Novel Tyrosine Kinase Substrates from Rous Sarcoma Virus-transformed Cells Are Present in the Membrane Skeleton

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Abstract. We have previously reported the production of monoclonal antibodies directed against phosphotyrosine, which is the modification induced by many oncogene products and growth factor receptors. One of these antiphosphotyrosine antibodies (py20) was used in affinity chromatography to isolate phosphotyrosine (PY)-containing proteins from Rous sarcoma virus-transformed chick embryo fibroblasts (RSV-CEFs). Mice were immunized with these PY-proteins for the production of monoclonal antibodies to individual substrates. Fifteen antibodies were generated in this way to antigens with molecular masses of 215, 76, 60, and 22 kD. Antibodies to individual substrates were used to analyze the subcellular location in normal and RSV-CEFs. Antibodies to the 215- and 76-kD antigen stained focal contacts when used in immunofluorescence microscopy while anti-22-kD protein antibodies resulted in punctate staining concentrated in the margins of cells and in parallel arrays. Both distributions were altered in transformed cells. When cells were extracted with nonionic detergent under conditions that stabilize the cytoskeleton, 50% of the 76-kD protein and >90% of the 22-kD protein fractionated with the cytoskeleton. This study offers a new approach to both the identification of membrane skeletal proteins in fibroblasts and changes that occur upon transformation by an activated tyrosine kinase.

MANY oncogenes are known to encode tyrosine-specific protein kinases and presumably exert their effect by phosphorylating proteins present in normal cells (6, 32, 44). The target substrates of tyrosine kinases have proven difficult to identify and characterize since they are generally minor proteins present in cultured cells. A variety of tyrosine kinase substrates are known. These include the cytoskeletal proteins vinculin (34, 50) and talin (15, 42) together with integrin (31) that are thought to participate in a cytoskeleton to membrane linkage (for reviews, see references 8, 33). Proteins termed calpactins (also referred to as p35 and p36 or lipocortins) are also known to be phosphorylated by both the transforming tyrosine protein kinases (10, 16, 45) and the epidermal growth factor receptor kinase (24, 49). Calpactins have been shown to associate with membrane lipids (22, 24) and the cytoskeletal proteins actin and spectrin (20, 24) in a Ca^{++}-dependent manner. They have been localized just under the membrane in a number of cell types (2, 13, 28, 35, 40). Other tyrosine kinase substrates include calmodulin (18), microtubule associated proteins (1), glycolytic enzymes (12), and a 50-kD protein (21).

Thus far there is little evidence suggesting a functional connection between the phosphorylation of the known substrates and any of the properties of transformed cells. Indeed, recent studies have shown that cells expressing the nonmyristylated mutants of the transforming protein of Rous sarcoma virus, $pp60^{src}$, are not transformed (30, 37). In this system, nonmyristylated p60src is no longer targeted to the membrane (14, 37), but it still phosphorylates the known substrates. Other studies have demonstrated a good correlation between transformation and the association of the kinase with the cytoskeleton (29). Clearly, the identification of other tyrosine kinase substrates is needed to evaluate the crucial cytoskeletal targets.

Early attempts to identify tyrosine kinase substrates relied on two dimensional gel analysis, followed by partial alkaline hydrolysis (i.e., reference 11). Other studies have tested antibodies to proteins that are known to reside in the same cytoskeletal network (focal contacts) as pp60 itself (31, 42, 46, 50). Antiphosphotyrosine (anti-PY)[1] antibodies have gained widespread use in recent years for the detection of tyrosine kinase substrates in transformed and growth factor treated cells (9, 17, 30, 41, 48, 52). The use of anti-PY antibodies in immunofluorescence microscopy has shown that normal cells possess a low level of PY proteins that are concentrated in focal contacts (39), and cells in development have PY proteins in close apposition to their plasma membrane (38, 51). We now report the use of an anti-PY monoclonal antibody to purify substrates from Rous sarcoma virus-transformed chick embryo fibroblasts (RSV-CEFs). The mixture of substrates was used to immunize mice for the

1. *Abbreviations used in this paper*: PY, phosphotyrosine; RSV, Rous sarcoma virus; RSV-CEF, Rous sarcoma virus-transformed chick embryo fibroblasts.

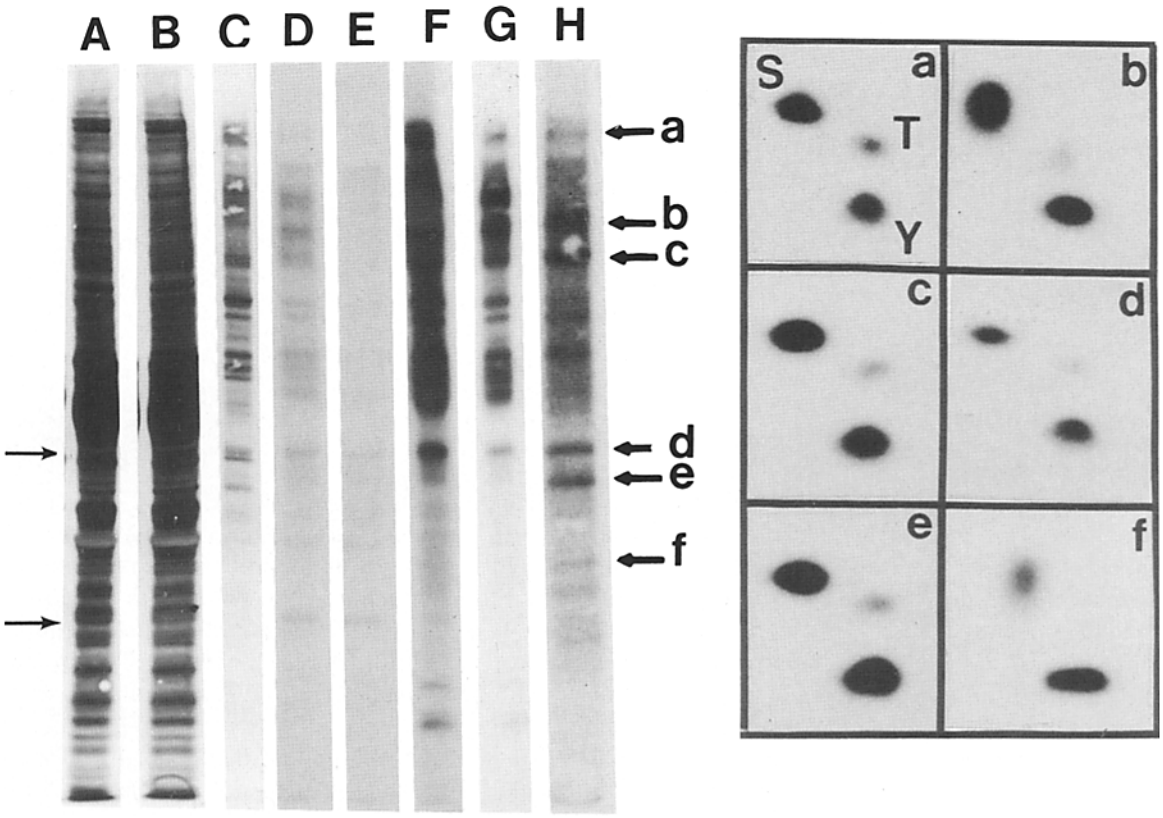

Figure 1. Immunoaffinity purification of tyrosine kinase substrates from RSV-CEF. An extract (*A* and *D*) was applied to a column of py20 sepharose. The column was washed extensively and eluted with 5 mM phosphotyrosine. The unretarded protein (lanes *B* and *E*) and protein eluted with phosphotyrosine (lanes *C*, *F*, and *G*) were run on SDS-PAGE (7.5% gel), transferred to immobilon and stained with India ink (lanes *A–C*) followed by Western blot analysis with ^{125}I-py20 (lanes *D–G*). The position of phosphotyrosine-containing proteins reactive with anti-pp60^{src} (36) and anti-calpactin I (55) antibodies are indicated by arrows. For lanes *D–F*, exposure to the x-ray film was 6 h whereas a 30-min exposure is represented in lane *G* to compare directly the py20 reactivity with the protein (lane *C*) and the ^{32}P-labeled proteins (lane *H*). RSV-CEF cells were metabolically labeled with $^{32}PO_4$, extracted, and passed through a column of py20-sepharose. The ^{32}P-labeled protein was eluted with phosphotyrosine, subjected to SDS-PAGE, and transferred to immobilon. Lane *H* shows the pattern of phosphoproteins on the autoradiogram. Randomly selected ^{32}P-labeled bands (*a–f*, *right*) were excised from the blot, hydrolyzed, and processed for two-dimensional phosphoamino acid analysis. The positions of phosphoserine (*S*), phosphothreonine (*T*), and phosphotyrosine (*Y*) are shown for reference in *a*.

production of monoclonal antibodies to individual proteins. Antibodies to novel proteins of molecular masses of 215, 76, and 22 kD were detected. Immunofluorescence microscopy and detergent extraction results suggest that these proteins are present in discrete regions of the plasma membrane cytoskeleton.

Materials and Methods

Affinity Chromatography and Hybridoma Production

Primary cultures of chick embryo fibroblasts were infected with RSV and grown at 41°C in DME containing 2% tryptone phosphate broth and 4% calf serum. Cells were lysed directly on the tissue culture plate with buffer A (10 mM imidazole, 0.5 M NaCl, 1% Triton X-100, 0.2 mM vanadate, 0.2 mM PMSF, 2 mM NaN_3, pH 7.3) at 4°C for 30 min (6–7 ml buffer/150 mm plate). The solution was clarified by centrifugation (100,000 *g*, 1 h), and the soluble protein was incubated with 1.5 ml py20-sepharose/50 ml lysate of the specified monoclonal antibodies coupled to CNBr-activated sepharose 4B (4 mg antibody/ml sepharose). After 1 h at 4°C the sepharose was collected by centrifugation, loaded into a column, and washed extensively with buffer B (0.1 M NaCl, 10 mM imidazole, 0.1% Triton X-100). The phosphoproteins were then eluted with buffer B containing 5 mM phosphotyrosine, and the column was monitored by SDS-PAGE and Western blots using [^{125}I]-labeled py20 as described (26).

Protein from RSV-CEF cells that specifically bound to the anti-PY antibody column (see Fig. 1) was used to immunize mice for the production of monoclonal antibodies. Antigen injections consisted of 0.2–0.5 mg protein subcutaneously and intramuscularly at 1-mo intervals followed by four consecutive daily intravenous injections of 100–200 μg protein. 2 d after the last injection, the spleen was harvested, and cells were fused with the nonsecreting myeloma, PAI (27). Cells were grown in hypoxanthine aminopterin thymidine media and tested after 8 d by an ELISA assay using the antigen protein adsorbed to the wells of 96-well immulon II plates (Dynatech Laboratories, Inc., Alexandria, VA). Hybridomas testing positive by the ELISA asssay were tested by Western blots again on protein specifically bound to the anti-PY column. Selected hybridomas that tested positive by Western blots were cloned by limiting dilution and screened as above.

Other Methods

For immunofluorescence microscopy, cells were grown on glass coverslips and fixed 6 h after plating. For anti-76-kD antibodies, cells were fixed in 4% formaldehyde overnight at 4°C and permeabilized with 0.5% Triton X-100 for 5 min. Anti-22-kD and anti-215-kD antibodies were fixed and permeabilized by treatment with methanol/acetone (1:1, vol/vol) at −20°C for 5 min, and air dried. Coverslips were treated with the hybridoma culture supernatants described in the legend to Fig. 2 for 30 min at 37°C, washed

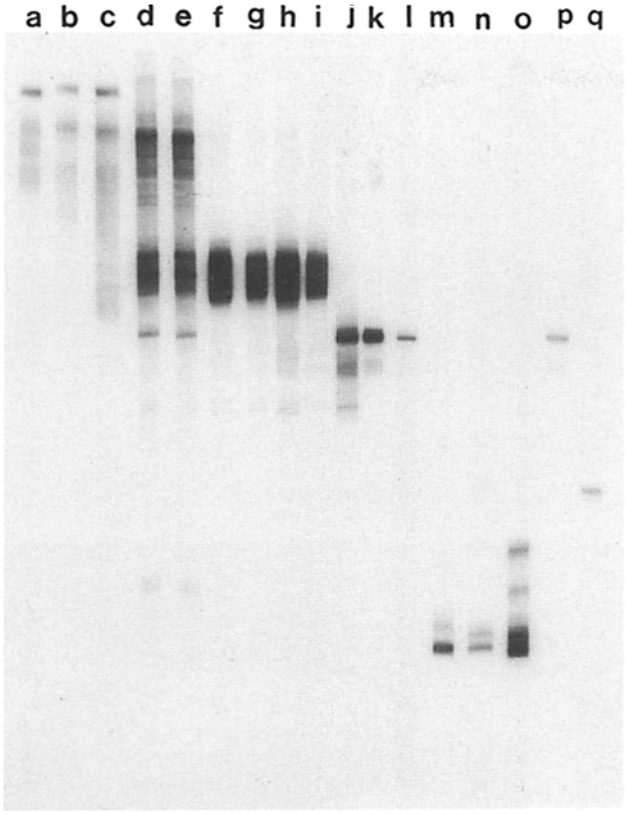

Figure 2. Monoclonal antibodies to proteins bound to the py20 column and eluted with phosphotyrosine. The protein specifically eluted from the py20 column (see lane *C* of Fig. 1) was used to immunize mice. Hybridomas derived from the fusion of immune spleen cells with a nonsecreting myeloma cell line were screened by ELISA and Western blots on the immunizing antigen. Cells were cloned and retested. This figure shows the results with 15 different monoclonal antibodies (lanes *a–o*) tested in a Western blot on protein bound to the anti-PY column. Protein was run on a single slot gel, transferred to immobilon, and strips were then treated with culture supernatants of cloned hybridomas (lanes *a–o*). Antibodies to $pp60^{v\text{-}src}$, 60 kD (lane *p*), or calpactin I, 38 kD (lane *q*), were included as markers. Reactivity was then assessed with ^{125}I-labeled second antibody followed by autoradiography.

and further treated with FITC anti-mouse antibodies. Controls using culture supernatant of the parental myeloma cells were performed in parallel and were always negative.

ELISA assays were performed essentially as described by Glenney et al. (27), and Western blots were as described by Glenney (23). ^{32}P-labeling and phosphoamino acid analysis were performed as done by Cooper and Hunter (11), as described previously (22). Detergent extractions were performed as by Zokas and Glenney (55) using a buffer containing 48 mM K_2HPO_4, 14 mM NaH_2PO_4, 45 mM KH_2PO_4, pH 7.2. Talin was purified by the method of Burridge and Connell (8).

Sucrose gradient fractionation was performed as follows: PY-proteins were isolated from RSV-CEF cells by extraction and affinity chromatography as described above. The PY-proteins were then concentrated with a centricon filter (Millipore Continental Water Systems, Bedford, MA) to 20% of the original volume. The protein was layered over a 5–20% sucrose gradient in buffer B and centrifuged 14 h at 45,000 rpm using a rotor (model SW60; Beckman Instruments, Inc., Palo Alto, CA). Fractions were collected from the bottom of the gradient (the pellet was resuspended separately and considered fraction 1); adjusted with SDS sample buffer; run on SDS-PAGE; transferred to immobilon; and stained with anti-PY, anti-76 or anti-22-kD, or anti-src (36) monoclonal antibodies. Reactivity was assessed using ^{125}I-labeled anti-mouse followed by autoradiography and quantitated with a densitometer.

Results

Primary cultures of RSV-CEFs were used as the starting material to isolate proteins phosphorylated on tyrosine residues. Anti-PY coupled to Sepharose removed >50% of the PY proteins that were then eluted from the columns with the inclusion of PY (Fig. 1). We compared the profile of proteins specifically eluted from the anti-PY column and stained with India ink to the proteins reactive with the anti-PY by Western blots and those metabolically labeled with ^{32}P. A good correspondence was observed between these profiles, suggesting that the majority of proteins bound and eluted from the anti-PY column are phosphoproteins. Phosphoamino acid analysis of randomly selected ^{32}P-labeled proteins demonstrated that the proteins contained PY although at different ratios to phosphoserine (Fig. 1). When normal and RSV-CEF proteins were metabolically labeled with [^{35}S]methionine and subjected to affinity chromatography, 0.6% of the total TCA insoluble radioactivity from RSV-CEF cells bound to the anti-PY antibody column whereas <0.1% of the radioactivity from normal fibroblasts bound to the column. None of the ^{32}P-labeled proteins that bound to the anti-PY column (Fig. 1) were observed when the same experiment was performed with normal chick fibroblasts (not shown) indicating that the tyrosine phosphorylations are transformation specific.

The mixture of proteins from RSV-CEF cells purified by immunoaffinity chromatography on the anti-PY column were used to immunize two BALB/c mice. The spleen cells from these mice were fused with a nonsecreting myeloma cell line, and the resulting hybrids were plated into 96-well tissue culture plates. Hybridoma culture supernatants were tested by an ELISA assay using test plates to which anti-PY-bound proteins were adsorbed. Antibodies that tested positive in this assay were further screened by immunoblotting against the affinity isolated tyrosine phosphoproteins. From two fusions, we observed several hundred hybridomas that gave rise to a positive reaction by the ELISA, and 50 of these antibodies were strongly reactive by Western blots. 15 different antibody secreting cells were then selected and cloned by limiting dilution for further study (Fig. 2).

The selected monoclonal antibodies reacted with proteins displayed on a Western blot (Fig. 2). Three antibodies reacted with a discrete high molecular mass (~215-kD) component and more diffuse lower molecular mass polypeptides (lanes *a*–c), reminiscent of proteolysis. These antibodies did not react with the 215-kD cytoskeletal protein talin isolated from chicken gizzard (8) when run in parallel lanes on the same blot (data not shown). Two antibodies react with a multitude of components between 60–200 kD (Fig. 2, lanes *d* and *e*). These may be reacting with shared epitopes such as the carbohydrate moiety on glycoproteins. Regardless of the reason for this broad specificity, these antibodies may be less useful than those that show a more limited reactivity. Four different antibodies react with a relatively diffuse component having an apparent molecular mass of 76 kD (lanes *f–i*). Three antibodies react with a 60-kD protein that co-migrates with the tyrosine kinase $pp60^{v\text{-}src}$ (compare lanes *j–l* to lane *p*). Finally, three antibodies (lanes *m–o*) react predominantly with a 22-kD component although they also react to a lesser extent with other higher molecular mass species. These higher species are not seen in total cell lysates (see Fig. 6).

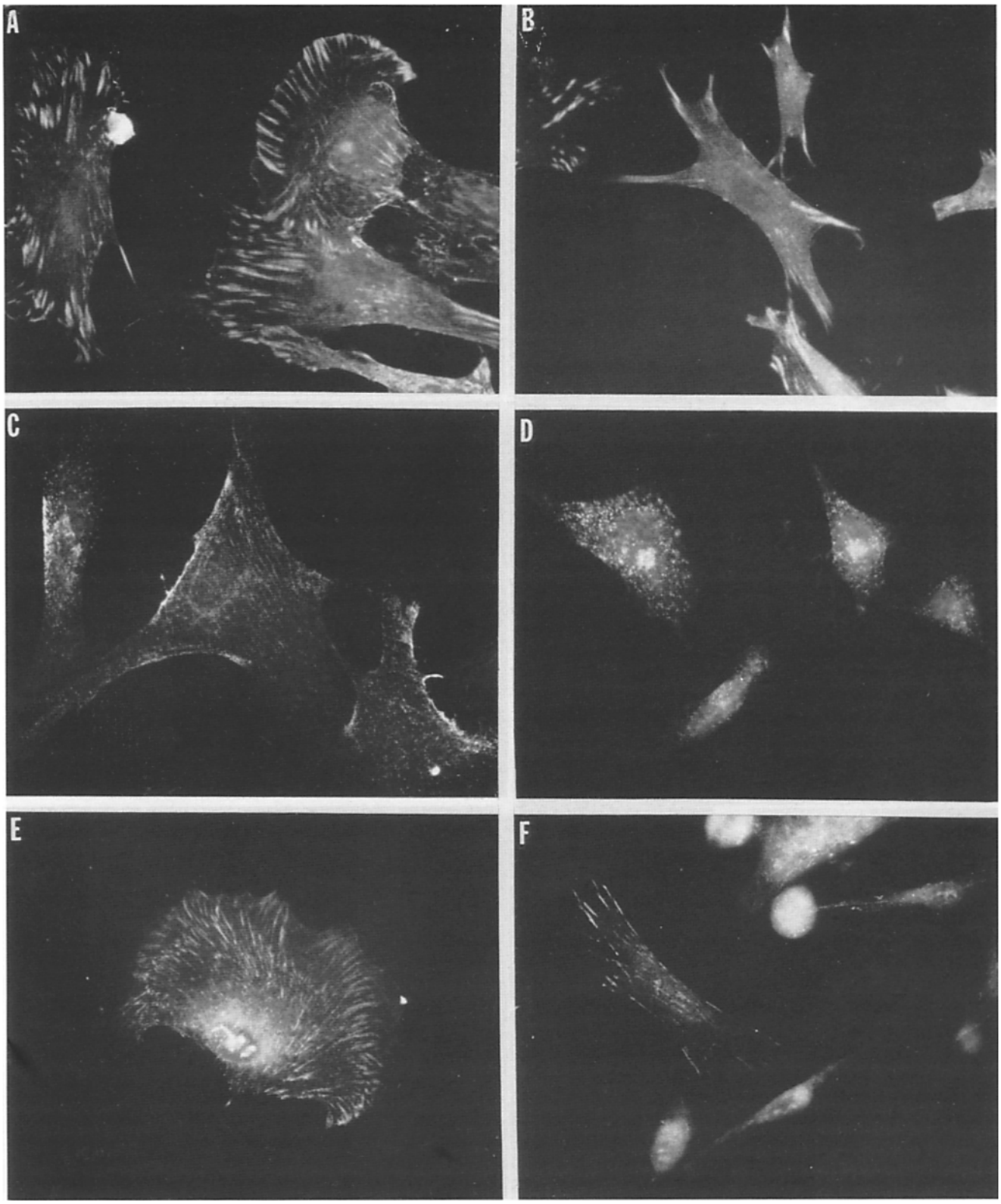

Figure 3. Immunofluorescence localization of antigens in normal and RSV-CEFs. Monoclonal antibodies against the 215-kD antigen (*E* and *F*), the 22-kD antigen (*C* and *D*), and the 76-kD antigen (*A* and *B*) (see Fig. 2, lanes *a*, *m*, and *f*, respectively) were used in indirect immunofluorescence microscopy on normal (*A*, *C*, and *E*) and RSV-transformed (*B*, *D*, and *F*) cells.

Antibodies that react with 215-kD (Fig. 2, lane *a*), 76-kD (Fig. 2, lane *h*), and 22-kD (Fig. 2, lane *m*) proteins were used to localize these components by immunofluorescence microscopy on normal and RSV-CEFs (Fig. 3). Antibodies to the 60-kD proteins have thus far been negative in immunofluorescence microscopy. The staining with anti–22-kD antibodies resulted in punctate fluorescence, in many cases concentrated in parallel arrays and at the cell margins.

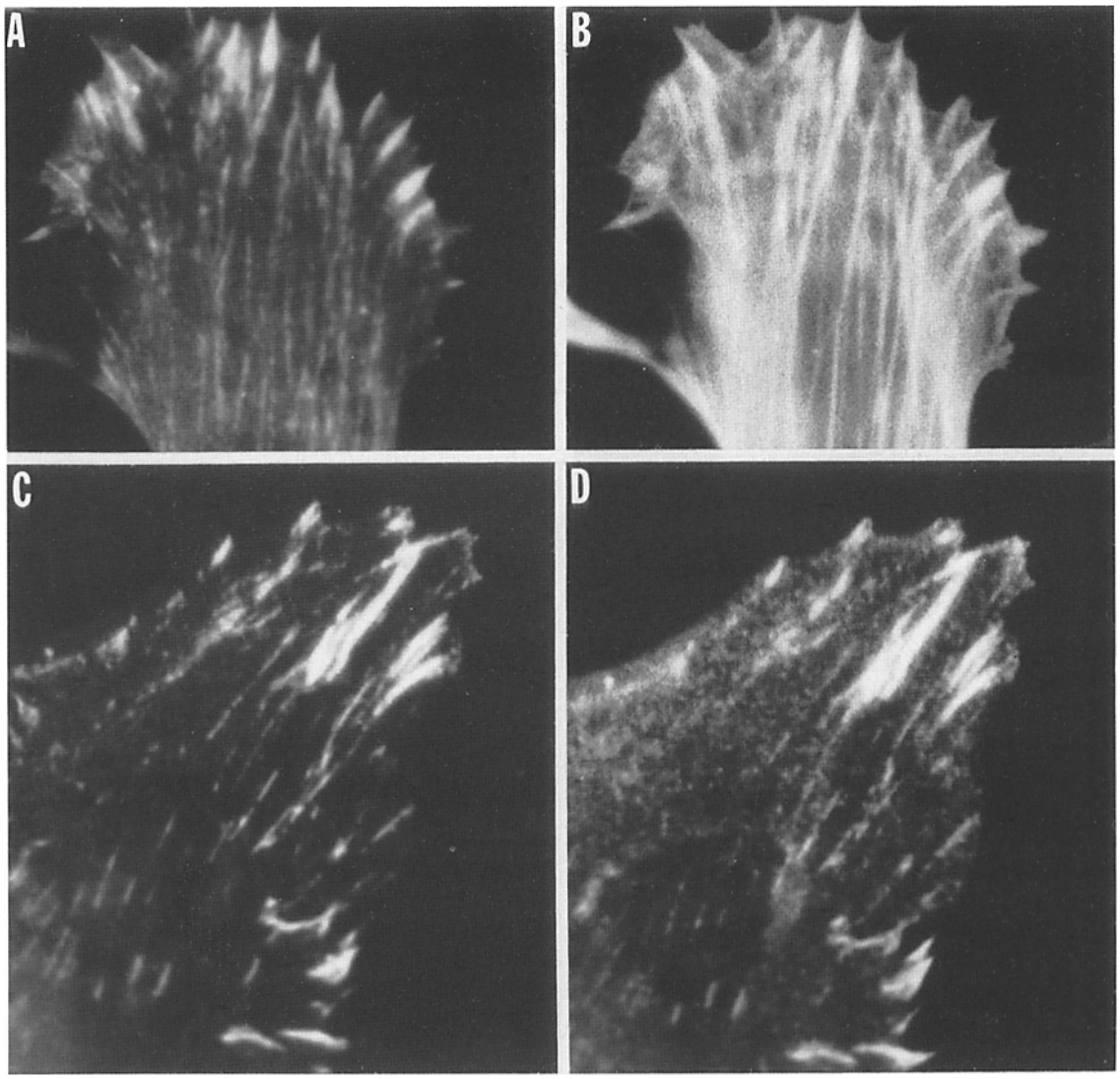

Figure 4. Double-label immunofluorescence microscopy with anti-76-kD antibodies. Anti-76-k monoclonal antibody was detected with a fluorescein anti-mouse second antibody (*A*, *C*) while the actin distribution was visualized with rhodamine-pholloidin (*B*). The distribution of vinculin was detected with rabbit anti-vinculin and rhodamine anti-rabbit second antibody (*D*). *A* and *B* represent images of the same cell as do *C* and *D*.

This staining gave the distinct impression of a submembranous location although unlike the fodrin or calpactin distribution previously observed in fibroblasts (55). In RSV-transformed cells, punctate fluorescence was again observed but without the concentrations at the margins or in parallel arrays.

Antibodies to both the 215-kD protein (Fig. 3, *E* and *F*) and to the 76-kD component (Fig. 3 *A* and *B*) reacted with structures predominantly on the ventral surface of the cell. Control monoclonal antibodies (see above) did not result in this pattern. The anti-76-kD staining was much more diffuse in transformed cells (Fig. 3). Since this antibody staining pattern was reminiscent of the staining of focal contacts, those specialized cell adhesions where actin filament bundles terminate close to the plasma membrane (3, 7, 19), we counterstained cells with rhodamine-conjugated phalloidin to detect the actin distribution or with anti-vinculin antibodies to observe the focal contact distribution. As shown in Fig. 4, anti-76-kD protein stained the ends of actin-containing stress fibers, giving rise to the so-called focal contact staining. This was confirmed by double label immunofluorescence that showed costaining of focal contacts with anti-76 kD and anti-vinculin.

The anti-76-kD antibodies were then used to immunoprecipitate a similar size phosphoprotein from ^{32}P-labeled RSV-CEF cells (Fig. 5). Higher molecular mass phosphoproteins were specifically coprecipitated as these bands were not observed when control mouse IgG was used (not shown). They may represent complexes with the 76-kD protein since they are not recognized by the antibody on a Western blot. Phosphoamino acid analysis demonstrated the presence of PY in the 76-kD protein (Fig. 5). Furthermore, it was found by Western blots that 20–30% of the total 76-kD protein in RSV-CEF cells would bind to the anti-PY column, pointing to a significant stoichiometry of tyrosine phosphorylation, while no 76-kD protein from normal CEF cells was bound to the column (not shown). Similarly, the immunoprecipitated 22-kD substrate protein has now been found to contain phosphotyrosine (not shown).

Since immunofluorescence microscopy suggested that these

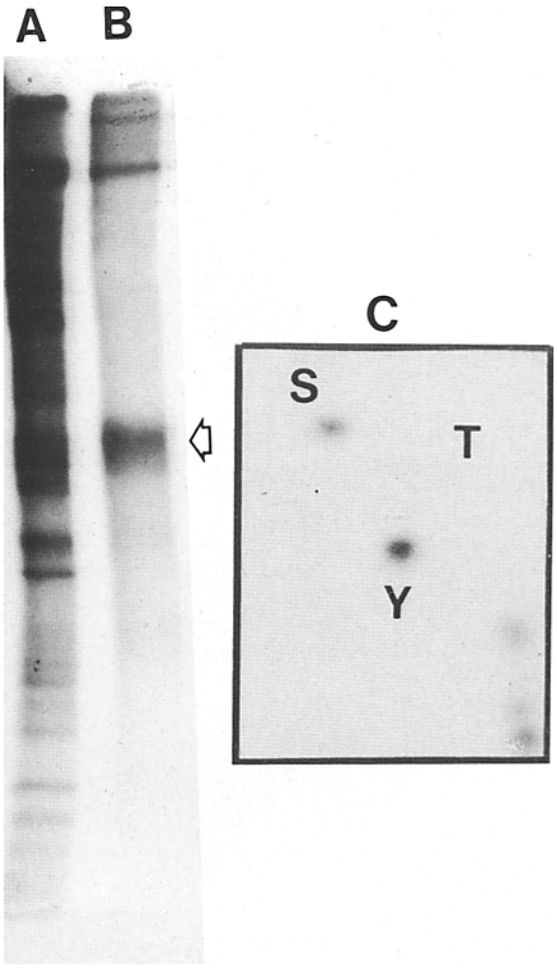

Figure 5. Immunoprecipitation of ^{32}P-labeled 76-kD protein. RSV-CEF cells were labeled overnight with $^{32}PO_4$, extracted, and the phosphotyrosine-containing proteins (*A*) were bound to the py20 antibody and eluted with phosphotyrosine. Anti-76-kD antibody was then used to immunoprecipitate the 76-kD antigen (*arrow*) that was subjected to phosphoamino acid analysis (*C*) as described in the legend to Fig. 2. When control mouse IgG was substituted, no precipitation of this band was observed.

antigens are in a cytoskeletal organization, we extracted cells with a detergent solution under conditions that stabilize the cytoskeleton. Surprisingly, two of the four anti-76-kD antibodies also recognized a 44-kD antigen in total cell lysates, but only the 76-kD protein was retained by the anti-PY column (Fig. 6). Fractionation of cellular protein into detergent soluble and cytoskeletal components revealed that ~50% of both the 76- and 44-kD proteins are soluble. By contrast, >90% of the 22-kD protein was found in the detergent resistant cytoskeleton under these conditions. There was no difference in the extractability of these proteins between normal and RSV-CEFs (not shown).

Discussion

The oncogenic tyrosine-specific protein kinases src (46) and abl (47) are found associated with focal contacts and the transforming ability correlates with its presence in the cytoskeleton (29). Many of the substrates of pp60^{src} are also known to be cytoskeletal proteins. These include calpactin, vinculin, talin, and integrin (see introduction for references). In addition, antibodies to phosphotyrosine have been shown to stain focal contacts in normal cells (9, 39). Indeed, alterations in the cytoskeleton are some of the earliest changes observed when cells become transformed (3, 43, 53, 54). Unfortunately, none of the previously described substrates are correlated with parameters associated with transformed cells (30, 37). Thus, although the cytoskeleton is tightly associated with the transforming ability of pp60^{src}, the relevant substrates have yet to be described.

The present study provides an alternative approach to the identification and study of cytoskeletal tyrosine kinase substrates. Rather than testing previously identified cytoskeletal proteins, we have generated monoclonal antibodies to a mixture of PY proteins phosphorylated in vivo and used the antibodies to test the association of these normal cellular proteins with other cellular structures. The anti-PY monoclonal antibody we have been using to isolate substrates has been found to be some 100-fold higher in affinity than the commercially available antibody 1G2 (Jamison and Glenney, manuscript in preparation). Previous studies demonstrated that this antibody would block the internalization of the activated epidermal growth factor receptor when microinjected into live cells (26). In the present study, we have used the anti-PY column to isolate the PY-proteins from RSV-CEFs followed by the generation of monoclonal antibodies to individual substrates. Given that the relevant substrates are also unknown for a variety of growth factor receptor tyrosine kinases, the approach presented here should find general application. Currently, we are using the same approach to generate antibodies to substrates of the epidermal growth factor receptor. Preliminary results have shown that the 76-kD protein described here is phosphorylated in mouse L cells that have been transfected with the epidermal growth factor receptor and treated with epidermal growth factor (data not shown).

In the present study, we have identified three new tyrosine-kinase substrate proteins of the membrane skeleton. A 215-kD protein (Fig. 2) is similar in molecular mass and subcellular distribution to another substrate, talin (8, 15, 42); however, antibodies to the 215-kD substrate did not react with gizzard talin (see Results). Antibodies to a 76-kD protein stain focal contacts in permeabilized cells (Figs. 3 and 4) and immunoprecipitate a PY protein from metabolically labeled transformed cells (Fig. 5). It is possible that the 76-kD protein is similar to an 82-kD protein present in focal contacts (4) or to the 82-kD Ca^{++} dependent protease that has also been found in focal contacts (5). Neither of these proteins has been reported to be phosphorylated on tyrosine in transformed cells. Two of the four anti-76-kD antibodies also recognize a 44-kD antigen among total cellular proteins. The relationship between the 76- and 44-kD proteins is unknown, but it seems unlikely that the 44-kD protein is derived from the 76-kD protein by proteolysis since the 44-kD protein is found in cells lysed in boiling sample buffer (Fig. 6) presumably denaturing endogenous proteases. Future studies will address the question of whether both the 76- and 44-kD proteins are present in focal contacts. The 76-kD protein is different in size than other known tyrosine kinase substrates. Anti-76-kD antibodies react with a broad band, suggesting

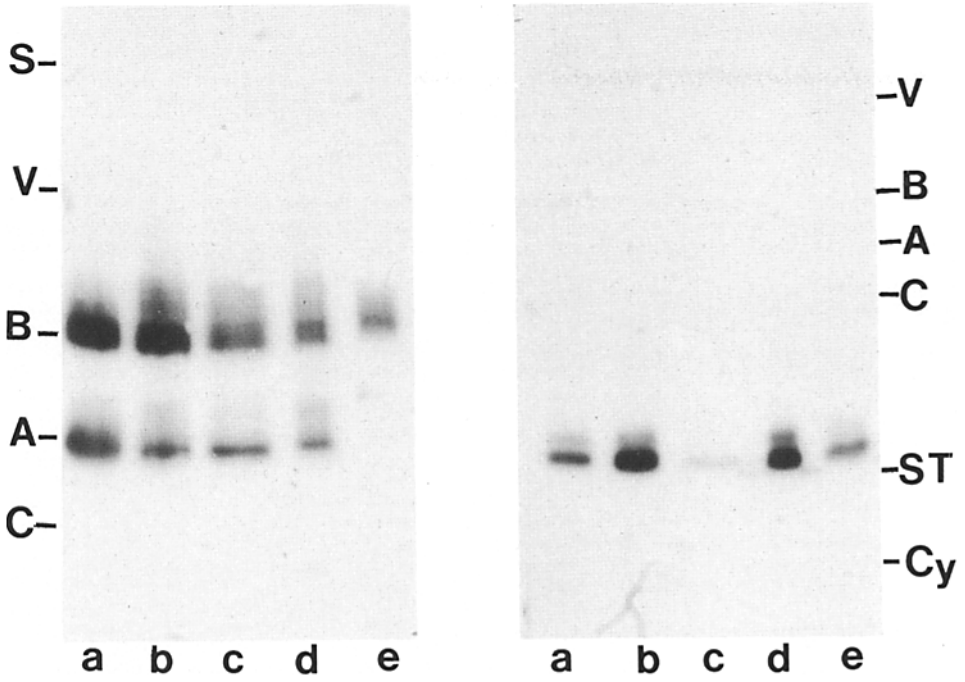

Figure 6. Identification of the 76- and 22-kD antigens in the cytoskeleton of RSV-CEF cells. Total CEF (*a*) or RSV-CEF (*b–d*) cells were lysed in SDS sample buffer (*a* and *b*) or a nonionic detergent solution and separated into an equal volume of soluble (*c*) and cytoskeletal (*d*) fractions and compared to the position of the antigen bound to the py20 column (*e*). Proteins were analyzed by Western blots using anti-76-kD (*left*, 7.5% gel) or anti-22-kD (*right*, 11.5% gel) antibodies. Lines indicate the position of the protein standards spectrin (*S*), vinculin (*V*), BSA (*B*), actin (*A*), calpactin (*C*), soybean trypsin inhibitor (*ST*), or cytochrome c (*Cy*) run on the same gel.

an extensive posttranslational modification such as glycosylation. The fibronectin receptor (integrin) is an example of an integral membrane protein that is thought to interact with cytoskeletal proteins inside the cell and to be phosphorylated on tyrosine residues (for review, see reference 33). Integrin polypeptides, however, have a significantly higher apparent molecular mass (31), and no integrin polypeptides of 70–80 kD have been described. Whereas integrin, vinculin, and talin are phosphorylated to a very low level (1%), preliminary studies have shown that 20–30% of the 76-kD protein is phosphorylated in RSV-transformed cells (see Results). We are currently analyzing the sites and extent of tyrosine phosphorylation of all of the proteins described here.

Antibodies were also generated to a 22-kD protein that was retained on the anti-PY column. The immunofluorescence staining pattern with anti–22-kD antibody is unlike any distribution previously seen. In normal fibroblasts, brightly fluorescent submembranous "patches" were found in parallel arrays and concentrated at the cell margins; while in transformed cells, no discernable organization of this punctate fluorescence was seen. All three of the anti–22-kD antibodies gave the same staining pattern. The cytoskeletal disposition of the 22-kD protein was confirmed by detergent extraction experiments (Fig. 6). Although the molecular mass of the 22-kD protein is similar to calmodulin, another of the tyrosine kinase substrates (18), calmodulin is not strictly cytoskeletal and is present at high levels in the brain, whereas the 22-kD protein is absent from brain (Glenney, unpublished observation). Furthermore, the 22-kD protein released with high salt sedimented in a position characteristic of a much higher native molecular mass (not shown). It is possible that the 22-kD substrate may be tightly associated with other subunits that may themselves be phosphorylated on tyrosine in transformed cells. Future studies will have to address the molecular associations that occur in these 22-kD–rich regions visualized by immunofluorescence microscopy.

The transforming ability of src requires an active protein tyrosine kinase activity in addition to the association with the membrane skeleton. Mutants of p60 have been generated, for instance, that lack the site of myristylation and are no longer associated with the membrane and cytoskeleton (14, 29, 37) and are not transforming. This has provided a crucial test of the relevance of the phosphorylation of known substrates to changes that occur in transformation. We are currently evaluating the phosphorylation of the substrates described here in cells expressing the nonmyristylated mutants of src. Preliminary results indicate that the phosphorylation of the 22-kD protein is correlated with transformation in this system. The approach described here should provide us with the tools needed to study the proteins that mediate the cytoskeletal changes in transformed cells.

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