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Differences in Mechanisms of Transformation by Independent Feline Sarcoma Virus Isolates

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The Gardner and Snyder-Theilen isolates of feline sarcoma virus (FeSV) have previously been shown to encode high-molecular-weight polyproteins with a transforming function and an associated tyrosine-specific protein kinase activity. Cells transformed by these viruses exhibited morphological alterations, elevated levels of phosphotyrosine, and a reduced capacity for binding epidermal growth factor. In addition, polyproteins encoded by both of these FeSV isolates bound to, and phosphorylated tyrosine acceptor sites within, a 150,000-molecular-weight cellular substrate (P150). McDonough FeSV-transformed cells resembled Gardner and Snyder-Theilen FeSV transformants with respect to morphological changes and a reduced capacity for epidermal growth factor binding. In contrast to the other two FeSV isolates, however, McDonough FeSV encoded as its major translational product a high-molecular-weight polyprotein with probable transforming function but without protein kinase activity detectable under similar assay conditions. Moreover, total cellular levels of phosphotyrosine remained unaltered in McDonough FeSV-transformed cells, and the major McDonough FeSV polyprotein translational product lacked binding affinity for P150. These findings argue for differences in the mechanisms of transformation by these independently derived FeSV isolates.

A number of mammalian type C RNA viruses with transforming activity are known to contain, within their genomic RNA, acquired cellular sequences with specificity for transformation (for a review, see reference 8). Insertion of such sequences frequently occurs in a preexisting open reading frame and results in the synthesis of high-molecular-weight polyproteins containing both virus structural and acquired sequence-encoded components (12, 15, 19, 21, 25, 34). The availability of these recombinant viruses and the ability to identify transformation-specific proteins encoded by their acquired cellular genes provide an approach for analysis of pathways involved in malignant transformation. Recombinant transforming viruses containing acquired sequences of many different mammalian species and associated with a broad spectrum of malignancies are now available (8). The availability of such viruses provides a means of identifying specific transforming events and studying them in a more controlled manner than is possible in studies involving spontaneous or chemically induced tumors.

Three independently derived recombinant type C virus isolates of feline origin have been shown to induce fibrosarcomas in vivo and to transform embryo fibroblasts in cell culture (10, 14, 24). The major translational products of such isolates are in each case high-molecular-weight (>100,000) polyproteins consisting of type C virus structural and acquired sequence-encoded components (2, 12, 21, 25, 30, 31). In the case of one of these isolates, Snyder-Theilen feline sarcoma virus (FeSV), two variants have been reported, one encoding a major polyprotein of 115,000 M_r (30) and the second encoding an 85,000-M_r polyprotein (2, 16, 21). McDonough FeSV and Gardner FeSV encode as their major translational products polyproteins of 170,000 M_r (2, 31) and 115,000 M_r (25, 30), respectively. Protein kinase activities with specificity for tyrosine residues closely associated with, or intrinsic to, the Gardner and Snyder-Theilen FeSV-encoded polyproteins have been identified and shown to recognize the polyproteins themselves as substrates (1, 18, 32). In the case of Snyder-Theilen FeSV, this involves a single tyrosine acceptor site within the polyprotein (4). The availability of transformation-defective mutants has made possible the demonstration that these virus-encoded polyproteins and their associated

protein kinase activities have transforming function (16). Finally, a 150,000-M_r cellular protein kinase with specificity for serine and threonine has been shown to exhibit binding affinity for Gardner FeSV P115 (17) and to represent a substrate for its associated tyrosine-specific protein kinase activity (18).

As reported previously (1, 16, 32) and confirmed in Fig. 1, the protein kinase activities associated with the Gardner and Snyder-Theilen FeSV-encoded polyproteins are subject to phosphorylation in the presence of [γ-^{32}P]ATP in immunoprecipitates. In contrast, McDonough FeSV P170 immunoprecipitated from nonproductively transformed CCL64 mink cells, when assayed under similar conditions, was not phosphorylated to any appreciable extent (Fig. 1, lane O). Analogous results were obtained by analyzing P170 immunoprecipitated from Fisher rat cells transformed by McDonough FeSV (data not shown). In further attempts to demonstrate McDonough FeSV P170-associated protein kinase, we modified assay conditions by substitution of [γ-^{32}P]GTP as the phosphate donor, varied immunoprecipitation and reaction times, and tested for reactivity in the presence and absence of cyclic nucleotides. Under none of these conditions was significant reactivity observed.

In an effort to resolve whether McDonough FeSV P170 actually lacks protein kinase enzymatic activity or simply represents an inactive substrate, we tested in vitro phosphorylation of an exogenous substrate, casein. Dephosphorylated casein labeled in the presence of Gardner FeSV P115, Snyder-Theilen FeSV P115, or Sny-

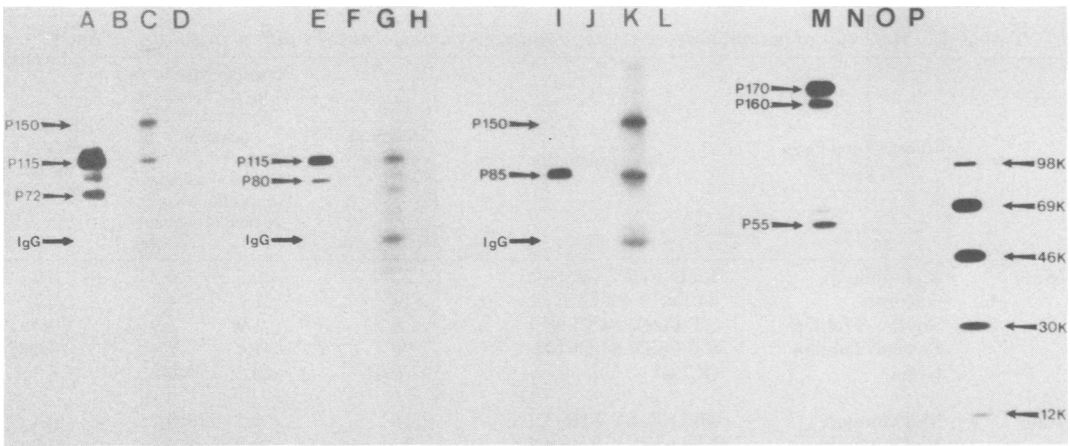

FIG. 1. *Analysis of protein kinase activities associated with polyproteins encoded by three independent isolates of FeSV. Cell lines have been described previously (16, 31) and include Gardner FeSV-transformed mink (64 FeSV) (A through D), Snyder-Theilen FeSV-transformed rat (ST-NRK) (E through H), Snyder-Theilen FeSV-transformed mink (ST-FeSV 64 Cl 56) (I through L), and McDonough FeSV-transformed mink (McD-FeSV Cl 15-1) (M through P). Metabolic labeling of exponentially growing cells for 2 h at 37°C with ^{14}C-amino acids (10 μCi/ml; 57 mCi/milligramatom; Amersham Corp., Arlington Heights, Ill.), immunoprecipitation by either goat anti-feline leukemia virus (A, E, I, and M) or control goat serum (B, F, J, and N), and analysis of immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as reported previously (16). For protein kinase assays, unlabeled cells were disrupted by repeated aspiration of 10^7 cells in 5 ml of 10 mM sodium phosphate (pH 7.2)–100 mM NaCl–1.0% Triton X-100–5 mM $MgCl_2$ (PBSTM) through a 25-gauge needle. Disrupted cells were clarified by centrifugation at 2,000 rpm for 10 min, preabsorbed by the addition of 50 μl of normal goat serum and 0.5 ml of a 10% (vol/vol) suspension of protein A–Sepharose Cl-4B, incubated overnight at 4°C, and clarified by centrifugation at 100,000 × g for 2 h. After the addition of 5 μl of either goat anti-feline leukemia virus (C, G, K, and O) or normal goat serum (D, H, L, and P), lysates (1 ml) were incubated at 4°C for 18 h, 50 μl of a 10% (vol/vol) protein A–Sepharose suspension was added, and incubation was continued for a further 2 h at 4°C. Immunocomplexes were collected by centrifugation for 10 min at 2,000 × g and washed in PBSTM. Protein kinase activity was assayed by resuspension of immunoprecipitates in 50 μl of PBSTM buffer containing 1 μCi of [γ-^{32}P]ATP (2,000 Ci mmol^{-1}) and incubation for 10 min at 30°C. Reactions were terminated by the addition of 3 ml of ice-cold 10 mM sodium phosphate (pH 7.2)–100 mM NaCl–1% Triton X-100–0.5% sodium deoxycholate–0.1% sodium dodecyl sulfate (PBSTDS), and the non-protein-bound ^{32}P label was removed by repeated centrifugation in excess PBSTDS. After the final wash, immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular weight standards included ^{14}C-labeled phosphorylase b (98,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome c (12,000).*

der-Theilen FeSV P85 exhibited readily detectable levels (1.7 to 4.2%) of phosphorylation in tyrosine (Table 1). In contrast, no detectable tyrosine phosphorylation in casein (<0.2%) was observed in immunoprecipitates of McDonough FeSV P170. Although phosphorylation of serine and threonine residues was observed in immunoprecipitates of all three virus-encoded polyproteins, this was probably attributable to trapping of cellular protein kinases in immunocomplexes. It should also be noted that, as shown in Fig. 1, Snyder-Theilen FeSV P115 (lane G)- and P85 (lane K)-associated protein kinases and, to a lesser extent, the Gardner P115 (lane C)-associated protein kinase phosphorylated the heavy chain of goat immunoglobulin. In each case this involved tyrosine acceptor sites (data not shown). In contrast, no detectable immunoglobulin phosphorylation was observed in immunoprecipitates of McDonough FeSV P170. Thus, under assay conditions by which protein kinase activities are readily demonstrated in association with polyprotein translational products of Gardner FeSV and Snyder-Theilen FeSV, McDonough FeSV P170 lacked detectable activity.

As a further comparison of mechanisms of transformation by the above virus isolates, levels of phosphotyrosine in mink and rat cell lines transformed by each were examined. As shown for representative clones in Fig. 2 and summarized in Table 1, total levels of cellular phosphotyrosine were 5- to 10-fold greater in cell lines nonproductively transformed by either the Gardner or the Snyder-Theilen strain of FeSV than they were in McDonough FeSV-transformed mink or rat cells. Thus, transformation by McDonough FeSV further differs from that by the other available FeSV isolates in that it

TABLE 1. *Properties of mammalian cells nonproductively transformed by different isolates of FeSV*[a]

Species	Transforming FeSV viral genome	Clone designation	Growth in soft agar (%)	Phosphotyrosine (% of total ^{32}P-labeled phosphoamino acids) in:		Ratio of EGF to insulin binding
				Total cell extract	Exogenous casein substrate	
Mink	McDonough	McD-FeSV Cl 15-1	23	<0.2	<0.2	0.9
	Gardner	64 FeSV F3 Cl 7	16	2.1	4.2	0.7
	Snyder-Theilen	ST-FeSV 64 Cl 56	18	1.9	1.9	0.9
	Snyder-Theilen	ST-FeSV 64 Cl 103	26	1.4	2.1	0.6
	None	CCL64	<0.01	<0.2	<0.2	8.3
Rat	McDonough	McD-FeSV FRE Cl 3	18	<0.2	<0.2	0.4
	McDonough	McD-FeSV FRE Cl 7	19	<0.2	<0.2	0.9
	Gardner	G-FeSV FRE Cl 4	24	2.1	3.5	0.6
	Snyder-Theilen	ST-FeSV FRE Cl 12	18	2.1	4.1	1.1
	Snyder-Theilen	ST-NRK	26	1.8	1.7	0.7
	None	FRE Cl 3	<0.01	<0.2	<0.2	5.9

[a] Efficiency of colony formation in soft agar was measured as previously described (16), and results are given as the number of colonies scored at 14 days expressed as a percentage of the input cell number. For phosphotyrosine analysis, cells (10^6) were incubated overnight in $^{32}P_i$-containing medium (100 μCi/ml) and disrupted in 10 mM sodium phosphate (pH 7.2)–100 mM NaCl–1% Triton X-100–0.5% sodium deoxycholate–0.1% sodium dodecyl sulfate (PBSTDS), phosphoproteins were prepared and hydrolyzed, and phosphoamino acids were subjected to two-dimensional separation on cellulose thin-layer plates as described in the legend to Fig. 2. Phosphotyrosine as a percentage of the total cellular phosphoamino acids was determined by scraping individual ^{32}P-labeled phosphoamino acids from cellulose thin-layer plates, and radioactivity was quantitated by liquid scintillation counting. Results are expressed as the ratio of ^{32}P label in total phosphoamino acids. In experiments involving the use of casein as an exogenous substrate, viral polyprotein translational products were immunoprecipitated in 10 mM sodium phosphate (pH 7.2)–100 mM NaCl–1.0% Triton X-100–5 mM $MgCl_2$ (PBSTM) as described in the legend to Fig. 1. Partially dephosphorylated casein (5 μg; Sigma Chemical Co., St. Louis, Mo.) and 100 μCi of [γ-^{32}P]ATP in 50 μl of PBSTM were added to immunoprecipitates and incubated at 25°C for 10 min. After the addition of 0.5 ml of PBSTDS, reaction mixtures were subjected to centrifugation at 2,500 rpm for 10 min at 4°C. ^{32}P-labeled casein in supernatant fluids was separated from excess [γ-^{32}P]ATP by gel filtration through a column (1 by 20 cm) of Bio-Gel P-10 (100 to 200 mesh; Bio-Rad Laboratories, Richmond, Calif.) preequilibrated with 10 mM ammonium bicarbonate, lyophilized, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel slices containing ^{32}P-labeled casein were washed in 10% methanol, lyophilized, hydrolyzed in 6 N HCl for 1 h at 100°C, mixed with unlabeled phosphoamino acid standards, and subjected to phosphoamino acid analysis as described above.

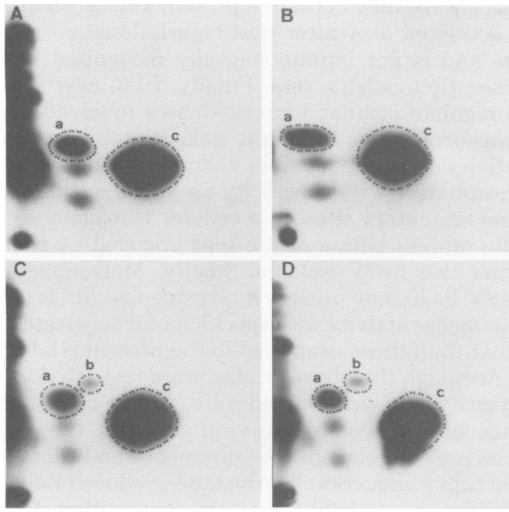

FIG. 2. *Identification of major phosphoamino acids in FeSV-transformed cells. Cells were radioactively labeled by overnight incubation in $^{32}P_i$-containing medium, disrupted, hydrolyzed, and subjected to two-dimensional phosphoamino acid analysis as described previously (16). To facilitate the identification of individual ^{32}P-labeled phosphoamino acids, unlabeled standards, including phosphothreonine (a), phosphotyrosine (b), and phosphoserine (c), were mixed at equimolar concentrations with ^{32}P-labeled hydrolysates of cellular phosphoproteins. Cell lines tested included McDonough FeSV-transformed mink (McD-FeSV Cl 15-1) (A) and rat (McD-FeSV FRE Cl 3) cells (B), Gardner FeSV-transformed mink cells (64 FeSV F3 Cl 7) (C), and Snyder-Theilen FeSV-transformed mink cells (ST-FeSV 64 Cl 56) (D).*

does not involve a generalized increase in total levels of cellular phosphotyrosine.

As indicated above, we previously described a 150,000-M_r cellular protein kinase that contains one or more tyrosine acceptor sites for the Gardner FeSV P115-associated protein kinase (18). This protein, designated P150, exhibits binding affinity for Gardner FeSV P115 and is efficiently incorporated into Gardner FeSV pseudotype virions (17). It was thus of interest to compare polyprotein translational products of Snyder-Theilen FeSV and McDonough FeSV for binding to and tyrosine phosphorylation of P150. As previously reported and confirmed in Fig. 1, in the absence of sodium dodecyl sulfate, mink cellular P150 was efficiently coimmunoprecipitated with Gardner FeSV P115 (Fig. 1, lane C) and Snyder-Theilen FeSV P85 (Fig. 1, lane K). The extent of P150 binding to Gardner FeSV P115 (Fig. 1, lane A) and to Snyder-Theilen FeSV P85 (Fig. 1, lane I) was considerably reduced in the presence of 0.1% sodium dodecyl sulfate. The inability to detect significant levels of P150 in immunoprecipitates of Snyder-Theilen FeSV P115 prepared from nonproductively transformed rat cells (Fig. 1, lane G) may indicate species differences in binding affinities between cellular P150s and FeSV-encoded transforming proteins.

In contrast to Snyder-Theilen FeSV P85, which was shown above to complex with mink cellular P150 as efficiently as did Gardner FeSV P115, McDonough P170 exhibited no apparent binding affinity for P150. This is indicated by the lack of detectable P150 (Fig. 1, lane O) in immunoprecipitates of McDonough FeSV P170 prepared in the absence of sodium dodecyl sulfate and analyzed by ^{32}P labeling under in vitro protein kinase assay conditions. As a control, goat anti-feline leukemia virus antiserum (Fig. 1, lane M), but not normal goat serum (Fig. 1, lane N), was shown to efficiently immunoprecipitate ^{14}C-amino acid-labeled McDonough FeSV P170. It should be noted that, if mink cellular P150 had been coimmunoprecipitated with McDonough FeSV P170 under these conditions, it would have been phosphorylated in serine and threonine acceptor sites by its associated protein kinase activity and thus would have been radioactively labeled.

Transformation of tissue culture cells by the Gardner and Snyder-Theilen strains of FeSV has previously been shown to result in a reduction in available epidermal growth factor (EGF) binding sites (7, 16, 28). As discussed above, transformation of cells by these two FeSV isolates is associated with an increase in tyrosine-specific protein kinase activity. In an effort to determine whether reduced EGF binding could occur independently of elevated protein kinase activity in FeSV-transformed cells, several McDonough FeSV-transformed clones were analyzed for EGF binding capacity. The results (Table 1) established that EGF binding, expressed as a ratio of EGF to insulin binding, was decreased around 8- to 10-fold in each of the transformed, as compared with nontransformed, clones. The fact that insulin binding did not vary to any appreciable extent among individual clones (data not shown) provides a convenient means of standardizing EGF binding. These findings strongly argue that a reduction in available EGF binding sites occurs to the same extent in McDonough FeSV-transformed cells and in cells transformed by each of the other virus isolates tested and that decreased EGF binding can occur independently of elevated total tyrosine phosphorylation.

The present findings establish that, despite similarities among the diseases they cause in vivo, different isolates of FeSV can induce trans-

formation by what appears to be at least two independent mechanisms. One such pathway involves phosphorylation of tyrosine acceptor sites by a virus-encoded protein kinase. Two independently derived FeSV isolates, Gardner and Snyder-Theilen, induce transformation through this mechanism. Of interest is the fact that the nonstructural components of polyproteins encoded by these two FeSV isolates share several methionine-containing peptides (2, 30) and are encoded by related acquired cellular sequences (9). The demonstration of homology between these sequences and acquired genetic sequences corresponding to nonstructural components of polyproteins encoded by the Fujinami and PRC II strains of avian sarcoma virus (22) argues that they are relatively conserved and thus of probable functional significance. Transformation by the Rous strain of avian sarcoma virus (5, 6, 11, 13, 20) and Abelson murine leukemia virus (3, 32, 33) also involves a virus-encoded protein kinase with specificity for tyrosine residues and is associated with the elevation of cellular phosphotyrosine levels. Of these, cells transformed by Abelson murine leukemia virus (3) and at least some Rous sarcoma virus-transformed clones (unpublished data) are characterized by a reduced capacity for EGF binding. Unlike transformations by the above viruses, McDonough FeSV-induced transformation is not associated with an overall increase in cellular levels of phosphotyrosine, and its major translational product, P170, does not exhibit apparent protein kinase activity. Although transformation by McDonough FeSV thus appears to involve a pathway different from that of the other two available FeSV isolates, these viruses resemble one another in that all are associated with a marked reduction in EGF binding. Additional evidence for differences in mechanisms for type C viral transformation derives from the recent demonstration of a GTP-specific protein kinase activity associated with the $p21^{src}$ protein of Harvey sarcoma virus, which exhibits specificity for threonine, rather than for tyrosine, acceptor sites (23). Another possibility is that each of these transforming proteins influences the same pathway, but interacts with different enzymatically linked components.

An important and as yet unresolved question raised by this study relates to possible functions of McDonough FeSV P170. One possibility is that P170 possesses a protein kinase activity not detected under assay conditions suitable for the Snyder-Theilen and Gardner FeSV polyprotein-associated enzymes. For instance, an activity with specificity for serine and threonine residues, but not capable of autophosphorylation, might be difficult to detect. Alternatively, McDonough FeSV P170 may contain a protein kinase which is activated only after post-translational cleavage and is not immunologically recognized by presently available sera. Finally, P170 may act to regulate cellular protein kinases involved in transformation although it lacks protein kinase activity itself. A possible example would be a phosphatase with specificity for serine or threonine regulatory sites on a cellular tyrosine-specific protein kinase of the type encoded by the other two FeSV isolates. Finally, McDonough FeSV P170 may possess a tyrosine-specific protein kinase activity with specificity for substrates other than those examined in the present study.

Although the above studies were restricted to an analysis of well-defined transforming proteins encoded by recombinant viral genomes, the results may be relevant to spontaneous and chemical tumor induction. For instance, reduced EGF binding, presumably due to competition for binding sites by an induced cellular growth factor, has been reported to be a property of some, but not all, chemically transformed cells (28). There is also a recent report that the cellular receptor for EGF contains a tyrosine-specific protein kinase activity which is activated by EGF binding (29). Thus, it should now be possible to begin to classify natural tumors with respect to mechanisms of transformation. Those spontaneous or chemically induced tumors with reduced EGF binding and elevated levels of cellular phosphotyrosine are the most likely to involve activation of cellular sequences analogous to those represented within the Gardner and Snyder-Theilen FeSV genomes. Conversely, at least a subset of tumors exhibiting reduced EGF binding associated with control levels of cellular phosphotyrosine may involve activation of cellular sequences more closely resembling those within the McDonough FeSV genome. The use of type C viruses as natural cloning vectors provides a means of isolating a broad range of cellular genes with transforming function and studying their mechanisms of action in a more highly controlled and precise manner than was previously possible (26).

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