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Mutagenic Analysis of the Roles of SH2 and SH3 Domains in Regulation of the Abl Tyrosine Kinase

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We have used in vitro mutagenesis to examine in detail the roles of two modular protein domains, SH2 and SH3, in the regulation of the Abl tyrosine kinase. As previously shown, the SH3 domain suppresses an intrinsic transforming activity of the normally nontransforming c-Abl product in vivo. We show here that this inhibitory activity is extremely position sensitive, because mutants in which the position of the SH3 domain within the protein is subtly altered are fully transforming. In contrast to the case in vivo, the SH3 domain has no effect on the in vitro kinase activity of the purified protein. These results are consistent with a model in which the SH3 domain binds a cellular inhibitory factor, which in turn must physically interact with other parts of the kinase. Unlike the SH3 domain, the SH2 domain is required for transforming activity of activated Abl alleles. We demonstrate that SH2 domains from other proteins (Ras–GTPase-activating protein, Src, p85 phosphatidylinositol 3-kinase subunit, and Crk) can complement the absence of the Abl SH2 domain and that mutants with heterologous SH2 domains induce altered patterns of tyrosine-phosphorylated proteins in vivo. The positive function of the SH2 domain is relatively position independent, and the effect of multiple SH2 domains appears to be additive. These results suggest a novel mechanism for regulation of tyrosine kinases in which the SH2 domain binds to, and thereby enhances the phosphorylation of, a subset of proteins phosphorylated by the catalytic domain. Our data also suggest that the roles of the SH2 and SH3 domains in the regulation of Abl are different in several respects from the roles proposed for these domains in the closely related Src family of tyrosine kinases.

Protein-tyrosine kinases play a central role in the regulation of cellular growth and differentiation of complex eukaryotes. The two major classes of tyrosine kinases are the transmembrane receptors, which are activated directly by binding of peptide growth factors and cytokines to their extracellular domains, and the nonreceptor kinases (for reviews, see references 5, 13, and 46). While the normal function of the receptors as transducers of extracellular signals is evident, the role of nonreceptor tyrosine kinases is in many cases unknown. Examples of both classes of kinases have been shown to function as dominant oncogenes, generally as a result of overexpression and structural alteration (see reference 14 for a review).

The Abl nonreceptor tyrosine kinase was originally isolated as the transforming gene of Abelson murine leukemia virus (12) and was subsequently shown to be involved in human leukemias by virtue of a chromosomal translocation resulting in the expression of a fusion protein containing N-terminal sequences from a second locus, *bcr* (28, 48). Overexpression of c-Abl does not result in efficient cell transformation or elevated tyrosine phosphorylation in vivo (10, 15), whereas structurally altered forms are able to efficiently induce transformation and elevated phosphotyrosine in a variety of cell types, suggesting that the activity of the normal kinase is tightly regulated. As in the Src family of tyrosine kinases, N terminal to the catalytic domain of Abl are two small modular domains termed the Src homology 2 and 3 (SH2 and SH3) domains. Previous work in a number of laboratories has suggested that the SH2 and SH3 domains of nonreceptor tyrosine kinases are involved in regulation of kinase activity in vivo.

SH2 and SH3 domains are found in a wide variety of proteins and are thought to function by mediating controlled protein-protein interactions (reviewed in references 24 and 34). SH3 domains are implicated in the repression of activity of Abl and the Src family kinases, because deletion or mutation of this domain generally activates the transforming activity of the proto-oncogenes (10, 15, 17, 39). Several SH3 domain-binding proteins have now been isolated, and a proline-rich binding motif for the Abl SH3 domain has been identified (4, 41). SH2 domains have been shown to bind specifically and with high affinity to tyrosine-phosphorylated proteins (1a, 20, 23, 25, 30) and are thought to mediate the association of signaling proteins in response to tyrosine phosphorylation. We have previously shown that activated Abl proteins with mutated SH2 domains that are no longer able to efficiently bind tyrosine-phosphorylated proteins are impaired in their ability to transform (26).

We have devised a mutagenesis scheme that facilitates the deletion, reiteration, swapping, and movement within the protein of various modular domains of the Abl tyrosine kinase. These mutants have been used to examine in detail the roles of the SH2 and SH3 domains in the regulation of Abl activity. As expected from previous results, we find that the SH2 and SH3 domains play very different roles: the SH3 domain appears to suppress the intrinsic transforming ability of Abl, while the SH2 is absolutely required for expression of the transforming activity of activated Abl genes. We extend these observations to show that in cells, the inhibitory activity of the SH3 domain is extremely sensitive to its position in the Abl molecule, but its presence or absence has no effect on the in vitro kinase activity of the purified protein, suggesting that the SH3 domain regulates kinase activity in vivo by a complex mechanism. We

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also find that the SH2 domain, not the catalytic domain, is largely responsible for determining the spectrum of proteins phosphorylated in vivo. We propose that the SH2 domain is required for transforming activity because it modulates the output of a relatively nonspecific catalytic domain by directly binding to, and enhancing the phosphorylation of, a subset of proteins phosphorylated by the catalytic domain.

MATERIALS AND METHODS

Construction of Abl Mutants. All Abl genes were constructed from modular fragments generated by PCR using wild-type murine type IV c-Abl cDNA (pPLcIV) (2, 15) and primers encoding unique restriction sites on their 5′ ends. *Pfu* DNA polymerase (Stratagene) was used as recommended by the manufacturer except that elongation times were occasionally extended to 5 min to generate longer fragments. PCR primers had 24 to 30 nucleotides (nt) of identity with template, 6 or 8 nt encoding restriction sites, and five or six G or C residues 5′ to clamp the ends of the fragment to facilitate restriction digestion. All PCR fragments were sequenced to approximately 200 nt from each end; no mutations were observed in any clone except in regions of primer binding. Only clones of the expected sequence were used for further studies.

The prototype modular c-Abl gene (termed construct F) consisted of three PCR fragments (all nucleotide numbers are relative to the A of the initiation codon of murine type IV c-Abl): N-terminal fragment A (*Not*I, nt −9 to 252, *Sac*II), SH3-plus-SH2 fragment F (*Sac*II, nt 256 to 723, *Bam*HI), and catalytic domain-plus-C-terminus fragment B (*Bam*HI, nt 730 to 3465, *Not*I). Deletions of SH2 or SH3 were generated by replacing fragment F with fragment E (*Sac*II, nt 256 to 417, *Bam*HI) or fragment G (*Sac*II, nt 427 to 723, *Bam*HI), to generate constructs E and G, respectively; deletion of both was effected by replacing fragment F with a short synthetic adaptor (top strand, 5′ TCGCGAACA; bottom strand, 5′ GATCTGT TCGCGAGC) to generate construct 9. For constructs with SH2 and/or SH3 domains C terminal to the catalytic domain, two other fragments were generated using construct 9 DNA as the template: N-terminal and catalytic domain fragment C (*Not*I, nt −9 to 1620, *Sac*II) and C-terminal fragment D (*Bam*HI, nt 1621 to 3465, *Not*I).

All constructs were initially manipulated while cloned into a modified vector derived from pBS-SK(−) (Stratagene), termed pBSΔ, in which the vector *Sac*II and *Bam*HI sites had been destroyed. For virus production, *Not*I Abl fragments were cloned into pGDN, a derivative of the retroviral vector pGD (7) in which the *Xho*I cloning site had been converted to a unique *Not*I site. All constructs were derived from the same cloned PCR fragments A, B, C, D, E, F, and G so that there could be no clone-to clone variation due to *Pfu* polymerase errors. Further details of the construction of all mutants described here can be provided on request.

For mutants in which portions of Abl are swapped with heterologous SH2 or catalytic domains, fragments were generated by PCR as described above with appropriate restriction sites. All SH2 domains contained 5′ *Sac*II and 3′ *Bam*HI sites and consisted of the following amino acids: murine N-terminal GTPase-activating protein (GAP) SH2 domain, corresponding to amino acids (aa) 178 to 274 of human GAP (52); murine Src SH2 domain, aa 153 to 255 of neuronal form (21); murine p85 phosphatidylinositol (PI) 3-kinase subunit C-terminal SH2 domain, aa 618 to 720 (8); and chicken c-Crk SH2 domain, aa 7 to 121 (40). The Src catalytic domain consisted of aa 249 to 524 of chicken c-Src (corresponding to aa 257 to 532 of murine neuronal form [21]), with 5′ *Bam*HI and 3′ *Bgl*II sites. This was ligated to the *Not*I-*Bam*HI N-terminal-plus-SH2 domain fragments containing various SH2 domains and the *Bam*HI-*Not*I C-terminal D fragment to generate the GSC series of mutants.

Generation of virus and assay of biological activity. Infectious virus expressing various Abl genes was produced by transient transfection of 293 cells. Generally 2 to 5 μg of pGDN-derived plasmid and 2 to 5 μg of pZAP, which encodes intact Moloney murine leukemia virus (15), were introduced by calcium phosphate coprecipitation into 4×10^6 293 cells in a 10-cm-diameter plate. In some experiments, chloroquine was added to 25 μM just prior to transfection. The medium was changed after approximately 8 h, and virus stocks were collected 2 days later. 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

Focus assays and G418-resistant colony assays were determined on NIH 3T3 cells. Only early-passage cells of a subclone chosen for flat morphology were used to assay transformation. 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum plus antibiotics. Six-centimeter-diameter dishes seeded with approximately 10^5 cells were infected with 1 ml of diluted virus and 1 ml of medium supplemented with 16 μg of Polybrene per ml. Virus was removed after 8 h and replaced with fresh medium. To select G418-resistant colonies, medium was changed to medium containing 1 mg of G418 per ml the next day. All plates were fed at 3-day intervals thereafter. Morphologically altered foci were scored 7 to 10 days postinfection, and G418-resistant colonies were scored 10 to 14 days postinfection.

Transient infections and protein characterization. For analysis of tyrosine-phosphorylated proteins induced by Abl SH2 domain mutants, 2.5×10^5 3T3 cells were infected in 10-cm-diameter dishes with 4 ml of undiluted virus stocks (multiplicity of infection of ~1). Two days postinfection, cells were lysed in 0.5 ml of Triton extraction buffer (TXB; 10 mM Tris [pH 7.4], 150 mM NaCl, 5 mM Na_2EDTA, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml) on ice for 30 min and centrifuged for 10 min in a microcentrifuge to clear, and protein concentrations were assayed by the Bradford method (Bio-Rad). For immunoblotting, equal amounts of cell protein were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose in 10 mM CAPS (pH 11)–20% methanol for 2 h at 500 mA. Filters were blocked in TBST (10 mM Tris [pH 8.0], 0.9% NaCl, 0.05% Tween 20) with 1% ovalbumin and 0.02% NaN_3 for several hours at 4°C and then incubated overnight at 4°C in blocking buffer containing either a combination of monoclonal antibodies 4G10 (Upstate Biochemical, Inc.) and PY20 (11) (for antiphosphotyrosine blotting) or affinity-purified rabbit polyclonal antibody directed against the Abl type IV-specific N-terminal sequences. After washing with TBST, filters were incubated with alkaline phosphatase-conjugated donkey anti-mouse immunoglobulin (for antiphosphotyrosine blotting) or either alkaline phosphatase-conjugated protein A or alkaline phosphatase-conjugated donkey anti-rabbit immunoglobulin, washed again, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium.

For anti-Abl immunoprecipitations, equal amounts of cell protein were incubated on ice with crude Abl-specific pEX-4 serum (19). Immunoprecipitates were collected on protein A-Sepharose beads, washed three times with lysis buffer, resuspended in sample buffer, boiled, and separated on SDS-polyacrylamide gels. For antiphosphotyrosine immunoprecipitates, Affi-Gel-Hz beads (Bio-Rad) were covalently coupled

to phosphotyrosine-specific monoclonal antibody PY20 for a final concentration of 2.5 mg of immunoglobulin per ml of beads, following the manufacturer's recommendations. Equal amounts of cell protein were incubated on ice with PY20 beads and then washed, electrophoresed, and immunoblotted as described above.

Production and characterization of GST-Abl fusion proteins. The glutathione *S*-transferase (GST) fragment from pGEX-2T (49) was modified by PCR so that the initiation codon was preceded by a eukaryotic Kozak consensus ribosome binding site and a 5′ *Bgl*II site. The *Bgl*II-*Bam*HI 0.7-kb fragment so generated was cloned into the unique *Bam*HI site of baculovirus vector pVL1393 (Invitrogen, Inc.) to generate plasmid pVLGS. The Abl genes of construct E, F, and G described above were inserted into pVLGS. To do this, an alternative N-terminal fragment A was synthesized by PCR, consisting of *Bam*HI, nt 4 to 252, *Sac*II. This fragment was ligated along with the *Sac*II-*Not*I fragments from pBSΔ-E, -F, or -G into pVLGS cut with *Bam*HI and *Not*I.

Recombinant baculovirus was generated by transfection of Sf9 cells with pVLGS derivatives along with wild-type *Autographa californica* nuclear polyhedrosis virus DNA, using standard protocols. In each case, several independent clones were plaque purified and characterized. For protein production, Sf21 cells (Invitrogen) were infected with high-titer virus in 1-liter roller bottles (approximately 20 ml of virus per 10^8 cells). After 90 min, 180 ml of Grace's medium (supplemented with 10% fetal bovine serum, 2% [vol/vol] yeastolate, and lactalbumin hydrolysate [GIBCO] plus antibiotics) was added, and bottles were incubated at 26°C for a further 2 days. Cells were harvested, washed in cold phosphate-buffered saline, and lysed in TXB with 1 mM dithiothreitol on ice for 30 min, and lysates were cleared by centrifugation at 12,000 × *g* for 15 min. GST fusion proteins were purified by incubating lysates with glutathione-agarose beads (Molecular Probes, Inc.) at 4°C for 90 min, washing extensively in TNGT buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol), and eluting with TNGT plus 20 mM reduced glutathione and 100 mM Tris (pH 8.8). For thrombin cleavage, washed glutathione-agarose beads bound to GST-Abl fusion proteins were incubated with an empirically determined amount of human thrombin (Calbiochem) in TNGT supplemented with 2.5 mM $CaCl_2$, and the eluted cleaved Abl proteins were separated from beads by pouring into a disposable column. Na_2EDTA was added to 2.5 mM, and phenylmethylsulfonyl fluoride was added to 1 mM to inactivate thrombin. Purified proteins were stored at −70°C.

For in vitro kinase assays, purified proteins were incubated in 50 mM *N*-2-hydroxyethy piperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–5 mM $MgCl_2$–0.5 mg of poly(Glu-Tyr) (4:1; Sigma P-0275) per ml–50 μg of acetylated bovine serum albumin (BSA) per ml–1 mM cold ATP–10 μCi of [γ-^{32}P]ATP in 20 μl (total volume) at 4°C for 20 min. Reactions were stopped with 5 μl of 5× Laemmli sample buffer, boiled, and run on SDS-polyacrylamide gels. Gels were stained with Coomassie blue, fixed in 10% acetic acid–20% methanol, dried, and exposed for autoradiography. Quantitative assays using angiotensin as a substrate were performed essentially as described previously (9). Reaction mixtures contained 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (~1,000 cpm/pmol), and 1 mg of [Val^5] angiotensin II (Sigma A-2900) per ml. Reactions were performed at 30°C, and 25-μl aliquots were stopped at various time points with 35 μl of 10% trichloroacetic acid and incubated on ice. Aliquots were briefly centrifuged, and 30 μl of the supernatant was spotted on a 4-cm^2 piece of phosphocellulose paper (Whatman P81). Filters were washed three times with ice-cold 0.5% phosphoric acid, washed once with acetone, and dried, and Cerenkov counts were determined. Data points shown are the mean of triplicate samples.

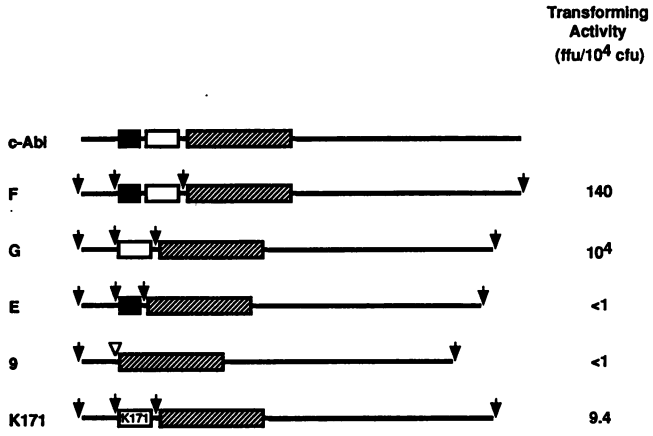

FIG. 1. Effects of SH3 and SH2 domains on transforming activities of c-Abl-derived mutants. Mutants are depicted diagrammatically. Black boxes, SH3 domain; white boxes, SH2 domain; hatched boxes, catalytic domain. Arrowheads indicated the position of unique restriction sites inserted by PCR. The white triangle indicates the position of short synthetic adaptor sequence. K171 denotes a mutant SH2 domain with an arginine-to-lysine mutation at position 171 that drastically reduces binding to phosphotyrosine. Transforming activity value given is the normalized average of morphologically transformed foci per 10^4 G418-resistant colonies for each mutant (see Table 1 and text for details). ffu, focus-forming units.

RESULTS

To analyze the roles of the SH2 and SH3 domains in Abl, we constructed a modularized Abl gene by inserting unique restriction sites flanking the SH3 and SH2 domains, using PCR (Fig. 1). This cassette system allowed individual SH2 or SH3 domains or the block of SH3 plus SH2 domains to be easily manipulated. For some constructs, another unique site was inserted C terminal to the catalytic domain, allowing insertion of sequences or swapping of catalytic domains. The location of all linkers was chosen to be in predicted loop regions between modular units of protein structure so that disruption of secondary and tertiary structure of the resulting proteins would be minimized. Furthermore, the spacing between elements of structure was conserved in the mutants. All of the constructs in this study encode the myristoylated type IV N terminus of Abl, because previous studies have shown that it is difficult to overexpress proteins encoding the nonmyristoylated type I N terminus (10, 15a).

All mutant Abl genes were inserted into a simple retroviral vector derived from pGD (7). This vector contains all sequences required in *cis* for expression and packaging of the genome and also encodes the neomycin resistance gene, allowing selection of infected cells with G418. When cotransfected with pZAP, which encodes a replication-competent Moloney murine leukemia virus helper, infectious particles encoding the mutant Abl genes are produced.

A transient transfection system was used to produce virus for these studies (36). 293 cells, a human kidney-derived cell line, were transfected with helper and pGD-derived plasmids, and virus was harvested 2 days posttransfection. This protocol minimizes mutation due to error-prone reverse transcription,

TABLE 1. Quantification of transformed foci and G418-resistant colonies

Mutant	Raw data[a]		Foci/10^4 CFU[b]	Normalized[c]	Avg[d]	n[e]
	Foci/ml	CFU/ml				
GDN	<10	7.2×10^5	<1	<1	<1	10
E	<10	6.8×10^5	<1	<1	<1	2
F	580	4.0×10^4	145	85	140	3
G	8.9×10^4	5.2×10^4	1.7×10^4	10^4	10^4	10
9	<10	7.4×10^5	<1	<1	<1	2
DE	ND[f]	ND			<4	2
DF	ND	ND			<10	2
DG	15	6.5×10^4	<3	<2	<2.5	2
D9	ND	ND			<2	2
K171	320	2.0×10^5	16	9.4	9.4	2
23	2.7×10^4	3.6×10^4	7.5×10^3	4.4×10^3	5.2×10^3	2
323	1.1×10^4	2.3×10^4	4.8×10^3	2.8×10^3	2.5×10^3	3
FN	3.5×10^4	2.2×10^4	1.6×10^4	9.4×10^3	6.1×10^3	5
GG	1.5×10^3	7.2×10^4	210	120	120	4
GS	5.2×10^3	8.5×10^4	620	360	510	3
GP	6.2×10^4	5.0×10^4	1.2×10^4	7.1×10^3	3.3×10^3	3
GC	1.4×10^5	1.4×10^4	10^5	5.9×10^4	2.2×10^4	4
GSC	2.4×10^3	4.6×10^4	520	310	460	3
GSC-G	2.6×10^3	4.7×10^4	550	320	340	2
GSC-S	1.1×10^4	5.5×10^4	2.0×10^3	1.2×10^3	1.2×10^3	2
GSC-P	2.7×10^4	2.8×10^4	9.6×10^3	5.6×10^3	4.9×10^3	2
GSC-C	9.2×10^3	1.3×10^3	7.1×10^4	4.2×10^4	1.9×10^4	3
GAG	ND	ND			9.4×10^3	2
GGA	ND	ND			1.2×10^4	2
2N3K2	2.0×10^4	2.0×10^4	10^4	5.9×10^3	5.8×10^3	2
K2N32	1.9×10^4	2.8×10^4	6.8×10^3	4.0×10^3	4.2×10^3	2

[a] Data from a single transfection and subsequent assay of morphologically transformed focus titer and G418 resistance colony titer.
[b] Transformed focus titer normalized to G418 resistance colony titer.
[c] Values from previous column normalized to give the mutant G a value of 10^4 foci/10^4 CFU (i.e., in this case dividing all values by 1.7).
[d] Mean normalized titer (averaged over the number of independent experiments given in the next column).
[e] Number of independent transfections used to generate average normalized titer (previous column).
[f] ND, not done in this experiment.

because at most two rounds of replication are undergone before the mutant genome is scored for biological activity. Transforming activity was assessed by infection of NIH 3T3 cells with dilutions of the 293 cell-derived virus and scoring parallel sets of dishes for morphologically transformed foci and G418-resistant colonies.

The raw numbers of transformed foci and G418-resistant colonies varied considerably from experiment to experiment, so data were normalized in two ways (Table 1). First, all transformation scores were normalized to virus titer by dividing transformed foci by G418-resistant colonies and expressed as transformed foci per 10^4 G418-resistant colonies. A second normalization was required so that data from separate transfections could be combined and averaged. This involved multiplying all scores within a given experiment by a normalization constant such that a fully transforming benchmark mutant (GDN-G) had a score of 10^4 foci per 10^4 G418-resistant colonies (in practice, the normalization constant had values of between 0.5 and 2). All transformation data reported in this paper were derived from at least two independent transfections. Raw data from one representative experiment is shown in Table 1 for reference.

For all mutants, transfected 293 cells used for production of virus were also analyzed by Western blotting (immunoblotting) with Abl-specific antisera. This analysis confirmed that the mutant proteins were of the expected molecular weight and were produced in reasonable quantities (data not shown). In vitro kinase assays were also performed on immunoprecipitates from the 293 cell lysates to ensure that the mutant proteins were active as protein-tyrosine kinases (data not shown).

Effect of SH2 and SH3 domain deletion. The first series of mutants was constructed to test whether our mutagenic approach was viable and to confirm the presumed inhibitory role of the SH3 domain and positive role of the SH2 domain in transformation by Abl (Fig. 1). When the linker-modified c-Abl construct (termed GDN-F) was assayed for transforming activity, only very low activity was observed (less than 150 foci per 10^4 G418-resistant colonies). We believe that this weak transforming activity was detected in our experiments, unlike earlier studies (10, 15, 32), because our expression strategy did not involve long-term selection of cells expressing c-Abl, which is strongly cytostatic (15, 15a, 42) and we assayed morphological alteration and not soft-agar colony growth. It is also possible that the insertion of two restriction sites by PCR had a slight activating effect. This c-Abl background was very small, however, relative to the transforming activity of a mutant in which the SH3 domain was deleted (GDN-G), which was approximately 100-fold higher. In a number of separate experiments, focus-forming titers with this virus were approximately equal to G418 resistance titers suggesting that virtually every infected cell produced a morphologically transformed focus. These foci were in general larger than the few foci seen with the undeleted c-Abl-expressing construct.

When the SH2 domain was deleted, either in the presence or in the absence of the SH3 domain (GDN-E and -9, respectively), transforming activity was undetectable (Fig. 1). Furthermore, when the SH2 domain of a mutant lacking the SH3

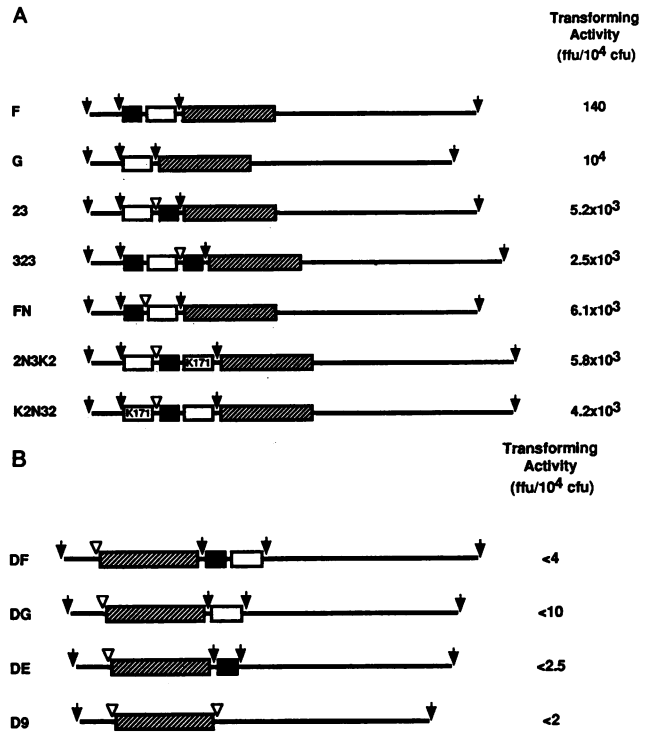

FIG. 2. Effects of altering positions of SH3 and SH2 domains on transforming activities of Abl mutants. Structures and transforming activities are depicted as in Fig. 1. (A) Mutants with SH2 domains N terminal to the catalytic domain. (B) Mutants with SH2 and SH3 domains deleted from their normal N-terminal positions and introduced C terminal to the catalytic domain. ffu, focus-forming units.

contained an arginine-to-lysine mutation at position 171 (R171K), which has been shown to be essential for binding to phosphotyrosine (26), transforming activity was drastically reduced relative to the same construct with an unmutated SH2 domain. These results are consistent with previously published results showing that deletion of the SH3 domain leads to activation of c-Abl and that a functional SH2 domain is required for transforming activity. Therefore, the insertion of linkers by PCR did not significantly alter the biological activity of Abl or mutants derived from it.

Position dependence of SH2 and SH3 domain function. The next series of mutants was constructed to test whether the negative function of the SH3 domain and the positive function of the SH2 domain were dependent on their positions within the Abl protein. Mutants were constructed in which the positions of the SH2 and SH3 domains were changed relative to each other and to the N terminus or catalytic domain. These mutants contained at least one functional SH2 and SH3 domain, and some contained an additional SH2 domain with the R171K mutation to render it nonfunctional. All of these mutants had transforming activities nearly as great as that of the SH3 domain-deleted positive control (Fig. 2A), demonstrating that the context of the SH3 domain is extremely important to its ability to inhibit transforming activity of Abl.

It is clear from these mutants that the relationship with more than one part of the protein is important, since in at least one of the mutants, the position of the SH3 domain is unchanged relative to either the N terminus, SH2 or catalytic domain, and C terminus. In mutant 23, the positions of the SH2 and SH3 domains are swapped, and the SH3 domain is in an altered position relative to the N terminus, SH2 domain, and catalytic domain. In mutant 323, a second SH3 domain is inserted between the SH2 and catalytic domains; the position of the N-terminal SH3 domain is altered only relative to the catalytic domain. In mutant FN, three amino acids were inserted between the SH3 and SH2 domains, subtly altering the position of the SH3 domain relative to the catalytic and SH2 domains. In mutant 2N3K2, the SH3 domain is in an altered position relative to the N terminus and the functional SH2 domain, and in mutant K2N32, a nonfunctional SH2 domain is inserted upstream of the SH3 domain, altering its position only relative to the N terminus.

Another implication of the results shown in Fig. 2A is that unlike the SH3 domain, the SH2 domain can function in different positions within the Abl protein. In mutant GDN-2N3K2, the functional SH2 domain is approximately 150 aa N terminal to its normal position relative to the catalytic domain, yet transforming activity is essentially equivalent to that of the positive SH3 domain-deleted control. To test whether the SH2 or SH3 domain could function in a radically different context, a series of mutants was constructed with the SH3 and SH2 domains deleted from their normal positions N terminal to the catalytic domain and inserted alone or in combination in a novel position C terminal to the catalytic domain (Fig. 2B). None of these constructs had significant transforming activity, although they produced proteins that were kinase active and present in amounts comparable to those shown in Fig. 2A (data not shown). This finding demonstrates that while there can be considerable flexibility in the position of the SH2 domain, it is not entirely position independent since it cannot effectively perform its positive role when C terminal to the catalytic domain.

Complementation of loss of the SH2 domain by heterologous SH2 domains. SH2 domains all have the ability to bind to tyrosine-phosphorylated proteins, but different SH2 domains bind to different specific binding sites (50). We were therefore interested in whether Abl transformation was dependent on the presence of the Abl SH2 domain, or whether SH2 domains from other proteins, with different binding specificities, would restore transforming ability in Abl mutants lacking the Abl SH2 domain. We constructed a series of mutants in which the SH3 domain was deleted to activate the transforming activity of c-Abl, and the Abl SH2 domain was replaced by the SH2 domain of Src or Crk, the N-terminal SH2 domain of GAP, or the C-terminal SH2 domain of the p85 PI 3-kinase subunit (Fig. 3A). When assayed, all of the mutants with heterologous SH2 domains had detectable transforming activity, whereas transformation was undetectable in the absence of any SH2 domain. Activities ranged from 100-fold less active (for the GAP SH2 domain) to at least as active (for the Crk SH2 domain) as the Abl SH2 domain-containing positive control (Fig. 3A). This finding demonstrates that transforming activity is not strictly dependent on the Abl SH2 domain, and that SH2 domains with very different binding specificities could function to promote transforming activity. The very low activity of the construct with the mutant Abl SH2 domain that is unable to bind to phosphotyrosine (GDN-K171 in Fig. 1) demonstrates that this requirement is not merely structural but requires the ability to bind to phosphotyrosine.

When viruses encoding the SH2 domain swap mutants described above were used to infect 3T3 cells and tyrosine-phosphorylated proteins were analyzed by immunoblotting, the pattern of tyrosine phosphorylation differed in the mutants (Fig. 4A). The pattern of phosphoproteins seemed to correlate with the proteins for which the specific SH2 domain had high affinity. For example, we have previously shown that the

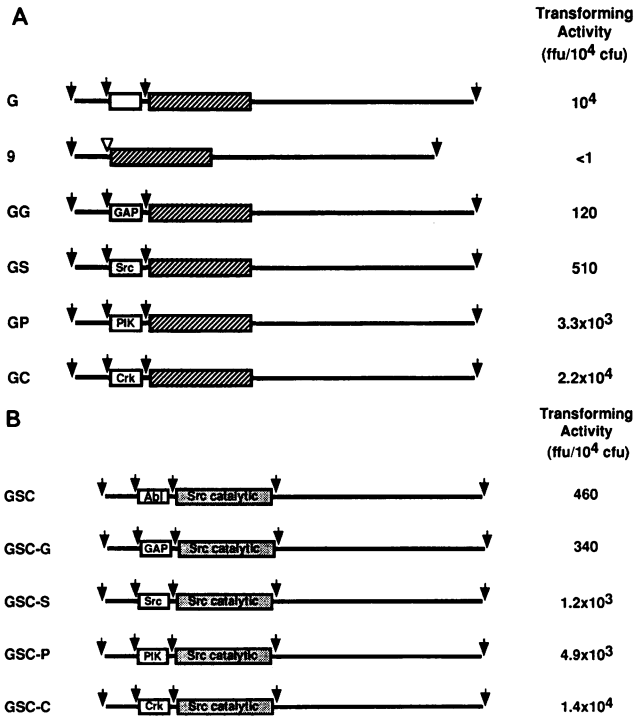

FIG. 3. Effects of heterologous SH2 and catalytic domains on transforming activities of Abl mutants. Structures and transforming activities are depicted as in Fig. 1. The origin of the SH2 domain is indicated within the white boxes. GAP, N-terminal SH2 domain of Ras-GAP; PIK, C-terminal SH2 of p85 subunit of PI 3-kinase. (A) Mutants with the Abl catalytic domain. (B) Mutants in which the Abl catalytic domain is replaced with that of Src. ffu, focus-forming units.

N-terminal GAP SH2 domain has a very high affinity for a protein of approximately 68 kDa (26), and a tyrosine-phosphorylated protein of this molecular mass is most prominent in cells infected by the GAP SH2 domain-containing mutant. Similarly, the Crk SH2 domain has high affinity for proteins in the 135- to 155-kDa range (3, 22, 24a), and tyrosine-phosphorylated proteins of this molecular mass are prominent in cells infected with the Crk SH2 domain-containing mutant. Surprisingly, there was no apparent correlation between overall phosphotyrosine levels and transforming efficiency.

We next examined whether any of the tyrosine-phosphorylated proteins were physically associated with the Abl proteins. We immunoprecipitated Abl from infected cell lysates, separated the immunoprecipitated proteins on gels, and immunoblotted them with a phosphotyrosine-specific antibody (Fig. 4C). In each lane, several tyrosine-phosphorylated proteins in addition to Abl itself were observed, suggesting that they were physically associated with the Abl product. Furthermore, the pattern of coprecipitating proteins varied with the SH2 domain, accentuating the differences seen in whole lysates and suggesting that these tyrosine-phosphorylated proteins might be bound to Abl by SH2-phosphotyrosine interactions and that this might be the cause of the difference in the overall pattern of tyrosine phosphorylation.

Effect of replacing the Abl catalytic domain. Since the experiments described above suggested that the pattern of phosphoproteins observed when activated Abl is overexpressed depends on the SH2 domain, we examined the role of the catalytic domain in determining this pattern. We generated a series of mutants with various SH2 domains, similar to those shown in Fig. 3A but with the Abl catalytic domain replaced with that of $p60^{c\text{-}src}$. As shown in Fig. 3B, all of these mutants had detectable transforming activity. In fact, the hierarchy of transforming activities was very similar to that seen with the Abl catalytic domain: the Crk SH2 domain was the most efficient, followed by that of PI 3-kinase, with the GAP SH2 being the least efficient. The two cases where the correlation broke down were when the Abl and Src SH2 domains were involved; in each case, the catalytic domain was most efficient when paired with its corresponding SH2 domain. It is remarkable that mutant GSC-C contained pieces of three proteins (N and C termini of Abl, SH2 domain of Crk, and catalytic domain of Src) and was as transforming as the most highly activated Abl variants, demonstrating the truly modular nature of these proteins.

The pattern of tyrosine-phosphorylated proteins was also examined in these mutants (Fig. 5A). Again, as in the case with

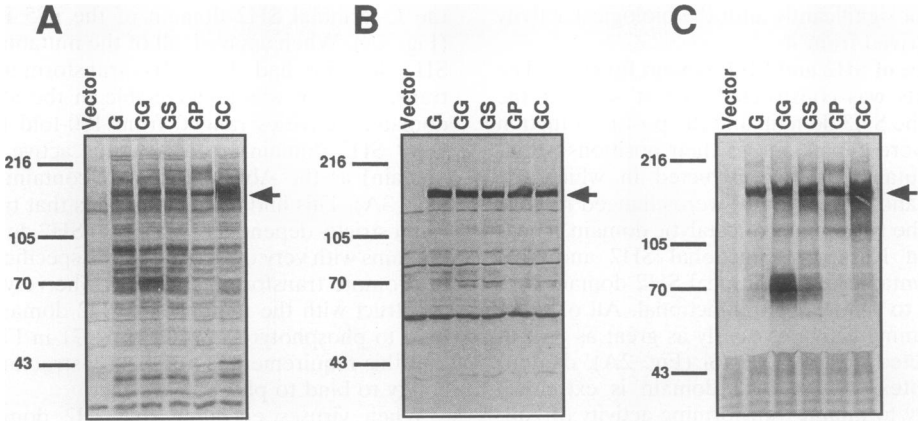

FIG. 4. Characterization of tyrosine-phosphorylated proteins in cells expressing SH2 domain swap mutants. 3T3 cells were infected with high-titer virus stocks expressing mutant Abl genes depicted in Fig. 3A, and lysates were prepared 2 days later. Lanes contain lysates from cells infected with virus as marked. Numbers on the left indicate apparent molecular mass markers in kilodaltons. Arrows indicate approximate positions of mutant Abl proteins. (A) Antiphosphophotyrosine immunoblot of whole cell lysates. (B) Immunoblot of same lysates with Abl-specific antiserum. (C) Antiphosphotyrosine immunoblot of lysates first immunoprecipitated with Abl-specific serum, showing tyrosine-phosphorylated proteins that coprecipitate with Abl protein.

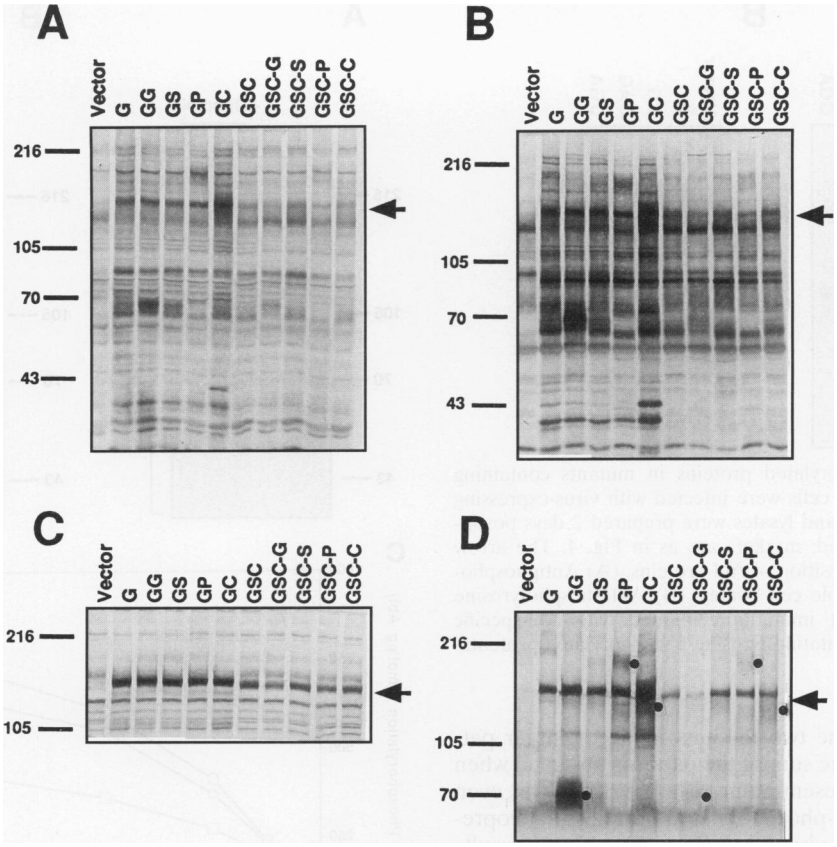

FIG. 5. Tyrosine-phosphorylated proteins in cells expressing mutants with heterologous SH2 and catalytic domains. 3T3 cells were infected with virus-expressing mutants depicted in Fig. 3, and lysates were prepared 2 days after infection. Lanes are as marked; molecular mass markers are as depicted as in Fig. 4. Approximate positions of Abl proteins are indicated by arrows. In panel D, dots indicate the positions of tyrosine-phosphorylated proteins characteristic of mutants containing the GAP, PI 3-kinase, or Crk SH2 domain (approximate molecular masses of 68, 180, and 135 to 155 kDa, respectively). (A) Antiphosphotyrosine immunoblot of whole cell lysates. (B) Antiphosphotyrosine immunoblots of lysates first partially purified by binding to beads covalently coupled to antiphosphotyrosine-specific antibody. (C) Immunoblot of whole cell lysates probed with Abl-specific antiserum. (D) Antiphosphotyrosine immunoblot of lysates first immunoprecipitated with Abl-specific antiserum, showing coprecipitating tyrosine-phosphorylated proteins.

the Abl SH2 domain, the pattern of tyrosine-phosphorylated proteins varied with the SH2 domain. This is more easily seen when the tyrosine-phosphorylated proteins were first immunoprecipitated with antiphosphotyrosine antibody beads (Fig. 5B) or when proteins that coprecipitated with Abl were analyzed (Fig. 5D) because it has proven difficult to get high-level expression of the mutants with the Src catalytic domain in transient infections (Fig. 5C). These results suggest that the SH2 domain plays at least as important a role in determining the pattern of tyrosine-phosphorylated proteins seen in cells infected with these Abl mutants as the catalytic domain does.

Role of multiple SH2 domains. We have shown that the transforming potency and ultimate substrate specificity of Abl mutants is highly dependent on the SH2 domain. We were further interested in whether the effect of multiple SH2 domains would be additive, or whether the properties of one SH2 domain would be dominant in a construct that encoded multiple SH2 domains. For this purpose, we constructed two mutants that contained both the Abl and N-terminal GAP SH2 domains, in the order Abl-GAP or GAP-Abl (Fig. 6). We used these two SH2 domains because they had the greatest disparity in both transforming activity (100-fold) and the pattern of tyrosine-phosphorylated proteins observed (where the GAP SH2 induces the prominent 68-kDa phosphoprotein). Both of these mutants had transforming activities essentially indistinguishable from that of the positive control encoding the Abl SH2 domain, suggesting that both the proximal and distal SH2 domains were able to function to promote transformation (Fig. 6). Furthermore, when the pattern of tyrosine-phosphorylated

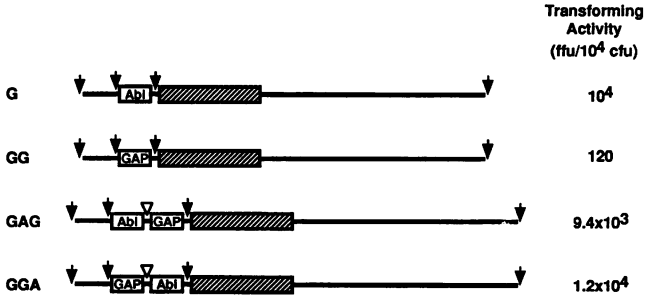

FIG. 6. Mutants containing multiple SH2 domains. Structures and transforming activities of mutants are depicted as in Fig. 1. The origin of each SH2 domain (Abl or N-terminal Ras-GAP SH2 domain) is indicated in a white box. ffu, focus-forming units.

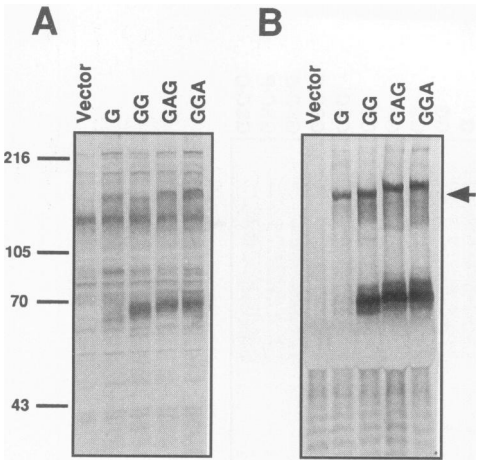

FIG. 7. Tyrosine-phosphorylated proteins in mutants containing multiple SH2 domains. 3T3 cells were infected with virus-expressing mutants depicted in Fig. 6, and lysates were prepared 2 days postinfection. Lanes are as marked; markers are as in Fig. 4. The arrow indicates the approximate position of Abl proteins. (A) Antiphosphotyrosine immunoblot of whole cell lysates. (B) Antiphosphotyrosine immunoblot of lysates first immunoprecipitated with Abl-specific antiserum, showing coprecipitating tyrosine-phosphorylated proteins.

proteins was analyzed, the two mutants induced similar patterns that seemed to be the sum of the patterns observed when each SH2 domain was present alone (Fig. 7A). This was even clearer when the tyrosine-phosphorylated proteins that coprecipitated with Abl were visualized (Fig. 7B). These results demonstrate that the SH2 domains function independently and that proximity to the catalytic domain does not appear to affect their activities significantly. The SH2 domains function additively in that properties of each (efficient transformation by the Abl SH2, phosphorylation of the 68-kDa protein by that of GAP) are conferred on proteins containing both of them.

Effect of SH2 and SH3 domains on purified protein. It is clear from the data presented above that the SH2 and SH3 domains have a profound effect on in vivo activity of the Abl protein. To examine the effect of deletion of these domains on the in vitro activity of purified protein, we have constructed GST fusion proteins encoding the Abl inserts of GDN-F, -G, and -E (intact, SH3 domain-deleted, and SH2 domain-deleted c-Abl, respectively; Fig. 1). In each case, GST was fused directly upstream of the position 2 glycine of Abl, so that cleavage with thrombin generated the natural Abl N terminus except for the lack of the N-terminal myristoyl moiety. The fusion proteins were expressed either in insect cells by using baculovirus vectors or in human 293 cells by using an expression vector driven by the elongation factor 1α promoter (29). In both cases, large quantities of protein were easily purified on glutathione-agarose beads and either eluted with glutathione or cleaved with thrombin.

When the in vitro kinase activities of the three different Abl proteins were compared, there was no apparent difference (Fig. 8). This was true whether or not the proteins were cleaved from GST (Fig. 8A) or whether proteins were synthesized in insect cells or human cells (not shown). Kinase activity was measured using Abl itself or poly(Glu-Tyr) random copolymer as the substrate and visualized on gels (Fig. 8A) or by using angiotensin II or [Val^5] angiotensin II in a quantitative filter paper assay (Fig. 8C); in all cases, the three proteins had essentially identical kinase activities per microgram of Abl protein (differences of less than a factor of 2). With use of

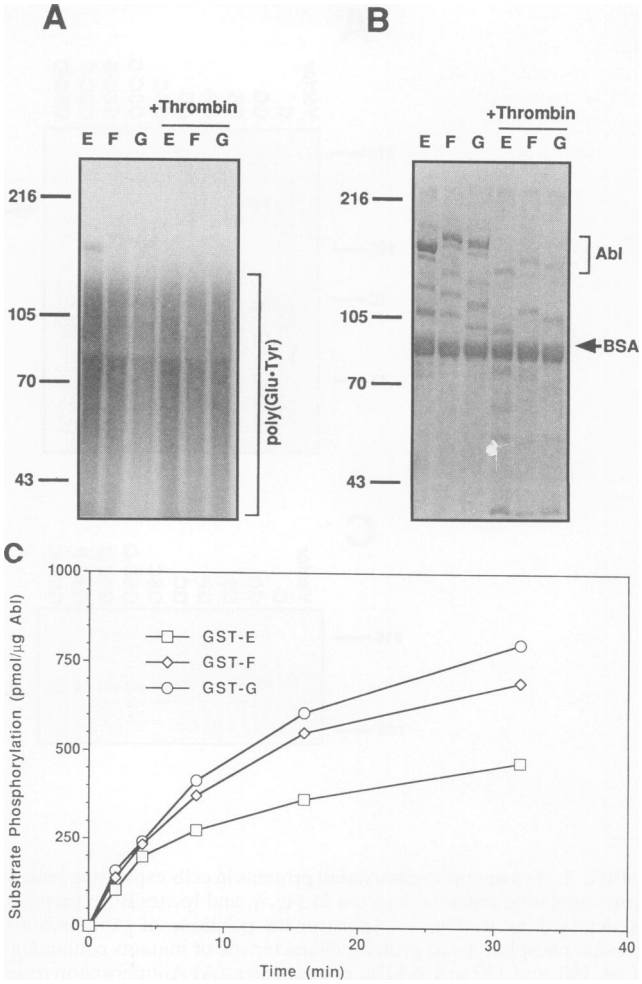

FIG. 8. Effects of SH2 and SH3 domains on in vitro kinase activity of purified GST-Abl fusion proteins. GST-Abl fusion proteins encoding the Abl genes of mutants E, F, and G (see Fig. 1) were produced in insect cells and purified on glutathione-agarose. Proteins were either eluted or cleaved with thrombin to remove GST, as marked. (A and B) Approximately equal amounts of the six purified protein samples were incubated with [γ-^{32}P]ATP and poly(Glu-Tyr) random copolymer to assess kinase activity. Reactions were run on SDS-polyacrylamide gels, stained with Coomassie blue, fixed, dried, and autoradiographed (A) or photographed directly (B). Phosphorylated substrate poly(Glu-Tyr) appears as a smear in panel A. The band at ~85 kDa in panel B (arrow) is acetylated BSA carrier protein. The approximate position of the full-length Abl proteins is indicated on panel B. Molecular mass markers are as in Fig. 4. (C) Rates of reaction of purified GST fusion proteins were assessed in vitro, using [Val^5] angiotensin II as the substrate. Results were normalized for protein content.

[Val^5] angiotensin II at 1 mM, initial reaction rates ranged from approximately 7 to 11 pmol of phosphate per min per pmol of Abl protein (Fig. 8C and data not shown). These results suggest that the SH3 and SH2 domains do not function to regulate the in vivo kinase activity of the Abl kinase in *cis* but require other cellular factors to exert the differential modulatory activities seen in cells.

DISCUSSION

In this study, we have constructed a series of mutants of the Abl kinase designed to probe the function of the SH2 and SH3

domains. We find that the SH3 domain suppresses the intrinsic transforming activity of the c-Abl protein in cells but that this suppression is extremely position dependent and is not apparent in vitro with use of purified proteins. The SH2 domain, on the other hand, is absolutely required for transforming activity of Abl mutants; furthermore, the SH2 domain appears to indirectly determine the spectrum of proteins phosphorylated in cells. Below, we outline models that are consistent with these data.

These studies start from the observation that the wild-type c-Abl protein is poorly transforming, even at relatively high levels of overexpression, while mutated forms such as v-Abl and Bcr-Abl have a greatly increased ability to induce the uncontrolled growth characteristic of transformation and have elevated in vivo tyrosine kinase activity (10, 15, 32). This finding suggests that the c-Abl product is tightly regulated in cells and that mutated forms can escape this regulation. By analyzing the transforming activity of c-Abl mutants, two issues are simultaneously addressed: first, the nature of the machinery that normally suppresses c-Abl kinase activity, and second, the functions required to allow derepressed Abl variants to transform. While the first issue is clearly relevant to the normal function of c-Abl, the second issue may not be. We assume, however, that in some (presently unknown) context, c-Abl kinase activity might be activated, and that this presumably transient activated state is mimicked by constitutively activated transforming variants. Furthermore, understanding the factors required for Abl transformation has obvious clinical relevance in diseases such as chronic myelogenous leukemia, in which activated Abl is strongly implicated.

The transformation assay used in this study is morphological alteration of monolayer 3T3 cells. While there are many parameters of cell transformation, we chose morphological change because it is rapidly and easily scored. One caveat of this type of analysis should be mentioned, however. It has been reported that both c-Abl and its transforming variants have a toxic or cytostatic effect when overexpressed (15, 42). This observation implies that the scores we report for G418 titers and focus titers might not reflect the behavior of the majority of infected cells, since all cells that die or never grow up to a discernible focus or colony are not scored. Indeed, G418 resistance titers for Abl-containing viruses were generally lower than titers for the empty virus (GDN), and SH2 domain-containing variants tended to give lower titers than those lacking a functional SH2 domain (see Table 1 for a representative experiment). Given the inherent difficulties in studying toxicity, however, we leave a more careful analysis of the roles of SH2 and SH3 domains in this phenomenon for future studies.

Role of the SH3 domain. It has been known for several years that deletion of the c-Abl SH3 domain is sufficient to activate its transforming potential (10, 15). Because SH3 domain mutations can also activate c-Src (17, 39), it is likely that SH3 domains act to inhibit an intrinsic transforming activity in many tyrosine kinases that contain them. Our data show that the ability of the SH3 domain to repress Abl activity is extremely position sensitive. Moving the SH3 domain relative to other parts of the protein (the N terminus, SH2 domain, and catalytic domain) activates the transforming ability of c-Abl at least 20-fold. Many of the positional changes are quite subtle, yet they result in almost complete derepression of Abl. These results provide important clues to the possible mechanisms whereby the SH3 domain might repress catalytic activity in vivo. Consistent with our results, another group has found that substituting heterologous SH2 domains (from GAP or Arg) for that of c-Abl activates c-Abl transforming activity (31), perhaps by subtly altering the relationship of the SH3 domain to the kinase and SH2 domains.

The results from the series of mutants shown in Fig. 2A make it likely that the physical relationship between the SH3 domain and more than one other part of the protein is important for repression. If the SH3 domain functioned by interacting directly or indirectly with a single part of the protein, one would expect that only mutations affecting the relationship between the SH3 domain and that part of the protein would activate. This prediction is not borne out by our data. Our results also strongly suggest that the SH3 domain does not function by a simple *trans* mechanism, for example by binding to a structural protein and thereby tethering c-Abl in a subcellular compartment incompatible with transformation. If this were the case, it is unlikely that subtle changes in the position of the SH3 domain would affect its activity.

Another possible mechanism for SH3 domain function would be for it to act in *cis* to inhibit kinase activity, for example by binding to some other part of the protein to hold the protein in a catalytically inactive conformation (reviewed in reference 56). The SH3 domain of c-Src is proposed to collaborate with the SH2 domain to inhibit activity by such a mechanism (51). The Abl SH3 domain is known to bind to proline-rich sequences, and a 9- or 10-aa high-affinity binding site has been identified (4, 41). While the C-terminal half of c-Abl is relatively proline rich, it does not bind with high affinity to the Abl SH3 domain (40a). Furthermore, if the SH3 domain functioned in *cis* by binding to Abl itself, the in vitro kinase activity of c-Abl protein would be expected to be lower than that of variants lacking the SH3 domain. This is not the case: using several substrates, we found no difference in the kinase activity of purified Abl proteins, whether or not the SH3 domain was present.

How, therefore, does the SH3 domain repress the in vivo activity of c-Abl? Any viable model must explain the position dependence of the SH3 domain and its inability to inhibit the activity of purified proteins. The most likely possibility is that the SH3 domain binds to a cellular factor that inhibits catalytic activity. The existence of such a factor has been previously suggested (37). SH3 domains are known to function as protein association domains during signaling (27), and several high-affinity Abl SH3 domain ligands have been isolated (4, 41). The observation that in vitro kinase activities are not SH3 domain dependent suggests that such an inhibitor does not bind tightly enough to copurify; consistent with this, we have found that it is difficult to coimmunoprecipitate Abl and known Abl SH3 domain-binding proteins under standard conditions (1). The extreme position sensitivity of the effect of the SH3 domain could be explained if the putative inhibitor had to make specific three-dimensional contacts with multiple surfaces of the protein in order to inhibit the kinase. For example, it might be necessary for the inhibitor to precisely interact with the catalytic active site, and moving the SH3 would make this interaction sterically unfavorable or even make the SH3 domain inaccessible for binding. Previous work has revealed an Abl catalytic site mutation that can activate transforming potential in the presence of a normal SH3 domain, consistent with such a model (16).

While the model outlined above is clearly speculative, it is consistent with the known behavior of c-Abl and its variants. It also suggests how the system might be regulated—by modification either of the inhibitor or of Abl itself—allowing for an activated state for c-Abl under some physiological conditions. It is possible that in such an activated state, the Abl SH3 domain might bind other proteins, such as 3BP-1 (4), which contains a domain with GAP activity for the Rho/Rac family of

Ras-like GTP-binding proteins, serving to tie c-Abl into other signaling pathways. Of course, such potential interactions must not be required for cell transformation, since SH3 domain-deleted mutants are fully activated for cell transformation.

Role of the SH2 domain in transformation. Unlike the SH3 domain, the SH2 domain of the Abl kinase plays a positive role in transformation. If the SH2 domain is deleted, or if it is mutated at a residue that is critical for binding to phosphotyrosine, transforming activity is almost completely lost, even in the absence of the SH3 domain. Abl proteins that lack a functional SH2 domain retain wild-type levels of in vitro kinase activity, assayed with purified proteins made either in insect cells (Fig. 8) or in immunoprecipitates from infected 293 or 3T3 cells (not shown), suggesting that the SH2 domain does not function by modulating the kinase domain directly.

These results raise the question of why a tyrosine kinase would require an SH2 domain, whose function is to tightly bind to tyrosine-phosphorylated proteins, in order to transform. Several possibilities will be considered. First, since the nontransforming c-Abl protein is localized largely in the nucleus (54), it is possible that the SH2 domain functions (when the kinase domain is activated, for example by SH3 deletion) to tether Abl via SH2-phosphotyrosine interactions in the cytoplasm, where it must be in order to transform. However, SH3 domain-deleted Abl mutants bearing the R171K mutation that abolishes SH2 domain function are localized in the cytoplasm (53a), suggesting that a functional SH2 domain is not necessary for cytoplasmic localization.

A second possibility is that the SH2 domain functions to tether substrate proteins to the plasma membrane. In signal transduction by receptor tyrosine kinases, a critical step of signaling is the binding of normally cytoplasmic signaling proteins, such as phospholipase C-γ, GAP, PI 3-kinase, and GRB-2, to the membrane by interaction of their SH2 domains with phosphotyrosine residues on the activated receptor (reviewed in references 33, 35, and 46). Since transforming Abl variants are myristoylated and at least partially localized on the plasma membrane (15, 54), the same effect would be achieved if the Abl kinase phosphorylated such proteins on tyrosine and then tightly bound them via its SH2 domain. We have tested this hypothesis by expressing two Abl variants in one cell, the first containing the R171K mutant SH2 domain and lacking the SH3 domain, and the second containing a functional SH2 domain but bearing an ATP binding site mutation (K290M) that inactivates catalytic activity (15a). Each of these mutants is nontransforming when expressed in cells. If binding phosphorylated substrate protein to the membrane were critical, the two mutants would be expected to complement each other in *trans* to induce transformation. We found, however, that expression of the K290M mutant did not increase the transforming potential of the R171K mutant (not shown), arguing against such a model.

An important clue to SH2 domain function was obtained from the SH2 domain swap experiments. We showed that heterologous SH2 domains could complement the lack of the Abl SH2 domain and, more importantly, that cells infected with mutants bearing heterologous SH2 domains displayed different patterns of tyrosine-phosphorylated proteins. In fact, the pattern of phosphoproteins correlated roughly with the known specificities of the different SH2 domains; i.e., the phosphorylation of proteins with high affinity for a particular SH2 domain was enhanced in cells infected with the Abl mutants bearing that SH2 domain. This was true even if the catalytic domain was swapped for that of Src, suggesting that much of the substrate specificity of the nonreceptor tyrosine kinases might be conferred by the SH2 domain and not the catalytic domain.

How does the SH2 domain affect substrate specificity? We propose that what is actually being modulated is not substrate specificity per se, but the prolonged phosphorylation of proteins that bind to the SH2 domain. Unlike the case for serine-threonine kinases, there is little in vitro evidence for substrate specificity for the tyrosine kinases, and it is possible that they can phosphorylate virtually any phosphotyrosine residue that is accessible. At the same time, cellular tyrosine phosphatase activities are very high, so most proteins are rapidly dephosphorylated in vivo. If the site that is phosphorylated had high affinity for the SH2 domain, however, it could tightly bind to it; once bound, it would probably be protected from phosphatases, since several studies have shown that binding to SH2 domains can protect phosphotyrosine residues from dephosphorylation in vitro (3, 44). Furthermore, if the protein is tethered to the kinase via the SH2 domain, the local concentration of the catalytic domain would be extremely high and it is likely that the protein would be repeatedly phosphorylated on multiple sites. Both of these mechanisms (protection from dephosphorylation and repeated phosphorylation) are likely to play a role, since addition of vanadate, a tyrosine phosphatase inhibitor, to the culture media of cells infected with an SH2 domain-deleted mutant increased tyrosine phosphorylation on cell proteins, but not to the level seen if the SH2 domain was present (not shown).

The model outlined above is consistent with our observation that the SH2 domain is largely position independent when N terminal to the catalytic domain, because it is unlikely that precise positioning would be required to bind to proteins phosphorylated by the catalytic domain, given its high local concentration. Furthermore, such a model would predict that SH2 domains would function independently and additively, consistent with our data from mutants containing both the Abl and GAP SH2 domains. It is possible that the SH2 domains of other families of nonreceptor tyrosine kinases perform a similar role, since SH2 domain mutations in v-Fps and v-Src can drastically affect transforming activity and substrate phosphorylation (18, 43, 45, 55). However, as discussed below, there are also important differences, at least in the case of Src, in which the SH2 domain also has an inhibitory role.

The model described above implies that the stoichiometry of tyrosine-phosphorylated substrates to Abl protein itself should be roughly equimolar, which is consistent with the levels of proteins seen in antiphosphotyrosine immunoblots. Therefore, as is the case with the receptor class of tyrosine kinases, it appears that the Abl nonreceptor kinase signals via stoichiometric complexes of proteins without signal amplification. This picture is very different from the traditional paradigm, taken from serine-threonine kinases, that kinases amplify signals by phosphorylating many substrate proteins when activated.

A further implication of the SH2 domain swap experiments is that many sets of tyrosine-phosphorylated proteins can induce transformation. The specificities of the SH2 domains used in these experiments, and thus the spectrum of proteins that would be phosphorylated in infected cells, are quite different, yet all of the constructs have considerable transforming activity. It is possible that there are common substrates that are relatively SH2 domain independent; in fact, all of the transforming mutants examined increase the phosphorylation of a protein of approximately 85 kDa (Fig. 4A and 5A), which does not efficiently coimmunoprecipitate with Abl and thus is probably not bound via the SH2 domain. It is likely, however, that there is no one critical substrate for transformation and that many combinations of tyrosine-phosphorylated proteins

can lead to growth. An analogy could be made to the growth factor receptors, which contain the binding sites for many SH2 domain-containing proteins; binding of several different proteins individually or in combination can be sufficient to signal DNA synthesis (53).

The models proposed above for SH2 and SH3 domain function in Abl are different in several respects from the functions proposed for these domains in the closely related Src family of nonreceptor tyrosine kinases (see reference 6 for a review). In these proteins, the SH2 domain has a largely inhibitory role on the activity of c-Src, since it interacts with a regulatory phosphotyrosine (Y-527 in Src) that holds the c-Src protein in an inactive conformation. The c-Abl protein has no Y-527 equivalent and no detectable tyrosine phosphorylation in vivo (38), so such an inhibitory role for the SH2 domain is implausible. In Src, the positive role for the SH2 domain in transformation is apparently less important; although SH2 domain mutations can alter and weaken the transformed phenotype, mutants in which the SH2 domain has been completely deleted have been reported to be highly transforming (47). The SH3 domain in Src, like that in Abl, is thought to have an inhibitory role, but unlike the case of Abl, Src SH3 domain mutants have elevated in vitro kinase activity (17). When c-Src is expressed in *Schizosaccharomyces pombe*, the SH3 domain cooperates with the SH2 domain to mediate binding to the regulatory C-terminal phosphotyrosine (51). These results indicate that in Src, the SH3 domain functions in *cis*, which our data suggest is not the case for Abl. These differences highlight how diversity of activity and regulation can evolve from very similar combinations of simple modular units.

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