

Abl protein–tyrosine kinase selects the Crk adapter as a substrate using SH3-binding sites

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To understand the normal and oncogenic functions of the protein–tyrosine kinase Abl, the yeast two-hybrid system has been used for identifying proteins that interact with it. One interacting protein is Crk-I, an SH3/SH2-containing adapter protein that was originally identified as the oncogenic element in the avian sarcoma virus CT10. Direct interaction between the Crk-I SH3 and Abl at novel, ~10 amino acid sites just carboxy-terminal to the Abl kinase domain occurs *in vitro* and in mammalian cells. There is a nearby site specific for binding another adapter, Nck, and these sites also bind Grb-2. When bound to Abl, Crk-I was phosphorylated on tyrosine. Thus, the SH3-binding sites on Abl serve as substrate recognition sites for the relatively nonspecific kinase of Abl. In Crk-I-transformed cells, Crk-I associates with endogenous c-Abl and is phosphorylated on tyrosine. The association of Crk and Abl suggests that Abl could play a role in v-Crk and Crk-I transformation and that normal Abl function may be partly mediated through bound adapter molecules.

[Key Words: Abl; Crk; Nck; Grb2; SH3; phosphorylation]

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The *abl* oncogene was first identified as the oncogenic element in the Abelson murine leukemia virus (for review, see Rosenberg and Witte 1988; Wang 1993). It is also an oncogenic element in the Philadelphia chromosome, a fusion of a portion of the breakpoint cluster region (*bcr*) gene with *c-abl* found first in cells of human chronic myelogenous leukemia (for review, see Daley and Ben-Neriah 1991).

Although all cells contain some c-Abl, the protein may play a particular role in certain developmental events. *Drosophila* Abl mutants have a disrupted axonal organization and die in the pupal stage of development (Henkemeyer et al. 1990). Mice with a homozygous disruption of the *c-abl* gene—either through a null mutation or a deletion of the carboxy-terminal 1/3 of the protein—are variably affected, but some display increased perinatal mortality, runtedness, lymphopenia, and abnormal head and eye development (Schwartzberg et al. 1991; Tybulewicz et al. 1991).

The amino-terminal half of c-Abl is similar to that of many Src family kinases in which Src homology regions SH3 and SH2 abut the kinase (Pawson 1988). SH2 and SH3 domains are modular units present in a very large group of proteins (for review, see Mayer and Baltimore 1993; Pawson and Gish 1992). SH2s, ~100 amino acids long, bind to short peptide segments containing a phosphorylated tyrosine residue. Frequently, SH2 binding

links activated growth factor receptors to downstream signal transduction proteins (Pawson and Gish 1992). SH3 domains contain ~50–60 amino acids and mediate protein–protein interactions among signal transduction proteins by binding to proline-containing sites (Cicchetti et al. 1992; Egan et al. 1993; Gout et al. 1993; Li et al. 1993; Liu et al. 1993; Ren et al. 1993; Rozakis-Adcock et al. 1993; Weng et al. 1993).

Although c-Abl is largely nuclear, some is associated with the plasma membrane or with actin filaments (Van Etten et al. 1989, 1993; McWhirter and Wang 1991). It is different from most tyrosine kinases in having a long carboxyl terminus that is encoded by a single long exon. In this region of >600 amino acids, there is a DNA-binding domain, an actin-binding domain, and a nuclear localization signal (Jackson and Baltimore 1989; Van Etten et al. 1989; Wang 1993). The complexity of c-Abl structure and localization in cells suggests that it may either fulfill multiple cellular functions or that it integrates multiple events. Overproduction of c-Abl does not result in cell transformation or elevated tyrosine phosphorylation in cells, suggesting that the c-Abl protein–tyrosine kinase activity is tightly controlled *in vivo* (Franz et al. 1989; Jackson and Baltimore 1989). Instead, overproduction leads to inhibition of cell growth during G₁, suggesting that c-Abl can interact with the machinery of cellular growth control (Jackson et al. 1993a). In contrast to c-Abl, transforming variants of Abl are largely cytoplasmic and their kinase activity is constitutively activated (Franz et al. 1989; Jackson and Baltimore

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1989; Van Etten et al. 1989; Jackson et al. 1993b; Muller et al. 1991; Goga et al. 1993).

One way to develop a better understanding of the normal and oncogenic functions of Abl is to look for proteins that regulate c-Abl or are regulated by it. In this study the entire carboxy-terminal half of Abl was used as a bait to screen a HeLa cDNA expression library using the yeast two-hybrid system (Fields and Song 1989; Zervos et al. 1993). One clone isolated was a fragment of the Crk-I protein, an SH2- and SH3-containing adapter that was originally identified as the oncogenic element of avian sarcoma virus CT10 (Mayer et al. 1988). Crk-I and Abl interaction occurs *in vitro* and in mammalian cells and, when bound to Abl, Crk-I is phosphorylated on tyrosine. Binding is a consequence of short, linear peptide sequences in Abl that bind to SH3 regions of Crk. A site for binding the SH3-containing Nck molecule was also found. These studies show that SH3 can be a binding region for a kinase substrate, extend our knowledge of SH3 binding-specificity, suggest that Abl may play a role in Crk transformation, and that c-Abl may use adapter molecules in its normal function.

Results

Proteins that interact with the Abl carboxy-terminal region

A yeast two-hybrid system was used to identify proteins that interact with c-Abl. The two-hybrid system, originally described by Fields and Song (1989), depends on a transcriptionally derived signal for identification of a gene encoding a protein that interacts with a protein of interest. One hybrid consists of a DNA-binding domain fused to the protein of interest, and the other consists of a transcriptional activation domain fused to a cDNA library. The two-hybrid system used in this study was developed by R. Brent and colleagues (Zervos et al. 1993).

For identifying proteins that interact with the carboxy-terminal half of c-Abl (AblC'), a cDNA fragment encoding amino acids 545–1149 of c-Abl was cloned in-frame into the pEG202 vector so it would be made as a fusion protein with the LexA DNA-binding domain (LexA/AblC'). The LexA/AblC'-encoding plasmid was transformed into yeast EGY48 along with a reporter plasmid that contains the β -galactosidase gene under LexA-operator control, *LexAop-lacZ*; the yeast genome also contained an integrated *LexAop-LEU2* reporter gene. The expression of the LexA/AblC' fusion protein was confirmed by Western blot analysis using PEX4 anti-Abl antibody (data not shown). Although the yeast contained LexA-controlled genes for synthesis of β -galactosidase and the *LEU2* protein, neither gene was expressed, indicating that the AblC' region provided no transcriptional activating activity.

To identify proteins that interact with AblC', we used a HeLa cDNA library cloned into a conditional expression vector pJG45. In the presence of galactose but not glucose, this vector produce cDNA-encoded proteins fused to an epitope tag, a nuclear localization sequence,

and an acidic transcription activation domain. Five million library transformants were plated onto five galactose–Leu⁻ selection plates. Colonies that were prototrophic for leucine were replica-plated onto X-gal plates, where β -galactosidase-producing colonies turn blue. About 100 galactose-dependent, blue colonies were picked, of which 10 were analyzed further in this study. These clones represent ~500,000 library transformants screened.

The 10 library cDNA plasmids were isolated through genetic selection in *Escherichia coli*. To test whether the library cDNAs truly encoded proteins that interacted specifically with AblC', the library plasmids were cotransformed back into yeast with vectors that produced either the LexA-binding domain alone or the binding domain fused to the entire c-Abl or AblC'. Nine of the clones produced β -gal activity only when c-Abl or AblC' was present (Table 1), suggesting that these cDNAs encode proteins that interact with the carboxy-terminal half of c-Abl. The remaining one clone was active even when cotransformed with the LexA DNA-binding domain alone, indicating that this clone did not encode a c-Abl-interacting protein.

Crk cDNA identified

DNA sequence analysis revealed that the nine c-Abl-interacting clones identified by the two-hybrid screen belonged to three genes. The DNA sequences were compared with sequences from GenBank. Two of them (clone A, representing six independent clones, and clone C) were found to be novel genes. The DNA sequence of clone B, representing 2 of 10 primary clones, was identical to part of the human proto-oncogene Crk-I cDNA (Fig. 1).

The Crk oncogene was originally isolated as the oncogenic element of avian retrovirus CT10 (Mayer et al. 1988) and ASV-1 (Tsuchie et al. 1989). The human c-Crk proto-oncogene encodes two proteins, Crk-I and Crk-II, through alternative splicing pathways (Matsuda et al. 1992). Crk-I and Crk-II proteins are adapter molecules containing primarily SH2 and SH3 domains: Crk-II has one SH2 and two SH3 domains; Crk-I lacks the carboxy-

Table 1. Clones interacting with carboxy-terminal portion of Abl in yeast two-hybrid system

Clone	Frequency (in 5×10^5 transformants)	β -Gal activity in yeast ^a		
		with pEG202/ AblC'	with pEG202/ Abl	with pEG202
A	6	+	+	–
B	2	+	+	–
C	1	+	+	–
D	1	+	+	+

^a β -Gal activity in yeast was detected in X-gal plates. Entries indicate the presence or absence of blue color with transformants.

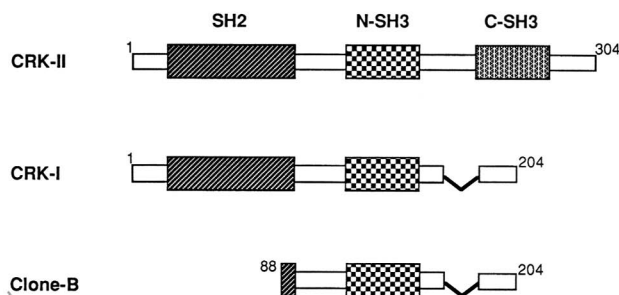


Figure 1. Schematic representation of the c-Abl-interacting Crk protein identified by genetic screening. The domain structures of human Crk-I and Crk-II [Matsuda et al. 1992] are compared with the primary structure of clone B found to interact in the two-hybrid screen with Abl.

terminal SH3 domain. The Abl-interacting clone B cDNA lack the sequence encoding the amino-terminal 87 amino acids of *Crk-I* cDNA, thus producing a partial Crk-I protein from which the SH2 domain was mostly deleted (Fig. 1). The Crk/Abl interaction therefore appeared to be mediated by the Crk-I SH3 domain (CrkI-SH3).

CrkI-SH3 binds c-Abl in vitro

To examine whether CrkI-SH3 bound Abl directly, an *in vitro* filter assay was employed. For this assay, the cloned partial Crk-I protein was expressed as a glutathione *S*-transferase fusion protein in *E. coli* (referred as GST/CrkI-SH3). GST/CrkI-SH3 was purified using glutathione-agarose beads, biotinylated as described [Mayer et al. 1991], and used as a probe on filters containing Abl protein.

The cDNA encoding AblC' was expressed in *E. coli* by cloning it in-frame carboxy-terminal to GST. Crk binding to the GST/AblC' fusion protein was examined following electrophoretic fractionation of crude *E. coli* extracts, transfer to a filter, and probing with biotinylated GST/CrkI-SH3. As revealed by anti-GST antibody (Fig. 2) and PEX4 anti-Abl antibody (data not shown), the GST/AblC' fusion protein expressed in bacteria had an apparent molecular mass of 110 kD. However, the majority of the GST/AblC' fusion protein was degraded into polypeptides with various sizes. Because AblC' was tagged with GST, this natural degradation of GST/AblC' facilitated mapping of the binding sites for Crk and other SH3-containing proteins.

The GST/CrkI-SH3 probe bound to the full-length GST/AblC' fusion protein and to a number of its degradation products (Fig. 2). Binding of the GST/CrkI-SH3 probe to GST and the GST probe to the GST/AblC' fusion protein was not detected, indicating that the interaction of GST/CrkI-SH3 and GST/AblC' was through the binding of CrkI-SH3 to AblC'. The GST/AblC' degradation products that bound to CrkI-SH3 were similar to those bound to anti-GST antibody, suggesting that the CrkI-SH3-binding site is located in the amino-terminal portion of AblC'.

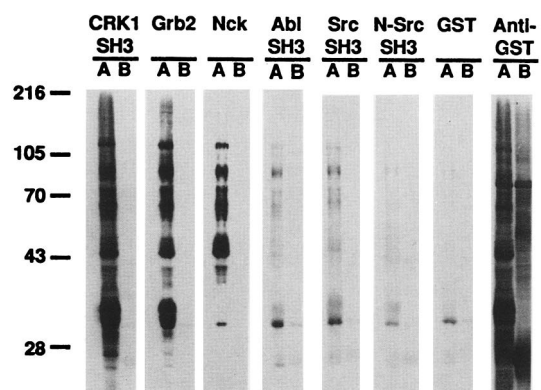


Figure 2. *In vitro* binding of the c-Abl carboxy-terminal half to various SH3 domains and SH3-containing proteins. Proteins from induced lysates of bacteria that expressed the GST-AblC' fusion protein (A) or GST (B) were probed with anti-GST antibody or with biotinylated GST or GST fusion proteins as indicated above each pair of lanes. Numbers (left) represent the molecular size in kilodaltons.

The CrkI-SH3 probe used to examine the Crk-Abl interaction *in vitro* contained flanking sequences of the SH3 domain. To confirm that the Crk-Abl interaction was through the Crk-I SH3 domain alone, the first SH3 domain of murine c-Crk [Cicchetti et al. 1992] was tested for binding to AblC'. The murine Crk SH3 bound to AblC' as effectively as did CrkI-SH3 (data not shown). Both the murine Crk SH3 domain and cloned human CrkI-SH3 could also bind overexpressed full-length c-Abl from NIH-3T3 cells (data not shown).

CrkI-SH3-binding sites are in a region between the Abl kinase domain and its nuclear localization signal

The pattern of CrkI-SH3 binding to the degradation products of the GST/AblC' fusion protein suggested that the SH3-binding site was located in the amino-terminal region of AblC'. Sequential deletion analysis of AblC' from its carboxyl terminus mapped the CrkI-SH3-binding site to a 56-amino-acid region (Table 2). In this region there are three proline-containing peptides (APELPT-KTR, EPAVSPLLPRK, and APDTPPELLHTK) that drew

Table 2. Mapping of the Crk, Nck, and Grb2 SH3-binding sites

GST fusion peptides	SH3 binding ^a		
	CrkI-SH3	Nck	Grb2
AblC' (545-1149) ^b	+	+	+
AblC'ΔSal (545-987)	+	+	+
AblC'ΔXho (545-763)	+	+	+
AblC'115 (545-659)	+	+	+
AblC'56 (551-606)	+	-	+

^aDetermined by filter-binding assay.

^bNumbers indicate the amino acid sequences in type IV c-Abl.

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our attention because other SH3s bind to sites rich in proline (Ren et al. 1993). The binding sites for the CrkI-SH3 domain were determined by preparing GST fusions of these peptides and expressing them in bacteria. Filter-binding assays showed that CrkI-SH3 bound strongly to peptide APELPTKTR and weakly to peptide EPAVSPLLPRK (Fig. 3A). These two peptides are termed Abl-binding sites for SH3 (AB3-1 and AB3-2). No binding to a third peptide, APDTPELLHTK, was detected (data not shown). Deletion of the two CrkI-SH3-binding sites abolished the association between Crk-I and c-Abl in cells (see below), indicating that in Abl there are two CrkI-SH3 binding sites located between the Abl kinase domain and the nuclear localization signal (Fig. 3B).

SH2- and SH3-containing adapter proteins Grb2 and Nck bind to Abl in vitro

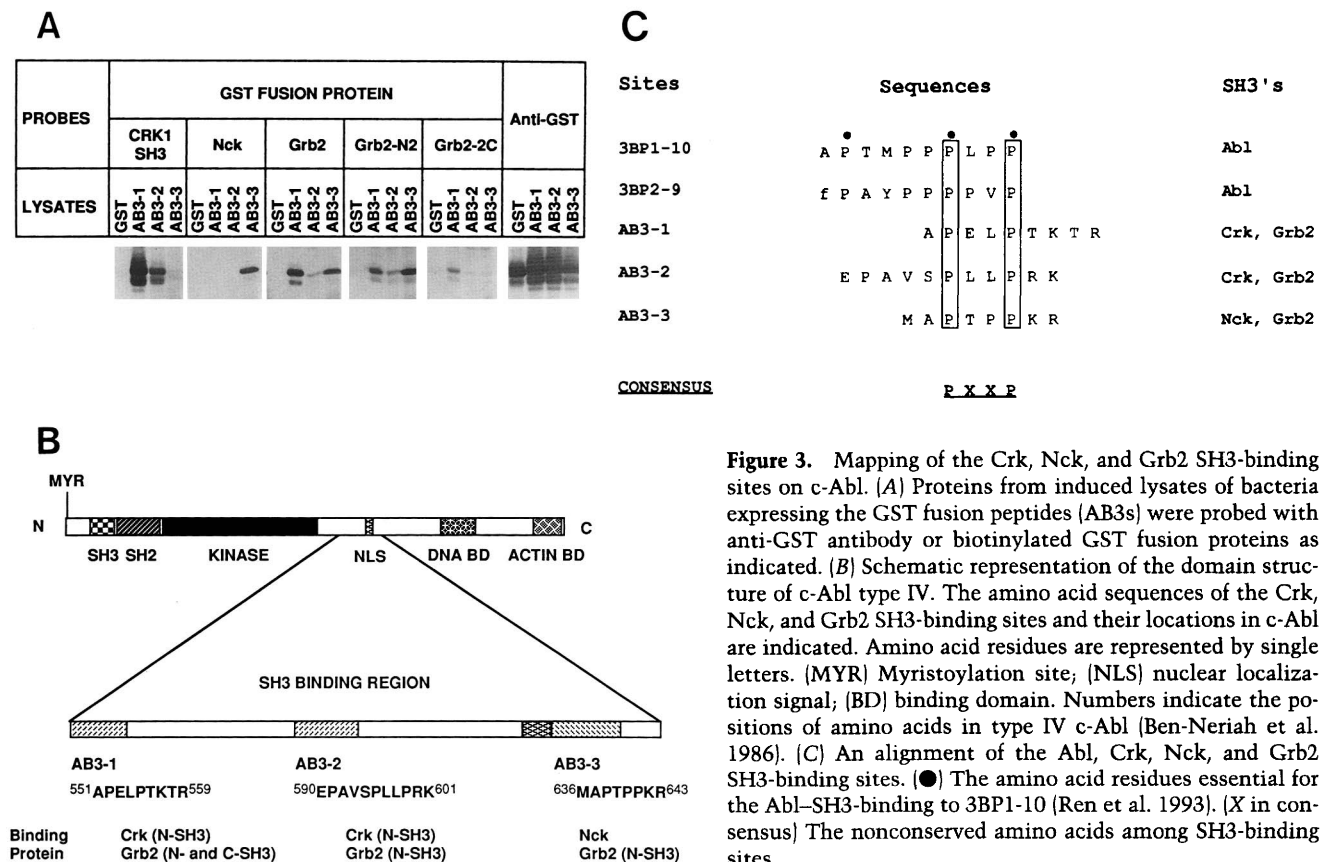
To examine the specificity of Crk-Abl interaction, the binding potential of different SH3 domains or SH3-containing proteins to Abl was tested by the filter-binding assay. The Abl-SH3 and Src-SH3 domains bound to GST/AbIC' very weakly, but above the signal of GST alone (Fig. 2). No binding was detected for neural-Src (N-Src). According to the binding pattern to the degradation products of GST/AbIC', the binding site for Abl-SH3 seems to be located carboxy-terminal to the Crk and Nck SH3-binding sites. This low affinity interaction

may be significant for intramolecular interaction and may play a role in the control Abl kinase activity and/or autophosphorylation.

Interestingly, both of the SH2- and SH3-containing adapter proteins, Grb2 and Nck, bound to AbIC' as strongly as did CrkI-SH3 (Fig. 2). The pattern of Grb2 binding to GST/AbIC' and its degradation products was similar to that of CrkI-SH3, suggesting that Grb2-binding sites are located very close to those of CrkI-SH3. However, Nck bound only to the larger degradation products of GST/AbIC', indicating that Nck binds in a region carboxy-terminal to the CrkI-SH3 sites.

We mapped the Nck-binding site to a 53-amino-acid region (606-659) (Table 2). The ability of Nck to bind the proline-containing peptide MAPTPPKR (AB3-3) in this region, expressed as a GST fusion protein in bacteria, was tested by the filter-binding assay. Nck bound AB3-3 specifically, but in spite of the three SH3 domains in Nck, no binding of Nck to AB3-1 and AB3-2 was detected (Fig. 3A). CrkI-SH3 bound very weakly to AB3-3.

Binding of Grb2 to the three AB3s was also examined. Grb2 bound to AB3-1 and AB3-3 strongly and to AB3-2 weakly (Fig. 3A). Because Grb2 contains two SH3 domains, the binding of different SH3 domains of Grb2 to AB3s was determined using Grb2 mutants. Grb2-N2 is Grb2 with the carboxy-terminal SH3 domain deleted. Grb2-2C is Grb2 with the amino-terminal SH3 domain deleted (Lowenstein et al. 1992). Grb2-N2 bound all



three AB3s: AB3-3 was strongest and AB3-2 weakest (Fig. 3A). The binding of Grb2-N2 to AB3-1 was weaker than that of wild-type Grb2. Grb2-2C bound weakly and only to AB3-1. The different specificities of the two SH3 domains of Grb2 suggested that they may bind Abl cooperatively in a specific orientation.

The locations of the CrkI-SH3, Grb2, and Nck-binding sites in c-Abl are shown in Figure 3B.

Two proteins that bind specifically to the Abl-SH3 domain were isolated previously by screening a λ gt11 cDNA expression library using the GST/Abl-SH3 fusion protein (Cicchetti et al. 1992). The SH3-binding sites of the two SH3-binding proteins were localized to a 10-amino-acid stretch very rich in proline residues (Ren et al. 1993). In the 3BP1 site, proline residues at positions 2, 7, and 10, were crucial to the binding. A number of SH3-binding proteins have been identified since then (Egan et al. 1993; Gout et al. 1993; Li et al. 1993; Liu et al. 1993; Rozakis-Adcock et al. 1993; G. Cheng, S.-Z. Ye, and D. Baltimore, in prep.; K. Alexandropoulos and D. Baltimore, unpubl.). All of the SH3-binding sites have been characterized by multiple proline residues. Although the Abl-SH3- and CrkI-SH3-binding sites are very different in amino acid sequence, they can be aligned with two conserved proline residues spaced by two nonconserved amino acids (Fig. 3C). The Crk, Nck, and Grb2 SH3-binding sites share basic amino acids at their carboxyl terminus not found in the Abl sites.

Crk-I associates with both c-Abl and transforming Abl in mammalian cells

The studies described above demonstrated that truncated Crk, consisting of the SH3 domain and its flanking sequences, interacts with c-Abl both in yeast and in vitro. To determine whether Crk associates with Abl in vivo, full-length human *Crk-I* cDNA was cloned by reverse transcriptase (RT)-PCR. For identification purposes, the Crk-I protein was tagged with an influenza virus hemagglutinin (HA) epitope at its amino terminus,

producing the HACrkl chimera. The gene for this chimeric protein was subcloned into mammalian expression vector pGD, in which the Moloney leukemia viral long terminal repeat (LTR) directs RNA synthesis.

To study the interaction of Crk and Abl in cells, we first examined a transforming allele of Abl. Human kidney 293 cells were either cotransfected with pGD/HACrkl and pGD/c-abl Δ XB, which encodes SH3-deleted transforming Abl, or transfected with pGD/c-abl Δ XB alone. After 48 hr the cells were lysed and the lysates were immunoprecipitated with either anti-HA monoclonal antibody (12CA-5), the anti-Abl polyclonal antibody PEX4, or control antibodies. The immunoprecipitates were then subjected to an in vitro kinase assay, using [γ - 32 P]ATP to label proteins in vitro. Labeled proteins were separated electrophoretically by SDS-PAGE and detected by autoradiography. A 140-kD phosphorylated protein was precipitated by anti-HA antibody, but not control monoclonal antibody, from cells cotransfected with pGD/HACrkl and pGD/c-abl Δ XB (Fig. 4A). This 140 kD protein comigrated with Abl Δ XB, as detected by anti-Abl antibody PEX4 (Fig. 4A). Abl Δ XB was not precipitated by anti-HA antibody from cells transfected with either pGD/HACrkl (data not shown) or pGD/c-abl Δ XB alone. Thus the antibody to HACrkl coprecipitated the Abl protein as detected by its autophosphorylation activity.

A phosphorylated 28-kD protein was precipitated by both anti-HA antibody and anti-Abl antibody, but not control antibody, from cells cotransfected with pGD/HACrkl and pGD/c-abl Δ XB but not from cells transfected with pGD/c-abl Δ XB alone (Fig. 4A). This 28-kD protein migrated identically to tyrosine-phosphorylated Crk-I protein as detected by Western blot using anti-HA antibody (data not shown). 32 P incorporation into HACrkl was much less following precipitation by anti-HA antibody than by anti-Abl antibody. This may be attributable to a blocking effect of the bound anti-HA antibody.

To determine whether Crk interacts with c-Abl, hu-

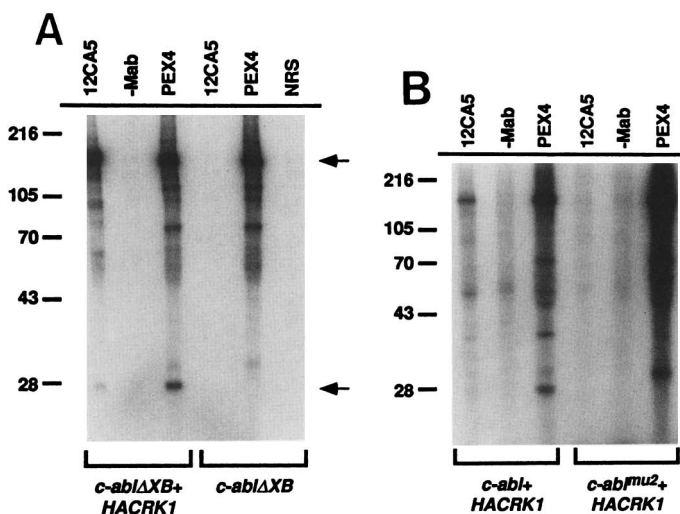


Figure 4. Intracellular association of Crk-I and Abl. (A) Immunoprecipitation (IP) and in vitro kinase analysis of 293 cells expressing the transforming c-Abl Δ XB or c-Abl Δ XB plus HACrkl. (B) IP kinase analysis of 293 cells expressing c-Abl plus HACrkl or c-Abl^{mu2} plus HACrkl. IP antibodies are indicated over the lanes. (12CA5) Anti-HA antibody (IgG_{2b}); (-Mab) anti-RAG2 monoclonal antibody (IgG_{2b}) as a control; (PEX4) anti-Abl antibody; (NRS) normal rabbit serum. Gels were exposed to X-ray film overnight. The positions of Abl tyrosine kinase and phosphorylated HACrkl protein on the gels are indicated (right). Numbers (left) represent the molecular size in kilodaltons.

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man kidney 293 cells were cotransfected with pGD/*HACrkI* and pGD/*c-abl*, and ^{32}P -labeled, immunoprecipitated proteins were detected after phosphorylation in vitro. As with transforming Abl, labeled Crk-I and c-Abl were specifically precipitated by both anti-HA and anti-Abl antibody (Fig. 4B), indicating that Crk-I associates with c-Abl in cells.

The SH3-binding sites on Abl are needed for Crk interaction in cells

The in vitro studies mapped the Crk SH3-binding site on Abl to a region between the Abl kinase domain and its nuclear localization signal. To determine whether the association of Crk and Abl in vivo was directly mediated by this interaction, the CrkI-SH3-binding sites were mutated in c-Abl by deletion. Mutation of the higher affinity CrkI-SH3 binding site, AB3-1, gave the c-Abl^{mu1} mutant Abl protein. Mutation of both CrkI-SH3-binding sites, AB3-1 and AB3-2, gave c-Abl^{mu2} mutant Abl protein. The expression and in vitro kinase activity of the mutant Abl proteins were similar to that of c-Abl (Fig. 4B). To examine whether HACrkI interacts with the mutant Abl proteins, they were coexpressed in 293 cells. Immunoprecipitation and in vitro kinase assay showed that HACrkI and c-Abl^{mu1} still form a complex in cells (data not shown). Association of HACrkI and c-Abl^{mu2} however, was not detected by immunoprecipitation and kinase assay (Fig. 4B), indicating that Crk-I interacts with Abl through the CrkI-SH3 domain's binding to the defined sites in Abl.

Crk is phosphorylated on tyrosine upon binding to Abl

The immunoprecipitation studies showed that Crk-I protein and Abl were coprecipitated by both anti-HA antibody and anti-Abl antibody. The Crk-I protein was phosphorylated as shown by ^{32}P incorporation, suggesting that Crk-I is an Abl kinase substrate. To examine whether Crk-I is phosphorylated by Abl kinase in vivo, 293 cells were either transfected with pGD/*HACrkI* or cotransfected with pGD/*HACrkI* and pGD/*c-abl*. After 48 hr, cells were metabolically labeled with $^{32}\text{P}_i$. $^{32}\text{P}_i$ -labeled cells were then lysed, and the lysates were subjected to immunoprecipitation with anti-HA monoclonal antibody. The immunoprecipitates were separated electrophoretically by SDS-PAGE and detected by autoradiography. In cells overexpressing HACrkI alone, it was very weakly phosphorylated and migrated with an apparent molecular mass of 24 kD (Fig. 5A). In cells coexpressing HACrkI and c-Abl, HACrkI was hyperphosphorylated and its electrophoretic mobility was quantitatively shifted to an apparent molecular mass of 28 kD (Fig. 5A). Autophosphorylation of c-Abl in cells was not detectable. Phosphoamino acid analysis demonstrated that the hyperphosphorylated HACrkI contained mainly phosphotyrosine and some phosphoserine (Fig. 5B). The phosphoamino acid(s) in the 24-kD HACrkI from cells not transfected with c-Abl could not be detected under this condition.

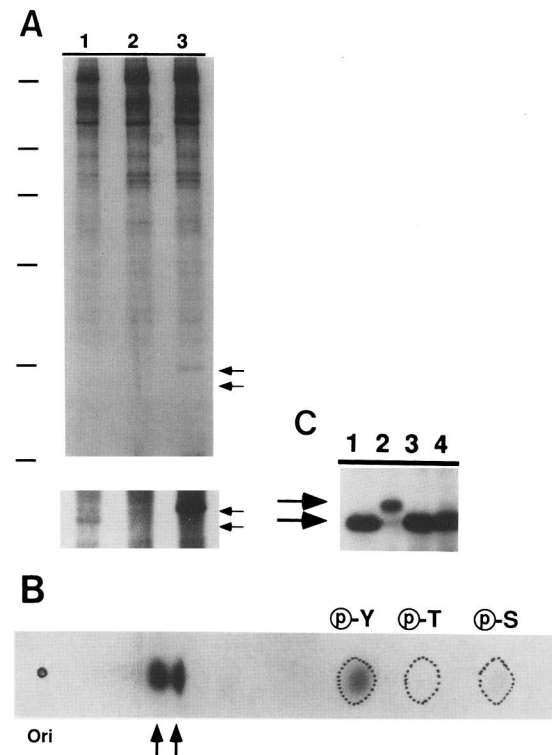


Figure 5. HACrk-I is phosphorylated on tyrosine by c-Abl. (A) Immunoprecipitation of the ^{32}P -labeled 293 cells expressing HACrkI (lane 1), c-Abl (lane 2), and HACrkI plus c-Abl (lane 3) with anti-HA antibody. The top and bottom autoradiograph represent short and long exposures of the gel. Positions of molecular size markers of 216, 105, 70, 43, 28, and 18 kD are marked at left from top to bottom, respectively. (B) Phosphoamino acid analysis of HACrkI immunoprecipitated from 293 cells expressing HACrkI plus c-Abl. Positions of the P-Ser, P-Thr, and P-Tyr phosphoamino acid standards are indicated. Arrows indicate incompletely hydrolyzed phosphopeptides. (Ori) Origin of the spotted sample. (C) Western blot analysis with anti-HA antibody of the anti-HA immunoprecipitates from 293 cells expressing HACrkI (lanes 1,3) and HACrkI plus c-Abl (lanes 2,4) in the presence (lanes 1,2) or absence (lanes 3,4) of sodium orthovanadate. The blot was developed with HRP-conjugated goat anti-mouse IgG_{2b} and enhanced chemiluminescence (ECL, Amersham).

To examine whether the slower migration rate of the hyperphosphorylated HACrkI was attributable entirely to its tyrosine phosphorylation, cells expressing HACrkI or HACrkI plus c-Abl were lysed and immunoprecipitated with anti-HA antibody in the presence or absence of sodium orthovanadate, a compound that was included in all of the analyses described previously to inhibit endogenous tyrosine-specific phosphatases. The immunoprecipitates were then subjected to Western blot analysis with anti-HA antibody. In the presence of vanadate, the HACrkI protein from cells coexpressing HACrkI and c-Abl migrated more slowly than it did in the absence of c-Abl (Fig. 5C, lanes 1,2). In the absence of vanadate, the HACrkI protein migrated similarly whether derived from cells expressing HACrkI alone or HACrkI and c-Abl

(lanes 3,4). Only the slower migrating HACrKI contained phosphotyrosine, as detected by antiphosphotyrosine antibody (data not shown). These data indicate that the Abl-generated electrophoretic mobility shift of HACrKI in SDS-PAGE is attributable to tyrosine phosphorylation.

Using the mutants of c-Abl described above, we could show that the phosphorylation of Crk depended on its ability to interact with Abl. Compared with wild type (Fig. 6, lane 2), less HACrKI protein was tyrosine phosphorylated in cells coexpressing HACrKI and c-Abl^{mu1} (lane 3) and very little phosphorylated form was evident when both CrkI-SH3-binding sites were deleted in c-Abl^{mu2} (lane 4). The expression of c-Abl and mutant c-Abl proteins in these cells was about equal (data not shown), directly demonstrating that the HACrK/Abl SH3-mediated interaction makes HACrK a substrate for the Abl kinase domain.

Crk-I associates with c-Abl and is phosphorylated in Crk-I-transformed fibroblast cells

Chicken embryo fibroblasts infected with the avian retrovirus CT10 become rapidly transformed in tissue culture (Mayer et al. 1988). Cells transformed by CT10 virus display elevated tyrosine phosphorylation on a number of proteins (Matsuda et al. 1990; Mayer and Hanafusa 1990a), suggesting that there is a tyrosine kinase involved in v-Crk transformation. Overexpression of human Crk-I, but not Crk-II, leads to transformation of mammalian fibroblasts (Matsuda et al. 1992). To examine whether the endogenous c-Abl is a Crk-I-associated tyrosine kinase in Crk-I-transformed cells, we established an HACrKI-transformed NIH-3T3 cell line (see Materials and methods). In the HACrKI-transformed cells, HACrKI induces and associates with a number of tyrosine phosphorylated proteins typically seen in v-Crk and Crk-I-transformed cells (data not shown; Matsuda et al. 1992). Western blot analysis using anti-Abl monoclonal antibody showed that c-Abl was precipitated by anti-HA antibody from a transformed cell line (Fig. 7A, lane 1) but not by an isotype-matched control monoclonal antibody (lane 3). Anti-Abl rabbit antibody PEX4 precipitated more Abl (lane 4), and normal rabbit serum did not precipitate the protein (lane 2). Precipitation of Abl by anti-HA antibody was seen in an independent transformed line (data not shown).

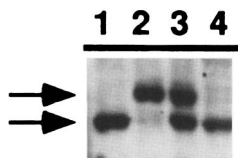


Figure 6. Western blot analysis with anti-HA antibody of the 293 cells expressing HACrKI (lane 1), HACrKI plus c-Abl (lane 2), HACrKI plus c-Abl^{mu1} (lane 3), and HACrKI plus c-Abl^{mu2} (lane 4). Arrows indicate positions of tyrosine-phosphorylated and non-tyrosine-phosphorylated HACrKI proteins on filter. Developed with HRP-conjugated goat anti-mouse IgG_{2b} and ECL.

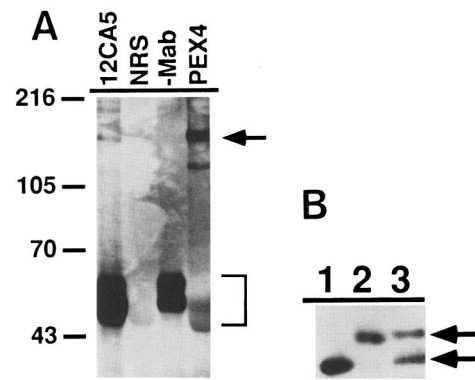


Figure 7. Association of HACrKI with Abl and phosphorylation of HACrKI in the HACrKI-transformed cells. (A) The HACrKI-transformed cell lysates were immunoprecipitated with antibodies indicated over the lanes (see Fig. 4A). The immunoprecipitates were subjected to Western blot analysis with anti-Abl monoclonal antibody and developed with HRP-conjugated goat anti-mouse IgG and ECL. Arrow indicates the position of the Abl tyrosine kinase; the bracket indicates immunoglobulin heavy chain on the blot. Numbers (left) represent the molecular size in kilodaltons. (B) Western blot analysis with anti-HA antibody of the 293 cells expressing HACrKI (lane 1), HACrKI plus c-Abl (lane 2), and HACrKI-transformed NIH-3T3 cells (lane 3). Arrows indicate positions of tyrosine-phosphorylated and non-tyrosine-phosphorylated HACrKI proteins on filter. Developed with HRP-conjugated goat anti-mouse IgG_{2b} and ECL.

In 293 cells transfected with both HACrKI and Abl, we showed that HACrKI is phosphorylated on tyrosine when it can bind to Abl. The phosphorylated HACrKI migrates more slowly than the unphosphorylated form on SDS-PAGE. In HACrKI-transformed cells, the electrophoretic mobility of a portion of HACrKI was shifted to this higher apparent molecular weight (Fig. 7B, lane 3), and this form reacted with antiphosphotyrosine antibody (data not shown). Thus, in Crk-I-transformed cells a portion of Crk-I associates with endogenous c-Abl and is phosphorylated on tyrosine.

Discussion

Using the yeast two-hybrid screen, *in vitro* binding assays, and interaction assays in mammalian cells, we have found that Abl contains selective binding sites for SH3 domains of Crk and Nck. The binding sites for Crk can mediate HACrK phosphorylation, implying that they serve the function of substrate binding sites. This work demonstrates that SH3 domains are versatile units for bringing together cellular proteins in a highly specific manner, that SH3 units can serve as substrate binding sites for enzymes, that Abl function must involve previously unsuspected interactions with adapter proteins, and that Crk may function at least partly through Abl.

We reported previously that SH3 domains can bind with high affinity to target proteins using as binding sites small, linear, proline-rich determinants (Ren et al. 1993). The present study extends that concept by show-

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ing that many SH3s have binding sites, each with a different specificity not necessarily involving more than two prolines. Thus, SH3 domains can provide the specificity to mediate aggregation of many highly specific protein complexes. Studies of the interaction of SH3 domains on Grb2 with proline-rich sites on Sos protein have also extended the range of action of SH3 regions (ref. in McCormick 1993). There, it appears that the function of SH3 is to bring the Sos protein to the cell membrane where it can find its substrate Ras. Similarly, SH3 domains were implicated in directing cellular localization of PLC γ and Grb2 (Bar-Sagi et al. 1993). The GTPase dynamin binds to and is activated by SH3 domains on other proteins (Gout et al. 1993). Furthermore, we have found recently that SH3 domains on Fyn, Lyn, and Hck can bring these protein-tyrosine kinases to the Btk kinase (G. Cheng, Z.-S. Ye, and D. Baltimore, in prep.). Thus, SH3 binding can apparently serve to bring substrates to enzymes, to organize protein complexes at the cell membrane and probably elsewhere in the cell, and to regulate enzymatic activities. Given the nature of molecules that contain SH3s, the purpose of these interactions must be to facilitate the transmission of signals through the cytoplasm and nucleus of the cell.

The function of the Crk-SH3 interaction with Abl appears to be, at least in part, to bring one or more tyrosines of Crk into the catalytic site of the Abl kinase domain. In vitro studies with the Abl kinase (Foulkes et al. 1985; Frackelton 1985), as well as its extensive phosphorylation of bacterial proteins (Wang et al. 1982), led us to conclude previously that it was a kinase with very little intrinsic substrate specificity. We can now suggest that this relative nonspecificity is a characteristic of the active site but that specificity can be mediated by interactions elsewhere in the molecule. Abl not only has its own SH2 and SH3 regions amino-terminal to the kinase—the SH2 having been implicated previously in bringing substrates to the kinase (Mayer and Baltimore, 1994)—but it has the SH3-binding sites carboxy-terminal to the kinase, which capture substrates. In both cases, the substrates would be expected to dissociate from the enzyme relatively slowly compared with dissociation from enzymes that have substrate binding sites as integral parts of their catalytic machinery. This has three consequences: The substrate will stay near the enzyme so that phosphorylation can predominate over dephosphorylation by phosphatases; the enzyme will have a very low turnover number because the substrate will not dissociate easily; and other substrates can also be brought to the enzyme through secondary domain associations. Crk in particular has an SH2 domain that can interact with other proteins, potentially mediating formation of complex, multiprotein complexes. It has been shown previously that the SH2 domains can mediate complex formation, particularly on receptor protein-tyrosine kinases, and we now must see SH3 as equally important in such interactions, especially because the Abl example shows us that one protein can have both an SH3 domain and an SH3-binding site.

The limitation of turnover number caused by separat-

ing the catalytic site from the substrate site has an important implication for signal transduction pathways: A signal will only be passed in a limited fashion, with relatively little amplification. By independent evolution of the substrate binding sites in transduction pathways, the extent of signal amplification can be controlled. A similar example is the Raf-Mek interaction, where Raf kinase stably binds its substrate Mek kinase through its carboxyl catalytic domain (Crews and Erikson 1993; Van Aelst et al. 1993).

In cells coexpressing HACrkl and c-Abl, HACrkl was phosphorylated by c-Abl as described; however, autophosphorylation of c-Abl in cells was not detected. This observation suggests that c-Abl may be activated without autophosphorylation, and the autophosphorylation, often seen in transforming variants of Abl, may provide other signals, for example, recruiting SH2-containing signaling molecules.

Crk structure and function

The human *crk* proto-oncogene encodes two proteins through alternative splicing (Matsuda et al. 1992). In this study the association of Crk-I and Abl through the SH3 interaction was demonstrated. Crk-II contains the Crk-I SH3 domain, making it likely that Crk-II will also associate with Abl in cells. Consistent with this possibility, the endogenous Crk-II proteins become phosphorylated on tyrosine in cells overexpressing transforming Abl (B. Mayer, unpubl.). Crk-II is the predominant form of Crk in most cells and differs in having a second SH3 domain of unknown specificity (Matsuda et al. 1992; Tanaka et al. 1993). Overexpression of human Crk-I leads to transformation of mammalian fibroblasts, whereas Crk-II is not transforming and therefore the second SH3 could serve to modulate the function of the first (Matsuda et al. 1992). Microinjection of Crk-I into PC-12 cells induces neuronal differentiation, whereas Crk-II has a much weaker effect in PC-12 cells (Tanaka et al. 1993), again suggesting that the second SH3 is inhibitory to Crk function. Thus, if Crk-I is a mediator of growth and differentiation events, Crk-II may have a more temperate activity.

Role of the Crk/Abl association in v-Crk transformation

Avian v-Crk is composed of viral Gag protein fused to a Crk-I-like domain containing one SH2 domain and one SH3 domain (Mayer et al. 1988). Chicken embryo fibroblasts infected with the avian retrovirus CT10 become rapidly transformed in tissue culture and induce tumors when injected into chickens (Mayer et al. 1988). Although v-Crk protein does not have a tyrosine kinase domain, cells transformed by CT10 virus display elevated tyrosine phosphorylation on a number of proteins (Matsuda et al. 1990; Mayer and Hanafusa 1990a), suggesting that there is a tyrosine kinase involved in v-Crk transformation. Tyrosine kinase activity was found to associate with Crk (Mayer and Hanafusa 1990a), and

both Crk SH2 and SH3 domains were found to be required for transformation (Mayer and Hanafusa 1990b). The demonstration of Crk and Abl association in this study indicates that the c-Abl tyrosine kinase is a Crk-associated tyrosine kinase and may play a critical role in Crk transformation.

Binding of other adapter proteins to Abl

Nck and Grb2 both interact with Abl *in vitro*. Nck was originally isolated from a melanoma expression cDNA library (Lehmann et al. 1990). It contains primarily three SH3 and one SH2 domains and is oncogenic. Overexpression of Nck leads to transformation of mammalian fibroblasts (Chou et al. 1992; Li et al. 1992). Nck is phosphorylated on both tyrosine and serine and threonine in response to epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF) stimulation, in response to activation of T-cell receptor and membrane IgM receptor and in v-Src-transformed cells (Chou et al. 1992; Li et al. 1992; Meisenhelder and Hunter 1992; Park and Rhee 1992). It is a common target for many receptor and nonreceptor tyrosine kinases. Nck binds to tyrosine-phosphorylated EGF receptor or PDGF receptor through its SH2 domain (Li et al. 1992). Studies here suggest that Nck associates with c-Abl through one or more of its SH3 domains. This association may also lead to tyrosine phosphorylation of Nck and could be important for signal transduction as well as Nck transformation.

Grb2 (Lowenstein et al. 1992), also known as ASH, Sem5, or Drk (Clark et al. 1992; Matuoka et al. 1992; Simon et al. 1993) is an SH2- and SH3-containing adapter that links activated protein-tyrosine kinase receptors to the Ras activator protein Sos. Consistent with our *in vitro* data, Pendergast et al. recently showed that both Grb2 SH3 domains can bind to c-Abl *in vitro* (Pendergast et al. 1993). The functional significance of this binding is uncertain because Grb2 is involved in other interactions with its SH3 domains, particularly with the Ras activator protein Sos. The Grb2 SH2, however, binds to a tyrosine-phosphorylated site in Bcr-Abl that is critical for transformation (Pendergast et al. 1993), providing the opportunity for a multipoint interaction among these proteins.

The Crk, Nck, and Grb2 SH3-binding sites were mapped close to the nuclear localization signal. The association of these adapter molecules with Abl may block the translocation of Abl into the nucleus and, therefore, retain Abl in cytoplasm. Alternatively, the association of Crk with c-Abl may bring Crk into nucleus. Translocation of proteins in cells is an important mechanism for transducing signals.

c-Abl has one close relative, Arg, that might also be a target for binding by the adapters that bind to Abl. Arg is also a protein-tyrosine kinase with SH2, SH3, and a long carboxy-terminal extension (Kruh et al. 1986). The carboxy-terminal portion of Arg is less homologous to c-Abl than the amino terminus, but the Crk, Nck, and Grb2 SH3-binding sites identified in c-Abl are conserved, sug-

gesting that these adapter molecules should also bind Arg.

Characteristics of SH3-binding sites

The solution and crystal structure of the SH3 domains of spectrin, Src, Fyn, p85 α , and PLC γ have been solved (Musacchio et al. 1992; Yu et al. 1992; Booker et al. 1993; Kohda et al. 1993; Koyama et al. 1993; Noble et al. 1993). All of these SH3 domains are formed from five antiparallel β -strands. The perturbation of resonances by bound proline-rich peptides indicates that the binding site is a hydrophobic surface region of the SH3 domain that consists of aromatic residues flanked by two charged loops (Booker et al. 1993; Yu et al. 1992).

Like the Abl-SH3-binding sites, the Crk, Nck, and Grb2 SH3-binding sites in c-Abl were mapped to proline-containing short stretches of amino acids. The CrkI-SH3-binding sites were proved to mediate the association of Crk-I and Abl in cells. The specificity of the SH3 interactions is evident. However, is there any common structural motif in the SH3-binding sites that might fit into a conserved element of SH3 structure? An alignment of the Abl, Crk, Nck, and Grb2 SH3-binding sites shows that two proline residues spaced by two nonconserved amino acids are conserved. The consensus PXXP was also observed among some other SH3-binding sites (Egan et al. 1993; Gout et al. 1993; Li et al. 1993; Liu et al. 1993; Rozakis-Adcock et al. 1993; G. Cheng, Z.-S. Ye, and D. Baltimore, *in prep.*; K. Alexandropoulos, *unpubl.*), suggesting that PXXP may serve as a scaffold for the SH3 interaction. The amino acids around these two highly conserved proline residues might then determine SH3-binding specificity. For example, the nonconserved sequences amino-terminal to the PXXP consensus, particularly the critical proline at position 2 in the 3BP1 site, may play an important role for Abl-SH3-binding specificity. The basic amino acid(s) carboxy-terminal to PXXP in the CrkI, Grb2, and Nck SH3-binding sites could play a similar role.

Materials and methods

Cells and antibodies

293T cells (Pear et al. 1993) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. Mouse monoclonal antibody 12CA-5 (Berkeley Antibody Company), anti-Abl monoclonal antibody 24-21 (Oncogene Science), and goat anti-mouse IgG-HRPO and IgG2b-HRPO (Southern Biotechnology Associates, Inc) were used in this study. Abl-specific PEX4 antiserum was described previously (Konopka et al. 1984). The control monoclonal antibody used was an anti-RAG2 monoclonal antibody (Spanopoulou et al. *unpubl.*).

HACrki-transformed cell lines were established by infecting NIH-3T3 cells with an HACrki retrovirus. The helper-free HACrki retrovirus was generated by transiently transfecting BOSC-23 cells with pGD/HACrki as described (Pear et al. 1993). NIH-3T3 cells (2×10^5) infected with 5×10^5 HACrki retrovirus were transferred into 0.3% soft agar 2 days after infection. Colonies were picked after 3 weeks. The transformed cells

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were purified twice. Three independent transformed cell lines were analyzed. All of these cell lines expressed a high level of HACrkl.

DNA constructs

Plasmid DNAs were grown in *E. coli* DH5 α and purified by CsCl centrifugation (Ausubel et al. 1990). DNAs were cleaved with restriction endonucleases under conditions recommended by the manufacturer (Boehringer Mannheim Biochemicals). Restriction fragments were purified by electrophoresis in low-gelling-temperature agarose gels (Ausubel et al. 1990). Ligations were performed according to the instructions of the manufacturer of T4 DNA ligase (Boehringer Mannheim Biochemicals).

For the two-hybrid screen, cDNA fragment encoding the carboxy-terminal portion (amino acid 545–1149) of c-Abl (Ben-Neriah et al. 1986), excised from plasmid pBS/D20 (B. Mayer and D. Baltimore, unpubl.) by *Bam*HI and *Not*I restriction enzyme digestion, was cloned in-frame into pEG202 vector with an *Eco*RI–*Bam*HI linker. This plasmid was designated as pEG/*ablC'*.

For the in vitro SH3-binding assay, the cDNA fragment encoding the carboxy-terminal portion (amino acid 545–1149) of c-Abl was cloned in-frame into the pGEX-1ZT vector (modified pGEX-1 λ T (Pharmacia) by inserting multiple restriction sites into *Eco*RI site of pGEX-1 λ T). pGEX–AblC' Δ Sal and pGEX–AblC' Δ Xho were constructed by subcloning the *Bam*HI–*Sal*I and *Bam*HI–*Xho*I cDNA fragments from pBS/D20 in-frame into the pGEX-1ZT vector. A DNA fragment encoding AblC'-115 and AblC'-56 was constructed with synthetic oligonucleotide-directed PCR of the *c-abl* cDNA under the conditions recommended by Perkin-Elmer Cetus. *Pfu* DNA polymerase (Stratagene) was used for PCR. The PCR reaction was carried out for 15 cycles at 95°C for 30 sec, 55°C for 1 min, and 72°C for 2 min. Oligonucleotides contain 22 bp of identical sequence for hybridization and a *Bam*HI site (for the sense oligonucleotide) or *Xho*I site (for the anti-sense oligonucleotide) with four extra base pairs at the 5' end for stability and recutting efficiency. The PCR fragments were cloned in-frame into the *Bam*HI–*Xho*I site of the pGEX-1ZT vector. The rest of DNA fragments encoding the peptides AB3-1 to AB3-3 were derived from the synthetic oligonucleotides. The oligonucleotides that were cloned into the *Bam*HI–*Eco*RI site of the pGEX2T vector contained coding sequences plus 5'-GATCC-3' at the 5' end of the sense strand and 5'-AATT-3' at the 5' end of the anti-sense strand. The 5' end of the DNA fragments derived from these oligonucleotides were in-frame with the GST of pGEX2T and the 3' ends were out of frame, resulting in amino acid residues NSS at the carboxyl terminus of the GST/peptide fusion proteins. The synthetic oligonucleotides were phosphorylated using T4 polynucleotide kinase under conditions recommended by the manufacturer (New England Biolabs). The sense and anti-sense strands were annealed after phosphorylation by boiling for 2 min and then cooling down slowly to room temperature (about 25°C). The DNA fragment was cloned into the pGEX-2T vector.

The human Crk-I gene was cloned from HeLa cDNA by PCR using the 5' primer AAGGATAGGCGGCCCGCCACCATGGT-TGGATACCCCTACGACGTCGCCGACTACGCCGAGTT-ATGGCGGGCAACTTGGACTCG (where the underlined segment encodes the HA epitope and the ATG indicates the natural start codon) and the 3' primer AAGGATAGGCGGCCCGCACCAGCTGAAGTCCTCATCGGGATTCTG. The PCR product was digested with *Not*I and cloned into the *Not*I site of pGDN, a derivative of the retroviral vector pGD (Daley et al. 1990). Crk-I cDNA was confirmed by sequence analysis. The

Crk-I cDNA in right orientation in pGDN vector was selected and used in studies.

The pGD/*abl^{mu1}* was constructed by first subcloning *Bam*HI (744, nucleotide number according to murine type IV *c-abl*) and *Xho*I (2285) from Δ BS-F (Mayer and Baltimore 1994) into pBS (Stratagene). The *Pst*I (1645) to *Pst*I (1681) segment (encoding LQAPELPTKTRTC) was replaced by a linker encoding LQAE-FRTC. The *Bam*HI and *Nar*I fragment containing the mutation was cloned back to Δ BS-F and then the *Not*I fragment containing the *c-abl^{mu1}*-coding sequences was cloned into pGDN. The pGD/*abl^{mu2}* was constructed by replacing PLLPRK (596–601, amino acid number in type IV c-Abl) into ID by oligo-directed PCR mutagenesis as described (Higuchi 1990). All of the mutations were confirmed by sequence analysis.

The two-hybrid screen

A genetic screen using the two-hybrid system was performed as described (Zervos et al. 1993). The bait plasmid pEG/*ablC'* was transformed into yeast strain EGY48 with a reporter plasmid pSH18-34 that contains the *LexAop*–*LEU2* reporter gene. This strain was transformed with the HeLa interaction library (Zervos et al. 1993). Yeast transformation was performed by the lithium acetate method (Ausubel et al. 1990) except that 10% dimethylsulfoxide (DMSO) was included during 42°C heat shock. Ten million primary library transformants were amplified, of which 5 million were screened. The candidate AblC' interacting clones were selected as meeting the following criteria: (1) They grew on Ura[–]His[–]Trp[–]Leu[–]-galactose plates, and (2) they turned blue on Ura[–]His[–]Trp[–]X-gal-galactose plates but not on Ura[–]His[–]Trp[–]X-gal-glucose plates. Plasmids from the candidate yeast strains were isolated (Hoffman and Winston 1987), and the library plasmids were selected through KC8 cells. To test whether the library cDNAs truly encoded proteins that interacted specifically with AblC', the library plasmids were retransformed back into yeast with either EG202/*ablC'* or EG202. Sequencing of the interacting clones was performed using Sequenase according to the manufacturer's directions (U.S. Biochemical).

Filter-binding assay

To examine SH3 binding in vitro, a filter-binding assay was performed as described (Ren et al. 1993). GST/CrkI-SH3 was constructed by subcloning the *Eco*RI–*Xho*I cDNA fragment encoding the c-Abl-interacting partial Crk-I protein from the pJG45 vector in-frame into pGEX-1ZT vector. The fusion protein was purified using glutathione-agarose beads and biotinylated as described (Mayer et al. 1991). The GST/Abl-SH3, GST/Src-SH3, GST/N-Src-SH3, and GST/Crk-SH3 probes were prepared as described (Cicchetti et al. 1992). The GST/Grb2, GST/GrbN2, GST/Grb2C, and GST/Nck fusion proteins were generous gifts from J. Schlessinger (New York University). Biotinylation of these proteins was performed as described (Mayer et al. 1991).

Transfection

Calcium phosphate transfection was performed as described (Pear et al. 1993). 293 T cells (1.5×10^6) were seeded in 60-mm plastic cell culture plates 1 day before use. Each plate was treated with 0.5 or 1 ml of a DNA-calcium phosphate coprecipitate containing 3 μ g each of the plasmid DNAs. After 18 hr of incubation at 37°C, the medium was replaced and incubation was continued for 24 hr.

Immunoprecipitation

Cells (1×10^7) were washed once in ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium orthovanadate and then lysed in 1 ml of ice-cold lysis buffer (50 mM HEPES at pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl₂, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM freshly made phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin). The cell debris was removed by centrifugation at 14,000 rpm in Eppendorf centrifuge for 10 min at 4°C. The cell lysate was incubated with 5 μl of normal rabbit serum and 25 μl of protein G-plus/protein A-agarose (Oncogene Science) for 1 hr and then clarified by centrifugation at 14,000 rpm in Eppendorf centrifuge for 2 min. The clarified cell lysate was incubated with 5 μg of the appropriate monoclonal antibody or 5 μl of the polyclonal antiserum at 4°C for 2–5 hr. The immunocomplexes were then collected after the addition of 25 μl of protein G-plus/protein A-agarose and incubation at 4°C for 30 min. The pellets of agarose beads were washed three times with 1 ml of lysis buffer and then subjected to kinase reaction or immunoblotting.

Kinase reaction

The kinase reaction was carried out as described (Konopka et al. 1984). The pellets of agarose beads obtained from immunoprecipitation described above were transferred to a fresh Eppendorf tube and washed twice with 1 ml of kinase buffer (20 mM HEPES at pH 7.4, 5 mM MgCl₂). The kinase reaction was carried out in 100 μl of kinase buffer containing 10 μM ATP and 0.2 μl of [γ -³²P]ATP (7000 Ci/mmol) (ICN) at 30°C for 30 min. The pellets of agarose beads were washed with PBS twice. The bound proteins were eluted in Laemmli sample buffer at 95°C for 5 min, and then the phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

Western blot analysis

Cells were lysed as described above. The cell extracts or anti-HA immunoprecipitates were boiled in Laemmli sample buffer, electrophoretically separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Filters were blocked in 10 mM Tris-HCl at pH 8.0/0.9% NaCl/0.05 Tween 20 (TBST buffer) plus 2% nonfat dry milk. Filters were blotted with 1 μg/ml of anti-HA antibody or anti-Abl monoclonal antibody in TBST/milk buffer at room temperature for 2 hr. After wash in TBST, filters were incubated with anti-mouse IgG2b- or IgG-conjugated horseradish peroxidase at a dilution of 1:2000 in TBST buffer at room temperature for 1 hr. Filters were then washed and developed with ECL (Amersham), as described by the manufacturer.

Cell metabolic labeling and phosphoamino acid analysis

After 48 hr of transfection, 293 cell monolayers were washed twice with phosphate-free DMEM (GIBCO) and incubated in phosphate-free DMEM containing 10% dialyzed fetal bovine serum (GIBCO) for 2 hr. Cells were washed twice with phosphate-free DMEM again and incubated in phosphate-free DMEM containing 10% dialyzed fetal bovine serum and ³²P_i (NEN, 1 mCi/ml per 60-mm dish) for 2 hr. The ³²P-labeled cells were then washed once with ice-cold PBS and harvested, lysed, and immunoprecipitated with anti-HA antibody as described above. The immunoprecipitates were separated electrophoretically in SDS-PAGE and detected by autoradiography.

Phosphoamino acid analyses were performed as described

(Naim and Greengard 1987; Li et al. 1992). Briefly, phosphorylated protein bands were excised from dried SDS gels, rehydrated in 50 mM NH₄HCO₃, minced, and boiled in 0.1% SDS/5% β-mercaptoethanol for 5 min. The mixture was incubated at 37°C with agitation overnight. The gel debris was removed by centrifugation. The supernatants were mixed with 50 μg of BSA and precipitated in 15% trichloroacetic acid on ice for 90 min. The precipitates were washed twice with cold acetone. The protein was hydrolyzed in 6 N HCl at 110°C for 1 hr. Hydrolysis was stopped by adding 1 ml of H₂O and drying in a Speed-Vac. The sample was washed with H₂O, dried twice, and then dissolved in 5 μl of electrophoresis buffer (pH 1.9). Phosphoamino acid standards (2 μg, phosphoserine, phosphothreonine, and phosphotyrosine) and 1 μl of 0.08% phenol red were added into the sample. The mixture was spotted on thin-layer chromatography (TLC), cellulose without fluorescent indicator (Kodak). The wetted TLC plate was run in pH 1.9 buffer at 500 V for 1 hr and then transferred to pH 3.5 buffer and run at 500 V for 1–1.5 hr. The TLC plate was then stained with 1% ninhydrine to show phosphoamino acid spots and exposed to X-ray film with an intensifying screen at –70°C for 3 days.

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Abl protein-tyrosine kinase selects the Crk adapter as a substrate using SH3-binding sites.

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