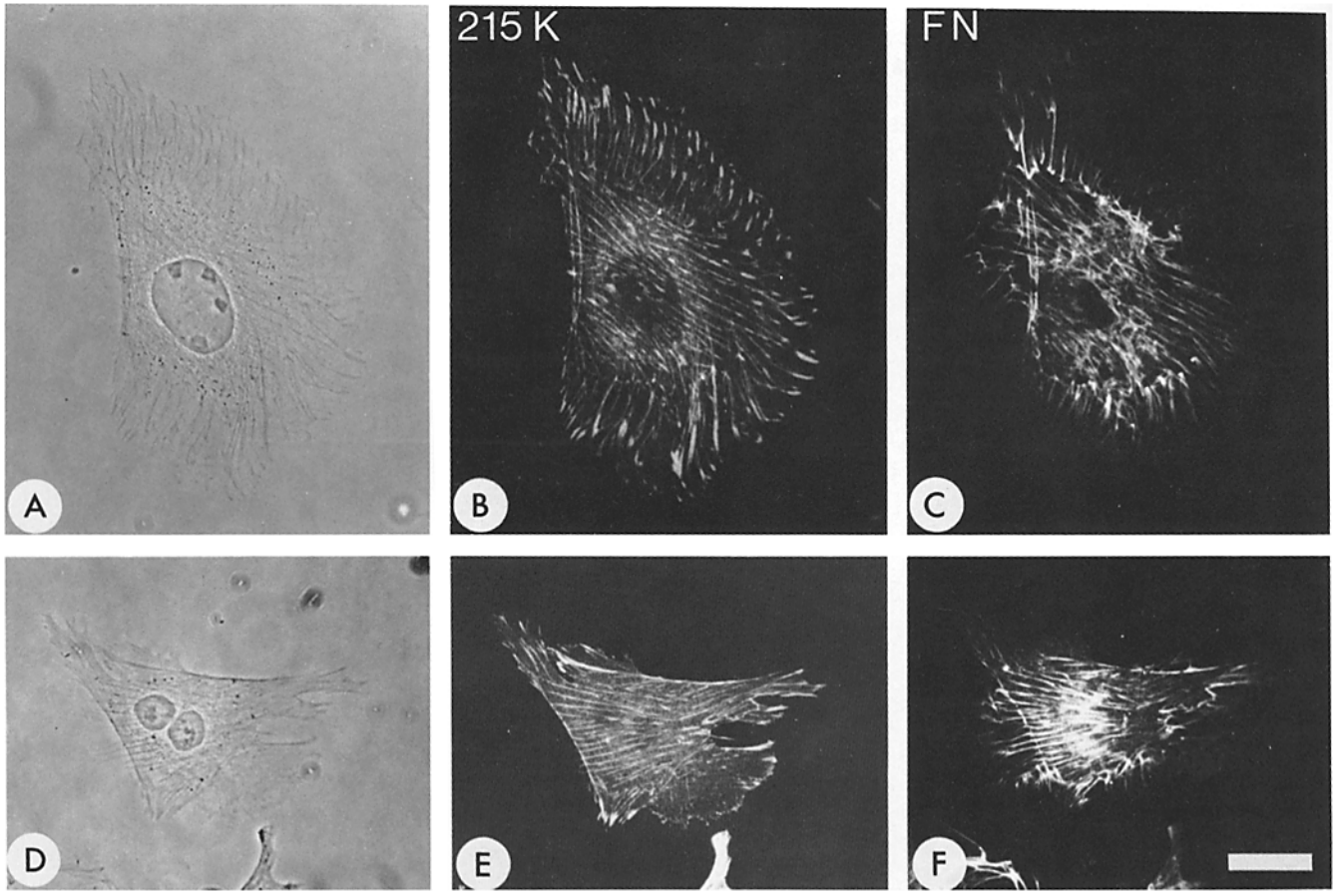

FIGURE 7 Comparison of the distribution of 215K and fibronectin (*FN*). *A* and *D* show phase micrographs of chick embryo fibroblasts stained with affinity-purified rabbit anti-215K (*B* and *E*) and mouse monoclonal antifibronectin (*C* and *F*). Fluorescein- and rhodamine-labeled second antibodies were used to reveal the distributions of the two different primary antibodies. Note that there are many regions of apparent coincidence between the distributions of the two proteins, but this coincidence is not complete. There are areas where fibronectin extends beyond the cell border whereas 215K remains confined to the cell. Similarly the prominent focal distribution of 215K seen in the periphery of the cell in *B* is barely detected when the fibronectin is visualized in this cell (*C*). Bar, 20 μm.

This protein is associated with both adhesion plaques and ruffling membranes.[2] Initially, our interest in this protein was generated because it is extracted from smooth muscle along with vinculin, α-actinin, and a number of other cytoskeletal components. It does not appear to be a major component of smooth muscle compared with α-actinin and vinculin and it has been difficult to purify sufficient quantities of the protein for detailed biochemical studies. By sedimentation analysis we have not detected a direct binding to actin filaments, nor have we found 215K to affect the decrease in F-actin viscosity induced by vinculin (our unpublished results). Although not abundant in smooth muscle, it appears to be quite prominent in chicken embryo fibroblasts as indicated by the immunoprecipitation experiments where gel analysis of the supernatants showed a reduction in a band at 215,000 mol wt following the immunoprecipitation of 215K (Fig. 3). Although this has not been quantitated, it would appear from autoradiographs to be close to the level of vinculin in such cells.

[2] Because of its prominent location in adhesion plaques we suggest the name "Talin" for this protein, deriving from the Latin, talus, meaning ankle. The region coinciding with the end of a microfilament bundle and the cytoplasmic face of the adhesion plaque would seem equivalent to an ankle, if the adhesion plaque is viewed as the cell's foot. We mean to imply no function by this name.

The pure 215K was used to raise specific antibodies with which we studied the distribution of the protein in various cell types. The protein was identified in chick embryo fibroblasts, NRK cells, and gerbil fibroma cells, both by immunofluorescence and by immunochemical analysis. Using immunofluorescence we detected 215K in primary cultures of embryonic chicken hepatocytes, but failed to detect the protein in two mammalian epithelial cell lines (Madin-Darby canine kidney and Madin-Darby bovine kidney); however this may reflect a lack of immunological cross-reactivity. Our antibodies raised against chicken smooth muscle 215K reacted only weakly with 215K from mammalian cells. We also failed to detect by immunofluorescence 215K in chicken myofibrils.

We observed three distinct locations for 215K in cultured cells: in adhesion plaques, in fibrillar structures co-aligning both with cell surface fibronectin and with stress fibers, and in ruffling membranes. No labeling was seen if the cells were not first permeabilized, consistent with the extraction data that indicated that 215K was a cytoplasmic protein and not a transmembrane protein. In NRK cells the most prominent distribution was in the adhesion plaques, usually close to the cell margins (Fig. 4, *A* and *B*). On the other hand, with chick embryo fibroblasts all three distributions were readily observed (Fig. 4, *C–H*), possibly because these cells have much

more fibronectin and also typically display many more membrane ruffles than do NRK cells. The presence of this protein in adhesion plaques and close to cell surface fibronectin was similar to the distribution of vinculin in such cells (6, 14, 24), and we confirmed the similarities of the two distributions using double-label immunofluorescence microscopy (Fig. 5, *C* and *D*). The alignment, however, of 215K with fibronectin (and simultaneously with stress fibers) appeared more pronounced than that of vinculin with fibronectin. This latter alignment is more apparent when fluorescent vinculin is micro-injected into cells than when intrinsic vinculin is simply visualized by immunofluorescence (6). It will be interesting to compare the relative positions of 215K and vinculin at the ultrastructural level and to determine which is closer to the membrane and to surface components such as fibronectin.

A marked difference between the distribution of 215K and that of vinculin was the presence of 215K in membrane ruffles, where vinculin is usually not detected. Finding 215K in membrane ruffles was reminiscent of the distribution of fimbrin (3), a 68,000-mol wt protein purified initially from brush border microvilli, but identified subsequently both in adhesion plaques and in ruffling membranes. In the microvillus, fimbrin appears to be involved in the bundling of microfilaments and it probably serves this function in other locations as well (2, 3). It could be that 215K also has a bundling function or it could be that it has a role in the membrane attachment of actin filaments, as has been suggested for vinculin.

The identification of 215K in adhesion plaques adds another protein to the growing list of proteins (α-actinin, vinculin, fimbrin, and $pp60^{src}$) that have been found concentrated in these structures, and emphasizes the complexity of these regions of actin-membrane interaction. Recently, Chen and Singer (9) have used immunoelectron microscopy to analyze

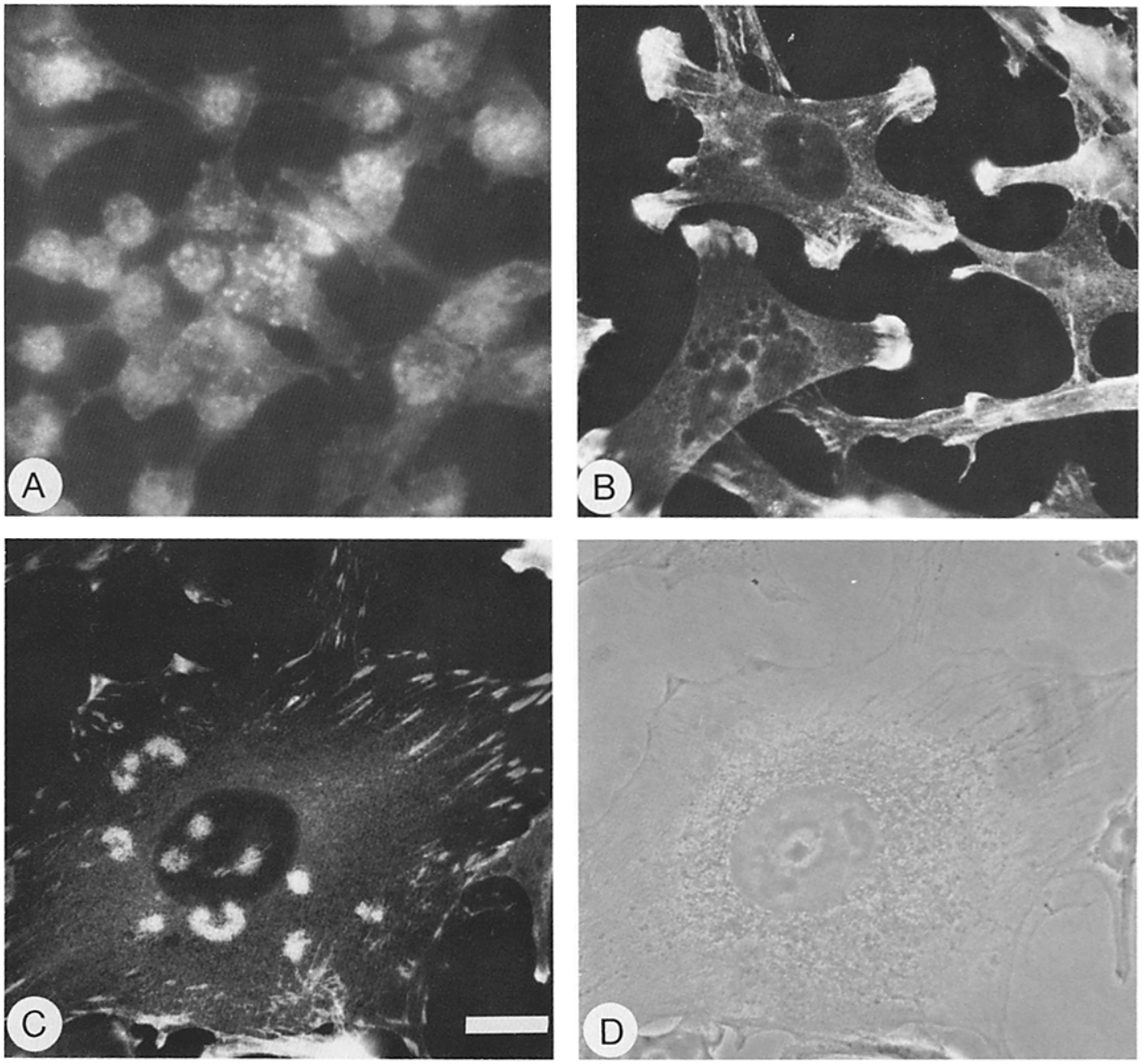

FIGURE 8 Effect of RSV-transformation on the distribution of 215K. *A* shows a fluorescent micrograph of RSV-transformed rat fibroblasts stained with anti-215K. *B* and *C* show fluorescent micrographs of chick embryo fibroblasts transformed by RSV. In *D* the phase micrograph corresponding to *C* is shown. Note the bright fluorescent structures visible in the cell in *C* and that these are not visible as phase-dense structures in *D*. Bar, 20 μm.

the distribution of α-actinin and vinculin in adhesion plaques, as well as the distribution of these proteins relative to fibronectin at the cell surface. They have suggested detailed models for the arrangement of these components at adhesion plaques and at other plasma membrane specializations. In this instance, model construction may be premature because constituents at these sites are still being identified (e.g., 215K), and it is not clear that all the critical components have been recognized. To understand how microfilament bundles are assembled and anchored at regions such as adhesion plaques, it will be important to determine the critical components and how they interact with each other and with actin, as well as with components in the plasma membrane.

The finding that $pp60^{src}$, the transforming protein kinase of RSV, is also concentrated in adhesion plaques (22) is of considerable interest with respect both to transformation and to the way in which transforming genes may disrupt the microfilament bundles. The subsequent finding that vinculin is a phosphoprotein containing phosphotyrosine, the level of which increases following RSV transformation (23), suggests that vinculin is a likely substrate for this tyrosine-specific protein kinase. We found that 215K was also a phosphoprotein (Fig. 3), and it will be important in future work to determine whether 215K contains phosphotyrosine and whether this is also affected by transformation. We observed that RSV transformation could alter the distribution of 215K in cells drastically (Fig. 8). Well-spread transformed cells continued to show 215K in adhesion plaques, but in rounded rat fibroblasts transformed by RSV, much of the 215K appeared as fluorescent dots in the cytoplasm. These were similar to the structures observed with antibodies against α-actinin and vinculin in similarly transformed rat cells (12). Most RSV-transformed chick embryo fibroblasts also showed fewer adhesion plaques and a lack of fibronectin when compared with the untransformed counterparts. 215K continued to be prominent in the membrane ruffles of these cells (Fig. 8 *B*) and was seen in adhesion plaques when these were present (Fig. 8 *C*). In these transformed cells, however, bizarre structures were sometimes seen labeled with this antibody (Fig. 8 *C*). These appeared as small, brightly fluorescent structures often with a dark, nonfluorescent core, and they tended to form much larger aggregates that were often "C"-shaped and sometimes "S"-shaped (Fig. 8 *C*). Using immunofluorescence, we also found actin and vinculin in these aggregates (unpublished observations). What is the significance of these structures? Currently, this is uncertain, but it will be interesting to determine whether any viral proteins are associated with these structures and to learn more about their development and any possible relationship they have with the transformed phenotype.

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REFERENCES

1. Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. W. Webb. 1980. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin. *Proc. Natl. Acad. Sci. USA.* 77:980–984.
2. Bretscher, A. 1981. Fimbrin is a cytoskeletal protein that cross-links F-actin *in vitro*. *Proc. Natl. Acad. Sci. USA.* 78:6849–6853.
3. Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335–340.
4. Burridge, K. 1976. Changes in cellular glycoproteins after transformation: identification of speific glycoproteins and antigens in sodium dodecylsulfate gels. *Proc. Natl. Acad. Sci. USA.* 73:4457–4461.
5. Burridge, K. 1978. Direct identification of specific glycoproteins and antigens in sodium dodecyl sulfate gels. *Methods Enzymol.* 50:54–64.
6. Burridge, K., and J. R. Feramisco. 1980. Microinjection and localization of a 130K protein in living fibroblasts: a relationship to actin and fibronectin. *Cell.* 19:587–595.
7. Burridge, K., and J. R. Feramisco. 1981. Non-muscle α-actinins are calcium-sensitive actin-binding proteins. *Nature (Lond.).* 294:565–567.
8. Burridge, K., and J. R. Feramisco. 1982. α-actinin and vinculin from nonmuscle cells: calcium-sensitive interactions with actin. *Cold Spring Harbor Symp. Quant. Biol.* 46:587–597.
9. Chen, W-T., and S. J. Singer. 1982. Immunoelectron microscopic studies of the sites of cell-substratum and cell-cell contacts in cultured fibroblasts. *J. Cell Biol.* 95:205–222.
10. Connell, L., and K. Burridge. 1982. A new protein of the adhesion plaque. *J. Cell Biol.* 95(2, pt. 2): 299*a*. (Abstr.)
11. Curtis, A. S. G. 1964. The mechanism of adhesion of cells to glass. A study by interference reflection microscopy. *J. Cell Biol.* 20:199–215.
12. David-Pfeuty, T., and S. J. Singer. 1980. Altered distributions of the cytoskeletal proteins vinculin and α-actinin in cultured fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 77:6687–6691.
13. Feramisco, J. R., and K. Burridge. 1980. A rapid purification of α-actinin, filamin, and a 130,000 dalton protein from smooth muscle. *J. Biol. Chem.* 255:1194–1199.
14. Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193–205.
15. Glenney, J. R., P. Kaulfus, and K. Weber. 1981. F-actin binding and bundling properties of fimbrin, a major cytoskeletal protein of microvillus core filaments. *J. Biol. Chem.* 256:9283–9289.
16. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of Iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.).* 194:495–496.
17. Izzard, C. S., and L. R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J. Cell Sci.* 21:129–159.
18. Jockusch, B. M., and G. Isenberg. 1981. Interaction of α-actinin and vinculin with actin: opposite effects on filament network formation. *Proc. Natl. Acad. Sci. USA.* 78:3005–3009.
19. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* 115:1617–1624.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.
21. Lazarides, E., and K. Burridge. 1975. α-Actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell.* 6:289–298.
22. Rohrschneider, L. R. 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the *src* gene product. *Proc. Natl. Acad. Sci. USA.* 77:3514–3518.
23. Sefton, B. M., T. Hunter, E. H. Ball, and S. J. Singer. 1981. Vinculin, a cytoskeletal target of the transforming protein of Rous sarcoma virus. *Cell.* 24:165–174.
24. Singer, I. I., and P. R. Paradiso. 1981. A transmembrane relationship between fibronectin and vinculin (130 Kd protein): serum modulation in normal and transformed hamster fibroblasts. *Cell.* 24:481–492.
25. Tokuyasu, K. T., A. H. Dutton, B. Geiger, and S. J. Singer. 1980. Ultrastructure of chicken cardiac muscle as studied by double immunolabeling in electron microscopy. *Proc. Natl. Acad. Sci. USA.* 78:7619–7623.
26. Wilkins, J. A., and S. Lin. 1982. High-affinity interaction of vinculin with actin filaments *in vitro*. *Cell.* 28:83–90.