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The Common *src* Homology Region 2 Domain of Cytoplasmic Signaling Proteins Is a Positive Effector of v-*fps* Tyrosine Kinase Function

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A conserved noncatalytic domain SH2 (for *src* homology region 2) is located immediately N terminal to the kinase domains of all cytoplasmic protein-tyrosine kinases. We found that the wild-type v-*fps* SH2 domain stimulated the enzymatic activity of the adjacent kinase domain 10-fold and functioned as a powerful positive effector of catalytic and transforming activities within the v-*fps* oncoprotein (P130$^{gag\text{-}fps}$). Partial proteolysis of P130$^{gag\text{-}fps}$ and supporting genetic data indicated that the v-*fps* SH2 domain exerts its effect on catalytic activity through an intramolecular interaction with the kinase domain. Amino acid alterations in the SH2 domain that impaired kinase function interfered with association of the SH2 domain with the kinase domain. Deletion of a conserved octapeptide motif converted the v-*fps* SH2 domain from an activator to an inhibitor of tyrosine kinase activity. This latent inhibitory activity of v-*fps* SH2 has functional implications for phospholipase C-γ and p21^{ras} GTPase-activating protein, both of which have two distinct SH2 domains suggestive of complex regulation. In addition to regulating the specific activity of the kinase domain, the SH2 domain of P130$^{gag\text{-}fps}$ was also found to be required for the tyrosine phosphorylation of specific cellular proteins, notably polypeptides of 124 and 62 kilodaltons. The SH2 domain therefore appears to play a dual role in regulation of kinase activity and recognition of cellular substrates.

The SH2 (*src* homology 2) domain was originally defined as a noncatalytic region of 80 to 100 amino acids that is conserved among all nonreceptor protein-tyrosine kinases (PTK), regardless of their cellular or viral origin, and is positioned immediately N terminal to their kinase domains (22, 29). Even though it does not contribute directly to catalysis or subcellular localization, mutations that perturb the SH2 domain compromise the transforming activity of v-*fps* and v-*src* oncoproteins (6–8, 21, 25, 29, 33, 41, 44, 48). On the basis of the properties of a v-*fps* host range mutant (AX9m) that transforms avian but not rat cells, we have proposed that the SH2 domain forms a binding site for a cellular product that is important for transforming activity of the P130$^{gag\text{-}fps}$ oncoprotein (8). Similar suggestions have recently been made for p60$^{v\text{-}src}$ (21, 41, 48).

Cytoplasmic PTKs encoded by genes such as *fps/fes*, *src*, and *abl* are confined to the inside of the cell, where they are generally associated with the plasma membrane or cytoskeleton (10). Although their normal functions are largely unclear, the enzymatic relationship of cytoplasmic PTKs to growth factor receptors and their potential oncogenicity suggest a role in signal transduction. Consistent with this possibility, stimulation of fibroblasts with platelet-derived growth factor elicits an increase in p60$^{c\text{-}src}$ kinase activity (9, 24). The related p56^{lck} protein-tyrosine kinase apparently interacts in T cells with cytoplasmic domains of the CD4 and CD8 cell surface receptors, is enzymatically stimulated upon cross-linking of CD4, and may therefore be involved in T-cell activation (27, 39, 40).

In addition to this functional evidence, there is a structural basis for implicating cytoplasmic PTKs in signal transduction. Sequence elements closely related to the PTK SH2 domains have been described in phospholipase C-γ (PLC-γ; previously PLC-148 or PLC II) (32, 35), in the related avian v-*crk* oncoprotein (18), and in the p21^{ras} GTPase-activating protein (GAP) (38, 42). Indeed, both PLC-γ and GAP have two adjacent SH2 domains. Although both PLC-γ and GAP are involved in signal transduction, they are functionally quite distinct from one another and from cytoplasmic PTKs. PLC catalyzes the breakdown of polyphosphoinositides and therefore regulates an important intracellular response to many hormonal signals (5, 16). In contrast, GAP stimulates p21^{ras} GTPase activity, interacts with its effector loop, and may also be its biological target. Functional p21^{ras}, and hence GAP, is required for the mitogenic and transforming activities of both receptorlike and cytoplasmic PTKs (20, 31). The SH2 domain appears to be a common feature of cytoplasmic signaling proteins and may therefore be of general importance in regulating signal transduction. As a prototype to explore the function of the SH2 domain, we have used the oncogenic P130$^{gag\text{-}fps}$ PTK. Here we provide direct evidence that the SH2 domain is a critical positive effector of v-*fps* kinase activity and indicate a mechanism by which the SH2 domain regulates enzymatic function.

MATERIALS AND METHODS

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis of v-*fps* coding sequence was performed on a uracil-substituted phagemid vector (pTZ1.4) as described previously (14, 19). Mutations were confirmed by dideoxy DNA sequencing and then were substituted for wild-type v-*fps* sequences in the simian virus 40-based mammalian expression vector pIV2.3 (19). The deletion mutants TO2 and TO5 were created by 33-mer oligonucleotides corresponding to the 15 nucleotides 5′ and 18 nucleotides 3′ of the sequences

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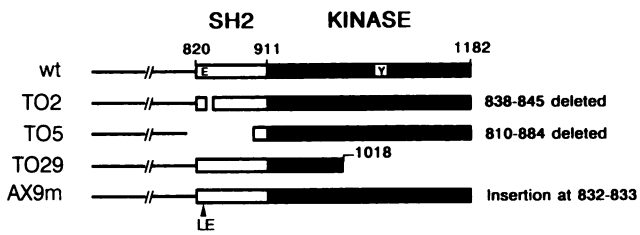

FIG. 1. v-*fps* SH2 and kinase domain mutants. The structures of v-*fps* deletion, insertion, and truncation mutants are indicated. The locations of Glu-832 (E) and Tyr-1073 (Y), both the sites of single amino acid substitutions, are shown on wt P130$^{gag\text{-}fps}$.

to be deleted (Fig. 1 and 2) such that the intervening residues were looped out upon annealing to the template; 18-mers were used to introduce the various point mutations. TO29 was created by changing the Lys-1018 codon (AAG) to the *amber* codon TAG. The Glu-832 codon GAG was changed to GAC (Asp), GCG (Ala), or AAG (Lys), and the Tyr codon at 1073 (TAT) was changed to TTT (Phe). The TO2 and TO5 deletions and the codon 832 substitutions were introduced into pIV2.3 as *Bst*XI-*Hin*dIII fragments; the TO29 stop codon and the Phe-1073 substitution were subcloned as *Apa*I-*Hin*dIII fragments.

Cells and viruses. Rat-2 cell lines were maintained in Dulbecco modified Eagle medium containing 50 U of penicillin and 50 μg of streptomycin per ml supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2. To assess focus-forming ability, cells at approximately 30% confluence in 10-cm plates were transfected with 150 ng of the appropriate pIV2.3 plasmid and 10 μg of carrier Rat-2 DNA by the calcium-phosphate precipitate method as described previously (33). Once the cells had attained confluence, they were maintained in antibiotic-supplemented Dulbecco modified Eagle medium containing 5% calf serum for up to 9 weeks. Cell lines expressing transformation-defective mutants were isolated by cotransfecting 1 μg of pSV2neo and 10 μg of pIV2.3 plasmid DNAs and selecting for cells resistant to the antibiotic G418 (400 μg/ml) as described previously (8). G418-resistant colonies were isolated and expanded.

Cloning of transformed cells in soft agar was performed as described previously (47). To quantify colony-forming ability, clonal cell lines were seeded into soft agar at 10^4, 5×10^4, and 10^5 cells per 3 ml of 0.3% agar and scored for colony formation 8 days later.

Antibodies. The mouse anti-*gag* monoclonal antibody R254E has been described elsewhere (11). Polyclonal anti-*fps* antiserum was raised by immunizing rabbits with a *trpE-fps* bacterial fusion protein (pTF822) containing P130$^{gag\text{-}fps}$ residues 822 to 1182 (28). Rabbit antiphosphotyrosine antibodies were raised and affinity purified as described by Kamps and Sefton (12). Immunoreactivity of antiphosphotyrosine antibodies was blocked by 10 mM phosphotyrosine but not by phosphothreonine or phosphoserine.

Radiolabeling and in vitro kinase reactions. Cells were metabolically labeled by growth in methionine-free medium containing 5% dialyzed fetal calf serum and 100 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp.) per ml for 4 h. *gag-fps* proteins were collected from labeled cells by immunoprecipitation with the anti-*gag* mouse monoclonal antibody R254E as described previously (19, 45). Specific kinase activities were measured by incubating one half of each immunoprecipitate in an immune complex-kinase reaction containing 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and 5 μg of acid-denatured enolase as an exogenous substrate. ^{32}P incorporation into P130$^{gag\text{-}fps}$ or enolase was quantified by densitometric analysis and normalized to the amount of ^{35}S-labeled P130$^{gag\text{-}fps}$ in the other half of the immunoprecipitate, also measured by densitometric analysis of autoradiograms, as described previously (19).

Western blot (immunoblot) analysis. Cells at approximately 80% confluence were rinsed with phosphate-buffered saline and then lysed by addition of 0.5 ml of phosphate-buffered sample buffer (5 mM sodium phosphate [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol, 0.05% bromphenol blue) at 100°C and were immediately incubated at 100°C for 5 min. After sonication for 10 s to reduce the viscosity of the lysate, a sample was taken for protein determination by a modified Lowry method (23), and the lysate was quickly frozen in a dry ice-ethanol bath and stored at −80°C. Frozen samples were thawed in a 15°C water bath, and 25 μg of protein was immediately loaded onto a 7.5% or 10% SDS-polyacrylamide gel. After electrophoresis, gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol) and transferred to nitrocellulose sheets (37). Immunoblotting with phosphotyrosine-specific antibodies followed by ^{125}I-protein A treatment was performed essentially as described by Kamps and Sefton (12) except that ^{125}I-protein A (8.3 μCi/μg; Dupont, NEN Research Products) was used at 2 μCi per 10 ml of blocking solution. Immunoblotting with anti-*gag* monoclonal antibody was performed by using a 10^{-3} dilution of R254E ascites fluid in blocking solution consisting of 5% Carnation skim milk powder in TBS (20 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.02% sodium azide) for 1 h at room temperature. Blots were subsequently incubated with a 0.5×10^{-3} dilution of rabbit anti-mouse immunoglobulin antibody (Organon Teknika) followed by ^{125}I-protein A (as described above), both for 1 h at room temperature. Each incubation was followed by successive 5-min washes in blocking solution and in TBS containing 0.05% Nonidet P-40 and two washes with TBS. Air-dried blots were then exposed to film at −80°C with an intensifying screen.

Partial proteolysis. Cells at approximately 80% confluency in 10-cm plates were lysed with kinase lysis buffer (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate) and immunoprecipitated with the R254E anti-*gag* monoclonal antibody. Immune complexes were washed twice with lysis buffer, followed by two washes in pH 9.0 buffer (10 mM Tris hydrochloride [pH 9.0], 100 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5% Nonidet P-40). The immune complexes were resuspended in 30 μl of pH 9.0 buffer in the presence or absence of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Diagnostics) as described previously (45) and incubated on ice for 15 min. Reactions were stopped by the addition of bovine pancreas trypsin inhibitor (Sigma Chemical Co.) to a molar concentration equal to twice that of the trypsin concentration. Soluble fragments of v-*fps* proteins that were liberated from the immune complex by proteolysis were separated from the immunoprecipitate by centrifugation. The resulting supernatant fraction was mixed with an equal volume of 2× SDS sample buffer (125 mM Tris hydrochloride [pH 6.8], 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.1% bromphenol blue), and the pellet was suspended in a corresponding 1× sample buffer. The supernatant and pellet fractions were then resolved by electrophoresis through 12.5 or 15% (supernatant) or 7.5% (pellet) polyacrylamide gels and trans-

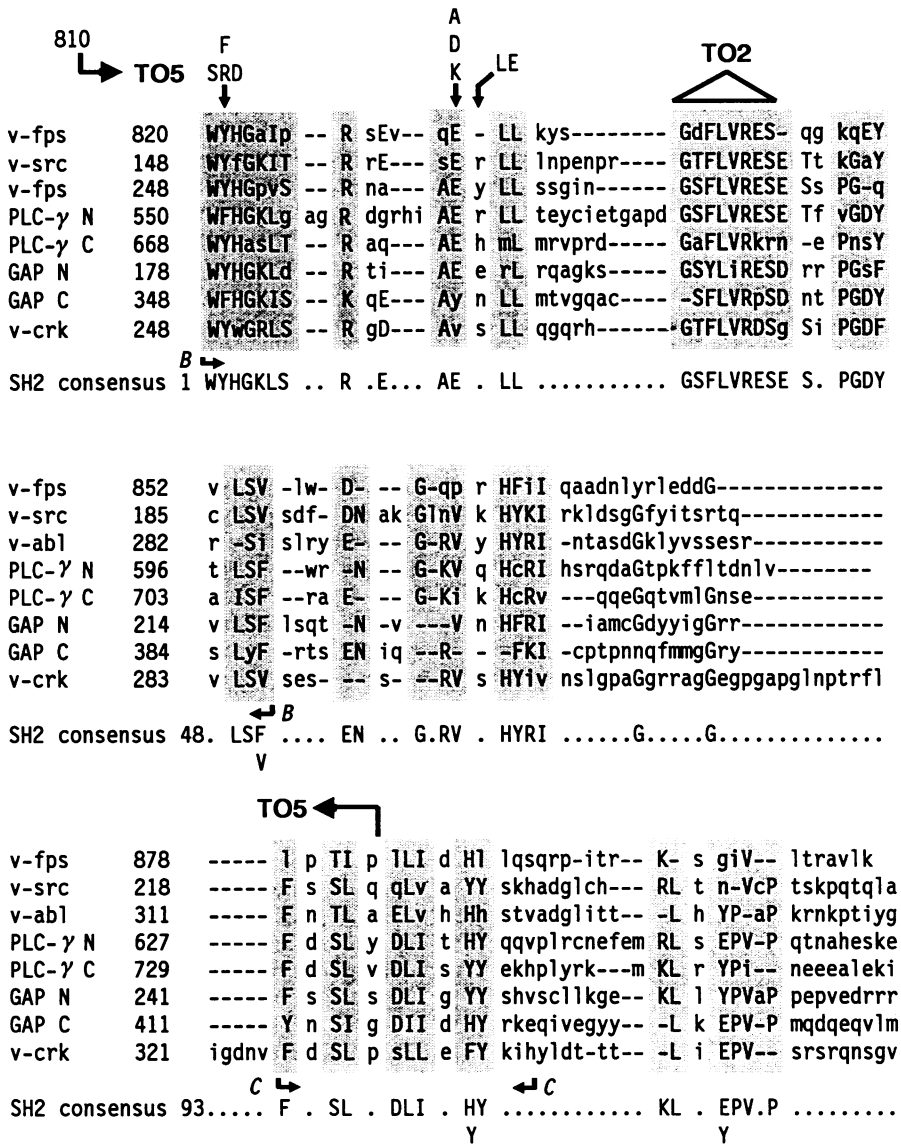

FIG. 2. SH2 domains of P130$^{gag\text{-}fps}$ (30), p60$^{v\text{-}src}$ (36), P120$^{gag\text{-}abl}$ (26), bovine PLC-γ (32), p21^{ras} GAP (42), and P47$^{gag\text{-}crk}$ (18). The more N-terminal and C-terminal SH2 domains of PLC-γ and GAP are indicated as N and C, respectively. Residues shared between four or more SH2 domains at any position are in the uppercase letters; conserved motifs are shaded. Conservative substitutions (Y/F, R/K, L/I, and E/D) have been allowed. Gaps are identified with hyphens. The N terminus of SH2 is defined by an invariant tryptophan. A consensus sequence is given, with periods indicating residues showing no obvious conservation. Sequence alignments were made by eye. The positions of insertions (SRD in RX15m; LE in AX9m), amino acid substitutions, and deletions in the v-*fps* SH2 are indicated. The locations of the B and C regions defined by Stahl et al. (32) are identified.

ferred to nitrocellulose membranes as described above. Soluble proteolytic fragments from the supernatant fraction were localized on blots with the *fps*-specific antiserum, followed by goat anti-rabbit immunoglobulin antibodies conjugated to alkaline phosphatase. Polypeptides from the pellet fractions were immunoblotted with anti-*gag* antibody R254E and detected by using a goat anti-mouse immunoglobulin antibody-alkaline phosphatase conjugate. These blots were preblocked with 5% skim milk powder in TBS (overnight at 4°C) before incubation with the primary and secondary antibodies for 1 h at room temperature. Antibody incubations were followed by two 15-min washes in TBS. Antibody complexes were visualized by incubating blots for 5 to 30 min in 100 mM Tris hydrochloride (pH 9.5)–100 mM NaCl–5 mM $MgCl_2$ containing 6.6 μl of nitroblue tetrazolium per ml (50 μg/ml in 70% dimethylformamide) and 3.3 μl of 5-bromo-4-chloro-3-indolyl phosphate per ml (50 μg/ml in 100% dimethylformamide). Alkaline phosphatase-conjugated antibodies and the color reaction substrates were from Sigma.

RESULTS

Regulation of v-*fps* catalytic and transforming activity by the SH2 domain. To test the activity of the v-*fps* kinase domain in the absence of SH2 sequences, we used oligonucleotide-directed mutagenesis to delete codons 810 to 884 of P130$^{gag\text{-}fps}$, thereby removing the majority of the SH2 domain (Fig. 1 and 2). This deletion mutant (TO5) failed to induce foci on Rat-2 cells within 4 weeks (Table 1). Upon more prolonged incubation, foci appeared on TO5-trans-

TABLE 1. Anchorage independence and focus-forming efficiency of Rat-2 cells expressing v-*fps* proteins

v-*fps*	Soft agar colony formation[a]	Focus formation[b]
Untransfected[c]	$<10^{-5}$	$<6.7 \times 10^{0}$[d]
wt	3.0×10^{-1}	3.1×10^{2}
TO5	1.0×10^{-2}	$<6.7 \times 10^{0}$[e]
TO2	$<10^{-5}$	$<6.7 \times 10^{0}$
Lys-832	9.0×10^{-2}	3.3×10^{1}
Asp-832	2.0×10^{-1}	1.2×10^{2}
Ala-832	ND	1.3×10^{2}

[a] Number of agar colonies per cell plated. Clonal cell lines were seeded into soft agar at 10^4, 5×10^4, and 10^5 cells per 3 ml of 0.3% agar and scored 8 days after plating. Results shown are from a single experiment; hence, standard errors were not derived. Similar results were obtained in additional experiments. ND, Not determined.

[b] Number of foci induced on Rat-2 cells 29 days posttransfection per microgram of plasmid DNA.

[c] Rat-2 cell line used as the negative control.

[d] pSV2neo plasmid DNA used as the negative control.

[e] TO5 induced foci with a longer latency period than 29 days (see text).

fected Rat-2 cells, but with very low efficiency and with an extended latent period of approximately 6 weeks, compared with 2 weeks for wild-type (wt) v-*fps*. A cell line established from such a TO5 focus contained a high level of TO5 P122$^{gag\text{-}fps}$ (Fig. 3A). Consistent with the results obtained by Western blotting, pulse-chase experiments indicated that the TO5 protein was not less stable than wt P130$^{gag\text{-}fps}$ (data not shown). Cells from the rare TO5-transformed lines were less rounded and refractile, were less efficient in colony formation, and formed smaller colonies in soft agar than did wt-transformed cells (Table 1; Fig. 4). Rat-2 lines expressing TO5 P122$^{gag\text{-}fps}$ isolated on the basis of G418 resistance showed only a very subtle morphological change (data not shown). The TO5 mutant is therefore seriously impaired in transforming activity.

The 122-kilodalton (kDa) TO5 protein immunoprecipitated from stably transfected Rat-2 cells retained only about 10% of wt P130$^{gag\text{-}fps}$ in vitro kinase activity, as judged by autophosphorylation and enolase phosphorylation (Fig. 3B; Table 2). Similar results were obtained by using Cos-1 cells in transient transfection assays (data not shown). Western blot analysis of whole-cell lysates by using antiphosphotyrosine antibody showed a spectrum of novel bands in wt-transformed cells compared with normal Rat-2 cells, of which the most prominent were P130$^{gag\text{-}fps}$ itself and a 62-kDa polypeptide (Fig. 3C). TO5-expressing cells showed a general decrease in tyrosine phosphorylation of both cellular and *gag-fps* proteins, consistent with the diminished in vitro activity of the TO5 protein. Although the majority of phosphotyrosine-containing species appeared in common between wt and TO5-expressing cells, there was a disproportionate decrease in the 62-kDa band as well as a specific loss of bands of 124 and 49 kDa (Fig. 3C). These results indicate that the v-*fps* SH2 domain is a positive regulatory element essential for full kinase activity and oncogenicity that may also be important for the recognition of specific cellular substrates.

A highly conserved SH2 motif is important in kinase activation. Residues 838 to 845 of P130$^{gag\text{-}fps}$ (GDFLVRES) represent the most highly conserved motif in SH2, with seven of eight residues identical between *fps*, *src*, and *abl* proteins (Fig. 2). This motif is similarly conserved in P47$^{gag\text{-}crk}$ and in one of the GAP and PLC-γ SH2 domains (Fig. 2). We deleted the coding sequence for these eight residues in v-*fps* and assayed the mutant protein for enzymatic and transforming activities. Transfection of the TO2 mutant into Rat-2 fibroblasts failed to elicit focus formation even when the transfected cells were maintained for extended periods of time. A series of Rat-2 cell lines expressing the TO2 mutant, which were isolated by selecting for transfectants with the antibiotic G418, appeared morphologically normal and were anchorage dependent (Table 1). The levels

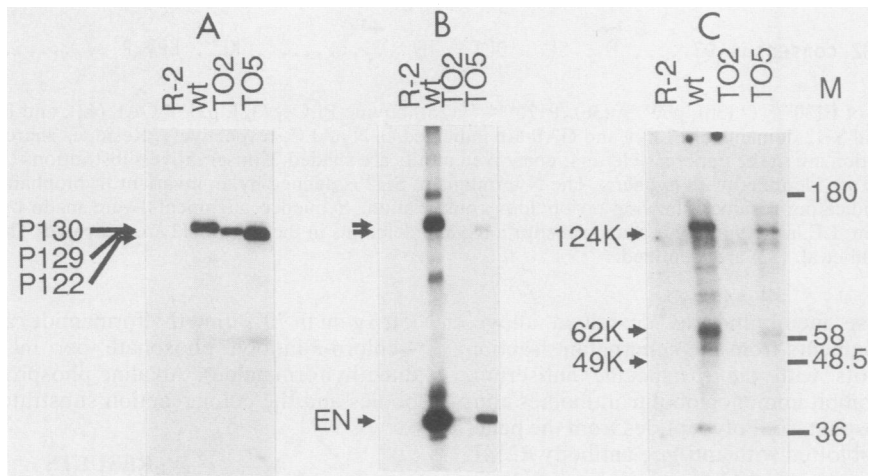

FIG. 3. Expression, kinase activity, and cellular substrates of v-*fps* SH2 deletion mutants. Parental Rat-2 cells (R-2) or cells expressing wt P130$^{gag\text{-}fps}$ (wt), TO2 P129$^{gag\text{-}fps}$ (TO2), or TO5 P122$^{gag\text{-}fps}$ (TO5) were analyzed as follows. (A) Analysis of the levels of *gag-fps* proteins in 25 μg of whole-cell lysates by Western blotting, using the anti-*gag* monoclonal antibody R254E. Antibody was detected with rabbit anti-mouse immunoglobulin followed by ^{125}I-protein A. (B) Determination of in vitro tyrosine kinase activities of wt and mutant *gag-fps* proteins after immunoprecipitation with anti-*gag* antibody. Immunoprecipitates were introduced into immune complex kinase reactions containing [γ-^{32}P]ATP and 5 μg of enolase (EN). (C) Identification of cellular phosphotyrosine-containing proteins by Western blot analysis of whole-cell lysates, using rabbit anti-phosphotyrosine antibodies. Antibodies were visualized with ^{125}I-protein A. All lysates were normalized for protein content before analysis. Immunoreactive bands that were selectively decreased or lost in TO5-expressing cells are arrowed. The mobilities of size markers (M) are indicated in kilodaltons.

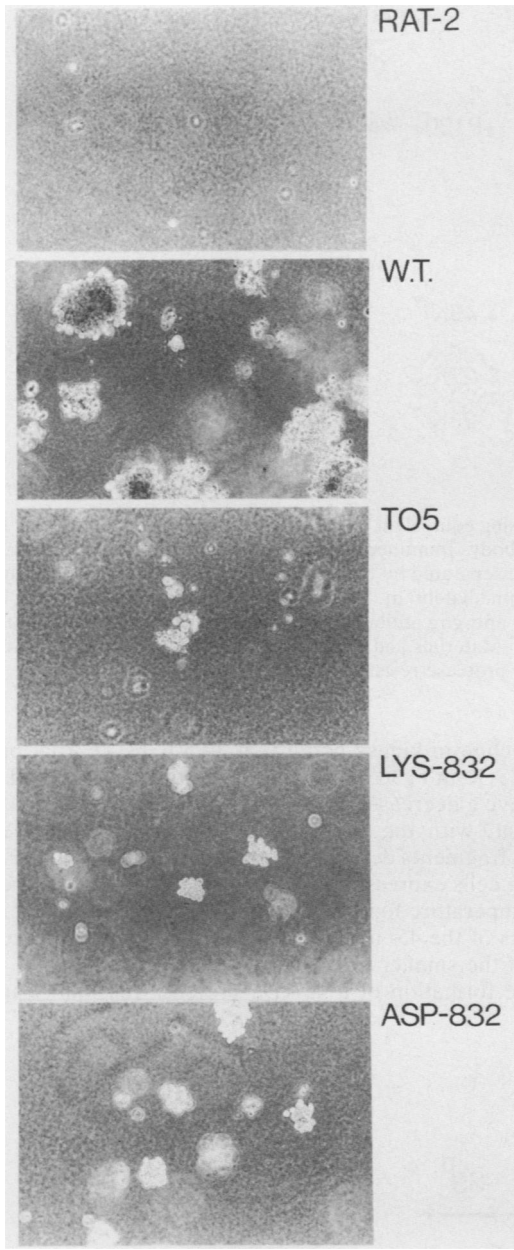

FIG. 4. Colony formation in soft agar by Rat-2 cells expressing wt and SH2 mutant *gag-fps* proteins. Colonies were photographed at 8 days.

of TO2 P129$^{gag\text{-}fps}$ in these cells were similar to the levels of wt P130$^{gag\text{-}fps}$ in transformed Rat-2 cells, as judged by Western blot analysis (Fig. 3A). TO2 P129$^{gag\text{-}fps}$ immunoprecipitated from such cells had little kinase activity that could be measured either by autophosphorylation or by enolase phosphorylation in an in vitro kinase reaction (Fig. 3B; Table 2). Antiphosphotyrosine antibody Western blots of TO2-expressing cells showed no obvious increase in cellular phosphotyrosine-containing proteins compared with Rat-2 cells (Fig. 3C), consistent with the lack of in vitro kinase activity.

The conserved motif deleted in TO2 P129$^{gag\text{-}fps}$ is therefore essential for the positive action of SH2 on enzymatic activity. Indeed, removal of these amino acids led to an inhibition of kinase activity, suggesting that the mutant SH2 domain of TO2 P129$^{gag\text{-}fps}$ represses enzymatic function. Thus, a modest sequence alteration can convert v-*fps* SH2 from a positive to a negative effector of catalytic activity.

TABLE 2. In vitro kinase activities of v-*fps* proteins in Rat-2 cells

gag-fps protein	Kinase sp act[a]	
	Autophosphorylation	Enolase
wt	100	100
TO2	0	2
TO5	9	17
Lys-832	12	22
Asp-832	31	32
Ala-832	46	80

[a] Determined as described in Materials and Methods and are expressed as percent of wild-type activity. Results shown are from a single experiment; hence, standard errors were not derived.

Interactions between the SH2 and kinase domains. Functional protein domains, including those of PTKs such as P130$^{gag\text{-}fps}$ and p60$^{v\text{-}src}$, are frequently folded into globular structures that are resistant to proteolysis and are released from the intact protein upon exposure to protease (3, 15, 45). To investigate the structural basis for the effects of the SH2 mutations on kinase activity, we immunoprecipitated wt or mutant P130$^{gag\text{-}fps}$ proteins from Rat-2 cells with an anti-*gag* monoclonal antibody and then incubated the immune complexes briefly with trypsin. Soluble polypeptides released by this mild protease digestion were immediately separated by gel electrophoresis, transferred to nitrocellulose, and probed with polyclonal rabbit antibodies (anti-*fps*) raised against P130$^{gag\text{-}fps}$ C-terminal residues 822 to 1182 (representing most of the SH2 and kinase domains). In the absence of trypsin, intact P130$^{gag\text{-}fps}$ was retained in the immune complex pellet, and no anti-*fps*-immunoreactive material was seen in the supernatant. Inclusion of trypsin in the incubation led to complete loss of intact P130$^{gag\text{-}fps}$ from the pellet and quantitative recovery of anti-*fps*-immunoreactive material as a single soluble 45-kDa polypeptide (Fig. 5), as observed previously with autophosphorylated P130$^{gag\text{-}fps}$ (45). The size, antigenicity (Fig. 5), kinase activity, and peptide composition (data not shown; see reference 45) of this 45-kDa trypsin-resistant fragment indicated that it contained both SH2 and kinase domains. In contrast, an identical digest of TO5 P122$^{gag\text{-}fps}$ yielded soluble immunoreactive fragments of 23, 24, 27, and 30 kDa (Fig. 5). The absence of the 45-kDa protease-resistant fragment is explained by the deletion of SH2 sequences from the TO5 protein. The 23- to 30-kDa fragments were apparently derived from the catalytic domain, and indeed the 30-kDa polypeptide was catalytically active (data not shown).

The integrity of a C-terminal 45-kDa fragment during proteolysis of wt P130$^{gag\text{-}fps}$ suggests an intramolecular interaction between the SH2 and kinase domains through which the SH2 domain stimulates catalytic activity. Consistent with this model, trypsin digestion of the TO2 *gag-fps* protein, which has only an 8-amino-acid deletion in SH2 and yet lacks catalytic activity, did not yield any protease-resistant fragment corresponding to the 45-kDa fragment of wt P130$^{gag\text{-}fps}$ (Fig. 5). The TO2 protein yielded prominent kinase domain fragments of 23, 24, and 27 kDa upon exposure to trypsin, which comigrated with those released from the TO5 polypeptide. However, the 30-kDa fragment, representing the functional kinase domain, was barely detectable after trypsin digestion of the TO2 protein. This finding

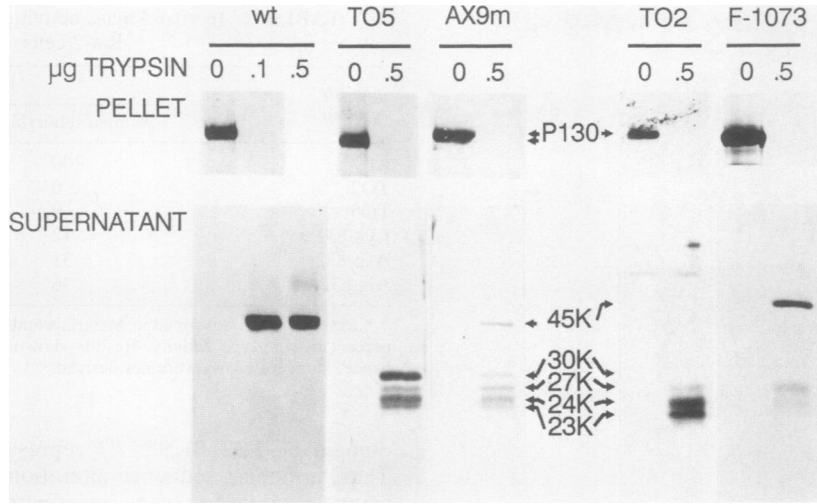

FIG. 5. Protease resistance of wt or mutant v-*fps* proteins. Rat-2 cells expressing equivalent amounts of wt, TO5, AX9m, TO2, or F-1073 *gag-fps* proteins were immunoprecipitated with mouse anti-*gag* monoclonal antibody. Immunoprecipitates were incubated on ice for 15 min in 30-μl incubation mixtures containing 0, 0.1, or 0.5 μg of trypsin. Samples were separated by centrifugation into residual insoluble immune complexes (pellet) or soluble proteolytic fragments (supernatant), boiled immediately in SDS sample buffer, and separated by gel electrophoresis. Polypeptides were transferred to nitrocellulose and probed with anti-*gag* antibody (pellet) or with anti-*fps* rabbit antibodies raised to C-terminal P130$^{gag\text{-}fps}$ residues 822 to 1182 (supernatant) as described in Materials and Methods. Mobilities of intact wt and mutant *gag-fps* protein (P130) and of the 45-, 30-, 27-, 24-, and 23-kDa immunoreactive protease-resistant fragments are shown.

suggested that the mutant SH2 region, through an altered association with the kinase domain, suppressed its ability to adopt a catalytically active conformation.

The temperature-sensitive AX9m host range mutant encodes a P130$^{gag\text{-}fps}$ protein with a Leu-Glu insertion between residues 832 and 833 (Fig. 1 and 2). This insertion results in a 10-fold decrease in P130$^{gag\text{-}fps}$ kinase activity at 34°C in Rat-2 cells (29), in a marked decrease or loss of cellular phosphotyrosine-containing proteins, as judged by antiphosphotyrosine antibody analysis (data not shown), and a further decline in kinase activity at 39.5°C (8). Partial proteolysis of AX9m P130$^{gag\text{-}fps}$ from phenotypically normal Rat-2 cells gave a decreased yield of the protease-resistant 45-kDa fragment, with the concomitant appearance of the 23- to 30-kDa fragments derived from the catalytic domain (Fig. 5). Shifting cells expressing AX9m P130$^{gag\text{-}fps}$ to the nonpermissive temperature for kinase activity induced a further selective loss of the 45- and 30-kDa fragments and increased the yield of the smaller bands (Fig. 6).

If the formation of a 45-kDa protease-resistant fragment

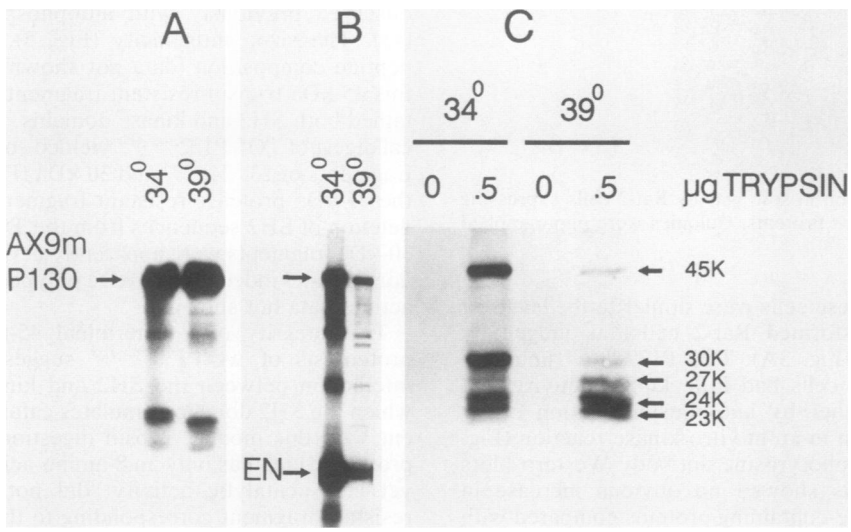

FIG. 6. Temperature-dependent changes in P130$^{gag\text{-}fps}$ protease resistance and kinase activity induced by an SH2 insertion mutation. Rat-2 cells expressing AX9m P130$^{gag\text{-}fps}$ were passaged, cultured overnight at 34 or 39°C, and analyzed as follows. (A) Analysis of whole-cell lysates by Western blot analysis with anti-*gag* monoclonal antibody. (B) Immunoprecipitation of cell lysates with anti-*gag* antibody. P130$^{gag\text{-}fps}$ was analyzed for autophosphorylation and enolase (EN) phosphorylation in an immune complex kinase reaction. (C) Incubation of anti-*gag* immunoprecipitates with 0 or 0.5 μg of trypsin. Soluble protease-resistant fragments (arrows) were identified by immunoblot analysis with anti-*fps* antiserum. K, Kilodaltons.

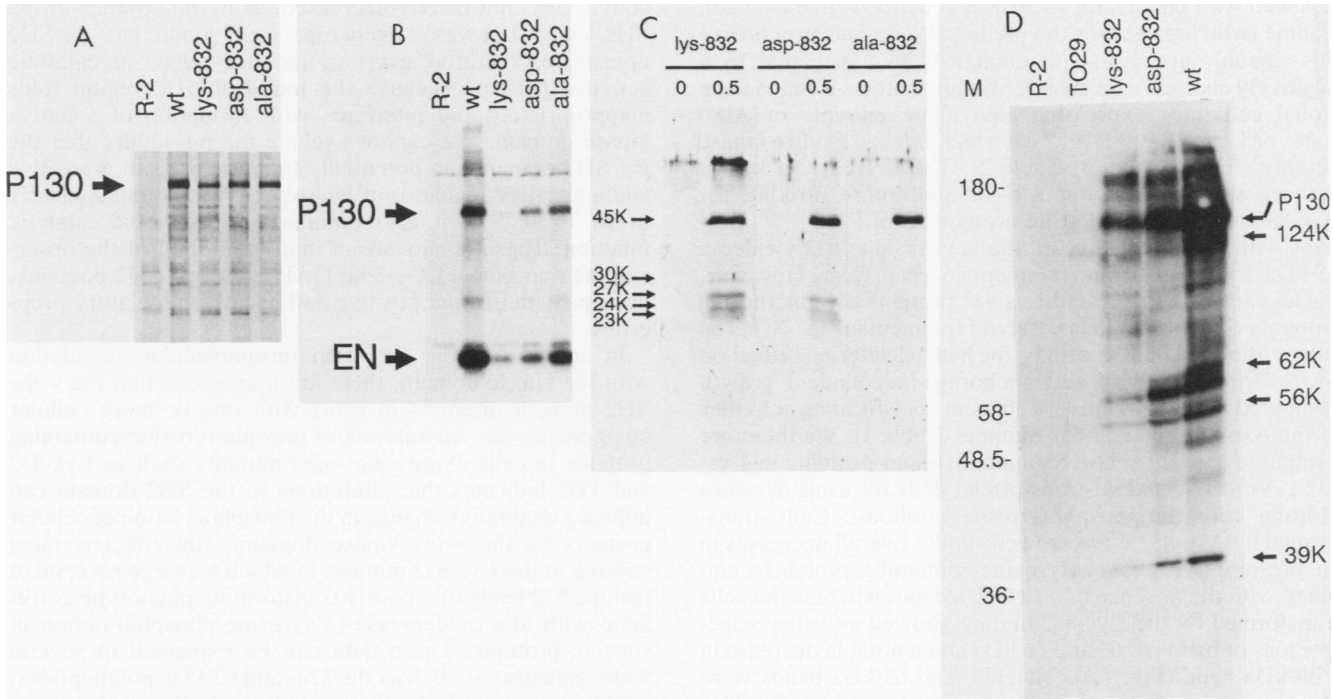

FIG. 7. Demonstration that substitution of a conserved SH2 residue (Glu-832) modifies v-*fps* tyrosine kinase activity, protease resistance, and cellular protein phosphorylation. Parental Rat-2 cells (R-2) or cells expressing wt, Lys-832, Asp-832, or Ala-832 P130$^{gag\text{-}fps}$ proteins were analyzed as follows. (A and B) Cells were metabolically labeled with [^{35}S]methionine and immunoprecipitated with anti-*gag* antibody. Immunoprecipitates were divided into two portions and either analyzed directly (A) or introduced into an immune complex kinase reaction containing enolase (B). (C) *gag-fps* proteins were immunoprecipitated with anti-*gag* antibody and exposed to 0 or 0.5 μg of trypsin. Soluble protease-resistant fragments were identified by Western blot analysis with anti-*fps* antiserum. (D) Cell lysates were subjected to immunoblot analysis with antiphosphotyrosine antibody and ^{125}I-protein A. The mobilities of size markers (M) and P130$^{gag\text{-}fps}$ are indicated, as are bands selectively lost in the Asp-832 or Lys-832 cells. K, Kilodaltons.

results from a reciprocal interaction between the SH2 and kinase domains, then it might also be perturbed by amino acid alterations in the catalytic domain. Within the kinase domain, substitution of Tyr-1073 (equivalent to p60^{src} Tyr-416) with phenylalanine (mutant F-1073) has been shown to cause a fivefold decrease in P130$^{gag\text{-}fps}$ kinase activity and reduced transforming potential, apparently as a result of loss of the principal autophosphorylation site (19, 47). Trypsin digestion of immunoprecipitated F-1073 P130$^{gag\text{-}fps}$ gave a reduced yield of the 45-kDa protease-resistant fragment and the concomitant appearance of the smaller fragments (Fig. 5). Thus, the conformation of the 45-kDa structure was affected by modifications to both SH2 and kinase domains.

These changes in protease sensitivity could be due to altered accessibility to trypsin resulting from differential binding of cellular proteins or to variable phosphorylation, or they could reflect conformational differences intrinsic to P130$^{gag\text{-}fps}$ itself. The similar effects of quite distinct mutations in the SH2 or kinase domains on trypsin digestion favor the latter possibility. These results indicate that the SH2 domain undergoes an intramolecular interaction with the catalytic domain. Mutations in the SH2 domain can apparently alter the association of this domain with the kinase domain and thereby reduce or inhibit enzymatic function and transforming activity.

The phenotype of v-*crk*-transformed cells, in which cellular tyrosine phosphorylation is elevated, has raised the possibility that P47$^{gag\text{-}crk}$ interacts in *trans* with an endogenous PTK (18, 22). To investigate whether the v-*fps* SH2 domain had any transforming activity in the absence of an intact kinase domain, we made a mutant (TO29) with a translation termination codon replacing codon 1018 (Fig. 1). This mutant encoded a 110-kDa protein lacking the C-terminal 165 amino acids of the catalytic domain that was stably expressed in Rat-2 cells (data not shown). The TO29 mutant was entirely transformation defective on Rat-2 cells and failed to complement the TO5 mutant (whose product lacks most of the SH2 domain) in inducing efficient transformation. Rat-2 cells expressing TO29 P110$^{gag\text{-}fps}$, isolated by selection for G418 resistance, were morphologically normal and had phosphotyrosine-containing proteins identical to those of parental Rat-2 cells (Fig. 7D). The v-*fps* SH2 domain therefore appears unable to *trans*-activate endogenous PTKs or its own catalytic domain. This view is consistent with the conclusion that the v-*fps* SH2 domain functions through an intramolecular interaction with the kinase domain.

Substitution of a conserved glutamate residue in SH2 affects v-*fps* kinase activity, transforming potential, and phosphorylation of cellular proteins. The properties of the TO5 and AX9m v-*fps* mutants suggested that the SH2 domain, in addition to an intramolecular interaction with the kinase domain, may be required for recognition of v-*fps* targets or regulators. We attempted to dissociate, as far as possible, the *cis*-acting effects of v-*fps* SH2 on kinase activity from any *trans*-acting recognition of cellular proteins by making single amino acid substitutions in SH2. For this purpose, we selected Glu-832 of P130$^{gag\text{-}fps}$, which is conserved in the SH2 domains of all cytoplasmic PTKs and PLC-γ and in one of the GAP SH2 elements. The codon for Glu-832 was

replaced with codons for aspartate (a conservative change), alanine (which preserves the predicted α-helical structure of this region), and lysine (a change from a negative to a positively charged side chain). Mutant proteins isolated from clonal cell lines expressing equivalent amounts of Ala-, Asp-, or Lys-832 P130$^{gag\text{-}fps}$ each had reduced in vitro kinase activity (Fig. 7A and B; Table 2). The decrease in kinase activity was modest for Ala-832 and more striking for Asp-832 and Lys-832. Partial proteolysis of P130$^{gag\text{-}fps}$ proteins with amino acid substitutions at residue 832 yielded a 45-kDa trypsin-resistant fragment in each case. However, the less active Asp-832 and Lys-832 proteins gave increasing amounts of 23- to 30-kDa catalytic fragments (Fig. 7C). The substitution of Glu-832 with lysine had a deleterious effect on focus-forming activity and anchorage-independent growth compared with the relatively efficient transforming activities of the Asp-832 and Ala-832 mutants (Table 1). We therefore compared the phosphotyrosine-containing proteins in Lys-832-, Asp-832-, and wt-transformed cells by using Western blotting with antiphosphotyrosine antibody. Cells transformed by Asp-832 v-*fps* showed similar overall decreases in all presumptive phosphotyrosine-containing proteins combined with the absence of a 124-kDa band, whereas the cells transformed by the Lys-832 mutant showed a further selective loss of bands of 62 and 56 kDa and a notable decrease in a 39-kDa band (Fig. 7D). The 124- and 62-kDa bands were also missing or reduced in Rat-2 cells expressing the TO5 (Fig. 3C) and AX9m (data not shown) mutants.

DISCUSSION

The v-*fps* SH2 domain is a noncatalytic effector of kinase activity and substrate recognition. These data indicate that the SH2 domain is a positive regulator of v-*fps* catalytic activity. Deletion of the SH2 domain, as in the TO5 mutant, results in 10-fold decreased enzymatic activity and depressed transforming potential. We infer that the basal catalytic activity of the v-*fps* kinase domain, manifested by the TO5 protein, is greatly enhanced in wt P130$^{gag\text{-}fps}$ by the adjacent SH2 domain. The resistance of a 45-kDa C-terminal fragment of wt P130$^{gag\text{-}fps}$, containing both the SH2 and kinase domains, to partial trypsinolysis strongly suggests a direct intramolecular interaction between these regions by which the SH2 domain stimulates catalytic activity. Analysis of P130$^{gag\text{-}fps}$ phosphorylation sites has shown that Tyr-836 in the SH2 domain is autophosphorylated in vivo, although without any obvious biological consequence, consistent with a direct association of the SH2 and catalytic domains (28, 46). This notion is supported by the observation that amino acid alterations in SH2 that reduce kinase activity induce a conformational change in P130$^{gag\text{-}fps}$, as judged by the loss of the 45-kDa SH2 and kinase protease-resistant fragment and the appearance of smaller tryptic fragments characteristic of the catalytic domain alone. In all of the mutant v-*fps* proteins we have analyzed, there is a positive correlation between the level of kinase activity and the extent to which the 45-kDa fragment is retained during proteolysis. P130$^{gag\text{-}fps}$ encoded by the temperature-sensitive AX9m mutant undergoes a coordinate change in protease resistance and kinase activity after a shift to the nonpermissive temperature. We conclude that mutations in the SH2 domain can interfere with the interaction of this domain with the kinase domain and thereby with enzymatic function and transforming activity.

In the case of the TO2 mutant protein, which lacks a small conserved octapeptide motif in the SH2 domain, kinase activity was not merely decreased, as in the absence of the SH2 region, but was actively repressed. A mutant v-*fps* SH2 domain can therefore exert an inhibitory effect on catalytic activity, perhaps because the modified SH2 region folds inappropriately and interferes with formation of a native kinase domain. We cannot exclude the possibility that the *fps* SH2 domain can potentially function as both a positive and a negative regulator of kinase activity, although clearly in wt P130$^{gag\text{-}fps}$ it is a dominant activator of catalytic function. These results are of interest in light of the observations that both PLC-γ and GAP have two SH2 domains, perhaps with distinct positive and negative regulatory properties.

In addition to the proposed intramolecular association with the kinase domain, there are indications that the v-*fps* SH2 domain interacts in *trans* with one or more cellular components (8). An analysis of phosphotyrosine-containing proteins in cells expressing v-*fps* mutants such as Lys-832 and TO5 indicates that alterations to the SH2 domain can induce a qualitative change in the phosphorylation of cellular proteins by the v-*fps* kinase domain. The effect is most striking in the Lys-832 mutant, in which a charge reversal at residue 832 leads to a poorly transforming phenotype correlated with loss or decrease of tyrosine phosphorylation of specific proteins. These data can be explained in several ways. Substrates, such as the 124- and 62-kDa polypeptides, could recognize a high-affinity binding site formed by juxtaposition of the SH2 and kinase domains. Alternatively, the SH2 domain may directly and independently recognize one or more cellular proteins, which are then phosphorylated by the kinase domain. Finally, the SH2 domain may be essential for recognition of a ligand that localizes the kinase domain adjacent to appropriate substrates. Although there is no direct evidence that these cellular substrates are important targets, there is a close correlation in a number of systems between v-*fps* transformation of Rat-2 cells and phosphorylation of the 62-kDa SH2-dependent substrate (M. Moran, unpublished observation).

The SH2 domain of P130$^{gag\text{-}fps}$ therefore appears to play a dual role as a regulator of kinase activity and in substrate recognition. Even in mutant proteins such as Lys-832, these activities cannot be fully divorced. We speculate that in normal cytoplasmic PTKs, stimulation from an upstream signal is accompanied by a transient conformational change that brings the SH2 and kinase domains together in an activated structure capable of recognizing and phosphorylating cellular targets. This interaction might be regulated by autophosphorylation within the kinase domain or by binding of an allosteric regulator to the SH2 domain.

A conserved domain in cytoplasmic regulators of signal transduction. The relationship between cytoplasmic PTKs, PLC-γ, GAP, and P47$^{gag\text{-}crk}$ extends throughout their SH2 sequences, including but not limited to residues in the B and C motifs described by Stahl et al. (32) (Fig. 2). Pairwise comparisons of SH2 domains generally show a sequence identity of 30 to 35%, which increases to approximately 40% when conservative substitutions are allowed. In at least one case, this is clearly a legitimate manipulation, as substitution of Tyr-821 with phenylalanine has no effect on P130$^{gag\text{-}fps}$ kinase or transforming activities (I. Sadowski, unpublished results). Although these proteins are all implicated in signal transduction, they are functionally quite distinct, suggesting that their SH2 domains may provide some common mode of regulation or interaction. As in the cytoplasmic PTKs, the SH2 domains of PLC-γ are apparently not critical for enzymatic activity (2a) and indeed are absent from some other

PLC isozymes (2, 13, 34). Similarly, the C-terminal 343 residues of GAP stimulate p21^{ras} GTPase activity, and the N-terminal SH2 domains are therefore not essential for GAP catalytic function (17). If the precedent established for the v-*fps* tyrosine kinase extends to PLC-γ and GAP, it is likely that their SH2 domains interact in *trans* with cellular factors and in *cis* with their respective catalytic domains to modify PLC or GAP activity. We and others have observed at least two proteins complexed with PLC-γ (18a, 43; C. Ellis and T. Pawson, unpublished observations); it will be of interest to determine whether these proteins bind to sites in the SH2 domains. Clearly, the identification of ligands for the SH2 domains of these different proteins is important in understanding how signal transduction is controlled at the plasma membrane.

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