MOLECULAR AND CELLULAR BIOLOGY, Mar. 1991, p. 1607–1613
0270-7306/91/031607-07$02.00/0

Vol. 11, No. 3

Identification of Domains of the v-*crk* Oncogene Product Sufficient for Association with Phosphotyrosine-Containing Proteins

MICHIYUKI MATSUDA,† BRUCE J. MAYER, AND HIDESABURO HANAFUSA*
The Rockefeller University, New York, New York 10021

Received 6 September 1990/Accepted 18 December 1990

The oncogene product of the avian sarcoma virus CT10, P47$^{gag\text{-}crk}$, contains the SH2, SH2′, and SH3 domains and binds proteins in a phosphotyrosine (ptyr)-dependent manner. In this study, we have determined the region of P47$^{gag\text{-}crk}$ essential for binding to ptyr-containing proteins. Mutant P47$^{gag\text{-}crk}$ proteins expressed in *Escherichia coli* that have the intact SH2 and SH2′ regions retained the capacity to bind ptyr-containing proteins obtained from cells transformed by *crk* and *src*. The deletion of SH2 resulted in the loss of binding activity. Other mutants that have altered SH2 or SH2′ bound few, if any, of the ptyr-containing proteins. Those mutants that bound ptyr-containing proteins associated with tyrosine kinase activity. We also found that polypeptides containing SH2, SH2′, and SH3 of p60$^{v\text{-}src}$ and p60$^{c\text{-}src}$ associated with ptyr-containing proteins from *crk*-transformed cells. Thus, the SH2 and SH2′ domains of P47$^{gag\text{-}crk}$ are responsible for their binding to ptyr-containing proteins.

Many proteins involved in signal transduction have been shown to contain three conserved domains, designated the SH3, SH2, and SH2′ domains (SH represents *src* homology) or the A, B, and C boxes, respectively (34, 40, 49). These domains, which we will refer to simply as SH2/3, were first found among non-receptor-type tyrosine kinases (48), and they were subsequently identified in the *crk* oncogene product (34), phosphatidylinositol-specific phospholipase C (PLC) (12, 49, 50), *ras* GTPase activator protein (GAP) (52, 54), and Nck protein, the function of which is not known (27). The SH3 domain alone has been found also in several proteins, some of which are associated with the membrane cytoskeleton (11, 27, 28, 30, 45, 51, 53, 55, 57).

The SH2/3 domains are regulatory rather than catalytic domains. The SH2/3 domains are dispensable for the catalytic activity of PLC (12), GAP (31), and non-receptor-type tyrosine kinases (3, 47). Mutations within the SH2 domain of p60$^{v\text{-}src}$ and P130$^{gag\text{-}fps}$ inhibit their transforming activity and produce transformation-defective, temperature-sensitive, or host-dependent mutants (4, 7, 9, 10, 43, 48, 56). However, recent studies have shown that some mutations in the SH2 domain may activate the transforming potential of p60$^{c\text{-}src}$, which is otherwise nontransforming (17, 39). Mutations in the SH3 domain have been known to activate the catalytic and transforming activity of p60$^{c\text{-}src}$ and p133$^{c\text{-}abl}$ (13, 14, 19, 20, 42). Morphological changes induced by p60$^{v\text{-}src}$ are also modulated by the SH2/3 domains (1, 2, 23).

The oncogene product of the CT10 virus, P47$^{gag\text{-}crk}$, is a fusion protein of a retroviral *gag* protein and c-*crk*, which consists almost entirely of the SH2/3 domains (43a). Cells transformed by P47$^{gag\text{-}crk}$ have an elevated level of phosphotyrosine (ptyr) by a mechanism that is yet to be clarified (34). We have shown previously that P47$^{gag\text{-}crk}$ immunoprecipitated by anti-*gag* or anti-*crk* antisera is associated with nearly all of the cellular proteins phosphorylated on tyrosine residues in CT10-transformed cells (35, 37). This association was found to be due to a unique capacity of P47$^{gag\text{-}crk}$ to bind ptyr-containing proteins (33). To determine the region of P47$^{gag\text{-}crk}$ essential for the association with ptyr-containing proteins, we have studied the binding of various *crk* mutants expressed in *Escherichia coli*. We also have shown that the amino-terminal region containing the SH2/3 domains of p60$^{v\text{-}src}$ and p60$^{c\text{-}src}$ also bind ptyr-containing proteins.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (CEF) were prepared, maintained, and infected as described previously (16). Preparation of avian sarcoma virus CT10, which encodes P47$^{gag\text{-}crk}$, and helper virus UR2AV (38) from molecularly cloned DNA was described elsewhere (36). Crk-3Y1 (30a) and SR-3Y1 (21) cells are rat 3Y1 cells (22) transformed by v-*crk* and v-*src*, respectively.

Plasmid constructions. A plasmid carrying the genomic DNA of CT10, pCT10, and its derivatives were described elsewhere (33a, 36). Briefly, p10-ΔSH2 lacks the SH2 and SH2′ domains (amino acids 208 to 338); p10-ΔSH3 lacks the carboxyl terminus of SH2′ and the SH3 domain (amino acid 340 to end); pOCC, pCOC, and pCCO lack the SH2 (amino acids 235 to 316), SH2′ (317 to 365) and SH3 (366 to 426) domains, respectively; p10-BSP has a 12-base insertion in the SH3 domain, which changes D386 to DRHAD in amino acid sequence; p10-MH has a 15-base insertion between the SH2′ and SH3 domains (L365 to LTQAWV); p10-ESP has a 12-base insertion in the SH2′ domain (E338 to ELACQ); p10-SMH has a 12-base insertion between the SH2 and SH3 domains (P316 to PPSLG); p10-R294 and p10-N273 both have 2-base substitutions in the SH2 domain which change His-294 to Arg and Arg-273 to Asn, respectively.

Expression vectors were constructed as follows; their structures are summarized in Fig. 1. The 160-bp *Sau*3AI fragment of pCT10 was inserted into a *Bam*HI site of the *E. coli* expression vector pET3a (46) to generate pET-SAB. This 160-bp fragment starts from 8 bases upstream of the translation initiation codon of P47$^{gag\text{-}crk}$ and contains the epitope domain recognized by monoclonal antibody (MAb) 3C2 (41). The *Bam*HI fragments of pCT10 and its derivatives, which extend from *gag* to the end of the *crk* gene, were subcloned into the *Bam*HI site of pET-SAB downstream of

* Corresponding author.
† Present address: Department of Pathology, National Institute of Health, Tokyo, Japan.

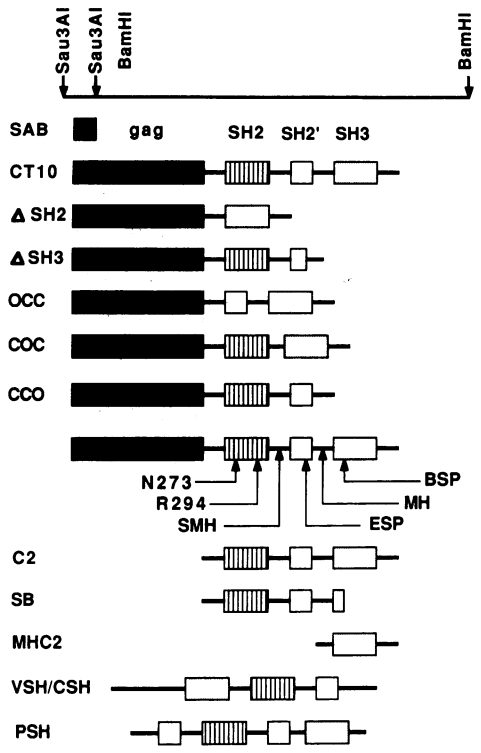

FIG. 1. The SH2/3 domains of *crk*, *src*, and PLC-γ expressed from pET3-derived vectors. pET-SAB (SAB) carries the amino-terminal region of $P47^{gag\text{-}crk}$ including an epitope for MAb 3C2 (anti-$p19^{gag}$). pET-CT10 (CT10) contains the entire SH2/3 domains of $P47^{gag\text{-}crk}$. pET-ΔSH2 (ΔSH2) lacks the SH2 and SH2′ domains. pET-ΔSH3 (ΔSH3) lacks SH3 and the carboxyl terminus of SH2′. pET-OCC, pET-COC, and pET-CCO lack SH2, SH2′, and SH3 domains, respectively. pET-BSP (BSP), pET-MH (MH), pET-ESP (ESP), and pET-SMH (SMH) have four- or five-amino-acid insertions within SH3, between SH2′ and SH3, within SH2′, and between SH2 and SH2′, respectively. pET-R294 (R294) and pET-N273 (N273) have one amino acid substitution in the SH2 domain. pET-C2 (C2) lacks the *gag* domain of $P47^{gag\text{-}crk}$. pET-SB (SB) contains only SH2, SH2′, and a small part of SH3. pET-MHC2 (MHC2) has only the SH3 domain. pET-VSH and pET-CSH (VSH/CSH) carry the amino-terminal halves of $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$, respectively. pET-PSH has the carboxyl-terminal one of the two SH2//SH2′ domains and one SH3 domain of PLC-γ.

the *gag* domain. The resulting plasmids were designated as pET-CT10, pET-ΔSH2, pET-ΔSH3, pET-OCC, pET-COC, pET-CCO, pET-BSP, pET-MH, pET-ESP, pET-SMH, pET-R294, and pET-N273. pET-C2, which is pET3a carrying the entire *crk* SH2/SH3 domains, was described previously (37). The 456-bp *Bam*HI-*Bgl*II fragment of p10-SHB (36), which contains the SH2 and SH2′ domains and a small part of the SH3 domain (M236 to D386) of *crk*, was inserted into the *Bam*HI site of pET3a to generate pET-SB. pET-MHC2 is derived from pET3a and contains the 296-bp *Mst*II-*Hae*II fragment of pCT10, which spans the SH3 domain of *crk*.

SH2/3 domains of v-*src*, c-*src*, and PLC-γ were also subcloned into the pET3 expression vectors. pSR-XD2 (8) and pHB5 (18), which carry the v-*src* and c-*src* genes, respectively, were cleaved with *Nco*I and *Mlu*I and blunt ended by DNA polymerase. The 774-bp fragments, which start from the initiation codon of the *src* gene and contain the entire SH2/3 domains, were inserted into pET3a by using a *Bam*HI 10-mer linker to generate pET-VSH (v-*src*) and pET-CSH (c-*src*). A DNA fragment containing the carboxyl-terminal one of two SH2/SH2′ domains and one SH3 domain of PLC-γ was first subcloned into pCT10 to generate pgag-PLC (37a) by substituting the SH2/3 domains of *crk* with the 744-bp *Bam*HI-*Ava*I fragment of the PLC-γ cDNA, which was a gift from J. L. Knopf (49). The 1.9-kb *Bam*HI fragment of pgag-PLC, which includes the SH2/3 domains of PLC-γ and noncoding sequences from CT10, was inserted into the *Bam*HI site of pET3c to generate pET-PSH.

Expression in *E. coli* and coupling of mutant $P47^{gag\text{-}crk}$ proteins to protein A-Sepharose. A T7 RNA polymerase expression system was used to produce *crk* peptides. *E. coli* BL21(DE3)pLysS bearing pET3-derived expression vectors was incubated with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h (46). Cells were lysed in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–1 mM EDTA–0.1% Triton X-100–1 mM phenylmethylsulfonyl fluoride (PMSF)–100 kallikrein inactivator units (KIU) of Trasylol per ml by incubation at 4°C for 1 h after freeze-thawing. Lysates were adjusted to 10 mM $MgCl_2$–50 μg of DNase I per ml–20 μg of RNase A per ml and were further incubated on ice for 4 h to digest nucleic acids. Peptides expressed from pET-SAB-derived plasmids, pET-SB, and pET-PSH were collected by centrifugation at 10,000 × *g* for 30 min, solubilized by boiling in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–2% sodium dodecyl sulfate (SDS), and reconstituted to RIPA buffer conditions (10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol). For the preparation of peptides expressed from pET-C2, pET-MHC2, pET-VSH, and pET-CSH, lysates were adjusted to 15 mM EDTA and clarified by centrifugation for 30 min at 10,000 rpm in an SS34 rotor, and the supernatants were reconstituted to RIPA.

MAb 3C2, recognizing $p19^{gag}$, was provided by D. Boettiger (University of Pennsylvania, Philadelphia) (41). The antibody was produced in BALB/c mice, purified by using DEAE Affi-Gel Blue (Bio-Rad), and bound covalently to protein A-Sepharose (Pharmacia) with dimethyl pimelimidate as described elsewhere (32).

The *crk*-coupled beads (*crk*-beads) were made as follows. The MAb 3C2-coupled beads were incubated in antigen excess with $P47^{gag\text{-}crk}$ peptides expressed in *E. coli*, washed sequentially with RIPA containing 300 and 10 mM NaCl, and cross-linked by dimethyl pimelimidate. Before the cross-linking, proteins bound to the beads were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. $P47^{gag\text{-}crk}$ and its derivatives were the only bands detected except immunoglobulin, and their concentrations were approximately 0.1 μg/μl of beads.

In vitro association of $P47^{gag\text{-}crk}$ with ptyr-containing proteins. Isotopic labeling, cell lysis, and immunoprecipitation have been described previously (34). Crk-3Y1 cells were labeled with 2.5 mCi of $^{32}P_i$ for 4 h in 2 ml of medium per 10-cm plate and lysed in 1 ml of RIPA buffer containing 0.5 mM Na_3VO_4, 0.05 mM Na_2MoO_3, 10 mM NaF, 1 mM PMSF, and 100 KIU of Trasylol per ml. Five microliters of *crk*-beads (see above) was preincubated with 5 μg of peptide expressed from pET-SAB to block unoccupied MAb 3C2. The binding of endogenous $P47^{gag\text{-}crk}$ from cell lysates to the beads was estimated to be less than 10% of total $P47^{gag\text{-}crk}$ under the following conditions. The beads were incubated for 2 h with 50 μl of ^{32}P-labeled cell lysates and washed sequentially with RIPA containing 300 and 10 mM NaCl. Proteins were dissociated from the beads by boiling in

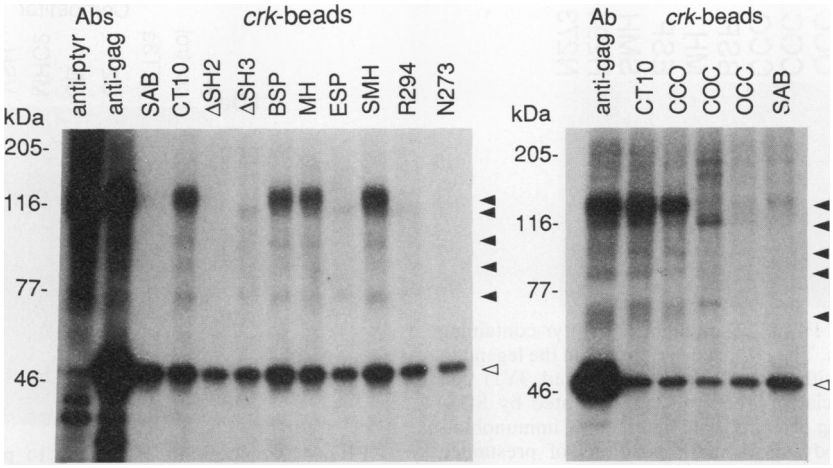

FIG. 2. Association of P47$^{gag\text{-}crk}$ with ptyr-containing proteins from Crk-3Y1 cells. P47$^{gag\text{-}crk}$ and its mutants expressed from pET3-derived vectors (Fig. 1) were covalently bound to protein A-Sepharose via MAb 3C2 (anti-p19^{gag}). These *crk*-beads were preincubated with peptide expressed from pET-SAB for 30 min and then incubated with Crk-3Y1 cell lysates labeled in vivo with $^{32}P_i$. Proteins associated with the immune complexes were analyzed by SDS-PAGE. To show the ptyr-containing proteins which bound in vivo to P47$^{gag\text{-}crk}$ expressed in Crk-3Y1 cells, the same cell lysates were precipitated with either anti-ptyr antibody (anti-ptyr) or MAb 3C2 (anti-*gag*). Symbols: ◀, major ptyr-containing proteins (135, 120, 94, 87, and 65 to 75 kDa) in Crk-3Y1 cells; ◁, P47$^{gag\text{-}crk}$.

Laemmli's sample buffer and analyzed by SDS-PAGE. Gels were treated with 1 N KOH at 55°C for 2 h before exposure.

A similar experiment was performed by using SR-3Y1 cells (*src*-transformed 3Y1 cells) except that nonlabeled cell lysates were used and associated proteins were detected by immunoblotting using anti-ptyr antibody (15).

Competition of P47$^{gag\text{-}crk}$ binding to ptyr-containing proteins by SH2/3 peptides. Peptides expressed from pET-C2, pET-MHC2, pET-SB, pET-VSH, and pET-PSH were extracted as described above. Lysates (50 μg) from Crk-3Y1 cells were preincubated for 30 min at 4°C with 50 μl of RIPA containing 5 to 20 μg of bacterially expressed peptides. P47$^{gag\text{-}crk}$ was precipitated with MAb 3C2, and associated proteins were analyzed by SDS-PAGE, followed by immunoblotting using anti-ptyr antibody.

In vitro association of P47$^{gag\text{-}crk}$ with tyrosine kinases. Crk-3Y1 cells (10^7) were lysed in 1 ml of Triton X-100 buffer (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM Na_3VO_4, 1 mM PMSF, 100 KIU of Trasylol per ml) for 30 min and clarified by centrifugation. The lysates (100 μl) were incubated for 2 h with 5 or 10 μl of *crk*-beads preincubated for 30 min at 4°C with 5 μg of peptide expressed from pET-SAB. After washing with Triton X-100 buffer containing 300 mM NaCl, the immune complexes were incubated in kinase buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM $MnCl_2$, 50 μM Na_3VO_4, 0.1% Triton X-100, 50 μM [γ-^{32}P]ATP [3 Ci/mmol]) containing 0.2 mg of poly(Glu,Tyr) (Sigma) per ml for 10 min at 30°C. Samples were combined with 2× Laemmli's sample buffer and analyzed by 12% SDS-PAGE. Gels were treated with 1 N KOH at 55°C for 1 h. Because poly(Glu,Tyr) is a random copolymer of 20 to 50 kDa, the areas of gels corresponding to 24 to 48 kDa were excised, and incorporation of ^{32}P into poly(Glu,Tyr) was quantitated by Cerenkov count. Radioactivity measured on MAb 3C2-coupled beads preincubated with peptide expressed from pET-SAB were subtracted from all counts as background, and the activity is presented as percentage of the counts obtained with authentic P47$^{gag\text{-}crk}$ precipitated by MAb 3C2.

In vitro association between ptyr-containing proteins and the SH2/3 domains of p60$^{v\text{-}src}$ and p60$^{c\text{-}src}$. An excess of peptides expressed from pET-VSH and pET-CSH was incubated with anti-*src* MAb 327 (29) covalently bound to protein A-Sepharose (gift from Y. Fukui). Five microliters of beads, which contains approximately 0.5 μg of *src* peptides, was incubated with 100 μg of lysate from Crk-3Y1 cells, and proteins bound to these beads were analyzed by SDS-PAGE, followed by immunoblotting using anti-ptyr antibody.

RESULTS

Association of P47$^{gag\text{-}crk}$ mutants with ptyr-containing proteins from Crk-3Y1 cells. The wild-type and mutant forms of P47$^{gag\text{-}crk}$ were expressed in *E. coli* (Fig. 1) and were covalently bound to protein A-Sepharose by means of anti-p19^{gag} MAb 3C2. These *crk*-beads were incubated with lysates of ^{32}P-labeled Crk-3Y1 cells, which were derived from a rat 3Y1 cell line transformed by P47$^{gag\text{-}crk}$. The association of ptyr-containing proteins with these *crk*-beads was determined by SDS-PAGE (Fig. 2). P47$^{gag\text{-}crk}$ mutants expressed from pET-BSP, pET-MH, and pET-SMH have insertions of five or six amino acids within SH3, between SH2′ and SH3, and between SH2 and SH2′, respectively. A mutant-expressed pET-CCO lacks the SH3 domain. These mutant proteins were similar to the wild-type P47$^{gag\text{-}crk}$, expressed either in rat cells or in *E. coli* from pET-CT10, in their association with four of five major ptyr-containing proteins: 135-, 94-, 87-, and 65- to 75-kDa proteins but not a 120-kDa protein. Mutant proteins expressed from pET-SAB (p19^{gag} portion of P47$^{gag\text{-}crk}$), pET-ΔSH2 (P47$^{gag\text{-}crk}$ without SH2 and SH2′), and pET-OCC (without SH2) did not associate with any of the ptyr-containing proteins. Other mutant proteins expressed from pET-ΔSH3 (P47$^{gag\text{-}crk}$ without SH3 and the carboxyl terminus of SH2′), pET-COC (without SH2′), pET-ESP (six-amino-acid insertion within SH2′), pET-R294 (His-294 substituted with Arg), and pET-N273 (Arg-273 with Asn) have small mutations within the SH2 or SH2′ domain. The association of these mutant

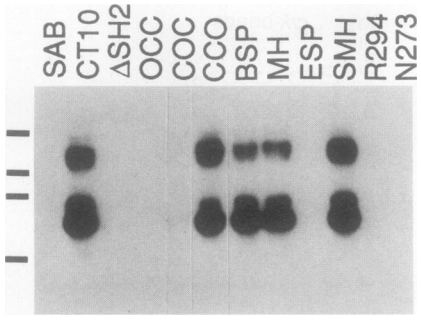

FIG. 3. Association of P47$^{gag\text{-}crk}$ mutants with ptyr-containing proteins from SR-3Y1 cells. The *crk*-beads described in the legend to Fig. 1 were incubated with SR-3Y1 (*src*-transformed 3Y1) cell lysates for 2 h, and associated proteins were separated by SDS-PAGE. The ptyr-containing proteins were detected by immunoblotting with anti-ptyr antibody. Bars mark positions of prestained molecular size markers: 205, 116, 77, and 46 kDa.

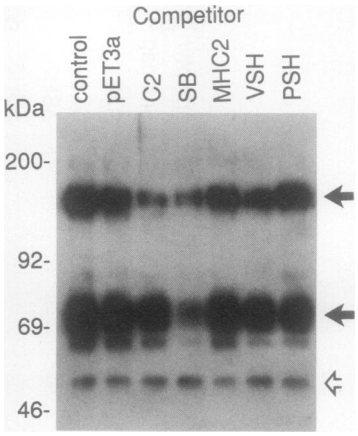

FIG. 4. Competition of binding to ptyr-containing proteins by polypeptides containing the SH2/3 domains. Crk-3Y1 cells were lysed and incubated with polypeptides expressed from pET3-derived expression vectors (Fig. 1). Lanes: pET3a, plasmid without insert; C2, pET-C2; SB, pET-SB; MHC2, pET-MHC2; VSH, pET-VSH; PSH, pET-PSH. P47$^{gag\text{-}crk}$ was precipitated with MAb 3C2 (anti-p19^{gag}), and associated proteins were analyzed by SDS-PAGE, followed by immunoblotting with anti-ptyr antibody. MAb 3C2 recognizes P47$^{gag\text{-}crk}$ from Crk-3Y1 cell lysates but does not bind the competitor peptides.

proteins with ptyr-containing proteins was weaker than that of the wild-type P47$^{gag\text{-}crk}$. Although small amounts of ^{32}P-labeled P47$^{gag\text{-}crk}$ still bound to these *crk*-beads as a result of unoccupied MAb 3C2 present on the beads, we could not detect any ptyr-containing proteins bound to the beads via the unoccupied MAb 3C2 (Fig. 2, lane SAB). These results indicate that the binding to ptyr-containing proteins requires the intact SH2 and SH2′ domains.

Association of P47$^{gag\text{-}crk}$ mutants with ptyr-containing proteins from SR-3Y1 cells. We examined whether P47$^{gag\text{-}crk}$ mutants could bind proteins phosphorylated on tyrosine by another oncogene product, p60$^{v\text{-}src}$ (Fig. 3). The ptyr-containing proteins (primarily 130, 78, and 65 kDa) were associated with the wild-type P47$^{gag\text{-}crk}$ expressed from pET-CT10, with mutant P47$^{gag\text{-}crk}$ proteins expressed from pET-CCO and pET-SMH, and slightly less with those expressed from pET-BSP and pET-MH. The ability of mutant P47$^{gag\text{-}crk}$ proteins to bind ptyr-containing proteins from *crk*-transformed cells correlated with their capacity to bind ptyr-containing proteins from *src*-transformed cells.

Competition in the association of P47$^{gag\text{-}crk}$ with ptyr-containing proteins by polypeptides containing the SH2/3 domains. To study directly the role of various regions of P47$^{gag\text{-}crk}$ in the binding to ptyr-containing proteins, the SH2/3 domains of P47$^{gag\text{-}crk}$, p60$^{v\text{-}src}$, and PLC-γ were expressed from pET3-derived vectors and were used as competitors (Fig. 4). Wild-type P47$^{gag\text{-}crk}$ was precipitated by an MAb against p19^{gag} (3C2) from untreated Crk-3Y1 cell lysates or from lysates preincubated with the same amount of various peptides. Two polypeptides of P47$^{gag\text{-}crk}$ expressed from pET-C2, carrying the entire SH2/3 domains, or pET-SB, spanning SH2, SH2′, and small part of SH3 (Fig. 1), substantially inhibited the association of wild-type P47$^{gag\text{-}crk}$ with the 120-kDa ptyr-containing protein. However, only peptides expressed from pET-SB inhibited the association with the 65- to 75-kDa ptyr-containing protein, indicating that the SH3 region may have some inhibitory effect on the binding to ptyr-containing proteins in the context of the *gag*-less *crk* protein. No substantial competition was observed when cell lysates were preincubated with the same amount of polypeptides expressed from the pET3a vector, pET-MHC2 (the SH3 domain of P47$^{gag\text{-}crk}$), pET-VSH (the amino-terminal half of p60$^{v\text{-}src}$ containing the SH2/3 domains), and pET-PSH (the carboxyl-terminal SH2/SH2′ domains and one SH3 domain of PLC-γ).

Association of tyrosine kinase activity with P47$^{gag\text{-}crk}$ mutants. Using poly(Glu,Tyr) as a substrate, we determined whether tyrosine kinase activity is associated with the proteins bound to the beads carrying the wild-type and mutant forms of P47$^{gag\text{-}crk}$. The results (Table 1) are presented as a percentage of enzyme activity associated with P47$^{gag\text{-}crk}$ expressed in Crk-3Y1 rat cells. Significant tyrosine kinase activity was associated with three mutant P47$^{gag\text{-}crk}$ proteins expressed from pET-BSP, pET-MH, and pET-SMH as well as the wild-type P47$^{gag\text{-}crk}$ expressed from pET-CT10. Less tyrosine kinase activity was measured in the immunoprecipitates of P47$^{gag\text{-}crk}$ expressed from pET-ESP. As in the case of association with ptyr-containing proteins, association with kinase activity was correlated with the intact SH2 and SH2′ domains.

Association of the amino-terminal half of p60$^{v\text{-}src}$ with ptyr-containing proteins. To determine whether the association between the SH2/3 domains and ptyr-containing proteins is a general phenomenon or is specific to P47$^{gag\text{-}crk}$, the

TABLE 1. Association of tyrosine kinase activity with P47$^{gag\text{-}crk}$ mutants

Mutant	Enzyme activity[a] (%)	
	5 μl[b]	10 μl
pET-CT10	16	31
pET-ΔSH2	0	2
pET-ΔSH3	0	0
pET-BSP	11	28
pET-MH	9	41
pET-ESP	1	14
pET-SMH	11	53
pET-R294	3	0
pET-N273	0	0

[a] Percentage of the tyrosine kinase activity associated with authentic P47$^{gag\text{-}crk}$ expressed in rat cells.

[b] Quantity of *crk*-beads incubated with cell lysates.

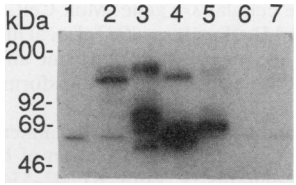

FIG. 5. Association of the amino-terminal halves of $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ with ptyr-containing proteins. Cell lysates from Crk-3Y1 (lanes 1 to 3), SR-3Y1 (lanes 4 and 5), and 3Y1 (lanes 6 and 7) cells were incubated with MAb 327 (lanes 1, 3, and 6), v-*src* peptide MAb 327 (lanes 2 and 7), and $P47^{gag\text{-}crk}$ MAb 3C2 (lane 3 and 5). Proteins associated with these immunoprecipitates were analyzed by SDS-PAGE, followed by immunoblotting using anti-ptyr antibody.

binding of ptyr-containing proteins to polypeptides containing the SH2/3 domains of $p60^{v\text{-}src}$ was examined. The polypeptides expressed from pET-VSH were incubated with Crk-3Y1 cell lysates. Two of the five major ptyr-containing proteins, 135- and 120-kDa proteins, were associated with the polypeptides (Fig. 5, lane 2). In contrast to the experiment using $P47^{gag\text{-}crk}$ polypeptides, the 120-kDa protein was the major protein associated with the *src* polypeptides. This 120-kDa protein was different in size from the ptyr-containing protein coimmunoprecipitated with $p60^{v\text{-}src}$ from SR-3Y1 cells (Fig. 5, lane 4) (26, 44). Similar results were obtained by using polypeptides containing SH2/3 domains of $p60^{c\text{-}src}$ and ^{32}P-labeled cell lysates (data not shown). We performed similar experiments using CEF transformed by CT10 and found that the major ptyr-containing proteins of 130 to 150 and 110 kDa associated with the *src* polypeptides (data not shown). When polypeptides containing the carboxyl-terminal SH2/SH2′ domains and one SH3 domain of PLC-γ expressed from pET-PSH were used, no significant association with ptyr-containing proteins was found (data not shown).

DISCUSSION

The data presented here demonstrate that the SH2 and SH2′ domains are necessary and sufficient to recognize ptyr-containing proteins (Table 2). Mutations in either SH2 (N273 and R294) or SH2′ (ESP and ΔSH3) significantly decreased the binding of $P47^{gag\text{-}crk}$ to ptyr-containing proteins. Compared with CCO, the ΔSH3 mutant lacks 26 amino acids, 3 of which (Y340, P353, and V354) are well conserved among the SH2/SH2′-containing proteins. On the other hand, an insertional mutation in the sequence between the SH2 and SH2′ domains (SMH) did not affect the association. Consistent with this finding, the distance between SH2 and SH2′ (from His-294 to Phe-330 of $P47^{gag\text{-}crk}$) varies from 35 amino acids ($P47^{gag\text{-}crk}$) (34) to 15 amino acids (GAP) (54) in various SH2/3-containing molecules, suggesting that this region comprises a spacer with variable length. In contrast to the SH2/SH2′ regions, the SH3 domain was dispensable for the association with ptyr-containing proteins (CCO, BSP, and SB). However, SH3 has a positive effect on transformation, because CCO does not transform CEF and BSP does only weakly (Table 2). $P47^{gag\text{-}crk}$ may bind to the membrane cytoskeleton through the SH3 domain, as has been suggested for other SH3-containing proteins (11).

The association with ptyr-containing proteins and tyrosine kinase activity appears to be an essential function of $P47^{gag\text{-}crk}$, because the association correlates well with transforming activity (Table 2). Previously, we showed that mutations in the SH2 and SH2′ domains of $P47^{gag\text{-}crk}$ abolished the transforming activity of CT10 in parallel with a relative decrease in the ptyr content of CEF infected with these mutant viruses (37). However, it was not clear whether $P47^{gag\text{-}crk}$ carried by these transformation-defective CT10 viruses could bind ptyr-containing proteins, because cells infected by these mutant viruses contained little detectable ptyr.

TABLE 2. Correlation of transforming activity with binding to tyrosine kinase activity and ptyr-containing proteins

Virus or protein	Transforming activity[a]	Association with:	
		ptyr-containing protein[b]	Kinase activity[c]
CT10	++	++	++
ΔSH2	−	−	−
ΔSH3	−	−	−
OCC	−	−	ND[d]
COC	−	−	ND
CCO	−	++	ND
BSP	+	++	++
MH	++	++	++
ESP	−	+/−	+/−
SMH	++	++	++
R294	−	+/−	−
N273	−	+/−	−
C2	ND	+	ND
SB	ND	+	ND
MHC2	ND	−	ND

[a] From reference 33a and 36.
[b] Assayed by using ptyr-containing proteins from Crk-3Y1 and SR-3Y1 cells.
[c] Assayed by using poly(Glu,Tyr) as a substrate.
[d] ND, Not determined.

The binding of SH2 and SH2′ domains to ptyr-containing proteins was also demonstrated by using the amino-terminal domains of $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$. These domains of $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ bind 135- and 120-kDa phosphoproteins from *crk*-transformed rat cells, whereas $P47^{gag\text{-}crk}$ binds the 135-kDa and three other ptyr-containing proteins but not the 120-kDa phosphoprotein. This difference suggests that each of the SH2 and SH2′ domains binds to a different spectrum of ptyr-containing proteins. This might explain why the carboxyl-terminal SH2/3 domains of PLC-γ did not bind ptyr-containing proteins from CT10-transformed cells. It has been shown that $p60^{v\text{-}src}$ associates with a 120-kDa ptyr-containing protein in *src*-transformed vole cells (25, 26) and 110- and 130-kDa ptyr-containing proteins in *src*-transformed CEF (44) and that mutations within the SH2 domain abolish the association (44, 58).

The SH2 and SH2′ domains associate with tyrosine kinase activity as well as ptyr-containing proteins (Table 1). This is not surprising, because all known tyrosine kinases have autophosphorylation sites, which may mediate the association. We have shown elsewhere that $P47^{gag\text{-}crk}$ binds to $p60^{v\text{-}src}$, $P90^{v\text{-}yes}$, and $gp68^{erb\text{-}B}$ in vitro and that the association with $p60^{v\text{-}src}$ is enhanced by autophosphorylation of $p60^{v\text{-}src}$ and is inhibited by its dephosphorylation (33).

How do SH2 and SH2′ domains recognize ptyr-containing proteins? We have shown that the ptyr monomer cannot compete with $P47^{gag\text{-}crk}$ in binding to ptyr-containing proteins, which suggests that the SH2 and SH2′ domains recognize some primary amino acid sequences together with ptyr residues (33). It is possible that the SH2 and SH2′ domains recognize polypeptide structures which are disclosed by conformational changes induced by tyrosine phosphorylation. $P47^{gag\text{-}crk}$ can bind ptyr-containing proteins that

have been denatured by boiling in 1% SDS (data not shown). This finding suggests that the SH2 and SH2′ domains recognize primary amino acid sequences including ptyr rather than some tertiary structures.

Although we have shown that binding to ptyr-containing proteins appears to be an essential function of P47$^{gag\text{-}crk}$, it is still unclear how the ptyr content is elevated in *crk*-transformed cells. Binding of P47$^{gag\text{-}crk}$ may activate tyrosine kinases as polyomavirus middle T antigen does (6, 24). The binding of middle T antigen to p60$^{c\text{-}src}$ induces dephosphorylation of the C-terminal tyrosine residue and tyrosine phosphorylation within the catalytic domain (5). It would be interesting to examine the specific activity and phosphorylation status of the tyrosine kinases associated with P47$^{gag\text{-}crk}$. Alternatively, P47$^{gag\text{-}crk}$ may compete with tyrosine phosphatases and thus cause accumulation of ptyr-containing proteins.

ACKNOWLEDGMENTS

We thank J. L. Knopf for PLC148 cDNA, J. S. Brugge for MAb 327, D. Boettiger for MAb 3C2, Y. Fukui and C. P. Marshall for technical assistance, and S. Kornbluth and D. W. Sternberg for critical reading of the manuscript.

This work was supported by grant CA44356 from the National Cancer Institute and grant 2517 from the Council for Tobacco Research. B.J.M. was supported by training grant AI 07233 from the National Institutes of Health.

ADDENDUM IN PROOF

Recently, the association between activated growth factor receptors and the SH2 domains of PCL-γ, GAP, *crk*, and *src* has been described by Anderson et al. (1a) and Moran et al. (37b). Consistent with our observation, these authors have found that the carboxyl-terminal SH2 domain of PLC-γ binds only weakly to ptyr-containing proteins; however, they have demonstrated that the carboxyl-terminal SH2 domain of PLC-γ augments binding of the amino-terminal SH2 domain (1a).

REFERENCES

1. **Anderson, D. D., R. P. Beckmann, E. H. Harms, K. Nakamura, and M. J. Weber.** 1981. Biological properties of "partial" transformation mutants of Rous sarcoma virus and characterization of their pp60^{src} kinase. J. Virol. **37**:445–458.
1a. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson.** 1990. Binding of SH2 domains of phospholipase Cγ1, GAP, and Src to activated growth factor receptors. Science **250**:979–982.
2. **Anderson, S. K., and D. J. Fujita.** 1987. Morph^{f} mutants of Rous sarcoma virus: nucleotide sequencing analysis suggests that a class of morph^{f} mutants was generated through splicing of a cryptic intron. J. Virol. **61**:1893–1900.
3. **Brugge, J. S., and D. Darrow.** 1984. Analysis of the catalytic domain of the phosphotransferase activity of two avian sarcoma virus transforming proteins. J. Biol. Chem. **259**:4550–4557.
4. **Bryant, D., and J. T. Parsons.** 1982. Site-directed mutagenesis of the *src* gene of Rous sarcoma virus: construction and characterization of a deletion mutant temperature sensitive for transformation. J. Virol. **44**:683–691.
5. **Courtneidge, S. A.** 1985. Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. EMBO J. **4**:1471–1477.
6. **Courtneidge, S. A., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the c-src gene. Nature (London) **303**:435–439.
7. **Cross, F. R., E. A. Garber, and H. Hanafusa.** 1985. N-terminal deletions in Rous sarcoma virus p60^{src}: effects on tyrosine kinase and biological activities and on recombination in tissue culture with the cellular *src* gene. Mol. Cell. Biol. **5**:2789–2795.
8. **Cross, F. R., and H. Hanafusa.** 1983. Local mutagenesis of Rous sarcoma virus: the major sites of tyrosine and serine phosphorylation of p60^{src} are dispensable for transformation. Cell **34**:597–607.
9. **DeClue, J. E., and G. S. Martin.** 1989. Linker insertion-deletion mutagenesis of the v-*src* gene: isolation of host- and temperature-dependent mutants. J. Virol. **63**:542–554.
10. **DeClue, J. E., I. Sadowski, G. S. Martin, and T. Pawson.** 1987. A conserved domain regulates interactions of the v-fps protein-tyrosine kinase with the host cell. Proc. Natl. Acad. Sci. USA **84**:9064–9068.
11. **Drubin, D. G., J. Mulholland, Z. Zhu, and D. Botstein.** 1990. Homology of a yeast actin-binding protein to signal transduction proteins and myosin-I. Nature (London) **343**:288–290.
12. **Emori, Y., Y. Homma, H. Sorimachi, H. Kawasaki, O. Nakanishi, K. Suzuki, and T. Takenawa.** 1989. A second type of rat phosphoinositide-specific phospholipase C containing a *src*-related sequence not essential for phosphoinositide-hydrolyzing activity. J. Biol. Chem. **264**:21885–21890.
13. **Espino, P. C., R. Harvey, R. L. Schweickgardt, G. A. White, A. E. Smith, and S. H. Cheng.** 1990. The amino-terminal region of pp60$^{c\text{-}src}$ has a modulatory role and contains multiple sites of tyrosine phosphorylation. Oncogene **5**:283–293.
14. **Franz, W. M., P. Berger, and J. Y. J. Wang.** 1989. Deletion of an N-terminal regulatory domain of the c-*abl* tyrosine kinase activates its oncogenic potential. EMBO J. **8**:137–147.
15. **Hamaguchi, M., C. Grandori, and H. Hanafusa.** 1988. Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis by use of anti-phosphotyrosine antibodies. Mol. Cell. Biol. **8**:3035–3042.
16. **Hanafusa, H.** 1969. Rapid transformation of cells by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA **63**:318–325.
17. **Hirai, H., and H. E. Varmus.** 1990. Site-directed mutagenesis of the SH2- and SH3-coding domains of c-*src* produces varied phenotypes, including oncogenic activation of p60$^{c\text{-}src}$. Mol. Cell. Biol. **10**:1307–1318.
18. **Iba, H., T. Takeya, F. R. Cross, T. Hanafusa, and H. Hanafusa.** 1984. Rous sarcoma virus variants that carry the cellular *src* gene instead of the viral *src* gene cannot transform chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **81**:4424–4428.
19. **Jackson, P., and D. Baltimore.** 1989. N-terminal mutations activate the leukemogenic potential of the myristoylated form of c-*abl*. EMBO J. **8**:449–456.
20. **Kato, J., T. Takeya, C. Grandori, H. Iba, J. B. Levy, and H. Hanafusa.** 1986. Amino acid substitutions sufficient to convert the nontransforming p60$^{c\text{-}src}$ protein to a transforming protein. Mol. Cell. Biol. **6**:4155–4160.
21. **Kawai, S.** 1980. Transformation of rat cells by fusion-infection with Rous sarcoma virus. J. Virol. **34**:772–776.
22. **Kimura, G., A. Itagaki, and J. Summers.** 1975. Rat cell line 3Y1 and its virogenic polyoma- and SV40-transformed derivatives. Int. J. Cancer **15**:694–706.
23. **Kitamura, N., and M. Yoshida.** 1983. Small deletion in src of Rous sarcoma virus modifying transformation phenotypes: identification of 207-nucleotide deletion and its smaller product with protein kinase activity. J. Virol. **46**:985–992.
24. **Kornbluth, S., M. Sudol, and H. Hanafusa.** 1987. Association of the polyomavirus middle-T antigen with c-*yes* protein. Nature (London) **325**:171–173.
25. **Lau, A. F.** 1986. Phosphotyrosine-containing 120,000-dalton protein co-immunoprecipitated with pp60$^{v\text{-}src}$ from Rous sarcoma virus-transformed mammalian cells. Virology **151**:86–99.
26. **Lau, A. F.** 1989. Evidence that a phosphotyrosine-containing 120,000 dalton protein from Rous sarcoma virus-infected cells is phosphorylated by pp60$^{v\text{-}src}$. Oncogene Res. **1**:185–194.
27. **Lehmann, J. M., G. Riethmuller, and J. P. Johnson.** 1990. Nck, a melanoma cDNA encoding a cytoplasmic protein consisting of the src homology units SH2 and SH3. Nucleic Acids Res. **18**:1048.
28. **Leto, T. L., K. J. Lomax, B. D. Volpp, H. Nunoi, J. M. G. Sechler, W. M. Nauseef, R. A. Clark, J. I. Gallin, and H. L. Malech.** 1990. Cloning of a 67-kD neutrophil oxidase factor with

similarity to a noncatalytic region of p60$^{c\text{-}src}$. Science **248**:727–730.

29. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48**:352–360.
30. **Lomax, K. J., T. L. Leto, H. Nunoi, J. I. Gallin, and H. L. Malech.** 1989. Recombinant 47-kilodalton cytosol factor restores NADPH oxidase in chronic granulomatous disease. Science **245**:409–412.

30a. **Marshall, C. P., and H. Hanafusa.** Unpublished data.

31. **Marshall, M. S., W. S. Hill, A. S. Ng, U. S. Vogel, M. D. Schaber, E. M. Scolnick, R. A. F. Dixon, I. S. Sigal, and J. B. Gibbs.** 1989. A C-terminal domain of GAP is sufficient to stimulate ras p21 GTPase activity. EMBO J. **8**:1105–1110.
32. **Matsuda, M., C. P. Marshall, and H. Hanafusa.** 1990. Purification of *crk* oncogene product, P47$^{gag\text{-}crk}$, from insect cells. J. Biol. Chem. **265**:12000–12004.
33. **Matsuda, M., B. J. Mayer, Y. Fukui, and H. Hanafusa.** 1990. Binding of oncoprotein, P47$^{gag\text{-}crk}$, to a broad range of phosphotyrosine-containing proteins. Science **248**:1537–1539.

33a. **Matsuda, M., et al.** Unpublished data.

34. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature (London) **332**:272–275.
35. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1989. Characterization of P47$^{gag\text{-}crk}$, a novel oncogene product with sequence similarity to a putative modulatory domain of protein-tyrosine kinases and phospholipase C. Cold Spring Harbor Symp. Quant. Biol. **53**:907–914.
36. **Mayer, B. J., and H. Hanafusa.** 1990. Mutagenic analysis of the v-*crk* oncogene: requirement for SH2 and SH3 domains, and correlation between increased cellular phosphotyrosine and transformation. J. Virol. **64**:3581–3589.
37. **Mayer, B. J., and H. Hanafusa.** 1990. Association of the v-*crk* oncogene product with phosphotyrosine-containing proteins and protein kinase activity. Proc. Natl. Acad. Sci. USA **87**: 2638–2642.

37a. **Mayer, B. J., and H. Hanafusa.** Unpublished data.

37b. **Moran, M. F., C. A. Koch, D. Anderson, C. Ellis, L. England, G. S. Martin, and T. Pawson.** 1990. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc. Natl. Acad. Sci. USA **87**:8622–8626.
38. **Neckameyer, W. S., and L.-H. Wang.** 1984. Molecular cloning and characterization of avian sarcoma virus UR2 and comparison of its transforming sequence with those of other avian sarcoma viruses. J. Virol. **50**:914–921.
39. **O'Brien, M. C., Y. Fukui, and H. Hanafusa.** 1990. Activation of proto-oncogene p60$^{c\text{-}src}$ by point mutations in the SH2 domain. Mol. Cell. Biol. **10**:2855–2862.
40. **Pawson, T.** 1988. Non-catalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene Res. **3**:491–495.
41. **Potts, W. M., M. Olsen, D. Boettiger, and V. M. Vogt.** 1987. Epitope mapping of monoclonal antibodies to *gag* protein p19 of avian sarcoma and leukemia viruses. J. Gen. Virol. **68**:3177–3182.
42. **Potts, W. M., A. B. Reynolds, T. J. Lansing, and J. T. Parsons.** 1988. Activation of pp60$^{c\text{-}src}$ transforming potential by mutations altering the structure of an amino terminal domain containing residues 90–95. Oncogene Res. **3**:343–355.
43. **Raymond, V. W., and J. T. Parsons.** 1987. Identification of an amino terminal domain required for the transforming activity of the Rous sarcoma virus *src* protein. Virology **160**:400–410.

43a. **Reichman, C. T., and H. Hanafusa.** Submitted for publication.

44. **Reynolds, A. B., S. B. Kanner, H.-C. R. Wang, and J. T. Parsons.** 1989. Stable association of activated pp60^{src} with two tyrosine phosphorylated cellular proteins. Mol. Cell. Biol. **9**:3951–3958.
45. **Rodaway, A. R. F., M. J. E. Sternberg, and D. L. Bentley.** 1989. Similarity in membrane proteins. Nature (London) **342**:624.
46. **Rosenberg, A. H., B. N. Lade, D.-S. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene **56**:125–135.
47. **Sadowski, I., and T. Pawson.** 1987. Catalytic and non-catalytic domains of the Fujinami sarcoma virus P130$^{gag\text{-}fps}$ protein-tyrosine kinase distinguished by the expression of v-*fps* polypeptides in Escherichia coli. Oncogene **1**:181–191.
48. **Sadowski, I., J. C. Stone, and T. Pawson.** 1986. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130$^{gag\text{-}fps}$. Mol. Cell. Biol. **6**:4396–4408.
49. **Stahl, M. L., C. R. Ferenz, K. L. Kelleher, R. W. Kriz, and J. L. Knopf.** 1988. Sequence similarity of phospholipase C with the non-catalytic region of *src*. Nature (London) **332**:269–272.
50. **Suh, P.-G., S. H. Ryu, K. H. Moon, H. W. Suh, and S. G. Rhee.** 1988. Inositol phospholipid-specific phospholipase C: complete cDNA and protein sequences and sequence homology to tyrosine kinase-related oncogene products. Proc. Natl. Acad. Sci. USA **85**:5419–5423.
51. **Tachibana, H., Y. Inoue, T. Kanehisa, and Y. Fukami.** 1988. Local similarity in the amino acid sequence between the noncatalytic region of Rous sarcoma virus oncogene product p60$^{v\text{-}src}$ and intermediate filament proteins. J. Biochem. **104**: 869–872.
52. **Trahey, M., G. Wong, R. Halenbeck, B. Rubinfeld, G. A. Martin, M. Landner, C. M. Long, W. J. Crosier, K. Watt, K. Koths, and F. McCormick.** 1988. Molecular cloning of two types of GAP complementary DNA from human placenta. Science **242**:1697–1700.
53. **Trueheart, J., J. D. Boeke, and G. R. Fink.** 1987. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. Mol. Cell. Biol. **7**:2316–2328.
54. **Vogel, U. S., R. A. F. Dixon, M. D. Schaber, R. E. Diehl, M. S. Marshall, E. M. Scolnick, I. S. Sigal, and J. B. Gibbs.** 1988. Cloning of bovine GAP and its interaction with oncogenic *ras* p21. Nature (London) **335**:90–93.
55. **Volpp, B. D., W. M. Nauseef, J. E. Donelson, D. R. Moser, and R. A. Clark.** 1989. Cloning of the cDNA and functional expression the 47-kilodalton cytosolic component of human neutrophil respiratory burst oxidase. Proc. Natl. Acad. Sci. USA **86**:7195–7199.
56. **Wang, H.-C. R., and J. T. Parsons.** 1989. Deletions and insertions within an amino-terminal domain of pp60$^{v\text{-}src}$ inactivate transformation and modulate membrane stability. J. Virol. **63**: 291–302.
57. **Wasenius, V.-M., M. Saraste, P. Salven, M. Eramaa, L. Holm, and V.-P. Lehto.** 1989. Primary structure of the brain α-spectrin. J. Cell Biol. **108**:79–93.
58. **Wendler, P. A., and F. Boschelli.** 1989. *Src* homology 2 domain deletion mutants of p60$^{v\text{-}src}$ do not phosphorylate cellular proteins of 120-150 kDa. Oncogene **4**:231–236.