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Localization and Characterization of Phosphorylation Sites of the Fujinami Avian Sarcoma Virus and PRCII Virus Transforming Proteins

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Fujinami sarcoma virus (FSV) and PRCII are avian sarcoma viruses which share cellularly derived v-*fps* transforming sequences. The FSV P140$^{gag\text{-}fps}$ gene product is phosphorylated on three distinct tyrosine residues in transformed cells or in an in vitro kinase reaction. Three variants of FSV, and the related virus PRCII which lacks about half of the v-*fps* sequence found in FSV, encode gene products which are all phosphorylated at tyrosine residues contained within identical tryptic peptides. This indicates a stringent conservation of amino acid sequence at the tyrosine phosphorylation sites which presumably reflects the importance of these sites for the biologic activity of the transforming proteins. Under suitable conditions the proteolytic enzymes p15 and V8 protease each introduce one cut into FSV P140, p15 in the *N*-terminal *gag*-encoded region and V8 protease in the middle of the *fps*-encoded region. Using these enzymes we have mapped the major site of tyrosine phosphorylation to the *C*-terminal end of the *fps* region of FSV P140$^{gag\text{-}fps}$. A second tyrosine phosphorylation site is found in the *fps* region of FSV P140 isolated from transformed cells, and a minor tyrosine phosphorylation site is found in the *N*-terminal *gag*-encoded region. Our results suggest that the *C*-terminal *fps*-encoded region is required for expression of the tyrosine-specific protein kinase activity.

Key words: tyrosine phosphorylation, transforming proteins, avian sarcoma virus

The avian sarcoma viruses (ASVs), like other acutely oncogenic RNA tumor viruses, have apparently arisen by recombination between nontransforming, replication-competent retroviral genomes and cellular nucleic acid sequences [1,2]. The acquisition of these specific transduced cellular sequences confers on the viruses the

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ability to rapidly induce both the neoplastic transformation of cells in tissue culture and tumors in the animal [3]. Among the ASVs four distinct cellularly derived transforming genes have been identified: v-*src* (Rous sarcoma virus); v-*fps* (Fujinami sarcoma virus, PRCII, URI); v-*yes* (Y73, Esh sarcoma virus); and v-*ros* (URII) [4–7]. While these genes are different as determined by RNA:DNA liquid hybridization, the amino acid sequences of their gene products as predicted from DNA sequence data show remarkable homology, especially in their *C*-terminal regions (ref. 8; M. Shibuya and H. Hanafusa, personal communication).

Consistent with this structural relationship is the observation that the transforming proteins encoded by the ASVs are all associated with a protein kinase activity which is specific for tyrosine residues [9–17]. Cells transformed by these viruses show elevated levels of phosphotyrosine resulting from the phosphorylation of a number of cellular proteins [9]. Variants of Rous sarcoma virus (RSV) or Fujinami sarcoma virus (FSV) which are temperature-sensitive for transformation also display a temperature-sensitive phosphorylation of cellular proteins at tyrosine and their transforming proteins lose their associated kinase activities at the nonpermissive temperature [16,18]. Some mutants of RSV show a decreased phosphorylation of specific cellular substrates [19]. These data have suggested that the transforming proteins may induce transformation by the agency of protein phosphorylation, thus affecting the regulation of cellular processes such as cell growth and morphology.

FSV and PRCII both possess *fps* sequences and have similar genetic structures. FSV encodes a single 140–150K protein ($P140^{gag\text{-}fps}$) which contains a short *N*-terminal region translated from a defective *gag* gene and a C-terminal *fps*-encoded region [16,20,21]. PRCII is an independently isolated virus which is lacking about half of the *fps* sequences found in FSV [22], as reflected in the smaller size of its 105K gene product ($P105^{gag\text{-}fps}$). FSV P140 immunoprecipitated with antiserum directed against antigenic determinants in its *N*-terminal *gag*-encoded region becomes phosphorylated at tyrosine residues following incubation with [γ-^{32}P]ATP and Mn^{2+}. In vivo P140 is phosphorylated at both tyrosine and serine residues in transformed cells [16]. Both FSV P140 and the putative cellular targets of its kinase activity show a temperature-sensitive tyrosine phosphorylation in cells infected with a variant of FSV which is temperature-sensitive for transformation [16]. While the kinase activity of P140 appears closely involved in neoplastic transformation by FSV, the mechanisms by which P140 induces transformation and the relationship of these mechanisms to oncogenicity remain unclear. To approach these questions we have investigated the phosphorylation of P140 in vivo in more detail and established the relationship between the in vivo and in vitro phosphorylation of P140.

MATERIALS AND METHODS

Cells and Viruses

The variants of FSV used here have been described previously [16,20,21]. Temperature-sensitive tsFSV clone L5 and its temperature-resistant derivative trFSV were both pseudotyped with Fujinami-associated helper virus (FAV) and encode a 140K protein (P140) [16,20]. FSV clone 12 encodes a 130K protein (P130) and comes from a different FSV stock [20,23]. PRCII rescued with ring-neck pheasant virus (RPV) was obtained from G.S. Martin. GS^- chick embryo fibroblasts (CEF) were

obtained from H & N Farms, and Japanese quail embryo fibroblast from the quail unit, University of British Columbia. Cells were infected with virus (5×10^7–10^8ffu/ml), passaged after 3 or 4 days, and used on the fourth or fifth day. All cells were maintained at 37°C during the course of these experiments.

Radiolabeling of Cells

Cells were seeded at a density of 2×10^5 cells in a 10-mm well (Linbro) and the following day were incubated with $^{32}P_i$ (2.0 mCi/ml; ICN, carrier free) in phosphate-free Dulbecco's modified Eagle's medium containing 1% calf serum and 1% heat inactivated chick 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% NP40, 0.5% sodium deoxycholate, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 2 mM ATP, and 0.01% wt/vol aprotinin (Sigma)]. The lysate was centrifuged at 4°C at 15,000 rpm for 30 min and the supernatant recovered and incubated with the appropriate antiserum.

Immunoprecipitation

Immunoprecipitation was performed essentially as described elsewhere [24] using rabbit anti-p19 antiserum (generously provided by V. Vogt). The immune complex was adsorbed to Staphylococcus aureus, washed three times, and finally resuspended in sodium dodecyl sulfate (SDS)–polyacrylamide gel sample buffer for 10 min at 30°C, following which the bacteria were pelleted and the supernatant prepared for electrophoresis.

Immune Complex Kinase Reaction

1–5 $\times$ 10^6 FSV- or PRCII-transformed cells were lysed, immunoprecipitated, and the immune complex incubated with 5–50 μCi [γ-^{32}P]ATP (2,500 Ci/mM; Amersham) in 10 mM $MnCl_2$, 20 mM Tris-HCl pH 7.5 at 20°C as described previously [16]. Following the incubation the immunoprecipitate was washed as described previously [16], except that the buffer contained 10 mM ATP and 10 mM EDTA, and was prepared for gel electrophoresis.

SDS–Polyacrylamide Gel Electrophoresis

Samples were treated at 100°C for 3 min in SDS sample buffer (2% SDS, 5% β-mercaptoethanol, 10 mM Tris-HCl pH 6.8, 10% vol/vol glycerol), and then subjected to electrophoresis through SDS–polyacrylamide slab gels at 3 watts 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. [25], with separating gels of 15% acrylamide cross-linked with 0.0867% bisacrylamide.

Proteolytic Cleavage With p15 and V8 Protease

Immunoprecipitates of *gag*-related proteins were incubated with NP40-disrupted virions (Prague-B RSV) as described previously [26,27]. For the cleavage of proteins phosphorylated in vitro, 10 mM ATP and 10 mM EDTA were included in all buffers following 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. [25]. Proteins were labeled with ^{32}P in vivo or in vitro and purified following immunoprecipitation by SDS–polyacrylamide gel electrophoresis. The pertinent bands were excised from wet gels, and treated exactly as described by Cleveland et al. [25].

Analysis of Tryptic Peptides and Phosphoamino Acids

[^{32}P]- or [^{35}S]methionine-labeled proteins were purified by SDS–polyacrylamide gel electrophoresis, eluted from the gel slices, oxidized, and digested with TPCK-treated trypsin as described previously [16]. Tryptic digests were separated in two dimensions using electrophoresis at pH 2.1 in the first dimension and chromatography in the second dimension, and analyzed as described elsewhere [16,27].

Phosphoamino acids obtained by acid hydrolysis of ^{32}P-labeled proteins or isolated tryptic phosphopeptides were identified as described previously following electrophoresis on thin-layer cellulose plates at pH 1.9 and pH 3.5 [16,28]. Phosphotyrosine for use as a marker was a generous gift of B. Sefton.

Cell-Free Translation

Isolation of poly (A)-containing heat-denatured FSV(FAV) 70S virion RNA, and its translation in the messenger-dependent rabbit reticulocyte lysate was as described previously [21,24].

RESULTS

Phosphotyrosine Residues in FSV P140$^{gag\text{-}fps}$

P140 from chick cells transformed with tsFSV L5 or its temperature-resistant derivative (referred to here as tsFSV and trFSV) was phosphorylated in the immune complex using [γ-^{32}P]ATP, digested with trypsin, and the tryptic peptides separated in two dimensions as described in Materials and Methods. FSV P140 phosphorylated in vitro contains phosphotyrosine as its sole phosphoamino acid [11,16]. Five labeled spots are generated in such a tryptic peptide analysis (Fig. 1) of which three spots (3a–c) apparently represent different forms of the same tryptic phosphopeptide. Our data indicate that tryptic phosphopeptides 1, 2, and 3a–c are located in physically separable regions of P140 (see below) suggesting that P140 is phosphorylated at three distinct tyrosine residues in vitro. Under some conditions of analysis spots 3a and b are not seen, but 3c is the major phosphorylated peptide. Reanalysis of spot 3c on a thin-layer cellulose plate of the same type used in the experiment described in Figure 1 generated spots 3a, b, and c. Spots 3a and b are thus probably derived by modification of the tryptic phosphopeptide 3c.

To compare the sites phosphorylated by the kinase activity in vitro to the residues of P140 which are actually phosphorylated in the cell, we isolated P140 by immunoprecipitation from FSV-transformed cells which had been incubated with $^{32}P_i$. FSV-transformed cells that have been radiolabeled with $^{32}P_i$ yield P140 possessing three sets of ^{32}P-labeled tryptic peptides — those in common with FAV Pr76^{gag}; P140-specific phosphoserine-containing peptides; and P140-specific phosphotyrosine-containing peptides [16]. In cells labeled with $^{32}P_i$ for 18 hr the phosphotyrosine residues of P140 are more intensively labeled than the phosphoserine residues (G. Weinmaster, unpublished observation). ^{32}P-labeled P140 from tsFSV-transformed cells labeled for 18 hr yields five major (and several minor) spots following tryptic

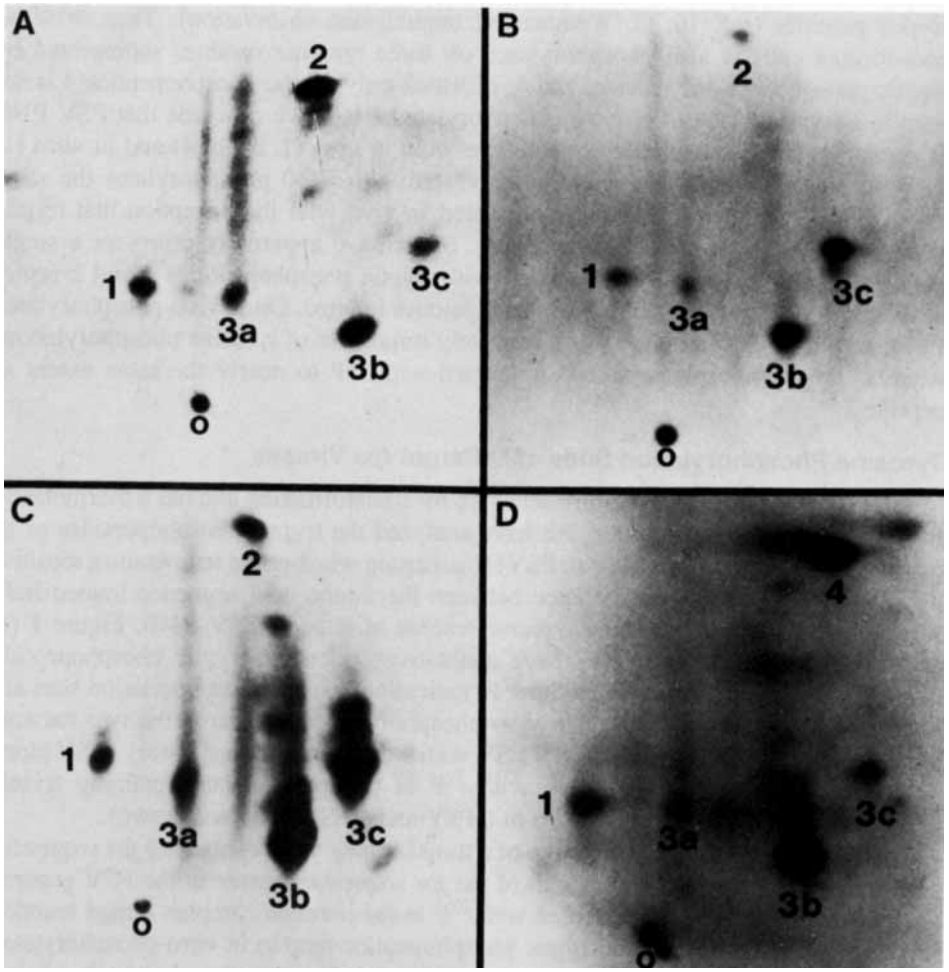


Fig. 1. Tryptic phosphopeptide analysis of FSV P140$^{gag\text{-}fps}$ and PRCII P105$^{gag\text{-}fps}$. FSV P140 and PRCII P105 were labeled with ^{32}P and isolated either (in vivo) by incubation of transformed CEFs with ^{32}P and subsequent immunoprecipitation of the labeled protein using anti-p19 serum, or (in vitro) by phosphorylation in an immune complex kinase reaction following immunoprecipitation with anti-p19 serum from transformed CEFs. Gel-purified P140 or P105 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 of: (A) trFSV P140 phosphorylated in vitro; (B) tsFSV P140 phosphorylated in vitro; (C) PRCII P105 phosphorylated in vitro; and (D) tsFSV P140 phosphorylated in vivo (18-hr $^{32}P_i$ labeling).

digestion and two-dimensional analysis (Fig. 1, D). Four of the five major spots comigrate with spots from in vitro-phosphorylated P140 and contain phosphotyrosine (1, 3a–c). Spot 2 is missing but a major new tryptic phosphopeptide (spot 4) is seen in in vivo phosphorylated P140, which also contains phosphotyrosine as its sole phosphoamino acid. The minor tryptic phosphopeptides of tsFSV P140 labeled in vivo with ^{32}P in this way represent either tryptic phosphopeptides in common with FAV Pr76^{gag} (the spots flanking peptide 4) or P140-specific phosphoserine-containing

tryptic peptides (ref. 16; G. Weinmaster, unpublished observation). Thus, P140 in transformed cells is also phosphorylated on three tyrosine residues represented by tryptic phosphopeptides 1, 3a–c, and 4, of which only tryptic phosphopeptide 4 is not seen in a tryptic digest of in vitro-phosphorylated P140. We conclude that FSV P140 is phosphorylated on multiple tyrosine sites both in vivo (1, 3a–c, 4) and in vitro (1, 2, 3a–c) and that the kinase activity associated with P140 phosphorylates the same sites on P140 in vitro as are phosphorylated in vivo with the exception that tryptic peptide 4 is only phosphorylated in vivo. Spots 3a–c apparently represent a single major site of tyrosine phosphorylation, with tryptic phosphopeptides 1 and 2 representing less strongly ^{32}P-labeled tyrosine residues in vitro. On in vivo-phosphorylated P140, tryptic phosphopeptide 1 is a relatively minor site of tyrosine phosphorylation, whereas tryptic phosphopeptide 4 is labeled with ^{32}P to nearly the same extent as peptide 3.

Tyrosine Phosphorylation Sites of Different *fps* Viruses

FSV clone L5 is temperature sensitive for transformation and has a thermolabile P140-associated kinase activity. We have analyzed the tryptic phosphopeptides of its temperature-resistant derivative (trFSV) to ascertain whether the temperature sensitivity of tsFSV reflects any difference between the amino acid sequence immediately surrounding the phosphorylated tyrosine residue of ts and trFSV P140. Figure 1 (A and B) shows that ts and trFSV have qualitatively identical tryptic phosphopeptide maps following in vitro labeling with ^{32}P, indicating that the phosphorylation sites are unchanged. This is also true of in vivo phosphorylated P140 from the two variants (data not shown). Another variant of FSV with a different passage history (FSV clone 12) is labeled in vivo or in vitro with ^{32}P at phosphotyrosine-containing tryptic peptides which comigrate with those of tsFSV and trFSV (data not shown).

PRCII is an independent isolate of a transforming virus containing *fps* sequences and is missing approximately 1.5 kb of the *fps* sequence present in the FSV genome [22]. However, PRCII P105 labeled with ^{32}P in the immune complex kinase reaction (Fig. 1, C) yields an identical tryptic phosphopeptide map to in vitro-phosphorylated FSV P140, showing that PRCII has retained the tyrosine phosphorylation sites found in FSV P140. The high degree of conservation of the amino acid sequences surrounding these phosphotyrosine residues, as reflected in the comigration of the tryptic phosphopeptides in a two-dimensional system from these different viruses, argues for the importance of the sequences in protein function.

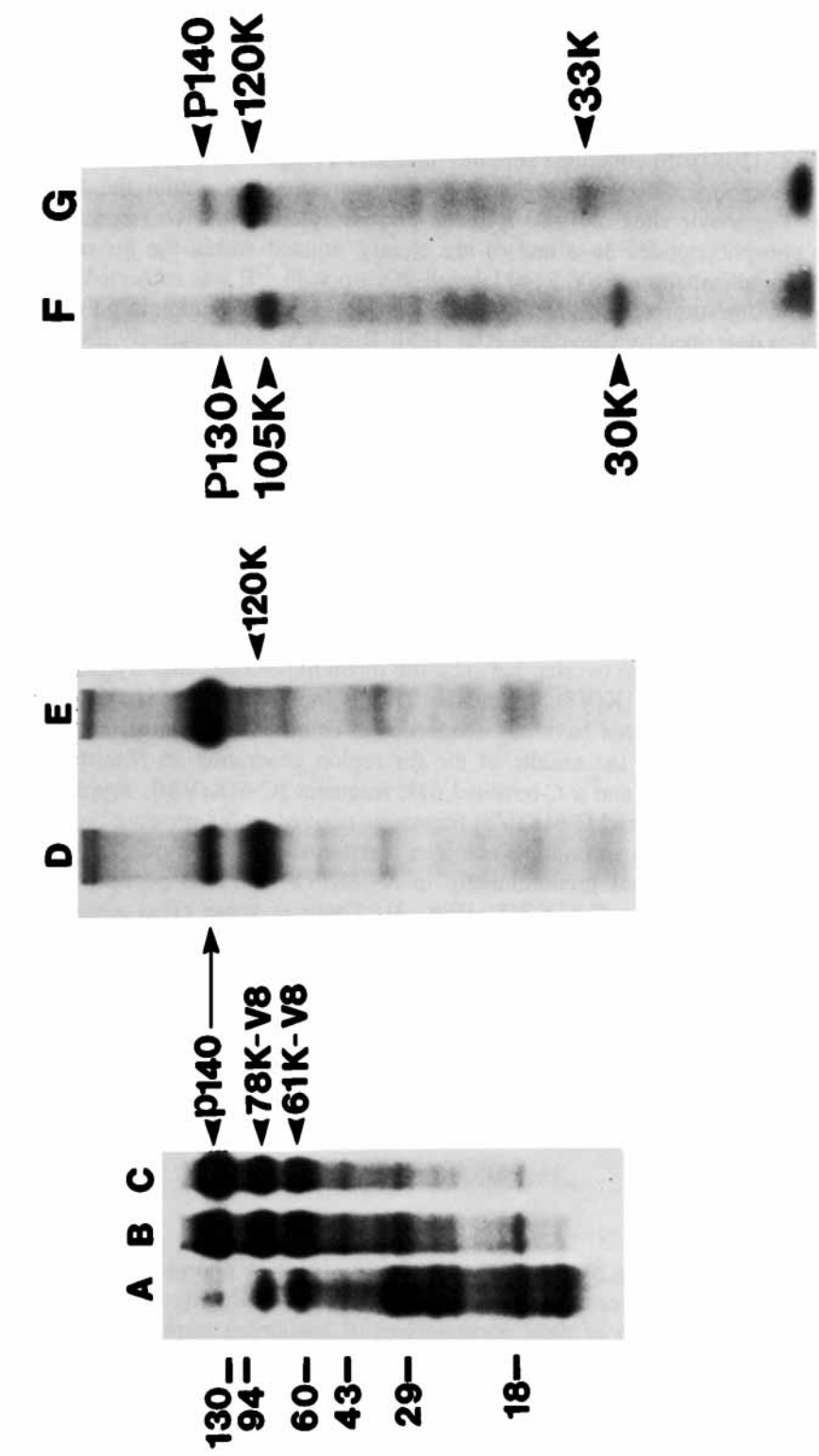
Localization of Phosphotyrosine Sites on P140$^{gag\text{-}fps}$

We have used proteolytic enzymes to cut FSV P140$^{gag\text{-}fps}$ into two or more fragments (diagrammed in Fig. 5), and these fragments have been mapped on the intact protein. By constructing such a proteolytic cleavage map of FSV P140$^{gag\text{-}fps}$ we have been able to localize phosphorylation sites to different regions of the protein. The cleavage of FSV P140 with avian retrovirus virion protease p15 has been previously described by us [27]. P15 cleaves P140 to an *N*-terminal 33K *gag*-encoded fragment [N-33K(p15)] and a 120K *C*-terminal fragment [C-120K(p15)] containing all of the *fps*-encoded sequences and a small portion of *gag* p27 sequence [27]. When tsFSV P140 phosphorylated either in vitro or in vivo was cleaved using NP40-disrupted RSV virus and the products analyzed by SDS–polyacrylamide gel electrophoresis (Fig. 2), both the 33K and the 120K fragments were labeled. Phosphoamino acid analysis of the 33K fragment from in vitro-phosphorylated P140 yielded solely

phosphotyrosine, whereas that from in vivo-labeled P140 yielded phosphoserine and phosphotyrosine (data not shown). Tryptic peptide analysis of the 33K and 120K fragments showed that of the phosphotyrosine-containing tryptic peptides described above for in vivo-phosphorylated P140, N-33K(p15) contained peptide 1, in addition to minor tryptic phosphopeptides comigrating with those found in FAV Pr76^{gag}, whereas C-120K(p15) contained peptides 3a–c and 4 (Fig. 3). These data suggest that tryptic phosphopeptide 1 represents a phosphotyrosine site within the P140 *gag* region. The major sites of P140 tyrosine phosphorylation in vivo (represented by tryptic phosphopeptides 3a–c and 4) are clearly situated within the *fps* region. To extend this information, FSV P140 labeled in vitro with ^{32}P was subjected to partial proteolytic digestion with V8 protease [25,29] using *in situ* digestion in polyacrylamide gels as described by Cleveland et al. [25]. Digestion with relatively low concentrations of V8 protease (Fig. 2), yields two major products with apparent molecular weights of 78K and 61K [78K(V8); 61K(V8)]. Limited V8 protease cleavage of C-120K(p15) gave the 61K but not the 78K V8 protease products, suggesting that the 61K fragment was *C*-terminal and the 78K fragment *N*-terminal (data not shown). To investigate this further we digested [^{35}S]methionine-labeled P140 with V8 protease and analyzed the products in parallel with V8 protease-digested ^{32}P-labeled P140 (Fig. 4). The [^{35}S]methionine-labeled P140 fragments were subjected to tryptic peptide mapping. Figure 3 shows that the 78K(V8) fragment contains all of the [^{35}S]methionine-labeled tryptic peptides previously identified as *gag*-encoded [27], whereas the 61K(V8) fragment contains only tryptic peptides previously identified as *fps*-specific. There is no overlap between the methionine-containing tryptic peptides of the 78K(V8) and 61K(V8) fragments. This is important since if these two V8 protease fragments do not have any sequences in common, they must result from a cut by V8 protease in the middle of the *fps* region generating an *N*-terminal 78K fragment (N-78K(V8)) and a *C*-terminal 61K fragment [C-61K(V8)]. Trypsin digestion of the N-78K(V8) and C-61K(V8) fragments derived by V8 protease digestion of P140 phosphorylated in an immune complex reaction shows that tryptic phosphopeptides 1 and 2 are found predominantly in N-78K(V8) whereas peptides 3a-c are predominantly found in C-61K(V8) (Fig. 3). There is some cross-contamination between the ^{32}P-labeled V8 protease fragments, which appears unavoidable. These results suggest that the major site of P140 tyrosine phosphorylation (represented by tryptic phosphopeptides 3a–c) resides in the *C*-terminal domain of the *fps*-encoded region of P140. In vivo an additional phosphorylated tyrosine residue (peptide 4) is located in the P140 *fps*-encoded domain, and preliminary data show that this residue, as well as the major *fps*-specific phosphorylated serine residue of P140, is located in the *C*-terminal V8 protease fragment.

DISCUSSION

We have shown by peptide mapping that the tyrosine phosphorylation sites of the gene products of three FSV variants and of PRCII are highly conserved on the basis of the comigration of their phosphotyrosine-containing tryptic peptides. FSV and PRCII are independent isolates of transforming viruses containing *fps* sequences [22]. The PRCII genome is missing approximately 1.5 kb of RNA sequence present at the 5′ end of the FSV *fps* gene, but retains those sequences necessary to encode the same phosphotyrosine sites on PRCII P105 as are found on FSV P140. PRCII P105


P140
120K
33K
G
F
P130
105K
30K
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E
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p140
78K-V8
61K-V8
A B C
130
94
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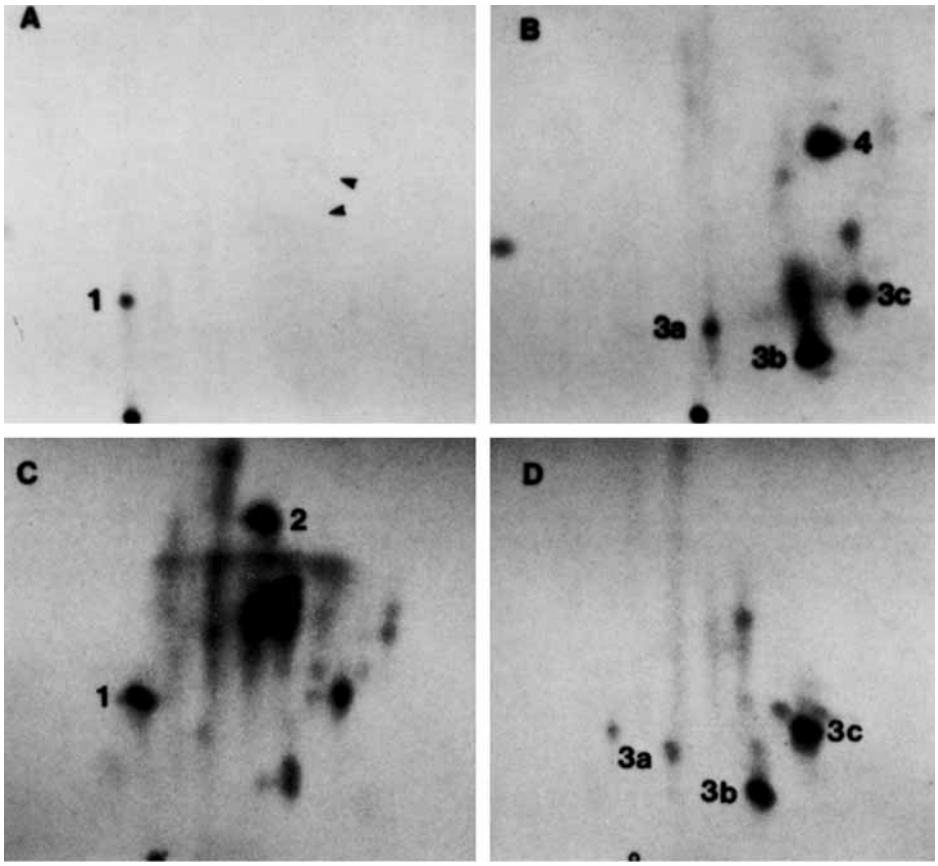

Fig. 3. Tryptic phosphopeptide analysis of p15 and V8 protease cleavage fragments of FSV P140. Polypeptides were gel purified, digested with trypsin, and analyzed by two-dimensional separation on thin-layer cellulose plates. Protein fragments analyzed were: A. N-33K(p15) obtained by p15 cleavage of tsFSV P140 phosphorylated in vivo. The arrows indicate tryptic phosphopeptides also found in Pr76. B. C-120K(p15) obtained by cleavage of tsFSV P140 phosphorylated in vivo. C. N-78K(V8) obtained by V8 protease cleavage of tsFSV P140 phosphorylated in vitro. D. C-61K(V8) obtained by V8 cleavage of tsFSV P140 phosphorylated in vitro.

Fig. 2. Cleavage of FSV P140$^{gag\text{-}fps}$ with V8 protease and p15. Lanes A–C: tsFSV P140 was labeled with ^{32}P during an immune complex kinase reaction, purified by SDS-polyacrylamide gel electrophoresis, and then subjected to in situ digestion with V8 protease in a new gel with a 15% polyacrylamide separating gel. Lane A, 500 ng V8 protease; B, 100 ng V8 protease; C, 50 ng V8 protease. Lanes D–G; FSV P130 or P140 was phosphorylated in an immune complex kinase reaction using [γ-^{32}P]ATP, washed extensively, incubated at 37°C for 60 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: Lane D, tsFSV P140 + NP40-disrupted RSV (the 33K *gag*-encoded cleavage product was electrophoresed off the gel); lane E, tsFSV P140, no additions; lane F, FSV cl.12 P130 + NP40-disrupted RSV; lane G, tsFSV P140 + NP40-disrupted RSV.

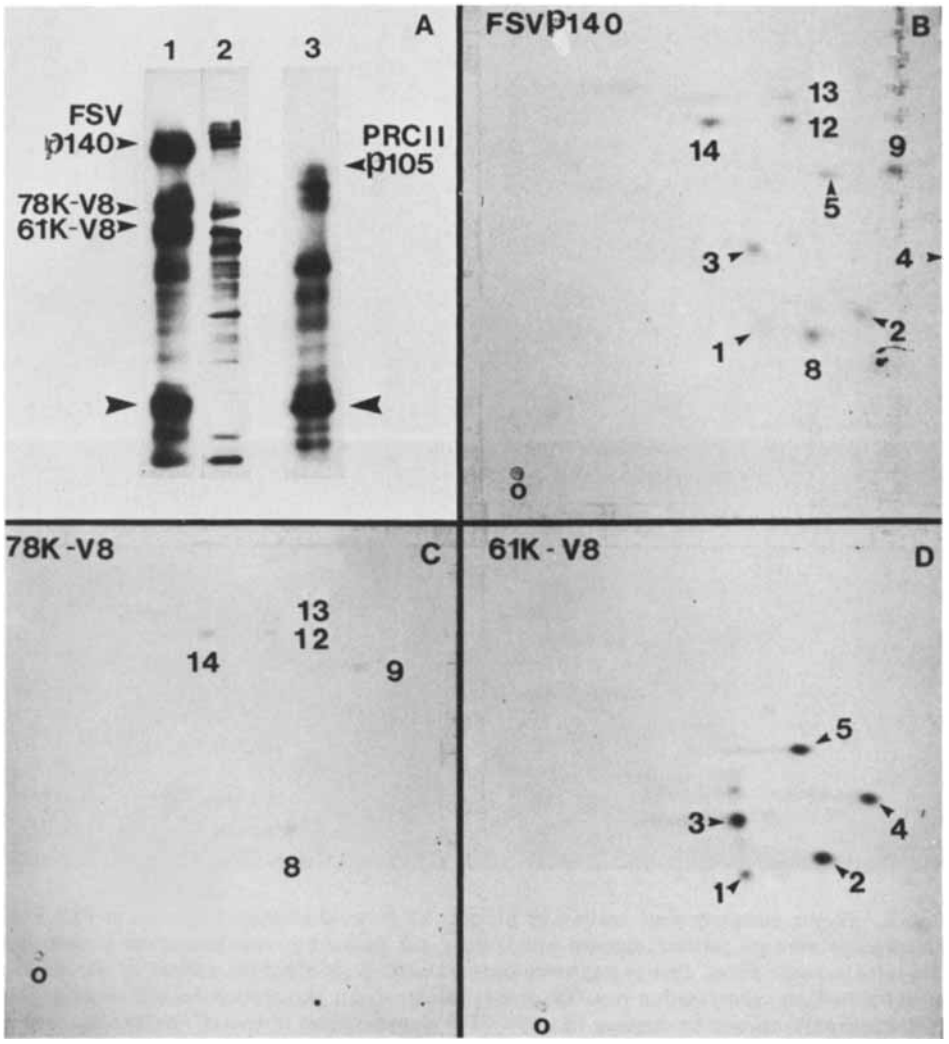

Fig. 4. Trypic peptide analysis of [^{35}S]methionine-labeled tsFSV P140$^{gag\text{-}fps}$and its V8 protease cleavage products. A. ^{32}P- or ^{35}S-labeled proteins were isolated from preparative gels and subjected to in situ digestion with V8 protease (50 ng per well) followed by electrophoresis through a 15% SDS–polyacrylamide separating gel. V8 protease digestions were of: lane 1, tsFSV P140 labeled with ^{32}P in vitro; lane 2, [^{35}S]methionine-labeled tsFSV P140 obtained by cell-free translation of FSV(FAV) poly(A)-selected heat-denatured 70S virion RNA in a messenger-dependent rabbit reticulocyte lysate; and lane 3, PRCII P105 labeled with ^{32}P in vitro. A common 15K V8 protease cleavage fragments of FSV P140 and PRCII P105 is indicated with large arrows. Proteins were analyzed in parallel in the same gel. B–D. Two-dimensional tryptic peptide maps of [^{35}S]methionine-labeled polypeptides. (B) tsFSV P140; (C) 78K(V8); and (D) 61K(V8). Numbering of methionine-containing peptides is according to Pawson et al. [27]. *Fps*-specific tryptic peptides (1–5) are arrowed, whereas *gag*-specific peptides are not.

has an associated kinase activity and transforms cells in tissue culture but is poorly oncogenic in animals relative to FSV [30]. Patschinsky et al. [31] have recently compared the phosphotyrosine sites from a number of different viral transforming proteins and cellular proteins using microsequencing. Their data indicate that there is some homology between the (usually acidic) amino acid sequences *N*-terminal to

tyrosine phosphorylation sites. Characteristically, a glutamic acid is found one and/or four residues *N*-terminal to the phosphorylated tyrosine, and a basic amino acid is located seven residues *N*-terminal to the tyrosine. For PRCII P105 phosphorylated in vitro the major tyrosine phosphorylation site has a glutamic acid four residues, and a basic amino acid seven residues *N*-terminal to the phosphotyrosine [31,32]. A comparison of this result with the amino acid sequence for FSV P130, deduced from DNA sequencing of the viral genome (M. Shibuya and H. Hanafusa, personal communication) indicates that this tyrosine is located 1,073 residues from the *N*-terminus of the 1,182-residue-long protein. Since the *N*-terminal amino acid of the tryptic peptide containing this sequence is glutamine, cyclization of the glutamine residue resulting from hydrolysis of the amide group during the tryptic mapping procedure will lead to a modified, more negatively charged peptide at pH 2.1. This suggestion has been advanced recently by Neil et al. [32] to account both for the appearance of the major in vitro phosphorylation site of PRCII P105 in more than one peptide during two-dimensional mapping, and for low yields during microsequencing. Our data are consistent with this observation, suggesting that tryptic phosphopeptides 3a–c represent this tyrosine phosphorylation site. Our localization of peptides 3a–c to the *C*-terminal domain of the *fps* sequence supports the conclusion of peptide and DNA sequence data. In addition, we find that FSV P140 phosphorylated in transformed cells contains a second major site of tyrosine phosphorylation within the *fps* region and a minor tyrosine phosphorylation site within the *gag* region. The relative importance of these three sites remains to be determined. In particular, it is unclear whether tyrosine phosphorylation within the *gag* region results fortuitously from the location of P140 within the cell, or whether it has a functional role.

Our results show, nonetheless, that all three tyrosine phosphorylation sites are strictly conserved between the different *fps* virus-transforming proteins studied, as defined by the comigration of the tryptic phosphopeptides, implying that they serve an important role. By contrast, the P140-specific tryptic peptides containing phosphoserine residues show considerable variations in electrophoretic mobility between different FSV isolates (G. Weinmaster, unpublished results). This implies that sequence variation may readily occur within the protein.

The finding that FSV P140 and PRCII P105 are multiply phosphorylated at tyrosine is consistent with the observation that PRCII P105 exists in a soluble, poorly phosphorylated form and an insoluble, highly phosphorylated cytoskeletal form which shows an increase in apparent molecular weight of 5K when compared with the soluble protein [33].

Recent observations from oligonucleotide mapping of virion RNA and hybridization of restriction fragments of cloned FSV DNA to viral RNA have shown that the 3′ half of the v-*fps* gene is homologous for FSV and PRCII, but that PRCII lacks

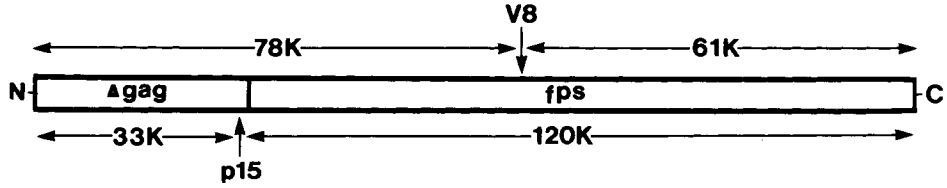

Fig. 5. Cleavage sites for p15 and V8 protease on FSV P140, yielding the fragments discussed in the text.

sequences found at the 5′ end of FSV v-*fps* [6,23]. Our data suggest that the 3′ v-*fps* sequences are conserved in FSV and PRCII because they encode that domain of the protein which contains the major tyrosine phosphorylation sites and the kinase-active site. It is possible that the 5′ *fps* region deleted in PRCII modulates the tyrosine-specific kinase activity or affects substrate recognition, since PRCII is poorly oncogenic.

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