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The noncatalytic *src* homology region 2 segment of *abl* tyrosine kinase binds to tyrosine-phosphorylated cellular proteins with high affinity

(phosphotyrosine/signal transduction/transformation/oncogenes)

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ABSTRACT **Several proteins implicated in the regulation of cell proliferation contain a common noncatalytic domain, *src* homology region 2 (SH2). We have used the bacterially expressed SH2 domain of *abl* protein-tyrosine kinase to evaluate the ability of this domain to bind to cellular proteins. *abl* SH2 specifically bound to a number of tyrosine-phosphorylated proteins from cells transformed by tyrosine kinase oncogenes in a filter-binding assay and to a subset of those proteins in solution. The SH2 probe bound almost exclusively to tyrosine-phosphorylated proteins, and binding was eliminated by dephosphorylation of cell proteins. Free phosphotyrosine could partially disrupt SH2 binding, suggesting that phosphotyrosine is directly involved in the binding interaction. These results demonstrate that an SH2 domain is sufficient to confer direct, high-affinity phosphotyrosine-dependant binding to proteins and suggest a general role for SH2 domains in cellular signaling pathways.**

The *abl* gene product is a member of the nonreceptor class of protein-tyrosine kinases and can induce malignant transformation when altered during retroviral transduction or chromosomal translocation (1–4). The amino terminus of the *abl* kinase contains an 80-amino acid domain, termed *src* homology region 2 (SH2) (5, 6) that is found in all known nonreceptor tyrosine kinases, a phosphatidylinositol-specific phospholipase C (PLC-γ), the *ras* GTPase activator protein (GAP), and the *crk* oncogene product (7–12); each of these proteins has been implicated in growth-control pathways. SH2 domains are not required for catalytic activity of enzymes in which they are found (12–16), but mutations in this domain can have profound effects on biological activity (6, 17–20). PLC-γ, GAP, and the *src* family of tyrosine kinases have recently been shown to bind to growth-factor receptors after ligand binding (21–26), and the *crk* protein binds phosphotyrosine-containing proteins and protein kinase activities (27, 28), raising the possibility that SH2 domains might mediate protein–protein interactions important to growth control. We therefore investigated whether the isolated SH2 domain of *abl* could specifically bind to cellular proteins *in vitro*.

MATERIALS AND METHODS

Generation of Bacterially Expressed SH2 Probe. A *Bam*HI 12-mer linker was inserted at the *Hinc*II site at amino acid 144 of type IV c-*abl*, and the 0.3-kilobase (kb) *Bam*HI–*Hin*PI fragment was subcloned into pGEX-2T (29) cut with *Bam*HI and *Sma* I. Bacteria were grown and proteins purified essentially as described (29).

Bacterial proteins were coupled at room temperature for 4 hr with biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma) in 100 mM sodium borate, pH 8.8, at a bacterial peptide concentration of 3 mg/ml and biotin ester at 50 μg/mg of peptide. Biotinylated peptide was purified by gel filtration.

Binding Assays. Tissue-culture cells were lysed on ice in Triton extraction buffer [10 mM Tris, pH 7.4/150 mM NaCl/5 mM EDTA/10% (vol/vol) glycerol/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/aprotinin at 0.25 trypsin inhibitor unit per ml/1 mM sodium orthovanadate, unless specified]. Equal amounts of protein were boiled in Laemmli sample buffer, separated on 8.5% acrylamide gels (except Fig. 3*B*, which is a 7–10% gradient gel), and transferred to nitrocellulose in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11/20% (vol/vol) methanol. Filters were blocked in 10 mM Tris, pH 8.0/0.9% NaCl/0.05% Tween 20 (TBST buffer) plus 0.2% gelatin or 1% ovalbumin (Sigma fraction V). Biotinylated probes were added in the same buffer at 1–2 μg/ml, incubated at room temperature 1–2 hr, and washed extensively in TBST. Filters were incubated with avidin-conjugated alkaline phosphatase at 2 μg/ml in TBST, washed, and developed with Nitro blue tetrazolium and X-phosphate (Promega Biotec).

For immunoblotting with monoclonal anti-ptyr antibody, filters were treated as above, except affinity-purified PY20 immunoglobulin was used at 2 μg/ml instead of biotinylated peptides, and bound antibody was detected with goat anti-mouse-conjugated alkaline phosphatase (Promega Biotec).

SH2 and the glutathione *S*-transferase fusion partner lacking *abl* sequences (GEX) peptides were coupled to Affi-Gel 10 beads (Bio-Rad) at 2 mg of peptide per ml of beads following the manufacturer's recommendations. Cell proteins were incubated in Triton extraction buffer with coupled beads for 1 hr at 4°C (≈50 μg of cell protein per μl of beads or equivalent fraction of purified phosphotyrosine-containing proteins) and then washed with Triton extraction buffer and the same buffer containing 10 mM NaCl before SDS/PAGE.

RESULTS

To generate a probe to identify possible SH2-binding proteins, we expressed a 100-amino acid fragment that contains the entire SH2 domain of c-*abl* in *Escherichia coli*. The *abl* sequences were fused downstream of the glutathione S-transferase gene in the pGEX expression vector, allowing rapid purification of the fusion protein on glutathione agarose (29). The purified fusion protein (termed SH2) and the GEX

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Abbreviations: SH2, *src* homology region 2; PLC, phospholipase C; GAP, *ras* GTPase activator protein; GEX, glutathione *S*-transferase fusion partner lacking *abl* sequences.

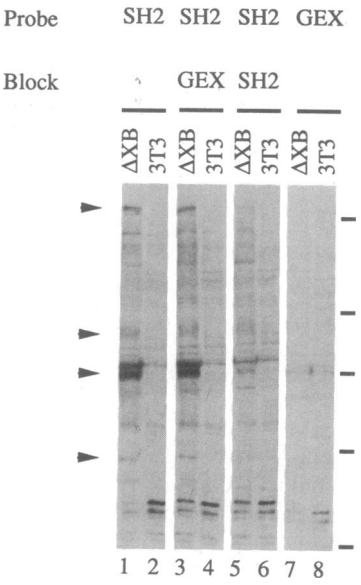

FIG. 1. *abl* SH2 peptide specifically binds to proteins in *abl*-transformed cells. Lysates were prepared from NIH 3T3 cells (lanes 2, 4, 6, 8) or 3T3 cells transformed by the activated c-*abl* mutant ΔXB (lanes 1, 3, 5, 7). Equal amounts of protein were separated by SDS/PAGE and transferred to nitrocelluose filters. Identical filters were incubated with biotinylated SH2 probe (lanes 1 and 2), GEX probe (lanes 7 and 8), or SH2 probe plus 100-fold excess of unbiotinylated GEX (lanes 3 and 4) or SH2 (lanes 5 and 6) peptide, and bound proteins were detected with avidin-conjugated alkaline phosphatase. Arrows indicate prominent specific SH2-binding proteins. Bars indicate the positions of molecular mass standards: 200, 106, 71, 44, and 28 kDa.

protein were biotinylated, permitting detection with avidin-conjugated alkaline phosphatase.

We developed a filter-binding assay in which lysates of tissue-culture cells were separated by SDS/PAGE, transferred to nitrocellulose membranes, and probed with the biotinylated bacterial proteins. A number of proteins were labeled by the SH2 probe but not by the GEX probe (Fig. 1, lanes 1 and 7). Binding was eliminated by competition with 100-fold excess of unlabeled SH2 peptide but not by the GEX peptide (lanes 3 and 5), demonstrating that binding was not an artifact of biotinylation. The majority of the labeled proteins were observed only in lysates from cells transformed by an activated *abl* tyrosine kinase (ΔXB; ref. 30) and were not observed in lysates of parental 3T3 cells (compare lanes 1 and 2). The precise number of bands specifically labeled by the SH2 probe varied slightly in different experiments, but prominent bands of ≈210, 85, 65–70, 38, and 34 kDa were consistently seen.

Because specific labeling by SH2 was seen with *abl*-transformed but not normal cell lysates, we examined whether tyrosine phosphorylation was involved in recognition by SH2. We prepared identical lysates from parallel dishes of cells with the exception that the potent tyrosine phosphatase inhibitor sodium orthovanadate (31, 32) was included in one set. Lysates were incubated on ice for several hours to allow dephosphorylation of tyrosine residues by endogenous phosphatases in the lysates lacking vanadate. When the paired lysates were assayed for binding to SH2, much less binding to proteins prepared without vanadate was observed (Fig. 2*A*, lanes 5 and 6), suggesting that SH2 binding depended on tyrosine phosphorylation. Protease inhibitors were included in both lysates, and no differences in nonspecific background bands were seen, so differences in SH2 binding were probably not due to proteolysis. An identical immunoblot probed with a monoclonal antibody that specifically recognizes phosphotyrosine residues (anti-ptyr) (33, 34) demonstrated that dephosphorylation had occurred; furthermore, anti-ptyr labeled major proteins with the same apparent molecular masses as those labeled by SH2, again suggesting that those proteins that bound SH2 were tyrosine-phosphorylated (Fig. 2*A*, lanes 1 and 2).

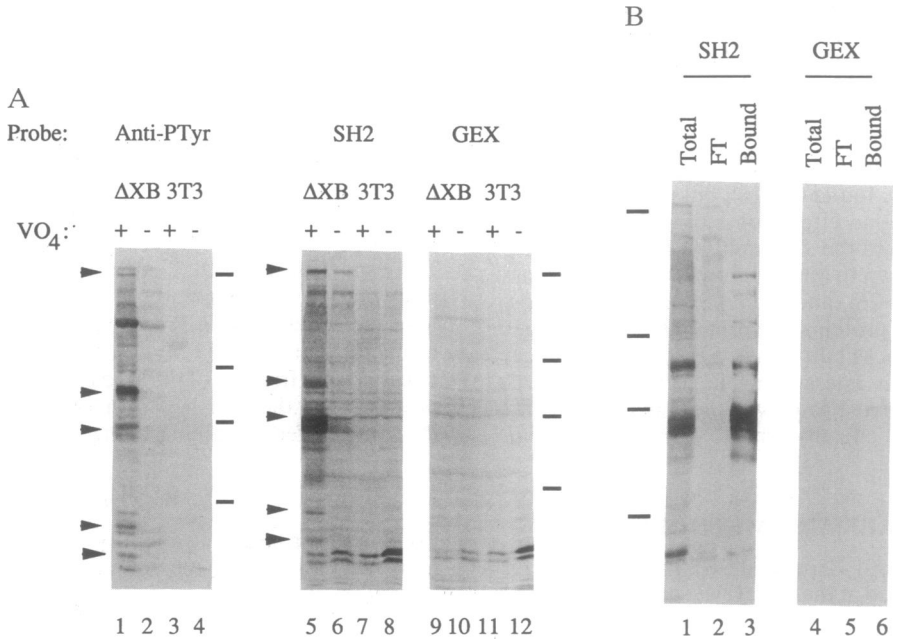

FIG. 2. The SH2 peptide binds to tyrosine-phosphorylated proteins. (*A*) ΔXB or 3T3 lysates were prepared in Triton X-100 lysis buffer with or without 1 mM sodium orthovanadate (VO_4) as marked and incubated on ice for 2 hr. After SDS/PAGE and transfer to nitrocellulose, identical filters were immunoblotted with anti-ptyr (Anti-Ptyr) (33) (lanes 1–4) or probed with biotinylated SH2 (lanes 5–8) or GEX (lanes 9–12) peptides as in Fig. 1. Arrows indicate prominent phosphotyrosine-containing or SH2-binding proteins. (*B*) ΔXB lysates were fractionated by affinity chromatography on anti-ptyr beads into flow-through (FT) and bound fractions (33, 34). Total (lanes 1 and 4), flow-through (lanes 2 and 5), and bound (lanes 3 and 6) fractions were separated by SDS/PAGE and transferred to nitrocellulose, and identical filters were probed with biotinylated SH2 (lanes 1–3) or GEX (lanes 4–6) as in Fig. 1. Protein in bound fractions represents 3.5 times the fraction of total lysate used in total and flow-through lanes.

To demonstrate more directly that the SH2 probe recognized proteins that contained phosphotyrosine residues, we prepared phosphotyrosine-containing proteins from ΔXB-transformed cells by incubating lysates with anti-ptyr coupled to beads and eluting bound proteins with free phosphotyrosine (33, 34). The SH2 probe bound few, if any, proteins in the anti-ptyr flow-through fraction (Fig. 2*B*, lane 2) but bound to many proteins in the anti-ptyr-bound fraction (lane 3). In contrast, most total protein was in the flow-through fraction. These results strongly suggest that SH2 binds exclusively to tyrosine-phosphorylated proteins.

We have also examined whether the *abl* SH2 domain could recognize tyrosine-phosphorylated proteins from cells expressing tyrosine kinase oncogenes other than *abl*. The *abl* SH2 probe bound specifically to several proteins from lysates of 3T3 cells transformed by the v-*src* and v-*fps* oncogenes. The bands labeled corresponded to major phosphotyrosine-containing proteins identified by anti-ptyr immunoblotting, and binding was abrogated by incubation of lysates in the absence of vanadate or by blocking binding reactions with excess unbiotinylated SH2 peptide (data not shown). The SH2 probe also specifically bound to tyrosine-phosphorylated proteins from an *abl*-transformed murine pre-B cell line (data not shown).

Cell proteins in the filter-binding assay are presumably partially or completely denatured and are separated from other proteins to which they might be bound in intact cells. To examine the ability of the *abl* SH2 peptide to bind to native proteins, SH2 and GEX peptides were covalently coupled to beads and incubated with Triton X-100 lysates of ΔXB-transformed cells; bound phosphotyrosine-containing proteins were detected by immunoblotting with anti-ptyr. In contrast to the approximate equivalence between the SH2-bound and phosphotyrosine-containing proteins in the filter-binding assay, only a small subset of the phosphotyrosine-containing proteins bound to the SH2 beads (Fig. 3*A*, lane 3). A 65- to 68-kDa doublet (and occasionally a 34-kDa species) were the only proteins quantitatively retained by the beads. Similarly, when purified phosphotyrosine-containing proteins (prepared by immunoaffinity chromatography as in Fig. 2*B*) were incubated with SH2 beads, the 65- to 68-kDa doublet was the major species bound (Fig. 3*B*, lane 4). Thus, in solution, the *abl* SH2 domain has a much higher affinity for the 65- to 68-kDa species than for other tyrosine-phosphorylated proteins. The apparent difference between the binding specificities observed in solution and in the filter-binding assay implies an inaccessibility of SH2-binding sites in solution; for example, these sites might already be bound to endogenous SH2-containing proteins or the proteins might be folded so as to make access difficult. However, when ^{32}P-labeled ΔXB lysates were bound to SH2 beads, prominent higher molecular mass SH2-binding phosphoproteins were observed, including 85- and 210-kDa species (data not shown), indicating that SH2 binding in solution is not entirely restricted to the 65- to 68-kDa species.

To examine whether free phosphotyrosine could compete with phosphotyrosine-containing proteins for binding to SH2, SH2 beads containing bound phosphotyrosine-containing proteins from ΔXB-transformed 3T3 lysates were incubated with 20 mM phosphotyrosine. These conditions are sufficient to liberate phosphotyrosine-containing proteins bound to anti-ptyr antibodies (refs. 33 and 34 and data not shown). A fraction of the phosphotyrosine-containing proteins was eluted from the SH2 beads by phosphotyrosine but not by phosphoserine at the same concentration (Fig. 4; compare lanes 3 and 4). We have found that it is very difficult to release proteins bound to SH2 beads under a variety of conditions; for example, treatment with 100 mM glycine, pH 2.5, released only a small fraction of bound proteins (data not shown). We therefore feel that the amount of bound protein released by phosphotyrosine is significant. The inefficient yet specific elution with free phosphotyrosine suggests that phosphotyrosine is directly involved in SH2 binding but does not constitute the entire binding site. Consistent with this, we have also found that 1 mM phosphotyrosine decreased but

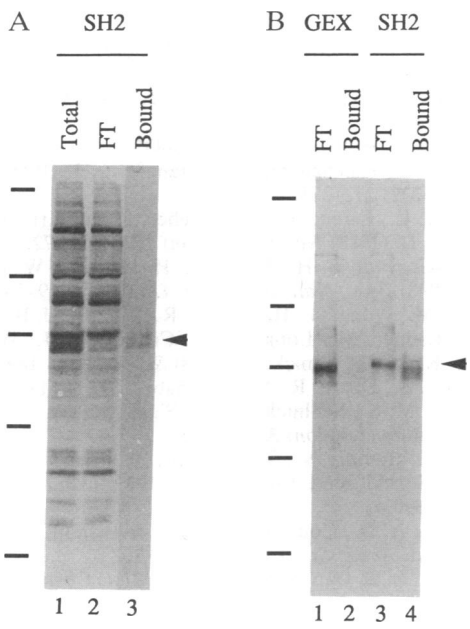

FIG. 3. SH2 peptide coupled to beads binds a subset of tyrosine-phosphorylated proteins (*A*) Lysates were prepared from ΔXB cells and incubated with beads covalently coupled to *abl* SH2 peptide. Total (lane 1), flow-through (FT) (lane 2), and bound (lane 3) fractions were separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-ptyr. Arrow denotes major SH2-binding species. (*B*) Phosphotyrosine-containing proteins were prepared by affinity chromatography on anti-ptyr, eluted, and incubated with beads coupled to GEX (lanes 1 and 2) or SH2 (lanes 3 and 4) peptide as marked. Proteins in the flow-through (FT) (lanes 1 and 3) and bound (lanes 2 and 4) fractions were immunoblotted with anti-ptyr. Arrow indicates major SH2-binding species (65- to 68-kDa doublet).

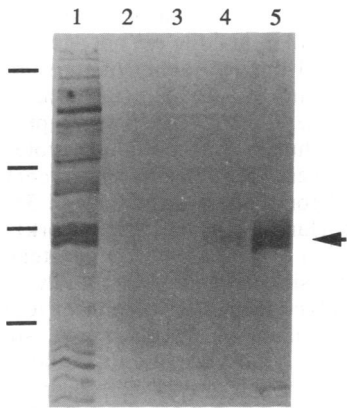

FIG. 4. Phosphotyrosine partially disrupts SH2 binding. ΔXB lysates were incubated with GEX beads or SH2 beads as in Fig.3. After binding to cell proteins, SH2 beads were incubated in Triton X-100 lysis buffer containing 20 mM phosphoserine, the eluate was then collected, and the beads were further incubated in 20 mM phosphotyrosine. Proteins in each fraction were boiled in SDS sample buffer and analyzed by immunoblotting with anti-ptyr. Lanes: 1, total lysate; 2, proteins bound to GEX beads; 3, proteins eluted from SH2 beads with phosphoserine; 4, proteins subsequently eluted from SH2 beads with phosphotyrosine; 5, proteins remaining bound to SH2 beads after elution with phosphoserine and phosphotyrosine. Arrow indicates major SH2-binding species.

did not eliminate SH2 binding in the filter-binding assay, whereas phosphoserine had no effect (data not shown).

DISCUSSION

We have shown that the SH2 domain of the *abl* tyrosine kinase can tightly associate with tyrosine-phosphorylated proteins both on nitrocellulose filters and in solution. The filter-binding assay demonstrates that SH2 binding to phosphotyrosine-containing proteins is direct and not mediated by unidentified associated proteins because cell lysates were separated by SDS/PAGE before transfer to filters. We have not analyzed the kinetics of SH2 binding in the filter assay, but the affinity must be very high because the concentration of SH2 probe in the binding reaction was $<10^{-7}$ M and the filters were washed extensively after binding. Although the *abl* SH2 binds to virtually all phosphotyrosine-containing proteins in the filter assay, only a subset of these bind in solution. This result suggests both that SH2 domains have the potential to bind to many proteins and that this potential could be modulated *in vivo* by such factors as accessibility of binding sites and local concentration.

We propose that tyrosine phosphorylation is required for high-affinity binding of proteins to the *abl* SH2 domain based on the following evidence. All bands labeled by the SH2 probe comigrate with major phosphotyrosine-containing proteins detected by anti-ptyr; dephosphorylation of these proteins abrogates SH2 binding. Furthermore, in ΔXB lysates, virtually all SH2-binding proteins are also bound by anti-ptyr (Fig. 2). We have performed phosphoamino acid analysis on two proteins that bind to SH2 beads, and in both cases at least 50% of phosphate was on tyrosine residues (data not shown). We have shown that phosphotyrosine, and not phosphoserine, can partially elute phosphotyrosine-containing proteins from SH2 beads (Fig. 4) and can partially block SH2 binding in the filter assay (data not shown). In sum, these data suggest that SH2 directly recognizes phosphotyrosine in cell proteins, as opposed to a phosphorylation-induced conformational change.

The demonstration that an SH2 domain can directly bind to tyrosine-phosphorylated proteins has general implications for the mechanisms of cellular signal transduction. SH2 domains have been found to date only in proteins implicated in the modulation of growth control: the nonreceptor tyrosine kinases, PLC-γ, GAP, and the *crk* oncogene product (6–12). PLC-γ, GAP, and the *src* family of tyrosine kinases have been shown to bind to activated (tyrosine-phosphorylated) growth-factor receptors (21–26), and *crk* protein binds to a number of phosphotyrosine-containing proteins (27, 28). In many of these cases, SH2 domains have recently been shown to be sufficient for binding (refs. 35 and 36; M. Matsuda, B.J.M., and H. Hanafusa, unpublished work). It is likely that a general function of SH2 domains in proteins is to mediate the phosphotyrosine-dependent assembly of multiprotein complexes involved in cellular responses to liganded receptors. A tyrosine-phosphorylation event, such as growth-factor receptor autophosphorylation, could trigger the colocalization of a whole class of SH2-binding proteins to a specific cellular site. The fact that both PLC-γ and GAP have two SH2 domains and are themselves tyrosine phosphorylated upon growth-factor stimulation (37–40) suggests that these proteins could form multimeric networks based on SH2–phosphotyrosine interaction.

There is no evidence yet that c-*abl* protein or its transforming variants can complex with growth-factor receptors, so the specific function of the *abl* SH2 domain is unclear. Because most overexpressed c-*abl* protein is found in the nucleus (41), unlike v-*abl* or other SH2-containing proteins that are cytosolic or membrane-associated, in normal cells the c-*abl* SH2 could participate in a specific set of phosphotyrosine-mediated interactions. The *abl* SH2 domain appears to bind to virtually all phosphotyrosine-containing proteins in the filter assay; we have not yet attempted to identify any of these SH2-binding proteins. The major protein that binds to the *abl* SH2 domain in solution, a 65- to 68-kDa doublet, is reminiscent of proteins of approximately the same molecular mass that have been reported to bind to GAP and *crk* (27, 28, 35, 40).

Because we have shown that the *abl* SH2 domain has a much higher affinity for tyrosine-phosphorylated proteins than for unphosphorylated potential substrates, the presence of SH2 domains in the nonreceptor tyrosine kinases is intriguing. This result suggests that the nonreceptor kinases would bind tightly to their reaction products, making them poorly suited to rapidly phosphorylate a number of substrates. It is possible, however, that the SH2 domains of the tyrosine kinases are actually sequestered in inter- or intramolecular interactions that regulate activity of the catalytic domain, especially given that all of the nonreceptor tyrosine kinases have multiple tyrosine phosphorylation sites (42–44). *In vitro* studies with purified nonreceptor tyrosine kinases, their SH2 domains, and natural substrates will be required to assess the significance of SH2–phosphotyrosine interactions in the regulation of kinase activity.

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