Tyrosyl Kinases Acquired from Anchorage-independent Cells by a Membrane-enveloped Virus

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ABSTRACT Tyrosyl kinase activity in vesicular stomatitis virus (VSV) acquired from host cells that differ in morphology was investigated. VSV grown in baby hamster kidney (BHK) cells with rounded morphology and a high efficiency of colony formation in soft agar (Rous sarcoma virus [RSV]-transformed and suspension BHK cells) was compared with VSV grown in BHK cells with a flattened morphology and lower efficiency of colony formation in soft agar (RSV-infected revertant and control BHK cells). Tyrosyl kinase activity measured with the substrates angiotensin II peptide or casein was found at 7–10-fold higher levels in virus released from the anchorage-independent BHK cells. Most of the VSV-associated tyrosyl kinases acquired from the RSV-transformed BHK cells reacted with antiserum to pp60src, whereas the activity acquired from the suspension BHK cells was unaffected by anti-src serum. The overall levels of tyrosyl kinase in subcellular fractions of the host BHK cells were also measured. Like the VSV released from them, the RSV-transformed cell extracts contained high levels. The suspension cells, however, contained the same low levels of tyrosyl kinase as was found in the control BHK cell extracts. Therefore, tyrosyl kinase was concentrated and acquired by VSV from the anchorage-independent suspension BHK cells. VSV-associated protein kinases acquired from other cell types followed a similar pattern. Tyrosyl kinase levels were high in VSV released from suspension cultures (Chinese hamster ovary and HeLa) and from virally transformed cells (Kirsten murine sarcoma virus-transformed rat kidney cells) and low in VSV released from an anchorage-dependent primary cell culture (chick embryo fibroblasts).

Tyrosine-specific protein kinases have been shown to play an important role in growth control and malignant transformation of cells. At least five retroviruses have been found to have tyrosyl protein kinase activities associated with their transforming proteins (cf. reference 1). Protein kinases with specificity for tyrosine have also been observed in normal cells. These include an enzyme purified from normal rat liver (2), the protein products of cell homologues to retroviral oncogenes (1) as well as the activities associated with the plasma membrane receptors for epidermal growth factor (3, 4) and insulin (5–7). The plasma membrane receptor for the platelet-derived growth factor may also be associated with a tyrosyl kinase (8, 9).

The mechanism whereby altered tyrosine phosphorylation in cell proteins causes changes in the growth properties of cells is currently unknown. It has been proposed that protein phosphorylation may alter cellular morphology and thereby convey growth regulatory information to cells. Both enhanced tyrosine phosphorylation of cell proteins and alterations in the cytoskeletal structure occur after transformation by retroviruses that encode tyrosyl kinases (10–12) or after addition of epidermal growth factor or platelet-derived growth factor to cells (13–16). Amplified tyrosine phosphorylation in proteins, however, is not always observed in cells that undergo alterations in growth properties and morphology. For example, transformation by chemical carcinogens, DNA tumor viruses, and some retroviruses does not lead to a stable increase in phosphotyrosine in total cell proteins (10, 17). It is possible that cellular tyrosyl kinases may alter protein phosphorylation without causing a stable increase in overall phosphotyrosine levels either by a transient increase in activity or a relocation of the enzyme within the cell where different substrate proteins are phosphorylated.

Knowledge of the intracellular location of the tyrosyl kinases is of importance in understanding their functions in normal and transformed cells. Cytological as well as biochemical techniques have been used to localize most viral and cellular tyrosyl kinases at or near the plasma membrane (cf. reference 1). The viral transforming proteins with kinase activity fractionate either with the detergent-insoluble, cyto-

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skeleton-containing fraction (18–20) or with the plasma membrane (21) depending on the fractionation technique employed, indicating that they are attached both to the cytoskeletal structure and to the plasma membrane. pp60src tyrosyl kinase encoded by Rous sarcoma virus (RSV)[1] has been more specifically localized at focal adhesions and tight junctions between adjacent cells (22–24).

The tyrosyl kinase activity of viral pp60src has also been localized at the maturation sites of two enveloped viruses, vesicular stomatitis virus (VSV) (25, 26) and RSV (27, 28) and is found in the membrane envelope fraction of both of these purified viruses. For VSV, the maturation process at the plasma membrane is highly regulated because most cell surface proteins are excluded from purified virions (29), whereas glycoproteins encoded by some retroviruses (30–32), as well as tumor antigens from HeLa cells (33), are specifically acquired by VSV. Furthermore, the VSV maturation sites in epithelial cells are apparently confined to distinct plasma membrane domains that differ from the maturation sites of some other enveloped viruses (34–37).

Since pp60src from RSV-transformed cells was found associated with VSV, we used VSV as a probe to investigate whether other cells contain tyrosyl kinases at VSV maturation sites. Elevated tyrosyl kinase activity was observed only when VSV matured from virally transformed cells and from other cells that are anchorage independent for growth. The tyrosyl kinase relative to other protein kinase activities was enriched more than 20-fold in VSV compared with the host cell for at least one anchorage-independent cell type. These results indicate a specific acquisition by VSV of tyrosyl kinases from anchorage-independent cells but not from anchorage-dependent cells.

MATERIALS AND METHODS

Cells and Viruses: Baby hamster kidney (BHK), Chinese hamster ovary, and HeLa cells were grown in suspension cultures in Joklik's modified Eagle's medium supplemented with 5% fetal calf serum and nonessential amino acids. Monolayer cultures of BHK cells, chick embryo fibroblasts, and rat kidney cells (RKC) were grown in Eagle's mimimal essential medium supplemented with 5% fetal calf serum (media and sera were from KC Biological, Lenexa, KS). Cells were infected with cloned and sucrose-gradient purified standard VSV of the Indiana serotype (San Juan Strain) as previously described (26).

Fractionation of Cells into Detergent-soluble and -insoluble Components: Monolayer cell cultures (60-mm dishes) were washed twice with phosphate-buffered saline and the detergent-soluble fraction was extracted at 4°C by the addition of 0.25 ml of extraction buffer containing 100 mM KCl, 10 mM piperazine-*N,N'-bis* 2-ethane sulfonic acid, disodium salt (PIPES, Sigma Chemical Co., St. Louis, MO) pH 6.8, 0.5% NP-40, 300 mM sucrose, 1% Trasylol (Aprotonin, Sigma Chemical Co.), and 3 mM $MgCl_2$. After 3 min at 4°C, the soluble fraction was removed and the residue was washed with 0.5 ml of extraction buffer which was then discarded. The detergent-insoluble material was scraped from the plate in 0.25 ml of extraction buffer and both detergent-soluble and -insoluble fractions were assayed for protein kinase activity. For BHK suspension cultures, ~2 × 10^6 cells were washed by centrifugation, and the detergent-soluble fraction was extracted with 0.25 ml of extraction buffer and removed from the insoluble material by centrifugation at 2,000 *g* for 5 min. The detergent-insoluble material was washed once and suspended in 0.25 ml of extraction buffer. To ensure that equal amounts of cell protein were assayed in each reaction, an aliquot of the soluble fraction was assayed with Bio-Rad protein dye (Bio-Rad Laboratories, Richmond, CA). Equal amounts of protein from the soluble fraction and an equivalent aliquot from the insoluble fraction were assayed.

Tyrosyl Protein Kinase Assays: For the assays using angiotensin as substrate (38), purified VSV (12 μg) was added to a 100-μl kinase reaction containing 10 mM Tris, pH 8.0, 10 mM $MnCl_2$, 5 mM dithiothreitol, 1% NP-40, 10 μCi of [γ-^{32}P]ATP (New England Nuclear, Boston, MA), 20 μM ATP, and 100 μg of angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, Sigma Chemical Co.). The reaction was incubated at 34°C and 25-μl aliquots were removed, incubated in a boiling water bath for 2 min, and centrifuged in a microfuge for 3 min. Aliquots (10 μl) were spotted on No. 3 MM (Whatman, Ltd., Kent, England) filter paper, and the phosphorylated angiotensin was resolved from other components by electrophoresis toward the anode at 1,500 V for 45 min in a buffer at pH 3.5, containing 5% glacial acetic acid and 0.5% pyridine as described (38). A control containing all components, except angiotensin, did not contain ^{32}P-labeled materials, above background, that comigrate with ^{32}P-labeled angiotensin. For quantitation, the ^{32}P-labeled angiotensin spots were cut from the paper and analyzed by Cerenkov counting.

For the assays using casein as substrate, 150 μg of casein was added to the kinase reaction mixture. After incubation at 34°C, 5–10-μl aliquots were spotted onto strips of phosphocellulose paper, and the paper was washed in 0.42% phosphoric acid to remove unincorporated radioactivity (39), and ^{32}P incorporation into the bound protein was quantitated by Cerenkov counting. To quantitate phosphorylation of serine, threonine, and tyrosine residues, the kinase reaction was precipitated with 10% trichloroacetic acid, the pellet was washed twice with 100% acetone, and the phosphoamino acids were analyzed.

Phosphoamino Acid Analysis: ^{32}P-labeled proteins were suspended in 6 N HCl, hydrolyzed, and analyzed by two-dimensional paper electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second, as previously described (26).

RESULTS

Tyrosyl Kinase Activity Is Amplified in VSV Virions from BHK Cells That Exhibit Rounded Morphology and Anchorage Independence

To investigate tyrosyl kinases at VSV maturation sites in cells with different morphologies, we compared enzyme activity in VSV released from continuous cell lines that differ in anchorage dependence for growth but have similar growth rates. The virus was grown in control BHK cells (40), in BHK cells transformed by RSV, in revertant BHK cells (41, 42), and in BHK cells adapted for growth in suspension culture (43). The control BHK cells had a more flattened morphology and oriented alignment compared with the rounded RSV-transformed cells and the suspension BHK cells. Furthermore, the BHK cells with rounded morphology exhibited a higher efficiency of colony formation in soft agar (41, 44, 45 and Fig. 1). The revertant BHK cells had a morphology that was similar to the control BHK cells, and the colonies formed in soft agar were smaller and three- to fourfold fewer than those observed for the suspension and RSV-transformed BHK cells (reference 41 and unpublished observations).

To measure tyrosyl kinase activity in VSV grown in these cells, equivalent amounts of purified virions (12 μg of virus protein) from the BHK cells were disrupted with nonionic detergent, and incubated in a reaction mixture containing [γ-^{32}P]ATP and casein as substrate (see Materials and Methods). The total ^{32}P-labeled protein product was bound to phosphocellulose papers (39) and quantitated by Cerenkov counting. The kinetics of the reaction indicated a linear rate of protein phosphorylation for at least 80 min (data not shown). In the absence of casein, protein phosphorylation was reduced about 15-fold indicating that viral proteins were minor substrates. The rate of casein phosphorylation by the VSV-associated protein kinases was not significantly different when VSV from the different BHK cells was assayed. Phosphoamino acid analyses were conducted of the ^{32}P-labeled products from the casein containing reaction obtained at 10 min of incubation. Quantitation of acid hydrolyzed ^{32}P-labeled phosphoamino

[1] *Abbreviations used in this paper:* BHK, baby hamster kidney; Ki-MSV, Kirsten murine sarcoma virus; RKC, rat kidney cells; RSV, Rous sarcoma virus; VSV, vesicular stomatitis virus.

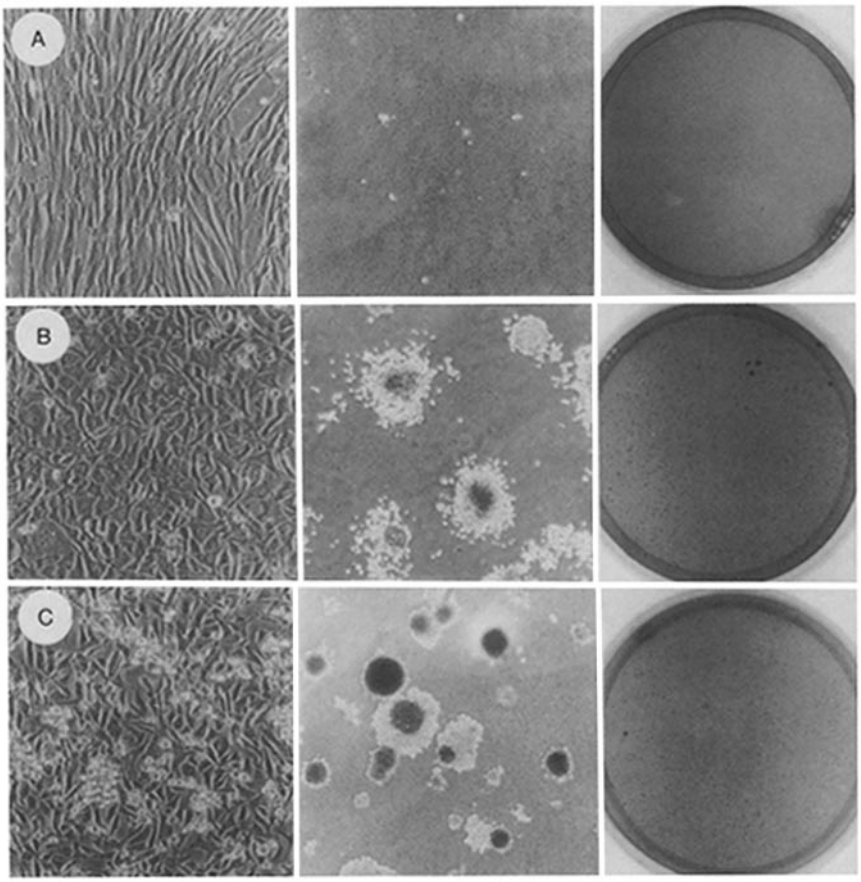

FIGURE 1 Morphology and anchorage-independent growth of BHK cells. Shown in the left panel is the morphology of cells that were grown to confluency in monolayers and photographed through a phase-contrast microscope at × 40. For growth in soft agar suspension (46), a 7-ml base layer of 0.5% Difco agar in minimal essential medium containing 10% fetal calf serum was poured into 60-mm plastic dishes. Cells (5×10^4, 10^4, 5×10^3, 2×10^2) were mixed with 0.3% Difco agar in minimal essential medium with 10% fetal calf serum and each dilution was plated onto the base layer. The cells were grown at 37°C in a humidified 5% CO_2 atmosphere for 10 d. The middle panel shows a phase-contrast micrograph (× 40) of the soft agar colonies in the plate containing 10^4 cells. The far right panel is a photograph of the soft agar colonies stained with crystal violet in the plates that contained 10^4 cells. (A) BHK control cells; (*B*) RSV-transformed BHK cells; (C) BHK suspension cells.

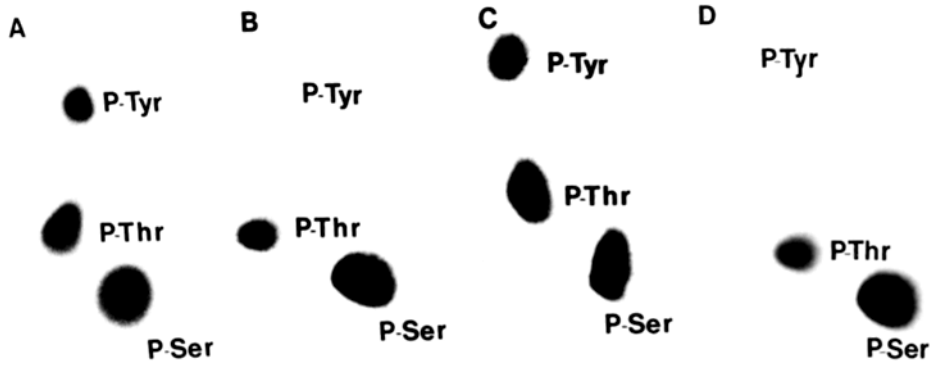

FIGURE 2 Amino acids phosphorylated by VSV-associated protein kinases. Purified virus was assayed in a protein kinase reaction containing casein as the phosphate acceptor. The products of the reaction were partially acid hydrolyzed and analyzed by high-voltage paper electrophoresis in two dimensions. The ^{32}P-labeled amino acids were detected by autoradiography. The phosphoamino acid markers were localized by ninhydrin staining. (*A*) VSV grown in RSV-transformed BHK cells; (*B*) VSV grown in control BHK cells; (*C*) VSV grown in BHK suspension cultures; (*D*) VSV grown in BHK revertant cultures.

acids does not reflect total casein-phosphorylating activity, in that only a fraction of the total phosphorylated amino acids can be detected after acid hydrolysis. Phosphoamino acid analyses are of value however, in comparing the relative levels of serine-, threonine-, and tyrosine-phosphorylating activities provided the substrate is in excess and the products are compared when the rates of the enzyme reactions are linear. The levels of serine and threonine phosphorylation by the protein kinases that co-purify with VSV (25, 26) were similar regardless of the host cell (Fig. 2). Tyrosine phosphorylation, however, was amplified by kinases in VSV grown in RSV-transformed cells (Fig. 2*A*) compared with the control and revertant BHK cells (Fig. 2, *B* and *D*). This was not surprising because it was previously shown that VSV incorporates pp60src tyrosyl kinase activity (26), which is amplified in RSV-transformed cells. VSV released from BHK cells that were not transformed by chemicals or viruses, but were selected for anchorage-independent growth in suspension culture also contained amplified tyrosyl kinase activity (Fig. 2*C*) similar to the levels observed in VSV released from RSV-transformed BHK cells. The results obtained from six separate VSV infections revealed that ^{32}P-labeled phosphotyrosine represented 21.5 ± 5.5% of the three acid-stable, ^{32}P-labeled phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine) when VSV from the suspension cells was assayed, 20.7 ± 4.2% when VSV from RSV-transformed BHK cells was assayed, and 2.5 ± 1.0% when VSV from the control BHK cells was assayed. Kinase assays of two separate prepa-

rations of VSV grown in the revertant BHK cells gave values of 3% and 2% of [^{32}P]phosphotyrosine. These results indicated that VSV-associated tyrosine, but not serine- or threonine-phosphorylating activities, were amplified in virus grown in the BHK cells with rounded morphologies and with high levels of growth in soft agar.

To investigate the kinetics of tyrosine phosphorylation using an alternative substrate, VSV-associated enzyme was assayed using angiotensin II peptide as the tyrosyl kinase substrate (38). The ^{32}P-labeled angiotensin in the product from the kinase assay was isolated by high-voltage paper electrophoresis and phosphate transfer was measured by quantitation of ^{32}P incorporation. The rate of tyrosine phosphorylation of angiotensin by VSV-associated protein kinase was linear for at least 80 min at 34°C (Fig. 3). The addition of vanadate (Fisher Scientific Co., Fair Lawn, NJ), a phosphotyrosyl phosphatase inhibitor (38, 47) at 25 μM did not alter the rate of the reaction (not shown). The kinetics of the VSV-associated tyrosyl kinase assay without vanadate (linear for at least 80 min) indicated that most cellular phosphotyrosyl phosphatases and other inhibitors of phosphorylation are excluded from VSV virions. In contrast, the rate of angiotensin phosphorylation by kinases in BHK cell extracts that contain phosphatases and other interfering components plateaus or decreases by 5–10 min of incubation and the extent of phosphorylation is doubled by inclusion of vanadate (48). In agreement with results obtained using casein as substrate, virions from RSV-transformed and suspension BHK cells catalyzed tyrosine phosphorylation in angiotensin at five to seven times the rate compared with VSV grown in the control BHK cells (Fig. 3).

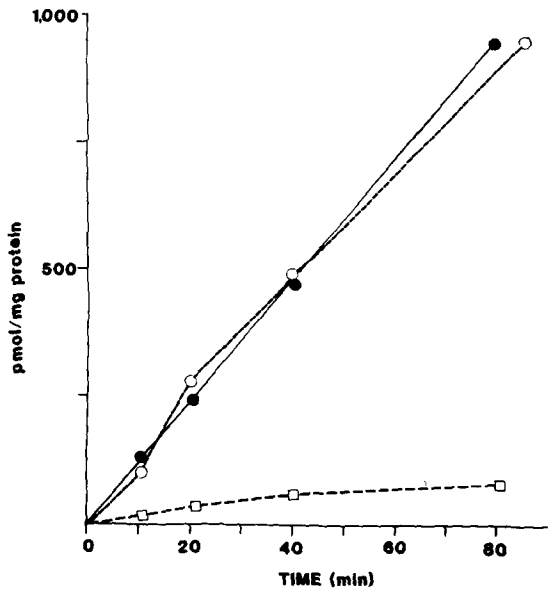

FIGURE 3 Tyrosine-specific phosphorylation of angiotensin II by VSV-associated protein kinase. Purified VSV was incubated in a reaction containing [γ-^{32}P]ATP and angiotensin II as substrate (see Materials and Methods). The ^{32}P-labeled angiotensin in the product was fractionated by paper electrophoresis and quantitated by Cerenkov counting. The activity was quantitated as picomole of phosphate transferred per milligram of VSV protein. (□) VSV grown in BHK control cells; (●) VSV grown in BHK suspension cells; (○) VSV grown in RSV-transformed BHK cells.

VSV-associated Tyrosyl Kinases from Anchorage-independent BHK Cells Are Unrelated

Incorporation of comparable levels of tyrosyl kinase activity into VSV grown in BHK cells that are anchorage independent for growth (suspension and RSV-transformed BHK cells) indicated that the kinases may be similarly localized where VSV buds through the cell membrane. To compare the identity of the tyrosyl kinases obtained from both BHK cell lines, the enzymes were tested for immunological relatedness. Purified VSV virions were reacted with serum from a tumor-bearing rabbit infected with RSV (anti-src serum), which recognizes both viral and cellular pp60src, or reacted with control serum from normal rabbits. After removal of the immune complexes, the remaining material was assayed in a kinase reaction containing casein as substrate (described in the legend to Fig. 4). Reaction of VSV from RSV-transformed BHK cells with control serum did not detectably alter the phosphorylation of casein (Fig. 4*B*). When virions grown in RSV-transformed BHK cells were immunoprecipitated with anti-src serum, most of the tyrosine-phosphorylating activity was eliminated. The loss of tyrosine-phosphorylating activity was evidenced by a 10-fold decrease in ^{32}P-labeled phosphotyrosine in the product from the kinase reaction (Fig. 4*A*). The tyrosyl kinase activity associated with VSV grown in BHK suspension cultures, on the other hand, was not greatly affected after reaction with either anti-src (15% [^{32}P]phosphotyrosine) or normal serum (18% [^{32}P]phosphotyrosine) (Fig. 4, *C* and *D*). These observations demonstrate that most of the tyrosyl kinase at VSV maturation sites in RSV-transformed cells was due to pp60src, whereas most of the cellular tyrosyl kinase activity at VSV maturation sites in the suspension BHK cells was unrelated to pp60src.

Tyrosyl Kinase Activity Is Not Amplified in Suspension BHK Cells

To determine whether the overall cellular tyrosyl kinase activity was amplified in the anchorage-independent BHK cells, or whether activity was amplified only at VSV maturation sites, BHK cell extracts were tested for overall tyrosyl kinase activity using the same assay conditions used with VSV virions. The rate of tyrosine phosphorylation in angiotensin as well as in endogenous cell proteins with added casein (per milligram of protein or relative to serine- and threonine-phosphorylating activity) was about fivefold lower in suspension and control compared with RSV-transformed BHK cell extracts (reference 48 and unpublished observations). This result indicated that tyrosyl kinase activity was amplified only in RSV-transformed cells. It was next tested whether cellular tyrosyl kinase activity might be more soluble in the suspension cultures due to the disorganization of the cytoskeleton. Increased solubility of cellular tyrosyl kinases may cause increased activity in VSV because of random entrapment of enzymes. The control and transformed BHK cells in monolayers and the suspension BHK cells were fractionated into their detergent-soluble fraction and insoluble cytoskeleton-containing matrix, and each was assayed in a reaction with added casein to ensure substrate excess. The kinase products at 5 min of incubation, when the reaction rate was linear, were acid hydrolyzed and the ^{32}P-labeled phosphoamino acids were analyzed (Fig. 5). The tyrosine-phosphorylating activity was enriched (~80%) in the detergent-insoluble matrix of

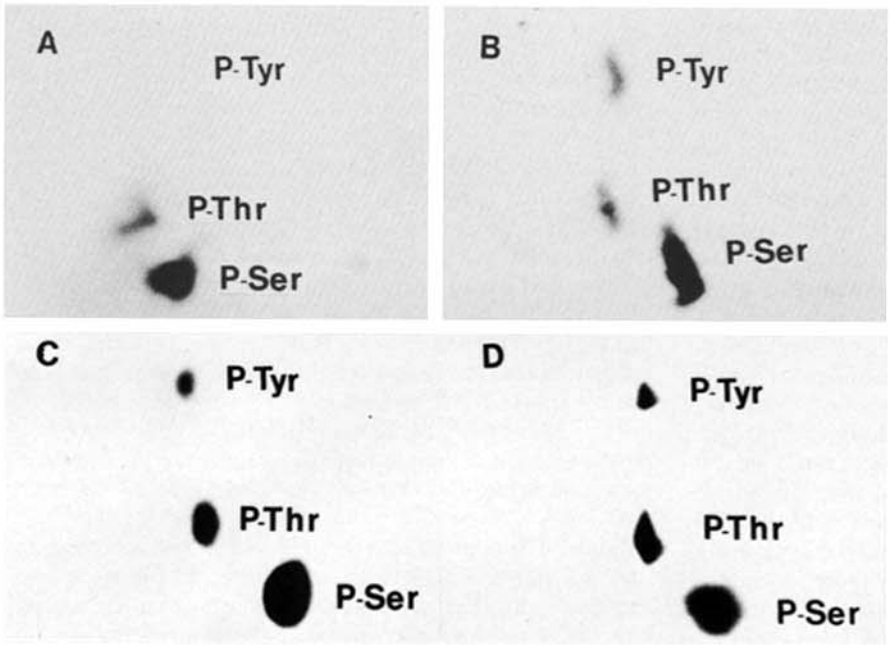

FIGURE 4 Amino acids phosphorylated by VSV-associated kinases after treatment with antiserum. Purified virions were reacted with 2.5 μl of antiserum on ice for 30 min in the kinase reaction mixture with the substrate and ATP omitted. The immune complexes were removed by binding to 25 μl of a 10% suspension of *Staphylococcus aureus* (IgG Sorb, The Enzyme Center, Inc., Malden, MA). The remaining material in the supernatant was assayed in a kinase reaction and the products were analyzed exactly as in Fig. 2. Autoradiography of the phosphoamino acids was for 16 h. (A) VSV from RSV-transformed BHK cells reacted with anti-src serum; (*B*) VSV from RSV-transformed BHK cells reacted with control serum; (*C*) VSV from BHK suspension cultures reacted with anti-src serum; (*D*) VSV from BHK suspension cultures reacted with control serum.

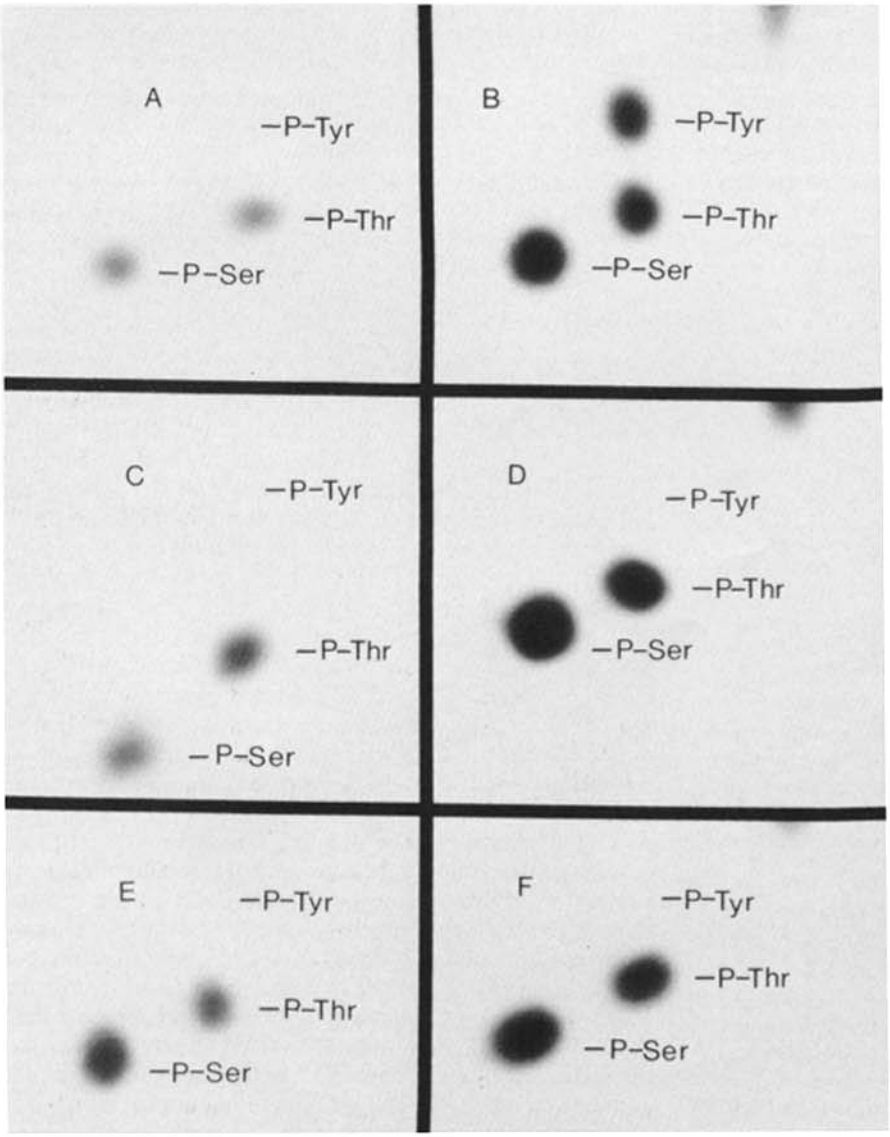

FIGURE 5 Amino acids phosphorylated by enzymes in detergent-soluble and -insoluble cell fractions. The detergent-soluble and -insoluble cell fractions from monolayer cultures were prepared as described in Materials and Methods. 12 μg of the detergent-soluble fraction of RSV-transformed BHK cells (*A*), control BHK cells (*C*), BHK suspension cells (*E*), and an equivalent proportion of the detergent-insoluble fraction from the RSV-transformed BHK cells (*B*), control BHK cells (*D*), and BHK suspension cells (*F*), were incubated in a reaction and ^{32}P-labeled phosphoamino acids were analyzed as described in Fig. 2.

RSV transformed cells (Fig. 5 *B*) and was due exclusively to pp60src tyrosyl kinase (48). Quantitations of the [^{32}P]phosphoamino acids showed ~7% [^{32}P]phosphotyrosine in the soluble fraction and 27% [^{32}P]phosphotyrosine in the detergent-insoluble fraction of the transformed BHK cells. Although a [^{32}P]labeled phosphotyrosine spot cannot be observed in this autoradiogram of BHK control and suspension cell assay products, longer exposure times revealed radioactive phosphotyrosine at levels above background. The tyrosine-phosphorylating activity from the suspension cultures was not increased either in the detergent soluble (0.98% [^{32}P]phosphotyrosine) or insoluble matrix (0.45% [^{32}P]phosphotyrosine) compared with the control BHK cells (1.1% [^{32}P]phosphotyrosine in the soluble and 0.52% in the insoluble fractions) (compare Fig. 5, *C* and *D* with *E* and *F*). The presence of inhibitors did not account for low activity in the soluble fraction of the suspension cultures since the soluble fraction did not depress tyrosyl kinase activity when added to the insoluble fraction (not shown). Measurements of tyrosyl kinase activity in the crude membrane and the cytosolic fraction from dounce homogenized cells also failed to reveal a higher level of tyrosyl kinase in suspension BHK cells (data not shown). Phosphotyrosine levels in cellular proteins, another indication of tyrosyl kinase expression, were found at similar levels in the control and suspension BHK cells and were about sixfold lower than levels found in the RSV-transformed cells (Clinton, G. M., unpublished observations). These observations all indicated that, although tyrosyl kinase levels were amplified in VSV virions, the host suspension BHK cells did not contain amplified activity either in whole cell extracts or in the detergent-soluble or -insoluble subcellular fractions compared with the control BHK cells. These data also indicate that the relative tyrosine-phosphorylating activity was greatly enriched in VSV (suspension) compared with the suspension cell extracts (21.5% in VSV compared to <1% in cell extracts). We do not believe that this differential activity was caused by agents that interfere with in vitro tyrosyl kinase detection in cell extracts, because we analyzed the products when the reaction rate was linear and in the presence of vanadate. Furthermore, the tyrosyl kinase activity was standardized to serine and threonine kinases; therefore ATPases and other components that interfere with protein kinase assays in cell extracts were compensated for.

Tyrosyl Kinase Activity in VSV Grown in Other Cell Types

It was of interest to determine whether high tyrosyl kinase levels at VSV maturation sites correlated with rounded morphology and anchorage-independent growth in cell lines other than BHK cells. CHO and HeLa cells, both of which are continuous cell lines that grow in suspension (49, 50) were infected with VSV. The relative level of tyrosyl kinase activity was determined by quantitation of the amount of ^{32}P incorporated into phosphotyrosine relative to phosphoserine and phosphothreonine in a kinase reaction containing casein and 12 μg of purified VSV. Enzymes in VSV virions grown in CHO and HeLa cells were found to catalyze a similar level of tyrosine phosphorylation (~22% of the total acid stable, ^{32}P-labeled phosphoamino acids) as was observed in VSV from BHK suspension cultures and from RSV-transformed BHK cells (Table I). An additional anchorage-independent cell line, RKC, transformed by temperature-sensitive, Kirsten murine sarcoma virus (Ki-MSV) (51), a virus whose transforming gene product is a guanine nucleotide binding protein with no associated tyrosyl kinase activity (52), were also infected with VSV and the released virus was purified. The virus yields from the RKC cells were about fivefold lower than yields from the BHK, Chinese hamster ovary, or HeLA cells. Nevertheless, the specific activity of protein kinases in the virions grown in transformed RKC cells was similar to levels observed in virions from BHK cells (not shown). The rate of tyrosine phosphorylation using angiotensin as substrate was similar to that observed in assays in virus from either the suspension or transformed BHK cells (not shown). When casein was used as the substrate, ^{32}P-labeled phosphotyrosine represented about 25% of the total phosphoamino acids (Table I). Because the RKC cells are temperature sensitive for transformation, we attempted to determine whether the association of tyrosyl kinase activity with maturing VSV was also temperature sensitive. However, the yields of VSV from the RKC cells, which were very low at the permissive temperature (34°C) were further reduced at the nonpermissive temperature (39°C), making it technically difficult to collect enough VSV for kinase assays. In contrast, VSV from anchorage-dependent cells in monolayer cultures including the control and, revertant BHK cells as well as a primary culture of chick embryo fibroblasts contained 3% or less of the kinase activity that phosphorylated tyrosine residues.

TABLE I

VSV-associated Tyrosyl Kinase Activity

Host cell	[^{32}P]Phosphotyrosine
	%
BHK (suspension)	21.5
BHK-RSV	20.7
BHK (revertant)	2.5
BHK (control)	2.5
Chinese hamster ovary	22.0
Chick embryo fibroblasts	3.0
HeLa	21.5
RKC-KiMSV	25.2

Purified VSV virions grown in the different cells were assayed in a kinase reaction containing [γ-^{32}P]ATP and casein as substrate. The products were partially acid hydrolyzed and analyzed by high-voltage paper electrophoresis in two dimensions. The % [^{32}P]phosphotyrosine was quantitated by dividing the counts per minute of ^{32}P incorporated into phosphotyrosine by the total counts per minute of ^{32}P incorporated into phosphoserine, phosphothreonine, and phosphotyrosine.

DISCUSSION

The percentage of tyrosine relative to serine- and threonine-phosphorylating activities associated with VSV grown in several different cell types is summarized in Table I. The activity from all eight cell types was found either at high (~20%) or low (~3%) levels as if these two levels were critical for the functions that these enzymes play. The high tyrosyl kinase activity was found in VSV from cells that were transformed by viruses (RSV and Ki-MSV), from tumorigenic cells that grow in suspension cultures (Chinese hamster ovary and HeLa), or from cells selected for anchorage independent growth in suspension cultures (BHK suspension). On the other hand, the VSV released from continuous cell lines that had not been transformed by chemicals or viruses and grow

efficiently only when attached in monolayer cultures (BHK control and revertant BHK) and VSV from primary cultures (chick embryo fibroblasts) contained the low levels of tyrosyl kinase activity. In general, high tyrosyl kinase activity in VSV correlated with capability for anchorage-independent growth and rounded morphology of the host cell. Growth in suspension was not necessary for acquisition of high kinase activity. VSV released from BHK suspension cells that were grown attached to culture dishes contained high levels of tyrosyl kinase (data not shown). Although the suspension cells were attached to the dish, they maintained their rounded morphology. VSV from RSV-transformed BHK or Ki-MSV-transformed RKC cells both in monolayers also contained high levels of tyrosyl kinase. Whether VSV was from cells that had been immortalized as continuous cell lines or from primary cultures or from cells with different growth rates did not apparently affect the tyrosyl kinase levels. The only other factor that we found to affect the VSV-associated tyrosyl kinase levels was the fetal calf serum used in the growth medium for the cells. For some lots of serum (about one in five) VSV grown in the RSV-transformed and suspension BHK cells contained low levels of tyrosyl kinase activity. VSV from the control and revertant BHK cells, however, was never found to contain levels >3.5%. Possibly factors in the serum affected either the localization of tyrosyl kinases or the VSV maturation sites from anchorage-independent cells.

The differences in the VSV-associated tyrosyl kinase activities that we measured were not caused by phosphatases or other components that interfere with protein kinase measurements. The phosphorylated angiotensin was stable for at least 60 min during incubation with detergent-disrupted VSV (Clinton, G. M., and P. Lobelle-Rich, manuscript in preparation). The kinetics of the kinase reactions (linear for at least 80 min) with either angiotensin or casein as substrates and the lack of an effect by vanidate point to the absence of interfering agents. Furthermore, assays of the VSV-associated kinases fractionated from most other viral components by column chromatography also indicated nearly 10-fold higher activity from the suspension cultures and from the RSV-transformed cells compared with monolayer BHK cells (Clinton, G. M., and P. Lobelle-Rich, manuscript in preparation). Moreover, equal amounts of VSV from BHK suspension and monolayer cultures mixed together gave additive values for tyrosyl kinase activity (data not shown) arguing against the presence of inhibitors or activators causing the differences in levels of enzyme measured.

The relative levels of tyrosyl kinase acquired by VSV virions did not always reflect the overall levels measured in the host cell. Whereas extracts of RSV-transformed BHK cells contained elevated levels of tyrosyl kinase like the virus released from them, the BHK suspension cell extract contained low levels of activity. This was concluded from in vitro protein kinase measurements in cell extracts and in subcellular fractions as well as in vivo measurements of phosphotyrosine content in cell proteins. Moreover, measurements of phosphotyrosine content in cell proteins conducted by others indicate that primary cells, continuous cell lines, chemically transformed cells, as well as cells transformed by Ki-MSV all contain similar and low levels compared to RSV-transformed cells (10, 17). The discrepancy between enzyme levels acquired by VSV and levels in the host cell may be explained by specific localization of cellular tyrosyl kinases in anchorage-independent cells at sites where VSV matures at the plasma membrane. A different location of kinases within the plasma membrane or within the cytoskeleton of cells upon losing their anchorage dependence may not be detected by conventional subcellular fractionation techniques but could lead to alterations in protein phosphorylation. In situ localization by immunological techniques should conclusively determine whether tyrosyl kinase is relocalized and concentrated at specific sites in anchorage-independent cells.

Several observations argue for specific acquisition rather than for random entrapment of cellular tyrosyl kinases into VSV (at least from BHK suspension cultures). First, the percentage of tyrosine relative to serine- and threonine-phosphorylating activities was enriched in VSV compared with extracts of the host suspension cells (22% in VSV compared with <1% in BHK suspension cells). If random entrapment occurred, several tyrosyl kinases would be captured in VSV virions since several separate tyrosyl kinases have been found in cells at or near the plasma membrane. This is not the case. A single major peak of activity has been observed after phosphocellulose chromatography of detergent disrupted VSV from BHK suspension cultures (Clinton, G. M., and P. Lobelle-Rich, manuscript in preparation). Whole cell extracts, on the other hand contain tyrosyl kinase activities that fractionate into several peaks. Finally, most evidence indicates that VSV maturation is highly regulated since most cell surface proteins and enzymes are excluded, while a few cellular proteins are enriched in VSV virions (29).

Although VSV maturation from anchorage-independent cells results in acquisition of tyrosyl kinases, these enzymes do not have an obvious effect on the growth cycle of VSV in tissue culture (26). One of the VSV structural proteins, the M protein, which functions in assembly and maturation of the virus at the membrane, is a substrate of tyrosyl kinases (25, 26). Amplification of tyrosine phosphorylation by 20-fold in the M protein does not significantly alter its function (26) or subcellular localization (Clinton, G. M., unpublished observation). It is possible that acquisition of tyrosyl kinases, as well as HeLA tumor antigens (33) and viral glycoproteins (30–32), occurs because these components are all localized at the same sites near the plasma membrane where VSV assembles and buds out from the cell. This membrane domain may be important in regulation of cell morphology as well as for providing receptors for maturation of some enveloped viruses. Analyses of tyrosyl kinases and tumor antigens acquired by VSV is of use in determining the presence of these components in a variety of cell types.

It is possible that the tyrosyl kinases found at VSV maturation sites function in cell morphology such that tyrosine phosphorylation in proteins causes alterations in the cytoskeletal structure leading to rounded morphology. Elements of this hypothesis have been previously proposed (11, 12, 22, 23), and there is some circumstantial evidence in its favor. For example, morphological changes are amongst the first alterations observed after amplified tyrosine phosphorylation when cells are transformed by RSV or when the epidermal growth factor or platelet-derived growth factor are added to cells. Moreover vinculin, a cytoskeletal protein believed to be a determinant of cell shape, is a substrate of src tyrosyl kinase (11). The data presented here, however, do not rule out the possibility that the elevation of tyrosyl kinase activity at VSV maturation sites is an indirect result of rather than a determinant of the altered organization of the cytoskeleton in cells with rounded morphology.

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