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Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases

(transformed phenotype/phosphotyrosine/adhesion plaques/cytoskeleton/extracellular matrix)

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ABSTRACT **The fibronectin (FN) receptor in avian cells has been characterized previously as a complex of three membrane glycoproteins of about M_r 160,000, M_r 140,000, and M_r 120,000 (simply termed protein band 1, band 2, and band 3, respectively). Monoclonal antibodies to the band 3 protein of the complex prevent FN and laminin binding both *in vivo* and *in vitro* and enable the detection of the receptor proteins in the plasma membrane and in adhesion plaques. Association of the FN receptor proteins with the adhesion-plaque protein talin also has been reported. We now find that the band 2 and band 3 proteins in the complex are phosphorylated in Rous sarcoma virus-transformed chicken cells but not in normal chicken cells. Phosphorylation occurs predominantly on tyrosine and is accompanied by a reorganization of the receptor complex in the membrane of the transformed cells. Whereas normal cells contain the FN receptor in focal contacts and cellular processes between cells, v-*src*-transformed cells exhibit a more diffuse distribution of this receptor. In addition to the viral v-*src* oncogene, cells transformed by other avian oncogenes that also encode tyrosine kinases (v-*fps*, v-*erbB*, and v-*yes*) also express the receptor complex proteins in the phosphorylated state regardless of whether the transforming protein is detectable in adhesion plaques. These results suggest that the altered FN and laminin receptor proteins may contribute to the transformed phenotype, but their significance and role in the transformed state remain to be established.**

Transformation by Rous sarcoma virus (RSV) is initiated and maintained by the protein product of the viral v-*src* gene (1, 2). This protein, designated pp60^{src}, possesses an inherent tyrosine kinase activity essential for transformation and is situated along the cytoplasmic face of the plasma membrane and concentrated within cell–substratum adhesion sites (3). These latter sites are termed focal contacts or adhesion plaques and are associated either directly or indirectly with several functions that are altered in RSV-transformed cells. Focal contacts mediate not only the physical associations of cells to each other and to substrata (via extracellular molecules) but also serve as organizing centers for attachment of extracellular matrix protein and intracellular focal points for stress-fiber termination (4–10). These sites are very specialized regions of the plasma membrane, and proteins within these structures are logical targets for such RSV-induced alterations as decreased cellular adhesiveness, loss of cell surface fibronectin (FN), abnormal cell migration, dissolution of stress-fiber bundles, and ultimately the rounded transformed cell morphology.

Little is known of the molecular composition and complexity of adhesion plaques; however, several proteins have been identified as constituents of these structures (7, 11–16). Of these proteins, vinculin has received considerable attention because it was shown to contain increased levels of phosphotyrosine in RSV-transformed cells (17). It was initially speculated that vinculin linked stress fibers to the plasma membrane within focal contacts (7), and the increased phosphorylation of vinculin on its tyrosine residues might be responsible for the general decrease of stress-fiber organization seen in these transformed cells (17). Although attractive, the phosphorylation of vinculin was not related to actin cable organization (18–20), and it is possible that the phosphorylation of tyrosine in vinculin and several other cellular substrates of pp60^{src} is simply a fortuitous event (21).

Recently, several laboratories have identified an additional adhesion-plaque protein complex with properties consistent with its being a receptor for extracellular matrix proteins (22–30). *In vitro* experiments have demonstrated a direct association between this complex and FN (30–31). In the developing embryo, these receptor proteins are associated with the expression of FN (32) and are found in and around adhesion plaques of cultured fibroblasts (26, 32, 33). In highly locomotory embryonic cells, however, the receptor proteins are distributed diffusely over the cell surface (32).

In avian cells, this receptor complex binds to FN and laminin (31) and is a complex of three integral membrane glycoproteins of approximately M_r 160,000, M_r 140,000, and M_r 120,000 (simply designated bands 1–3, respectively, and collectively termed the FN receptor). A distinct site for binding talin, a cytoskeletal protein found in adhesion plaques, also is present in the receptor complex (34). These results suggest that this FN receptor may function as a transmembrane link between the extracellular matrix and the intracellular cytoskeleton via the adhesion plaques. As a first step in determining the role of this receptor complex in transformation mechanisms, we have explored the possibility that these receptor glycoproteins serve as potential substrates for oncogenes that encode tyrosine kinase.

MATERIALS AND METHODS

Cells, Viruses, and Immune Reagents. Chicken embryo fibroblasts (CEF) of phenotype C/E were obtained from H and N Laboratories (Redmond, WA) and were cultured as described (19). The following viruses were from standard laboratory stocks: Schmidt–Ruppin strains A and D of RSV (SRA and SRD, respectively), tsNY68, avian erythroblastosis virus (AEV), Fujinami sarcoma virus (FuSV), and Yamaguchi 73 virus (Y73). A monoclonal antibody (mAb) to the avian FN and laminin receptor is termed CSAT mAb

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Abbreviations: FN, fibronectin; RSV, Rous sarcoma virus; SRA, Schmidt–Ruppin A strain of RSV; SRD, Schmidt–Ruppin D strain of RSV; CEF, chicken embryo fibroblasts; SR-CEF, SRA transformed CEF; mAb, monoclonal antibody; AEV, avian erythroblastosis virus; FuSV, Fujinami sarcoma virus; Y73, Yamaguchi 73 virus.

(standing for cell substratum attachment) and has been described (23).

Radioactive Labeling. Cells grown on 60-mm plates were labeled metabolically with 100 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per ml for 4 hr in methionine-free medium. [^{32}P]Orthophosphate labeling was performed for 4 hr in the presence of 50 μM sodium vanadate (35) in phosphate-free medium. Cell surface labeling with ^{125}I was performed as described (36).

Immunoprecipitation. Cells were extracted on ice with 0.7 ml of CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} detergent buffer (10 mM Tris·HCl, pH 7.6/100 mM NaCl/2 mM EDTA/50 μM Na_3VO_4/0.5% CHAPS), and lysates were centrifuged at 15,600 × *g* to pellet particulates. The clear extracts were preabsorbed with *Staphylococcus aureus* for 30 min on ice and centrifuged again; aliquots of the lysate were treated with 4 μg of either CSAT mAb or normal mouse immunoglobulin as described (37). Immunocomplexes collected after a 30-min incubation with fixed *S. aureus* were washed three times with the CHAPS buffer, once with a high-salt buffer (1 M $MgCl_2$/10 mM Tris·HCl, pH 7.5/50 μM Na_3VO_4) and once with Nonidet P-40 buffer (10 mM Tris·HCl, pH 7.5/100 mM NaCl/ 0.5% Nonidet P-40). All steps to this point were conducted at 4°C. Samples were electrophoresed on 7.5% polyacrylamide gels as described but with methylenebisacrylamide as the cross-linker (37).

Immunofluorescence. Details of indirect immunofluorescence on methanol-fixed cells have been described (37).

Phosphoamino Acid Analysis. These analyses were performed exactly as described by Cooper and Hunter (38), as was the NaOH treatment of gels to enhance detection of phosphotyrosine-containing proteins.

RESULTS

Detection of FN Receptor in CEF and SRA-Transformed CEF (SR-CEF). Immunoprecipitates from [^{35}S]methionine-labeled CEF and SR-CEF were prepared with CSAT mAb or control mouse IgG and analyzed by $NaDodSO_4$/PAGE under nonreducing conditions (Fig. 1). Four bands labeled 1, 2, 3, and 4 were present in both CEF and SR-CEF and, in our gel system, corresponded to proteins of M_r 160,000, M_r 140,000, M_r 120,000, and M_r 110,000, respectively. Bands 1, 2, and 3 corresponded to the three distinct integral membrane glycoproteins previously shown to be precipitated from extracts of CEF by the CSAT mAb (23–25). Band 4 was not detected previously and may be either a precursor or proteolytic fragment of band 3 (see *Discussion*).

Analysis of the results shown in Fig. 1 by scanning densitometry revealed a quantitative increase in the incorporation of radioactivity into bands 1 and 2 (2-fold) and particularly into band 3 (4.5-fold) in SR-CEF compared to uninfected CEF. Immunoprecipitates were prepared from equal amounts of radioactivity in the CEF and SR-CEF extracts. The radioactivity detected in band 4 was the same in both CEF and SR-CEF. A decrease in the electrophoretic mobility of bands 2 and 3 also was observed in SR-CEF relative to CEF. This may be due to phosphorylation (see below) or to differences in glycosylation between the normal and transformed cells. Therefore, although both CEF and SR-CEF expressed the four protein bands of the FN receptor complex, both qualitative and quantitative differences in their expressions were detected between these normal and transformed cells.

Cell Surface Expression. The expression of the FN receptor on the surface of CEF and SR-CEF was assessed by cell surface iodination (36). Bands 1, 2, and 3 were each expressed on the surface of both CEF and SR-CEF (Fig. 2), while band 4 was not detectable at the cell surface. Band 2

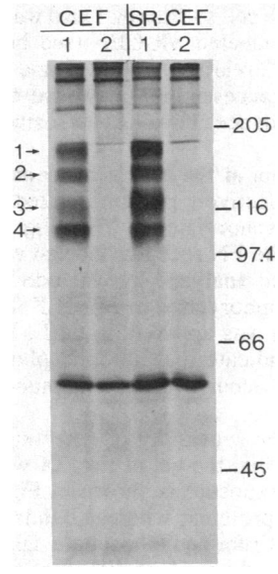

FIG. 1. Detection of FN receptor proteins in normal and RSV-transformed CEF. Normal CEF and SR-CEF were labeled for 4 hr with [^{35}S]methionine, and detergent extracts containing equal amounts of acid-precipitable radioactivity were treated with CSAT mAb (lane 1) or control mouse IgG (lane 2). Immunoprecipitates were separated on a 7.5% polyacrylamide gel under nonreducing conditions. Numbers on the left refer to protein bands detected, and numbers on the right represent the molecular mass × 10^{-3} of marker proteins.

appeared to be somewhat sharper and larger in molecular mass than did the corresponding [^{35}S]methionine-labeled protein. We believe this is due to the fact that the [^{35}S]methionine-labeled band 2 was comprised of two proteins separable on longer gels (not shown). The larger of these band 2 proteins (i.e., slower migrating) was the species

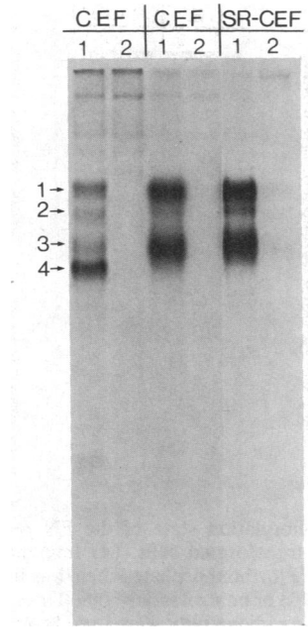

FIG. 2. Cell surface expression of proteins in the FN receptor complex. Detergent extracts of CEF and SR-CEF were treated with either CSAT mAb (lanes 1) or control mouse IgG (lanes 2). The left two CEF lanes were prepared from [^{35}S]methionine-labeled cells to mark the location of the four protein bands. The right four lanes were from cells whose surface proteins were labeled with ^{125}I.

detectable on the cell surface and also was the band 2 protein detected in ^{32}P-labeled SR-CEF (see below). The weaker labeling of band 2 relative to bands 1 and 3 may reflect either its more limited accessibility or exposure on the cell surface or perhaps abundance of available tyrosines and histidines for iodination.

Phosphorylation of the FN Receptor on Tyrosine. The FN receptor was selectively phosphorylated *in vivo* in SR-CEF but was not phosphorylated in CEF. Fig. 3*A* shows immunoprecipitates of the FN receptor labeled with [^{32}P]orthophosphate *in vivo* and analyzed by NaDodSO$_4$/PAGE. Bands 2 and 3 were phosphorylated in SR-CEF (lane 1), whereas no phosphorylation was apparent in CEF. Preliminary *in vitro* kinase assays indicate that autophosphorylation of the FN receptor did not occur within the immunocomplex (data not shown).

To increase the sensitivity of detecting phosphotyrosine-containing proteins, the gel in Fig. 3*A* was treated with hot alkali, and a reexposure is shown in Fig. 3*B*. CEF did not contain labeled proteins, whereas bands 2 and 3 of the FN receptor clearly retained phosphate label in SR-CEF. In addition, a trace amount of ^{32}P was detected in band 1. Phosphorylation of bands 2 and 3 appeared to be specific because band 4 was not labeled and band 1 contained only trace amounts of radioactivity.

Results of phosphoamino acid analysis of bands 2 and 3 from SR-CEF are shown in Fig. 3*C*. Band 2 contained approximately equivalent amounts of phosphotyrosine and phosphoserine. Band 3 contained a majority of phosphotyrosine, some phosphoserine, and a trace amount of phosphothreonine. Phosphoamino acid analysis was not performed on band 1 because too little radioactivity was incorporated.

Localization of the FN Receptor. The FN receptor was detected in methanol-fixed CEF and SR-CEF by indirect immunofluorescence. The results in Fig. 4*A* demonstrate the localization of the receptor protein complex in CEF, where

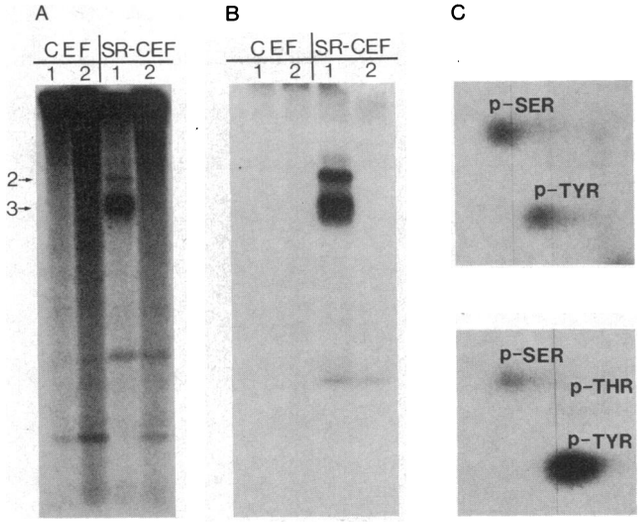

FIG. 3. Phosphorylation state of the FN receptor proteins in normal and RSV-transformed cells. (*A*) Immunoprecipitates were prepared from [^{32}P]orthophosphate-labeled cells by using either CSAT mAb (lanes 1) or control mouse IgG (lanes 2). Equal amounts of acid-precipitable radioactivity were used from CEF and SR-CEF extracts. (*B*) The same gel shown in *A* was treated with NaOH to increase detectability of phosphotyrosine-containing proteins. (*C*) Two-dimensional separation of phosphoamino acids in proteins recovered from SR-CEF shown in *A*. (*C Upper*) Protein band 2. (*C Lower*) Protein band 3. Phosphotyrosine, phosphoserine, and phosphothreonine are indicated. The vertical streaks are imperfections in the developed film.

it was found in the plasma membrane and in the vicinity of adhesion-plaque sites (Fig. 4*A Inset*) as described (23–25). It also was seen at contact points between cells and at cellular processes that stretch out toward other cells. These latter sites may represent points of FN attachment. In SR-CEF (Fig. 4*B*), the receptor antigen appeared to be more diffuse, in general, and not markedly associated with cell–substratum or cell–cell contact sites. High concentrations of FN receptor antigen were seen in the ruffled membranes of the SR-CEF. These results are similar to those reported for a FN receptor in normal vs. RSV-transformed mammalian cells (39).

Phosphorylation of the FN Receptor in Cells Transformed by Oncogenes that Encode Other Tyrosine Kinases. Cells infected with tsNY68, Y73, AEV, and FuSV were labeled with [^{32}P]orthophosphate *in vivo*, and immunoprecipitates with CSAT mAb are shown in Fig. 5. The phosphorylation of the FN receptor was temperature dependent in cells transformed by the tsNY68 temperature-sensitive mutant of v-*src*. At the permissive temperature (34°C), tsNY68-infected CEF contained phosphorylated band 2 and 3 proteins of the FN receptor (Fig. 5, lane 3) similar to those found in SR-CEF (lane 2). At the nonpermissive temperature (42°C) (lane 4), these bands were not phosphorylated, and only a background comparable to uninfected CEF was observed (lane 1). The phosphorylation of the FN receptor proteins on tyrosine residues correlates with the expression of pp60^{src} in adhesion plaques and the plasma membrane of tsNY68-infected CEF (3).

We next determined whether the same receptor proteins were phosphorylated in cells transformed by distinct but related avian sarcoma viruses. The transforming proteins of Y73 (P90$^{gag\text{-}yes}$), AEV (gp74^{erbB}), and FuSV (P140$^{gag\text{-}fps}$) all have tyrosine kinase activity, but each was derived from a unique protooncogene, and each exhibits a slightly different intracellular localization (40–42). P90$^{gag\text{-}yes}$ localizes in adhesion plaques and along the internal surface of the plasma membrane. gp74^{erbB} is homologous to the EGF receptor, and the mature protein is localized on the cell surface. P140$^{gag\text{-}fps}$ has been localized in the cytoplasm and on the internal surface of the plasma membrane. Cells transformed by P90$^{gag\text{-}yes}$ (Fig. 5, lane 5), gp74^{erbB} (lane 6), and P140$^{gag\text{-}fps}$ (lane 7) all showed phosphorylation of the FN receptor protein complex. In AEV-transformed CEF, the phosphorylation of bands 1 and 2 was increased with respect to band 3, whereas in Y73- and FuSV-transformed CEF, the phosphorylation of receptor proteins was similar to that seen in SR-CEF. These phosphorylations appeared to be specific because cells transformed by MC29, a virus that does not encode a tyrosine kinase, did not contain detectable phosphorylation of the receptor proteins (not shown). These results suggest that the FN receptor protein complex is a target of several tyrosine kinases, each of which exhibits a slightly different subcellular localization.

DISCUSSION

The results presented here demonstrate that a complex of proteins having both FN and laminin binding capabilities (24, 25, 31) is phosphorylated within cells transformed by a number of avian oncogenes that encode tyrosine kinase proteins. An increase in both phosphotyrosine and phosphoserine was observed in the FN receptor proteins of cells transformed by any of RSV, FuSV, Y73, or AEV (unpublished data). Three of the receptor proteins, bands 1–3 (M_r 160,000, M_r 140,000, and M_r 120,000, respectively), are expressed on the surface of both normal and transformed cells, and two of these species (bands 2 and 3) are selectively phosphorylated in the transformed cells but not in the normal cells. Also, we observed that band 2 is resolved into two

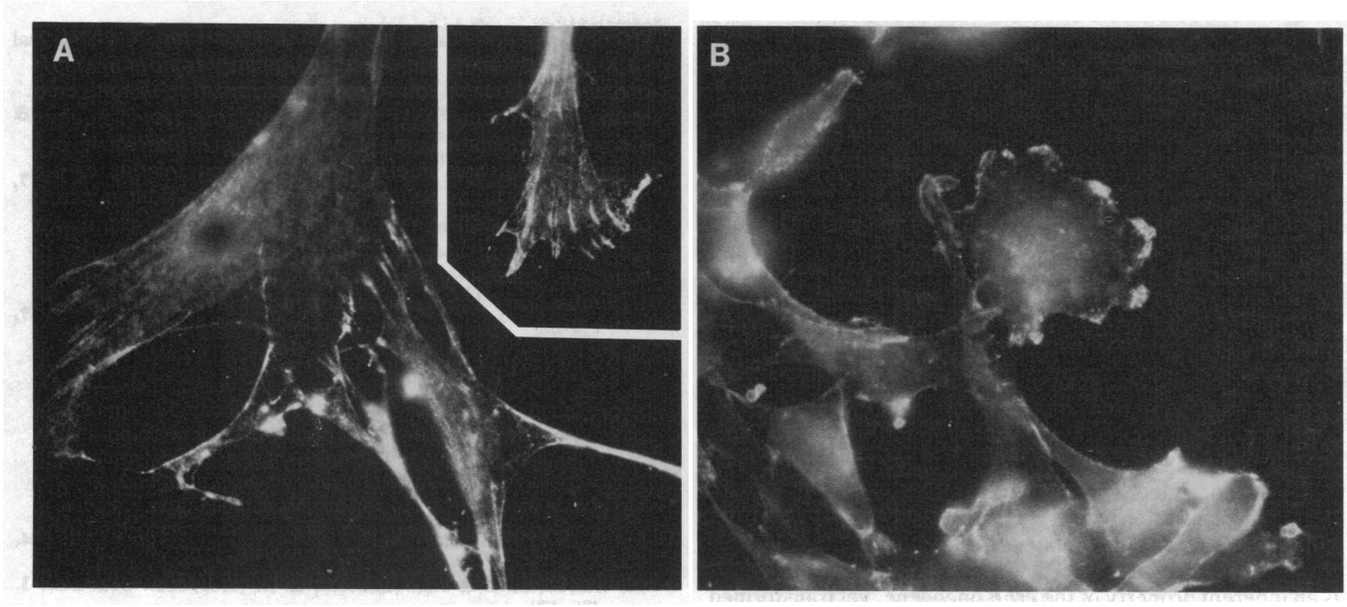

FIG. 4. Cellular distribution of the FN receptor complex in normal and RSV-transformed cells. Indirect immunofluorescence with CSAT mAb (25 μg/ml) was used to detect the receptor protein antigen in methanol-fixed normal CEF (*A*) and SR-CEF (*B*).

species on longer gels. The slightly slower migrating protein of this band is the species that is cell-surface-expressed and phosphorylated in the transformed cells (unpublished observation). Another protein (band 4) of about M_r 110,000 was detected in our experiments that has not been reported previously (22, 26). Band 4 may be derived by proteolysis from one of the other bands; however, it behaves similarly to band 3 on reelectrophoresis under reducing conditions, is still detectable in the presence of protease inhibitors, and is neither phosphorylated nor expressed on the cell surface (unpublished observations). We believe band 4 may be a precursor to the band 3 protein.

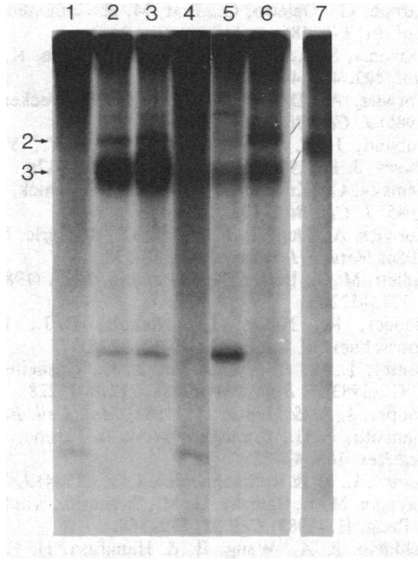

FIG. 5. Phosphorylation of the FN receptor proteins in cells transformed by a temperature-sensitive mutant of v-*src* or by v-*yes*, v-*erbB*, or v-*fps* oncogenes. Cells were labeled for 4 hr with [^{32}P]orthophosphate, and detergent extracts were treated with CSAT mAb. Immunocomplexes were electrophoresed on a 7.5% polyacrylamide gel. Lanes: 1, uninfected CEF; 2, SR-CEF; 3, tsNY68-infected CEF at the permissive temperature (34°C); 4, tsNY68-infected CEF at the nonpermissive temperature (42°C); 5, Y73 (v-*yes* oncogene)-transformed CEF; 6, AEV (v-*erbB* oncogene)-transformed CEF; 7, FuSV (v-*fps* oncogene)-transformed CEF. Lane 7 was from a separate gel. The migration positions of bands 2 and 3 of the receptor complex are shown on the left.

The three proteins comprising the avian FN receptor presumably span the plasma membrane, with the extracellular domain binding to FN and laminin and the intracellular domain forming attachments to the cytoskeleton through talin (34). These proteins appear to function as an oligomeric complex, perhaps a heterotrimer (24, 25, 31). Phosphorylation of the tyrosine residues of the band 2 and 3 receptor proteins presumably occurs within their cytoplasmic domain because this is the principal cellular localization of the tyrosine kinase-containing proteins (43). The increased phosphorylation of serine residues of band 2 and 3 proteins also may occur within the cytoplasmic domain, and phosphorylations of both serine and tyrosine could regulate the various functions of the FN receptor complex.

It is surprising that neither of the two phosphotyrosine-containing proteins of the FN receptor was detected previously. RSV-transformed cells have been examined extensively for the presence of new phosphotyrosine-containing proteins (21), and the glycoproteins of these cells have been specifically analyzed by lectin column methods (44). The fact that the band 2 (M_r 140,000) and band 3 (M_r 120,000) proteins were not detected may be explained by the possibility that these glycoproteins are not easily resolved or readily detectable by two-dimensional gel methods. Phosphorylated vinculin and pp60^{src}, both known to contain phosphotyrosine in RSV-transformed cells, have been difficult to detect by similar analyses (38). Also, glycoproteins analyzed on lectin columns may be overlooked if they lack the exact carbohydrate configuration for binding or if they bind too tightly and are not eluted. In addition, the amount of phosphorylated FN receptor relative to other phosphoproteins (especially in the absence of vanadate) may be low, making detection difficult.

Although we do not yet know whether the FN receptor proteins are direct substrates for pp60^{src} or other related avian oncogene proteins, there are several pieces of evidence that point in this direction. First, both pp60^{src} and the cytoplasmic domains of the FN receptor colocalize. This is most evident in the adhesion plaques and probably extends to the plasma membrane distribution as well. Second, the FN receptor proteins are phosphorylated on tyrosine upon RSV transformation, and the phosphorylation was temperature dependent in cells infected with a temperature-sensitive *src*

mutant. Phosphorylation of the receptor proteins was not detectable in cells transformed by an oncogene (*myc*) that does not encode a protein with tyrosine kinase activity (unpublished data). This supports an enzyme–substrate relationship but does not prove it. Alternatively, this could reflect a secondary event resulting from transformation. Third, fortuitous phosphorylations occur with several other phosphotyrosine-containing proteins found in RSV-transformed cells, and this also could account for the FN receptor phosphorylation. This seems less likely, however, because substrates for pp60^{src} are believed to reside in the plasma membrane (45); also, there is a logical reason to suspect that alterations of the FN receptor complex could be involved in the transformation mechanism. Fourth, specificity of FN receptor phosphorylation differs from the specificity of vinculin phosphorylation. In cells transformed by the *fps* oncogene of FuSV, the two proteins of the receptor complex are phosphorylated, whereas vinculin, which is also found in adhesion plaques, is not hyperphosphorylated on tyrosine (20). A final fifth point relates to the FN receptor proteins within AEV-transformed cells. Here, tyrosine kinase activity is an inherent property of the *erbB* oncogene, yet transformed cells and even specific phosphotyrosine-containing proteins exhibit only marginal increases in relative abundance of phosphotyrosine (46, 47). However, proteins of the FN receptor demonstrated a dramatic increase in phosphorylation with an even more noticeable increase in phosphorylation of the M_r 140,000 (band 2) species over the M_r 120,000 (band 3) protein species.

Both pp60^{src} and FN receptor proteins are adhesion-plaque proteins, and potential phosphorylations could occur within these subcellular sites. This need not be the general case, however, since the *fps* gene product does not localize to adhesion plaques (unpublished observation), yet cells transformed by *fps* contain phosphorylated FN receptor proteins. However, both the *fps* protein and receptor proteins do occupy locations along the cytoplasmic face of the plasma membrane as does pp60^{src} (43). This may suggest that phosphorylation by the tyrosine kinase activities of oncogene proteins could occur while in the more fluid state of the plasma membrane.

An important aspect of these results is that they may lead potentially to a molecular explanation of specific transformation parameters induced in RSV-infected cells. The FN receptor complex functions both as a receptor for extracellular matrix proteins (22–31) and as a link with cytoskeletal elements (34). Several alterations within RSV-transformed cells are associated with these structures. Certainly the loss of matrix FN and general stress-fiber disorganization are two primary transformation parameters that should be analyzed in relation to FN receptor phosphorylation. Fusiform mutants of RSV that transform but still express matrix FN will be valuable in this respect (48). More subtle variations in cell behavior (migration and metastasis) also may result from FN receptor phosphorylation.

Overall, the data presented in this paper suggest that the FN receptor protein complex may be a substrate target for the actions of a number of oncogenes that encode tyrosine kinase proteins. The results are intriguing and open new avenues of investigation.

Note Added in Proof. Analysis of the cloned gene for the CSAT band 3 protein has shown that it is a transmembrane protein with a tyrosine phosphorylation acceptor site within the cytoplasmic domain (49).

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