

Phosphorylation of Cellular Proteins in Rous Sarcoma Virus-Infected Cells: Analysis by Use of Anti-Phosphotyrosine Antibodies

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The protein substrates for the tyrosine protein kinases in cells transformed by avian sarcoma viruses were analyzed by gel electrophoresis in combination with immunoblotting or immunoprecipitation by antibodies against phosphotyrosine. We found that >90% of phosphotyrosine-containing cellular proteins can be immunoprecipitated by these antibodies. The level of phosphotyrosine-containing cellular proteins detectable by this method markedly increased upon transformation with Rous sarcoma virus, and more than 20 distinct bands of such proteins were found in lysates of Rous sarcoma virus-transformed cells. Most of these phosphotyrosine-containing proteins had not been identified by other methods, and their presence appeared to correlate with morphological transformation in cells infected with various Rous sarcoma virus mutants and Y73, PRCII, and Fujinami sarcoma viruses. However, considerably different patterns were obtained with cells infected with nontransforming Rous sarcoma virus mutants that encode nonmyristylated *src* kinases, indicating that most substrates that correlate with transformation can only be recognized by p60^{v-src} associated with the plasma membrane.

Cell transformation by Rous sarcoma virus (RSV) is mediated by the *v-src* gene product, p60^{v-src}, a tyrosine protein kinase (5, 20, 30) which is primarily located at the internal face of the plasma membrane (10, 23, 28). While a number of cellular proteins have been shown to be phosphorylated in RSV-transformed cells (7, 8), it remains largely unclear whether these proteins represent major substrates of the *src* kinase or whether their phosphorylation is critical for cell transformation. The analysis of phosphoamino acids of total cell proteins has indicated that phosphotyrosine (PTYR) represents only 0.03% of the total phosphoamino acids in proteins in uninfected chicken embryo fibroblasts (CEF), but that this level increases about 10 times upon transformation by RSV (20). Therefore, tyrosine phosphorylation should serve as an excellent marker for identification of cellular substrates of p60^{v-src} protein kinase.

Proteins phosphorylated on tyrosine residues have been found in RSV-infected cells by three different approaches. First, some proteins, such as p50 (20, 39) or a 120-kilodalton (kDa) protein (29), were found to be associated with p60^{v-src}. Second, certain proteins, e.g., calmodulin (14), vinculin (45), ezrin (16), talin (41), and fibronectin receptor (19), have been shown to contain increased levels of PTYR by analysis of the proteins purified or immunoprecipitated with antisera specific to each protein. Third, a more general approach to obtain a picture of overall changes in phosphorylation of cellular protein has been the analysis of phosphoproteins by two-dimensional gels (7, 42). After separation of proteins, the gels were treated with alkali to distinguish phosphorylation of tyrosine from that of serine and threonine residues (7). A 36-kDa protein (42) and glycolytic enzymes (9) have been recognized by this method. However, proteins analyzed by the two-dimensional gels were limited to those having neutral pI and of relatively low molecular weight (7).

Attempts were made to develop specific sera that recognize PTYR residues (6, 13, 38, 40, 43; 47) in order to identify PTYR-containing proteins more systematically. We have examined the utility of these sera in analysis of substrates of

p60^{v-src} kinase. In this paper, we report that these antisera can effectively recognize most, if not all, PTYR-containing proteins. Using this technique, we were able to identify a number of new proteins as substrates, some of which appear to be specific for transforming virus-infected cells.

MATERIALS AND METHODS

Cells and viruses. CEF were prepared, maintained, and infected as described previously (17). The viruses analyzed included a wild-type strain of RSV, Schmidt-Ruppin strain (SR-RSV); a deletion mutant of SR-RSV, NY309 (10); nonmyristylation mutants NY314 and NY315 (10); temperature-sensitive mutants *ts*NY68 (25) and *ts*NY72-4 (33); *c-src*-containing virus NY5H (31); transforming mutants of *c-src*-containing viruses, NYCHB and NY501T7 (21, 31); recovered avian sarcoma viruses rASV157 and rASV1702 (15, 27); Fujinami sarcoma virus (FSV) (12, 18); and avian sarcoma viruses Y73 (26), 16L (36), and PRCII (3, 37). Efficiency of infection was examined by [³H]leucine labeling and immunoprecipitation of oncogene products as described before (17).

Preparation of anti-PTYR antibodies. Anti-PTYR antibodies were prepared by two methods. First, antibody against *N*-bromoacetyl-*O*-phosphotyramine was prepared as described previously (40). Antibody was obtained in rabbits and affinity purified with *O*-phosphotyramine-coupled Sepharose (40). Second, as described by Wang (47), *v-abl*-encoded protein expressed in bacteria, which can autophosphorylate on tyrosine residues (48), was used to immunize rabbits, and antibody specifically recognizing PTYR was purified from the sera by affinity chromatography, using PTYR-coupled Sepharose as described previously (13, 47).

Immunoblotting. Cell cultures were washed with ice-cold Tris-buffered saline, scraped off the plate, quickly centrifuged, and frozen with dry ice and ethanol. Frozen pellets were suspended in Sol buffer (10 mM Tris hydrochloride, pH 7.4, 1% sodium dodecyl sulfate [SDS], 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 1 mM phenylmethylsulfonyl fluoride) which was immediately heated in boiling water for 5 min. Concentration of protein was assayed with

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a protein assay kit (Bio-Rad Laboratories), and 100 μ g of proteins was analyzed by SDS-7.5% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were transferred electrophoretically to a nitrocellulose filter with a transfer buffer (25 mM Tris glycine, pH 8.6, 20% methanol) and stained with anti-PTYR followed by 125 I-labeled protein A (Amersham Corp.), as described before (44, 46, 47). The molecular weight of stained bands was estimated by comparison with 14 C-labeled protein molecular weight standards (Bethesda Research Laboratories, Inc.). Autoradiography was performed on X-ray film with an intensifying screen at -80°C for 24 h, unless indicated otherwise.

Immunoprecipitation of ^{32}P -labeled proteins. Cells were labeled with ^{32}P , as described previously (12). Cell proteins were extracted with Sol buffer and heated in boiling water as described for immunoblotting. One milliliter of RIPA buffer without SDS (10 mM Tris hydrochloride, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 1% Trasylol, 1 mM phenylmethylsulfonyl fluoride) was added to the lysate in 100 μ l of Sol buffer, and the mixture was clarified by centrifugation at $15,000 \times g$ for 15 min. A 10- μ g portion of affinity-purified anti-PTYR or anti-vinculin was added to the supernatant, which was immunoprecipitated as described previously (1, 10). Proteins immunoprecipitated were analyzed by SDS-PAGE.

Partial protease mapping by *Staphylococcus aureus* V8. Analysis of peptides by partial digestion with *S. aureus* V8 protease was described previously (35).

Phosphoamino acid analysis. ^{32}P -labeled cell lysates were extracted with chloroform, and proteins in interphase between chloroform and buffer were precipitated with ethanol. ^{32}P -labeled proteins immunoprecipitated with anti-PTYR were eluted from the immune complex with 10 mM phenylphosphate in RIPA buffer and were precipitated with ethanol. Proteins remaining in the supernatant after immunoprecipitation were treated with chloroform and precipitated in ethanol. Proteins recovered by ethanol precipitation were subjected to tryptic digestion followed by acid hydrolysis as described before (12). Hydrolysates were analyzed by two-dimensional thin-layer electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension as described previously (20). ^{32}P -labeled phosphoamino acids were detected by autoradiography.

RESULTS

Characterization of antibodies against PTYR. We have prepared anti-PTYR by two different methods. The first preparation (anti-PTYR_{tyrm}) was raised against *O*-phosphotyramine conjugated with keyhole limpet hemocyanin as an antigen by the method of Pang et al. (40). The second (anti-PTYR_{abl}) was produced against a *v-abl* oncogene-encoded protein expressed in bacteria as described by Wang (47). Both antibodies were purified by affinity chromatography with phosphotyramine- or PTYR-coupled Sepharose. The two preparations of antibodies reacted in essentially the same manner to proteins from RSV-transformed cells in the experiments described below. Therefore, we do not distinguish between these two antibody preparations in the text.

Uninfected CEF or rat 3Y1 cells and their RSV transformants were first analyzed for PTYR-containing proteins by immunoblotting. Cell proteins were denatured by boiling immediately after extraction with SDS-containing buffer. Many bands were detectable in lysates from RSV-trans-

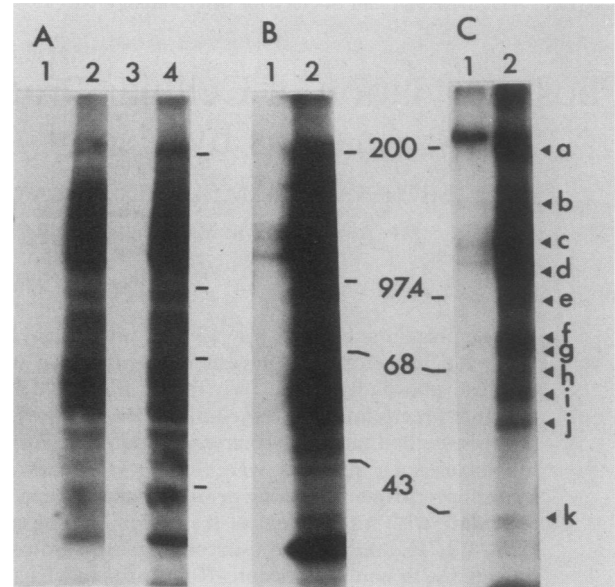


FIG. 1. Detection of PTYR-containing proteins in RSV-transformed CEF and 3Y1 cells with two types of anti-PTYR antibodies. (A) A 100- μ g portion of each cell lysate was analyzed by immunoblotting with anti-PTYR_{tyrm} (lanes 1 and 2) or anti-PTYR_{abl} (lanes 3 and 4). Lanes 1 and 3, Uninfected CEF; 2 and 4, SR-RSV-transformed CEF. (B) Immunoblotting of cell lysate of uninfected rat 3Y1 cells (lane 1) and SR-RSV-transformed 3Y1 cells (lane 2). (C) ^{32}P -labeled cell lysates from uninfected CEF (lane 1) and CEF transformed with SR-RSV (lane 2) immunoprecipitated with anti-PTYR and analyzed by SDS-PAGE. Proteins: a, 215; b, 150; c, 130; d, 110; e, 95; f, 82; g, 78; h, 65; i, 61; j, 54; and k, 42 kDa. Positions of protein molecular mass standards (200, 97.4, 68, and 43 kDa) are indicated.

formed cells (Fig. 1), whereas almost nothing was shown in lysates from uninfected cells by the same exposure of the films. Both antibody preparations recognized more than 20 bands from transformed cells, with the most intense bands at about the 110- to 150-, 78- to 84-, 61- to 65-, and 34-kDa regions. Analysis of gels with shorter exposures revealed that the 150-, 130-, and 110-kDa bands each consists of more than one band. After an extended exposure, bands of 180, 150, 130, 65, 54, and 34 kDa were detectable in uninfected CEF (data not shown). Binding of these antibodies to the cellular proteins was inhibited with PTYR or its analog phenylphosphate, but not with phosphoserine or phosphothreonine (data not shown).

We examined the efficiency of blotting of substrates to nitrocellulose filters by transferring ^{32}P -labeled bands immunoprecipitated with anti-PTYR as described later. Since we detected only a few weak signals of less than 34 kDa, 7.5% gels were used to facilitate the transfer of high-molecular-weight proteins. We found that a basic buffer (pH 8.6) facilitated the transfer. Under these conditions, the blotting was nearly complete except for some high-molecular-weight bands (150,000, 130,000, and 110,000), about one-tenth of which still remained in the gels after transfer. Therefore, in immunoblotting, the intensity of these bands was slightly underestimated.

Immunoprecipitation of ^{32}P -labeled proteins by anti-PTYR. To further examine the specificity of the antibodies, lysates from cells labeled with ^{32}P were immunoprecipitated with anti-PTYR. To avoid possible modification of proteins, such as by phosphorylation, dephosphorylation, and proteolysis

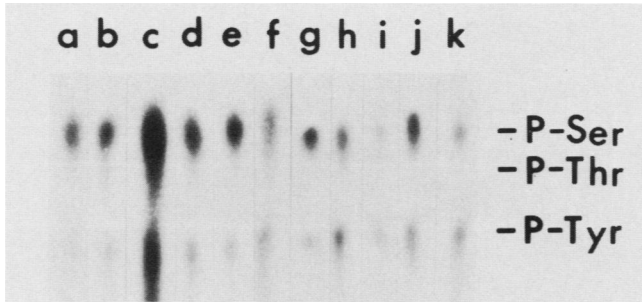


FIG. 2. Analysis of phosphoamino acid of proteins immunoprecipitated by anti-PTYR. Protein bands shown in Fig. 1C (lane 2, indicated by arrowheads) were eluted and analyzed. P-Ser, Phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

during immunoprecipitation, lysates were also heated at 100°C in the presence of SDS to inactivate cellular enzymes as in the case of immunoblotting, and then a nonionic detergent buffer was added to adjust the buffer to a composition suitable for immunoprecipitation. This extraction method was also found to be effective in solubilizing PTYR-containing proteins. Compared with extraction with RIPA buffer, we recovered approximately 50% more radioactivity in lysates by this method, and the protein profile was more reproducible than in the case of RIPA-extracted samples. Compared with immunoblots, immunoprecipitated samples gave similar protein species, but they were less distinct (Fig. 1C). While bands detected by immunoblotting represent only PTYR, those obtained by immunoprecipitation contain both phosphoserine and PTYR (see Fig. 2). This difference contributes to the difference of the profiles obtained by these two methods. At least 11 bands were discernible in precipitates, and these ^{32}P -labeled proteins, except for the largest (about 220 kDa), could be completely eluted from the immune complex by 10 mM phenylphosphate, indicating that the antibody directly recognized PTYR. Phosphoamino acid analysis of these protein bands confirmed that they contained PTYR (Fig. 2).

To further examine the efficiency of immunoprecipitation with anti-PTYR, the level of phosphoamino acids was analyzed for proteins in the pellet and supernatant fractions. Most of PTYR detected in the whole-cell lysate was found in the precipitate (Fig. 3), while <8% of the PTYR in whole lysates was found in the supernatant after two cycles of immunoprecipitation. These results indicate that the anti-PTYR recognizes almost all PTYR-containing proteins irrespective of the structures surrounding the PTYR residues. Interestingly, we found a very low level of phosphothreonine in anti-PTYR immunoprecipitates, suggesting the possibility that phosphorylations of tyrosine and threonine are mutually exclusive in a given protein.

The 130- and 110-kDa bands precipitated by anti-PTYR (Fig. 1, bands b and c) were subjected to partial digestion with *S. aureus* V8 protease, and the fragments produced were compared with the V8 fragments of vinculin obtained by immunoprecipitation with antivinculin (1). V8 profiles of the 130- and 110-kDa proteins (Fig. 1C, bands c and d) were different from that of vinculin (Fig. 4). A similar comparison showed that a 54-kDa protein (Fig. 1C, band j) precipitated by anti-PTYR is identical to p60^{v-src} in V8 cleavage pattern. V8 partial digestion analysis also revealed that the 82- and 78-kDa proteins (Fig. 1C, bands f and g) are related to each other, while the 130-, 110-, 82-, 65-, and 54-kDa species (Fig. 1C, bands c, d, f, h, and j) appear to be unrelated (Fig. 4).

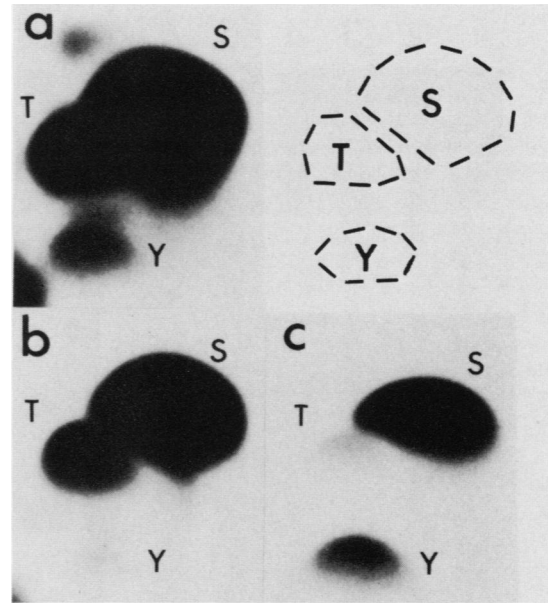


FIG. 3. Phosphoamino acid analysis of proteins in the precipitates and supernatants after immunoprecipitation with anti-PTYR. ^{32}P -labeled lysate of RSV-transformed CEF was immunoprecipitated with anti-PTYR. Phosphoamino acids were separated by two-dimensional electrophoresis on thin-layer plates: a, cell lysate before immunoprecipitation; b, supernatant after two cycles of immunoprecipitation; c, immunoprecipitated fraction. S, Phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

(Although vinculin and p60^{v-src} have been reported to have molecular weights of 130,000 and 60,000, molecular weights of these proteins immunoprecipitated with specific antibodies were 115,000 and 54,000 on the basis of their mobility compared with molecular weight standards in 7.5% SDS-PAGE under the conditions used. All molecular masses described in this paper are based on this comparison.)

Temperature-dependent phosphorylation of substrates in tsNY68-infected cells. Protein phosphorylation was studied in cells infected with a temperature-sensitive mutant of RSV, tsNY68, by immunoblotting with anti-PTYR (Fig. 5). In cells maintained at a permissive temperature (lane 7), protein species phosphorylated were essentially the same as those of wild-type RSV-infected cells. We found that, when temperature was raised to a nonpermissive temperature, these substrates were dephosphorylated. Six hours after shiftup (lane 8), when cells were well flattened, most of the substrates were underphosphorylated to a level close to that of cells maintained at a nonpermissive temperature (lane 2), in which only a few proteins (215, 150, and 130 kDa) remained weakly phosphorylated. Upon temperature shiftdown, phosphorylation of these substrates was quickly induced. By 2 h, when morphological transformation began to appear, the number of phosphorylated substrates and the intensity of each band were greatly increased (lane 5). Thus, the results show that a clear correlation exists between the level of PTYR in cellular proteins and the extent of cell transformation.

Most of the PTYR-containing proteins present in wild-type RSV-transformed cells were reversibly phosphorylated upon temperature shift. However, some differences were detectable among proteins in the kinetics of phosphorylation. In temperature shiftdown, 70- and 34-kDa proteins showed a transient peak of phosphorylation at 2 and 6 h,

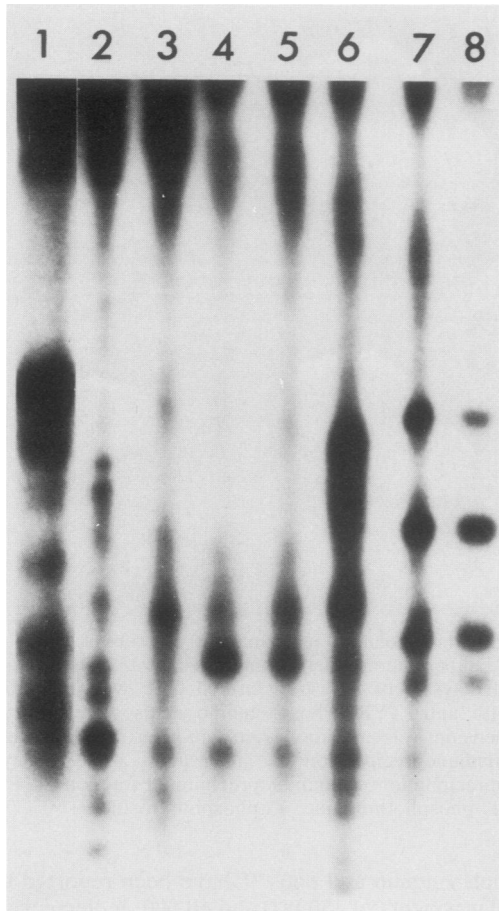


FIG. 4. Partial digestion of PTYR-containing proteins by V8 protease. Proteins immunoprecipitated from ^{32}P -labeled cell lysate (Fig. 1C) and $\text{p60}^{\text{v-src}}$ and vinculin immunoprecipitated with their specific antisera were subjected to partial digestion with *S. aureus* V8 protease. Lanes: 1, 130-kDa protein; 2, vinculin; 3, 110-kDa protein; 4, 82-kDa protein; 5, 78-kDa protein; 6, 65-kDa protein; 7, 54-kDa protein; 8, $\text{p60}^{\text{v-src}}$.

respectively. The phosphorylation of 78-, 65-, 54-, and 52-kDa proteins reached a plateau at 2 h, while bands of 110, 82, and 61 kDa were most prominent in cells maintained at the permissive temperature for more than 12 h. The 130-, 110-, 70-, and 65-kDa proteins were phosphorylated early after temperature shiftdown and may represent direct substrates of $\text{p60}^{\text{v-src}}$.

Similar results were obtained with another temperature-sensitive mutant, *tsNY72-4* (data not shown).

Phosphorylation of cellular proteins in various mutant-infected cells. PTYR-containing proteins in cells infected with various mutants of RSV were examined (Fig. 6). The profile of phosphorylation in cells infected with NY5H, a mutant encoding $\text{p60}^{\text{c-src}}$ instead of $\text{p60}^{\text{v-src}}$, was essentially similar to that of uninfected cells except for the presence of a strong band of overproduced $\text{p60}^{\text{c-src}}$ and weak bands at the 130- to 110-kDa region (lane A-2). On the other hand, in cells infected with NY501T7 or NYCHB, mutants of the *c-src* virus which have been converted to strongly transforming viruses by single mutations and have elevated kinase activities, the pattern of PTYR-containing proteins was similar to that in wild-type RSV-infected cells (lanes A-3, B-2, and C-2). A minor variation was found in the 150- to 130-kDa region in these mutant-infected cells. Instead of the

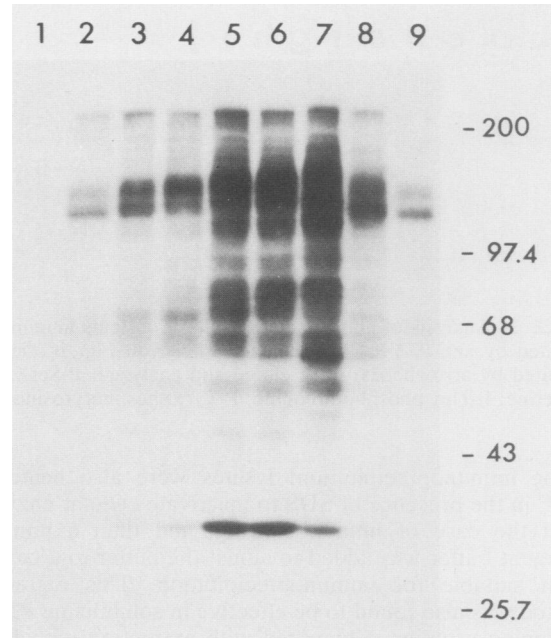


FIG. 5. Changes in pattern of protein phosphorylation of *tsNY68*-infected cells upon temperature shift. Cell lysates of normal CEF and *tsNY68*-infected CEF under various conditions were prepared, and PTYR-containing proteins were analyzed by immunoblotting. Lanes: 1, normal CEF; 2 and 7, *tsNY68*-infected CEF maintained at 41 and 36°C, respectively; 3, 4, 5, and 6, *tsNY68*-infected CEF at 10 min, 30 min, 2 h, and 6 h after shiftdown, respectively; 8 and 9, *tsNY68*-infected cells at 6 and 12 h after shiftup.

two bands at 150 and 130 kDa seen in wild-type RSV-infected cells (lanes B-3 and C-3), one broad band at 140 kDa was shown in NYCHB-infected cells (lanes B-2 and C-2). A similar protein profile with a slightly lower degree of phosphorylation was seen with cells infected with mutant NY309 (lane A-4), which encodes $\text{p60}^{\text{v-src}}$ with a deletion of amino acids 15 to 81 and produces fusiform transformed cells.

A substantially different pattern was found with cells infected with two nonmyristylation mutants, NY314 and NY315 (lanes B-1, C-1, and D-1). The *src* proteins of these mutants are active in protein kinase but lack N-terminal myristylation and, as a consequence, are unable to associate with plasma membranes and induce cell transformation (10). Moreover, recently we found that these mutant *src* proteins are soluble by Triton X-100 extraction, unlike wild-type $\text{p60}^{\text{v-src}}$ (17). Approximately 20 bands were found in mutant-infected cells, compared with about 25 bands found in wild-type RSV-infected cells. Of the 20 bands, 9 (205, 185, 173, 72, 63, 61, 54, 51, and 34 kDa) in mutant-infected cells shared mobility with corresponding bands from wild-type RSV-transformed cells. A number of proteins well phosphorylated in wild-type RSV-transformed cells (215, 150, 130, 110, 100, 95, 82, 78, and 65 kDa; filled arrowheads, Fig. 6) were not detected or were faintly phosphorylated in these mutant-infected cells. On the other hand, two unique bands (225 and 103 kDa; open arrowheads, Fig. 6) were strongly phosphorylated in mutant-infected cells but not in wild-type RSV-infected cells.

Two recovered avian sarcoma viruses, rASV157 and rASV1702, were also examined. The N termini of these *src* proteins are not myristylated, but are substituted with virus-derived sequences (15). These *src* proteins are capable of

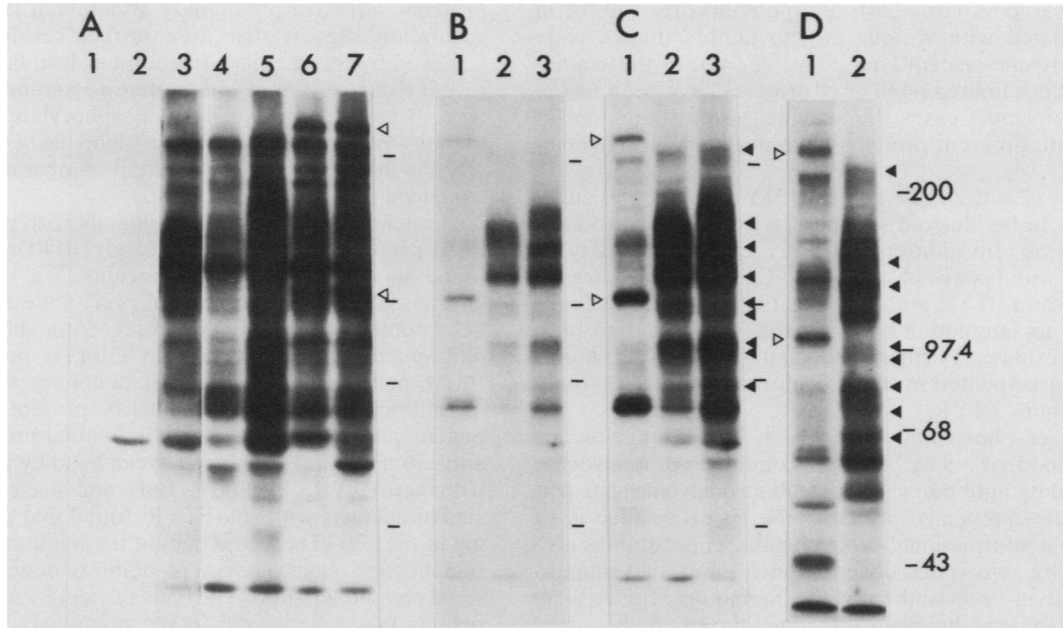


FIG. 6. Proteins phosphorylated on tyrosine in various RSV mutant-infected cells. PTYR-containing proteins in cell lysates were analyzed by immunoblotting. Lanes: A-1, uninfected CEF; A-2, NY5H-infected CEF; A-3, NY501T7-transformed CEF; A-4, NY309-transformed CEF; A-5, B-3, C-3, and D-2, SR-RSV-transformed CEF; A-6, rASV157-transformed CEF; A-7, rASV1702-transformed CEF; B-1 and C-1, NY315-infected CEF; B-2 and C-2, NYCHB-transformed CEF; D-1, NY314-infected CEF. Proteins unique for recovered avian sarcoma viruses and nonmyristylation mutants (225 and 130 kDa) are indicated by open arrowheads. Proteins specifically detected in transformed cells (215, 150, 130, 110, 100, 95, 82, 78, and 65 kDa) are indicated by filled arrowheads. X-ray films of panels A, C, and D were exposed for 24 h, whereas that of panel B was exposed for 12 h. A 6% gel was used for panel D, and 7.5% gels were used for the others.

transforming cells by uniquely interacting with focal adhesion plaques rather than plasma membranes in infected cells (27). These proteins have been found to be moderately associated with cytoskeletal structures (17). rASV157- and rASV1702-infected cells showed patterns basically similar to that of wild-type RSV-infected cells, but contained two additional bands (225 and 103 kDa), which may correspond to bands of similar size found in NY314- and NY315-infected cells.

Cells transformed with other oncogene products. To see whether common cellular proteins are phosphorylated in transformation by other oncogene products having tyrosine-protein kinase activity, cells transformed with avian sarcoma viruses Y73, FSV, PRCII, and 16L were analyzed by immunoblotting. These viruses encode P90^{gag-yes} (26), P130^{gag-fps} (12), P105^{gag-fps} (37), and P142^{gag-fps} (36), respectively. Many PTYR-containing proteins were found in these transformed cells (Fig. 7). Although the relative intensity of bands substantially varies, many proteins, especially those in the range between 60 and 100 kDa, are found in common in these transformed cells. In addition, each viral transforming protein was highly phosphorylated on tyrosine (arrowheads). In Y73-transformed cells, the level of phosphorylation of 150- and 110-kDa proteins was extremely low compared with that in RSV-transformed cells. The 130- to 140-kDa bands overlapped with the oncogene products in FSV- and 16L-infected cells, but were detectable in PRCII-infected cells. The intensity of bands was generally lower with FSV-transformed cells, which have the least pronounced morphological alteration among the viruses examined. A strong band at about 70 kDa was found in PRCII-transformed cells, the nature of which is not clear.

DISCUSSION

Antibodies against PTYR have been shown to be useful in detection of some phosphoproteins in cells infected with RSV (6, 38, 47), Abelson murine leukemia virus (13, 43, 47), avian erythroblastosis virus (47), simian sarcoma virus (47),

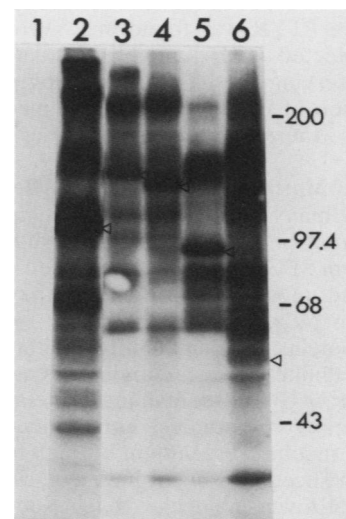


FIG. 7. Cellular proteins phosphorylated on tyrosine in cells infected with Y73, FSV, PRCII, and 16L viruses. PTYR-containing proteins were analyzed by immunoblotting. Lanes: 1, uninfected CEF; and 2, PRCII-; 3, 16L-; 4, FSV-; 5, Y73-; and 6, SR-RSV-infected CEF. Arrowhead indicates transforming protein of each virus.

SM-feline sarcoma virus (34), and polyomavirus (50) or in cells stimulated with various growth factors that activate receptor tyrosine-protein kinase (11, 22, 49). In these studies, however, a limited number of proteins have been recognized, and in most cases the specificity and the extent of reaction with different proteins have not been clearly demonstrated.

Specificity of anti-PTYR. The anti-PTYR used in this study was shown to be blocked specifically by PTYR but not by phosphoserine. In addition, the very limited reactivity of anti-PTYR with lysates of uninfected CEF indicates that the reaction of anti-PTYR with proteins from transformed cells is not due to interaction with phosphoserine or phosphothreonine residues. Furthermore, analysis of several bands of immunoprecipitated proteins confirmed that each protein indeed contains PTYR.

Analysis of phosphoamino acids in immunoprecipitates demonstrated that >90% of PTYR-containing proteins were precipitated by antibodies. This result strongly suggests that the antibodies recognize primarily the PTYR residue itself rather than conformational determinants. This notion is also supported by two other observations. First, the antigens were effectively released from the immunoprecipitates by addition of phenylphosphate. Second, the antibodies used were prepared against two different antigens: phosphotyramine conjugated to a carrier protein and the *abl* protein kinase expressed in bacteria; the two preparations gave the same results with lysates of RSV-transformed cells. The excellent recognition of PTYR residues by antibodies may be due partly to the unfolding of proteins by heating in SDS buffer, which has allowed essentially all PTYR residues to be accessible.

The analysis of transformed cells showed tyrosine phosphorylation of more than 20 cellular proteins, and the level of tyrosine phosphorylation was found to be greater in RSV-transformed CEF than in uninfected CEF. This is basically consistent with the previous observation that at least 10 times more PTYR is present in RSV-transformed cells (20). In fact, the difference in the level of total PTYR-containing proteins detectable by immunoblotting was even greater than that seen by PTYR analysis in acid hydrolysates: about 20 times more PTYR was found in RSV-transformed cells than in uninfected CEF (unpublished data). One critical factor in preserving the PTYR-containing proteins may be the inactivation of cellular enzymes immediately after the lysis of cells, thereby preventing further modification of proteins.

Previously, Martinez et al. (32) and Beemon et al. (2) showed that many proteins smaller than p60^{src} contain PTYR by direct phosphoamino acid analysis of gel slices recovered from SDS-PAGE of transformed-cell proteins. On the other hand, most of the proteins we have found distributed between 50 and 200 kDa. Since >90% of PTYR-containing proteins were immunoprecipitated in our analysis, it seems unlikely that the antibodies recognized only a subpopulation of PTYR-containing proteins. It is possible that, in the previous studies, either incomplete protease digestion or incomplete elution of high-molecular-weight proteins from sliced gels might have contributed to relative enrichment for lower-molecular-weight substrates.

PTYR-containing proteins in transformed cells. It is difficult to estimate the exact number of protein species detectable in RSV-transformed cells by this method. Some bands, particularly of 150-, 130-, and 110-kDa proteins, appear actually to consist of several bands. Clearly, more analysis is needed to establish an accurate account of PTYR-containing

proteins, although preliminary attempts at two-dimensional separation suggest that little further resolution could be achieved by this method. In addition, further analysis might reveal a correlation of one protein to another by posttranslational modification such as phosphorylation. The altered mobility of 150- and 130-kDa proteins as seen in lysates of *tsNY68*-infected cells at different temperatures might be explained by such modification.

In attempts to compare some bands with previously identified proteins, the 150-, 130-, and 110-kDa proteins were found to be different from vinculin. We also found that anti-fibronectin receptor cannot precipitate the 130- or 110-kDa proteins and that fibronectin receptor showed a mobility different from that of 130- and 110-kDa proteins in SDS-PAGE in reducing conditions (data not shown). Both vinculin and fibronectin receptor must be present as minor components, however, since vinculin immunoprecipitated by its antiserum was detectable as a weak band by immunoblotting with anti-PTYR. Recently, Burr and his colleagues, in a similar analysis with anti-PTYR, found that proteins migrating in the 130-kDa region cannot be immunoprecipitated by specific sera against vinculin or fibronectin receptor (personal communication). The bands detected at 81- and 34-kDa regions may correspond to the previously identified ezrin (16) and p36 (42). Other known substrates, talin (215 kDa) (41), enolase (46 kDa) (9), phosphoglycerate mutase (28 kDa) (9), and lactate dehydrogenase (35 kDa) (9), seem to be relatively less abundant components, and we did not find any intense bands corresponding to the molecular weight of these proteins. Thus, most of the protein species detected by either immunoblotting or immunoprecipitation with anti-PTYR appear to be unidentified by other methods, but some may correspond to 130-, 70-, and 60-kDa proteins detected in previous studies with anti-PTYR (6, 38, 47; J. Burr, personal communication).

Substrates for cell transformation. Cells transformed by various RSV mutants were analyzed in the hope that some differences in protein profile may correlate with the different phenotypes the mutant viruses induce. However, we found few differences among transformed cells, including those that assumed a fusiform morphology. This was the case even with cells transformed by viruses encoding *yes* and *fps* protein kinase oncogenes. We should point out, however, that 110-kDa protein phosphorylation was extremely low in Y73-transformed cells, suggesting that this protein phosphorylation may not be essential for transformation. Although these results might suggest that different phenotypes of transformed cells are not results of phosphorylation of different substrates, further analysis of PTYR-containing proteins is required to make such a conclusion. In addition, it is quite possible that less abundant PTYR-containing proteins play an important role in cell transformation.

Kamps et al. (24) examined the PTYR contents of several substrates such as vinculin, ezrin, glycolytic enzymes, p50, and p36 in cells infected with nonmyristylation mutants, and these substrates were found to be well phosphorylated, although the mutant-infected cells were not transformed. They did not find substantial difference of phosphorylation between cells infected with wild-type RSV and those infected with nonmyristylation mutants. In contrast, clearly different species of PTYR-containing proteins were observed in cells infected with NY314 or NY315, which encode nontransforming p60^{src} lacking N-terminal myristylation. About 10 PTYR-containing proteins detectable in these nontransformed cells were not present in wild-type transformed cells, and conversely, a different set of nine proteins

detectable in wild-type RSV-transformed cells were not found in NY314- or NY315-infected cells. It is likely that some of the PTYR-containing proteins detectable specifically in transformed cells are involved in cell transformation. The drastic differences between NY314- or NY315-infected and wild-type RSV-transformed cells in PTYR-containing proteins corroborate the notions that key substrates for the wild-type kinase are not accessible to the cytosolic mutant protein kinases (10, 24) and the biochemical events leading to cell transformation can only be initiated near the plasma membranes with p60^{src} in proper protein configurations (17).

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LITERATURE CITED

1. Antler, A. M., M. E. Greenberg, G. M. Edelman, and H. Hanafusa. 1985. Increased phosphorylation of tyrosine in vinculin does not occur upon transformation by some avian sarcoma viruses. *Mol. Cell. Biol.* 5:263-267.
2. Beemon, K., T. Ryden, and E. A. McNelly. 1982. Transformation by avian sarcoma viruses leads to phosphorylation of multiple cellular proteins on tyrosine residues. *J. Virol.* 42:742-747.
3. Breitman, M. L., J. C. Neil, C. Moscovici, and P. K. Vogt. 1981. The pathogenicity and defectiveness of PRC II: a new type of avian sarcoma virus. *Virology* 108:1-12.
4. Burr, J. G., G. Dreyfuss, S. Penman, and J. M. Buchanan. 1980. Association of the *src* gene product of Rous sarcoma virus with cytoskeletal structures of chicken embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* 77:3484-3488.
5. Collett, M. S., and R. L. Erikson. 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. *Proc. Natl. Acad. Sci. USA* 75:2021-2024.
6. Comoglio, P. M., M. F. DiRenzo, G. Tarone, F. G. Giancotti, L. Naldini, and P. C. Marchisio. 1984. Detection of phosphotyrosine-containing proteins in the detergent insoluble fraction of RSV-transformed fibroblasts by azobenzene phosphonate antibodies. *EMBO J.* 3:483-489.
7. Cooper, J. A., and T. Hunter. 1981. Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells. *Mol. Cell. Biol.* 1:165-178.
8. Cooper, J. A., and T. Hunter. 1983. Regulation of cell growth and transformation by tyrosine-specific protein kinases: the search for important cellular substrate proteins. *Curr. Top. Microbiol. Immunol.* 107:125-161.
9. Cooper, J. A., N. A. Reiss, R. J. Schwartz, and T. Hunter. 1983. Three glycolytic enzymes are phosphorylated at tyrosine in cells transformed by Rous sarcoma virus. *Nature (London)* 302:218-223.
10. Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa. 1984. A short sequence in the p60^{src} N terminus is required for p60^{src} myristylation and membrane association and for cell transformation. *Mol. Cell. Biol.* 4:1834-1842.
11. Ek, B., and C.-H. Heldin. 1984. Use of an antiserum against phosphotyrosine for the identification of phosphorylated components in human fibroblasts stimulated by platelet-derived growth factor. *J. Biol. Chem.* 259:11145-11152.
12. Feldman, R. A., T. Hanafusa, and H. Hanafusa. 1980. Characterization of protein kinase activity associated with the transforming gene product of Fujinami sarcoma virus. *Cell* 22:757-765.
13. Frackelton, A. R., A. H. Ross, and H. N. Eisen. 1983. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus-transformed cells and growth factor-stimulated cells. *Mol. Cell. Biol.* 3:1343-1352.
14. Fukami, Y., T. Nakamura, A. Nakayama, and T. Kanehisa. 1986. Phosphorylation of tyrosine residues of calmodulin in Rous sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA* 83:4190-4193.
15. Garber, E. A., and H. Hanafusa. 1987. NH₂-terminal sequences of two src proteins that cause aberrant transformation. *Proc. Natl. Acad. Sci. USA* 84:80-84.
16. Gould, K. L., J. A. Cooper, A. Bretscher, and T. Hunter. 1986. The protein-tyrosine kinase substrate, p81, is homologous to a chicken microvillar core protein. *J. Cell Biol.* 102:660-669.
17. Hamaguchi, M., and H. Hanafusa. 1987. Association of p60^{src} with Triton X-100-resistant cellular structure correlates with morphological transformation. *Proc. Natl. Acad. Sci. USA* 84:2312-2316.
18. Hanafusa, T., L.-H. Wang, S. M. Anderson, R. E. Karess, W. S. Hayward, and H. Hanafusa. 1980. Characterization of the transforming gene of Fujinami sarcoma virus. *Proc. Natl. Acad. Sci. USA* 77:3009-3013.
19. Hirst, R., A. Horwitz, C. Buck, and L. Rohrschneider. 1986. Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 83:6470-6474.
20. Hunter, T., and B. M. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA* 77:1311-1315.
21. Iba, H., F. R. Cross, E. A. Garber, and H. Hanafusa. 1985. Low level of cellular protein phosphorylation by nontransforming overproduced p60^{src}. *Mol. Cell. Biol.* 5:1058-1066.
22. Kadowaki, T., S. Koyasu, E. Nishida, K. Tobe, T. Izumi, F. Takaku, H. Sakai, I. Yahara, and M. Kasuga. 1987. Tyrosine phosphorylation of common and specific sets of cellular proteins rapidly induced by insulin, insulin-like growth factor I, and epidermal growth factor in an intact cell. *J. Biol. Chem.* 262:7342-7350.
23. Kamps, M. P., J. E. Buss, and B. M. Sefton. 1985. Mutation of NH₂-terminal glycine of p60^{src} prevents both myristylation and morphological transformation. *Proc. Natl. Acad. Sci. USA* 82:4625-4628.
24. Kamps, M. P., J. E. Buss, and B. M. Sefton. 1986. Rous sarcoma virus transforming protein lacking myristic acid phosphorylates known polypeptide substrates without inducing transformation. *Cell* 45:105-112.
25. Kawai, S., and H. Hanafusa. 1971. The effect of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma virus mutant. *Virology* 46:470-479.
26. Kawai, S., M. Yoshida, K. Segawa, H. Sugiyama, R. Ishizaki, and K. Toyoshima. 1980. Characterization of Y73, an avian sarcoma virus: a unique transforming gene and its product, a phosphopolyprotein with protein kinase activity. *Proc. Natl. Acad. Sci. USA* 77:6199-6203.
27. Krueger, J. G., E. A. Garber, S. S.-M. Chin, H. Hanafusa, and A. R. Goldberg. 1984. Size-variant pp60^{src} proteins of recovered avian sarcoma viruses interact with adhesion plaques as peripheral membrane proteins: effects on cell transformation. *Mol. Cell. Biol.* 4:454-467.
28. Krueger, J. G., E. A. Garber, and A. R. Goldberg. 1983. Subcellular localization of pp60^{src} in RSV-transformed cells. *Curr. Top. Microbiol. Immunol.* 107:51-124.
29. Lau, A. F. 1986. Phosphotyrosine-containing 120,000-dalton protein coimmunoprecipitated with pp60^{v-src} from Rous sarcoma virus-transformed mammalian cells. *Virology* 151:86-99.
30. Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell* 15:561-572.
31. Levy, J. B., H. Iba, and H. Hanafusa. 1986. Activation of the

- transforming potential of p60^{c-src} by a single amino acid change. Proc. Natl. Acad. Sci. USA **83**:4228-4232.
32. Martinez, R., K. D. Nakamura, and M. J. Weber. 1982. Identification of phosphotyrosine-containing proteins in untransformed and Rous sarcoma virus-transformed chicken embryo fibroblasts. Mol. Cell. Biol. **2**:653-665.
 33. Mayer, B. J., R. Jove, J. F. Krane, F. Poirier, G. Calothy, and H. Hanafusa. 1986. Genetic lesions involved in temperature sensitivity of the *src* gene products of four Rous sarcoma virus mutants. J. Virol. **60**:858-867.
 34. Morrison, D. K., P. J. Browning, M. F. White, and T. Roberts. 1988. Tyrosine phosphorylation in vivo associated with *v-fms* transformation. Mol. Cell. Biol. **8**:176-185.
 35. Nagai, Y., M. Hamaguchi, K. Maeno, M. Iinuma, and T. Matsumoto. 1980. Proteins of Newcastle disease virus. A comparison by partial protease digestion among the strains of different pathogenicity. Virology **102**:463-467.
 36. Neel, B. G., L.-H. Wang, B. Mathy-Prevot, T. Hanafusa, H. Hanafusa, and W. S. Hayward. 1982. Isolation of 16L virus: a rapidly transforming sarcoma virus from an avian leukosis virus-induced sarcoma. Proc. Natl. Acad. Sci. USA **79**:5088-5092.
 37. Neil, J. C., M. L. Breitman, and P. K. Vogt. 1981. Characterization of a 105,000 molecular weight *gag*-related phosphoprotein from cells transformed by the defective avian sarcoma virus PRC II. Virology **108**:98-110.
 38. Ohtsuka, M., S. Ihara, R. Ogawa, T. Watanabe, and Y. Watanabe. 1984. Preparation and characterization of antibodies to o-phosphotyrosine and their use for identification of phosphotyrosine-containing proteins. Int. J. Cancer **34**:855-861.
 39. Oppermann, H., A. D. Levinson, L. Levintow, H. E. Varmus, J. M. Bishop, and S. Kawai. 1981. Two cellular proteins that immunoprecipitate with the transforming protein of Rous sarcoma virus. Virology **113**:736-751.
 40. Pang, D. T., B. R. Sharma, and J. A. Shafer. 1985. Purification of the catalytically active phosphorylated form of insulin receptor kinase by affinity chromatography with o-phosphotyrosyl-binding antibodies. Arch. Biochem. Biophys. **242**:176-186.
 41. Pasquale, E. B., P. A. Maher, and S. J. Singer. 1986. Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA **83**:5507-5511.
 42. Radke, K., T. Gilmore, and G. S. Martin. 1980. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell **21**:821-828.
 43. Ross, A. H., D. Baltimore, and H. N. Eisen. 1981. Phosphotyrosine-containing proteins isolated by affinity chromatography with antibodies to a synthetic hapten. Nature (London) **294**:654-656.
 44. Saga, S., M. Hamaguchi, M. Hoshino, and K. Kojima. 1985. Expression of meta-vinculin associated with differentiation of chicken embryonal muscle cells. Exp. Cell Res. **156**:45-56.
 45. 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.
 46. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350-4354.
 47. Wang, J. Y. J. 1985. Isolation of antibodies for phosphotyrosine by immunization with a *v-abl* oncogene-encoded protein. Mol. Cell. Biol. **5**:3640-3643.
 48. Wang, J. Y. J., and D. Baltimore. 1985. Localization of tyrosine kinase-coding region in *v-abl*-encoded proteins in bacteria. J. Biol. Chem. **260**:64-71.
 49. White, M. F., R. Maron, and C. R. Kahn. 1985. Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. Nature (London) **318**:183-186.
 50. Yonemoto, W., A. J. Filson, A. E. Queral-Lustig, J. Y. Wang, and J. S. Brugge. 1987. Detection of phosphotyrosine-containing proteins in polyomavirus middle tumor antigen-transformed cells after treatment with a phosphotyrosine phosphatase inhibitor. Mol. Cell. Biol. **7**:905-913.