

## Point Mutations in the *abl* SH2 Domain Coordinately Impair Phosphotyrosine Binding In Vitro and Transforming Activity In Vivo

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We have constructed a series of point mutations in the highly conserved FLVRES motif of the *src* homology 2 (SH2) domain of the *abl* tyrosine kinase. Mutant SH2 domains were expressed in bacteria, and their ability to bind to tyrosine-phosphorylated proteins was examined in vitro. Three mutants were greatly reduced in their ability to bind both phosphotyrosine itself and tyrosine-phosphorylated cellular proteins. All of the mutants that retained activity bound to the same set of tyrosine-phosphorylated proteins as did the wild type, suggesting that binding specificity was unaffected. These results implicate the FLVRES motif in direct binding to phosphotyrosine. When the mutant SH2 domains were inserted into an activated *abl* kinase and expressed in murine fibroblasts, decreased in vitro phosphotyrosine binding correlated with decreased transforming ability. This finding implies that SH2-phosphotyrosine interactions are involved in transmission of positive growth signals by the nonreceptor tyrosine kinases, most likely via the assembly of multiprotein complexes with other tyrosine-phosphorylated proteins.

The *src* homology 2 (SH2) domain is an approximately 100-amino-acid region found in a variety of proteins implicated in growth control, including nonreceptor protein-tyrosine kinases, phospholipase C- $\gamma$ , the *ras* GTPase activator protein (GAP), the *crk* oncogene product, and the 85-kDa phosphatidylinositol 3-kinase subunit (p85) (reviewed in reference 29). Because SH2 domains are found in a wide variety of contexts and are not required for catalytic activity, it has been suggested that SH2 domains confer a modular function on the proteins in which they are found (29, 35, 44). Evidence from a number of groups has suggested that SH2 domains bind with high affinity to tyrosine-phosphorylated cellular proteins (1, 32-34, 36, 38, 39, 51). It is thought that the SH2 domains of phospholipase C- $\gamma$ , GAP, and p85 mediate the binding of these proteins to growth factor receptors of the tyrosine kinase class, which rapidly auto-phosphorylate on tyrosine upon ligand binding (1, 32, 39; reviewed in references 29 and 55). While the specific role of SH2-mediated association with growth factor receptors is still under investigation, altered access to substrates or increased enzymatic activity due to phosphorylation by the associated receptor are likely possibilities (16, 26, 40, 41).

In nonreceptor tyrosine kinases, mutations in the SH2 domain modulate biological activity. In most cases, SH2 mutations decrease the transforming activity of activated kinases (references 8, 18, 24, 30, and 58 and references therein), although several point mutations in SH2 have been shown to activate the transforming potential of normally nontransforming proto-oncogene products (18, 42). Furthermore, SH2 mutations in nonreceptor tyrosine kinases have been reported to confer host-dependent transforming activ-

ity (8, 9, 19, 49, 56) and to generate dominant-negative inhibitors of transformation (20), suggesting a role for SH2 interactions with cellular factors. The mechanism whereby SH2 mutations modulate the activity of tyrosine kinase oncogene products is unclear. While the clearest activity of SH2 domains is phosphotyrosine binding, it is not obvious how this property is involved in the regulation of biological activity. The possibility remains that SH2 domains confer a crucial function that is independent of phosphotyrosine binding.

In this work, we have generated a series of conservative point mutations in the SH2 domain of the *abl* oncogene product, a nonreceptor tyrosine kinase (17, 48). We show that several mutations in the highly conserved FLVRES motif drastically reduce in vitro binding to phosphotyrosine itself and to tyrosine-phosphorylated proteins, implicating this region in binding to phosphotyrosine. Reduced binding to tyrosine-phosphorylated proteins in vitro correlates with decreased transforming activity in vivo when the mutations are present in a retrovirally expressed activated *abl* protein. These data suggest that binding to phosphotyrosine is involved in transformation by nonreceptor tyrosine kinases.

### MATERIALS AND METHODS

**Mutagenesis.** The *dut ung* bacterial strain and protocols from the Muta-Gene kit (Bio-Rad) were used to generate oligonucleotide-directed point mutations in the *abl* SH2 domain. For mutants in the glutathione *S*-transferase (GST)-SH2 expression vector, the *Bam*HI-*Eco*RI fragment from wild-type pGEX-SH2 (38) was subcloned into M13mp18, and mutagenesis was performed on uracil-substituted viral DNA. Mutant *Bam*HI-*Eco*RI fragments were excised from M13 replicative-form DNA and reinserted into pGEX-2T (52). For SH2 mutations in full-length *abl*, the 2.5-kbp *Hinc*II fragment containing the SH2 and catalytic domains of *c-abl* was subcloned into pTZ19U (Bio-Rad), and mutagenesis was performed on uracil-substituted phagemid DNA. Mutant

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*HincII* fragments were subcloned into pPL- $\Delta$ XB-HA (see below).

Mutagenic primers were 28 to 51 nucleotides in length with one to three mismatches. All resulting mutants were sequenced to verify their structures.

**Construction and expression of mutant *abl* genes.** Mutant SH2 domains were inserted into pPL- $\Delta$ XB-HA, a derivative of the retroviral expression vector pPL- $\Delta$ XB, which encodes an SH3-deleted type IV *c-abl* (21). pPL- $\Delta$ XB-HA differs from its parental vector by the addition of a C-terminal epitope tag from the influenza virus HA protein (11; details to be published elsewhere). pPL- $\Delta$ XB-K290M-HA differs from pPL- $\Delta$ XB-HA by the presence of a point mutation in the ATP binding site, changing lysine 290 to methionine (23).

pPL- $\Delta$ XB-HA-derived plasmids were transfected into NIH 3T3 fibroblasts by calcium phosphate coprecipitation along with pZAP, which encodes a Moloney murine leukemia virus helper, as described previously (21). Briefly, 10  $\mu$ g of *abl*-encoding plasmid and 0.5  $\mu$ g of pZAP were mixed in calcium phosphate buffer, and dilutions of the mixture were transfected onto duplicate plates. Foci were scored from the 1:5 dilution plates 12 days posttransfection. Three independent plasmid isolates were analyzed for the V170L mutant, two were analyzed for R171K and S173C, and one was analyzed for the wild type (wt), E172Q, E174Q, and S175C.

For transient transfections, 4  $\mu$ g of pPL- $\Delta$ XB-HA-derived plasmids was transfected by calcium phosphate into subconfluent human 293 cells, and lysates were made 44 h posttransfection.

**Bacterial expression.** All SH2 domains were expressed in *Escherichia coli* NB42 by using the GEX expression system and purified by using glutathione-agarose as described previously (52). To generate cleaved SH2 peptides for nuclear magnetic resonance (NMR) spectroscopy, bacterial lysates containing GST-SH2 fusion proteins were incubated with glutathione-agarose beads (Molecular Probes), and then the beads were washed with TN buffer (50 mM Tris [pH 8.0], 150 mM NaCl) and incubated at room temperature for 2 h in TN buffer with 2.5 mM CaCl<sub>2</sub> and 125 U of human thrombin (Calbiochem) per liter of original bacterial culture. The eluate and wash from the cleaved beads were concentrated with a Centriprep-10 (Amicon), and contaminating proteins were removed by gel filtration on Superose-12 (Pharmacia).

**Cloning and expression of GAP SH2.** The N-terminal SH2 domain of murine GAP was cloned from NIH 3T3 total RNA by using reverse transcription and polymerase chain reaction. The amplified fragment contained sequences corresponding to amino acids 175 to 280 of human GAP (54) as well as N-terminal *Bam*HI and C-terminal *Eco*RI sites in the proper reading frame and orientation for expression from pGEX-2T. The amplified fragment was digested with *Eco*RI and *Bam*HI and cloned into pGEX-2T, and the resulting clones were sequenced to confirm their identities. The murine SH2 sequence encodes a protein that is identical in amino acid sequence to the published human clone.

**SH2 binding assays.** Phosphotyrosine-agarose and phosphoserine-agarose were made by coupling phosphotyrosine or phosphoserine (Sigma) to Affi-Gel 15 beads (Bio-Rad) according to the manufacturer's recommendations. Briefly, 20 mM phosphoamino acids were prepared in 100 mM morpholine propanesulfonic acid (MOPS; pH 7.5), 50  $\mu$ mol of phosphoamino acid was added per ml of Affi-Gel, and the mixture was incubated at room temperature for 2 h. To assay binding of GST-SH2 fusion proteins to phosphotyrosine, 20  $\mu$ g of fusion protein in 100  $\mu$ l of phosphate-buffered saline (PBS) plus 1% Triton X-100 was added to 20  $\mu$ l of phos-

phoamino acid-agarose or 10  $\mu$ l of glutathione-agarose bead slurry (50% beads by volume). The mixture was incubated at 4°C for 40 min, then the beads were quickly washed twice with 1 ml of cold PBS and resuspended in sample buffer, and half of the sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13% polyacrylamide gels).

Filter-binding assays were performed with biotinylated GST-SH2 fusion proteins (1  $\mu$ g/ml) as described previously (38) except that 1% ovalbumin was used for blocking and peptide binding. All incubations and washes were carried out at 4°C, and filters were incubated with biotinylated SH2 peptides overnight. For titration experiments, tyrosine-phosphorylated proteins were purified from an *abl*-transformed pre-B cell line ( $\Delta$ XB-BM [20a, 21]) by immunoaffinity chromatography on monoclonal antibody PY20-agarose beads as described previously (14, 15). Binding assays were performed as described above at various GST fusion peptide concentrations, with the exceptions that the biotinylated probes were diluted fivefold with unbiotinylated peptide to decrease their specific activity and dithiothreitol was present at 10 mM in the blocking and binding reactions. The GST-SH2 fusion proteins were labeled with biotin as described previously (38) under identical conditions. To ensure that labeling efficiencies were similar, the relative specific activity of the biotinylated probes was assessed by side-by-side comparison of both the intensity of Coomassie blue staining and labeling with avidin-conjugated alkaline phosphatase. In all cases, the specific activities were indistinguishable.

**Immunoblotting.** Immunoblotting was performed by standard protocols as described elsewhere (38), using monoclonal antibodies PY20 (15), 19-84 (50), and 12CA5 (11), which recognize phosphotyrosine, *abl* protein, and influenza virus HA protein epitope tag, respectively. Bound antibody was detected with alkaline phosphatase-conjugated anti-mouse immunoglobulins.

For coprecipitation experiments, 293 cells were lysed in Triton X-100 lysis buffer containing 1 mM sodium orthovanadate, and equal amounts of protein were immunoprecipitated with polyclonal anti-*abl* serum pEX-4 (31). Immune complexes were collected on protein A-Sepharose beads and washed three times with lysis buffer, and aliquots were immunoblotted and probed with monoclonal antibody PY20 or 19-84 as described above.

**NMR spectroscopy.** NMR spectra were acquired on a General Electric OMEGA 500-MHz NMR system operating at 500.115 MHz at 25°C. Native samples (see Fig. 6A) were approximately 1 mM in PBS plus 10% D<sub>2</sub>O. A spectral width of 7,017.54 Hz was used with a water-presaturating single-pulse sequence of 1.3-s presaturation and an acquisition time of 1.17 s in 8,192 data locations for 512 cycles. The wt sample contained approximately 200  $\mu$ M 3-(trimethylsilyl)-1-propanesulfonate, whose principal resonance appears at 0.0 ppm in the upper spectrum of Fig. 6A. Spectra were apodized by applying an exponential function with a line broadening of 2 Hz. The denatured sample (see Fig. 6B) was 110  $\mu$ M wt *abl* SH2-6 M guanidinium-HCl in 99.96% D<sub>2</sub>O. A total of 2,048 scans were acquired with a sweep width of 6,006 Hz. No apodization function was applied.

## RESULTS

We have previously shown that the *abl* SH2 domain, when expressed in *E. coli* as a fusion protein with GST, binds with high affinity to tyrosine-phosphorylated cell proteins (38). To

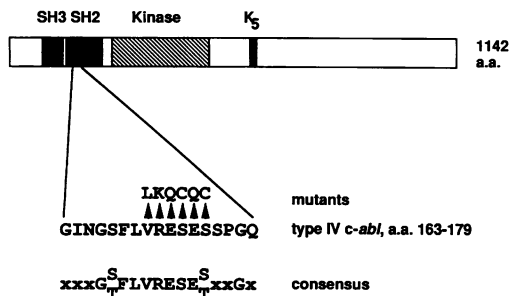


FIG. 1. SH2 mutations used in this study. The diagram at the top is a schematic representation of type IV *c-abl* protein, showing to scale the locations of the SH3 and SH2 domains, conserved kinase catalytic domain, and major nuclear localization signal ( $K_5$ ). Below is shown the sequence of the *abl* SH2 domain in the vicinity of the highly conserved FLVRES motif. Amino acids (a.a.) 170 through 175 were individually mutated to the those shown in the line above (arrowheads). The consensus sequence consists of amino acids present in at least 50% of all SH2 domains; X denotes less-conserved residues.

identify residues critical for phosphotyrosine binding and to probe the biological relevance of this property to *abl* gene function, we generated a series of conservative amino acid changes within the SH2 domain. We chose to mutate residues within the FLVRES motif of SH2, because this is the best-conserved region among all SH2 domains (29). Oligonucleotide-directed mutagenesis was used to create single amino acid changes designed to maintain the approximate size and hydrophilicity of the wt residues. The six mutants used in this study are shown in Fig. 1. These mutations were initially constructed into the GST expression vector to study the properties of the isolated, bacterially expressed SH2 domains in vitro.

We first examined whether the mutant SH2 domains bound to phosphotyrosine itself. We have previously shown that a fraction of tyrosine-phosphorylated proteins bound to the wt *abl* SH2 domain could be eluted with high concentrations of phosphotyrosine (38), suggesting that phosphotyrosine is directly involved in binding; other studies, however, have found no effect of phosphotyrosine (33). When the bacterially expressed GST-SH2 fusion protein was incubated with agarose beads covalently linked to phosphotyrosine, a significant fraction bound to the beads, while none bound to similar beads linked to phosphoserine (Fig. 2B and C, lanes 2). GST protein lacking SH2 sequences did not bind (Fig. 2B, lane 1). These results demonstrate that the *abl* SH2 domain can bind directly and stably to phosphotyrosine and not to phosphoserine under these conditions. From experiments in which phenyl phosphate, a phosphotyrosine analog, was used to block association of tyrosine-phosphorylated cell proteins with the *abl* SH2 domain, we estimate the  $K_i$  for phenyl phosphate to be between 1 and 10 mM (not shown). This low-affinity binding to phosphotyrosine is probably a common property of functional SH2 domains, because we have also observed binding to phosphotyrosine beads for bacterially expressed *src* and GAP SH2 domains (not shown).

When the mutant *abl* SH2 domains were examined for phosphotyrosine binding, three of the mutants were indistinguishable from wt: V170L, E172Q, and E174Q (by convention, mutants are designated by wt amino acid, residue number, and mutant amino acid) (Fig. 2B). Three of the mutants, R171K, S173C, and S175C, showed no detectable

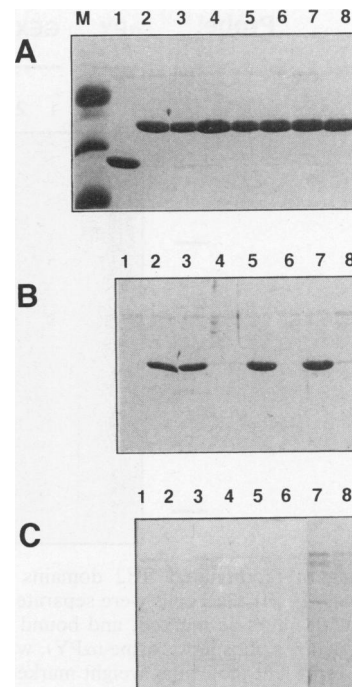


FIG. 2. Binding of bacterially expressed SH2 domains to phosphotyrosine-agarose. Purified GST-SH2 fusion proteins were incubated with glutathione-agarose (A), phosphotyrosine-agarose (B) or phosphoserine-agarose (C), and the bound fraction was separated by SDS-PAGE and visualized by staining with Coomassie blue. Lanes: M, molecular weight markers (45, 29, and 18 kDa apparent molecular mass); 1, GST alone; 2, wt *abl* SH2; 3, V170L; 4, R171K; 5, E172Q; 6, S173C; 7, E174Q; 8, S175C.

binding to phosphotyrosine, suggesting that these mutations affect residues directly involved in interaction with phosphotyrosine. The fusion proteins were all present at the same concentration and could be bound by glutathione agarose with the same efficiency (Fig. 2A). The arginine at wt position 171 of *abl* is absolutely conserved in all known SH2 domains; substitution of lysine at this position (which maintains the positive charge) eliminated stable phosphotyrosine binding, suggesting that the specific structure of this arginine residue is critical to the binding interaction.

To examine whether the SH2 mutants retained high-affinity binding to tyrosine-phosphorylated cell proteins, we used biotinylated GST-SH2 fusion proteins at a low concentration (approximately 25 nM) to bind to total protein from *abl*-transformed fibroblasts which had been separated by SDS-PAGE and transferred to nitrocellulose filters (38). Consistent with results from binding to phosphotyrosine, three mutants (V170L, E172Q, and E174Q) bound to tyrosine-phosphorylated proteins as well as did the wt, while three mutants (R171K, S173C, and S175C) showed no specific binding (Fig. 3, lanes 1). No changes were seen in the profile of binding to various tyrosine-phosphorylated proteins for any of the mutants that retained activity; like wt SH2, these mutants bound well to a subset of tyrosine-phosphorylated proteins and relatively poorly to others, such as *abl* protein itself and a prominent protein of approximately 34 kDa. As is the case with wt SH2, all of the mutants specifically bound few proteins in lysates from untransformed 3T3 cells, in which the levels of tyrosine-phosphorylated proteins are low (Fig. 3, lanes 2).

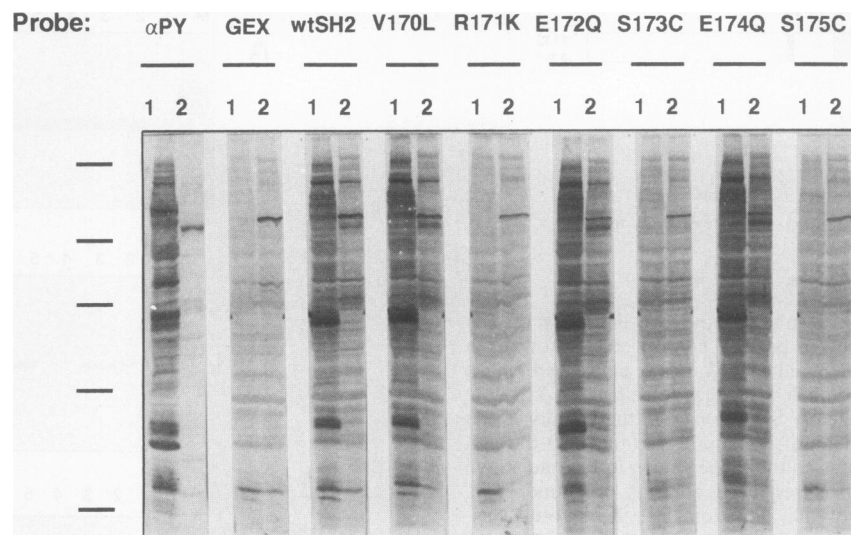


FIG. 3. Binding of biotinylated SH2 domains to tyrosine-phosphorylated cell proteins. Lysates from *abl*-transformed (lane 1) or untransformed (lane 2) NIH 3T3 cells were separated by SDS-PAGE and transferred to nitrocellulose. Biotinylated GST-SH2 fusion proteins were incubated with filters as marked, and bound probe was detected with avidin-conjugated alkaline phosphatase. PY20, a monoclonal antibody that recognizes phosphotyrosine ( $\alpha$ PY), was used to detect total tyrosine-phosphorylated proteins. GEX, GST protein lacking SH2 sequences. Bars represent molecular weight markers (196, 106, 71, 44, and 28 kDa apparent molecular mass).

A more sensitive assay was used to determine whether any of the mutants retained a low level of residual binding activity. We purified tyrosine-phosphorylated proteins from *abl*-transformed murine bone marrow cells by immunoaffinity chromatography, blotted these proteins to nitrocellulose, and assayed binding of the mutant SH2 domains. Biotinylated SH2 fusion proteins were incubated with filters at concentrations ranging from 0.1 to 62.5  $\mu$ g/ml (approximately 2.5 nM to 1.5  $\mu$ M). Titration experiments such as these give an approximate value for the association constant for various phosphoproteins (the concentration at which binding is half-maximal). In the case of wt *abl* SH2, the apparent half-maximal binding value was less than 100 nM for some proteins, and the V170L, E172Q, and E174Q mutants were indistinguishable from wt (Fig. 4). The S173C and S175C mutants also bound specifically to tyrosine-phosphorylated proteins, but only when present at high concentrations. Thus, these mutations decrease the apparent binding affinity by at least 2 orders of magnitude but do not completely eliminate binding. Little specific binding was seen with the R171K mutant, even at the highest concentration examined.

While several of the SH2 mutants shifted the binding curve drastically, none of the mutants appeared to significantly change the relative affinity for various tyrosine-phosphorylated proteins. To show that the assay would detect such differences in binding specificity, we used a biotinylated GST fusion protein containing the N-terminal SH2 domain of GAP (54, 57). The profile of phosphoprotein binding was quite different when GAP was compared with *abl* (Fig. 5, lanes 1 to 5; compare with profile of wt *abl* in Fig. 4). In particular, the apparent affinity for a 68-kDa protein was much higher for the GAP SH2, while the apparent affinity for 34- and 36-kDa proteins was somewhat higher for the *abl* SH2. This finding is consistent with the results of Moran et al., who found that in solution, different SH2 domains have different apparent specificities and that the GAP SH2 has high affinity for a 62-kDa protein (39). In the

concentration range examined, neither the *abl* or GAP SH2 domain bound to all the tyrosine-phosphorylated proteins detected by an antiphosphotyrosine antibody (Fig. 5, lane 6). From this result, we conclude that the filter-binding assay is able to detect relative affinity differences and that the FLVRES mutations most likely affect only binding to phosphotyrosine and not the interactions that determine the profile of relative binding to specific tyrosine-phosphorylated proteins.

A potential pitfall of mutagenesis experiments is that even conservative amino acid changes can have global effects on protein conformation, leading to incorrectly folded or unfolded mutant proteins. All of the bacterially expressed SH2 mutants used in this study were as soluble as wt, suggesting that there were no gross abnormalities in folded structure (in contrast, several mutant *abl* SH2 domains with more drastic alterations were clearly less soluble than wt [37a]). NMR spectroscopy is a very sensitive probe for protein conformation (59). We therefore prepared wt and R171K mutant fusion proteins, cleaved them from the GST fusion partner, purified the SH2 domains to homogeneity, and obtained one-dimensional NMR spectra. The upfield portions of the two spectra were virtually superimposable, with only a few minor differences observed in the chemical shifts (Fig. 6A). This region of the NMR spectrum contains resonances from methyl and other aliphatic protons, which are normally found in the hydrophobic core of the polypeptide; their shifts are perturbed by the ring current effects of internalized aromatic residues. Any major change in folded structure would be expected to cause drastic changes in the spectrum. The similarity of the two spectra in this upfield fingerprint region demonstrates that the R171K mutant has a folded structure similar to that of the wt SH2 domain. To give a sense of the magnitude of changes in the NMR spectrum induced by unfolding, we also present the spectrum of the wt *abl* SH2 domain in 6 M guanidinium hydrochloride. As seen in Fig. 6B, many of the dispersed signals of the spectrum are now absent and there is no recognizable similarity with the

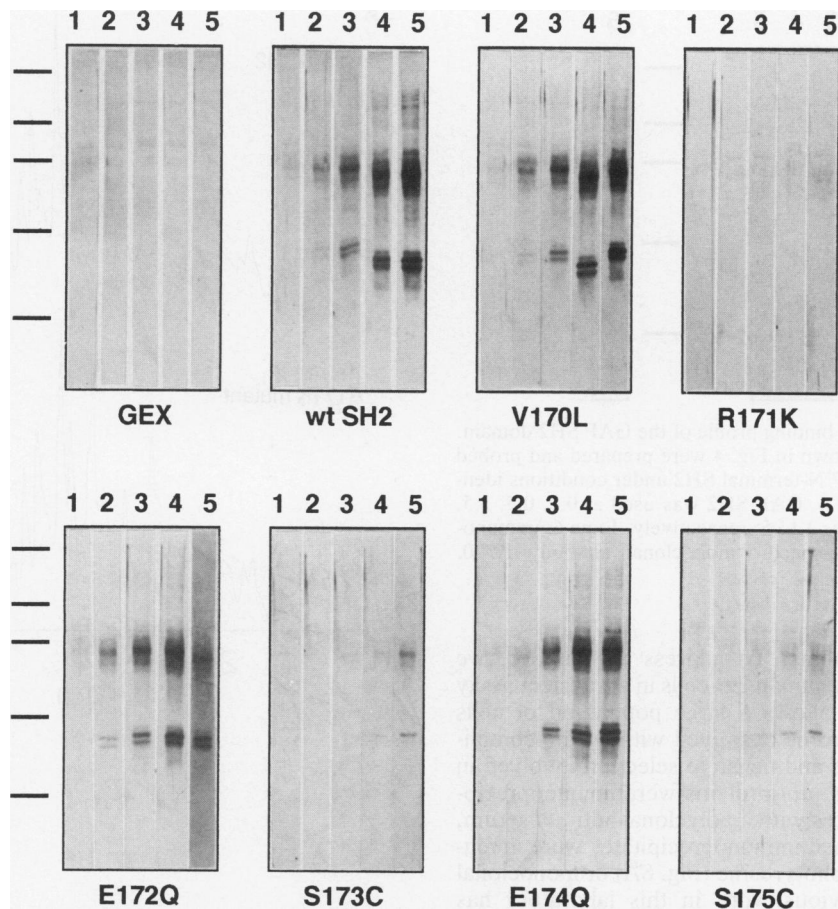


FIG. 4. Binding of mutant SH2 domains to purified tyrosine-phosphorylated proteins. Tyrosine-phosphorylated proteins were purified from *abl*-transformed murine bone marrow cells, separated by SDS-PAGE, and blotted to identical nitrocellulose strips. Strips were incubated with biotinylated GST-SH2 fusion proteins as marked, at concentrations of 0.1  $\mu\text{g/ml}$  (lane 1), 0.5  $\mu\text{g/ml}$  (lane 2), 2.5  $\mu\text{g/ml}$  (lane 3), 12.5  $\mu\text{g/ml}$  (lane 4), or 62.5  $\mu\text{g/ml}$  (lane 5), and detected with avidin-conjugated alkaline phosphatase. Markers are as in Fig. 3.

native spectrum, even though the samples are identical in primary structure. The diminished phosphotyrosine binding of the mutant is therefore probably not due to a substantial disruption of protein conformation but is more likely due to specific alterations in the binding region.

To examine the role of phosphotyrosine binding in the transforming activity of *abl*, the various FLVRES point mutations were constructed into an activated *abl* gene. A type IV *c-abl* gene containing a deletion of the SH3 domain was used, which is highly transforming for both fibroblasts and pre-B cells (21). An epitope tag from influenza virus was appended to the C terminus to facilitate detection of the protein with a highly specific monoclonal antibody (11); the epitope tag does not significantly affect transforming activity (20a). The various mutant *abl* genes were inserted into a retroviral expression vector and transfected into NIH 3T3 fibroblasts along with helper virus. The three mutants containing SH2 domains with wt phosphotyrosine-binding activity in vitro were similar to wt in focus-forming activity (decreased by less than twofold relative to wt), while the mutants containing SH2 domains impaired in phosphotyrosine binding had significantly decreased focus-forming activity (35-, 10-, and 4-fold relative to wt for R171K, S173C, and S175C, respectively) (Table 1). The relative decrease in transforming activity roughly paralleled the decrease in in vitro phosphotyrosine binding, in that little or no binding

was seen with R171K, while some binding was evident with S173C and S175C, especially the latter (Fig. 4). The few transformed foci from the R171K mutant were smaller and more fusiform than wt foci and were difficult to establish as cell lines. Cells transformed by mutant S173C were also morphologically distinguishable from wt, while the other mutants gave foci with morphology and growth properties similar to those of wt (not shown). All of the mutants had greater transforming activity than normal (unactivated) *c-abl*, which has undetectable focus-forming activity in this assay (Table 1).

A possible explanation for the decreased transforming activity of the *abl* genes containing mutant SH2 domains could be a defect in protein production. Plates transfected with the various mutants were maintained for 25 days to allow viral spread, and lysates were prepared and immunoblotted to detect *abl* proteins. Roughly comparable amounts of the mutant proteins were detected (Fig. 7; compare lanes 1 to 6 with lanes 7), using antibodies that recognize the influenza virus epitope tag (Fig. 7A) or *abl* protein (Fig. 7B). In vitro kinase assays performed on immunoprecipitated mutant proteins demonstrated that all mutants also had similar kinase activities (data not shown).

If the defect in the less-transforming mutants was a failure to associate with tyrosine-phosphorylated proteins, this difference should be apparent in an analysis of proteins bound

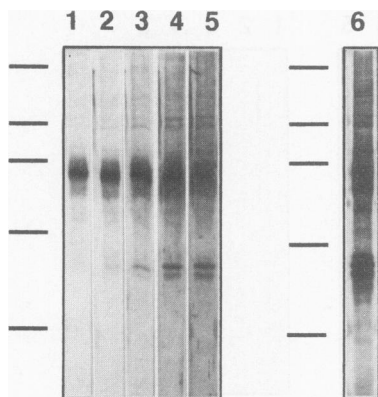


FIG. 5. Phosphoprotein binding profile of the GAP SH2 domain. Strips identical to those shown in Fig. 4 were prepared and probed with biotinylated GST-GAP N-terminal SH2 under conditions identical to those used for Fig. 4. GAP SH2 was used at 0.1, 0.5, 2.5, 12.5, and 62.5  $\mu\text{g/ml}$  in lanes 1 to 5, respectively. Lane 6, immunoblot with phosphotyrosine-specific monoclonal antibody PY20. Markers are as in Fig. 3.

to the mutant *abl* proteins. To address this matter, we expressed the mutants in human 293 cells in a transient assay system. This approach allows a large population of cells expressing the mutants to be examined without the complicating effects of positive and negative selection involved in isolating clonal cell lines. *abl* proteins were immunoprecipitated from 293 cell lysates with a polyclonal anti-*abl* serum, and aliquots of the washed immunoprecipitates were immunoblotted with antiphosphotyrosine (Fig. 8A) or monoclonal anti-*abl* (Fig. 8B). Previous work in this laboratory has demonstrated that transforming *abl* proteins coimmunoprecipitate with a 65- to 68-kDa protein that comigrates with the major tyrosine-phosphorylated protein that associates with GAP (10, 39, 50a). This protein also coimmunoprecipitated with the FLVR mutants that retain *in vitro* binding to phosphotyrosine (Fig. 8A, lanes 3, 5, and 7). By contrast, those mutants with decreased *in vitro* binding coimmunoprecipitated little if any 65- to 68-kDa protein (Fig. 8A, lanes 4, 6, and 8). Blotting with *abl*-specific antiserum showed that similar levels of mutant *abl* proteins were present in all immunoprecipitates (Fig. 8B). Blotting of whole cell lysates with antiphosphotyrosine showed that phosphotyrosine levels were increased in 293 cells expressing all FLVR mutants (data not shown). A kinase-inactive *abl* mutant with a point mutation in the ATP binding site also coimmunoprecipitated a small amount of 65- to 68-kDa protein (Fig. 6A, lane 2), presumably via its intact SH2 domain, even in the absence of increased phosphotyrosine due to *abl* kinase activity.

## DISCUSSION

We have demonstrated in this study that point mutations in the highly conserved FLVRES motif of the *abl* SH2 domain drastically decreased SH2 binding to phosphotyrosine and tyrosine-phosphorylated proteins *in vitro*. Decreased binding was paralleled by a coordinate decrease in the transforming activity of activated *abl* genes containing the SH2 mutations. Using NMR spectroscopy, we were able to show that the folded structure of the mutant most impaired in its ability to bind phosphotyrosine was not drastically affected by the mutation. These results strongly implicate phosphotyrosine binding, and not some unknown

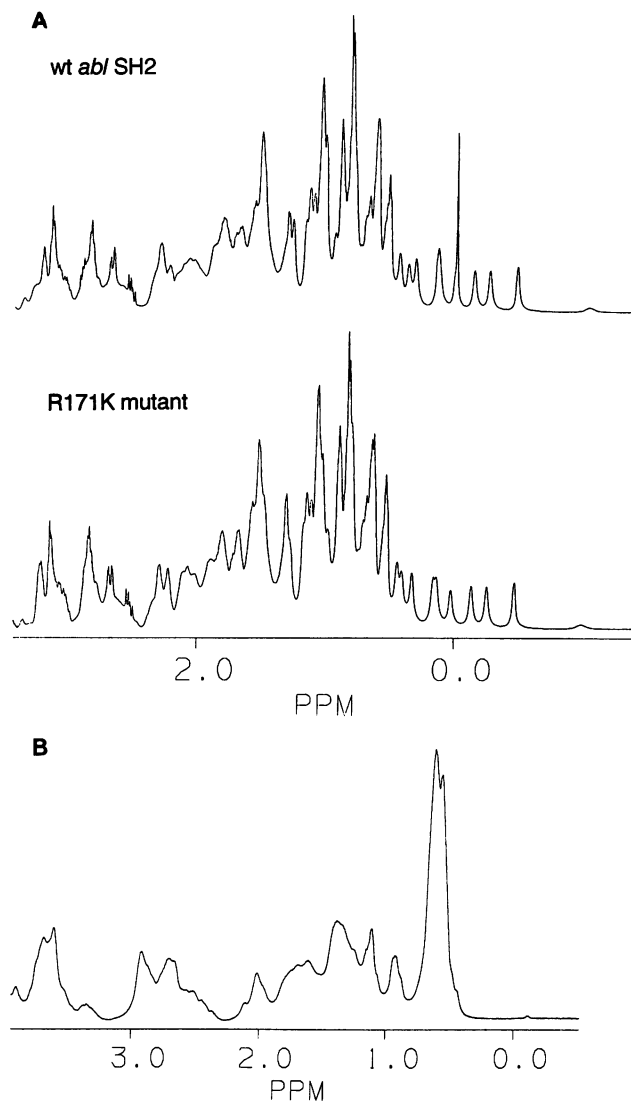


FIG. 6. One-dimensional NMR spectra of purified wt *abl* and R171K mutant SH2 domains. The upfield fingerprint region of each spectrum is shown. (A) Spectra of native wt and R171K mutant SH2 domains in PBS. The sharp peak at 0.0 ppm in the wt spectrum is the 3-(trimethylsilyl)-1-propanesulfonate chemical shift standard. (B) Spectrum of the wt SH2 domain in 6 M guanidinium hydrochloride.

function of SH2 domains, in modulation of the biological activity of nonreceptor tyrosine kinases. Our results also suggest that *in vitro* studies with isolated, bacterially expressed SH2 domains, which are easily manipulated and whose binding interactions can be analyzed quantitatively, can be extrapolated to the behavior of SH2 domains in intact proteins.

The region of the SH2 domain that we chose to mutate, the FLVRES motif, is very highly conserved among all SH2 domains (over 30 to date [29]). We propose that SH2 binding to tyrosine-phosphorylated peptides involves two distinct interactions: a low-affinity binding to phosphotyrosine itself, mediated at least in part by the FLVRES motif, and subsequent interaction with peptide residues surrounding the phosphotyrosine, in which nonconserved SH2 amino acids confer high-affinity binding. We have found that SH2 domains interact with phosphotyrosine and phenyl phosphate

TABLE 1. Properties of *abl* SH2 mutants

Mutant	Phosphotyrosine binding <sup>a</sup>	Phosphoprotein binding <sup>b</sup>	Transforming activity <sup>c</sup>
wt <sup>d</sup>	+	+	2,310 (1)
V170L	+	+	1,350 (1.7)
R171K	-	-	66 (35)
E172Q	+	+	1,286 (1.8)
S173C	-	+/-	225 (10.3)
E174Q	+	+	1,425 (1.6)
S175C	-	+/-	563 (4.2)
c- <i>abl</i> <sup>e</sup>	+	+	<1 (>2,000)

<sup>a</sup> Binding of purified GST-SH2 fusion protein to phosphotyrosine-agarose.

<sup>b</sup> Binding of biotinylated GST-SH2 fusion protein to purified tyrosine-phosphorylated cell proteins in a filter-binding assay.

<sup>c</sup> Number of morphologically transformed foci per dish scored 12 days posttransfection; fold decrease relative to the activated *abl* gene with the wt SH2 domain is given in parentheses.

<sup>d</sup> Activated *abl* containing an SH3 deletion and C-terminal influenza virus epitope tag (see text).

<sup>e</sup> Normal type IV c-*abl*.

directly, but that the apparent dissociation constant for phenyl phosphate is in the millimolar range (Fig. 2 and data not shown). In other experiments, we have found a similar low affinity for a nonspecific tyrosine-phosphorylated peptide (not shown). The affinity for some tyrosine-phosphorylated proteins is much higher; binding of *abl* or GAP SH2 domains to specific tyrosine-phosphorylated proteins on filters (Fig. 4 and 5) or to autophosphorylated epidermal growth factor receptor in a solid-phase enzyme-linked immunosorbent assay (2) give apparent half-maximal binding values of 1 to 10 nM for some binding sites. We have not observed binding of SH2 domains to unphosphorylated proteins, suggesting that the low-affinity interaction with phosphotyrosine is required to initially position the two proteins so that contacts conferring high-affinity binding can form. However, Pendergast et al. have found recently that the *abl* SH2 domain binds with high affinity in vitro to a region of the BCR protein, which is fused to *abl* via chromosomal translocation in some human leukemias (45). Binding occurred in the absence of detectable phosphotyrosine on the BCR protein but was shown to be dependent on phosphorylation. It will be of interest to determine whether the FLVR mutations described here, which disrupt phosphotyrosine-dependent binding, also disrupt interaction with BCR.

An important implication of this work is that data demonstrating the binding of SH2 domains to specific tyrosine-phosphorylated proteins must be evaluated critically. Because SH2 domains can bind to phosphotyrosine itself, albeit with low affinity, it follows that any SH2 domain has the potential to bind to any tyrosine-phosphorylated protein if the concentrations are high enough. Titration experiments on nitrocellulose filters (Fig. 4 and 5) show that binding is relatively specific at low SH2 concentrations, with a wider range of phosphoproteins binding at higher SH2 concentrations. The local concentration of SH2 domains and tyrosine-phosphorylated proteins, coupled with the relative affinities of the SH2 domains for various proteins, will determine what interactions are kinetically relevant in vivo. Furthermore, the accessibility and orientation of SH2 domains, which could be sequestered in intramolecular interactions, may be important.

The properties of the FLVRES mutants reported here provide clues to the nature of the SH2-phosphotyrosine

binding interaction. The observation that replacement of the invariant arginine at position 171 drastically diminishes phosphotyrosine binding suggests an important role for this residue. Positive charges on the SH2 domain might be expected to interact electrostatically with the negatively charged phosphate group. While the R171K mutation maintains the positive charge, the specific orientation or charge distribution of arginine is apparently important for stable binding. The abolition of activity on substitution of arginine by lysine has been observed in several other systems, most recently RNA recognition (4). Others have noted that there are two other relatively invariant basic amino acids in the SH2 domain, corresponding to arginine 153 and histidine 192 of *abl*, which might also be involved in phosphate binding (29). In the *crk* oncogene product (which does not itself encode a tyrosine kinase domain), mutation of the FLVRES arginine or the invariant histidine abolished transforming activity and decreased binding to tyrosine-phosphorylated proteins (34, 37).

We have shown that for activated *abl* kinase, transforming activity correlates with the phosphotyrosine-binding capacity of the SH2 domain that it encodes. Our results are generally consistent with previous observations in which SH2 mutations in *src* and *fps* decreased the transforming activity of the activated kinases (8, 18, 24, 30, 58). We cannot conclude that phosphotyrosine binding is absolutely required for transformation by *abl* because the R171K mutant retains a low level of morphological transforming activity. In the case of *src* and *fps*, it has been clearly shown that deletions spanning the FLVRES motif decrease but do not necessarily abolish transforming activity (3, 8, 27, 30, 43, 58). It is possible that there are multiple modes of transformation by tyrosine kinases, a major subset of which are dependent on SH2-phosphotyrosine interactions.

Previous studies of mutations in the SH2 domains of

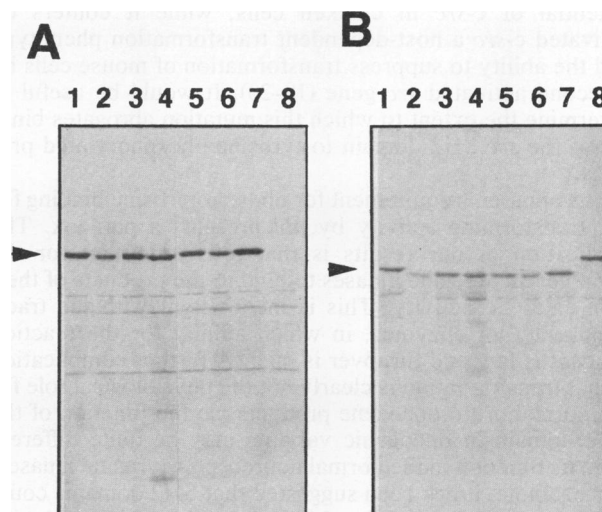


FIG. 7. Expression of *abl* proteins with mutant SH2 domains. Lysates were prepared from NIH 3T3 cells transfected with retroviral vectors containing activated *abl* genes harboring SH2 point mutations. Total cell protein (50  $\mu$ g per lane) was immunoblotted and detected with monoclonal antibody 12CA5 to the influenza virus epitope present on the *abl* protein (A) or monoclonal 19-84, which recognizes *abl* protein itself (B). Arrowheads denotes positions of *abl*-specific bands. Lanes: 1, V170L; 2, R171K; 3, E172Q; 4, S173C; 5, E174Q; 6, S175C; 7, wt activated *abl*; 8, wt activated *abl* lacking the epitope tag.

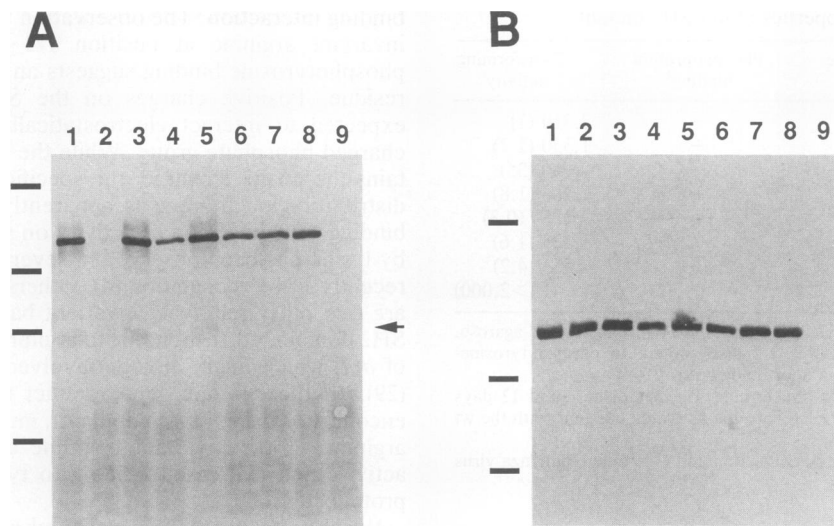


FIG. 8. Coimmunoprecipitation of tyrosine-phosphorylated proteins with *abl* SH2 mutants. One milligram of lysate from transiently transfected 293 cells was immunoprecipitated with polyclonal *abl* antiserum (pEX-4), and aliquots of washed immunoprecipitates were immunoblotted with monoclonal antiphosphotyrosine antibody PY20 (A) or anti-*abl* antibody 19-84 (B). Gels were 10% (A) and 7.5% (B) acrylamide. Lanes: 1, wt activated *abl*; 2, K290M kinase-defective mutant; 3, V170L; 4, R171K; 5, E172Q; 6, S173C; 7, E174Q; 8, S175C; 9, mock transfected. Arrow in panel A indicates major coprecipitating phosphoprotein. Bars represent molecular weight markers (in panel A, 196, 106, 71, and 44 kDa apparent molecular mass; in panel B, 196, 106, and 71 kDa).

tyrosine kinase oncogenes have been complicated by a lack of data about the specific effects of the mutations. Several mutations of *src* and *fps* in the vicinity of the FLVRES sequence confer interesting properties, but it is not known whether these mutations diminish phosphotyrosine binding, alter other SH2-protein interactions, or result in global conformation changes. For example, a mutation in the *src* SH2 domain changing the invariant arginine (corresponding to position 171 in *abl*) to leucine activates the transforming potential of *c-src* in chicken cells, while it confers on activated *c-src* a host-dependent transformation phenotype and the ability to suppress transformation of mouse cells by a second activated *src* gene (18–20). It would be useful to determine the extent to which this mutation abrogates binding of the *src* SH2 domain to tyrosine-phosphorylated proteins.

The apparent requirement for phosphotyrosine binding for full transforming activity by *abl* presents a paradox. The implication of our results is that it is important for the nonreceptor tyrosine kinases to bind to the products of their own catalytic activity. This is inconsistent with the traditional view of enzymes, in which affinity for the reaction product is low and turnover is high. A further complication is that transformation is clearly not the physiological role for the normal proto-oncogene products, so the function of the SH2 domain in oncogenic variants may be quite different from its function in the normal nonreceptor tyrosine kinases. For example, it has been suggested that SH2 domains could regulate catalytic activity by interaction with regulatory phosphorylation sites on the kinases themselves (5, 29, 30, 42). For *v-src* and activated *abl*, this is clearly not the case, because the major sites of tyrosine autophosphorylation can be eliminated without greatly diminishing transforming activity (6, 22, 53). But because SH2 mutations have been shown, in contrast, to partially activate the transforming activity of *c-src* (18, 42), it is possible that intramolecular binding is in some cases a normal role for SH2 domains. Consistent with this, in weakly activated *c-src* variants,

phosphorylation of a catalytic site tyrosine plays a positive role in transforming activity (28, 46).

A potential role for SH2 domains in transformation is in localizing the active kinase to a specific subcellular compartment or multiprotein complex. N-terminal myristoylation of the *src* or *abl* oncogene product is in most cases essential for transforming activity, even though the nonmyristoylated kinases retain high catalytic activity (6, 21, 23, 25). This implies that localization plays a critical role in the transforming activity of activated nonreceptor kinases. Once directed to the membrane by the myristoylated N terminus, the activated kinases might phosphorylate membrane-associated substrates and subsequently bind tightly to them via the SH2 domain; assembly of this complex would facilitate the transmission of positive growth signals. Consistent with this view, it has been shown that an intact SH2 domain is required for association of *v-src* protein with the detergent-insoluble matrix, the p85 phosphatidylinositol kinase subunit, and an unidentified 130-kDa protein (12, 13, 47).

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