Immunofluorescent Localization of a 39,000-dalton Substrate of Tyrosine Protein Kinases to the Cytoplasmic Surface of the Plasma Membrane

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ABSTRACT The intracellular distribution of p39, a 39,000-dalton substrate for a number of tyrosine protein kinases, has been determined by indirect immunofluorescence microscopy. No binding of anti-p39 antibodies to intact cells was observed, indicating that this protein is not accessible to antibody on the cell surface. Following detergent permeabilization of formaldehyde-fixed cells, a reasonably uniform cytoplasmic labeling was observed. This fluorescence was most pronounced in membrane ruffles, especially in the leading lamellae of migrating cells, and in areas of cell-cell contact. Brief permeabilization of cells with detergent prior to formaldehyde fixation resulted in the appearance of a reticular lattice. An identical staining pattern was observed when fluorescently-labeled lectins were used as plasma membrane markers, but not when antibodies to a variety of cytoskeletal proteins were used. Taken together, these results indicate that p39 is, at least in part, located at the cytoplasmic surface of the plasma membrane. Immunolabeling of Rous sarcoma virus-transformed cells with anti-p39 antibodies resulted in fluorescent staining patterns indistinguishable from those observed in untransformed cells. It is conceivable that p39 plays some structural role within a protein network underlying the plasma membrane.

Various RNA tumor viruses express transforming gene products with protein kinase activities specific for tyrosine residues on cellular target proteins (1). Similarly, two growth factors, epidermal growth factor (EGF) (2) and platelet-derived growth factor (PDGF) (3), have recently been showed to stimulate cellular tyrosine protein kinase activities. In the case of EGF, this activity may reside within the membrane receptor for the growth factor (4, 5). It is likely that modification of tyrosines in specific cellular proteins plays a major role in certain cases of viral transformation and cell growth control. To understand the molecular events leading to cell transformation and growth stimulation, it will therefore be necessary to identify and characterize intracellular target proteins for tyrosine protein kinases.

One protein, variously estimated between 34,000 and 39,000 daltons (6–9), hereinafter named p39, has been described as a major substrate for the tyrosine protein kinases induced by both sarcoma viruses (6–13) and EGF (14–16). In Rous sarcoma virus (RSV)-transformed fibroblasts, ~7–10% of the population of p39 is found to be phosphorylated on a single tyrosine residue and a similar fraction is phosphorylated at serine (7, 8, 12, 17). Moreover, it has been shown that purified p39 can act as a substrate for the isolated Rous transforming protein, $pp60^{src}$ in vitro (7). The same tyrosine residue in p39 becomes phosphorylated under either in vitro or in vivo conditions (7). p39 is not phosphorylated, however, in untransformed cells or in cells transformed by agents that do not induce tyrosine protein kinase activities (6, 11).

p39 is a relatively abundant cellular protein, representing ~0.3% of the total methionine in cellular polypeptides (7, 12, 17), yet its function(s) in the cell is completely unknown. Initial efforts to characterize p39 have mainly been aimed at determining its intracellular location. Cell fractionation studies have indicated that a p39 population may be associated with membranes (18) and/or detergent-insoluble cytoskeletal structures (18, 19). There is no evidence for posttranslational alterations in the size of p39, suggesting that it is neither extensively glycosylated nor is it synthesized with a cleavable signal peptide

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(S. Staprans and J. A. Cooper, unpublished data).

Here, using antibodies raised against partially purified p39 (17), we have investigated the intracellular distribution of p39 by indirect immunofluorescence microscopy. Our results indicate that p39 is associated with the cytoplasmic surface of the plasma membrane in cultured fibroblasts and epithelial cells.

MATERIALS AND METHODS

Cell Culture: Normal rat kidney cells (NRK cells) and RSV B77-transformed NRK cells were grown as described previously (20). Dog kidney cells (MDCK) were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum and antibiotics. Chick embryo fibroblasts were prepared and cultured as described previously (21) and 1881 lymphoma cells were grown in DME supplemented with 10% calf serum and 5×10^{-5} M 2-mercaptoethanol.

Immunochemical Reagents and Fluorescein-conjugated Lectins: The purification of p39 and the production of rabbit anti-p39 antisera were described previously (17). Antiserum was also raised to p39 purified through an SDS gel. For this purpose carboxymethyl-cellulose-purified material from $\sim 2 \times 10^9$ chick embryo fibroblasts was lyophilized, dissolved in SDS gel sample buffer, and electrophoresed through a 15% polyacrylamide slab gel. The p39 band was excised, homogenized in 8 ml saline and 1-ml samples, emulsified with 2 ml of complete Freund's adjuvant, and injected into a New Zealand white rabbit at 4-wk intervals. The positive serum was obtained 1 wk after the second injection. IgG fractions were prepared by passing sera over DEAE-Affigel Blue (Bio-Rad Laboratories, Richmond, CA), according to the manufacturer's instructions, and concentrated by vacuum dialysis to a concentration of ~15 mg/ml. For preabsorption on 1881 lymphoma cells, 20 μl serum was incubated for 30 min at 0°C with 10^8 cells that had been fixed for 5 min with 4% formaldehyde in Tris-buffered saline at 25°C and permeabilized for 5 min with 0.5% Nonidet P-40 (Shell Chemical Company, New York, NY) in buffered sucrose (18). Guinea pig antibodies to chicken vimentin were raised against vimentin extracted from 14-d chick embryos (22) and purified by preparative gel electrophoresis. The specificity of the antibodies for vimentin was confirmed by immunoprecipitation and immunoblotting (E. A. Nigg and S. J. Singer, unpublished data). Actin was fluorescently labeled using 7-nitrobenz-2-oxa-1,3-diazole phallacidin (NBD-phallacidin) (Molecular Probes, Inc., Junction City, OR) at twice the concentration recommended by the manufacturer. Rhodamine- and fluorescein-conjugated goat antibodies against rabbit IgG and guinea pig IgG were prepared as described by Brandtzaeg (23). Fluorescein-conjugated concanavalin A (Con A) and wheat germ agglutinin (WGA) were prepared as described (24), and were a kind gift of Dr. Pamela Maher, (University of California, San Diego).

Indirect Immunofluorescence Experiments: Cells were cultured on 18- or 22-mm square coverslips for at least 40 h prior to immunofluorescent labeling. They were treated in either of two ways: (*a*) Prefixation: cells were fixed for 5 min at room temperature with 3% formaldehyde, 2% sucrose in phosphate-buffered saline (PBS), pH 7.6, washed three times in PBS, and extracted for 5 min with ice-cold 0.5% Triton X-100 in 20 mM HEPES, 300 mM sucrose, 3 mM $MgCl_2$, 50 mM NaCl, pH 7.4; (*b*) pre-extraction: cells were extracted for exactly 30 s with the ice-cold Triton X-100 buffer described above, then washed quickly in the 3% formaldehyde fixative described above, and fixed in this fixative for 5 min. The fluorescence intensity of p39 antibodies was not greatly affected by the permeabilization procedure. However, since the local fluorescent signal reflects not only p39 concentration but also accessibility to antibodies, the fluorescence intensity is not a good index of p39 retained through extraction. When chick embryo cells, grown on coverslips and labeled with [^{35}S] methionine, were extracted under these conditions, 48% of immunopreciptable p39 remained. However, since removing the soluble fraction from the residual material took an additional 30 s after the original 30-s extraction, the fraction of p39 in the cells prepared for immunofluorescence may be underestimated.

Prefixed and pre-extracted cells were then washed three times with PBS prior to incubation with antibodies. Anti-p39 IgG was used at ~450 μg/ml of total IgG when labeling mammalian cells and at 150 μg/ml when labeling chicken cells. Cells were incubated with antibodies for 10 min at room temperature. Between incubations with primary and secondary antibodies and after incubations with secondary antibodies, the coverslips were washed three times for 5 min with PBS. For double indirect immunofluorescent staining, the two primary antibodies were mixed, and the two secondary antibodies were mixed, before being applied to the cells. Secondary antibodies were used at 10–20 μg/ml. For actin-staining, NBD-phallacidin was included together with rhodamine-conjugated goat antibodies against rabbit IgG. Fluorescence microscopy was performed with a Zeiss photoscope III. The stained cells were observed through a × 63 oil immersion objective with filter settings for rhodamine- and fluorescein-fluorescence. Photographs were taken on Kodak Tri-X film.

RESULTS

Specificity of Anti-p39 Antibodies

The partial purification of the 39,000-dalton protein, the production of antisera, and a characterization of their specificity have been published (17). These antibodies immunoprecipitate p39 together with small amounts of proteins of molecular weights ~50,000 and 35,000 but react specifically with p39 when denatured cell proteins are immobilized on nitrocellulose (17, 25). For the present immunofluorescence studies, antibodies raised to partially purified p39 were enriched for p39-specific molecules by preabsorption on fixed and permeabilized 1881 lymphoma cells that lack p39 but contain the contaminating proteins (25).

Fig. 1 illustrates the effect of the lymphoma cell preabsorption step on the immunofluorescent staining patterns produced by anti-p39 antibodies in cultured NRK cells. The nonabsorbed sera gave rise to some fluorescent staining of 1881 lymphoma cells, which is presumably caused either by nonspecific striking of IgG or by the presence of antibodies reacting with proteins contaminating the p39 used for immunization (Fig. 1 *A*). As would be anticipated, this immunofluorescent staining was almost completely abolished by extensive preabsorption on 1881 lymphoma cells (Fig. 1 *B*). In contrast, when immunofluorescent labeling was carried out in NRK cells that were either fixed with formaldehyde prior to permeabilization with Triton X-100 (prefixed cells) (Fig. 1, *C* and *D*) or briefly exposed to Triton X-100 (30 s at 4°C) before fixation with formaldehyde (preextracted cells) (Fig. 1, *E* and *F*), the only obvious effect of preabsorption was a reduction of diffuse staining in the region of the nucleus.

More striking was the dependence of the observed immunofluorescence patterns upon the experimental conditions chosen to prepare the cells for incubation with anti-p39 antibodies. In cells that had been fixed prior to permeabilization by detergent (Fig. 1, *C* and *D*), a relatively diffuse fluorescence was apparent over most of the cell. Labeling was most pronounced around the nucleus and in membrane ruffles, particularly in the leading lamellae of migrating cells (arrows in Fig. 1, *C* and *D*). Similar results were obtained when cells were fixed and permeabilized simultaneously by a sequential treatment with methanol for 5 min at −20°C and acetone for 20 s at −20°C (not shown). By contrast, in cells that had been exposed to detergent for 30 s and then promptly fixed (Fig. 1, *E* and *F*), a reticular network extending over the whole cell became apparent. Because the reticular pattern was very characteristic, we chose the corresponding experimental conditions to extend our tests of the specificity of the anti-p39 antibodies.

Similar serpiginous immunofluorescence staining patterns were obtained with prepermeabilized cells from several different species using antibodies from two rabbits, even though the animals had been immunized with widely different preparations of p39: One of the rabbits had been injected with a preparation of p39, partially purified by ion-exchange chromatography (17), whereas the other animal had been immunized with p39, purified further by preparative SDS polyacrylamide gel electrophoresis and elution from a gel slice (see Materials and Methods). Antibodies from the former rabbit recognized p39 both in immunoprecipitation and in immunoblotting assays and were used for all the immunofluorescence experiments shown here. Antibodies from the latter rabbit did not immunoprecipitate native p39 but did react with this protein in immunoblots and gave very similar immunofluores-

cent staining patterns. Serum from a nonresponsive rabbit failed to recognize p39, either in immunofluorescence or in immunochemical assays. Antibodies from a rabbit immunized with a 46,000-dalton protein unrelated to p39, but also identified as a substrate of certain viral tyrosine protein kinases (17), produced immunofluorescent staining patterns entirely distinct from those observed with the anti-p39 reagents. Moreover, the staining by anti-46,000-dalton protein antibodies was not dependent on the order of cell permeabilization and fixation (not shown).

Fig. 2 shows additional specificity controls. A reticular network, similar to that seen in NRK cells, was also observed in

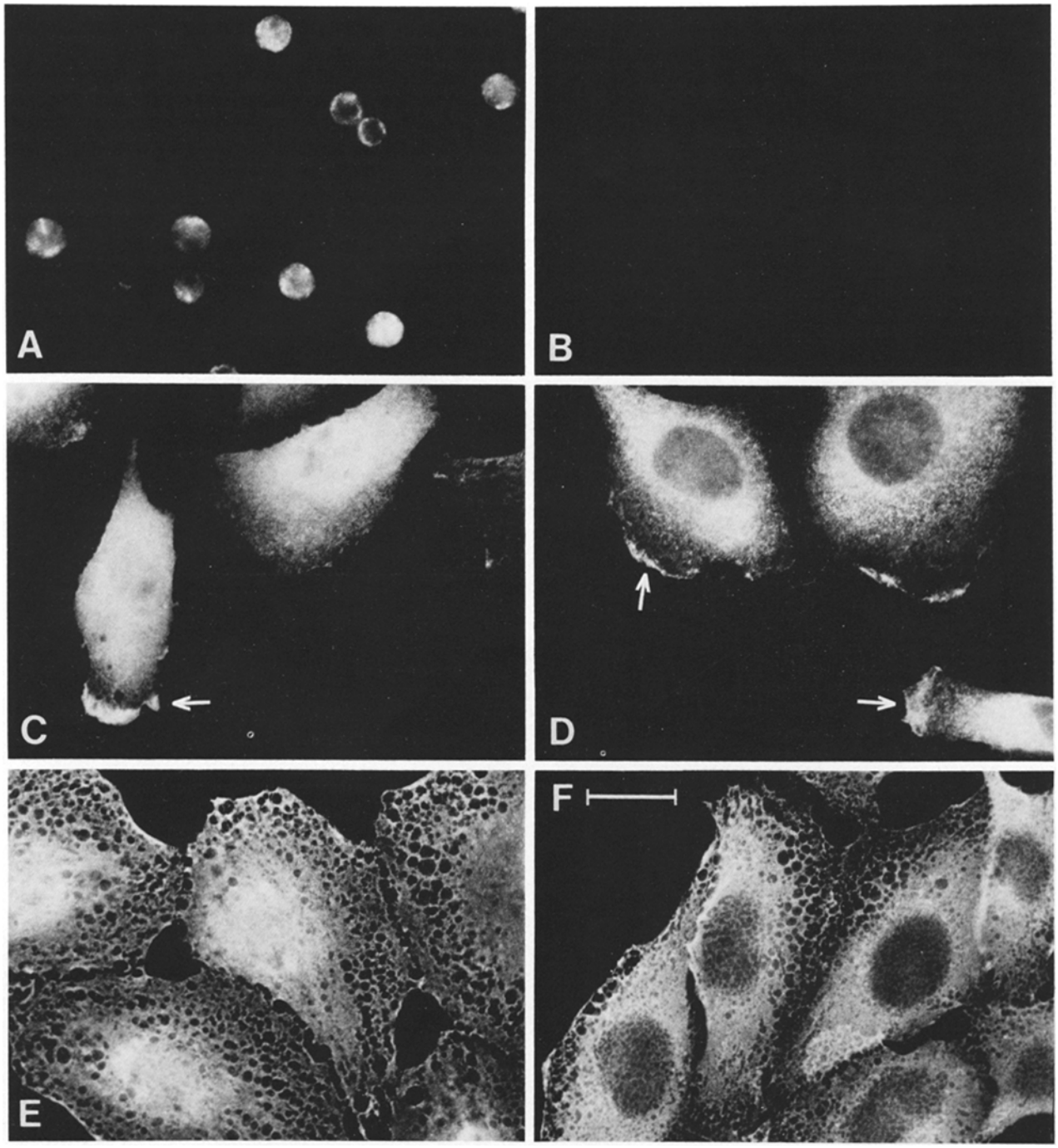

FIGURE 1 Immunofluorescent staining produced by anti-p39 antibodies before and after absorption on 1881 lymphoma cells. Cells were either prefixed and then permeabilized with detergent (*A–D*), or pre-extracted with detergent and subsequently fixed (*E* and *F*), and finally stained as described in Materials and Methods. Anti-p39 antiserum was absorbed on fixed and permeabilized 1881 lymphoma cells as described in Materials and Methods. Both unabsorbed (*A*, *C*, and *E*) and absorbed (*B*, *D*, and *F*) sera were used at a final dilution of 1:25. Secondary antibodies were rhodamine-conjugated goat antibodies against rabbit IgG. (*A*) 1881 lymphoma cells stained with anti-p39 serum after pre-fixation; (*B*) 1881 lymphoma cells stained with absorbed anti-p39 serum after prefixation; (*C*) NRK cells stained with anti-p39 serum after prefixation; (*D*) NRK cells stained with absorbed anti-p39 serum after prefixation; (*E*) NRK cells stained with anti-p39 serum after pre-extraction; (F) NRK cells stained with absorbed anti-p39 serum after pre-extraction. The plane of focus was at the bottom of the cells. Bar, 20 μm. × 700.

pre-extracted chick embryo fibroblasts (Fig. 2*A*). When preimmune serum was used, an extremely low level of very diffuse background staining was apparent in the cytoplasm, and some faint labeling of nuclei could be seen (Fig. 2*B*). Most importantly, immunofluorescent labeling by anti-p39 antibodies was completely abolished after preincubation of the antibodies with an excess of highly purified p39 (Fig. 2*C*). The extent of purity of the p39 used in this competition experiment is illustrated by the gel electropherogram in Fig. 2*E*. On the basis of these experiments we conclude that the antibodies used in the present study are indeed highly specific for p39 and that the reticular lattice stained in pre-extracted cells must contain p39.

Association of p39 with Cytoplasmic Surface of the Plasma Membrane

While studying the immunofluorescent labeling of p39 in NRK cells and in chick embryo fibroblasts, we occasionally observed marked labeling of cell-cell contacts. To examine this location of p39 more closely, we turned to truly epithelial cells, characterized by polygonal morphology and extensive, readily visualized cell-cell contact areas. The anti-p39 immunofluorescent labeling patterns of MDCK cells that had been either prefixed (Fig. 3*A*) or pre-extracted for 2 min at 4°C with 0.5% Triton X-100 (Fig. 3*B*) both showed a pronounced labeling of cell-cell contact areas. No fluorescence was detectable after immunolabeling of intact MDCK cells by anti-p39 antibodies (Fig. 3, *C* and *D*). Similarly, negative results were obtained when labeling intact chick embryo fibroblasts or RSV-transformed NRK cells (not shown). Fluorescence was never observed unless the cells were extracted with detergent, indicating that p39 is absent (or at least inaccessible to antibody) at the cell surface.

To investigate the origin of the serpiginous structures stained by anti-p39 antibodies in pre-extracted cells, we first carried out a series of double indirect immunofluorescence experiments using reagents directed against known cytoskeletal proteins together with antibodies to p39 (Fig. 4). Little detailed correspondence was observed between the distributions of p39 and vimentin (Fig. 4, *A* and *B*), vinculin (not shown), or actin (Fig. 4, *C* and *D*). Moreover, pre-extraction of cells with detergent for prolonged times (>10 min) resulted in almost complete loss of fluorescence arising from anti-p39 antibodies, whereas actin filaments could still be readily seen in the same cells (not

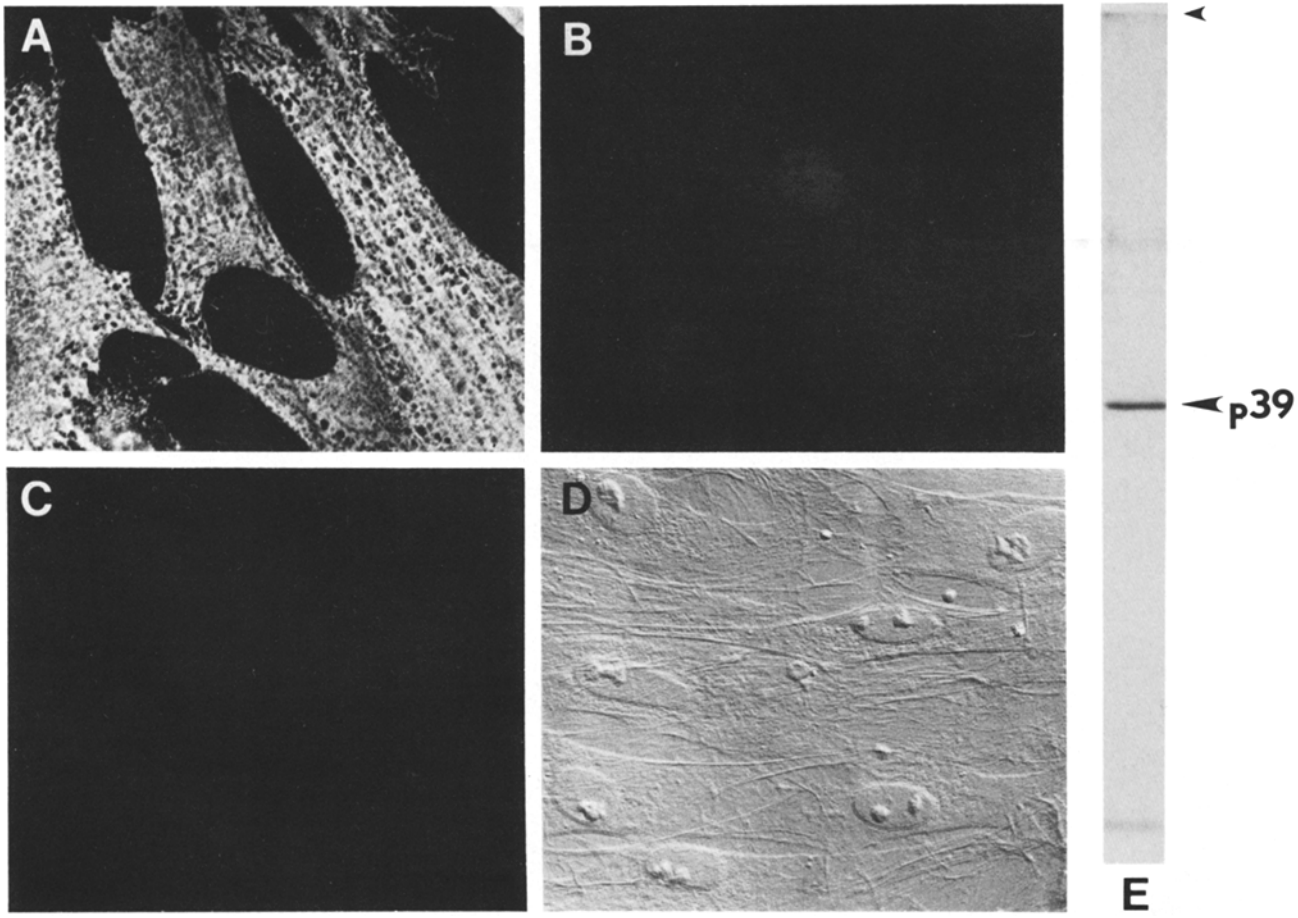

FIGURE 2 Specificity of immunofluorescent staining pre-extracted chick embryo fibroblasts by anti-p39 antibodies. Chick embryo fibroblasts fixed after pre-extraction with detergent were stained as described in Materials and Methods. Anti-p39 serum was preabsorbed on 1881 lymphoma cells and used for immunolabeling at 1:75 dilution (*A* and *C*). Preimmune IgG was used at 150 μg/ml (*B*). Secondary antibodies were rhodamine-conjugated goat antibodies against rabbit IgG. (*A*) Cells stained with preabsorbed anti-p39 serum; (*B*) cells stained with preimmune IgG; (*C*) cells stained with preabsorbed anti-p39 antiserum that had been incubated for 10 min at 4°C with 1 μg of highly purified p39 antigen prior to use. (*D*) The same field as shown in *C* viewed by Nomarksi optics. The scale and magnification are the same as in Fig. 1. (*E*) SDS gel electropherogram stained by Coomassie Brilliant Blue, illustrating the extent of purity of the p39 antigen used for the blocking experiment shown in *C* and *D*. 0.4 μg protein was applied to the gel. The small arrowhead denotes the top of the gel.

shown). Occasionally, however, there seemed to be some overall alignment between the reticular arrays and microfilament bundles (Fig. 4, *C* and *D*, arrows; Fig. 2 *A*).

While characterizing the nature of the spongiform network stained by anti-p39 antibodies, we learned about the immunofluorescence results obtained independently by other groups ([25a], K. Shriver, personal communication) that, together with our own cell fractionation data (18), suggested that p39 might be associated with the plasma membrane. To examine the possibility that the reticular lattice seen after immunolabeling of pre-extracted cells with anti-p39 antibodies might represent remnants of the plasma membrane, we carried out fluorescent labeling studies with reagents directed to the plasma membrane. When fluorescently labeled lectins were used as markers for plasma membrane glycoproteins, very similar patterns to those produced by anti-p39 antibodies were observed (Fig. 5). Double-labeling of NRK cells with anti-p39 antibodies (Fig. 5 *A*) and fluorescent WGA (Fig. 5 *B*) produced virtually superimposable images (note patterns indicated by arrows). Most important, the same dependence of the immunofluorescent labeling patterns on the experimental conditions (i.e., prefixation vs. pre-extraction) was observed for WGA, Con A, or anti-p39 antibodies as exemplified by the labeling of NRK cells by fluorescent Con A after pre-extraction (Fig. 5 *C*) and prefixation (Fig. 5 *D*). Neither WGA nor Con A can be considered absolutely specific markers for the plasma membrane under the conditions used here, because they also label glycoproteins in the extracellular matrix and in endomembranous systems. Nevertheless, the plasma membrane is clearly a major site of reaction for Con A and almost the exclusive site of reaction for WGA (26). Moreover, when intact cells were briefly incubated with fluorescein-conjugated WGA or Con A and washed prior to detergent extraction and formaldehyde fixation, the typical spongiform lattice was also observed, whereas virtually no labeling of glycoproteins occurred in the cell interior (not shown).

In addition, we found that antibodies specific for components of the rough endoplasmic reticulum and the Golgi apparatus (27) produced patterns of immunofluorescent labeling different from one another and the reticular structures seen with anti-p39 antibodies in pre-extracted cells. On the basis of these results, we conclude that it is the plasma membrane that is stained in similar ways by both anti-p39 antibodies and fluorescently labeled lectins. Clearly, however, p39 resides on the cytoplasmic surface of the membrane, whereas the carbohydrate moieties recognized by lectins are exposed at the external surface of the plasma membrane.

In other experiments (not shown), we studied the partitioning

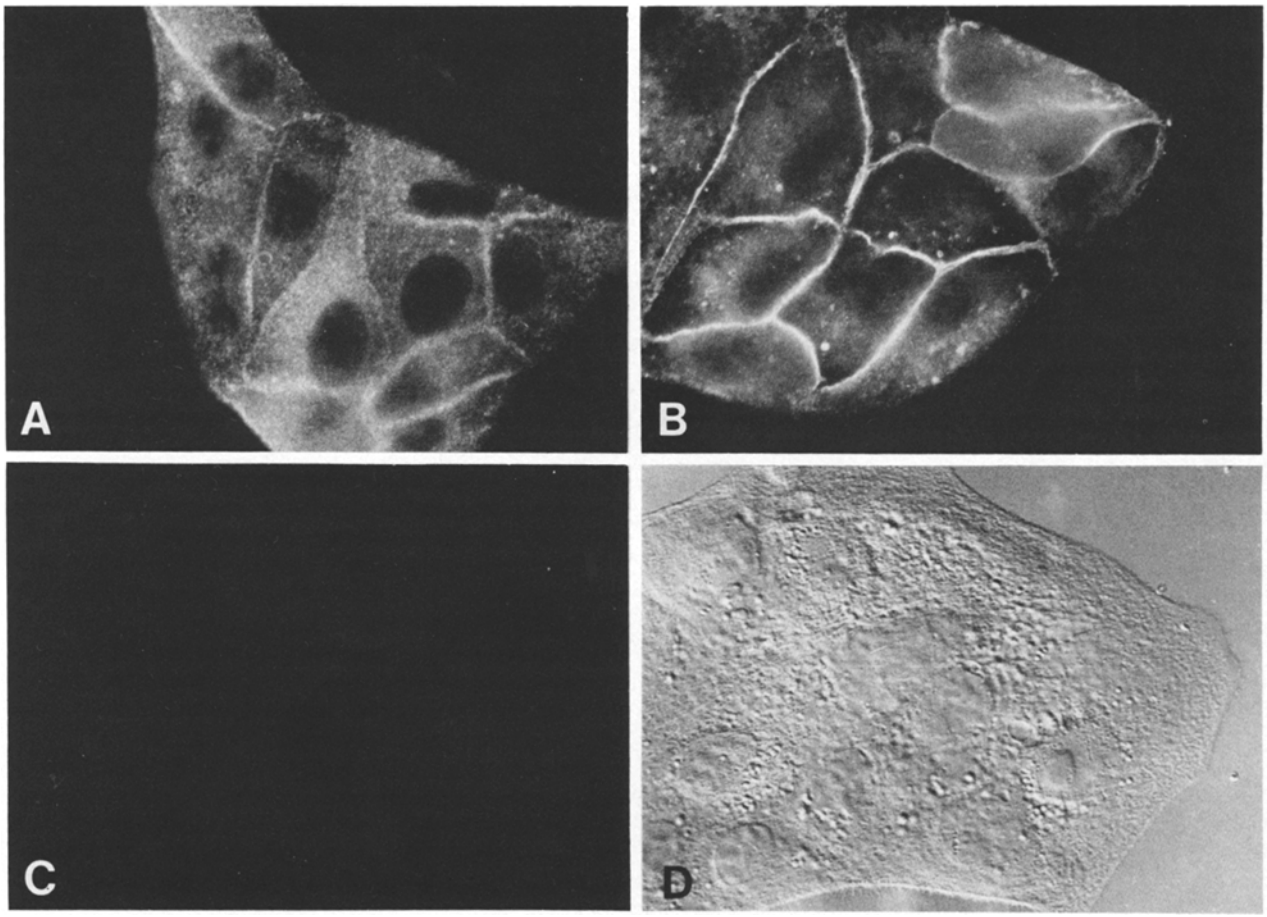

FIGURE 3 Indirect immunofluorescent labeling of MDCK cells by anti-p39 antibodies. MDCK cells were either prefixed and subsequently permeabilized with detergent (*A*), pre-extracted with detergent and then fixed (*B*), or fixed for 5 min with 3% formaldehyde but not subsequently permeabilized (*C* and *D*), as described in Materials and Methods, except that pre-extraction was done for 2 min instead of 30 s. Anti-p39 serum preabsorbed on 1881 lymphoma cells was used at 1:25 dilution. Secondary antibodies were rhodamine-conjugated goat antibodies against rabbit IgG. (*A*) Prefixed cells stained with anti-p39 serum; (*B*) pre-extracted cells stained with anti-p39 serum; (*C*) intact cells stained with anti-p39 serum. (*D*) The same field as in *C* viewed by Nomarski optics. The scale and magnification are the same as in Fig. 1.

of p39 in a detergent-water phase-separation assay (28) designed to provide an operational criterion for distinguishing integral from peripheral membrane proteins. In this assay, an originally homogeneous aqueous solution containing the protein(s) of interest and the nonionic detergent Triton X-114 is warmed above 20°C. Above this temperature, the solution separates into two distinct phases, an aqueous phase and a detergent phase, which can be physically separated and analyzed for their protein content. It has been demonstrated that a series of bona fide integral membrane proteins, when present during the formation of the two phases, partition into the detergent phase, whereas peripheral membrane proteins or other water-soluble proteins partition into the aqueous phase (28). In similar experiments, p39 partitioned almost exclusively into the aqueous phase, which suggests that it is not an integral membrane protein but rather is peripherally associated with the membrane and/or with cytoskeletal proteins underlying the membrane.

Distribution of p39 in RSV-transformed Cells

A p39 population becomes phosphorylated on tyrosine residues when cells are transformed by certain sarcoma viruses or after some cultured cells are treated with EGF or PDGF. It therefore was of interest to examine whether or not changes in the intracellular distribution of p39 might be detected in appropriate cells. Immunofluorescent labeling of p39 in prefixed NRK cells transformed by the B77 strain of RSV showed uniform labeling over the entire cell body (Fig. 6*A*), whereas the typical reticular network appeared after fluorescent staining of pre-extracted RSV-transformed cells (Fig. 6*B*). Cell-cell contact staining was also occasionally apparent under either experimental condition, when crowded cultures were examined (not shown). Analysis of several pairs of normal and transformed cells indicates that, at the resolution level of immunofluorescence microscopy, no major differences in the intracellular distribution of p39 can be detected between transformed and untransformed cells.

DISCUSSION

The determination of the cellular location of p39 may give some clues as to the function(s) of this protein and to the effect of tyrosine phosphorylation on these functions. Here, we have

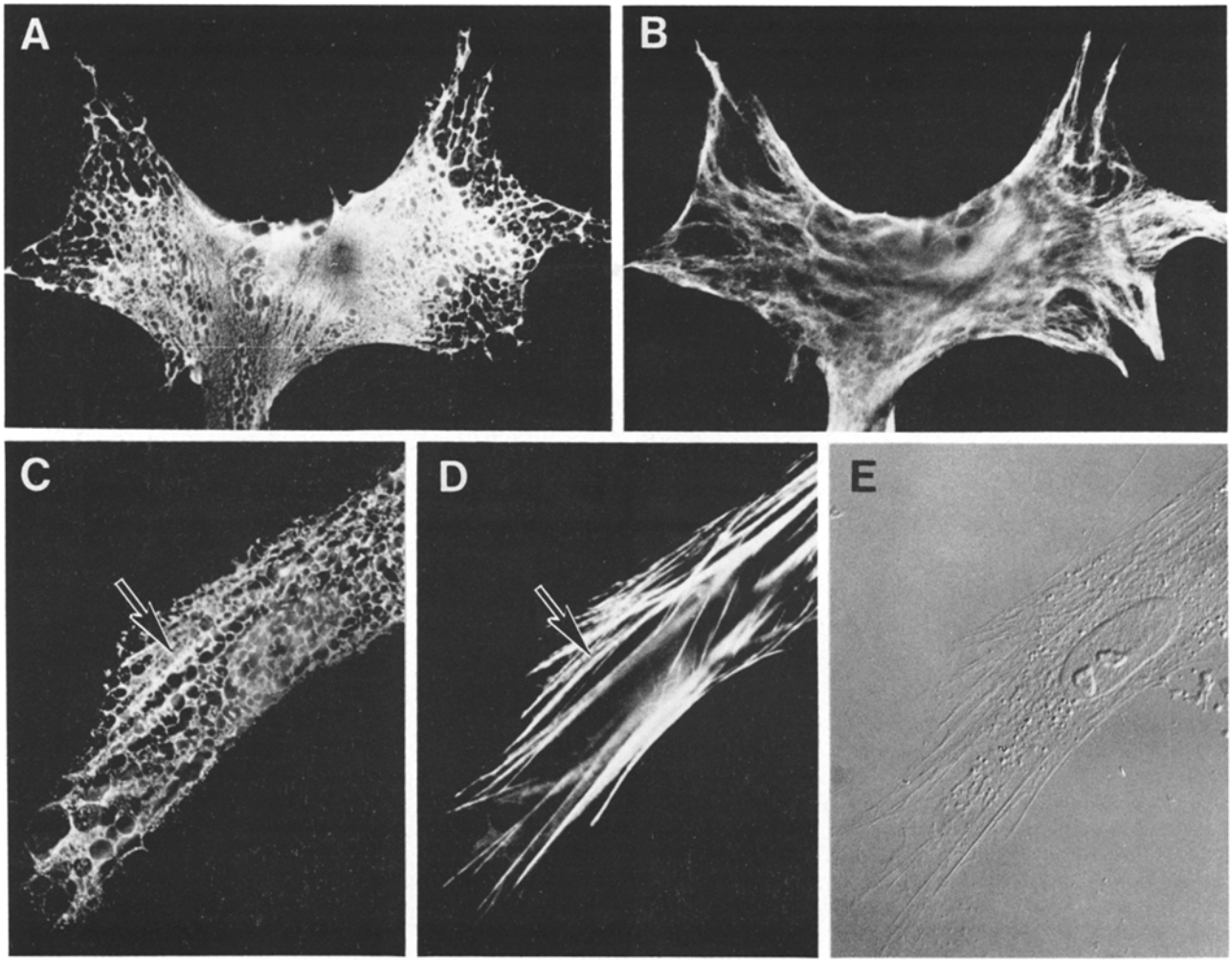

FIGURE 4 Double indirect immunofluorescent labeling of p39 and cytoskeletal proteins in chick embryo fibroblasts. Chick embryo fibroblasts fixed after pre-extraction with detergent were stained as described in Materials and Methods. (*A* and *C*) Cells stained with rabbit anti-p39 IgG at 150 μg/ml, followed by rhodamine-conjugated goat antibodies against rabbit IgG. (*B*) The same field of cells as in *A*, stained with guinea pig anti-vimentin serum at 1:35 dilution, followed by fluorescein-conjugated goat antibodies against guinea pig IgG. (*D*) The same field of cells as in *C*, stained with MBD-phallacidin for actin. (*E*) The same field as in *C* and *D* viewed by Nomarski optics. The scale and magnification are the same as in Fig. 1. Arrows in *C* and *D* indicate a region where there is alignment between the p39- and actin-staining patterns.

investigated the intracellular distribution of p39 by using indirect immunofluorescence microscopy. No p39 could be detected by labeling intact cultured cells, indicating that p39 is not accessible to antibodies and is most probably not exposed at the cell surface. This conclusion agrees with our previous finding that p39 is not degraded during trypsin treatment of intact cells (18). However, two lines of evidence from this study suggest that a significant population of p39 is associated with the plasma membrane. First, with cells prepared conventionally for immunofluorescence, a rather uniform labeling of the entire cell was observed. Staining was most pronounced in regions of high plasma membrane density: in membrane ruffles, especially at the leading lamellae of moving cells, and in cells of epithelial origin, at cell-cell contacts. Similar immunofluorescence observations have also been made by other workers ([25a, 28a] K. Shriver, personal communication). Second, the fluorescent patterns produced by anti-p39 antibodies and by reagents to other membrane proteins were altered concordantly if permeabilization preceded fixation.

Brief extraction of cells, under conditions designed to maximize structural preservation (29), prior to fixing and labeling with anti-p39 antibodies, revealed a striking reticular pattern that was distinct from the patterns of known cytoskeletal systems in the same cells. Fluorescent staining of cell-surface lectin-receptors revealed that the pre-extraction procedure resulted in the formation of the same reticular structures. This suggests that membrane-associated proteins are reorganized when membrane lipids are extracted: possibly they collapse onto one another or onto submembranous structures. This might explain the occasional alignment of actin-containing microfilament bundles and parts of the p39 lattice. When we consider that the glycoproteins recognized by the lectins are exposed at the cell surface, whereas p39 reacts exclusively with antibodies at the cytoplasmic surface of the plasma membrane, their congruent arrangement in pre-extracted cells is remarkable and certainly supports the conclusion that at least a fraction of p39 is associated with the plasma membrane.

A factor contributing to the appearance of the reticular network during pre-extraction may be the removal of a subpopulation of membrane-associated p39 with a diffuse localization. Our extraction conditions solubilize ~50% of p39 from chick embryo fibroblasts, as demonstrated by immunoprecipitation of the soluble and insoluble fractions. Potentially, solubilized proteins could associate artefactually with submembranous structures during the pre-extraction procedure, although we are not aware of any examples of this. Many other

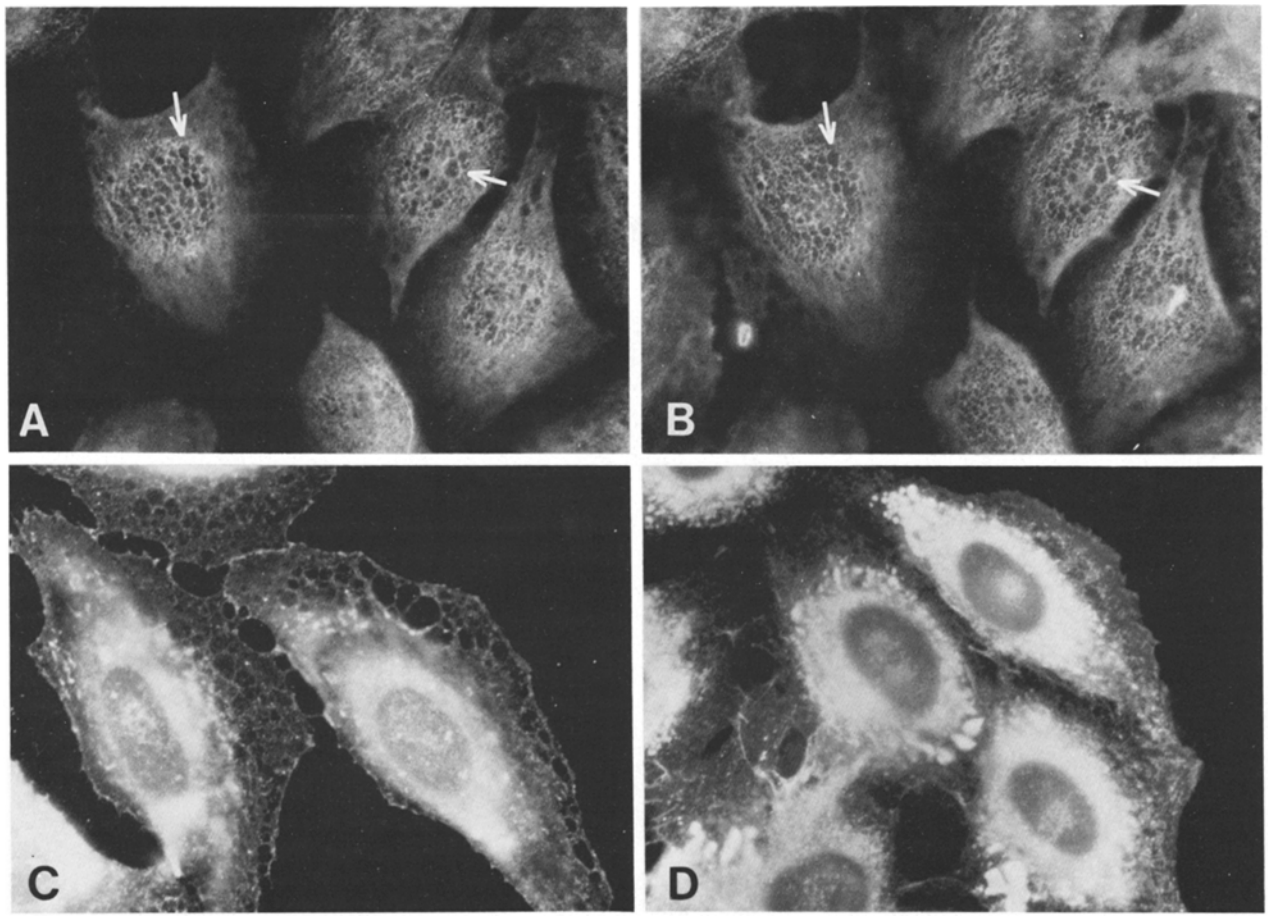

FIGURE 5 Correspondence between the reticular lattice stained by anti-p39 antibodies and fluorescently labeled lectins in NRK cells. NRK cells were either pre-extracted and subsequently fixed (*A*- *C*) or prefixed and then permeabilized (*D*), and finally stained as described in Materials and Methods. The fluorescent lectins were both used at 20 μg/ml. (*A*) Cells stained with rabbit anti-p39 IgG at 450 μg/ml followed by rhodamine-conjugated goat antibodies against rabbit IgG; (*B*) the same field of cells as in *A* stained with fluorescein-conjugated WGA simultaneously with the secondary antibody (in both *A* and *B*, the focal plane of the microscope was set to the top of the cells). (*C*) Pre-extracted cells stained with fluorescein-conjugated Con A; (*D*) prefixed cells stained with fluorescein-conjugated Con A. The scale and magnification are the same as in Fig. 1. Arrows in *A* and *B* indicate regions where there are particularly striking similarities between the p39 and WGA staining patterns.

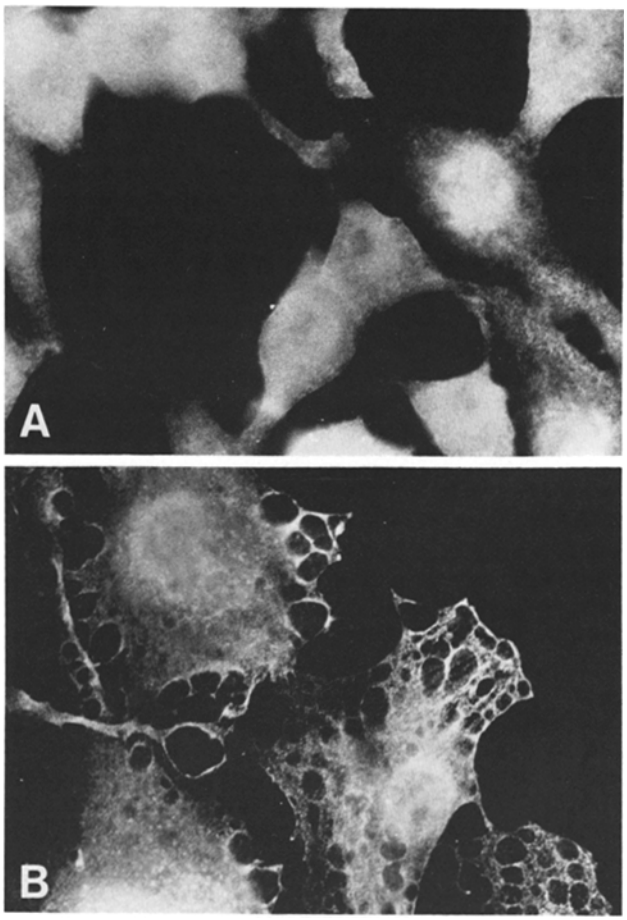

FIGURE 6 Indirect immunofluorescent labeling of RSV-transformed NRK cells by anti-p39 antibodies. NRK cells transformed by the B77 strain of RSV were either prefixed (*A*) or pre-extracted (*B*), and stained as described in Materials and Methods. Anti-p39 IgG were used at 450 μg/ml followed by rhodamine-conjugated goat antibodies against rabbit IgG. (*A*) Prefixed cells stained with anti-p39 antibodies; (*B*) pre-extracted cells stained with anti-p39 antibodies. The scale and magnification are the same as in Fig. 1.

proteins with isoelectric points similar to that of p39 are extracted quantitatively under our conditions (18). Moreover, the concentration of p39 in regions of plasma membrane activity in prefixed cells suggests that at least some of the p39 present in the reticular structures was membrane-associated before permeabilization.

The present immunofluorescence results agree well with recent data on the distribution of p39 during cell fractionation. Following hypotonic lysis of cells, a major fraction of p39 is found in the particulate pelletable material, whereas detergent extraction results in the solubilization of variable fractions of p39, depending on the exact conditions used for extraction (18, 19). Our studies and another report have indicated that p39 is associated with a cytoskeletal fraction, defined operationally as material adhering to the substratum after extraction of attached cells with nonionic detergents (18, 19). In contrast, when cells are removed from the substratum before extraction, much of the p39 population is soluble. The cell fractionation results can now be reconciled, since cytoskeletal residues remaining after detergent extraction of cells clearly contain remnants of the plasma membrane, as well as intracellular organelles. The detergent soluble fraction of p39 from suspended cells could also be membrane associated, since cytoskeletal/membrane contacts are organized differently in rounded and flattened cells (30). The small fraction of p39 that is not particulate following hypotonic lysis may constitute a soluble pool. A soluble population of p39 might contribute to the diffuse cytoplasmic immunofluorescence observed in prefixed cells, and possibly coexist with the membrane-associated protein in a dynamic equilibrium.

Besides p39, another substrate for tyrosine protein kinases, vinculin (31), is concentrated in membrane proximal structures (32–35). The transforming protein of RSV, pp60^{src}, is associated, at least in part, with the cytoplasmic surface of the plasma membrane (20, 36–39), and the tyrosine protein kinase activity stimulated by EGF presumably resides in a portion of the EGF-receptor complex that extends to the cytoplasmic face of the plasma membrane (4, 5). The co-localization of tyrosine protein kinases and their substrates on the cytoplasmic face of the plasma membrane may somehow reflect the occurrence there of events important in cell transformation and growth stimulation. On the other hand, there are substrates for tyrosine protein kinases that are not associated with the plasma membrane (18).

Transformation by RSV results in tyrosine phosphorylation of ~10–15% of the population of p39 (12, 17). Phosphorylated and unphosphorylated p39 co-distribute in cell fractionation experiments (18). We were unable to detect any differences in the immunofluorescent labeling patterns produced by anti-p39 antibodies in untransformed and RSV-transformed cells. The effect of phosphorylation on p39 function remains to be determined.

p39 may appear concentrated at sites of cell-cell contacts in polygonal cells and in membrane ruffles partly because of the accumulation of fluorescence that results from the opportunity to view these particular membrane areas tangentially. Actin, in addition to being arranged in microfilaments and distributed diffusely within the cytoplasm, is also concentrated in membrane ruffles, the leading lamellae of migrating cells, and in areas of cell-cell contact (40–42), much like p39. It is possible therefore that p39 is not directly associated with the plasma membrane but is bound to cytoskeletal structures underlying the membrane. Immunoelectron microscopy would be necessary to distinguish these possibilities.

In view of the relative abundance of p39 in fibroblasts, it would appear that a structural role for it within a protein matrix underlying the plasma membrane is plausible. From the amount of p39 in the cell, one can calculate that this single protein could account for as much as 3% of a monomolecular layer covering the entire inner surface of the plasma membrane. However, p39 is absent from certain lymphoma cell lines (25), and preliminary evidence has been obtained that p39 is also scarce in some tissues including brain, liver, and muscle, but is abundant in gut, lung, and thymus (K. L. Gould and J. A. Cooper, unpublished results). This argues against the suggestion that p39 is identical to malic dehydrogenase (43), an enzyme vital to basic cellular metabolism and unlikely to be absent from any cell type. Whatever function one wants to envisage for p39, this function has to be compatible with both its subcellular location and its tissue distribution.

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