MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7278–7287
0270-7306/93/127278-10$02.00/0

Vol. 13, No. 12

Binding of the Src SH2 Domain to Phosphopeptides Is Determined by Residues in both the SH2 Domain and the Phosphopeptides

KIRSTEN B. BIBBINS,[1,2] HELENE BOEUF,[1]† AND HAROLD E. VARMUS[1,2]*

Departments of Microbiology and Immunology[1] and Biochemistry and Biophysics,[2] University of California at San Francisco, San Francisco, California 94143

Received 26 May 1993/Returned for modification 12 July 1993/Accepted 26 August 1993

Src homology 2 (SH2) domains are found in a variety of signaling proteins and bind phosphotyrosine-containing peptide sequences. To explore the binding properties of the SH2 domain of the Src protein kinase, we used immobilized phosphopeptides to bind purified glutathione *S*-transferase–Src SH2 fusion proteins. With this assay, as well as a free-peptide competition assay, we have estimated the affinities of the Src SH2 domain for various phosphopeptides relative to a Src SH2-phosphopeptide interaction whose K_d has been determined previously (YEEI-P; K_d = 4 nM). Two Src-derived phosphopeptides, one containing the regulatory C-terminal Tyr-527 and another containing the autophosphorylation site Tyr-416, bind the Src SH2 domain in a specific though low-affinity manner (with about 10^4-lower affinity than the YEEI-P peptide). A platelet-derived growth factor receptor (PDGF-R) phosphopeptide containing Tyr-857 does not bind appreciably to the Src SH2 domain, suggesting it is not the PDGF-R binding site for Src as previously reported. However, another PDGF-R-derived phosphopeptide containing Tyr-751 does bind the Src SH2 domain (with an affinity approximately 2 orders of magnitude lower than that of YEEI-P). All of the phosphopeptides which bind to the Src SH2 domain contain a glutamic acid at position −3 or −4 with respect to phosphotyrosine; changing this residue to alanine greatly diminishes binding. We have also tested Src SH2 mutants for their binding properties and have interpreted our results in light of the recent crystal structure solution for the Src SH2 domain. Mutations in various conserved and nonconserved residues (R155A, R155K, N198E, H201R, and H201L) cause slight reductions in binding, while two mutations cause severe reductions. The W148E mutant domain, which alters the invariant tryptophan that marks the N-terminal border of the SH2 domain, binds poorly to phosphopeptides. Inclusion of the SH3 domain in the fusion protein partially restores the binding by the W148E mutant. A change in the invariant arginine that coordinates twice with phosphotyrosine in the peptide (R175L) results in a nearly complete loss of binding. The R175L mutant does display high affinity for the PDGF-R peptide containing Tyr-751, via an interaction that is at least partly phosphotyrosine independent. We have used this interaction to show that the R175L mutation also disrupts the intramolecular interaction between the Src SH2 domain and the phosphorylated C terminus within the context of the entire Src protein; thus, the binding properties observed for mutant domains in an in vitro assay appear to mimic those that occur in vivo.

Src homology 2 (SH2) domains are regions of approximately 100 amino acids that bind phosphotyrosine-containing peptide sequences (20, 29, 33). SH2 domains are implicated in the formation of signaling complexes, such as those that occur upon activation of the platelet-derived growth factor receptor (PDGF-R) and epidermal growth factor receptor. These receptors are autophosphorylated on several tyrosine residues and subsequently bind to the SH2 domains of the p85 subunit of phosphatidylinositol 3-kinase (PI3 kinase) (10, 16), the GTPase-activating protein (14, 17), growth factor receptor-bound protein 2 (24), and phospholipase Cγ (27, 31).

SH2-phosphopeptide interactions exhibit a significant degree of specificity. Although there are numerous phosphorylated sites on activated receptors and a given receptor will bind many SH2-containing proteins, each of the SH2-containing proteins will bind only a subset of the phosphorylated sites on the receptor with high affinity (10, 11, 15, 18, 36, 40). Specificity appears to be mediated by the residues immediately C terminal to the phosphotyrosine in the peptide target (11, 15, 36, 39) and presumably by the regions of variability in the SH2 domains (20, 39, 42, 43). For example, the SH2 domains in the p85 subunit of PI3 kinase interact with numerous proteins, each of which contains a similar sequence flanking the phosphotyrosine responsible for binding (3). This motif (pTyr-Met/Val-Xxx-Met) has been shown in peptide binding studies to have the highest affinity for both p85 SH2 domains (39).

Although the study of p85 interactions has served as a useful paradigm for the SH2 field, sequence motifs within the naturally occurring phosphopeptide targets to which other SH2 domains bind remain to be elucidated. Binding studies with randomly generated peptides have identified sequences that exhibit high-affinity binding to other SH2 domains, but many of these motifs have not been found in known protein sequences (39). The sequences within each SH2 domain important for binding specific phosphopeptides also remain to be determined. To pursue such issues, we chose to analyze phosphopeptide binding to the SH2 domain of $pp60^{c\text{-}src}$.

The Src SH2 domain is a worthy choice for further study for a number of reasons. First, the Src SH2 domain has been implicated in a novel type of phosphopeptide binding reaction, an intramolecular event associated with the regulation of Src's intrinsic enzymatic activity (3, 23, 28, 37). When phosphorylated, Tyr-527 at the C terminus of the Src protein acts as a negative regulator of protein tyrosine kinase

* Corresponding author.
† Present address: Laboratoire de Génétique Moléculaire des Eucaryotes, 67085 Strasbourg Cedex, France.

activity (5, 19, 34, 35). Mutational analysis (12, 32) as well as in vitro studies (23, 25, 37, 38) suggest that inhibition depends on binding of the phosphorylated C terminus to the SH2 domain. Second, like other SH2-containing proteins, the Src protein and other Src family members are reported to associate with activated receptors via their SH2 domains (1, 7, 8, 21). Third, many Src SH2 mutants have been assayed for their biological and enzymatic activities (9, 12, 32, 44). Thus, studies of these mutants in in vitro binding assays can be correlated with the known behavior of these mutants in biological assays and might provide a biochemical basis for their phenotypes. Finally, the crystal structure of the Src SH2 domain has recently been solved (42, 43). This has aided in the selection of informative mutants as well as interpretation of their behavior in various assays.

Because SH2 binding reactions can be mimicked with phosphopeptides and isolated SH2 domains, we chose to study the binding of Affi-Gel-coupled phosphopeptides to purified Src SH2 fused with glutathione *S*-transferase (GST). We have used free peptides as competitors to estimate the affinities of the Src SH2 domain for two Src phosphopeptides and for two PDGF-R phosphopeptides and found the affinities of the Src SH2 domain for the different phosphopeptides to vary over 5 orders of magnitude. We have also identified a glutamic acid at position −3 or −4 with respect to phosphotyrosine which is important for the affinity of the phosphopeptides for the Src SH2 domain. By analyzing the binding properties of SH2 mutants, we have defined residues within the SH2 domain important for binding to phosphopeptides, and our results raise provocative questions in light of the recent crystal structure of the Src SH2 domain complexed with different phosphopeptides. We have shown that a mutation in the arginine responsible for coordinating phosphotyrosine in the peptide abolishes binding to all phosphopeptides but reveals a binding site for a PDGF-R-derived phosphopeptide. Finally, we have shown that a change in this arginine appears to disrupt the intramolecular binding of the Src SH2 domain with the phosphorylated Src C terminus within the context of the entire Src protein.

MATERIALS AND METHODS

Expression and purification of GST fusion proteins. DNA fragments encoding domains of $pp60^{c\text{-}src}$ and Src mutants (12) were amplified by the polymerase chain reaction, sequenced, and cloned into the *Eco*RI site of the pGEX-1 vector (Pharmacia). All of the GST-SH2 fusion proteins contain residues 146 to 251 of Src. The GST-SH3 protein contains residues 87 to 146; the GST-SH3/SH2 protein spans residues 87 to 251. Constructs encoding these fusion proteins were used to transform *Escherichia coli* NB-42. Cultures of 100 ml were grown to an optical density of 0.6 and induced with 0.1 mM isopropyl thiogalactopyranoside (IPTG) for 3 h. Cells were pelleted and resuspended in 1 ml of lysis buffer (phosphate-buffered saline [PBS; per liter, 0.1 g of $CaCl_2$ and $MgCl_2$, 0.2 g of KCl, 8 g of NaCl, and 2.16 g of Na_2HPO_4], 1% Triton X-100, 100 mM EDTA, 1 mg of aprotinin per ml, 1 mg of leupeptin per ml, 2 mM phenylmethylsulfonyl fluoride). Resuspended cells were sonicated three times for 30 s each time on ice and cleared of debris by centrifugation for 5 min at 14,000 × *g*. Cleared lysates were incubated with 300 μl of 50% (vol/vol) glutathione-agarose (Sigma) for 30 min at 4°C. The agarose was washed with cold lysis buffer three times, and fusion proteins were eluted with two 150-μl washes with freshly made elution buffer (50 mM Tris [pH 8.0], 5 mM glutathione [Sigma]).

Each of the wild-type and mutant fusion proteins (except W148E) was produced at high levels with nearly 90% solubility. The amount of soluble protein obtained for the two W148E mutants was roughly a quarter of that obtained for the other fusions. In all cases (including W148E), the fusion proteins migrated as discrete bands and exhibited phosphotyrosine-dependent binding to peptides. The concentrations of the soluble fusion proteins were equalized for each experiment.

Peptides. All peptides were synthesized with *tert*-butoxycarbonyl-protected amino acids and exhibited greater than 90% purity as determined by high-pressure liquid chromatography (Biosynthesis, Inc.). Free peptides were diluted in PBS for the competition assays. Immobilized peptides were kindly provided by Andrew Laudano (University of New Hampshire), and the synthesis and coupling protocol was described previously (37). Immobilized peptides were synthesized with ^{14}C, which allowed determination of the coupling efficiency. The concentrations of peptides coupled to Affi-Gel were equalized by diluting the peptide-coupled Affi-Gel with blocked Sepharose CL4B (Sigma). The YEEI-P peptide (see Fig. 2A) was kindly provided by Zhou Songyang and Lewis Cantley (Harvard University).

Binding of fusion proteins with immobilized phosphopeptides. Purified GST fusion proteins were incubated with 30 μl of 50% (vol/vol) Affi-Gel coupled to peptides. Except where otherwise indicated, precipitations included 10 μl of GST fusions (0.01 mg/ml) in wash buffer (PBS, 1% Triton X-100) and 30 μl of a 50% slurry of peptide-coupled Affi-Gel. Free peptides were always added in a 10-μl volume. Free phosphotyrosine (Sigma) was diluted in PBS and also added in a 10-μl volume. The final reaction volume was 50 μl, and incubations were carried out for 30 min at 4°C. Beads were washed three times with wash buffer, and bound proteins were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer (0.0625 M Tris [pH 6.8], 10% [wt/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 2.3% SDS) for 5 min prior to SDS-polyacrylamide gel electrophoresis (PAGE) in 10% gels. Proteins were electrophoretically transferred to nitrocellulose membranes and blocked with 2% bovine serum albumin (BSA) in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.2% Tween 20) for 30 min. Membranes were incubated with anti-GST antibody (kindly provided by Doug Kellogg, University of California, San Francisco) at 1:5,000 dilution in TBST–2% BSA either for 1 h at room temperature or overnight at 4°C. After incubation, membranes were washed three times for 5 min each time in TBST. Goat anti-rabbit antibody coupled to horseradish peroxidase (Boehringer Mannheim) at 1:20,000 dilution in TBST–2% BSA was incubated with the membranes for 1 h at room temperature. Membranes were again washed three times for 5 min each time in TBST and developed with chemiluminescence reagents (Amersham).

To quantitate the binding observed in the Western blots (immunoblots), we used the preceding protocol but used 5 μCi of ^{125}I-labeled protein A (Amersham) as a secondary reagent. Radioactivity in the bands was counted by using a PhosphorImager (Molecular Dynamics).

Binding $pp60^{c\text{-}src}$ with immobilized phosphopeptides. NIH 3T3 cells expressing the Src protein and various mutants of Src were washed on ice with PBS and then lysed in TENT lysis buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl, 1% Triton X-100). Lysates (2 mg) were cleared by centrifugation at 14,000 × *g* for 5 min and then incubated with 50 μl of 50% slurry peptide-coupled Affi-Gel for 30 min at 4°C. The Affi-Gel was washed three times for 5 min each

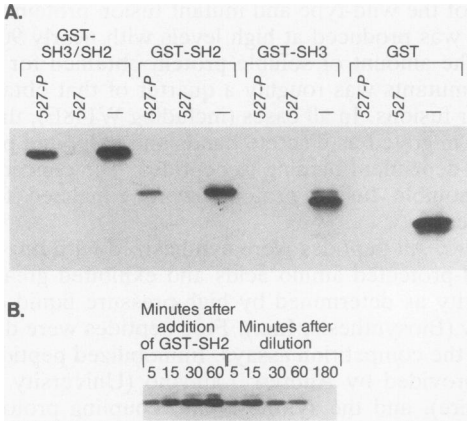

FIG. 1. (A) Binding of GST-SH3/SH2 and GST-SH2 fusion proteins to immobilized 527-P peptide. The indicated GST fusion proteins were precipitated with Affi-Gel-coupled 527-P peptide or 527 peptide, and eluted proteins were detected by immunoblotting with an anti-GST antibody as described in Materials and Methods. Lanes marked by − contain the amount of fusion protein added to each reaction. (B) Time course for the binding of the GST-SH2 to immobilized 527-P. GST-SH2 fusion proteins were incubated with immobilized 527-P for the indicated time periods, with a maximum of 1 h of incubation to determine the time course of association. Bound proteins were analyzed as described above. After 1 h of incubation, reactions were diluted into 10 ml of PBS–1% Triton X-100 and then incubated for the indicated time periods to determine the time course of dissociation.

time in TENT buffer; bound proteins were eluted in SDS sample buffer with 5% 2-mercaptoethanol for 5 min and then subjected to SDS-PAGE in 10% gels. Proteins were transferred to nitrocellulose and blocked with 2% BSA in TBST. Membranes were incubated with monoclonal antibody 327 (kindly provided by Joan Brugge, Ariad) at 1:5,000 dilution in TBST–2% BSA overnight at 4°C and subsequently washed three times for 5 min each time in TBST. ^{125}I-labeled goat anti-mouse secondary antibody (Amersham) was incubated with the membranes in TBST–2% BSA for 2 h at 4°C. Membranes were then washed three times for 5 min each time in TBST and analyzed by autoradiography.

RESULTS

Src SH2 and SH3-SH2 domains bind specifically to the phosphorylated form of the 527 peptide. To assay binding of the Src SH2 domain to phosphopeptides, we expressed and purified Src SH2 as a fusion protein with GST (GST-SH2). Peptides were synthesized with either tyrosine or phosphotyrosine and coupled to Affi-Gel. GST-SH2 proteins were incubated with Affi-Gel-coupled peptides, and the amount of GST-SH2 proteins bound to beads was determined by Western blotting. The efficiency of binding between the phosphopeptides and the fusion proteins was estimated by comparing the levels of input and bound protein (Fig. 1A).

Because the Src SH2 domain is thought to bind the phosphorylated C terminus of the Src protein, we tested whether this interaction would be observed in vitro. Beads carrying a 13-mer peptide spanning residues 521 to 533 of the Src protein and including phosphotyrosine at position 527 (called 527-P; see Fig. 2A) were incubated with GST-SH2 (Fig. 1A). Most of the GST-SH2 added to the reaction bound to the Affi-Gel-coupled 527-P peptide. Binding is rapid and reaches a maximum level within 30 min (Fig. 1B). The complexes formed are stable for several hours, although the reaction is completely reversible by dilution. Phosphorylation is required for binding; the GST-SH2 fusion proteins did not bind beads carrying the identical 13-residue peptide which lacks phosphotyrosine (called 527). In addition, GST alone did not bind to either peptide.

A fusion protein containing both the SH3 and SH2 domains of Src (GST-SH3/SH2) binds 1.5-fold more efficiently to Affi-Gel-coupled 527-P peptides than does the fusion protein containing the SH2 domain alone. Binding was again specific for the phosphorylated form of the 527-P peptide. GST-SH3, a fusion protein lacking the SH2 domain, does not bind appreciably to either peptide. Thus, the SH3 domain appears to increase the affinity of the SH2 domain for 527-P, although it does not itself bind 527-P.

The Src SH2 domain binds 527-P with low affinity. Because of the difficulty in determining the effective concentration of phosphopeptides coupled to Affi-Gel and the relatively high concentration of GST-SH2 fusion protein needed to assess binding in this assay, we were unable to derive a dissociation constant for the binding of the GST-SH2 fusion protein to 527-P. To determine a relative affinity of the Src SH2 domain for 527-P, we therefore devised a competition assay in which free peptides were tested for the ability to interfere with the binding of GST-SH2 to Affi-Gel-coupled 527-P. The findings were interpreted relative to the behavior of phosphopeptide YEEI-P (Fig. 2A), since the K_d for the interaction between this peptide and Src SH2 has been determined to be 4 nM (39). Approximately 10,000-fold-greater concentrations of free 527-P than YEEI-P were required to compete for half of the binding of GST-SH2 to immobilized 527-P (Fig. 2B). Thus the affinity of 527-P for the Src SH2 domain is about 10^4 less than that of YEEI-P for the Src SH2 domain (see Discussion). This affinity is relatively low, although not as low as the affinity of the domain for free phosphotyrosine, which is approximately 10^5 less than that of YEEI-P for the Src SH2 domain (data not shown).

The relatively weak affinity of a peptide known to interact with Src SH2 prompted us to measure the ability of other tyrosine-phosphorylated peptides to compete in this assay. Src protein is autophosphorylated on Tyr-416, the only tyrosine residue other than Tyr-527 that is abundantly phosphorylated. A 13-mer phosphopeptide representing the region surrounding Tyr-416 (416-P; Fig. 2A) interfered with binding of GST-SH2 to immobilized 527-P in a manner indistinguishable from that observed with free 527-P. This finding indicates that the two Src-derived phosphopeptides have similar and relatively weak affinities for Src SH2. This binding is consistent with the possibility that both of these phosphorylation sites interact intramolecularly with the Src SH2 domain (see Discussion).

Src protein is known to associate with activated PDGF-R, presumably through SH2 interactions with autophosphorylated sites in the receptor. We therefore tested two PDGF-R-derived phosphopeptides for the ability to compete with immobilized 527-P for binding to GST-SH2. A peptide containing the autophosphorylation site at Tyr-857 (PD857-P; Fig. 2A) failed to interfere with the binding of GST-SH2 to immobilized 527-P, even at concentrations 100,000-fold greater than required for half-maximal competition by YEEI-P. Thus, this peptide appears to have an extremely low affinity for Src SH2, since it is at least 10-fold less effective than Src-derived phosphopeptides in the competition assay. This was surprising because Tyr-857 has previously been reported to mediate the association of Src with

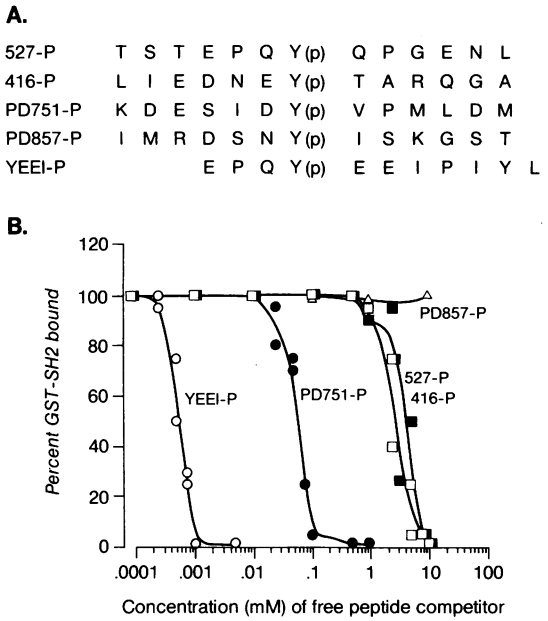

FIG. 2. Relative affinity of the GST-SH2 fusion protein for various phosphopeptides. (A) Sequences of the phosphopeptides used as competitors in panel B. (B) Competition assay with different phosphopeptides. Increasing concentrations of each of the free peptide competitors listed in panel A were added to 527-P previously coupled to Affi-Gel, mixed thoroughly, and then incubated with 0.1 μg of GST-Src SH2 fusion proteins for 30 min at 4°C. Bound proteins were eluted, subjected to SDS-PAGE, and detected by immunoblotting with a ^{125}I-labeled goat anti-rabbit antibody. Radioactivity in each band was quantitated with a PhosphorImager. One hundred percent binding was defined as the level of binding observed without any peptide competitor. Open circles, competition with YEEI-P; closed circles, competition with PD751-P; open squares, competition with 527-P; closed squares, competition with 416-P; open triangle, competition with PD857-P.

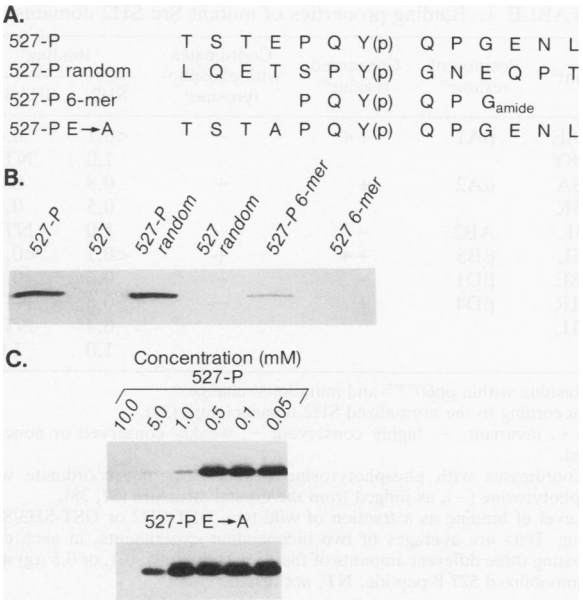

FIG. 3. Binding of GST-SH2 to altered versions of 527-P phosphopeptide. (A) Sequences of the phosphorylated forms of the peptides used in panels B and C. (B) Binding of GST-SH2 to a random form of 527-P but not to a short version of the peptide. 527, 527(R), and 527 6-mer peptides in both the phosphorylated and nonphosphorylated forms were used to bind GST-SH2, and the results were analyzed as described in the legend to Fig. 1. (C) Competition assay comparing wild-type 527-P and 527-P E→A peptides. 527-P and 527-P E→A were used to compete for binding of GST-SH2 to immobilized 527-P as described in the legend to Fig. 2.

the activated receptor (7, 8). In contrast, PD751-P, a phosphopeptide representing the autophosphorylation site that mediates the association of the p85 subunit of PI3 kinase with the activated receptor (10, 18), competes relatively efficiently; 100-fold less free PD751-P peptide than 527-P is required for half-maximal competition (Fig. 2B). These findings suggest that the region around Tyr-857 is likely not to mediate the association of the Src protein with the PDGF-R as previously reported, and they show that the competition assay can distinguish peptides with relative affinities for the Src SH2 domain that range over more than 5 orders of magnitude.

A glutamic acid N terminal to tyrosine 527 is important for binding to Src SH2. Although the peptide 527-P appears to have a relatively weak affinity for Src SH2, it binds more efficiently than other phosphopeptides (e.g., PD857-P). 527-P also binds more efficiently than the nonphosphorylated version of the peptide, suggesting that phosphorylation of Tyr-527 is required for binding. To seek other features of 527-P responsible for its ability to recognize Src SH2, we tested variant forms of the 527-P peptide for binding to GST-SH2.

To assess which sequences flanking the phosphotyrosine in 527-P play a role in binding to GST-SH2, we synthesized a shorter version of 527-P (527-P 6-mer; Fig. 3A). When this shorter form of 527-P was immobilized and incubated with GST-SH2, the amount of bound GST-SH2 was greatly diminished over that bound to immobilized 527-P (Fig. 3B). The low level of binding to 527-P 6-mer was still dependent on phosphate, since no binding was observed with the nonphosphorylated version of the peptide. These results suggest that in addition to the phosphorylation on tyrosine, the sequences surrounding the phosphorylated tyrosine in 527-P are important for binding GST-SH2.

We also generated a randomized version of 527-P [527-P(R); Fig. 3A] in which the position of each of the residues except the central phosphotyrosine was changed, but the total amino acid content and the charge balance on either side of the phosphotyrosine were retained. When this peptide was coupled to Affi-Gel and incubated with GST-SH2, binding to immobilized 527-P(R) and binding to wild-type 527-P were indistinguishable (Fig. 3B). Thus, it appears that the positional changes in 527-P(R) do not greatly affect the binding of GST-SH2 to this peptide, and the binding observed is still dependent on phosphotyrosine.

Taken together, these results indicate that residues flanking phosphorylated Tyr-527 may be important for binding to the GST-SH2 fusion protein, although the exact position of these residues may not be critical. We compared the N-terminal sequences of 527-P with those of other phosphopeptides that bind GST-SH2 (Fig. 2A) and noted that all of the phosphopeptides for which GST-SH2 has appreciable affinity, including 527-P(R), have a glutamic acid residue at position −3 or −4 with respect to the phosphotyrosine. PD857-P and 527-P 6-mer, which show reduced affinity for GST-SH2, both lack a glutamic acid in this region. To test the importance of this residue for binding of GST-SH2 to

TABLE 1. Binding properties of mutant Src SH2 domains

Mutant[a]	Position of residue[b]	Conserved residue[c]	Coordinates with phosphotyrosine[d]	Binding[e]	
				SH2	SH3/SH2
W148E	βA1	++	−	<0.1	0.4
W148Y				1.0	NT
R155A	αA2	+	+	0.8	0.8
R155K				0.5	0.5
P165L	AB2	−	−	1.0	NT
R175L	βB5	++	+	<0.1	<0.1
N198E	βD1	−	−	0.6	0.6
H201R	βD4	+	−	0.8	NT
H201L				0.4	NT
WT				1.0	1.0

[a] Residue within pp60$^{c\text{-}src}$ and mutational change.
[b] According to the normalized SH2 nomenclature (38).
[c] ++, invariant; +, highly conserved; −, weakly conserved or nonconserved.
[d] Coordinates with phosphotyrosine (+) or does not coordinate with phosphotyrosine (−), as judged from the crystal structure (37, 38).
[e] Level of binding as a fraction of wild-type GST-SH2 or GST-SH3/SH2 binding. Data are averages of two independent experiments, in each case incubating three different amounts of fusion protein (0.05, 0.1, or 0.5 μg) with the immobilized 527-P peptide. NT, not tested.

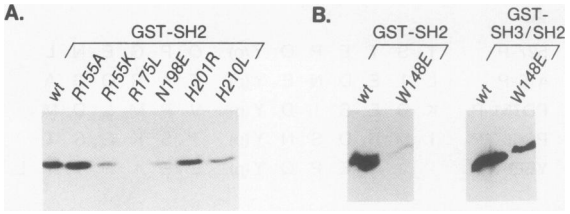

FIG. 4. (A) Mutant SH2 domains display different affinities for immobilized 527-P peptide. Indicated mutant GST-SH2 fusion proteins were bound to immobilized 527-P peptide and analyzed as described in the legend to Fig. 1. (B) The presence of the SH3 domain enhances binding of the W148E mutant to 527-P. The W148E GST-SH2 fusion and the W148E GST-SH3/SH2 fusion were bound to 527-P and analyzed as described in the legend to Fig. 1. wt, wild type.

527-P more directly, we synthesized a 13-mer phosphopeptide similar to 527-P with a Glu→Ala mutation at position −3 (Fig. 3A). Various concentrations of this mutant peptide were incubated with GST-SH2 and immobilized 527-P; 10-fold-higher concentrations of 527-P E→A than of wild-type 527-P were needed to inhibit the binding of GST-SH2 to Affi-Gel-coupled 527-P (Fig. 3C). These results suggest that a glutamic acid at position 524 has a significant role in the recognition of 527-P by GST-SH2.

Mutations in the Src SH2 domain and the presence of the Src SH3 domain affect binding to 527-P. Having identified some of the residues in 527-P important for binding to GST-SH2, we were interested in the residues within the SH2 domain which might be important for binding 527-P. Although 527-P binds weakly to GST-SH2, the binding is specific for the Src SH2 domain. The C-terminal SH2 domain of the p85 subunit of PI3 kinase and the SH2 domain of the Vav protein do not bind efficiently to 527-P (data not shown). To identify residues within the Src SH2 domain that might be responsible for this specific interaction with 527-P, we tested mutant SH2 domains for the ability to bind immobilized 527-P. Mutations have been made previously in the Src SH2 domain and assayed in vivo for their effects on the biological and enzymatic activities of pp60^{Y527F} (12). We made GST-SH2 fusion proteins with several of these mutants and compared the binding of mutant and wild-type GST-SH2 to 527-P (Table 1).

Arg-175 is invariant in all SH2 domains and coordinates twice with the phosphotyrosine in the peptides (42). Changing this residue to leucine produces the most severe reduction in binding of all mutants tested (Table 1 and Fig. 4A). Even at 100-fold-higher concentrations of this mutant domain, very little binding is observed (data not shown). Trp-148 is also invariant in SH2 domains; although it does not contact phosphotyrosine, this residue is critical for the tertiary structure of the domain. When Trp-148 is changed to glutamic acid, a severe reduction in binding is observed; however, the more conservative change of this tryptophan residue to a tyrosine had no effect on its ability to bind 527-P (Table 1).

Other mutations reduce binding of the SH2 domain to 527-P to 20 to 80% of wild-type binding. These include two changes in another conserved arginine that also contacts the phosphotyrosine in the peptide (R155A and R155K; Table 1 and Fig. 4A) (42). Changes in a conserved histidine (H201R and H201L), which does not contact phosphotyrosine but is important for maintaining active-site geometry (42), reduce binding (Table 1 and Fig. 4A). The H201L mutant shows only 40% of wild-type binding, while the more conservative H201R mutant displays closer to wild-type levels of binding. Although Asn-198 is not well conserved, the mutant N198E domain binds at only 60% of wild-type levels (Table 1 and Fig. 4A). The P165L mutant, with a change at another nonconserved residue, displays wild-type binding (Table 1).

Many of these mutants were also assayed in the context of the GST-SH3/SH2 fusion proteins. For the most part, the mutants behaved the same in GST-SH3/SH2 fusion proteins as they did in the context of GST-SH2 alone (data not shown), with one notable exception. The affinity of W148E GST-SH3/SH2 for 527-P is significantly greater than the affinity of W148E GST-SH2 for the immobilized phosphopeptide (Fig. 4B). Although the binding of W148E GST-SH3/SH2 is not at wild-type levels, the presence of the SH3 domain appears to enhance the ability of the W148E mutant to bind 527-P.

R175L binds with high affinity to PD751-P. To determine whether the SH2 mutations would affect binding of the GST-SH2 fusion to other phosphopeptides, we assayed the binding of the mutant SH2 domains to the phosphopeptides whose affinity for the wild-type SH2 domain we had already estimated (Fig. 2). The phosphorylated and nonphosphorylated forms of these peptides were coupled to Affi-Gel and incubated with the mutant GST-SH2 fusion proteins. Binding of the mutant domains to immobilized 416-P, PD751-P, and PD857-P was similar to that observed for each of these mutant domains binding to immobilized 527-P (although slight variation may not have been detected; data not shown). The one exception was the mutant R175L domain: it binds poorly to 527-P but binds at near wild-type levels to PD751-P (Fig. 5). The R175L mutant domain binds with the same kinetics as the wild-type domain but exhibits a slightly faster time course for dissociation, resulting in a 10-fold-reduced affinity compared with the affinity of the wild-type domain for PD751-P in a competition assay (data not shown).

The wild-type SH2 domain also binds the nonphosphorylated version of PD751 (Fig. 5). Competitions using free PD751 and PD751-P to inhibit binding of the wild-type Src SH2 domain to PD751 suggest that the affinity of the wild-type domain for PD751-P is approximately 50-fold greater

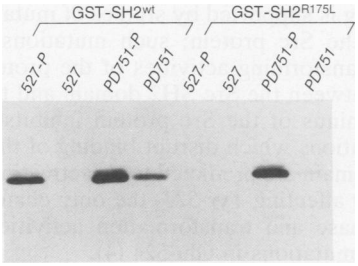

FIG. 5. Binding of wild-type and R175L mutant SH2 domains to 527-P and PD751-P phosphopeptides. Wild-type (wt) and R175L mutant GST-SH2 fusion proteins were bound to phosphorylated and nonphosphorylated forms of 527 and PD751 and analyzed as described in the legend to Fig. 1.

than the affinity of the domain for the nonphosphorylated version of the peptide (data not shown). The binding of the R175L mutant domain to PD751-P, as well as the binding of the wild-type domain to both PD751-P and PD751, suggests that this binding is at least partly independent of phosphotyrosine (see Discussion).

The SH2 domain of pp60^{R175L} is accessible for phosphopeptide binding. pp60$^{c\text{-}src}$ is extensively phosphorylated at Tyr-527. Previous studies (37) have shown that pp60$^{c\text{-}src}$ cannot be precipitated from whole cell lysates with 527-P peptide coupled to Affi-Gel, presumably because the SH2 domain of pp60$^{c\text{-}src}$ is associated with the phosphorylated Tyr-527 on the same molecule (Fig. 6A). When Tyr-527 is changed to phenylalanine to prevent the intramolecular SH2 interaction, pp60^{Y527F} can be precipitated from whole cell lysates with immobilized 527-P (Fig. 6A). Src protein binding to immobilized phosphopeptides has thus proven a useful assay to determine the conformation of Src and Src family members within lysates (37, 38).

We have described an interaction between the Src SH2 domain and PD751-P that is not strictly dependent on phosphotyrosine, since a mutant which abolishes phosphotyrosine binding (R175L) retains affinity for this peptide (Fig. 5). This interaction provides a useful means to determine the conformation of Src proteins that contain lesions in their SH2 domains that affect phosphotyrosine binding. If the in vitro assays that we describe mimic the Src SH2 interactions that occur in vivo, mutations that disrupt binding of the GST-SH2 fusion protein to the 527-P peptide should disrupt the interaction of the Src SH2 domain with the phosphorylated C terminus in context of the entire protein. As a result of this disruption, the SH2 domain in these mutant proteins would be expected to be accessible for binding to certain immobilized peptides, such as PD751-P. The properties of the R175L mutant SH2 domain provided a means to test this prediction, because the mutant does not bind 527-P but can interact with PD751-P. We would expect that the SH2 domain of pp60^{R175L} should no longer complex with Tyr-527 and should therefore be available to bind to immobilized PD751-P (Fig. 6A).

Whole cell lysates of NIH 3T3 cell expressing various mutant Src proteins were incubated with PD751-P coupled to Affi-Gel, and precipitated proteins were examined for the level of binding of the different Src mutants. As predicted from earlier experiments, immobilized PD751-P does not bind wild-type pp60$^{c\text{-}src}$ but does bind pp60 proteins with a mutation in Tyr-527 (pp60^{Y527F}) (Fig. 6B). Proteins with two point mutations (pp60$^{R175L/Y527F}$) also bind immobilized PD751-P, as predicted by the ability the mutant R175L

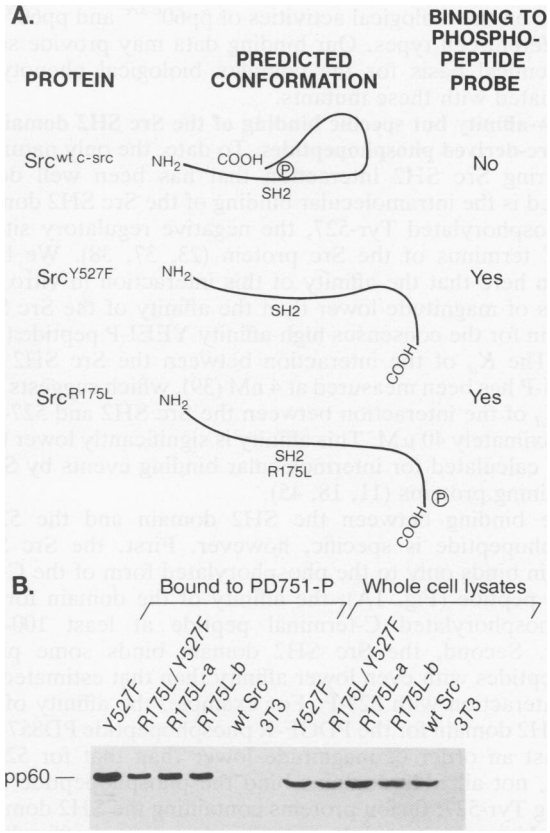

FIG. 6. (A) Predicted conformations of three different Src proteins and their abilities to bind immobilized PD751-P phosphopeptide. wt, wild type. (B) Binding of mutant Src proteins to PD751-P. Whole cell lysates expressing various mutant Src proteins were incubated with immobilized PD751-P peptide, and the bound proteins were detected by immunoblotting with monoclonal antibody 327 and a ^{125}I-labeled secondary antibody. One-twentieth of the amount of protein added to each precipitation was loaded in the lanes labeled "Whole cell lysates" to indicate the level of Src protein expressed in each of the lysates. R175L-a and R175L-b are two different clones of NIH 3T3 cells expressing the mutant Src protein.

GST-SH2 fusion protein to bind this peptide. Interestingly, proteins with the single mutation in the SH2 domain (pp60^{R175L}) also bind immobilized PD751-P, consistent with such a mutation disrupting the interaction between the Src SH2 domain and the phosphorylated C terminus.

DISCUSSION

By estimating the affinity of the Src SH2 domain for various phosphopeptides, we have provided evidence that bears directly on the selection of biologically relevant targets by the Src SH2 domain, both intramolecularly and intermolecularly. We have also defined residues within both the phosphopeptides and the SH2 domain important for binding and have identified a role for the adjacent SH3 domain in SH2-phosphopeptide binding. These results are of particular interest in light of the recent crystal structure of the Src SH2 domain as well as attempts to identify sequence motifs in phosphopeptides that might mediate high-affinity binding. Finally, the mutations in the SH2 domain that we have assayed for binding have been described previously for their

effects on the biological activities of $pp60^{c\text{-}src}$ and $pp60^{Y527F}$ in different cell types. Our binding data may provide some biochemical basis for the complex biological phenotypes associated with these mutants.

Low-affinity but specific binding of the Src SH2 domain to two Src-derived phosphopeptides. To date, the only naturally occurring Src SH2 interaction that has been well documented is the intramolecular binding of the Src SH2 domain to phosphorylated Tyr-527, the negative regulatory site at the C terminus of the Src protein (23, 37, 38). We have shown here that the affinity of this interaction in vitro is 4 orders of magnitude lower than the affinity of the Src SH2 domain for the consensus high-affinity YEEI-P peptide (Fig. 2B). The K_d of the interaction between the Src SH2 and YEEI-P has been measured at 4 nM (39), which suggests that the K_d of the interaction between the Src SH2 and 527-P is approximately 40 μM. This affinity is significantly lower than those calculated for intermolecular binding events by SH2-containing proteins (11, 18, 45).

The binding between the SH2 domain and the 527-P phosphopeptide is specific, however. First, the Src SH2 domain binds only to the phosphorylated form of the C-terminal peptide (Fig. 1A); the affinity of the domain for the nonphosphorylated C-terminal peptide at least 100-fold lower. Second, the Src SH2 domain binds some phosphopeptides with even lower affinity than that estimated for the interaction with 527-P. For example, the affinity of the Src SH2 domain for the PDGF-R phosphopeptide PD857-P is at least an order of magnitude lower than that for 527-P. Third, not all SH2 domains bind the phosphopeptide containing Tyr-527; fusion proteins containing the SH2 domains of the Vav protein and the C-terminal portion of p85 subunit of PI3 kinase do not bind efficiently to 527-P (data not shown).

A specific, low-affinity interaction between the SH2 domain and Tyr-527 is consistent with models for the regulation of $pp60^{c\text{-}src}$. Because the interaction is hypothesized to be intramolecular, the local concentrations of the components of the binding reaction are high, implying that relatively low affinities will permit efficient binding. Also, the inhibition of kinase activity associated with phosphorylation of Tyr-527 must be regulatable. A tight interaction between the SH2 domain and phosphorylated Tyr-527 might not permit derepression of enzymatic activity by the action of phosphatases on phosphorylated Tyr-527.

The Src SH2 domain may be involved in a second regulatory event mediated by the intramolecular binding of the SH2 domain to autophosphorylated Tyr-416 (22, 41). Our in vitro data suggest that such an interaction is possible. The Src SH2 domain binds to a phosphopeptide containing Tyr-416 as avidly as it does to phosphopeptide 527-P (Fig. 2B). The binding is phosphotyrosine dependent and specific for the Src SH2 domain, since the SH2 domains of Vav and p85 do not bind appreciably to 416-P. This specific, low-affinity interaction is also consistent with a regulatable, intramolecular binding event. Definitive evidence that either of these interactions actually occurs within the context of the entire protein may require determination of the crystal structure of the Src protein.

Requirement for an N-terminal glutamic acid within the phosphopeptide target. All of the peptides to which the Src SH2 has been shown to bind have a glutamic acid residue at positions −3 or −4 with respect to phosphotyrosine (Fig. 2A and 3A). A single change of this residue to an alanine in 527-P results in reduced affinity of the peptide for the SH2 domain (Fig. 3A and C). The importance of this glutamic acid in binding is supported by studies of mutations in the C terminus of the Src protein; such mutations activate the kinase and transforming activities of the protein (4). If the interaction between the Src SH2 domain and the phosphorylated C terminus of the Src protein inhibits Src's kinase activity, mutations which disrupt binding of the C terminus to the SH2 domain are predicted to be activating. Other than those directly affecting Tyr-527, the only changes that activated the kinase and transformation activities of the Src protein were mutations in Glu-524 (4).

The region N terminal to the phosphotyrosine has not previously been considered an important factor in recognition by SH2 domains. The motif identified as mediating the binding between SH2 domain of the p85 subunit of PI3 kinase and the PDGF-R consists of a phosphotyrosine and three residues C terminal to the phosphotyrosine (11). Selection strategies to search for phosphopeptides that bind tightly to the SH2 domains of Src family members have focused exclusively on sequence motifs in the region C terminal to the phosphotyrosine (39). The crystal structure of the Src SH2 domain with a high-affinity peptide (43) identifies the phosphotyrosine and the isoleucine at position +3 in the phosphopeptide as mediating the contacts with the SH2 domain. Our results suggest that sequence motifs in the N-terminal portion of the phosphopeptides bear further examination.

Association of the Src SH2 domain with PDGF-R phosphopeptides. The binding site on the PDGF-R for Src and Src family members has been the subject of much investigation. We have tested two PDGF-R phosphopeptides and find that the Src SH2 domain binds with high affinity only to the phosphopeptide containing Tyr-751. However, we do not believe that this phosphorylation site mediates the association of Src with the PDGF-R. Tyr-751 has been reported by others to mediate the interaction of PI3 kinase with activated receptors (10, 18), and in our assay, the C-terminal SH2 domain of the p85 subunit of PI3 kinase has at least 10-fold-greater affinity for this phosphopeptide target (data not shown).

Phosphorylated Tyr-857 and the analogous residues on other receptors have been implicated previously in the association of Src and Src family members with activated receptors (7, 8). We believe the weak affinity between GST-SH2 and PD857-P observed in this in vitro reaction to be inconsistent with Tyr-857 mediating an intermolecular SH2 interaction between the PDGF-R and the Src protein. Such an interaction is likely to require the much higher affinities, as reported for the association of the p85 subunit of PI3 kinase (18) and the GTPase-activating protein (11) with the PDGF-R.

Others have recently reported the identification of a high-affinity juxtamembrane binding site for Src family members on the PDGF-R (30). The phosphorylation of Tyr-857 may serve only to ensure that the other tyrosines on the receptor are fully phosphorylated.

Residues within the SH2 domain and the presence of the SH3 domain affect binding to phosphopeptides. Our analysis of the residues in the SH2 domain important for phosphopeptide binding was aided greatly by the recent determination of the structure of the Src SH2 domain complexed with various phosphopeptides (42, 43). Most of the mutated residues are well conserved. Some are involved in binding the phosphotyrosine on the target, some are important structurally, and some may be implicated in the interaction of the domain with residues flanking the phosphotyrosine on the target.

The invariant tryptophan which marks the N-terminal boundary of all SH2 domains is important for the tertiary structure of the SH2 domain, with the side chain of Trp-148 packing against other conserved side chains to form the hydrophobic core of the structure. As predicted by this critical role in the formation of the hydrophobic core of the SH2 domain, the W148E mutation drastically reduces binding of the domain to phosphopeptide targets (Fig. 4B). Proteins containing W148E also exhibit altered mobility on SDS-PAGE (data not shown). A more conservative change, W148Y, does not impair binding, demonstrating that the invariant tryptophan can be replaced by a similar residue and still retain function.

The reduction in binding observed with W148E is partially restored by the inclusion of the SH3 domain in the fusion protein, suggesting that the SH3 domain might stabilize the SH2 domain and its interactions with phosphopeptides. The presence of the SH3 domain also increases the level of binding to the wild-type SH2 domain (Fig. 1A), although the effect is not as great. Interactions between the SH2 and SH3 domains have been postulated previously because these domains often appear together, usually with an SH3 domain followed immediately by an SH2 domain. Definitive proof of the interaction between these two domains will require crystallization together with appropriate phosphopeptides.

Arg-155 is involved in the simultaneous recognition of the phosphate group and the aromatic ring of the phosphotyrosine. Amino-aromatic interactions are comparable in strength with conventional hydrogen bonds between noncharged groups, and this particular amino-aromatic interaction is optimally constructed. Given the importance of this residue, it was somewhat surprising that the mutations R155K and R155A reduce binding only slightly. The adjacent position 156 in the Src SH2 domain is also an arginine and might substitute for Arg-155 in coordinating the phosphotyrosine. It is also possible that Arg-155 can be functionally replaced other residues; in fact, some SH2 domains do have a lysine in this position. Marengere and Pawson (26) have mutated the equivalent residue in the GTPase-activating protein SH2 domain and find that a number of different substitutions at this position have little effect on the binding properties of this domain.

His-201 is strongly conserved and makes contacts that may be critical for the maintenance of active-site geometry. Although theoretical predictions suggest that changing this residue to a leucine might preserve the interactions attributed to the histidine (42), we find that such a change impairs binding to phosphopeptides. The more conservative mutation, H201R, still preserves 80% of wild-type binding, suggesting that another basic residue might partially substitute for His-201. Arginine at position 201 is also hypothesized to make novel contacts with the phosphopeptide that are not possible for the histidine (42).

It has been suggested that the loops between the conserved α helices and β strands might contact the regions flanking the phosphotyrosine and mediate specificity. Two of the tested mutant domains change residues in loops: Pro-165 in the loop between β strands A and B, and Asp-198 at the edge of the CD loop that contains an insertion specific to Src family members. The P165L mutant domain binds with wild-type affinity, while the N198E mutant domain exhibited 60% of wild-type binding. The position of Asn-198 at the edge of the Src-specific loop allows us to postulate a role for this residue and the Src insertion in mediating contacts that might be important for SH2 interactions specific for the Src protein. The effect of this mutation on the biology of $pp60^{Y527F}$ is consistent with such a role (12, 13). While the parental protein transforms both chicken and mouse cells, the N198E mutant no longer transforms chicken cells efficiently, suggesting that an SH2-mediated contact important for transformation by Src in chicken cells might have been disrupted.

Arg-175 and its role in SH2 binding. Arg-175 plays a critical role in binding the phosphotyrosine in peptide targets. This residue forms an ion pair with the phosphate group, with specific hydrogen-bonding interactions between the two terminal nitrogens and two of the phosphate oxygens. Such a dual interaction is possible only with an arginine residue. Consistent with this unique role, the R175L mutant SH2 domain displays the most drastic reduction in binding to phosphopeptides of all mutants tested (Table 1 and Fig. 4A).

We have tested the R175L mutation in the context of $pp60^{c\text{-}src}$ for the accessibility of the SH2 domain, and $pp60^{R175L}$ has also been assayed for biological and enzymatic activities. Results in chicken cells are consistent with the possibility that the R175L mutation disrupts the intramolecular interaction between the Src SH2 domain and the phosphorylated C-terminal Tyr-527. $pp60^{R175L}$ displays elevated levels of kinase activity as well as the ability to transform chicken cells (12). As predicted for such a disruption, the SH2 domain of $pp60^{R175L}$ is accessible and binds immobilized PD751-P (Fig. 6A).

When the R175L mutation is placed in the context of the activated $pp60^{Y527F}$ protein, the phenotypes vary in different cell types. While the parental $pp60^{Y527F}$ protein is able to transform both chicken and mouse cells, $pp60^{R175L/Y527F}$ no longer transforms mouse cells yet transforms chicken cells better than the parental protein (12, 13). This host range phenotype cannot be readily explained; the mutant R175L SH2 domain binds poorly to most tyrosine-phosphorylated proteins in both cell types (1a). We have made one intriguing observation that may bear on the complex phenotype associated with R175L. While unable to bind most other phosphopeptides, the mutant R175L SH2 domain is able to bind the PDGF-R phosphopeptide PD751-P. Since Arg-175 mediates the binding observed in the crystal structure, the affinity of the mutant domain for this peptide suggests a novel type of binding event.

We believe that the binding of the Src SH2 domain to the Tyr-751-containing peptide is at least partly phosphotyrosine independent. Others have reported phosphotyrosine-independent binding events associated with SH2 domains (6, 33a), and we have observed that the wild-type Src SH2 domain binds the nonphosphorylated version of PD751 (Fig. 5). The R175L mutant domain displays higher affinity for the nonphosphorylated PD751 than for other phosphopeptides, including YEEI-P (data not shown). Others have shown that a deletion (Δ144–175) that abolishes binding of activated Src to most phosphopeptides (37) does not affect binding of the activated protein to PD751-P (2). This result suggests that the binding site for the PD751-P peptide is in the C-terminal portion of the Src SH2 domain, unlike the binding site for other phosphopeptides. Consistent with the possibility of two binding sites is the observation that the nonphosphorylated version of PD751 is unable to compete for binding of the wild-type SH2 domain to 527-P (data not shown).

Although these data point to a mode of binding that is not dependent on phosphotyrosine, we do observe greater levels of binding of both the wild-type and R175L mutant domains to the phosphorylated version of the PD751 peptide. This may be explained simply by nonspecific electrostatic interactions between the PD751-P peptide (which is relatively

acidic) and the SH2 domain (which contains a number of basic residues). We believe that these nonspecific electrostatic interactions may increase the affinity of a primarily phosphotyrosine-independent binding event. Consistent with this hypothesis is our observation that although free phosphotyrosine competes completely for the binding of the SH2 domain to the 527-P and YEEI-P phosphopeptides, it competes only partially for the binding of the SH2 domain to PD751-P (data not shown).

The phosphotyrosine-independent binding of the SH2 domain to the PD751 peptide and the affinity of the R175L mutant for this peptide may offer some explanation for the host range phenotype associated with pp60$^{R175L/Y527F}$. Certain SH2 binding events which are phosphotyrosine independent and therefore do not require Arg-175 might occur and allow for transformation in some cell types. Arg-175-mediated SH2 interactions would be abolished, eliminating transformation in other cell types.

ACKNOWLEDGMENTS

We are indebted to Andrew P. Laudano (University of New Hampshire) for the gift of immobilized phosphopeptides and numerous helpful discussions and suggestions throughout the duration of this project. We thank A. Klippel and L. T. Williams (University of California, San Francisco [UCSF]) for DNA encoding the GST–C-terminal p85 SH2 fusion protein, S. Katzav (St. Judes) for DNA encoding the GST-Vav SH2 fusion protein, and Peter Jackson (UCSF) for DNA encoding the GST-Abl SH2 fusion protein. We also thank Tony Pawson (Mount Sinai Hospital, Toronto, Ontario, Canada) and Peter Jackson for useful advice regarding the production of GST fusion proteins. We express much gratitude to those who contributed helpful comments in the preparation of the manuscript: J. Murphy, K. Kaplan, I. Taylor, K. Willert, and P. Sorger (UCSF).

This work was supported by a grant from NIH. K.B.B. is supported by a Howard Hughes predoctoral fellowship. H.B. was on leave from CNRS LGME, Strasbourg, France, and was supported by an NIH-CNRS exchange program (Fogarty International Center) and by the Philippe Foundation. H.E.V. is an American Cancer Society Research Professor.

REFERENCES

1. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson.** 1990. Binding of SH2 domains to phospholipase Cγ1, GAP and Src to activated growth factor receptors. Science **250:**979–982.

1a. **Boeuf, H., J. E. Murphy, K. B. Bibbins, and H. E. Varmus.** Unpublished data.

2. **Brodeur, S., D. B. Eisen, S. L. Groskoph, A. J. Tanner, X. Zhang, D. Shalloway, and A. P. Laudano.** Unpublished data.

3. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64:**281–302.

4. **Cheng, S. H., H. Piwnica-Worms, R. W. Harvey, T. M. Roberts, and A. E. Smith.** 1988. The carboxy terminus of pp60$^{c\text{-}src}$ is a regulatory domain and is involved in complex formation with the middle-T antigen of polyomavirus. Mol. Cell. Biol. **8:**1736–1747.

5. **Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter.** 1986. Tyr527 is phosphorylated in pp60$^{c\text{-}src}$: implications for regulation. Science **231:**1431–1434.

6. **Cooper, J. A., and A. Kashishian.** 1993. In vivo binding properties of SH2 domains from GTPase-activating protein and phosphatidylinositol 2-kinase. Mol. Cell. Biol. **13:**1737–1745.

7. **Courtneidge, S. A., R. Dhand, G. M. Twamley, M. D. Waterfield, and M. F. Roussel.** 1993. Activation of Src family kinases by colony stimulating factor-I and their association with the receptor. EMBO J. **12:**943–950.

8. **Courtneidge, S. A., R. M. Kypta, J. A. Cooper, and A. Kazlauskas.** 1991. Platelet-derived growth factor receptor sequences important for binding of src family members. Cell Growth Differ. **2:**483–486.

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. **Escobedo, J., D. R. Kaplan, W. M. Kavanaugh, C. W. Turck, and L. T. Williams.** 1991. A phosphatidylinositol 3-kinase binds to platelet-derived growth factor receptors through a specific receptor sequence containing phosphotyrosine. Mol. Cell. Biol. **11:**1125–1132.

11. **Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. D. Rosario, F. McCormick, and L. T. Williams.** 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell **69:**413–423.

12. **Hirai, H., and H. Varmus.** 1990. Site-directed mutagenesis of the SH2 and SH3 coding domains of c-*src* produces varied phenotypes, including oncogenic activation of pp60$^{c\text{-}src}$. Mol. Cell. Biol. **10:**1307–1318.

13. **Hirai, H., and H. E. Varmus.** 1990. Mutations in the src homology regions 2 and 3 of activate chicken c-src that result in preferential transformation of mouse or chicken cells. Proc. Natl. Acad. Sci. USA **87:**8592–8596.

14. **Kaplan, D. R., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** 1990. PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell **61:**125–133.

15. **Kashishian, A., A. Kazlauskas, and J. A. Cooper.** 1992. Phosphorylation sites in the PDGF receptor with different specificities. EMBO J. **11:**1373–1381.

16. **Kazlauskas, A., and J. A. Cooper.** 1989. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. Cell **58:**1121–1133.

17. **Kazlauskas, A., C. Ellis, T. Pawson, and J. A. Cooper.** 1990. Binding of GAP to activated PDGF receptors. Science **247:**1578–1581.

18. **Klippel, A., J. Escobedo, W. J. Fantl, and L. T. Williams.** 1992. The C-terminal SH2 domain of p85 accounts for the high affinity and specificity of the binding of phosphatidylinositol 3-kinase to phosphorylated platelet-derived growth factor β receptor. Mol. Cell. Biol. **12:**1451–1459.

19. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60$^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49:**65–73.

20. **Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson.** 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science **252:**668–674.

21. **Kypta, R. M., Y. Goldberg, E. T. Ulug, and S. A. Courtneidge.** 1990. Association between the PDGF Receptor and members of the src family of tyrosine kinases. Cell **62:**481–492.

22. **Liebl, E. C., L. J. England, J. E. DeClue, and G. S. Martin.** 1992. Host range mutants of v-*src*: alterations in kinase activity and substrate interactions. J. Virol. **66:**4315–4324.

23. **Liu, X., S. R. Brodeur, G. Gish, Z. Songyang, L. C. Cantley, A. P. Laudano, and T. Pawson.** 1993. Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. Oncogene **8:**1119–1126.

24. **Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E. Y. Skolnik, D. Bar-Sagi, and J. Schlessinger.** 1992. The SH2 and SH3 domain-containing protein GRB-2 links receptor tyrosine kinases to ras signalling. Cell **70:**431–442.

25. **MacAuley, A., and J. A. Cooper.** 1989. Structural differences between repressed and depressed forms of pp60$^{c\text{-}src}$. Mol. Cell. Biol. **9:**2648–2656.

26. **Marengere, L. E. M., and T. Pawson.** 1992. Identification of residues in GTPase-activating protein src homology 2 domains that control binding to tyrosine phosphorylated growth factor receptors and p62. J. Biol. Chem. **267:**22779–22785.

27. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A.**

Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger. 1989. EGF induces tyrosine phosphorylation of phospholipase C-II; a potential mechanism for EGF receptor signaling. Cell **57:**1101–1107.

28. **Matsuda, M., B. J. Mayer, Y. Fukui, and H. Hanafusa.** 1990. Binding of transforming protein p47gag-crk to a broad range of phosphotyrosine containing proteins. Science **248:**1537–1539.
29. **Mayer, B. J., and D. Baltimore.** 1993. Signaling through SH2 and SH3 domains. Trends Cell Biol. **3:**8–13.
30. **Mori, S., L. Ronnstrand, K. Yokote, S. A. Courtneidge, L. Claesson-Welsh, and C.-H. Heldin.** 1993. Identification of two juxtamembrane autophosphorylation sites in the PDGF β-receptor; involvement in the interaction with Src family tyrosine kinases. EMBO J. **12:**2257–2264.
31. **Morrison, D. K., D. R. Kaplan, S. G. Rhee, and L. T. Williams.** 1990. Platelet-derived growth factor (PDGF) receptor-dependent association of phospholipase C-γ with the PDGF receptor signaling complex. Mol. Cell. Biol. **10:**2359–2366.
32. **O'Brien, M. C., Y. Fukui, and H. Hanafusa.** 1990. Activation of the proto-oncogene $pp60^{c\text{-}src}$ by point mutations in the SH2 domain. Mol. Cell. Biol. **10:**2855–2862.
33. **Pawson, T., and G. D. Gish.** 1992. SH2 and SH3 domains: from structure to function. Cell **71:**359–362.

33a. **Pendergast, A. M., A. J. Muller, M. H. Havlik, Y. Maru, and O. N. Witte.** 1991. BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. Cell **66:** 161–171.

34. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of $pp60^{c\text{-}src}$. Cell **49:**75–82.
35. **Reynolds, A. B., J. Vila, T. J. Lansing, W. M. Potts, M. J. Weber, and J. T. Parsons.** 1987. Activation of the oncogenic potential of the avian cellular src protein by specific structural alteration of the carboxy terminus. EMBO J. **6:**2359–2364.
36. **Rotin, D., B. Margolis, M. Mohammadi, R. J. Daly, G. Dawn, N. Li, E. H. Fischer, W. H. Burgess, A. Ullrich, and J. Schlessinger.** 1992. SH2 domains prevent tyrosine dephosphorylation of the EGF receptor: identification of Tyr 992 as the high affinity binding site for SH2 domains of phospholipase Cγ. EMBO J. **11:**559–567.
37. **Roussel, R. R., S. R. Brodeur, D. Shalloway, and A. P. Laudano.** 1991. Selective binding of activated $pp60^{c\text{-}src}$ by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60c-src. Proc. Natl. Acad. Sci. USA **88:**10696–10700.
38. **Sieh, M., J. B. Bolen, and A. Weiss.** 1993. CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. EMBO J. **12:**315–322.
39. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and L. C. Cantley.** 1993. SH2 domains recognize specific phosphopeptide sequences. Cell **72:**767–778.
40. **Valius, M., C. Bazenet, and A. Kazlauskas.** 1993. Tyrosine 1021 and 1009 are phosphorylation sites in the carboxy terminus of the platelet-derived growth factor receptor β subunit and are required for binding of phospholipase Cγ and a 64-kilodalton protein, respectively. Mol. Cell. Biol. **13:**133–143.
41. **Verderame, M., and H. E. Varmus.** Unpublished data.
42. **Waksman, G., D. Kominos, S. R. Robertson, N. Pant, D. Baltimore, R. B. Birge, D. Cowburn, H. Hanafusa, B. J. Mayer, M. Overduin, M. D. Resh, C. B. Rios, L. Silverman, and J. Kuriyan.** 1992. Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. Nature (London) **358:**646–653.
43. **Waksman, G., S. E. Shoelson, N. Pant, D. Cowburn, and J. Kuriyan.** 1993. Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. Cell **72:**779–790.
44. **Wang, H. 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.
45. **Wood, E. R., O. B. McDonald, and N. Sahyoun.** 1992. Quantitative analysis of SH2 domain binding. J. Biol. Chem. **267:** 14138–14144.