Mapping of Multiple Phosphorylation Sites Within the Structural and Catalytic Domains of the Fujinami Avian Sarcoma Virus Transforming Protein

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Received 12 July 1982/Accepted 17 December 1982

The phosphorylation sites of the P140\(^{gag-fps}\) gene product of Fujinami avian sarcoma virus have been identified and localized to different regions of this transforming protein. FSV P140\(^{gag-fps}\) isolated from transformed cells is phosphorylated at least three distinct tyrosine residues and one serine residue, in addition to minor phosphorylation sites shared with Pr76\(^{gag}\). Partial proteolysis with virion protease p15 or with Staphylococcus aureus V8 protease has been used to generate defined peptide fragments of P140\(^{gag-fps}\) and thus to map its phosphorylation sites. The amino-terminal gag-encoded region of P140\(^{gag-fps}\) contains a phosphotyrosine residue in addition to normal gag phosphorylation sites. The two major phosphotyrosine residues and the major phosphoserine residue are located in the carboxy-terminal portion of the fps-encoded region of P140\(^{gag-fps}\). P140\(^{gag-fps}\) radiolabeled in vitro in an immune complex kinase reaction is phosphorylated at only one of the two C-terminal tyrosine residues phosphorylated in vivo and weakly phosphorylated at the gag-encoded tyrosine and at a tyrosine site not detectably phosphorylated in vivo. Thus, the in vitro tyrosine phosphorylation of P140\(^{gag-fps}\) is distinct from that seen in the transformed cell. A comparative tryptic phosphopeptide analysis of the gag-fps proteins of three Fujinami avian sarcoma virus variants showed that the phosphotyrosine-containing peptides are invariant, and this high degree of sequence conservation suggests that these sites are functionally important or lie within important regions. The P105\(^{gag-fps}\) transforming protein of PRCII avian sarcoma virus lacks one of the C-terminal phosphotyrosine sites found in Fujinami avian sarcoma virus P140\(^{gag-fps}\). Partial trypsin cleavage of FSV P140\(^{gag-fps}\) immunoprecipitated with anti-gag serum releases C-terminal fragments of 45K and 29K from the immune complex that retain an associated tyrosine-specific protein kinase activity. This observation, and the localization of the major P140\(^{gag-fps}\) phosphorylation sites to the C-terminal fps region, indicate that the kinase domain of P140\(^{gag-fps}\) is located at its C terminus. The phosphorylation of P140\(^{gag-fps}\) itself is complex, suggesting that it may itself interact with several protein kinases in the transformed cell.

The avian sarcoma viruses (ASVs) are a group of acutely oncogenic RNA tumor viruses. They rapidly induce the neoplastic transformation of cells such as fibroblasts in tissue culture and the formation of sarcomas in animals (4, 10). Like other transforming RNA tumor viruses, the ASVs have apparently arisen by recombination between a cellular gene and a nontransforming, replication-competent viral genome, usually resulting in the deletion of viral replicative coding sequences and their substitution by the transduced cellular sequence (23, 37). The proteins encoded by the cellularly derived transforming genes of these viruses are directly responsible for the initiation and maintenance of the transformed phenotype of infected cells (4, 10). Among the ASVs, four distinct transforming genes have been identified: src (Rous sarcoma virus), fps (Fujinami avian sarcoma virus [FSV], PRCII), yes (Y-73), and ros (UR2) (15, 17, 18, 37, 39, 43, 44). The amino acid sequences of their gene products, predicted from DNA sequence data, show remarkable homology in their carboxy-terminal 300 amino acids, whereas their amino termini are largely unrelated (17, 36). Reflecting this structural relationship, the ASV transforming proteins are all associated with protein kinase activities specific for tyrosine residues and are themselves phosphorylated at both serine and tyrosine sites (11, 12a, 13, 24, 29, 30, 35). Cells transformed by these viruses show elevated levels of phosphotyrosine resulting from the phosphorylation of a number of cellular proteins (9, 20, 30, 35). The sets of
cellular proteins phosphorylated at tyrosine in response to transformation by the different classes of ASV show considerable overlap, and although a few have been identified (6, 22, 27, 34) the function of most of these cellular targets and the relationship between their phosphorylation and the phenotypic changes seen in transformed cells is unclear. Variants of FSV and Rous sarcoma virus which induce a temperature-sensitive transformed cell phenotype are also temperature sensitive for the phosphorylation of cellular proteins (14, 30, 35), and temperature-sensitive FSV (tsFSV) encodes a transforming protein that rapidly loses its kinase activity and undergoes dephosphorylation of its tyrosine and serine residues at the nonpermissive temperature (30). Transformation-defective mutants of Snyder-Theilen feline sarcoma virus, which is closely related to FSV (2, 38), are deficient in protein kinase activity (3). These data have suggested that the ASV transforming proteins induce cellular transformation by modulating the control of cell growth, structure, and gene expression through the pleiotropic effect of protein phosphorylation.

The 4.5-kilobase genomic FSV RNA encodes a 140 to 150K protein (P140\(^{gag-fps}\)) synthesized from a defective gag gene and the fps gene (15, 18). The nondefective gag gene of a replication-competent virus encodes a precursor (Pr\(T^6\)) to the five virion core proteins (40). We have previously shown that P140\(^{gag-fps}\) possesses an N-terminal sequence of approximately 40K corresponding to the gag proteins p19, p10, and part of p27 and a C-terminal sequence of 100 to 110K synthesized from the fps gene (31). FSV P140\(^{gag-fps}\) immunoprecipitated with antiserum directed against antigenic determinants in its gag or fps regions is itself phosphorylated exclusively at tyrosine residues after incubation in vitro with [\(\gamma\)-\(^{32}\)P]ATP and Mn\(^{2+}\) (12, 21, 31). In contrast, P140\(^{gag-fps}\) isolated from transformed cells is phosphorylated at both serine and tyrosine residues, of which the major sites are found in the fps-encoded region (12, 30, 31). The tyrosine phosphoepitopes of P140\(^{gag-fps}\) phosphorylated in vitro are related to those from in vivo-phosphorylated P140\(^{gag-fps}\) (30). Here we have investigated the phosphorylation of FSV P140\(^{gag-fps}\) in detail and have established the relationship between the in vivo and in vitro phosphorylation of P140\(^{gag-fps}\) and the location of these sites within different structural and functional regions of the protein.

**MATERIALS AND METHODS**

**Cells and viruses.** The variants of FSV used here have been described previously (15, 18, 30). Temperature-sensitive tsFSV clone L5 and its temperature-resistant derivative, trFSV, were both pseudotyped with FSV-associated helper virus (FAV) and encode a 140K protein (P140\(^{gag-fps}\)) (30). FSV clone 12 encodes a 130K protein (P130\(^{gag-fps}\)) and comes from a different FSV stock (14, 15). PRCII rescued with ring-neck pheasant virus was obtained from G. S. Martin. gs\(^{+}\)-chicken embryo fibroblasts (CEF) were obtained from H & N Farms, and Japanese quail embryo fibroblasts were obtained from the quail unit, University of British Columbia. Cells were infected with virus (5 \(\times\) 10\(^{5}\) to 1 \(\times\) 10\(^{6}\) focus-forming units per ml), passaged after 3 or 4 days, and used on the day 4 or 5. Unless otherwise specified, all cells were maintained at 37°C.

**Radiolabeling of cells.** Cells were seeded at a density of 2 \(\times\) 10\(^{5}\) cells in a 10-mm well (Linbro) and the following day were incubated with \(^{32}\)P, (2.0 mCi/ml, carrier free; ICN Pharmaceuticals, Inc.) in 0.5 ml of phosphate-free Dulbecco modified Eagle medium containing 1% calf serum and 1% heat-inactivated chicken serum. After the labeling period the dish was transferred to ice, and the cells were washed two times with ice-cold phosphate-buffered saline and then lysed in a total of 750 µl of lysis buffer (1% Nonidet P-40 [NP40], 0.5% sodium deoxycholate, 10 mM Tris-hydrochloride [pH 7.5], 100 mM NaCl, 1 mM EDTA, 2 mM ATP, and 1% [w/vol] aprotinin [Sigma Chemical Co.]). The lysate was centrifuged at 4°C at 27,000 \(\times\) g for 30 min, and the supernatant was recovered and incubated with the appropriate antiserum. Cells were labeled with \(^{35}\)S)methionine according to a similar protocol, except that the cells were incubated with \(^{35}\)S)methionine (400 µCi/ml, 1,000 Ci/mmol; Amersham Corp.) in 0.5 ml of methionine-free Dulbecco modified Eagle medium containing 1% calf serum and 1% heat-inactivated chicken serum. ATP was omitted from the lysis buffer.

**Immunoprecipitation.** Pepsin cell lysates were incubated with rabbit serum (2 to 4 µl) for 30 min and then with 15 volumes of a 10% suspension of *Staphylococcus aureus* strain Cowan 1 (IgSorb; the Enzyme Center) in cell lysate buffer for another 30 min. The immune complex was then pelleted in a microfuge and washed successively with 1 M NaCl-10 mM Tris-hydrochloride (pH 8.0)—0.1% NP40, with 0.1 M NaCl-1 mM EDTA-10 mM Tris-hydrochloride (pH 8.0)—0.1% NP40—0.1% sodium dodecyl sulfate (SDS), and with 10 mM Tris-hydrochloride (pH 8.0)—0.1% NP40. Immunoprecipitates of \(^{32}\)P-labeled cells were washed once more with 1.5 M NaCl-1 mM EDTA-10 mM Tris-hydrochloride (pH 7.5)—0.1% NP40, which eliminated nonspecific absorption of RNA. All of these steps were performed at 4°C. Immunoprecipitates were prepared for SDS-polyacrylamide gel electrophoresis as described elsewhere (30).

**Immune complex kinase reaction.** Samples of 1 \(\times\) 10\(^{6}\) to 5 \(\times\) 10\(^{6}\) FSV- or PRCII-infected cells were lysed and immunoprecipitated, and the immune complex was washed and incubated with 5 to 50 µCi of [\(\gamma\)-\(^{32}\)P]ATP (2,500 Ci/mmol; Amersham) in 10 mM MnCl\(_2\)—20 mM Tris-hydrochloride (pH 7.5) at 20°C as described previously (30). After the incubation the immune complex was washed as described previously (30), except that the buffers contained 10 mM ATP and 10 mM EDTA, and prepared for gel electrophoresis.

**SDS-polyacrylamide gel electrophoresis.** Samples were heated at 100°C for 3 min in SDS sample buffer (2% SDS, 5% β-mercaptoethanol, 10 mM Tris-hydrochloride [pH 6.8], 10% [w/vol] glycerol), and then subjected to electrophoresis through SDS-polyacrylamide...
amide slab gels at 3 W per gel. Separating gels usually contained 7.5% acrylamide cross-linked with 0.2% bisacrylamide. Gels used for V8 protease digestion and separation of the resulting cleavage products were as described by Cleveland et al. (7), with separating gels of 15% acrylamide cross-linked with 0.867% bisacrylamide. After electrophoresis, gels of 32P-labeled proteins were either covered with Saran Wrap and exposed to film (Kodak XAR-5) while wet at 4°C or stained with Coomassie blue to locate molecular weight markers of known size. Fixed and stained gels were dried, and the sensitivity of detection of 32P was increased by using an intensifying screen (DuPont; Lightning-Plus) with XAR-5 film at -80°C. Gels of [35S]methionine-labeled proteins were impregnated with En3Hance (New England Nuclear Corp.) before drying and exposed at -80°C, unless the proteins were to be analyzed further, in which case fluorography was omitted.

Partial proteolytic cleavage with p15, V8 protease, and trypsin. Immunoprecipitates of gag-related proteins were incubated with NP40-disrupted virions (Prague B Rou5 sarcoma virus) as described previously (31, 41). For the cleavage of proteins phosphorylated in vitro, 10 mM ATP and 10 mM EDTA were included in all buffers after the termination of the kinase reaction.

Limited proteolysis with V8 protease was performed in situ in SDS-polyacrylamide gels as described by Cleveland et al. (7). Proteins were labeled with 32P in vivo or in vitro and purified after immunoprecipitation by SDS-polyacrylamide gel electrophoresis. The pertinent bands were excised from wet gels, equilibrated with buffer (125 mM Tris-hydrochloride [pH 6.8], 1 mM EDTA, 0.1% SDS), and applied to the sample well of a fresh gel with a 5-cm-long stacking gel and a 15% polyacrylamide separating gel. The sample was overlaid, first with this buffer containing 20% glycerol and then with buffer containing 10% glycerol and V8 protease. Electrophoresis was performed at 2 W until the dye front approached the separating gel, when the current and the cooling system were turned off for 30 min. Electrophoresis was then resumed at 3 W. Limited proteolysis with trypsin of immunoprecipitated proteins was performed essentially as described previously (33). Cells were lysed and immunoprecipitated as described above. The immune complex adsorbed to S. aureus was washed with three immunoprecipitation buffers as described above, suspended in 30 μl of pH 9 buffer (10 mM Tris-hydrochloride [pH 9], 100 mM NaCl, 5 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 0.5% NP40 [33]), and incubated with 30 μg of tolyslsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin on ice for 15 or 30 min.

To perform protein kinase reactions after 15 minutes of partial trypsin cleavage, the pH was adjusted to 7.5, and MnCl2 was added to a final concentration of 10 mM in the presence of 5 μCi of [γ-32P]ATP, and the incubation was continued for 15 minutes on ice. Alternatively, the immune complex was pelleted by centrifugation after trypsin digestion and suspended in 30 μl of 10 mM MnCl2-20 mM Tris-hydrochloride (pH 7.5), whereas the supernatant from the centrifugation was made to pH 7.5 and 10 mM MnCl2. Both incubations were continued on ice in the presence of [γ-32P]ATP. Reactions were terminated by the addition of SDS-gel sample buffer.

Analysis of tryptic peptides and phosphoamino acids. 32P-labeled proteins were purified by SDS-polyacrylamide gel electrophoresis, eluted from the gel slices, oxidized, and digested with TPCK-treated trypsin as described previously (30, 31). Tryptic digests were separated in two dimensions on 20- by 20-cm thin-layer cellulose plates (0.1 mm; E. Merck Labs) using electrophoresis at pH 2.1 (water–88% formic acid–acetic acid, 90:2.8:0.05 by volume) for 60 min at 1,000 V in the first dimension and chromatography in N-butanol–acetic acid–water–pyridine (75:15:60:50, by volume) in the second dimension. 32P was detected by exposing the plates to XAR-5 film at -80°C with the aid of an intensifying screen.

[35S]methionine-labeled proteins were eluted from gel slices from a preparative gel, oxidized, digested with TPCK-trypsin and analyzed as for 32P-labeled proteins (30). Thin-layer cellulose plates were sprayed with En3Hance and exposed to film at -80°C.

Phosphoamino acids obtained by hydrolysis of 32P-labeled proteins or isolated tryptic phosphopeptides in 6 N HCl at 110°C for 90 min in vacuo were identified as described previously after electrophoresis on thin-layer cellulose plates at pH 1.9 and pH 3.5 (30, 35). Phosphotyrosine for use as a marker was a generous gift of B. Sefton.

Cell-free translation. Isolation of polyadenylated, heat-denatured FSV(FAV) 70S virion RNA and its translation in the messenger-dependent rabbit reticulocyte lysate was as described previously (18, 30).

RESULTS

Tryptic phosphopeptides of P140<sup>agg</sup> and P140<sup>fps</sup>. To identify the sites of phosphorylation in the FSV transforming protein, we have analyzed the phosphopeptides produced by trypsin digestion of tsFSV L5 P140. P140 was either isolated by immunoprecipitation from FSV-transformed cells labeled with 32P or phosphorylated in vitro in the immune complex with [γ-32P]ATP as a phosphate donor. The sites phosphorylated in vivo were then compared with those labeled in vitro.

FSV P140 phosphorylated in vitro in the immune complex kinase reaction is labeled exclusively at phosphotyrosine (12, 30; G. Weinmaster, unpublished observation). Tryptic phosphopeptide analysis of tsFSV L5 P140 thus phosphorylated in the immune complex revealed five labeled spots (Fig. 1C) after electrophoresis at pH 2.1 and chromatography in a butanol-acetic acid-pyridine buffer on a thin-layer cellulose sheet. Under some conditions of analysis spots 3a and 3b were not seen, and 3c was the major phosphorylated peptide. Reanalysis of purified peptide 3c under the experimental conditions used in Fig. 1 generated peptides 3a and 3b, suggesting that these are derived by modification of peptide 3c, and that spots 3a through c represent different forms of the same tryptic peptide (data not shown). In contrast, the relatively minor tryptic phosphopeptides 1 and 2 apparently represent distinct sites of tyrosine
phosphorylation (see below), suggesting that P140 is phosphorylated in vitro at three different tyrosine residues.

To compare the P140 sites phosphorylated in vitro by its associated kinase activity with the residues actually phosphorylated in the transformed cell, P140 was isolated from $^{32}$P-labeled tsFSV L5-transformed chicken embryo fibroblasts and subjected to tryptic phosphopeptide analysis (Fig. 1A and B). P140 labeled in vivo yields five major phosphotyrosine-containing spots when analyzed in this way, of which four (peptides 1 and 3a through c) comigrate with those from in vitro phosphorylated P140. A major new tryptic phosphopeptide (peptide 4) which contains phosphotyrosine as its sole phosphoamino acid is present in the digest of in vivo-labeled P140, and peptide 2 of in vitro-phosphorylated P140 is missing. Mixing experiments on tryptic digests of P140 phosphorylated in vivo and in vitro show that peptides 2 and 4 migrate differently. At least two minor tryptic phosphopeptides of in vivo-labeled P140 comigrate with those found in FAV Pr76 gag, isolated from $^{32}$P-labeled, FSV(FAV)-infected cells, indicating that they represent normal sites of gag phosphorylation. In addition, P140 possesses a strongly labeled tryptic phosphopeptide containing phosphoserine (spot 5) which is not found in FAV Pr76 gag and thus represents specific phosphorylation of a serine residue on P140. These results indicate that tsFSV L5 P140 is phosphorylated in transformed cells on three tyrosine residues (contained within tryptic peptides 1, 3a through c, and 4), of which one (peptide 4) is not phosphorylated in vitro, and on several serine residues of which at least one (peptide 5) is specific to P140. Three minor FSV-specific tryptic phosphopeptides from in vivo-phosphorylated P140 (peptides 6 through 8) have not been analyzed in any detail, although peptide 6 is known to contain phosphotyrosine, and it is not known whether they represent additional minor sites of phosphorylation. We see little radioactivity remaining at the origin in these two-dimensional analyses and very little free phosphate, indicating that we have separated the majority of tryptic phosphopeptides. Peptide 1 must have an overall negative change, as it migrates to the positive electrode at pH 2.1, and it therefore either is very small or multiply phosphorylated or contains cysteic acid in addition to a phosphate group since the side chains of glutamic acid and aspartic acid are not ionized at this pH. All of the other peptides migrate toward the negative electrode in the electrophoretic dimension.

For in vivo-phosphorylated P140 the relative labeling of the tyrosine residues within tryptic phosphopeptides 1, 4, and 3a through c is approximately 0.2:0.6:1 (where the figure for 3a through c is the sum of these spots), although it is hard to know whether this reflects the actual steady-state levels in vivo. On in vitro-phosphorylated P140, spots 3a through c represent a single major site of tyrosine phosphorylation, with tryptic phosphopeptides 1 and 2 comprising relatively minor phosphorylation sites.

Localization of phosphorylation sites on P140 gag-fps. We have used proteolytic enzymes to cleave FSV P140 into two or more fragments, and these fragments have been mapped onto the

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**FIG. 1.** Tryptic phosphopeptide analysis of FSV P140$^{gag-fps}$. FSV P140 was labeled with $^{32}$P in vivo by incubation of transformed CEFs with $^{32}$P; and isolated by subsequent immunoprecipitation of the labeled protein with anti-p19 serum or phosphorylated in vitro in an immune complex kinase reaction after immunoprecipitation with anti-p19 serum from transformed CEFs. Gel-purified P140 was then digested with trypsin and separated in two dimensions on thin-layer cellulose plates. An "O" indicates the sample origin. Electrophoresis at pH 2.1 was from left to right with the anode on the left, and chromatography was from bottom to top. Tryptic digests were as follows: A, tsFSV P140, phosphorylated in vivo (18-h $^{32}$P labeling); B, tsFSV P140 phosphorylated in vivo (4-h $^{32}$P labeling); C, tsFSV P140 phosphorylated in vitro. Phosphoamino acid analysis of tryptic peptides from in vivo-labeled P140 showed that spots 1, 3a through c, and 6 contain predominantly phosphotyrosine, whereas spot 5 contains predominantly phosphoserine.
intact protein. By constructing such a proteolytic cleavage map of FSV P140 we have been able to localize phosphorylation sites to different regions of the protein (see Fig. 7). The cleavage of P140 by the avian retrovirus virion protease p15 has been previously described (31, 41, 42). p15 cuts P140 within its N-terminal gag region, yielding a 33K, N-terminal, gag-encoded fragment [N-33K(p15)] and a C-terminal, 120K fragment [C-120K(p15)] that contains a small region of gag p27 sequence and the entire C-terminal fps-encoded region (31). To localize phosphorylation sites to these two fragments, P140 was labeled in vivo with 32P or phosphorylated in vitro in the immune complex reaction and then cleaved with p15 by the addition of disrupted Rous sarcoma virus virions to the immunoprecipitated protein. Figure 2 shows that both the N-33K(p15) and the C-120K(p15) fragments of in vivo- and in vitro-phosphorylated P140 are labeled with 32P. Phosphoamino acid analysis of the N-terminal 33K fragment of in vitro-phosphorylated P140 revealed only phosphotyrosine, whereas the corresponding 33K fragment from P140 phosphorylated in transformed cells contained phosphoserine and phosphotyrosine in equivalent amounts (data not shown). Tryptic phosphopeptide analysis of the N-terminal 33K gag fragment of P140 phosphorylated in vivo yielded the acidic phosphotyrosine-containing tryptic peptide 1 in addition to phosphopeptides which comigrate with those of FAV Pr76gag (Fig. 3A). The N-33K(p15) fragment of in vitro-phosphorylated P140 is labeled only at tryptic phosphopeptide 1 (Fig. 3B). The C-terminal 120K p15 cleavage fragment of in vivo-phosphorylated P140 gives phosphotyrosine-containing tryptic peptides 3a through c and 4 and phosphoserine-containing peptide 5 (Fig. 3D), whereas the C-120K(p15) fragment of P140 labeled in the immune complex reaction contains only peptides 3a through c (Fig. 3E). These results indicate that P140 is phosphorylated in transformed cells at a tyrosine site (tryptic phosphopeptide 1) within the gag region in addition to sites of phosphorylation shared with nondefective Pr76gag. However, the major sites of tyrosine (peptides 3a through c and 4) and serine (peptide 5) phosphorylation are contained within the fps region. To verify the identity of the p15 cleavage fragments used in these experiments, [35S]methionine-labeled P140 isolated from FSV-infected cells was cleaved with p15 and coelectrophoresed with the 32P-labeled fragments, and the [35S]methionine-labeled 33K p15 fragment, intact FSV P140, and FAV Pr76gag were subjected to tryptic peptide analysis (Fig. 3). This confirmed that the 33K fragment contains only gag-encoded sequences.

To localize phosphorylation sites in C-120K(p15) more accurately within the fps-encoded region of FSV P140 we generated partial proteolytic cleavage fragments with S. aureus V8 protease (17) by the technique of Cleveland et al. (7). Digestion of [32P]-labeled P140 with low concentrations of V8 protease yields two major products with apparent molecular weights of 78K and 61K [78K(V8) and 61K(V8)] (Fig. 4), as does limited V8 protease cleavage of [35S]methionine-labeled P140 (data not shown). Tryptic peptide analysis of the V8 protease digestion products of [35S]methionine-labeled P140 (Fig. 5) showed that the 78K(V8) fragment contains all of the tryptic peptides previously identified as gag specific, whereas the 61K(V8) fragment contains only tryptic peptides previously identified as fps specific (31) (Fig. 3). In particular, there is no apparent overlap between these two V8 protease cleavage fragments, arguing that they represent a unique N-terminal 78K fragment and a unique C-terminal 61K fragment presumably separated at a single V8 protease cleavage site in the middle of the P140 fps region. To test this deduction, we isolated the C-120K(p15) fragment of in vitro-phosphorylated

FIG. 2. Cleavage of FSV P140[32P] with p15. FSV P140 was labeled in vivo with 32P, and isolated by immunoprecipitation with anti-p19 serum or phosphorylated in an immune complex kinase reaction with [γ-32P]ATP. Both samples were washed extensively, incubated at 37°C for 30 min in the presence or absence of 10 μg of NP40-disrupted RSV(Pr-B), and then prepared for electrophoresis on a 7.5% SDS-polyacrylamide gel. Lanes: A and B, tsFSV P140 phosphorylated in vivo (18-h labeling); A, uncleaved; B, cleaved; C and D, tsFSV P140 phosphorylated in vitro; C, uncleaved; D, cleaved.
FIG. 3. Tryptic peptide analysis of p15 cleavage fragments of FSV P140<sup>ts</sup>/fps. <sup>32</sup>P- or <sup>35</sup>S-labeled polypeptides were gel purified, digested with trypsin, and analyzed by two-dimensional separation on thin-layer cellulose plates. Protein fragments analyzed were as follows: A, N-33K(p15) obtained by p15 cleavage of <i>ts</i>FSV P140
This would generated this and within [N-78K(V8)] digestion. This concurs the terminal *gag* region and shows that the two major phosphotyrosine sites (peptides 3a through c and 4) and the major phosphoserine site (peptide 5) are clustered C terminally in the P140 *fps* region. A similar analysis of P140 phosphorylated in vitro in the immune complex kinase reaction (Fig. 5) demonstrates that N-78K(V8) contains tryptic phosphopeptides 1 and 2, whereas C-61K(V8) contains only tryptic phosphopeptides 3a through c. This is consistent with our observation that the N-78K(V8) from in vitro-phosphorylated P140 is more heavily labeled with $^{32}$P relative to C-61K(V8) than the corresponding fragment from in vivo-phosphorylated P140 (data not shown). It is possible that in vitro-phosphorylated peptide 2 is near the site of p15 cleavage, since it is not easily recovered in the p15 cleavage fragments.

The results of these mapping experiments are shown diagrammatically in Fig. 7.

**Phosphorylation of the transforming proteins of different *fps* viruses.** FSV L5 induces a temperature-sensitive, transformed phenotype and encodes a P140 with a thermolabile protein kinase activity (30). To determine whether this temperature sensitivity reflects any change in the amino acid sequences immediately surrounding the P140 phosphorylation sites, we analyzed the tryptic phosphopeptides of its temperature-resistant derivative, *tr*FSV. Figure 8 shows that *tr*FSV and *ts*FSV P140 isolated from $^{32}$P-labeled, FSV-transformed CEFs have tryptic phosphopeptide maps identical to those of their P140 proteins phosphorylated in the immune complex. A further variant of FSV with a different passage history (14, 15) was analyzed in the same way. The P130$^{ag-fps}$ from this variant is phosphorylated in vivo with $^{32}$P at phosphotyrosine-containing tryptic peptides which comigrate with those of *ts*FSV L5 and *tr*FSV. However, phosphoserine-containing peptide 5 is missing, and new phosphopeptides are seen, suggesting that there has been sequence divergence in the tryptic peptide encompassing the major serine phosphorylation site, or that the sites are completely different.

PRCII is an independent isolate of a transforming virus containing *fps* sequences, and its RNA genome lacks approximately 1.5 kilobases of sequence relative to the FSV *fps* gene (1, 5, 9, 43a). Its transforming protein P105$^{ag-fps}$ might therefore be expected to be missing about half of the *fps* sequence of FSV P140, probably toward phosphorylated in vivo; B, N-33K(p15) obtained by p15 cleavage of *ts*FSV P140 phosphorylated in vitro; C, N-33K(p15) obtained by p15 cleavage of *ts*FSV P140 labeled with $[^{35}]$methionine in vivo; D, C-120K(p15) obtained by p15 cleavage of *ts*FSV P140 phosphorylated in vivo; E, C-120K(p15) obtained by p15 cleavage of *ts*FSV P140 phosphorylated in vitro; F, FSV P140 isolated from *ts*FSV-transformed CEFs labeled with $[^{35}]$methionine; G, Pr76$^{ag}$ isolated from the same $[^{35}]$methionine-labeled cells. The numbering of methionine-containing peptides is by the method of Pawson et al. (31) and is differentiated by a superscript "S." Tryptic peptides 1* through 5* are *fps* specific.
FIG. 5. Tryptic peptide analysis of V8 protease cleavage fragments of FSV P140<sup>env/ves</sup>. <sup>32</sup>P- or <sup>35</sup>S-labeled polypeptides were gel purified, digested with trypsin, and analyzed by two-dimensional separation on thin-layer cellulose plates. Protein fragments analyzed were as follows: A, N-78K(V8) produced by V8 cleavage of <sup>[<sup>35</sup>S]</sup>methionine-labeled tsFSV P140 obtained by cell-free translation of FSV(FAV) polyadenylic acid-selected, heat-denatured 70S virion RNA in a messenger-dependent rabbit reticulocyte lysate; B, N-78K(V8) obtained by V8 protease cleavage of tsFSV P140 phosphorylated in vivo (18-h labeling); C, N-78K(V8) obtained by V8 protease cleavage of tsFSV P140 phosphorylated in vitro; D, C-61K(V8) produced by V8 protease cleavage of <sup>[<sup>35</sup>S]</sup>methionine-labeled tsFSV P140 obtained by cell-free translation of FSV(FAV) polyadenylic acid-selected, heat-denatured 70S virion RNA in a messenger-dependent rabbit reticulocyte lysate; E, C-61K(V8) obtained by V8 protease cleavage of tsFSV P140 phosphorylated in vivo (18-h labeling); F, C-61K(V8) obtained by V8 protease cleavage of tsFSV P140 phosphorylated in vitro. The numbering of methionine-containing peptides is by the method of Pawson et al. (31) and is differentiated by a superscript "S." Tryptic peptides 1<sup>+</sup> through 5<sup>′</sup>s are <i>ves</i> specific.
and the FSV cleaved with V8 fragment.

of possible ftsFSV A, P105.

tical separation on N-terminal in the phosphorylated transforming proteins examined. It is therefore possible that this site is contained within a region of FSV P140 that is deleted in PRCII P105.

Kinase activity of FSV P140<sup>agfps</sup> proteolytic cleavage fragments. We have used partial trypsin cleavage of FSV P140 to generate peptide fragments which retain protein kinase activity. FSV P140 was immunoprecipitated with anti-p19 serum, labeled in the immune complex with <sup>32</sup>P, washed extensively, and then digested on ice with trypsin for 15 or 30 min. The <i>S. aureus</i>-associated immune complex was then pelleted by centrifugation, and the P140 trypsin cleavage fragments remaining bound in the immune complex were compared with those released from the complex and left in the supernatant (Fig. 9). No intact P140 was seen after 15 min of partial tryptic digestion, but <sup>32</sup>P-labeled fragments of 45K, 36K, 29K, and 23K were generated (Fig. 9, lanes G through J). All four fragments were found in the immune complex after digestion (Fig. 9, lanes G and I), but only the 45K and 29K fragments were released from the complex (Fig. 9, lanes H and J). Increasing the digestion time had no qualitative effect on the cleavage fragments, although we have observed increased amounts of the 29K fragment corresponding with decreased amounts of the 45K fragment, suggesting that the 29K peptide may be a subfragment of 45K. Both the 29K and 45K fragments contain tryptic phosphopeptides 3a through c, indicating that they represent C-terminal fragments of P140, as might be expected from the observation that they are released from the immune complex by trypsin digestion. The 36K and 23K fragments are presumably N terminal as they are completely retained in the anti-gag immune complex after trypsin cleavage. To test the activity of these fragments in a protein kinase reaction, we isolated P140 from ftsFSV-transformed chicken cells maintained at 37°C by immunoprecipitation with anti-p19 serum, digested the P140 with trypsin as described above, and incubated the cleavage products with [γ-<sup>32</sup>P]ATP. The fragments released from the immune complex by trypsin retained the protein kinase activity of intact P140, and both the 45K and 29K fragments became phosphorylated (Fig. 9, lane D). The corresponding cleavage fragments of P140 isolated from ftsFSV-infected cells shifted to the nonpermissive temperature (41.5°C) 18 h before cell lysis showed little kinase activity (Fig. 9, lane F). Surprisingly, the immune complex pellet recovered after trypsin digestion has little kinase activity (data not shown). These results suggest that the tyrosine-

FIG. 6. Mapping V8 protease cleavage fragments of FSV P140<sup>agfps</sup>. The tsFSV P140 was labeled with <sup>32</sup>P, during an immune complex kinase reaction and cleaved with NP40-disrupted virions. Uncleaved P140 and the two p15 cleavage fragments 120K(p15) and 33K(p15) were recovered from the same gel and then subjected to in situ V8 protease digestion with 50 ng of V8 protease per sample followed by electrophoretic separation on a 15% SDS-polyacrylamide gel. Lanes: A, tsFSV P140; B, 120K(p15) fragment; C, 33K(p15) fragment.

FIG. 7. Cleavage sites for p15 and V8 protease on FSV P140<sup>agfps</sup> yielding the fragments described in the text. The numbers indicate the putative location of phosphorylation sites within FSV P140<sup>agfps</sup> as discussed in the text.
specific protein kinase domain of P140 is contained within a C-terminal 45K fragment that can be released from the immune complex by partial trypsin cleavage. At least one of the major tyrosine phosphorylation sites, represented by tryptic phosphopeptides 3a through c, is contained within this fragment, and this C-terminal location of the kinase domain is consistent with the C-terminal clustering of the major fps phosphorylation sites.

**DISCUSSION**

Our data show that FSV P140 is phosphorylated in transformed cells at multiple tyrosine residues, and comparative tryptic phosphopeptide analysis indicates that these residues lie within sequences that are highly conserved between the transforming proteins of different FSV variants. As previously reported (31), the N-terminal gag region of P140 contains minor phosphor-
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Figure 9. Identification and kinase activity of FSV P140gag-fps partial tryptic cleavage fragments. Lanes A through F: Lysates made from tsFSV-infected cells that had been maintained at 37°C (A through D) or 41.5°C (E and F) were immunoprecipitated with rabbit nonimmune serum (A and B) or with anti-p19 serum (C through F), and the immune complexes were incubated on ice for 15 min in the presence (B, D, and F) or absence (A, C, and E) of 30 μg of TPCK-trypsin per ml. Undigested samples (A, C, and E) were washed and incubated with [γ-32P]ATP-10 mM MnCl₂ and 20 mM Tris-hydrochloride (pH 7.5) for 15 min at 4°C. The trypsin-digested samples (B, D, and F) were centrifuged to pellet the immune complexes, and the supernatants were incubated with [γ-32P]ATP and 10 mM MnCl₂ at pH 7.5 for 15 min on ice. For lanes G through J, tsFSV-infected CEFs maintained at 37°C were lysed and immunoprecipitated with rabbit anti-p19 serum, and the immune complex was incubated with [γ-32P]ATP to label P140gag-fps. The immune complex was suspended in 30 μl of pH 9 buffer and incubated with 30 μg of TPCK-trypsin for 15 min (G and H) or 30 min (I and J). The immune complex was pelleted, and the fragments sedimenting in the immune complex (G and I) were analyzed separately from those remaining in the supernatant (H and J). Samples were separated by electrophoresis on a 10% polyacrylamide gel, which was stained, dried, and exposed to X-ray film in the presence of an intensifying screen at -80°C. The mobilities of markers of known size are shown with their molecular weights × 10⁻³.

ylation sites shared with Pr76gag, but surprisingly is also phosphorylated at a tyrosine residue contained within an acidic tryptic phosphopeptide. The importance of the gag phosphotyrosine site, and indeed of the fps phosphorylation sites, to the functional activity of the protein remains to be seen. Clearly, the tyrosine phosphorylation within the gag region is specific, but whether this represents fortuitous phosphorylation owing to the proximity of this sequence to a kinase active site or an important functional modification is unknown. The major fps-specific phosphorylation sites of FSV P140, including two phosphotyrosine residues and a phosphoserine residue, are clustered C terminally in the fps region, as represented by the 61K C-terminal V8 protease fragment. We have found that C-terminal fragments released from P140 by partial trypsin cleavage retain the protein kinase activity of P140 and must therefore contain the kinase domain. RSV pp60rc also yields a C-terminal fragment with kinase activity (19), indicating a functional homology between the C-terminal domains of these two ASV transforming proteins. The C-terminal localization of the major fps phosphorylation sites and the detection of trypsin fragments with kinase activity support the suggestion that the C-terminal sequences of the ASV transforming proteins are highly conserved because they encode the kinase domain. The conservation of sequence surrounding the P140 phosphotyrosine residues argues that these sites are important for the activity of the protein, although the relationship between the phosphorylation of P140 itself and its kinase activity is not yet clear. The extensive phosphorylation of FSV P140gag-fps is in contrast to RSV pp60rc, which is reported to contain only two phosphorylation sites—an N-terminal phosphoserine and a C-terminal phosphotyrosine (8, 35).

Several groups have compared the phosphotyrosine sites from a number of different virus transforming proteins and cell proteins by using microsequencing (25, 26, 28). There is substantial homology between the characteristically acidic amino acid sequences N terminal to the tyrosine phosphorylation sites. For PR_CII P105gag-fps and FSV P140gag-fps phosphorylated in vitro, the major tyrosine phosphorylation site has a glutamic acid four residues N terminal to the phosphotyrosine and a basic amino acid seven residues N terminal (28). Examination of the amino acid sequence of FSV P130gag-fps deduced from DNA sequence data shows a tyrosine at residue 1,073 from the N terminus of the 1,182-residue protein fulfilling these characteristics (36). The N-terminal amino acid of the tryptic peptide containing this sequence is glutamine, as initially suggested to account for poor yields in microsequencing (26). Cyclization of this glutamine residue after hydrolysis of its side chain amide group during the tryptic mapping procedure yields a more negatively charged species at pH 2.1. These observations suggest that tryptic peptides 3a through c, which we find as the major tyrosine phosphorylation site in vitro, correspond to this peptide.

P140 is also phosphorylated in vivo at a C-terminal tyrosine residue contained within peptide 4. It is intriguing that this residue is not phosphorylated in the immune complex kinase reaction, whereas a tyrosine residue in the N-
terminal region of the protein (peptide 2) is labeled in vitro, but not in vivo. Tryptic phosphopeptide 4 is also apparently absent from digests of PRCII P105 and is thus distinguished on these two counts from peptides 1 and 3a through c. The absence of this tyrosine phosphorylation site in PRCII P105 clearly does not limit its kinase activity, although whether it has anything to do with the decreased oncogenicity of PRCII (5) remains to be seen. These data raise the possibility that more than one kinase is involved in the phosphorylation of P140 tyrosine residues in transformed cells. Since the amino acid sequence of FSV P130<sup>prf-p</sup> is available, it will be possible to define the exact residues phosphorylated. A more detailed investigation of existing mutants and the construction of site-specific mutants will be required to reveal the relationship between the phosphorylation of FSV P140 and its enzymatic and transforming activities. The 98K product of the c-<i>fps</i> gene is reported to possess in vitro tyrosine-specific kinase activity, but to lack phosphorysine itself (21). It will be interesting to compare the peptide sequences and kinase domains of p98<sup>c-<i>fps</i></sup> and P140<sup>prf-p</sup>.

ACKNOWLEDGMENTS

We are indebted to G. S. Martin, P. Duesberg and H. Hanafusa for providing us with viruses; to V. Vogt and D. Bolognesi for generous gifts of anti-p19 serum; and to M. Lai, J. Neil, M. Shibuya, H. Hanafusa, and C. Sherr for communicating their results before publication.

This work was supported by grants from the Medical Research Council of Canada, the National Cancer Institute of Canada, and the B.C. Health Care Research Foundation. G.W. is a recipient of a Student Fellowship from the National Cancer Institute of Canada.

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