Abi-2, a novel SH3-containing protein interacts with the c-Abl tyrosine kinase and modulates c-Abl transforming activity

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A protein has been identified that interacts specifically with both the Src homologous 3 (SH3) domain and carboxy-terminal sequences of the c-Abl tyrosine kinase. The cDNA encoding the *Abl* interactor protein (Abi-2), was isolated from a human lymphocyte library using the yeast two-hybrid system with the Abl SH3 domain as bait. Abi-2 binds to c-Abl in vitro and in vivo. Abi-2 is a novel protein that contains an SH3 domain and proline-rich sequences critical for binding to c-Abl. A basic region in the amino terminus of Abi-2 is homologous to the DNA-binding sequence of homeo-domain proteins. We show that Abi-2 is a substrate for the c-Abl tyrosine kinase. Expression of an Abi-2 mutant protein that lacks sequences required for binding to the Abl SH3 domain but retains binding to the Abl carboxyl terminus activates the transforming capacity of c-Abl. The properties of Abi-2 are consistent with a dual role as regulator and potential effector of the c-Abl protein and suggest that Abi-2 may function as a tumor suppressor in mammalian cells.

[Key Words: Abl substrate; signaling; homeo-domain homology; tumor suppressor; cancer]

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The c-Abl protein, originally identified as the cellular homolog of the *v-abl* oncogene product of Abelson murine leukemia virus (A-MuLV) (Goff et al. 1980; Wang et al. 1984), is a tyrosine kinase of unknown function. Biochemical data suggest that c-Abl may regulate signal transduction events in the cytoplasm and processes in the nucleus. c-Abl is found primarily in the nucleus (Van Etten et al. 1989), but it is also found in association with the plasma membrane and bound to actin filaments in the cytoplasm (Van Etten et al. 1989; Van Etten et al. 1994). In several nontransfected hematopoietic cells, c-Abl is predominantly localized to the cytoplasm with some nuclear staining (Wetzler et al. 1993).

The c-Abl protein has a complex structure that includes several domains common to proteins implicated in signal transduction pathways. Among these domains are the noncatalytic Src homology 2 and 3 (SH2 and SH3) domains and the tyrosine kinase (SH1) domain. SH2 and SH3 domains are modular components present in a large number of proteins (Pawson 1995). These domains are critical in the formation of stable signaling protein complexes (Pawson 1995), and have also been shown to regulate protein function (Feller et al. 1994b; Cohen et al. 1995; Pawson 1995). The SH3 domain suppresses the intrinsic transforming activity of c-Abl in vivo (Franz et al. 1989; Jackson and Baltimore 1989) while the SH2 domain is required for the transforming function of acti-

vated *abl* genes (Mayer et al. 1992; Mayer and Baltimore 1994). The unique carboxy-terminal region of c-Abl, which is encoded by a single exon, contains several functional and structural domains that include a nuclear localization signal (NLS) (Van Etten et al. 1989), proline-rich sequences that have the potential to bind to SH3-domain-containing proteins (Feller et al. 1994a,b; Ren et al. 1994), a DNA-binding domain (Kipreos and Wang 1992), and an actin-binding domain (McWhirter and Wang 1993; Van Etten et al. 1994). Several serine/threonine residues within the carboxy-terminal exon are phosphorylated by the *cdc2* kinase (Kipreos and Wang 1990) and by protein kinase C (Pendergast et al. 1987). The presence of multiple structural and functional domains within the c-Abl tyrosine kinase and its localization to cytoplasmic and nuclear cellular compartments suggest a potential role for c-Abl in the regulation of transcription, DNA replication or cell cycle progression, as well as in the control of signaling events in the cytoplasm.

The tyrosine kinase activity of c-Abl is tightly regulated in vivo (Pendergast et al. 1991a; Mayer and Baltimore 1994). Overexpression of c-Abl at levels 5- to 10-fold over the endogenous c-Abl protein does not lead to cell transformation but causes growth arrest (Jackson and Baltimore 1989; Jackson et al. 1993; Sawyers et al. 1994). In contrast, structurally altered forms of Abl cause cell transformation and exhibit elevated tyrosine kinase activity when expressed at similar levels (Franz et al. 1989; Jackson and Baltimore 1989; Muller et al. 1991).

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Activation of the oncogenic potential of c-Abl has been shown to occur as a consequence of structural alterations in the amino- or carboxy-terminal sequences (for review, see Wang 1993). Three naturally occurring c-*abl*-derived oncogenes have been identified. They are the v-*abl* oncogene of A-MuLV (Goff et al. 1980), the v-*abl* oncogene of the Hardy-Zuckerman-2 feline sarcoma virus (HZ2-FSV) (Bergold et al. 1987), and the *bcr-abl* chimeric oncogene of Philadelphia chromosome-positive human leukemias (for review, see Kurzrock et al. 1988). Interestingly, the oncogenic activity of the v-*abl* oncogene of A-MuLV is a result of the deletion of the Abl SH3 domain and fusion with gag sequences (Franz et al. 1989; Jackson and Baltimore 1989; Muller et al. 1991) while activation of the v-*abl* oncogene of HZ2-FSV is associated with deletion of Abl carboxy-terminal sequences and fusion with viral sequences with retention of the Abl SH3 domain (Bergold et al. 1987). Mutants of c-Abl with deletions or alterations in the SH3 and carboxy-terminal sequences have also been generated experimentally that exhibit increased transforming activity (Franz et al. 1989; Jackson and Baltimore 1989; Goga et al. 1993; Mayer and Baltimore 1994). The transforming Abl proteins are constitutively active tyrosine kinases and are primarily localized in the cytoplasm.

Several possible mechanisms have been suggested for the inhibition of the c-Abl tyrosine kinase. The inhibitory effect of the Abl SH3 domain is extremely position sensitive (Mayer and Baltimore 1994). These results, together with the data described above, suggest that in addition to the SH3 domain, other regions of c-Abl may be required for repression. Two potential mechanisms have been proposed. First, it is possible that the SH3 domain functions in *cis* by binding to another region of Abl and effectively locking the protein in an inactive conformation. As mutations in both the SH3 and carboxy-terminal sequences result in release of the c-Abl protein from inhibition, it is possible that *cis*-inhibitory sequences are present in the carboxy-terminal domain. The Abl SH3 domain binds to proline-rich sequences (Cicchetti et al. 1992; Ren et al. 1993). Several proline-rich sequences have been identified in the carboxy-terminal domain of c-Abl (Ren et al. 1994). However, no appreciable binding has been detected between the Abl carboxy-terminal sequences and the Abl SH3 domain (Ren et al. 1994; Z. Dai and A.M. Pendergast, unpubl.). A second model consistent with the available data suggests that the c-Abl protein is negatively regulated by a *trans*-acting cellular inhibitor that exerts its effects by interacting with the Abl SH3 domain and a second region of the Abl protein (Mayer and Baltimore 1994).

To gain insight into the regulation and function of the c-Abl tyrosine kinase we sought to identify proteins that interact directly and specifically with the regulatory Abl SH3 domain. Here we report the identification, cloning, and characterization of Abi-2, an Abl-interactor protein from human lymphocytes. Abi-2 is a member of a novel family of SH3-containing proteins that exhibit homology to homeo-domain proteins (Scott et al. 1989; Pabo and Sauer 1992). Two other genes homologous to *abi-2* are

the *Xenopus laevis xlan 4* gene (Reddy et al. 1992) and mouse *abi-1* (Shi et al., this issue).

Results

Cloning of an Abl interacting protein by the yeast two-hybrid system

To identify proteins that interact with the Abl regulatory domains we employed the yeast two-hybrid system (Fields and Song 1989). A human lymphocyte library was screened, as genetic and biological data suggest that c-Abl plays a role in lymphocyte development (Caracciolo et al. 1989; Schwartzberg et al. 1991; Tybulewicz et al. 1991). Using the Abl SH3 domain as bait, we identified 24 positive clones out of 4×10^5 transformants. Among these, clone AS3B2 was demonstrated to confer a bait plasmid-dependent expression of *lacZ* in yeast strain Y190 and grew in *his*⁻, *trp*⁻, *leu*⁻ selection plates with 25 mM 3-aminotriazole (3-AT) (data not shown). Nucleotide sequence analysis revealed that AS3B2 contains a single long open reading frame (ORF). The complete coding sequence of AS3B2 encompasses 1203 nucleotides. The gene contained in AS3B2 was designated *abi-2* for Abl interactor 2. The corresponding protein product was referred to previously as Aip-1 (Abl interacting protein 1) (Feller et al. 1994b). The amino acid sequence of Abi-2 is shown in Figure 1A.

Abi-2 encodes an SH3-containing, proline-rich protein that exhibits homology to DNA-binding proteins

Analysis of the predicted amino acid sequence of Abi-2 reveals several interesting features (Fig. 1A,B). The carboxy-terminal region of the protein encodes an SH3 domain (amino acids 346–397). Alignment of the Abi-2 SH3 domain with SH3 domains found in other proteins reveals the highest identity with the SH3 domains from cortactin (53%), the hematopoietic specific protein HS1 (48%), vav (46%), phospholipase C- γ (47%), and the Nck adaptor protein (46%). Several proline-rich stretches are found in Abi-2 (Fig. 1A,B) that constitute potential binding sites for SH3 domain-containing proteins and contain the consensus PXXP sequence that is present in all high-affinity SH3 ligands identified to date (Rickles et al. 1994; Cohen et al. 1995). Also, a polyproline stretch is found upstream of the SH3 domain of Abi-2, which could function as a transcriptional activation domain (Tanaka et al. 1994). Sequences enriched in serine/threonine, glutamate/aspartate, and proline residues, designated PEST regions, are also found in Abi-2. Three PEST regions are identified in the central and carboxy-terminal portions of Abi-2 (Fig. 1A,B). PEST sequences have been implicated in increased susceptibility to protein degradation (Rogers et al. 1986). The amino-terminal region of Abi-2 is extremely basic with a calculated pI of 11.4, whereas the carboxy-terminal portion is markedly acidic with a pI of 3.5. A serine-rich region is found in the central portion of Abi-2 (Fig. 1A,B). The protein contains nine serine/thre-

Figure 1. Sequence and Northern blot analysis of Abi-2. (A) Predicted amino acid sequence of human Abi-2. Sequences in the carboxyl terminus corresponding to the SH3 domain are boxed. The three potential SH3-binding sites are in boldface type and a serine-rich region is bold and underlined. A potential c-Abl tyrosine phosphorylation site is doubly underlined, and the tyrosine is marked with an asterisk. Three PEST regions are bracketed with arrows and a homeo-domain homologous region at the amino terminus is underlined. A polyproline stretch is underlined with dashed lines. (B) Diagram of structural features of Abi-2. (C) Alignment of the Abi-2 homeo-domain homologous region with several homeo-domains. The consensus sequence is shown at the bottom. (D) Northern blot analysis of *abi-2* expression in human tissues. Two micrograms of poly(A)⁺ RNA from the indicated human tissues was hybridized to ³²P-labeled full-length *abi-2* cDNA probe. The RNA markers are indicated at left.

onine residues followed by proline, suggestive of potential phosphorylation by proline-directed protein kinases (Kemp and Pearson 1990). Three sites conform to the cdc2 kinase consensus sequence Ser/Thr-Pro-X-basic (Moreno and Nurse 1990). There are also 11 potential cAMP-dependent protein kinase sites and 9 potential protein kinase C sites (Kemp and Pearson 1990). Several tyrosines in the Abi-2 sequence are found in peptides that correspond to optimal peptide substrates for the Abl, Fps, and Src protein tyrosine kinases (Songyang et al. 1995).

Interestingly, the basic, amino-terminal region of Abi-2 exhibits 40%–50% similarity over a 53-amino-acid stretch to the DNA-binding region of homeo-domain proteins (Figure 1A–C) [Rushlow et al. 1987; Scott et al. 1989; Pabo and Sauer 1992]. Homeo-domain proteins have been implicated in specifying positional information in the embryo during development and in the

control of cell lineages by regulating the expression of cell type-specific genes (Scott et al. 1989). It is significant that all of the amino acids in the homeo domain that are implicated in contacting the DNA major groove are conserved in the basic region of Abi-2. Similarly, all of the amino acids that contact the DNA backbone, with the exception of an invariant tryptophan, are present in this domain of Abi-2 (Fig. 1C) (Pabo and Sauer 1992).

A search of the data base for homologous sequences revealed that the *abi-2* DNA sequence is 70% identical to that of the *X. laevis xlan 4* DNA (Reddy et al. 1992). The predicted amino acid sequence of the Xlan 4 protein is 93% identical to that of the last 286 amino acids of Abi-2. Remarkable conservation of the SH3 domain, PEST sequences, serine-rich region, proline-rich stretches, and phosphorylation sites is observed among the human Abi-2 and predicted *Xenopus* Xlan 4 proteins. The *xlan 4* gene is expressed as a maternal transcript and

localizes in the animal pole region of the oocyte. The expression of *xlan 4* is developmentally regulated (Reddy et al. 1992). More recently, a mouse gene with high homology to the human *abi-2* gene was cloned as a cDNA encoding an Abl-binding protein (Shi et al., this issue). The corresponding protein was designated Abi-1, Abl interactor 1, and its predicted amino acid sequence is 65% identical to that of Abi-2.

Abi-2 is widely expressed in human tissues

To investigate the expression pattern of *abi-2*, we performed Northern blot analysis on poly(A)-selected RNA from multiple human tissues. Using the *abi-2* cDNA as a probe, we detected two transcripts, 7 and 1.9 kb, respectively, in all tissues examined (Fig. 1D). The abundance of the two transcripts varies among tissues (Fig. 1D). The expression of the 7-kb transcript appears constant in most tissues examined, with the exception of colon and peripheral blood leukocytes where low levels of the transcript are detected. Although detectable in almost all tissues examined, the 1.9-kb transcript is relatively abundant in testes, ovary, thymus, and colon, with lower but detectable levels in prostate, peripheral blood leukocytes, and spleen. The entire *abi-2* cDNA obtained encompasses ~1800 nucleotides and contains in-frame termination codons in the 5'-untranslated region as well as consensus sequences for polyadenylation in the 3'-untranslated region. Thus, it is most likely that *abi-2* corresponds to the 1.9-kb transcript. The 7-kb transcript may be the product of a related gene or an alternative spliced form of the *abi-2* gene. Further experiments are needed to address these possibilities and identify other *abi-2*-related transcripts.

Abi-2 binds to c-Abl in vitro and in vivo

We have shown that Abi-2 binds to the Abl SH3 domain in yeast. To determine whether Abi-2 can interact with the full length c-Abl protein, a glutathione *S*-transferase (GST)-Abi-2 fusion protein was used in an *in vitro* binding assay with full-length c-Abl produced in baculovirus-infected insect cells (Pendergast et al. 1993). As shown in Figure 2A, the GST-Abi-2 protein interacts with full-length c-Abl in solution. The binding of Abi-2 to c-Abl appears to be selective, as shown by the failure of Abi-2 to form a complex with another SH3-containing protein, the guanosine triphosphatase-activating protein (GAP) of Ras (Fig. 2A, lanes 4–6; Trahey et al. 1988). To examine further the specificity of the interaction of the Abl SH3 domain with Abi-2, we compared a number of SH3 domains from distinct proteins for their ability to bind Abi-2. We failed to detect interactions between Abi-2 and the SH3 domains of Crk, Grb2, GAP, Src, and the p67 protein from neutrophils (data not shown). These results indicate that the interaction between Abi-2 and the Abl SH3 domain is highly specific.

To examine the interaction of Abi-2 with c-Abl *in vivo* we developed antibodies to the Abi-2 protein. Interestingly, analysis of the Abi-2 protein by SDS-PAGE re-

Figure 2. Abi-2 interacts with c-Abl. (A) Sf9 insect cells were infected with either c-Abl or GAP recombinant baculoviruses and labeled with [³⁵S]methionine. Cell lysates were incubated with equal amounts of immobilized GST alone (lanes 1,4), GST-Abi-2 (lanes 2,5), and anti-Abl (lane 3) or anti-GAP (lane 6) antibodies bound to protein A-Sepharose beads. After incubation for 90 min at 4°C, the beads were washed with RIPA buffer to remove unbound material. Bound proteins were separated on SDS-PAGE and visualized by fluorography. The positions of c-Abl and GAP are indicated. (B) Bosc 23 cells cotransfected with the pSRα/c-abl and pCGN/abi-2 expression vectors were lysed and immunoprecipitated with preimmune serum (lane 1), anti-Abi-2 (lane 2), and Anti-Abl antibodies (lane 3). The immunoprecipitated proteins were subjected to an *in vitro* kinase assay in the presence of [γ-³²P]ATP. The phosphorylated proteins were separated on SDS-PAGE and visualized by autoradiography. (C) Following immunoprecipitation (I.P.) and kinase assay as described in B, the bound proteins were eluted from the immune complexes by boiling in the presence of SDS-PAGE sample buffer and subjected to a second cycle I.P. with the indicated sera.

vealed that Abi-2 migrates aberrantly. While the calculated molecular mass of the protein encoded by the *abi-2* cDNA is 44 kD, the Abi-2 protein produced in bacteria or translated *in vitro* in a reticulocyte lysate migrates with an apparent molecular mass of 55 kD (Fig. 4B; data not shown). In addition to the major 55-kD band, two proteins of 44-kD and 75-kD are obtained following *in vitro* transcription/translation using the *abi-2* cDNA, which

are recognized by an anti-Abi-2 polyclonal antibody raised against full-length Abi-2 (data not shown). The smaller 44-kD protein is likely to be produced by translation from an internal initiation codon because it cannot be recognized by antibodies specific to the Abi-2 amino terminus (data not shown). The 75-kD protein may result from additional post-translational modifications. A 75-kD protein is the predominant product obtained following transfection of the *abi-2* cDNA in Bosc 23 human embryonic kidney cells (Fig. 2B,C). The 75-kD protein is recognized by anti-Abi-2 antibodies. The 75-kD protein and a minor 55-kD protein are also detected with the anti-Abi-2 polyclonal antibodies in lysates from human B lymphoid cells (data not shown). Aberrant protein migration has been reported for several proteins that belong to an emerging family of SH3-containing proteins. These proteins are rich in acidic amino acids and proline residues and contain basic amino-termini with one or more repeats of a helix–turn–helix motif (Kitamura 1989; Wu et al. 1991; Fukamachi et al. 1994; Takemoto et al. 1995). Members of this protein family include cortactin, HS1/SPY75, and the recently cloned LckBP1. The *LckBP1* cDNA encodes a protein with a predicted molecular mass of 54-kD, but analysis of the protein isolated from hematopoietic cells reveals that the apparent molecular mass of LckBP1 is 85 kD (Takemoto et al. 1995). Poor binding of SDS to the acidic portions of these proteins, coupled with the presence of additional post-translational phosphorylations, have been proposed to explain the aberrantly slow mobility of these proteins.

Interaction of Abi-2 with c-Abl in vivo was examined following overexpression of wild-type c-Abl and Abi-2 in Bosc 23 cells and immunoprecipitation with antisera to Abi-2 or c-Abl. Bosc 23 cells were transfected with pCGN/*abi-2* and pSR α /*c-abl* mammalian expression plasmids. After 2 ½ days the cells were lysed and the lysates were incubated with anti-Abl, anti-Abi-2, or preimmune sera. The immunoprecipitates were then subjected to in vitro kinase assays with [γ^{32} P] ATP to radiolabel the proteins. A 145-kD protein was precipitated by the anti-Abi-2 antibody but not the corresponding preimmune sera (Fig. 2B, lanes 1,2). The 145-kD protein comigrated with a protein of the same size, which was immunoprecipitated with anti-Abl antibodies from the same cells (Fig. 2B, lane 3). The identity of the 145-kD protein as c-Abl was confirmed by subjecting the precipitated proteins to a second round of immunoprecipitation with anti-Abl antibodies (Fig. 2C). Thus, the c-Abl wild-type protein is precipitated with antibodies to Abi-2 as detected by its in vitro autophosphorylation activity.

A radiolabeled protein of ~75-kD was detected in both anti-Abi-2 and anti-Abl immunoprecipitates from lysates of Bosc 23 cells cotransfected with the pCGN/*abi-2* and pSR α /*c-abl* expression plasmids (Fig. 2B, lanes 2,3). To examine whether this protein was Abi-2, a portion of the immunoprecipitates was boiled in the presence of SDS to denature the proteins and disrupt protein/protein interactions, diluted with buffer lacking SDS, and then incubated with anti-Abi-2 antibodies or the corresponding preimmune sera. A 75-kD protein was pre-

cipitated with anti-Abi-2, but not the preimmune sera from both the anti-Abi-2 and anti-Abl immunoprecipitates of the Bosc 23 cell lysates (Figure 2C, lanes 1,2,4,5). These results show that full-length Abi-2 interacts with c-Abl following overexpression of both proteins in Bosc 23 cells and that Abi-2 becomes phosphorylated in an in vitro kinase assay in the presence of the c-Abl tyrosine kinase. We have also observed that endogenous Abi-2 coimmunoprecipitates with the endogenous c-Abl protein in B cell lysates (data not shown).

Abi-2 binds to multiple surfaces on the c-Abl protein

To confirm that the Abl SH3 domain was responsible for the interaction between c-Abl and Abi-2, we examined whether a mutant c-Abl protein lacking the SH3 domain was deficient in binding to Abi-2. To our surprise, the c-Abl Δ SH3 mutant bound to Abi-2 as efficiently as wild-type c-Abl (Fig. 3A). This result suggested that in addition to the SH3 domain, other sequences in c-Abl may participate in binding to Abi-2. Using both the yeast two-hybrid system and in vitro binding assays, we identified a second region in the carboxyl terminus of c-Abl that interacted strongly with Abi-2 (Fig. 3B). The Abi-2-binding region in the carboxyl terminus of c-Abl maps to sequences near the NLS. Recently, it was reported that three distinct proline-rich sites in the Abl carboxyl terminus are binding sites for the SH3-containing Crk, Grb2, and Nck adaptor proteins (Ren et al. 1994). The presence of an SH3 domain in Abi-2 suggested that Abi-2 may also interact with c-Abl by direct binding of the Abi-2 SH3 domain with the proline-rich sequences in the carboxyl terminus of c-Abl.

To define more precisely the Abi-2 binding site in the Abl carboxyl terminus, additional c-Abl mutants were created and tested for binding to Abi-2 (Fig. 3C). Two of the three proline-rich stretches identified in c-Abl that bind to SH3 domains are found upstream of the nuclear localization sequence while the third proline stretch is downstream of this sequence (Ren et al. 1994). Deletion of the two proline-rich sites upstream of the NLS (Δ 544–601) did not diminish binding of c-Abl Δ SH3 to Abi-2 (Fig. 3C, lane 8). In contrast, deletion of additional sequences, including the third proline-rich site (Δ 544–637), completely abolished binding of c-Abl Δ SH3 to Abi-2 (Fig. 3C, lane 11). These results indicate that Abi-2 binds to c-Abl by interacting directly with the Abl SH3 domain and a proline-rich stretch downstream of the nuclear localization signal in the Abl carboxyl terminus.

c-Abl binds to the proline-rich amino terminus and carboxy-terminal SH3 domain of Abi-2

To map the sites on Abi-2 that interact with c-Abl, a series of Abi-2 deletion mutants was generated (Fig. 4A) and tested for binding to GST–fusion proteins containing the Abl SH3 domain and Abl carboxy-terminal sequences (Fig. 4B,C). Full-length and carboxy-terminal truncated Abi-2 proteins were synthesized by in vitro

Figure 3. Abi-2 binds to c-Abl at both SH3 and carboxy-terminal regions. (A) Interaction of Abi-2 with c-Abl is retained following deletion of the c-Abl SH3 domain. Lysates of COS cells transfected with expression vectors encoding wild-type c-Abl (pSRα/c-abl) (lanes 1–3) or an SH3 deletion mutant of c-Abl (pSRα/c-ablΔSH3) (lanes 4–6) were incubated with immobilized GST alone (lanes 1,4), GST-Abi-2 (lanes 2,5), or anti-Abl antibodies bound to protein A-Sepharose beads (lanes 3,6). Bound proteins were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Abl antibodies. (B) Mapping of the Abi-2-binding sites on c-Abl. Wild-type c-Abl and c-Abl deletion mutants are shown schematically. Binding of c-Abl fragments to Abi-2 was determined by the yeast two-hybrid system assay or by an in vitro binding assay, as indicated at right. (NLS) Nuclear localization sequence; (BD) binding domain. (C) Deletion of the Abl SH3 domain and Abl carboxy-terminal proline-rich sequences abolishes Abl binding to Abi-2.

2. The indicated in vitro-translated proteins were labeled with [³⁵S]methionine and incubated with GST alone, GST Abi-2, and anti-Abl antibodies bound to protein A-Sepharose beads, as indicated. Bound proteins were analyzed by SDS-PAGE and the [³⁵S]-labeled proteins were visualized by fluorography.

transcription/translation in a rabbit reticulocyte lysate. The predominant in vitro transcription/translation products generated from the full-length *abi-2* cDNA, migrate with apparent molecular masses of 55 and 44 kD (Fig. 4B). Deletion of the Abi-2 SH3 domain abrogates binding to the proline-rich Abl carboxy-terminal sequences encompassing amino acids 593–730 (Fig. 4B, lane 6). Further deletion of sequences upstream of the Abi-2 SH3 domain, which contain a polyproline stretch and a PEST domain does not affect binding to the Abl SH3 domain (Fig. 4B, lane 8).

To identify the sequences in Abi-2 that mediate binding to the Abl SH3 domain, we generated additional Abi-2 mutant proteins containing deletions of proline-rich sequences localized in the amino-terminal or central regions of the protein (Fig. 4A,C). The Abi-2 proteins

were produced in mammalian COS cells and tested for binding to Abl sequences. As shown in Figure 4C, deletion of the most amino-terminal proline-rich stretch of Abi-2 eliminates binding to the Abl SH3 domain (Fig. 4C, lane 5). Mutation of the individual prolines in this sequence is required to directly demonstrate that the interaction of the Abl SH3 domain with the amino-terminal sequences of Abi-2 is dependent on the presence of these proline residues. However, a 10-amino-acid proline-rich stretch within this region of Abi-2 exhibits strong similarity to the Abl SH3-binding site in 3BP-1 (Cohen et al. 1995), suggesting that this proline-rich sequence in Abi-2 is likely to bind to the Abl SH3 domain. Thus, our results indicate that the c-Abl tyrosine kinase contacts Abi-2 at a minimum of two sites: an amino-terminal proline-rich stretch and the Abi-2 SH3 domain.

Figure 4. c-Abl binds to the proline-rich amino terminus and the carboxy-terminal SH3 domain of Abi-2. (A) Schematic representation of Abi-2 and the Abi-2 deletion mutants constructed. The positions and names of the various structural domains in Abi-2 are indicated. (HHR) Homeo-domain homologous region. (B) cDNAs encoding *abi-2*, *abi-2* Δ SH3, and *abi-2* Δ 244-401 were subcloned into the pGEM vector for in vitro transcription/translation. The 35 S-labeled translated proteins were incubated with anti-Abi-2 antibodies bound to protein A (lanes 1,4,7), GST-Abi SH3 (lanes 2,5,8), and GST-Abi (593-730) (lanes 3,6). Bound proteins were washed and separated on SDS-PAGE, and 35 S-labeled proteins were visualized by fluorography. (C) cDNAs encoding amino-terminal deletion mutants (*abi-2* Δ 1-100 and *abi-2* Δ 1-157) or an internal deletion (*abi-2* Δ 158-243) of Abi-2 were subcloned in-frame into the pCGN vector and transfected into COS cells. Cell lysates were prepared at 2 $\frac{1}{2}$ days post-transfection, and the lysates incubated with GST alone (lanes 1,4,7), GST-Abi SH3 (lanes 2,5,8), and GST-Abi (593-730) (lanes 3,6,9). Bound proteins were separated on SDS-PAGE and analyzed by Western blotting with the anti-HA monoclonal antibody.

A model for the interaction between c-Abl and Abi-2 is shown in Figure 6D (below).

Abi-2 is a substrate for the c-Abl tyrosine kinase in vitro and in vivo

The strong association between Abi-2 and c-Abl suggested that Abi-2 may be a target for the tyrosine kinase activity of c-Abl. Additional support for this suggestion was provided by the observation that Abi-2 became phosphorylated in immune complex in vitro kinase assays following coprecipitation with the c-Abl tyrosine kinase from Bosc 23 cell lysates coexpressing the two proteins (Fig. 2B,C). Phosphoamino acid analysis of Abi-2 demonstrated that the phosphorylation was exclusively on tyrosine residues (data not shown). To determine whether the purified Abi-2 protein is a c-Abl substrate, wild-type and kinase defective forms of c-Abl were synthesized by in vitro transcription/translation in a reticulocyte lysate (Fig. 5A) and tested for their ability to phosphorylate purified GST or GST-Abi-2 proteins in vitro. Abi-2 was phosphorylated by wild-type but not kinase-defective c-Abl (Fig. 5A). A c-Abl mutant protein, c-Abl Δ SH3 Δ 544-637, which retains tyrosine kinase activity (data not shown) but is defective in binding to Abi-2 (Fig. 3C),

did not phosphorylate Abi-2 in this assay (Fig. 5A, lane 6). Next, we examined whether Abi-2 could be phosphorylated in vivo by c-Abl following expression in Bosc 23 cells. Coexpression of Abi-2 with wild-type c-Abl resulted in a marked increase in Abi-2 tyrosine phosphorylation (Fig. 5B, lane 4). These data show that Abi-2 can be phosphorylated by the activated c-Abl tyrosine kinase in vivo.

Expression of an Abi-2 mutant protein uncovers the transforming activity of c-Abl

The findings that Abi-2 binds to c-Abl via dual SH3 domain/proline-rich sequence interactions and that Abi-2 is a substrate of the c-Abl tyrosine kinase strongly suggested that Abi-2 may modulate the biological properties of c-Abl in the cell. Two biological assays are available to examine the effects of Abl expression in vivo. First, overexpression of c-Abl wild type in NIH-3T3 cells has been shown to elicit growth arrest and result in counterselection of the expression of the c-Abl protein (Jackson et al. 1993; Sawyers et al. 1994). Second, specific alterations in the c-Abl structure have been shown to result in cell transformation (Wang 1993). To examine whether Abi-2 can affect the biological properties of c-Abl, we trans-

Figure 5. Abi-2 is phosphorylated in vitro and in vivo by c-Abl. (A) In vitro phosphorylation. Immobilized GST alone (lanes 1,3,5) or GST-Abi-2 (lanes 2,4,6) were incubated at 4°C for 60 min with in vitro transcription/translation products (bottom) of c-Abl wild type (lanes 1,2), c-Abl K290R (lanes 3,4) and c-Abl Δ SH3 Δ 544–637 (lanes 5,6). Bound proteins were subjected to in vitro phosphorylation at 30°C in the presence of [γ - 32 P]ATP and MnCl₂. The phosphorylated proteins bound to beads were washed three times, separated on SDS-PAGE, and visualized by autoradiography. (B) In vivo phosphorylation. Bosc 23 cells were transfected with pCGN vector alone (lane 1), pSR α /c-abl (lane 2), pCGN/abi-2 (lane 3), and pSR α /c-abl plus pCGN/abi-2 (lane 4). Cell lysates were separated on SDS-PAGE and immunoblotted with the indicated antibodies.

fected NIH-3T3 cells with hygromycin-containing mammalian expression vectors encoding full-length or truncated forms of Abi-2. Expression of full-length Abi-2 could not be detected following transfection and hygromycin drug selection. Like the Abl proteins, full-length Abi-2 appears to exert cytotoxic and, possibly, cytostatic, effects on cells following overexpression (Ziegler et al. 1981; Renshaw et al. 1992; Jackson et al. 1993; Sawyers et al. 1994). In contrast, an amino-terminal truncated form of Abi-2 lacking the homeo-domain homologous region and the proline-rich sequences required for interaction with the Abl SH3 domain (Abi-2 Δ 1–157) was readily expressed in NIH-3T3 cells (Fig. 6A). Cells stably expressing the Abi-2 Δ 1–157 mutant or vector alone were infected with retroviruses encoding for wild-type c-Abl, a kinase-defective form of c-Abl, c-Abl K290R, or vector alone (Fig. 6B). A fraction of the cells was collected at 2 1/2 days postinfection, lysed, and subjected to Western blot analysis with anti-Abl antibodies. Similar levels of c-Abl wild-type and c-Abl K290R proteins were detected in control NIH-3T3 cells and in NIH-3T3 cells that stably expressed the Abi-2 Δ 1–157 mutant (Fig. 6B, top). At 2 1/2 days postinfection the cells were selected with G418. Surprisingly, analysis of drug-selected cells at 22 days postinfection revealed that while the c-Abl wild-type protein was counter-selected in control NIH-3T3 cells in accordance with previous observations (Sawyers et al. 1994), c-Abl was expressed to high levels in NIH-3T3 cells that coexpressed the Abi-2 Δ 1–157 mutant protein (Fig. 6B, lanes 2 and 5, bottom left). As shown previously, the kinase-defective c-Abl K290R protein was not counter-selected (Fig. 6B, lanes 3 and 6, bottom left). Furthermore, antiphosphotyrosine Western blot analysis revealed that expression of Abi-2 Δ 1–157 activates the tyrosine kinase activity of c-Abl in NIH-

3T3 cells (Fig. 6B, right). No enhanced tyrosine phosphorylation is detected in cells coexpressing Abi-2 Δ 1–157 and a kinase-defective form of c-Abl (Fig. 6B, right).

A striking difference was observed among the cells coexpressing c-Abl wild-type and Abi-2 Δ 1–157 and those cells expressing Abi-2 Δ 1–157 alone, c-Abl alone, vector control, or coexpressing Abi-2 Δ 1–157 and a kinase-defective form of c-Abl (Fig. 6C). Cells that coexpressed Abi-2 Δ 1–157 and c-Abl wild type were morphologically transformed and exhibited a similar morphology to those NIH-3T3 cells expressing the v-Abl oncoprotein (Fig. 6C, 3 and 6). To directly examine whether the cells coexpressing Abi-2 Δ 1–157 and c-Abl wild type were transformed, we assayed for colony formation in soft agar as a measurement of anchorage-independent growth (Muller et al. 1991). As shown in Table 1, cells coexpressing the Abi-2 Δ 1–157 mutant and c-Abl wild type exhibit a dramatic increase in soft agar plating efficiency compared with cells expressing either protein alone or cells expressing both the Abi-2 Δ 1–157 mutant and a kinase-defective form of c-Abl. These results demonstrate that expression of the Abi-2 Δ 1–157 mutant protein activates the tyrosine kinase and transforming activities of c-Abl in NIH-3T3 cells.

Discussion

Using a protein interaction cloning technique, we have identified a cDNA encoding a novel c-Abl-binding protein: Abi-2. Abi-2 interacts with at least two domains in c-Abl and is a substrate for its tyrosine kinase activity. We have shown that c-Abl can be converted into a transforming protein by coexpression with an amino-terminal truncated form of Abi-2 in the absence of structural alterations on the c-Abl molecule itself.

Figure 6. Expression of an Abi-2 mutant deficient in binding to the Abl SH3 domain activates the tyrosine kinase and transforming properties of c-Abl. (A) Expression of Abi-2 Δ 1-157 in NIH-3T3 cells. Cells were transfected with pCGN alone (lane 1) or pCGN/abi-2 Δ 1-157 (lane 2). Following hygromycin selection, cells were lysed in sample buffer and subjected to Western blot analysis using anti-HA antibody. The Abi-2 Δ 1-157 protein is indicated with the arrowhead. (B) Abi-2 Δ 1-157 reverses biologic selection against c-Abl overexpression. NIH-3T3 cells expressing the *neo* resistance gene alone, *c-abl* wild-type or *c-abl* K290R, as indicated. After 2 1/2 days, cells were selected with G418. (Left) Anti-Abl Western blots of lysates prepared from infected cells at 2 1/2 days (top) or 22 days postinfection (bottom). c-Abl is indicated by the arrowhead. (Right) An anti-pTyr Western blot of lysates from cells at 22 days postinfection. (C) Abi-2 Δ 1-157 activates the c-Abl-transforming activity. NIH-3T3 cells were transfected with either pCGN vector (panels 1-3) or pCGN/abi-2 Δ 1-157 (panels 4-6). After selection with hygromycin, the cells were infected with retroviruses expressing *neo* (1 and 4), wild type c-Abl (2 and 6), c-Abl K290R (5), or v-Abl (3). At 2 1/2 days postinfection the cells were incubated with media containing G418 and maintained under drug selection for 22 days. Morphological transformation was clearly observed in c-Abl-infected NIH-3T3 cells that had been transfected with Abi-2 Δ 1-157 but not in cells expressing vector control (cf. 2 and 6). (D) A model is proposed for the interaction of c-Abl and Abi-2 and the presence of c-Abl/Abi-2 complexes in unphosphorylated and tyrosine-phosphorylated states. Activation of the c-Abl tyrosine kinase leading to tyrosine phosphorylation of Abi-2 may occur by a variety of events, some of which are listed in the box.

(Fig. 1D) and murine Abi-1 (Shi et al., this issue) suggests that the two proteins may belong to a protein family. Moreover, whereas the expression pattern of murine Abi-1 shows markedly higher levels in the brain, the human Abi-2 is ubiquitously expressed. An additional distinction between the two proteins is the absence in Abi-2 of a second region of homology in murine Abi-1, which is found in a group of homeo-domain proteins (Shi et al., this issue). This region of homology lies outside the DNA-binding region and corresponds to a proline-rich segment of the protein. Our results indicate that the human *abi-2* gene localizes to a chromosome different

the *abi-1* gene (Z. Dai, T. Yang-Feng, and A.M. Pendergast, unpubl.).

A body of experimental data suggest that the tyrosine kinase and transforming activity of c-Abl is tightly regulated in vivo by a *trans*-acting protein inhibitor (Pendergast et al. 1991a; Mayer and Baltimore 1994). Several proteins have been identified that bind to specific domains in Abl and may be candidates for the c-Abl inhibitor. Two proteins, 3BP-1 and 3BP-2, have been identified by screening of a λ gt 11 cDNA expression library with the Abl SH3 domain (Cicchetti et al. 1992). However, neither protein has been shown to interact with full-length c-Abl in vivo (Mayer and Baltimore 1994). An in-

Table 1. Transformation of NIH-3T3 cells by coexpression of c-Abl and a truncated Abi-2 protein

Retroviruses ^a	No. of agar colonies ^b	
	NIH-3T3 vector ^c	NIH 3T3/Abi-2 Δ 1–157 ^c
c-Abl wild type	3	563
c-Abl K290R	2	3
Tkneo	4	3

^aHelper-free retroviruses were prepared by transient overexpression in Bosc 23 cells as described in Materials and methods.

^bNumber of agar colonies per 10⁴ cells. The average frequency of colony formation in soft agar was determined from two plates per assay. Colonies with a diameter of >0.5 mm were counted ~2 weeks after plating the cells.

^cNIH-3T3 cells stably transfected with either pCGN vector alone or pCGN/abi-2 Δ 1–157 were infected with the indicated helper-free retroviruses. At 2½ days postinfection the cells were selected with G148 and cultured for 3 weeks in the presence of the drug.

interaction of c-Abl with the retinoblastoma protein (Rb) has been reported (Welch and Wang 1993). Rb interacts with Abl by binding to the Abl tyrosine kinase domain and inhibits its kinase activity (Welch and Wang 1993). The observation that only 5%–25% of the total c-Abl is present in a complex with Rb while all of the c-Abl in the cell is inhibited suggests that additional mechanisms regulate the c-Abl tyrosine kinase, possibly through interactions with other cellular proteins. More recently, it was shown that c-Abl interacts with the Crk family of SH3-containing adaptor proteins (Feller et al. 1994a; Ren et al. 1994). The cytosolic Crk proteins bind to the c-Abl carboxy-terminal domain (Ren et al. 1994). No inhibitory effect of Crk binding on the c-Abl tyrosine kinase was reported. The results presented here show that Abi-2 forms a complex with c-Abl through direct contact with both the Abl SH3 and carboxy-terminal domains and modulates c-Abl activity. The proposed regulatory function of Abi-2 may involve stabilization of the inactive form of c-Abl or, alternatively, Abi-2 may block access to critical cellular protein substrates of the c-Abl tyrosine kinase. The latter hypothesis is consistent with the inhibitory effect of the related murine Abi-1 protein on the transforming activity of the P160^{v-abl} tyrosine kinase (Shi et al., this issue).

A second role for Abi-2 that is suggested from the data presented here is that of effector of the c-Abl protein tyrosine kinase. Abi-2 is a substrate for c-Abl in vitro and in vivo. At least two tyrosines within Abi-2 are found in sequences predicted to be optimal peptide substrates for the c-Abl tyrosine kinase (Songyang et al. 1995). The optimal phosphorylation site corresponds to the tyrosine in the sequence YSDP just upstream of the Abi-2 SH3 domain (Fig. 1A). A tyrosine in a similar context was reported to be the site phosphorylated by c-Abl on c-Crk (Feller et al. 1994a). These results support a role for the Abl SH3 and carboxy-terminal domains in substrate recruitment. Recruitment of c-Abl substrates through di-

rect protein interactions is also illustrated by the binding of Enabled to the *Drosophila* Abl protein, which is mediated by direct interaction of the proline-rich sequences in Enabled with the Abl SH3 domain (Gertler et al. 1995). The Enabled protein is phosphorylated by the Abl tyrosine kinase, and this phosphorylation is proposed to regulate Enabled function during axogenesis (Gertler et al. 1995).

In addition to the dual SH3–proline interactions within the c-Abl/Abi-2 complex, contacts may be made between tyrosine-phosphorylated sequences in Abi-2 and the Abl SH2 domain. It has been shown that protein tyrosine kinases preferentially phosphorylate peptide sequences that bind with high affinity to their own SH2 domains (Songyang et al. 1995). This type of interaction may protect Abi-2 from dephosphorylation and strengthen the interaction between Abi-2 and c-Abl. In this regard, a role for the Abl SH2 domain in substrate selectivity has been described recently. The Abl SH2 domain is required for the efficient in vitro hyperphosphorylation of multiple tyrosines present in the carboxy-terminal domain of RNA polymerase II and p130^{CAS} (Duyster et al. 1995; Mayer et al. 1995).

The most revealing result presented here is the finding that overexpression of a truncated form of Abi-2, which has lost the sequences required for binding to the Abl SH3 domain but retains binding to the Abl carboxyl terminus, converts c-Abl into a transforming protein. Several models may be proposed to explain this result. The Abi-2 Δ 1–157 protein may simply displace the endogenous Abi-2 protein and thereby allow constitutive phosphorylation of other protein targets by the c-Abl tyrosine kinase. Alternatively, binding of Abi-2 Δ 1–157 to the Abl carboxyl terminus may activate its tyrosine kinase activity and transforming potential. These two possibilities may not be mutually exclusive. An additional mechanism whereby Abi-2 Δ 1–157 could elicit transformation is by retaining c-Abl in the cytoplasm and thereby keeping c-Abl outside of the nucleus. It has been proposed that the growth inhibitory activity of overexpressed c-Abl requires localization to the nuclear compartment (Sawyers et al. 1994).

What is the cellular role of Abi-2 and related family members? The Abi proteins may function to coordinate the cytoplasmic and nuclear functions of the c-Abl tyrosine kinase. Subcellular fractionation reveals that Abi-2, like c-Abl, is found in both the cytoplasm and the nucleus (data not shown). Abi-2 contains protein motifs associated with cytoplasmic signaling proteins (SH3 domain) as well as with nuclear factors (homeo-domain homology domain, PEST sequences, and polyproline and acidic domains similar to those present in the activation domains of transcription factors). An attractive possibility is that Abi-2 may function in a manner analogous to that of the signal transducers and activators of transcription (STATs), which become tyrosine phosphorylated in response to extracellular stimuli and migrate into the nucleus where they bind to DNA and activate transcription (Darnell et al. 1994).

Abi-2 may represent a class of signaling molecules that

have evolved to specifically couple signals elicited by activated protein tyrosine kinases to specific changes in the nucleus, the cytoskeleton, and other cellular compartments. Although Abi-2 does not share significant sequence identity with other known proteins, it exhibits a very similar structural arrangement to that of proteins implicated in signaling by the Src family of tyrosine kinases: HS1 (Yamanishi et al. 1993) and cortactin (Wu et al. 1991). Both proteins are only 20% identical to Abi-2; however, all three have an SH3 domain at the carboxyl terminus, contain proline-rich sequences, a basic amino terminus with helix–turn–helix motifs and, significantly, all three proteins have a potential site of tyrosine phosphorylation just upstream of the SH3 domain. HS1 is expressed exclusively in hematopoietic cells and has been shown to be a major substrate of the Lyn tyrosine kinase upon B cell stimulation (Yamanashi et al. 1993). Like Abi-2, HS1 has been shown to localize to the cytoplasm and the nucleus (Yamanishi et al. 1993). Cortactin is a substrate of the Src tyrosine kinase and associates with the cytoskeleton (Wu et al. 1991). More recently, another member of this family, LckBP1, was cloned as a protein that interacts with the LckSH3 domain (Takemoto et al. 1995). These proteins may play critical roles in the transmission of growth, differentiation, stress, and apoptotic signals elicited by specific protein tyrosine kinases. Further work is necessary to determine whether Abi-2, HS1, LckBP1, and cortactin share similar functional roles.

The identification of Abi-2 as a substrate of the c-Abl tyrosine kinase, which like c-Abl is found in the cytoplasm and the nucleus, and the finding that alterations in Abi-2 can activate the c-Abl-transforming potential provide new directions in the investigation of c-Abl functions. A role for Abi-2 in cancer may be hypothesized. Mutations or deletions in Abi-2 could be associated with the progression of Philadelphia chromosome-positive human leukemias from the chronic to the blast crisis phases of the disease. Alterations in Abi-2 could also potentially be linked to the development of other cancers.

Materials and methods

Cells and viruses

Recombinant c-Abl baculovirus was prepared as described (Pendergast et al. 1991b). Recombinant GAP baculovirus (Trahey et al. 1988) was obtained from Drs. R. Clark and F. McCormick. Bosc 23 cells, obtained from Drs. W.S. Pear and D. Baltimore (Pear et al. 1993), were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS); COS and NIH-3T3 cells were grown in DMEM plus 5% FCS; Sf9 insect cells were grown in Grace media plus yeastolate and 10% FCS.

Antibodies

Polyclonal antibodies against Abi-2 were prepared by immunization of New Zealand White female rabbits with a GST–Abi-2 fusion protein or with a peptide corresponding to the amino terminus of Abi-2. Anti-Abl polyclonal antibodies and a mouse monoclonal antibody to Abl were described previously

(Konopka et al. 1984; Pendergast et al. 1991b). Antibodies against Ras GAP were obtained from Drs. R. Clark and F. McCormick. Monoclonal antibody against HA (12CA5) was purchased from Boehringer Mannheim. Monoclonal antibody against phosphotyrosine (PY69) was obtained from ICN Biochemicals, Inc.

Plasmid constructions

A cDNA fragment encoding the c-Abl SH3 domain (amino acids 47–147) was excised from pGEX3X(929-1029)P210[SH3] (Pendergast et al. 1991b) by *Bam*HI–*Aat*II restriction enzyme digestion and subcloned in-frame into pPC62 (obtained from P.M. Chevray) (Chevray and Nathans 1992). The resultant plasmid, designated pPC60/*abl*SH3, directs the synthesis of a fusion protein containing the Gal4 DNA-binding domain and the SH3 domain of c-Abl when expressed in yeast.

The pGEM vector (Promega) was used for in vitro transcription/translation of *abi-2*, *c-abl*, and their respective deletion mutants. The entire *abi-2*-coding sequence was subcloned into the pGEM vector at the *Bam*HI site. This plasmid, designated pGEM/*abi-2*, was subsequently digested with *Stu*I to remove the sequence encoding amino acids 244–401 from *abi-2* and ligated to generate pGEM/*abi-2* Δ 244–401. To generate the SH3 domain deletion of Abi-2, the DNA sequence encoding amino acids 161–321 of Abi-2 was amplified by polymerase chain reaction (PCR). The PCR-generated cDNA fragment was digested with *Kpn*I–*Hind*III and subcloned into pGEM/*abi-2* at the *Kpn*I and *Hind*III sites. The resultant plasmid was designated pGEM/*abi-2* Δ SH3.

To express *abi-2* in mammalian cells, a modified pCGN expression vector (Tanaka and Herr 1990) was used. The entire coding sequence of *abi-2* was subcloned in-frame into pCGN at the *Bam*HI site downstream of the sequence encoding the hemagglutinin (HA) tag. The resultant plasmid pCGN/*abi-2*, under the control of the cytomegalovirus (CMV) promoter, directs the synthesis of Abi-2 with an HA tag fused at its amino terminus. Construction of pCGN/*abi-2* Δ 1–100 was performed by cutting the pGEM/*abi-2* plasmid with *Xba*I and adding a *Bam*HI linker to the 5' end of *abi-2*. The cDNA fragment with a 5' deletion was then subcloned in-frame into the pCGN vector. To create pCGN/*abi-2* Δ 1–157, the pGEM/*abi-2* plasmid was cut with *Kpn*I, and following addition of a *Bam*HI linker at the 5', the *Bam*HI cDNA fragment of *abi-2* was subcloned into pCGN. The plasmid pCGN/*abi-2* Δ 158–243 was created by an internal deletion of pGEM/*abi-2* at the *Kpn*I and *Stu*I sites and subcloning of the deleted *abi-2* DNA into pCGN.

pGEM/*c-abl* and pGEM/*abl* Δ SH3 were constructed as described previously (Pendergast et al. 1991a). To generate carboxy-terminal deletion mutants of c-Abl a PCR-directed mutagenesis strategy was employed as described (Dai et al. 1992). The resultant plasmids, designated pGEM/*abl* Δ SH3 Δ 544–601 and pGEM/*abl* Δ SH3 Δ 544–637, respectively, bear double deletion of sequences encoding both SH3 and carboxy-terminal amino acids as indicated.

The pGEX 3X vector (Pharmacia) was used to express a GST–Abi-2 fusion protein. The entire coding sequence of *abi-2* was subcloned in-frame into pGEX 3X at the *Bam*HI site, and the plasmid created was designated pGEX/*abi-2*. cDNA fragments encoding the SH3 and SH2 domains of c-Abl were subcloned in-frame into pGEX 3X as described (Pendergast et al. 1991b). cDNA fragments encoding Abl carboxy-terminal amino acids 593–1149, 593–730, and 731–1149 were excised from pGEM/*c-abl* by appropriate restriction enzyme digestions and subcloned in-frame into pGEX 3X. These cDNA fragments of *c-abl*, together with the full-length *c-abl* and *c-abl* Δ SH3, were also sub-

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cloned in-frame into pAS-CYH2 (obtained from S. Elledge) (Harper et al. 1993) for testing the interaction by the yeast two-hybrid system.

Yeast two-hybrid screen

The yeast two-hybrid system was employed to screen a human lymphocyte cDNA library as described (Durfee et al. 1993). The bait plasmid pPC60/*ablSH3* was cotransformed with the cDNA library into yeast strain Y190 (Harper et al. 1993). Transformants (4×10^5) were screened for *lacZ* reporter gene expression by a filter lift assay (Durfee et al. 1993). Colonies that turned blue in 4 hr were replicated onto a minus tryptophan, leucine, and histidine plate containing 25 mM 3-AT and grown at 30°C for 3 days. Total DNA was isolated from the colonies that grew and used to transform the DH5 α bacterial strain. Plasmid DNA was isolated from ampicillin-resistant colonies and tested for bait-dependent reporter gene expression by retransforming back into yeast strain Y190 with pAS-CYH plasmid alone or bait plasmid pPC60/*ablSH3*. cDNAs that provided bait-dependent *lacZ* gene activation were subcloned into pBSK and subjected to dideoxy chain termination sequencing. PCR amplification of the *abi-2* 5'-untranslated sequence was performed using the human lymphocyte cDNA library as template. Amplified cDNA fragments were subcloned into pBSK. Plasmid DNA from three independent colonies was prepared and sequenced. All three colonies contained cDNA inserts that represent the 5'-untranslated sequence of *abi-2*. Sequence analysis was performed with the Genetics Computer Group program (Pearson and Lipman 1988). FASTA searches for homologous sequences were performed against the SwissProt, PirProtein, and GenBank data libraries. The accession number of the GenBank data base for the human *abi-2* [previously *aip-1*] cDNA sequence reported in this paper is U23435.

In vitro transcription and translation

In vitro transcription/translation was performed using the SP6 in vitro transcription/translation kit (Promega) according to manufacturer's specifications. The reaction was incubated at 30°C for 2 hr, and in vitro-translated protein products were analyzed directly by SDS-PAGE or subjected to immunoprecipitation/binding assay prior to SDS-PAGE as indicated in text.

Binding assays

Radiolabeled proteins from whole-cell lysates or translated in vitro in reticulocyte lysates were incubated with GST or GST fusion proteins attached to glutathione-Sepharose beads as described previously (Pendergast et al. 1993). Bound proteins were analyzed by SDS-PAGE.

Immunoblotting and in vitro kinase assay

Immunoblotting was performed as described (Pendergast et al. 1993). Immunoreactive proteins were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham) according to manufacturer's specifications. In vitro kinase assays were performed as described (Pendergast et al. 1993). Phosphorylation was carried out in the presence of [γ - 32 P]ATP and MnCl $_2$ for 30 min at 30°C.

Retrovirus infections and transformation assays

Helper-free retroviral stocks were prepared by transient hyperexpression in Bosc 23 cells as described previously (Muller et al.

1991; Pear et al. 1993). Infection of NIH-3T3 fibroblasts with the indicated retroviruses was performed using polybrene as described previously (Muller et al. 1991). Following a 4-hr infection period, the cells were placed in fresh medium (DMEM + 10% FCS) and cultured for 2 ½ days before selection with G418. Cells were selected for 22 days. Western blot analysis was performed on cell populations at 2 ½ days postinfection and after 22 days of G418 selection. Cell transformation was quantitated by growth in soft agar as described previously (Muller et al. 1991). The cells (1×10^4) were plated per 6-cm dish in duplicate. Agar colonies with a diameter of >0.5 mm were counted ~2 weeks after plating the cells.

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Note added in proof

The sequence data for *Abi-2* has been submitted to the GenBank data library under accession number U23435.

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