A Major Site of Tyrosine Phosphorylation within the SH2 Domain of Fujinami Sarcoma Virus P130^{gag-fps} Is Not Required for Protein-Tyrosine Kinase Activity or Transforming Potential

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Phosphorylation of the major autophosphorylation site (Tyr-1073) within Fujinami sarcoma virus P130^{gag-fps} activates both the intrinsic protein-tyrosine kinase activity and transforming potential of the protein. In this report, a second site of autophosphorylation Tyr-836 was identified. This tyrosine residue is found within a noncatalytic domain (SH2) of P130^{gag-fps} that is required for full protein-kinase activity in both rat and chicken cells. Autophosphorylation of this tyrosine residue implies that the SH2 region lies near the active site in the catalytic domain in the native protein and thus possibly regulates its enzymatic activity. Four mutations have occurred within the SH2 domain between the *c-fps* and *v-fps* proteins. Tyr-836 is one of these changes, being a Cys in *c-fps*. Site-directed mutagenesis was used to investigate the function of this autophosphorylation site. Substitution of Tyr-836 with a Phe had no apparent effect on the transforming ability or protein-tyrosine kinase activity of P130^{gag-fps} in rat-2 cells. Mutagenesis of both autophosphorylation sites (Tyr-1073 and Tyr-836) did not reveal any cooperation between these two phosphorylation sites. The implications of the changes within the SH2 region for *v-fps* function and activation of the *c-fps* oncogenic potential are discussed.

The 130-kilodalton (kDa) transforming protein of Fujinami avian sarcoma virus (FSV) is a protein-tyrosine kinase (PTK) and is itself phosphorylated on tyrosine and serine residues (35, 38). Neither the phosphorylated serine residues nor the protein-serine kinases responsible for their phosphorylations have been identified, and as yet the functions of the serine modifications are unknown. Most of the tyrosine phosphate is found within two tryptic peptides (3a-C+4), located in a C-terminal 61-kDa fragment of P130^{gag-fps} (35). Tryptic phosphopeptides 3a and 3c contain Tyr-1073, which is found in the kinase domain (30, 35, 38) and corresponds to a residue that is invariant in all PTKs (15). The high conservation of this tyrosine residue suggests a critical function. Consistent with this idea, we have previously reported that substitution of Tyr-1073 with a Phe, Ser, Thr, or Gly residue results in a decrease in both the kinase activity and the transforming ability of P130^{gag-fps} (36, 38).

There are other PTKs that provide examples for stimulation of biological activity through autophosphorylation of this highly conserved tyrosine residue, such as the insulin receptor (Tyr-1162/1163) (5, 12, 31) and pp60^{c-src} (Tyr-416) (20, 25). However, the activity of pp60^{c-src} can also be suppressed by phosphorylation of another highly conserved tyrosine residue (2, 3) unique to this family of cellular PTKs (*yes*, *lck*, *fgr*, *hck*, *fyn*, and *lyn*) (14). In the latter case the phosphorylation is believed to be mediated by another PTK (19). Therefore, tyrosine phosphorylation at different sites may have either a positive or a negative effect on the activity of certain PTKs.

To gain insight into the role of additional tyrosine phosphorylation sites in P130^{gag-fps}, we undertook the localization of the tyrosine residue phosphorylated in tryptic peptide 4 (38). Using a combination of sequence analysis of the ³²P-labeled tryptic peptide, secondary digestion with other proteases, and comigration with a phosphorylated synthetic peptide, we determined that tyrosine 836 is the other major

site of tyrosine phosphorylation within P130^{gag-fps}. To determine whether either the phosphorylation of Tyr-836 or the presence of a tyrosine at this position contributes to the transforming potential or kinase activity of P130^{gag-fps}, we chose to mutate this residue. In the work described in this report, we have assessed the effect of changing Tyr-836 to a phenylalanine on the enzymatic activity and biological function of P130^{gag-fps}. In addition, we have determined whether there is functional cooperativity between the two major sites of autophosphorylation within P130^{gag-fps} by constructing a double mutant containing Phe-836 and Phe-1073.

MATERIALS AND METHODS

Cells and labeling. Wild-type (WT) and mutant FSV-transformed cell lines and rat-2 cells were grown in Dulbecco-Vogt modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were plated at a density of 3×10^5 cells per 35-mm dish 24 h prior to labeling. Labeling was performed with 1 mCi of ³²P_i (ICN Pharmaceuticals Inc.) per ml for 18 to 20 h in phosphate-free DMEM supplemented with 2% FBS or with 100 μ Ci of [³⁵S]methionine (>1,000 Ci/mmol; Amersham Corp.) per ml or 200 μ Ci of [³⁵S]cysteine per ml for 18 h in methionine- or cysteine-free DMEM supplemented with 2% FBS, respectively. For preparative purposes, four confluent 10-cm dishes of FSV-transformed cells were labeled in 4 ml with 1.5 mCi of ³²P_i per ml for 18 h to isolate enough peptide 4 (ca. 6,000 cpm) for secondary digests and sequencing.

Immunoprecipitation of radiolabeled proteins. Radiolabeled cells on a 35-mm dish were lysed in 0.5 ml of ice-cold RIPA buffer (17) modified by the addition of 2 mM EDTA, 50 mM NaF, and 100 μ M Na₂VO₄ (Fisher Scientific Co.). Immunoprecipitation of radiolabeled P130^{gag-fps} with anti-p19^{gag} monoclonal antibody and analysis of immunoprecipitates by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis have been described elsewhere (35, 38).

Preparation of a synthetic peptide containing Tyr-836. A peptide corresponding to amino acids 828 to 843 of

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P130^{gag-fps} (SEVQELLKYSGEFLVR) was synthesized manually by using *t*-butyloxycarbonyl chemistry and purified by reverse-phase high-pressure liquid chromatography with a C18 column developed in a gradient of acetonitrile in 0.1% trifluoroacetic acid (32). The composition confirmed the structure of this peptide. Peptide (40 nmol), P130^{gag-fps} immunoprecipitated from 5×10^5 FSV-transformed cells, and 50 μ Ci of [γ -³²P]ATP (3,000 Ci/mmol) were incubated in the presence of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4)–10 mM MnCl₂, in a final volume of 20 μ l, for 10 min at 30°C. The phosphorylated peptide was isolated from the kinase reaction by electrophoresis on 100- μ m cellulose thin-layer plates (E. Merck, Darmstadt, Federal Republic of Germany) at pH 1.9 and digested with 5 μ g of trypsin for 2 h at 37°C as described previously (16). The resultant tryptic phosphopeptide was isolated and used in the comigration experiment described in the legend to Fig. 1.

In vitro protein kinase reaction. Normal or FSV-transformed cells were lysed in kinase lysis buffer (1% Nonidet P-40, 0.5% SDS, 10 mM Tris hydrochloride [pH 7.4], 100 mM NaCl, 1 mM EDTA) and immunoprecipitated with anti-p19 monoclonal antibody, and the immunoprecipitates were assayed for autophosphorylation or phosphorylation of acid-denatured enolase as previously described (35, 38). Each immune complex was suspended in 20 μ l of kinase reaction mixture containing 10 mM MnCl₂, 50 mM Tris hydrochloride (pH 7.4), 5.0 μ Ci of [γ -³²P]ATP, and 5 μ g of enolase that had been previously treated at 30°C for 5 min in 25 mM acetic acid (38) and incubated at 30°C for 15 min.

Site-directed mutagenesis and construction of FSV mutants. The origins of the M13mp10FSV and M13mp10FSV F-1073 clones used to produce the mutants described here have been published previously (38). Details of the oligonucleotide-directed mutagenesis reaction, screening and isolation of mutant bacteriophage used to generate M13mp10FSV F-836 and sequencing of mutant DNAs have also been described before (38).

The FSV F-836/F-1073 mutant genome was constructed from fragments contained within the M13mp10FSV F-1073 and M13mp10FSV F-836 genomes. Digestion of M13mp10FSV F-836 or M13mp10FSV F-1073 with *Eco*RI, *Not*I, and *Hind*III releases two FSV-containing fragments of 1.9 and 2.9 kilobase pairs (kbp) from the vector M13 sequences. The 1.9-kbp fragment from M13mp10FSV F-1073 and the 2.9-kbp fragment from M13mp10FSV F-836 were electrophoresed onto NA-45 paper (Schleicher & Schuell, Inc.), phenol extracted twice, and concentrated by ethanol precipitation. These purified fragments were ligated with *Eco*RI-*Hind*III-linearized pEMBL9⁺ DNA and transformed into competent *Escherichia coli*. Restriction analysis of the isolated recombinants confirmed the correct structure of the pEMBL-FSV F-836/F-1073 clones. Cells harboring this clone were superinfected with M13-K07 helper virus at a multiplicity of infection of 20 to induce the synthesis of single strands, which were subsequently sequenced, in the relevant regions, to confirm the presence of the two mutations within the recombinant clones.

Transfection of DNA onto rat-2 cells. Inserts containing FSV sequences were separated from vector sequences and purified for transfection onto rat-2 cells as previously described (38). The transfections were carried out exactly as described (38), except that transfected rat-2 cells were fed DMEM supplemented with 10% FBS rather than 5% calf serum and 0.5 μ M dexamethasone. Foci of transformed cells were initially isolated by using cloning cylinders and re-

cloned from a single colony growing in soft agar. Soft-agar colony formation was assayed by using 5×10^3 cells seeded in DMEM containing 10% FBS and 0.3% (wt/vol) Bacto-Agar (Difco Laboratories) in 60-mm dishes.

Additional proteolysis and Edman degradation. ³²P-labeled tryptic peptide 4 was isolated, purified, and digested further with *Staphylococcus aureus* V8 protease, *Pseudomonas fragi* protease, chymotrypsin, and thermolysin essentially as described (16). Edman degradation of ³²P-labeled tryptic phosphopeptides was carried out either manually as described previously (16) or by B. F. Tack, Scripps Clinic, La Jolla, Calif., with a Beckman 890C protein sequencer.

Tryptic peptide mapping and phosphoamino acid analysis. The phosphoamino acid content of proteins and peptides was determined as described (4). Labeled proteins were resolved on one-dimensional gels, extracted, and subjected to tryptic digestion as described (17, 35). ³²P-labeled tryptic peptides were separated in two dimensions on cellulose thin-layer plates by electrophoresis at 1 kV for 30 min at pH 1.9 followed by chromatography (35).

Immunoblotting of proteins with antiphosphotyrosine antibodies. Western immunoblotting was performed essentially as described by Towbin et al. (34) and modified by Kamps and Sefton (M. Kamps and B. M. Sefton, *Oncogene*, in press). Briefly, monolayers of cells on 9-cm plates were washed twice with 5 ml of 50 mM Tris hydrochloride (pH 7.4)–140 mM NaCl–3.3 mM KCl and lysed by the addition of boiling protein sample buffer (5 mM sodium phosphate [pH 6.8], 2% SDS, 0.1 M dithiothreitol, 5% 2-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue). The cell lysates were quickly scraped and transferred into tubes, which were boiled for 5 min. The samples were then sheared 10 times through a 22-gauge needle and 10 times through a 27-gauge needle and stored in liquid nitrogen until used. The relative concentration of proteins contained in each sample was determined by staining a test gel with Coomassie brilliant blue R (Sigma Chemical Co.). Equivalent amounts of cellular proteins were then fractionated on a second gel and transferred to a nitrocellulose filter (Schleicher & Schuell) by electrophoresis at 20 V for 3 h in transfer buffer (5.7 g of glycine, 12.0 g of Tris-base, 3.0 g of SDS; 0.37 g of Na₃VO₄, 800 ml of methanol, water to 4 liters). The transfer buffer was degassed extensively before use. Following transfer of the proteins, the filter was incubated overnight in blocking buffer, consisting of 5% bovine serum albumin (Sigma) and 1% ovalbumin dissolved in rinse buffer (10 mM Tris hydrochloride, [pH 7.2], 0.9% NaCl, 0.01% Na₂S₂O₃), to saturate sites on the nitrocellulose which would bind proteins non-specifically. The filters were then incubated with an antibody directed against a mixture of phosphotyrosine, alanine, and glycine, copolymerized, and attached to keyhole limpet hemocyanin, which was prepared and kindly provided by M. Kamps. Typically, the filters were incubated for 2 h at room temperature with 2 μ g of anti-phosphotyrosine antibody per ml of blocking buffer and then rinsed twice for 10 min in rinse buffer, once for 10 min in the same buffer containing 0.05% Nonidet P-40 and twice again in rinse buffer. The presence of the antiphosphotyrosine antibody was detected by incubating the filter with 200 μ Ci of ¹²⁵I-protein A (100 Ci/ μ g; New England Nuclear Corp.) in 40 ml of blocking buffer for 1 h at room temperature and then subjecting it to two 10-min washes in rinse buffer, one 10-min wash in rinse buffer containing 0.05% Nonidet P-40, and, finally, two 10-min washes in rinse buffer. Proteins that reacted with the antiphosphotyrosine antibody were visualized by exposing the filter to Kodak XAR film that had been presensitized.

^{14}C -labeled molecular weight markers (spectrin, 220,000; β -galactosidase, 116,000; phosphorylase, 97,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 22,000) were electrophoresed on the same gel so that we could calculate the apparent molecular mass of the reactive proteins.

RESULTS

Tyrosine 836 is phosphorylated on P130^{gag-fps} from transformed cells. The identity of the tyrosine residue phosphorylated within the ^{32}P -labeled tryptic peptide 4 was initially determined indirectly by using the following information. First, P130^{gag-fps} metabolically labeled with [^{35}S]methionine or [^{35}S]cysteine and analyzed by two-dimensional tryptic peptide mapping did not contain a peptide which comigrated with ^{32}P -labeled tryptic peptide 4 (data not shown). This result suggested that peptide 4 did not contain Met or Cys, assuming that the stoichiometry of phosphorylation of peptide 4 is high enough to allow the detection of the phosphorylated form of peptide 4 labeled with [^{35}S]cysteine or [^{35}S]methionine. Second, additional digestion of ^{32}P -labeled tryptic peptide 4 with various proteases revealed that it was not cleaved by *S. aureus* V8 protease (specific for Glu), but was cleaved by thermolysin (specific for Leu, Ile, and Val), chymotrypsin (specific for Tyr, Trp, and Phe) and *P. fragi* protease (specific for Asp and cysteic acid) (data not shown). Third, sequential Edman degradation of ^{32}P -labeled tryptic peptide 4 released phenylthiohydantoin-phosphotyrosine in the first cycle (data not shown). The only predicted P130^{gag-fps} tryptic peptide consistent with these data contains a single tyrosine at position 836 within residues 836 through 843 (YSGEFLVR). To confirm this identification we synthesized the peptide SEVQELLKYSGEFLVR, which contains the presumptive sequence of peptide 4 (YSGEFLVR). This synthetic peptide was phosphorylated in vitro by immunoprecipitated P130^{gag-fps} exclusively on tyrosine (data not shown). Trypsinization of this phosphorylated synthetic peptide generated a single phosphopeptide which comigrated with peptide 4 (Fig. 1C), derived by tryptic digestion of in vivo ^{32}P -labeled P130^{gag-fps} (see Fig. 6A).

Creation of M13 FSV-F-836 by using site-directed mutagenesis. Once the identity of the phosphorylated residue within peptide 4 was determined, we wanted to examine the effect of phosphorylation of this site on the protein-kinase activity and transforming potential of P130^{gag-fps}. For the mutagenesis we designed and synthesized the oligonucleotide 5'-CTGAAATTTAGCGGAGAC-3'. This oligonucleotide is identical with nucleotides 2878 to 2896 of the FSV genome as defined by Shibuya and Hanafusa (30), except that the TAC codon for Tyr has been changed to TTT encoding a Phe. A two-primer mutagenesis reaction was carried out and the product was used to transform competent *E. coli* JM101. Positive clones generated in the mutagenesis reaction were initially identified by differential hybridization of phage DNA with the ^{32}P -labeled mutagenic primer. Mutants were produced at a frequency of 21%. F-836 mutant clones were confirmed by sequencing the DNA in the region encompassing the mutation.

Construction of the F-836/F-1073 FSV mutant genome. To produce a P130^{gag-fps} protein that would lack both of the major autophosphorylation sites, we constructed a double FSV F-836/F-1073 mutant by using the existing M13FSV F-836 and M13FSV F-1073 genomes. This construction was facilitated by the presence of a single *NotI* site at nucleotide

FIG. 1. Comigration of P130^{gag-fps} tryptic peptide 4 with a synthetic phosphopeptide. Peptide 4 from in vivo ^{32}P -labeled P130^{gag-fps} and a phosphorylated synthetic peptide of identical composition were separated in two dimensions on cellulose thin-layer plates by electrophoresis at pH 1.9 for 10 min at 1.0 kV, with the anode on the left, and ascending chromatography as described in Materials and Methods. (A) Tryptic digest of phosphorylated synthetic peptide; (B) P130^{gag-fps} peptide 4; (C) mixture of A and B. The origin is indicated by the arrowhead. Cerenkov counts per minute loaded: (A) 290 cpm; (B) 334 cpm; (C) 290 cpm of A and 334 cpm of B. Exposure time was 22 h at -70°C with an intensifying screen.

3186, which lies between the codons for Tyr-836 (nucleotides 2885 to 2887) and Tyr-1073 (nucleotides 3595 to 3597) in the FSV sequence. Figure 2 is a diagrammatic representation of the F-836/F-1073 mutant construction as detailed in Materials and Methods.

Biological activity of mutant FSV genomes in rat-2 cells. We assessed the oncogenic potential of the F-836 and F-836/F-1073 mutants by measuring their ability to induce foci of transformed cells following transfection into rat-2 cells. For a comparison, the FSV WT or FSV F-1073 DNA was also transfected into rat-2 cells. The WT DNA transformed rat-2 cells, with a latency period of approximately 2 weeks, at an efficiency of >100 foci per μg of DNA. The F-836 mutant DNA transformed rat-2 cells with the same latency period and efficiency as those of WT DNA. The morphologies of the transformed cells produced by the WT and F-836 mutant DNA were strikingly similar (Fig. 3); the cells were round and refractile, in contrast to the flat parental rat-2 cells. Therefore, neither the Tyr at position 836 nor the phosphorylation of this residue is required for P130^{gag-fps} biological activity. Rat-2 cells transfected with F-836/F-1073 DNA showed some evidence of focus formation on monolayers of rat-2 cells. The morphological change induced by the F-836/F-1073 DNA was at first subtle, but within a few weeks of their initial detection the foci were more apparent. The final morphology of these transformed cells was not as pronounced in appearance as that of the WT P130^{gag-fps}-transformed cells, but they were definitely more rounded and refractile than the normal rat-2 cells (Fig. 3). As ex-

FIG. 2. Construction of the FSV F-836/F-1073 genome. The 1.9-kbp *NotI*-*HindIII* fragment from M13 FSV F-1073, containing the TTT codon for Phe at nucleotides 3595 to 3597, and the 2.9-kbp *EcoRI*-*NotI* fragment from M13 FSV F-836, containing the TTT codon for Phe at nucleotides 2885 to 2887, were isolated and subcloned into pEMBL+9 as described in Materials and Methods. The positions of the pertinent restriction endonuclease cleavage sites are indicated by the following abbreviations: H, *HindIII*; N, *NotI*; R, *EcoRI*; S, *SstI*.

pected, similar results were obtained with the F-1073 mutation alone. The fact that F-836/F-1073 DNA transformed rat-2 cells at the same efficiency and yielded a similar morphological phenotype to that seen with F-1073 DNA suggests that the loss of an additional tyrosine phosphorylation site within the F-1073 P130^{gag-fps} protein has no additional deleterious effect on its biological activity in rat-2 cells.

Several individual foci of transformed cells produced from each of the four DNAs tested were isolated and expanded. These cell lines were tested for their ability to grow in soft agar. Cells transformed by WT or F-836 DNA produced very large, robust colonies in soft agar within 2 weeks of seeding, in stark contrast to rat-2 cells, which cannot divide under these conditions (Fig. 3). Even though the morphological phenotype of the F-1073- or F-836/F-1073-transformed cells was not as altered as that of WT- or F-836-transformed cells, both of these cell types were able to grow in soft agar, indicative of a tumorigenic phenotype. The colony sizes produced by the F-1073 or F-836/F-1073 mutant-transformed cells were identical and both were consistently smaller than those induced by WT- or F-836-transformed cells (Fig. 3).

Expression and activity of P130^{gag-fps} proteins. To examine the expression of the mutant P130^{gag-fps} proteins, the transformed cell lines were incubated for 18 h with [³⁵S]methionine and P130^{gag-fps} proteins were isolated by immunoprecipitation with an anti-p19^{gag} monoclonal antibody and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). The amount of ³⁵S-labeled P130^{gag-fps} varied. This variation probably reflects different levels of gene expression resulting from the transfection technique rather than protein instability, since pulse-chase experiments indicated that the turnover rates of all P130^{gag-fps} proteins examined were approximately the same (half-life of about 12 h; data not shown). Close inspection revealed the presence of multiple forms of P130^{gag-fps}; these are presumably due to differential phosphorylation, with the upper bands being

more highly phosphorylated than the lower bands. F-836, F-1073, and F-836/F-1073 FSV P130^{gag-fps} proteins are missing one and two sites of tyrosine phosphorylation, respectively. This loss is reflected in a decrease of the upper forms and an increase in lower forms. The loss of Tyr-1073 appears to have a greater effect than the loss of Tyr-836 on the mobility of P130^{gag-fps}.

When immunoprecipitates were incubated with MnCl₂ and [³²P]ATP, all P130^{gag-fps} proteins became autophosphorylated (Fig. 4). Usually, under these conditions, the WT P130^{gag-fps} protein is phosphorylated only at Tyr-1073 and at a minor site located in the *gag* region of the protein (35, 38). It should be noted that the tyrosine residue phosphorylated within peptide 4 is not labeled during these in vitro kinase reactions. As expected, the level of ³²P incorporated into F-1073 and F-836/F-1073 P130^{gag-fps} was reduced compared with that of WT P130^{gag-fps} (see Table 1), since the Tyr-1073 autophosphorylation site has been lost in these mutants. F-836 P130^{gag-fps} autophosphorylated as efficiently as WT P130^{gag-fps}, suggesting that the ability to autophosphorylate Tyr-1073 has not been altered by the F-836 mutation. Indeed, tryptic peptide analysis indicated that the in vitro autophosphorylation pattern of F-836 P130^{gag-fps} was identical to that previously reported for WT P130^{gag-fps} (35, 38; data not shown). F-1073 P130^{gag-fps} has been shown previously to autophosphorylate mainly at a tyrosine site in the *gag* region of the protein (35, 36, 38), and the F-836/F-1073 mutant P130^{gag-fps} was also phosphorylated similarly (data not shown), indicating that the additional mutation within the F-1073 P130^{gag-fps} protein did not alter its kinase activity.

Autophosphorylation of P130^{gag-fps} is not a good measure of its intrinsic PTK activity, especially when the major autophosphorylation site is removed, as it is in the F-1073 and F-836/F-1073 mutant P130^{gag-fps} proteins. Therefore, immunoprecipitates of the WT and mutant P130^{gag-fps} proteins were assayed for their ability to phosphorylate the exogenous substrate enolase in an in vitro kinase reaction. The extent of enolase phosphorylation was normalized by analysis of ³⁵S-labeled P130^{gag-fps} proteins immunoprecipitated from sister cultures (Fig. 5). The average of four independent experiments yielded the following relative specific PTK activities: FSV WT, 100; F-836, 125; F-1073, 24; and F-836/F-1073, 12. The specific protein kinase activity of the F-836 mutant P130^{gag-fps} was similar to that of the WT P130^{gag-fps}, which is consistent with the unaltered biological properties of this mutant. The kinase activity of the F-1073 and F-836/F-1073 mutant P130^{gag-fps} proteins were four- to eightfold lower, respectively, than that of WT P130^{gag-fps}.

Phosphorylation of mutant P130^{gag-fps} proteins in FSV-transformed cell lines. P130^{gag-fps} is phosphorylated in transformed cells on a number of serine and tyrosine residues (35, 38). When WT or mutant FSV-transformed cells were incubated with ³²P_i for 18 h, all P130^{gag-fps} proteins were phosphorylated (Fig. 4), but the level of phosphorylation varied (Table 1). The removal of two tyrosine phosphorylation sites correlates with less detectable ³²P in the F-836/F-1073 P130^{gag-fps} protein than in the WT, F-836, and F-1073 P130^{gag-fps} proteins. A quantitative change in phosphotyrosine levels was also apparent after phosphoamino acid analysis (Table 2). The loss of the Tyr-1073 resulted in a greater decrease in phosphotyrosine than the loss of Tyr-836 did.

To confirm that substituting Tyr-836 with Phe does result in the loss of peptide 4 and to assess the effect of this mutation, alone and in conjunction with the F-1073 mutation, on the phosphorylation of P130^{gag-fps}, we excised

FIG. 3. Transformation of rat-2 cells following transfection with FSV DNAs. Rat-2 cells were transfected with WT, F-836, F-1073, or F-836/F-1073 FSV DNAs and examined for the appearance of foci of transformed cells, which were then cloned and expanded into stable cell lines. Phase-contrast photomicrographs of monolayers of rat-2 cells, WT-, F-836, F-1073, and F-836/F-1073 FSV-transformed cells are shown in the left panel. The appearance of colonies after 2 weeks in soft agar for normal rat-2 cells and WT, F-836, F-1073, and F-836/F-1073 FSV-transformed cells is shown in the right panel.

FIG. 4. Expression, phosphorylation, and autophosphorylation of FSV P130^{gag-fps} in WT- and mutant FSV-transformed cells. P130^{gag-fps} was isolated from equivalent numbers of unlabeled cells and cells labeled for 18 h with ³²P_i or [³⁵S]methionine with anti-p19^{gag} antibody as described in Materials and Methods. Unlabeled immunoprecipitates were phosphorylated in an in vitro kinase reaction as described in Materials and Methods. All immunoprecipitates were analyzed by electrophoresis on SDS-7.5% polyacrylamide gels followed by autoradiography. The lanes contain immunoprecipitates prepared from the following cell lines: lanes A, WT; lanes B, F-836; lanes C, F-1073; lanes D, F-836/F-1073; and lanes E, rat-2. Exposure time was 16 h.

³²P-labeled P130^{gag-fps} proteins (Fig. 4) from the gel and subjected them to tryptic peptide analysis (Fig. 6). The tryptic peptides designated 5, 6, 8, 9, and X in Fig. 6 all contain phosphoserine, whereas peptides 1, 3a, 3b, 4, and 7 contain only phosphotyrosine (data not shown). The major autophosphorylation site found in P130^{gag-fps} labeled in an immune complex kinase reaction and from ³²P-metabolically-labeled transformed cells is Tyr-1073, contained within peptides 3a and 3b (35, 38). The other minor site of autophosphorylation (peptide 1) has been mapped to the gag region of the protein (35), but the specific tyrosine residue phosphorylated is unknown. The two other presumptive autophosphorylation sites are contained within peptides 4 and 7, which have previously been mapped to the C-terminal half of P130^{gag-fps} (35, 38). The residue phosphorylated in peptide 7 is unknown. The absence of peptide 4 from the map of F-836 P130^{gag-fps} would confirm that Tyr-836 is the residue phosphorylated within peptide 4.

The distorted shape of peptide 4 from WT P130^{gag-fps} (Fig. 6A) had suggested the presence of more than one tryptic phosphopeptide in this spot. Phosphoamino acid analysis of the left portion of spot 4 yielded phosphotyrosine, whereas the right portion of spot 4 released phosphoserine and a trace of phosphotyrosine (data not shown). Therefore, spot 4 contains two poorly resolved phosphotryptic peptides: one containing phosphotyrosine (peptide 4) and one containing phosphoserine (peptide X). Phosphoamino acid analysis of the presumptive spot X isolated from F-836 P130^{gag-fps} (Fig. 6B) released only phosphoserine, indicating that it was indeed peptide X and not peptide 4. In addition, a mix of ³²P-labeled tryptic phosphopeptides from WT and F-836 P130^{gag-fps} contained two peptides, with similar mobilities to 4 and X, whereas the map of F-836 P130^{gag-fps} contained

FIG. 5. PTK activity exhibited by WT and mutant FSV P130^{gag-fps} proteins. P130^{gag-fps} from equivalent numbers of [³⁵S]methionine-labeled and unlabeled cells was immunoprecipitated with anti-p19^{gag} antibody from rat-2 cells and FSV-transformed cell lines. The unlabeled immunoprecipitates were incubated with [³²P]ATP and acid-denatured enolase as described in Materials and Methods. Equal volumes of each immunoprecipitate were analyzed on an SDS-7.5% polyacrylamide gel and then subjected to autoradiography. The lanes contain immunoprecipitates prepared from the following cell lines: lanes A, rat-2; lanes B, F-836/F-1073; lanes C, F-1073; lanes D, F-836; and lanes E, WT. Exposure time was 10 h.

only one spot at this location (data not shown). The close mobility of spot X and spot 4 is more easily seen in the map of F-1073 P130^{gag-fps} (Fig. 6C), since the phosphorylation of spot 4 is reduced in this mutant protein.

Examination of the tryptic peptide maps presented in Fig. 6 suggests that the other autophosphorylation sites within FSV F-836 P130^{gag-fps} (peptides 1, 3a, 3b, and 7) are phosphorylated to the same extent as in WT P130^{gag-fps}. The same WT autophosphorylation pattern (except for the loss of spot 4) seen with this mutant is consistent with its unchanged kinase activity. This is in contrast to the F-1073 mutation, in which the loss of the major autophosphorylation site affects

TABLE 1. Relative levels of phosphorylation^a

FSV P130 ^{gag-fps}	% Phosphorylation	
	In vivo ^b	In vitro ^c
F-836/F-1073	30	3
F-1073	55	3
F-836	77	58
WT	100	100

^a Duplicate cultures were labeled either with ³²P_i or with [³⁵S]methionine for 18 h, and P130^{gag-fps} was isolated by immunoprecipitation. P130^{gag-fps} was also immunoprecipitated from cell lysates prepared from unlabeled cultures and labeled with ³²P in an in vitro kinase reaction. Proteins were separated by SDS-gel electrophoresis (Fig. 4), and the relative level of phosphate to protein was determined by scintillation counting the gel-purified proteins and expressing these values as a ratio of ³²P counts per minute to ³⁵S counts per minute incorporated into the respective P130^{gag-fps} proteins.

^b ³²P incorporated during metabolic labeling of cells with ³²P_i. Values are expressed relative to WT (100%).

^c ³²P incorporated during an immune complex kinase assay. Values are expressed relative to WT (100%).

TABLE 2. Phosphoamino acid analysis of P130^{gag-fps} proteins^a

Phosphoamino acid	Amt of phosphoamino acid in ^b :			
	F-836/ F-1073	F-1073	F-836	WT
Phosphoserine	82	66	44	39
Phosphothreonine	7	14	18	15
Phosphotyrosine	11	20	38	46

^a Radioactivity in individual phosphoamino acids was quantitated either by scanning an autoradiogram of electrophoretically separated acid hydrolysates of P130^{gag-fps} with an LKB Ultrascan laser densitometer or by counting aspirated spots identified by marker phosphoamino acids.

^b The values of each phosphoamino acid are expressed as a percentage of total acid-stable phosphoamino acids recovered in that sample.

the kinase activity and results in a decrease in phosphorylation of peptides 1, 4, and 7. The serine phosphorylation sites (peptides 5, 6, 8, 9, and X) were unaffected by the substitution of Tyr-863 with a Phe, suggesting that this change had a subtle, if any, effect on the conformation of the F-836 P130^{gag-fps} protein.

Tryptic peptide mapping of F-836/F-1073 P130^{gag-fps} (Fig. 6D) and phosphoamino acid analysis of the putative spot X confirmed that both Tyr-1073 and Tyr-836 tryptic phosphopeptides were missing. Also, similarly to the situation for the F-1073 mutant, the phosphorylation of the other autophosphorylation sites (peptides 1 and 7) was decreased, whereas the serine phosphopeptides were labeled to a similar level as seen in WT P130^{gag-fps}, suggesting that the additional mutation had no obvious effect on protein structure.

Tyrosine phosphorylation in normal and FSV-transformed cell lines. The transformed phenotype induced by WT or F-836 P130^{gag-fps} in rat-2 cells appears to be more pronounced than that induced by F-1073 or F-836/F-1073 P130^{gag-fps}. Likewise, WT and F-836 P130^{gag-fps} proteins are more active as PTKs than F-1073 or F-836/F-1073 P130^{gag-fps} proteins are. To determine whether the PTK activity measured in vitro reflects that seen in the various FSV-transformed cells and whether it correlates with the phenotype of the cells, we examined protein phosphorylation on tyrosine in the different FSV-transformed cell lines. Antibodies directed against phosphotyrosine were used to probe immunoblots of lysates from WT-, F-836-, F-1073-, and F-836/F-1073-transformed cells and rat-2 cells (Fig. 7). The intensely reactive, approximately 130-kDa band seen in WT and F-836 lysates is probably P130^{gag-fps}, since ³²P-labeled P130^{gag-fps} run in the lane next to WT FSV lysates and blotted migrated to a similar location in the gel. This band was barely detectable in lysates immunoblotted from F-1073- or F-836/F-1073-transformed cells. The low level of reactivity of these mutant P130^{gag-fps} proteins with antiphosphotyrosine antibodies is partly accounted for by the loss of the Tyr-1073 phosphorylation site and the consequent absence of phosphotyrosine within these proteins (Table 2). Also, the Tyr-836 phosphorylation site may have a lower affinity for the antibody than the Tyr-1073 phosphorylation site, since F-1073 P130^{gag-fps} is not readily detected with this antibody, even though it is phosphorylated to some extent on Tyr-836 (Fig. 6C).

FIG. 6. Tryptic peptide analysis of P130^{gag-fps} encoded by WT and mutant FSVs. P130^{gag-fps} was isolated from WT- or mutant-transformed cells incubated with ³²P_i for 18 h. After digestion with trypsin, the tryptic peptides were separated in two dimensions as described in Materials and Methods. Electrophoresis was performed at pH 1.9 in the horizontal dimension with the anode on the left. Chromatography was from bottom to top. Arrowheads mark the points of the sample application. The P130^{gag-fps} proteins are as follows: (A) WT, (B) F-836, (C) F-1073, (D) F-836/F-1073. Cerenkov counts per minute loaded onto thin-layer plates and exposure times (at -70°C with intensifying screens) are as follows: (A) 5,340, 18 h; (B) 5,420, 18 h; (C) 3,225, 48 h; (D) 2,100, 72 h.

FIG. 7. Phosphotyrosine-containing proteins identified by immunoblotting cellular lysates with antiphosphotyrosine antibodies. Equivalent amounts of cell proteins were separated on an SDS-10% polyacrylamide gel, transferred to a nitrocellulose filter, and incubated with antibodies to phosphotyrosine as described in Materials and Methods. Lysates were prepared from the following cell lines: lane A, WT; lane B, F-836; lane C, F-1073; lane D, F-836/F-1073; and lane E, rat-2. MW indicates ¹⁴C-molecular weight markers, and the size, in thousands, is indicated on the side. Exposure time was 12 h at -70°C with an intensifying screen and presensitized Kodak XAR film.

At least 30 different bands present in lysates from WT or F-836 FSV-transformed cells reacted with the antiphosphotyrosine antibodies. Each band may consist of more than one phosphotyrosine-containing protein; conversely, a single protein could be represented by more than one band as a result of multiple phosphorylation or other posttranslational modifications. Consequently, the numbers represent an approximation of the proteins phosphorylated on tyrosine in these cells. About 17 proteins were detectably phosphorylated on tyrosine in rat-2 cells transformed by F-1073 or F-836/F-1073 P130^{gag-fps}, whereas only 7 proteins were phosphorylated in normal rat-2 cells. The qualitative and quantitative differences between WT and mutant F-1073- or F-836/F-1073-transformed cells probably reflect the lower level of tyrosine phosphorylation in these mutant cells owing to the impaired kinase activity of the mutated P130^{gag-fps} proteins (assuming equivalent levels of phosphotyrosine-specific phosphatases in the cells tested). However, three proteins with apparent molecular masses of 50, 66, and 70 kDa were detected in WT- and F-836-transformed cells, but were clearly absent in rat-2 cells and in F-836/F-1073- and F-1073-transformed cells (indicated by asterisks in Fig. 7). The phosphorylation of these proteins on tyrosine correlates with the more highly transformed phenotype seen with WT and F-836 FSV-expressing cells. Interestingly, there is a 120-kDa protein that was more highly reactive with the antibody in rat-2 cells than in any of the FSV-transformed cells tested. This protein may be similar to a protein found in quiescent mouse fibroblasts, which is also strongly reactive with antiphosphotyrosine antibodies (24).

DISCUSSION

We have identified Tyr-836 as a major site of tyrosine phosphorylation in FSV P130^{gag-fps}. Substitution of this

tyrosine residue with a phenylalanine appears to have little, if any, effect on the function of the FSV transforming protein. The transforming potential of the F-836 mutant P130^{gag-fps} in rat-2 cells is essentially the same as that of WT P130^{gag-fps}. In addition, the intrinsic PTK activity of this mutated P130^{gag-fps} protein appears largely unchanged compared with the WT P130^{gag-fps} activity. Furthermore, the array of proteins phosphorylated on tyrosine in these cells is comparable.

FSV P130^{gag-fps} appears to have at least four autophosphorylation sites. The major site at Tyr-1073 in the kinase domain and a minor unidentified site in the *gag* region are phosphorylated both in vitro and in vivo (35, 38). The two other presumptive autophosphorylation sites, which are contained within peptides 4 and 7, are not detected in vitro. However, there is indirect evidence establishing phosphotryptic peptides 4 and 7 as autophosphorylation sites. For example mutations within P130^{gag-fps} which either inactivate or impair its intrinsic PTK activity result in a loss or decrease, respectively, in phosphotryptic peptides 4 and 7 (36-38), suggesting that they are autophosphorylation sites. This is further substantiated by the fact that these sites are phosphorylated when bacterially expressed *v-fps* proteins are biosynthetically labeled with ³²P_i (27), even though bacteria do not have PTKs. Together, these data suggest that Tyr-836, the residue phosphorylated within peptide 4, is a bona fide autophosphorylation site within FSV P130^{gag-fps}. However, the possibility remains that Tyr-836 is phosphorylated in *trans* by P130^{gag-fps} as previously reported for Tyr-1073 (37).

Mutation of the Tyr-836 autophosphorylation site within the F-1073 mutant protein, which is missing the major autophosphorylation site, had no consequences in addition to that of the F-1073 mutation. The phosphorylation of Tyr-1073 is clearly not dependent upon the phosphorylation of Tyr-836, since the level of Tyr-1073 phosphorylation within F-836 P130^{gag-fps} is equivalent to that found in WT P130^{gag-fps}. Note that the reverse is not true, because phosphorylation of Tyr-1073 within P130^{gag-fps} activates its protein kinase activity (23, 38) and thereby increases phosphorylation of Tyr-836.

Tyrosine 836 lies upstream of the catalytic domain in a region conserved among several PTKs. This region contains approximately 100 amino acids (residues 810 to 900 in FSV P130^{gag-fps}) and has been designated SH2 for *src* homology 2 region (28). Apparently this domain is dispensable for phosphate transfer, since the entire SH2 domain can be removed without affecting the activity of the residual catalytic domain expressed in bacteria (27). However, Sadowski et al. (28) have recently described two FSV mutants that have dipeptide insertions at residues 821 and 832 in the SH2 domain of P130^{gag-fps}, both of which are transformation defective when expressed in rat-2 cells. Apparently this is a result of impaired kinase activity, even though when expressed in bacteria the *v-fps*-derived mutant proteins were found to be as active as the WT proteins in PTK activity (27). In addition, the AX9m mutation, which is 4 residues N terminal to Tyr-836, renders P130^{gag-fps} both host dependent and temperature sensitive for transformation (J. E. DeClue, I. Sadowski, G. S. Martin, and T. Pawson, Proc. Natl. Acad. Sci. USA, in press). On the basis of these results, the authors have suggested that the SH2 region is a domain within P130^{gag-fps} to which eucaryotic cellular proteins bind and thereby regulate or modulate the intrinsic kinase activity of P130^{gag-fps}. The fact that the SH2 domain contains the Tyr-836 autophosphorylation site implies that the SH2 do-

main normally lies near the active center of the catalytic domain in the native protein and could thereby regulate its enzymatic activity. However, we are as yet unable to demonstrate any difference in FSV WT and F-836 P130^{gag-fps} activity either in vitro or in vivo, even though one might have expected an effect from a phosphorylation that occurs within the conserved SH2 domain of FSV P130^{gag-fps}, which is required for full kinase activity in both rat and chicken fibroblasts (28; DeClue et al., in press). Perhaps the phosphorylation of Tyr-836 is merely fortuitous and completely without consequence, occurring because of the juxtaposition of the SH2 domain and the catalytic domain.

Of the 26 mutations that have occurred within the *v-fps* protein following transduction of the *c-fps* gene from chicken cells, 4 are found in the SH2 domain. Tyr-836 is one of these changes, being a Cys in *c-fps* (13) and a His in *c-fes* (26) and in *v-fes* (11). Since the SH2 domain may promote interactions between P130^{gag-fps} and other cellular proteins, it seems likely that at positions other than the highly conserved ones, more than a point mutation would be required to disrupt such a proposed protein-protein interaction. Indeed, only insertions in the SH2 domain (28, 33) or large deletions upstream of the SH2 domain (1) have been reported to affect the host range, transforming potential, and kinase activity of FSV P130^{gag-fps}. Even changing Tyr-821, the site of one of the FSV SH2 insertion mutants, to a Phe has no effect on P130^{gag-fps} function (I. Sadowski and T. Pawson, personal communication). The fact that there are multiple changes in the SH2 region may indicate that they have combined effects on P130^{gag-fps} function. Perhaps reversion of the additional mutations within the FSV F-836 P130^{gag-fps} SH2 domain and/or substitution of Tyr-836 with a Cys would affect the activity of P130^{gag-fps} and reveal a function for Tyr-836 phosphorylation in transformation by FSV.

Since there are 26 scattered amino acid changes between the *c-fps* and FSV *v-fps* proteins (13), some of these mutations must account for the differences in biological properties of these two proteins (29). The *c-fps/c-fes* proteins are unable to transform chicken (9), rat (10), or mouse (7) fibroblasts, even when expressed to high levels. However, *c-fps* can be activated to transform chicken embryo fibroblasts by the N-terminal addition of viral *gag* sequences (9). In contrast, removal of the viral *gag* sequences from FSV has little effect on its transforming potential in chicken (8) and rat (G. A. Weinmaster, unpublished data) cells. Therefore, either the addition of viral sequences or mutations within *c-fps* results in the activation of its transforming potential. Apparently, several mutations might be required to activate *c-fps*, since Foster et al. (9) did not recover any transforming mutants of *c-fps* during 2 months of passage of infected cells, whereas spontaneous transformants are recovered with a high frequency from cells infected with a *c-src*-containing virus (18). It seems likely that the initial step in the conversion of normal *c-fps* into the oncogenic *v-fps* involved a *gag-c-fps* fusion event which was followed by further mutagenesis to potentiate the *gag-v-fps* transforming activity, Tyr-836 being one of these mutagenic events.

Unlike the situation for the viral transforming proteins, it is difficult to detect the presence of phosphotyrosine within the *c-fps/fes* proteins (21, 22). However, the *c-fps/fes* proteins can be readily phosphorylated on tyrosine when assayed in an immune complex kinase reaction (6, 21, 22), suggesting that the kinase activity inherent in these cellular proteins is negatively regulated in the cell. Perhaps the mutations acquired by *v-fps* are involved in the release of this negative regulation. Therefore, it would be interesting to

introduce some of these *v-fps*-acquired mutations, either singly or in combination, into the *c-fps/fes* proteins to identify those involved in the regulation of its kinase activity and transforming potential. Since the SH2 domain within FSV P130^{gag-fps} appears to regulate its PTK activity and transforming ability, this region may also be important for *c-fps/fes* activity. Thus, systematic mutagenesis of the *c-fps* residues which are different between *v-fps* and *c-fps* in their SH2 domains may provide important clues to the regulation and function of the *c-fps* protein. The substitution of Cys-527 within the *c-fps* protein, the residue equivalent to Tyr-836 in FSV P130^{gag-fps}, with a tyrosine could be a revealing mutation.

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