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c-ABL Tyrosine Kinase Activity Is Regulated by Association with a Novel SH3-Domain-Binding Protein

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The c-ABL tyrosine kinase is activated following either the loss or mutation of its Src homology domain 3 (SH3), resulting in both increased autophosphorylation and phosphorylation of cellular substrates and cellular transformation. This suggests that the SH3 domain negatively regulates c-ABL kinase activity. For several reasons this regulation is thought to involve a cellular protein that binds to the SH3 domain. Hyperexpression of c-ABL results in an activation of its kinase, the kinase activity of purified c-ABL protein in the absence of cellular proteins is independent of either the presence or absence of a SH3 domain, and point mutations and deletions within the SH3 domain are sufficient to activate c-ABL transforming ability. To identify proteins that interact with the c-ABL SH3 domain, we screened a cDNA library by the yeast two-hybrid system, using the c-ABL SH3SH2 domains as bait. We identified a novel protein, AAP1 (ABL-associated protein 1), that associates with these c-ABL domains and fails to bind to the SH3 domain in the activated oncoprotein BCRABL. Kinase experiments demonstrated that in the presence of AAP1, the ability of c-ABL to phosphorylate either glutathione *S*-transferase–CRK or enolase was inhibited. In contrast, AAP1 had little effect on the phosphorylation of glutathione *S*-transferase–CRK by the activated ABL oncoproteins v-ABL and BCRABL. We conclude that AAP1 inhibits c-ABL tyrosine kinase activity but has little effect on the tyrosine kinase activities of oncogenic BCRABL or v-ABL protein and propose that AAP1 functions as a *trans* regulator of c-ABL kinase. Our data also indicate that loss of susceptibility to AAP1 regulation correlates with oncogenicity of the activated forms of c-ABL.

Protein phosphorylation is a dynamic cellular phenomenon, balanced between the activity of protein kinases, which catalyze substrate phosphorylation, and protein phosphatases, which specifically remove phosphate groups from molecules (reviewed in reference 42). Each of these processes is regulated in a precise manner; deregulated kinase activity can have severe effects on normal cellular metabolism and ultimately lead to oncogenic transformation. The control of protein kinases has evolved along several conserved pathways, mainly posttranslational modifications and protein-protein interactions. Protein kinases can be activated or inhibited upon phosphorylation by other protein kinases. A well-characterized example is the protein kinases involved in mitogen-activated protein kinase signal pathway. This signal cascade is dependent upon the successive phosphorylation of participating proteins. ERK and JNK are dually phosphorylated and activated by MEKs, which in turn are phosphorylated and activated by several MAPKK kinases, including RAF isoforms, c-MOS, and MEKK1 (19). The nonreceptor tyrosine kinase pp60 c-Src is down-regulated when phosphorylated on Tyr-527 by protein kinase C-CSK (reviewed in reference 4).

The binding of protein inhibitors to kinases is another means of controlling enzymatic activity. Protein kinase activity of a variety of cyclin–cyclin-dependent kinase complexes is inhibited by cyclin-dependent kinase inhibitors; these include CIP1, INK4, KIP1-2, and CIP2 (35). Cyclic AMP (cAMP)-dependent protein kinase A is inhibited by the binding of protein kinase inhibitor (8, 44), which functions as a competitive substrate antagonist (20). Studies with protein kinase C (20), myosin light-chain kinase (MLCK) (22), and calmodulin-dependent protein kinase II (32) revealed that these kinases are autoinhibited by a mechanism whereby part of their structure occupies the active site. In contrast with the inactive molecular complex of enzyme and bound inhibitor is the assembly of signaling heterocomplexes at specific locations within the cell. This acts as an additional layer of control that depends upon the spatial containment of the kinase and its signaling partners (9).

Phosphorylation and protein-protein associations combine to regulate cyclin-dependent kinase, whereby both association with the appropriate cyclin partner and phosphorylation status regulate kinase and biological activity. Phosphorylation of a specific residue can obscure substrate-binding sites (25) or potentially interfere with ATP binding (1). The inhibition of insulin signaling by the insulin receptor is a result the serine threonine phosphorylation of insulin receptor substrate 1, suggesting that the substrate-kinase interaction is dynamic and not unidirectional (16).

The c-*ABL* proto-oncogene encodes a nonreceptor protein tyrosine kinase, whose biological effects are mediated through its kinase activity. The oncogenic variants of the c-*ABL* gene, v-*ABL* and *BCRABL*, differ from c-*ABL* by an enhanced tyrosine kinase activity and the acquisition of transforming and tumorigenic properties (reviewed in reference 45). In contrast to the constitutive kinase activity of the activated oncoproteins v-ABL and BCRABL, c-ABL kinase activity is tightly controlled. Several lines of evidence have suggested the existence of a cellular inhibitor of c-ABL kinase. Purified c-ABL protein, in the absence of cellular factors, possesses kinase activity and is phosphotyrosine modified, whereas c-ABL isolated from cells contains no phosphotyrosine. Either deletion of or point mutations within the SH3 domain of c-ABL result in the constitutive tyrosine kinase activity and oncogenic activation (re-

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viewed in reference 45). Hyperexpression of c-ABL results in autophosphorylation in vivo, suggesting that the cellular inhibitory factor is present at a limited concentration (33). Unlike c-Src protein, c-ABL has no C-terminal tyrosine residue homologous to Tyr-527 responsible for its regulation. Instead, mutational analysis has implicated the SH3 domain as a potential regulator of its kinase activity (12, 18, 43). This model is consistent with the ability of SH3 domains to serve as sites for specific protein-protein interactions and is further supported by the fact that SH3 domains are known to function as protein association domains during cellular signaling (reviewed in reference 13). The observation that c-ABL in vitro kinase activity is independent of the SH3 domain suggests that the inhibitor does not bind tightly enough to be copurified by immunoprecipitation, hampering the search for such a c-ABL kinase inhibitor (26).

In this report, we present evidence that a novel cellular protein, ABL-associated protein 1 (AAP1), functions as an inhibitor of c-ABL protein tyrosine kinase. AAP1 associates with ABL via the c-ABL SH3 domain and fails to associate with BCRABL. The functional significance of AAP1 was demonstrated by its ability to inhibit the tyrosine phosphorylation of c-CRK and enolase by c-ABL but not by v-ABL, while phosphorylation by BCRABL was slightly inhibited. Thus, loss of susceptibility to AAP1 regulation correlates with the oncogenicity of the activated forms of c-ABL. This study directly implicates AAP1 as a *trans* inhibitor of c-ABL tyrosine kinase.

MATERIALS AND METHODS

Two-hybrid screening, *aap1* cloning, and analysis of expression. A PCR fragment containing the SH3SH2 region amplified from murine c-ABL (amino acids 58 to 220) (22) was inserted in frame into pGBT9 (Clontech) as a GAL4 DNA binding domain fusion protein. cDNA was prepared from HeLa mRNA and was cloned into the pGAD GH plasmid (Clontech). Yeast two-hybrid screening was carried out as has been previously described (11). Briefly, *Saccharomyces cerevisiae* host strain HF7C (*trp1 leu2*) (*HIS3* and *lacZ* reporter genes) was maintained in YPD medium (1% yeast extract and 2% Difco peptone). Fusion plasmids were cotransfected into the yeast strain by the lithium acetate method (14). His^+ Trp^+ Leu^+ transformants were selected in synthetic medium (0.67% Difco yeast nitrogen base–2% glucose) lacking appropriate supplements and transferred to a filter and assayed for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a substrate. Individual blue colonies were restreaked and again assayed for β-galactosidase activity. Colonies which turned blue in less than 20 min in the filter assay were scored positive. The pGAD424 plasmid was rescued by transforming the HB101 bacterial strain with yeast lysate derived from the positive colonies. The cDNA insert was sequenced with Sequenase dideoxy DNA sequencing reagents (United States Biochemical). To obtain full-length cDNA clones, a 5′-stretch λgt11 HeLa cDNA library (Clontech) was screened with *aap1* probes.

aap1 and c-*abl* cDNAs were ^{32}P labeled by random-primer synthesis (Life Technologies) and used as probes for multiple-human-tissue Northern (RNA) blots (Clontech). The amount of poly(A)$^+$ RNA in each lane was confirmed by subsequent hybridization with a β-actin probe (data not shown). The blots were hybridized overnight under stringent conditions, washed three times with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 50°C, and then exposed to X-ray film. The Northern blots were first probed with *aap1* ^{32}P-labeled cDNA and then stripped and rehybridized with c-*abl* ^{32}P-labeled cDNA.

Protein expression and SH3 binding analysis. Sequences encoding either the murine c-ABL SH3 (amino acids 58 to 115) (31), c-ABL SH3SH2 (amino acids 58 to 220) (31), or c-FGR SH3 (amino acids 125 to 189) (48) domain were inserted in frame into the *Bam*HI site of a pGEX-2T vector (Pharmacia) and modified to encode the heart muscle cAMP-dependent kinase phosphorylation site (Arg Arg Ala Ser Val) at the C terminus, and glutathione *S*-transferase (GST) fusion proteins were purified by glutathione affinity chromatography (41). *aap1* cDNA, encoding amino acids 151 to 463, was inserted into a baculovirus GST fusion vector (Invitrogen), the recombinant baculovirus was used to infect Sf9 insect cells at 10:1 ratio, and the expressed GST-AAP1 fusion protein was purified by glutathione affinity chromatography according to the procedures recommended by the manufacturer. Bacterially expressed and affinity-purified GST fusion proteins encoding the c-ABL SH3, c-ABL SH3 and SH2, and c-FGR SH3 domains were ^{32}P labeled with the catalytic subunit of cAMP-dependent kinase (Promega) in kinase buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, and 0.5 mM dithiothreitol (DTT); this was followed by the removal of unincorporated isotope by gel filtration. Five micrograms of either baculovirus-expressed GST or GST-AAP1 was spotted onto a nitrocellulose membrane, and 10^6 cpm of ^{32}P-labeled GST fusion protein probes was incubated in phosphate-buffered saline–1% Triton X-100–5% nonfat milk for 1 h at room temperature. The nitrocellulose filter was then washed with the same buffer three times at room temperature and exposed to X-ray film for 5 min.

For the liquid binding assay, nuclear lysate from the murine myeloblast cell line 32Dcl3 (15) was used as the source of c-ABL protein. Cells were first resuspended in hypotonic buffer (20 mM HEPES [pH 7.4], 15 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin-pepstatin-aprotinin per ml) and homogenized with 10 strokes in a type B Dounce homogenizer. Nuclei were pelleted and lysed in Tris-buffered saline–0.1% Nonidet P-40. To obtain p210 BCRABL protein, 32Dcl3 cells overexpressing BCRABL (49) were homogenized in hypotonic buffer and nuclei were removed by low-speed centrifugation. The cytoplasmic fraction was adjusted to 0.1% Nonidet P-40 and 150 mM NaCl. Equal amounts of either GST or GST-AAP1 were immobilized on glutathione-Sepharose beads (Pharmacia) and incubated with either 32Dcl3 nuclear lysate or 32Dcl3/BCRABL cytoplasmic lysates at 4°C for 1 h. The immobilized protein complexes were then extensively washed with Tris-buffered saline–0.1% Nonidet P-40, fractionated by SDS–8% polyacrylamide gel electrophoresis (PAGE), and transferred onto a nitrocellulose membrane. Western blotting (immunoblotting) was performed with anti-ABL monoclonal antibody (MAb) 8E9 as previously described (49). An immunoprecipitation with the same MAb was also run in parallel as a positive control. The immunoreactive proteins were visualized with chemiluminescence reagents (DuPont).

ABL kinase inhibition by AAP1. A cDNA fragment consisting of c-CRK sequences encoding amino acids 120 to 225 [CRK(120-225)] was isolated by reverse transcriptase PCR with HeLa total cellular RNA. The primer sequences were derived from the published c-CRK sequence (24) and were 5′-CGGGATCCAGATCCAGGCAGGGTAGTGG (sense) and 5′-CGGAATCCGCTGGGTTGGGCATAGGGGCC (antisense). A *Bam*HI site introduced for in-frame insertion into pGEX-2T (Pharmacia) is underlined. The affinity-purified GST-CRK(120-225) fusion protein was used as the substrate for the subsequent ABL kinase reactions. Ten milliliters of c-ABL recombinant baculovirus-infected Sf9 cells were lysed in Tris-buffered saline–0.1% Triton X-100, and c-ABL protein (~0.5 μg) was immunopurified by adding 5 μg of MAb 8E9. The c-ABL immune complex was immobilized on protein G-Sepharose (Pharmacia) and thoroughly washed in lysis buffer. One microgram of GST-CRK(120-225) was added together with increasing amounts of either GST-AAP1 or GST. Kinase reactions were assembled by resuspending immunopurified c-ABL in reaction buffer consisting of 20 mM HEPES (pH 7.0), 150 mM NaCl, 10 mM $MnCl_2$, and 0.5 mM DTT. The reaction was initiated by the addition of 1 μCi of [γ-^{32}P]ATP and then proceeded for 20 min at 30°C. The reaction was stopped by the addition of 2× Laemmli buffer and boiling for 5 min. Phosphorylated proteins were separated by SDS–13% PAGE and visualized by autoradiography. The phosphorylation of GST-CRK(120-225) was also quantitated on a PhosphorImager (Fuji).

pcDNA3/c-Abl(1B) and pcDNA3/v-Abl were transiently expressed in Cos-7 cells following Lipofectamine transfection (Life Technologies). The transfected Cos cells and 32Dcl3 cells expressing BCRABLp210 (49) were lysed in 20 mM HEPES (pH 7.4)–150 mM NaCl–1% Triton X-100–0.5 mM DTT including 1 μg of leupeptin-pepstatin-aprotinin and 1 mM phenylmethylsulfonyl fluoride. ABL proteins were immunoprecipitated by the addition of 1 μg of anti-ABL MAb Ab-3 (Oncogene Science) and immobilized on protein G-Sepharose (Pharmacia). The immune complexes were washed three times with lysis buffer and then twice with kinase buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, 10 mM $MnCl_2$, and 0.5 mM DTT). Increasing amounts of GST-AAP1 were added together with 1 μg of GST-CRK(120-225) substrate to each assembled kinase reaction mixture. The kinase reaction mixtures were incubated at 30°C for 20 min, and the reaction products were separated by SDS–13% PAGE.

Competition assays were performed with excess SH3 polypeptides. For these assays, 5×10^6 human 293 cells were lysed in 20 mM HEPES (pH 7.4)–150 mM NaCl–0.1% Triton X-100–1 mM phenylmethylsulfonyl fluoride–1 μg of leupeptin-pepstatin-aprotinin and endogenous c-ABL protein was immunoprecipitated with 5 μg of anti-ABL 8E9 and immobilized on protein G-Sepharose. Increasing amounts of GST-AAP1 were added, and 15 μg of either GST-ABL SH3 or GST-FGR SH3 was also included in the kinase reaction, as indicated in Fig. 6. One microgram of GST-CRK(120-225) was used in all kinase reactions as the substrate. The kinase reactions proceeded for 20 min at 30°C, and the products were then separated by SDS–13% PAGE.

RESULTS

Cloning and characterization of *aap1*. To identify a putative regulator of c-ABL kinase, we screened a HeLa cDNA library using the yeast two-hybrid system (11) with the SH3SH2 domains of c-ABL as bait. From a total of 5×10^5 transformants we identified five positive clones that when cotransfected into *S. cerevisiae* HF7c along with the pGT9-SH3SH2 plasmid re-

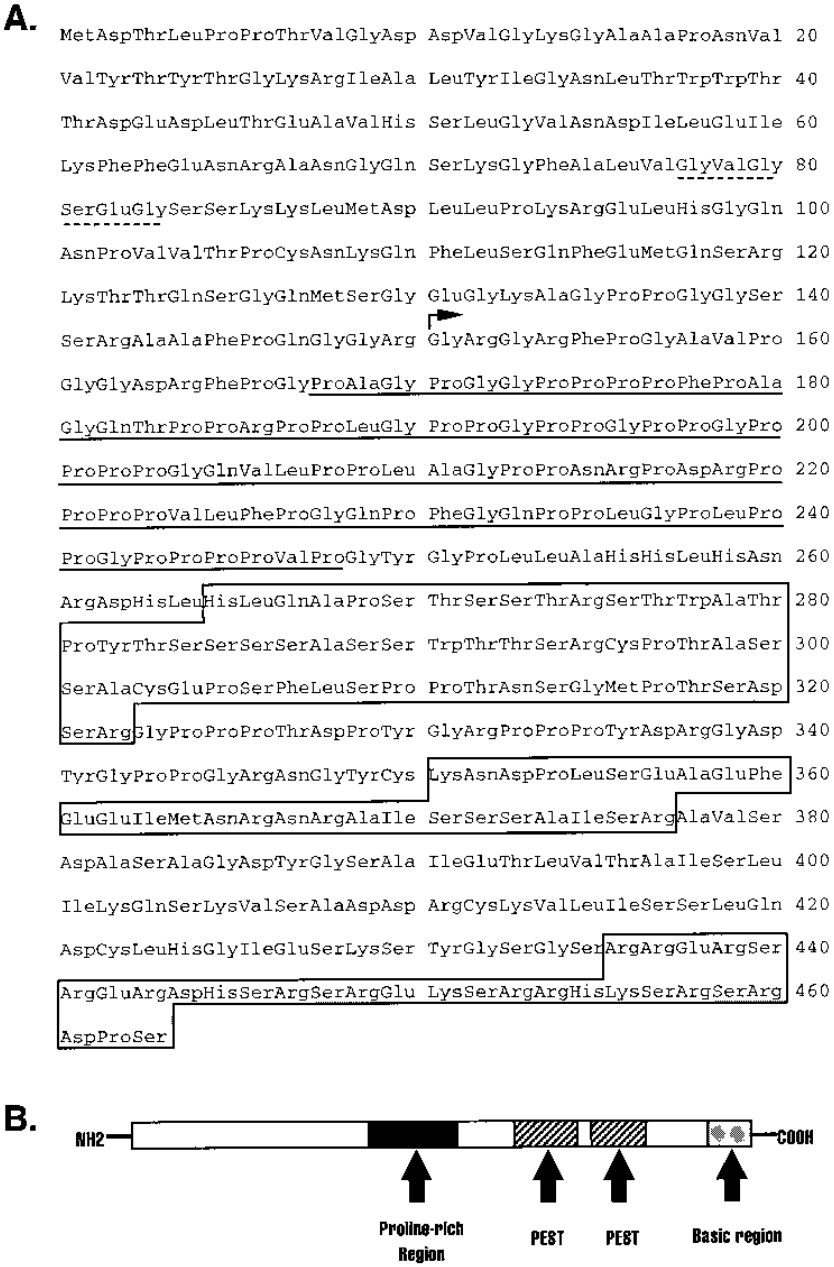

FIG. 1. (A) Amino acid sequence of full-length AAP1. A putative nucleotide binding motif (Gly Val Gly Ser Glu Gly) is encoded by residues 78 to 83 (dash underlined). A proline-rich domain is present between amino acids 168 and 248 (underlined); present within this stretch is the sequence Leu Pro Pro Gly Pro Pro Pro Pro Val Pro, which is identical at 7 of 10 positions with a sequence reported for the c-ABL SH3-binding protein BP-2 (2, 37) (identical residues are underlined). Two potential PEST domains (39) (boxed) are present between amino acids 265 and 322 and amino acids 350 and 376. The extreme COOH terminus, beginning at residue 436 and extending to the end of the protein (shaded box), is highly basic, with a calculated pI of 12.4. The GST-AAP1 fusion protein was constructed to express AAP1 sequences beginning at residue 151, which is indicated by the arrow. (B) Schematic diagram of the structural features present in AAP1.

sulted in the expression of β-galactosidase activity. The cDNA insert was used as a probe to screen a λgt11 HeLa cDNA library in order to isolate a full-length cDNA. The longest cDNA insert identified encodes a 463-amino-acid open reading frame. The frame remains open to the end of the insert, so we cannot rule out the possibility of additional coding sequences. The *aap1* gene has been found to reside in the long arm of chromosome 12 (data not shown). The amino acid sequence encoded by the cDNA clone is shown in Fig. 1A.

As expected from our screening approach, the AAP1 protein contains a highly proline-rich region. Within the proline-rich domain is the sequence Leu Pro Gly Pro Pro Pro Pro Val Pro, which is identical at seven positions to the binding sequence reported for the c-ABL SH3-binding protein 3BP-2 (37). Additional potential functional domains include two PEST domains (39) and a GXGXXG nucleotide binding motif. The protein encodes several putative phosphorylation sites; these consist of a CDC-2 kinase and four S/T-P proline-directed kinase sites (21). Searches of the databases failed to reveal any significant homologies to previously entered gene products; thus, it appears that *aap1* represents a novel gene.

To analyze the expression of *aap1*, we probed human

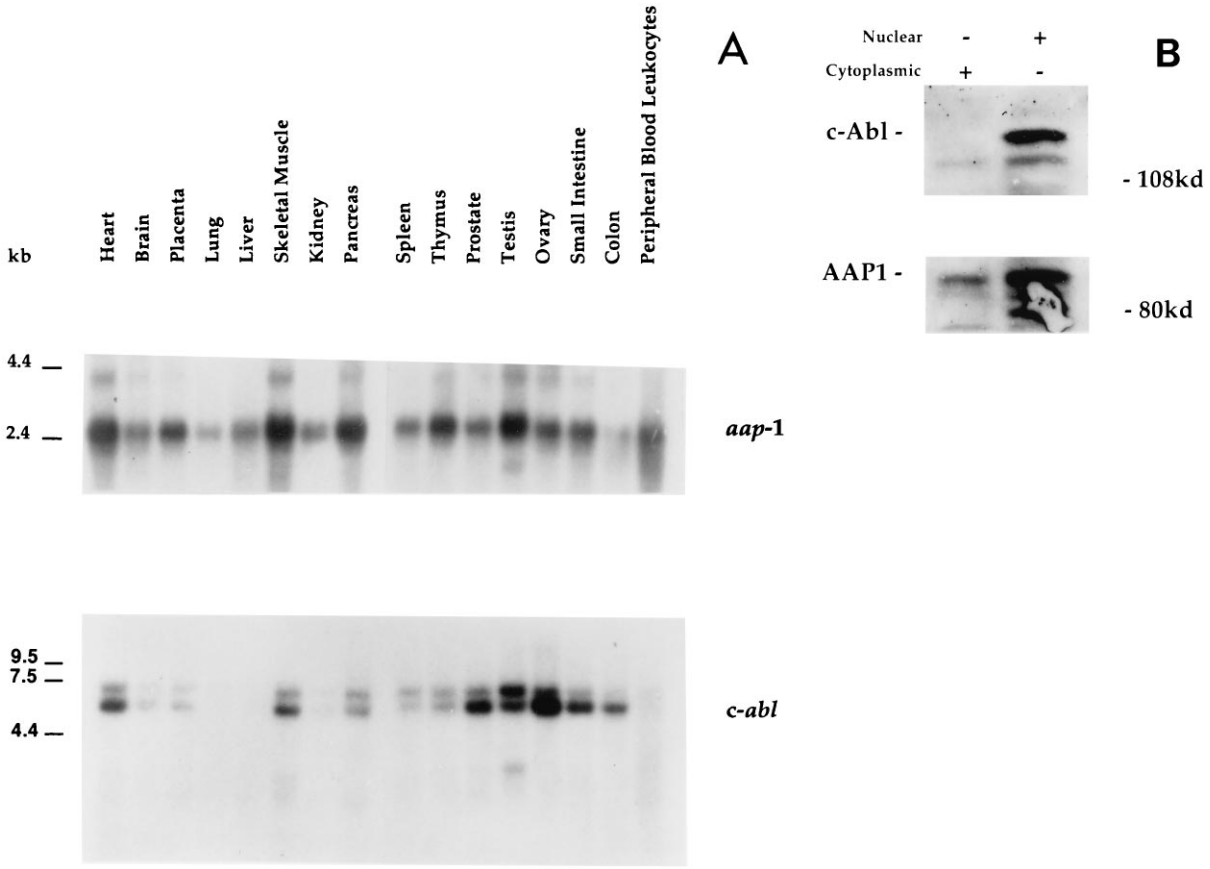

FIG. 2. *aap1* and c-*abl* mRNAs are coexpressed in normal human tissues, and the encoded proteins share a common subcellular localization within the nucleus. (A) *aap1* and c-*abl* cDNAs were ^{32}P labeled by random-primer synthesis (Life Technologies) and used to probe multiple-human-tissue Northern blots (Clontech). The level of poly(A)$^+$ RNA in each lane was confirmed by subsequent hybridization with a β-actin probe (data not shown). (B) Nuclear and subcellular fractions from 10^7 32Dcl3 murine myeloid cells were isolated by hypotonic lysis and resolved by SDS–10% PAGE. Western blots were probed with anti-ABL MAb 8E9 and an antiserum raised against GST-AAP1.

poly(A)$^+$ RNA samples with the *aap1* cDNA insert. A single 2.4-kb transcript is expressed in all the normal human tissues analyzed, with abundant levels in the placenta, kidney, pancreas, and testis. Expression closely coincided with c-*abl* mRNA expression patterns in most of tissues assayed (Fig. 2A).

Western blotting experiments using antisera raised against GST-AAP1 showed a single major reactive protein that migrated with a molecular mass of 92 kDa. To determine the subcellular localization of AAP1, we isolated nuclear and cytoplasmic fractions from the myeloid progenitor cell line 32Dcl3 and immunoprobed them with the AAP1 antisera. As shown in Fig. 2B, AAP1 is present predominantly in the nucleus, similar to c-ABL. Therefore, because both *aap1* and c-*abl* appear to be ubiquitously expressed and the encoded proteins are both present within the nucleus, this overlap in expression indicates that both gene products coexist within the same cellular compartment.

AAP1 associates with c-ABL but not BCRABL in vitro. To investigate the interaction between AAP1 and c-ABL, AAP1 was expressed as a GST fusion protein. AAP1 sequences including amino acids 151 to 463 were expressed by infection of Sf9 insect cells with recombinant baculoviruses, and GST-AAP1 was purified by affinity chromatography (Fig. 3A). To assess the specificity of the association between AAP1 and the c-ABL SH3 or SH2 domain, GST fusion proteins encoding the c-ABL SH3 and c-ABL SH3SH2 domains were purified from *Escherichia coli* and tested in a filter binding assay. The SH3 domain of murine c-FGR (48), a *src*-related tyrosine kinase, was also subcloned and expressed in bacteria as a GST fusion protein to test the specificity of AAP1 SH3 binding. Although the library screening used c-*abl* sequences encoding both SH3 and SH2 regions as bait, GST-AAP1 bound to GST-ABL SH3 alone, although stronger binding was observed in the presence of the SH2 domain. This suggests that although AAP1 binds to the SH3 domain, the SH2 domain may in fact cooperate in binding. Moreover, GST-FGR SH3 showed no binding to GST-AAP1, indicating that there is a specific association between AAP1 and the ABL SH3 domain and that AAP-1 does not associate nonspecifically with SH3 domains (Fig. 3B).

The *bcrabl* oncogene is activated by replacement of the first exon of c-*abl* with *bcr* sequences. Although the resulting gene product retains a wild-type SH3 domain, it possesses a high level of tyrosine kinase activity and transforms hematopoietic cells (29, 30). To determine whether the SH3 domain of BCRABL is capable of associating with AAP1, we performed a solution binding assay using cell lysates containing either p210 BCRABL or c-ABL protein. Cytoplasmic cell lysates prepared from p210 BCRABL-expressing murine myeloblast 32Dcl3 cells or nuclear lysates from the 32Dcl3 parental cells were incubated with either GST, GST-AAP1, or an anti-ABL MAb, 8E9. While GST-AAP1 could readily precipitate p150

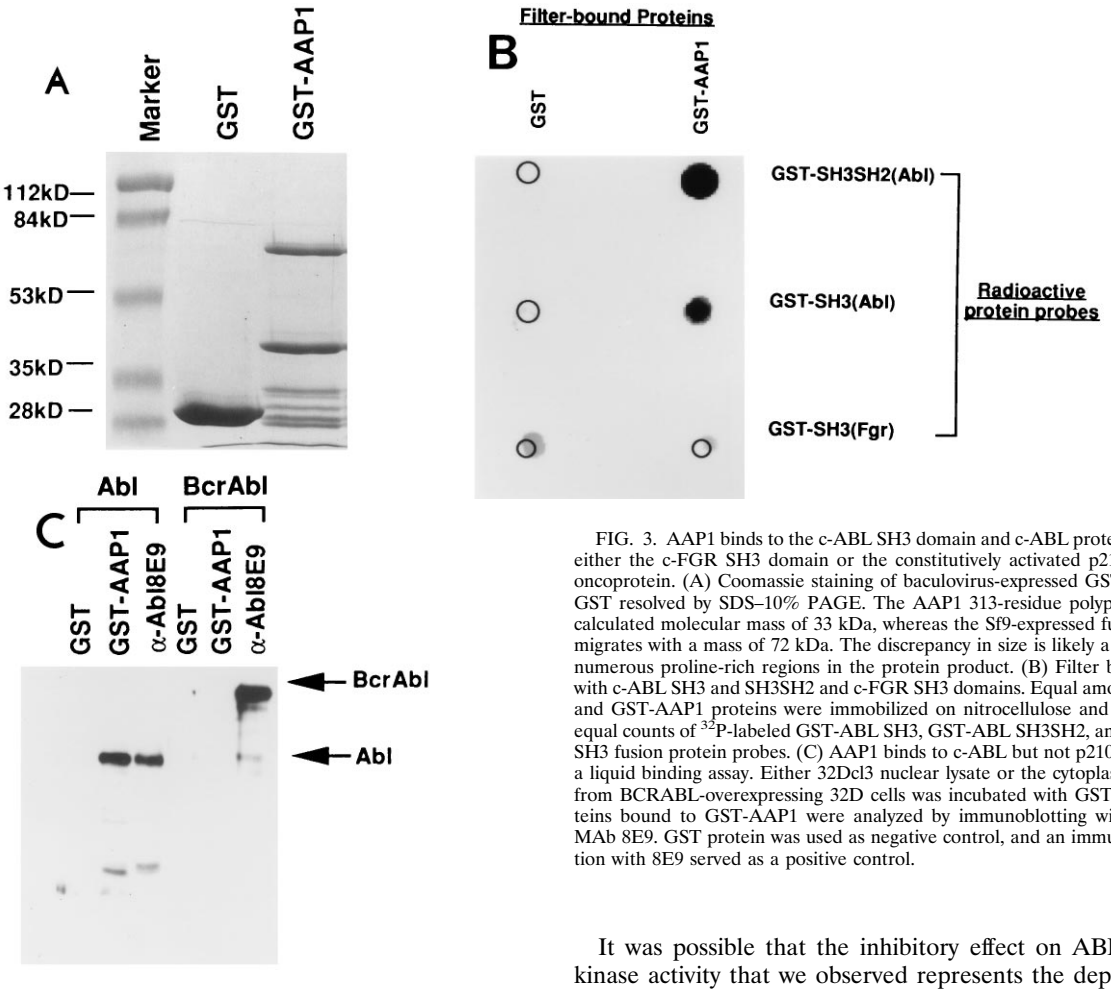

FIG. 3. AAP1 binds to the c-ABL SH3 domain and c-ABL protein but not to either the c-FGR SH3 domain or the constitutively activated p210 BCRABL oncoprotein. (A) Coomassie staining of baculovirus-expressed GST-AAP1 and GST resolved by SDS–10% PAGE. The AAP1 313-residue polypeptide has a calculated molecular mass of 33 kDa, whereas the Sf9-expressed fusion protein migrates with a mass of 72 kDa. The discrepancy in size is likely a result of the numerous proline-rich regions in the protein product. (B) Filter binding assay with c-ABL SH3 and SH3SH2 and c-FGR SH3 domains. Equal amounts of GST and GST-AAP1 proteins were immobilized on nitrocellulose and probed with equal counts of ^{32}P-labeled GST-ABL SH3, GST-ABL SH3SH2, and GST-FGR SH3 fusion protein probes. (C) AAP1 binds to c-ABL but not p210BCRABL in a liquid binding assay. Either 32Dcl3 nuclear lysate or the cytoplasmic fraction from BCRABL-overexpressing 32D cells was incubated with GST-AAP1. Proteins bound to GST-AAP1 were analyzed by immunoblotting with anti-ABL MAb 8E9. GST protein was used as negative control, and an immunoprecipitation with 8E9 served as a positive control.

c-ABL, we failed to observe any precipitated p210 BCRABL from the cell lysates (Fig. 3C). These data are consistent with the hypothesis that BCR obstructs the SH3 domain in BCRABL, possibly by the intramolecular association of BCR with the SH2 domain of c-ABL (34) and/or by the oligomerization of BCRABL proteins (28).

AAP1 specifically inhibits c-ABL kinase activity but not v-ABL. Because deletion of the SH3 domain from c-ABL is known to activate its kinase activity (12, 18) and AAP1 fails to associate with BCRABL, which encodes an activated tyrosine kinase, we speculated that AAP1 might function as a *trans* inhibitor of c-ABL tyrosine kinase activity. To test the effect of AAP1 on c-ABL tyrosine kinase activity, we isolated baculovirus-expressed c-ABL protein from Sf9 cells by immunoprecipitation using anti-ABL MAb 8E9. The kinase activity of the immunopurified c-ABL protein was assayed with GST-CRK(120-225) as a substrate. The c-CRK SH3 domain associates with ABL via a polyproline stretch in ABL, downstream of the kinase domain, and is then directly phosphorylated by ABL (10, 38). Increasing amounts of GST-AAP1 added to a kinase reaction resulted in a linear decrease in c-ABL kinase activity as quantitated by the decrease in ^{32}P phosphorylation of GST-CRK(120-225). With the addition of GST protein instead, no reduction in ABL tyrosine kinase activity was observed (Fig. 4A and B).

It was possible that the inhibitory effect on ABL tyrosine kinase activity that we observed represents the dephosphorylation of CRK due to a contaminating phosphatase activity in the GST-AAP1 protein preparation. To test this possibility, we phosphorylated GST-CRK(120-225) by incubating it with c-ABL and [γ-^{32}P]ATP in a kinase reaction. The radiolabeled ^{32}P-GST-CRK was then removed and incubated with increasing amounts of GST-AAP1. The results of this experiment indicate that a phosphatase is not responsible for the reduction of CRK phosphorylation, as only a slight decrease in GST-CRK(120-225) phosphorylation resulted during the incubation of phosphorylated GST-CRK(120-225) with GST-AAP1 (Fig. 4C).

We next sought to investigate the effect of AAP1 on c-ABL autophosphorylation, as well as substrate phosphorylation. Cos cells were transiently transfected with a c-ABL(1B) expression vector, and c-ABL was immunoprecipitated with anti-ABL MAb Ab-3, which recognizes an epitope within the COOH terminus of c-ABL. The immunopurified c-ABL protein was incubated in a kinase reaction with increasing concentrations of GST-AAP1 and 1 μg of GST-CRK, and the phosphorylated products were separated by SDS-PAGE and autoradiographed. These experiments indicated that both c-ABL autophosphorylation and substrate phosphorylation were inhibited in the presence of AAP1. Since it was possible that AAP1 was binding to CRK directly or inhibiting CRK binding to c-ABL, thereby preventing phosphorylation, we tested a second c-ABL substrate, enolase. When enolase was substituted for GST-CRK in kinase reactions, we also observed the inhibition of its phosphorylation in the presence of increasing amounts of

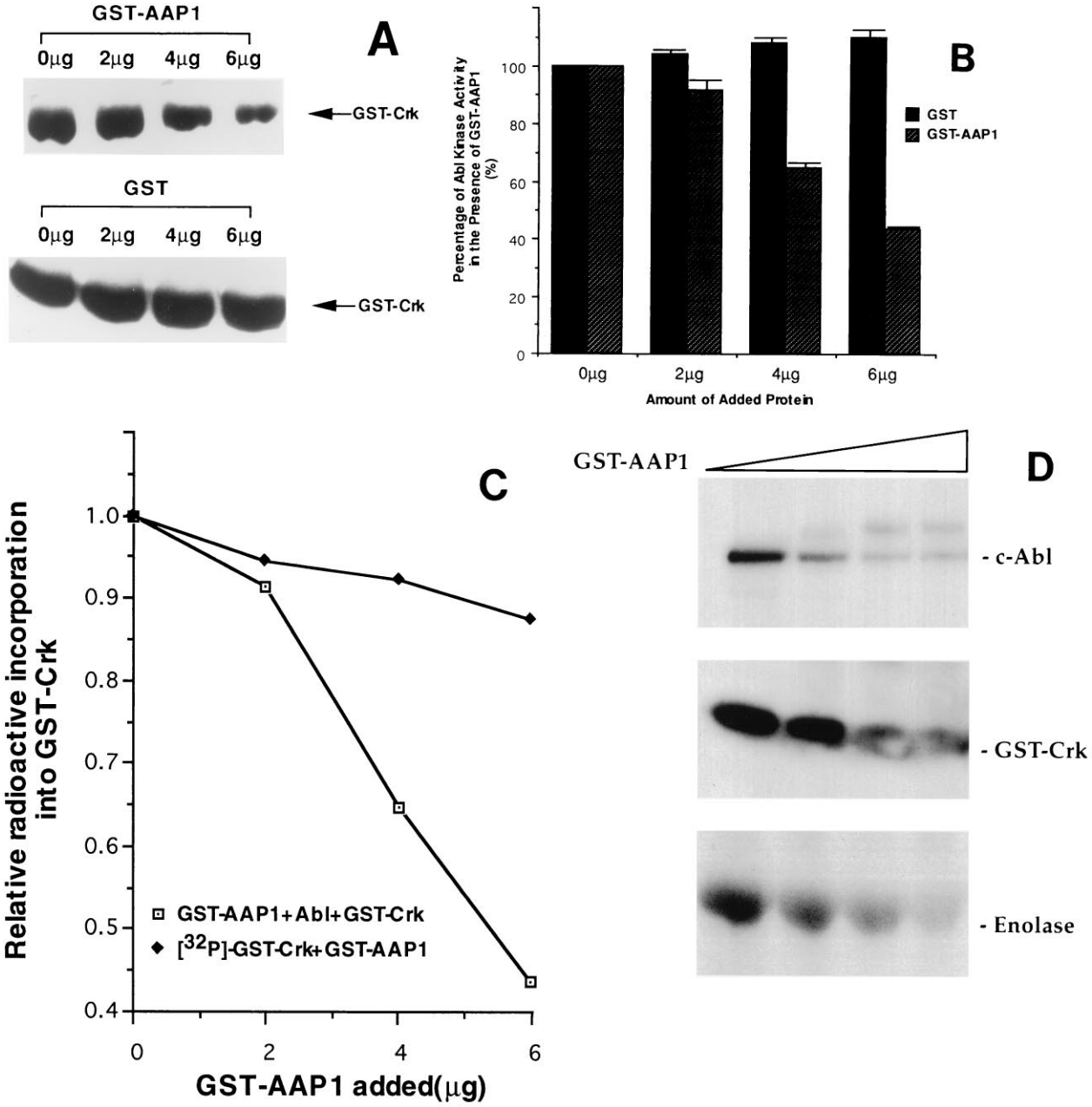

FIG. 4. Inhibition of c-ABL tyrosine kinase activity by AAP1. (A) Baculovirus-expressed c-ABL protein (~0.5 μg) was immunopurified with MAb 8E9. The c-ABL immune complex was immobilized on protein A-Sepharose (Pharmacia). GST-CRK(120-225) was added together with increasing amounts of either GST-AAP1 or GST. The reaction was initiated by the addition of [γ-^{32}P]ATP. The reaction products were separated by SDS–13% PAGE and visualized by autoradiography. Four experiments were performed in parallel, and a representative gel is shown. (B) The phosphorylation of GST-CRK(120-225) from panel A was quantitated on a PhosphorImager (Fuji). The bar graph was plotted as a percentage of the normal c-ABL kinase activity, that is, the kinase activity observed when no GST-AAP1 or GST proteins were added. The standard deviation calculated from four independent experiments is shown. (C) One microgram of GST-CRK(120-225) was added to c-ABL immune complex immobilized on protein A-Sepharose beads. [γ-^{32}P]ATP was added, and the reaction mixture was incubated at 30°C for 20 min. The supernatant containing phosphorylated GST-CRK(120-225) was transferred to fresh tubes and incubated with increasing amounts of GST-AAP1 for another 20 min at 30°C. As a positive control reaction, c-ABL immunocomplex was incubated together with GST-AAP1 and GST-CRK(120-225) for 20 min at 30°C. Reaction products were separated by SDS–13% PAGE, and the phosphorylation of GST-CRK(120-225) was quantitated on a PhosphorImager (Fuji) and plotted as a relative percentage. Three experiments were performed, and the average was plotted. (D) c-ABL(1B) was isolated from transiently transfected Cos-7 cells by immunoprecipitation with anti-ABL MAb Ab-3 (Oncogene Science). Kinase reactions were assembled with increasing amounts of GST-AAP1 (0, 2, 4, or 6 μg) and 1 μg of GST-CRK or 5 μg of acid-denatured enolase and processed as described previously. The phosphorylated proteins are indicated for each panel.

AAP1 (Fig. 4D). Together these results implicate AAP1 in the inhibition of c-ABL kinase.

The lack of AAP1 binding to the oncogenic form of c-ABL is a unique property of the *aap1*-encoded gene product. Two recently identified ABL-interacting proteins bind to both c-ABL and v-ABL, by an association between a proline-rich region in the carboxy terminus of ABL and the SH3 domain encoded by both ABI-1 and ABI-2 (7, 40). Because we failed to observe any significant association between AAP1 and oncogenically activated forms of ABL, we tested the role of SH3

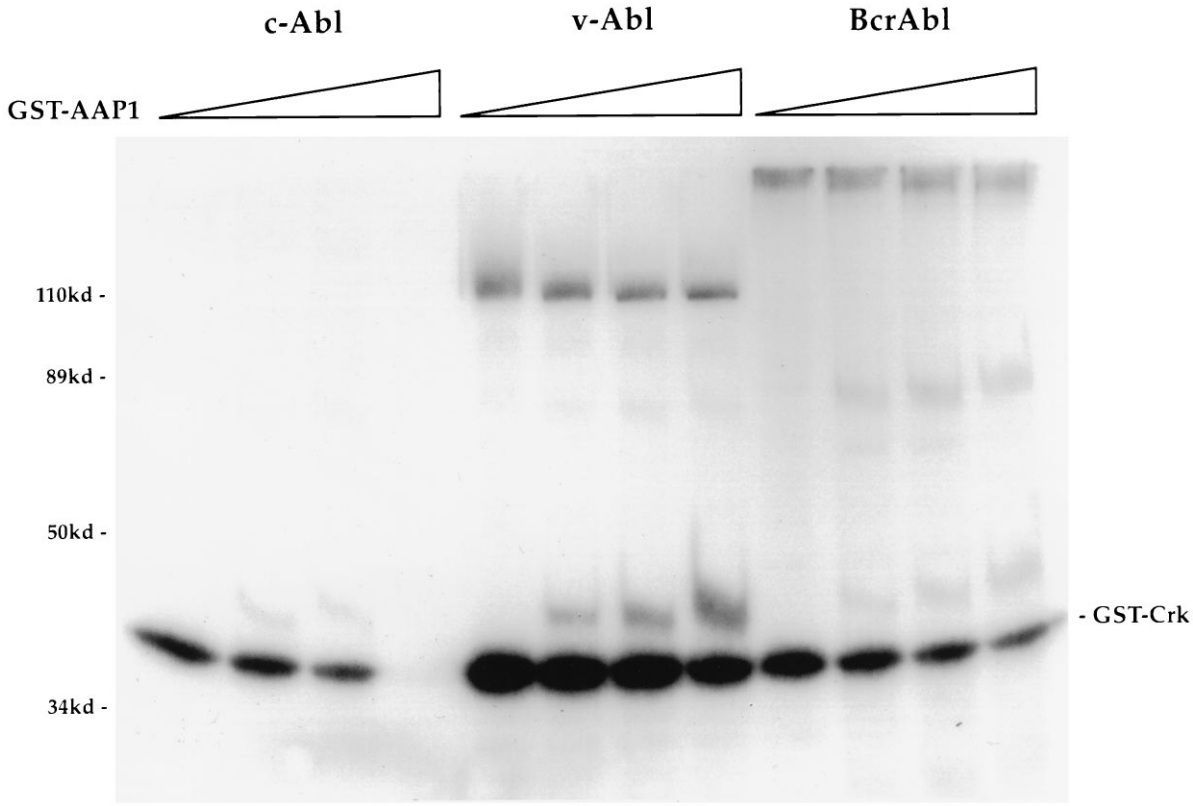

FIG. 5. Comparison of AAP1 inhibition of GST-CRK phosphorylation by c-ABL, v-ABL, and BCRABL. ABL(1B) and v-ABL p120 were isolated from transiently transfected Cos-7 cells, while BCRABL p210 was immunoprecipitated from a 32Dcl3-overexpressing cell line (49). Equivalent amounts of each ABL protein, as determined by Western blotting of total cell lysates, were immunoprecipitated with anti-ABL MAb Ab-3. Increasing amounts of GST-AAP1 (0, 2, 4, and 6 μg) were added together with 1 μg of GST-CRK(120-225) substrate to each assembled kinase reaction mixture. The positions of the GST-CRK(120-225) substrate are indicated.

binding by AAP1 upon the inhibition of ABL kinase. For these experiments, equivalent amounts of either ABL(1B), v-ABL, or BCRABL were used in kinase reactions which included increasing concentrations of GST-AAP1 (Fig. 5). In contrast to the inhibition of c-ABL phosphorylation of GST-CRK by AAP1, we did not observe inhibition of either autophosphorylation or GST-CRK phosphorylation with v-ABL, in which the SH3 domain has been deleted and replaced with a Gag polypeptide (36). Interestingly, we did note a decline in BCRABL phosphorylation of GST-CRK, although to a much lesser extent than c-ABL, suggesting that under the conditions of the kinase reaction, the SH3 domain in BCRABL gains some accessibility to AAP1 binding. In addition, we noted that AAP1 does not appear to be a substrate for either oncoprotein.

Therefore, inhibition of tyrosine kinase activity by AAP1 appears to be dependent on the ability of AAP1 to physically associate with the ABL SH3 domain, and oncogenic activation of ABL correlates directly with an altered sensitivity to AAP1 regulation.

Inhibition of c-ABL kinase activity requires association between AAP1 and the c-ABL SH3 domain. If inhibition specifically correlated with the SH3 binding ability of AAP1, we hypothesized that excess SH3 ABL polypeptide should compete for AAP1 binding and thereby block the inhibitory effect on c-ABL kinase. Kinase reactions were performed in the presence of excess amounts of competitor GST-ABL SH3 fusion protein. The inclusion of the competitor SH3 polypeptide prevented the down-regulation of c-ABL kinase activity, as predicted (Fig. 6). The c-FGR SH3 domain, which fails to associate with GST-AAP1 in a solid-phase binding assay (Fig. 3B), similarly failed to compete against the kinase inhibitory activity of AAP1 (Fig. 6). Moreover, during our yeast two-hybrid screening, another distinct c-ABL SH3-binding protein (which we have designated AAP2) was identified. When expressed as a GST fusion protein and added to the kinase reaction, GST-AAP2 failed to inhibit c-ABL kinase activity (data not shown). Therefore, the inhibition of c-ABL kinase activity is not merely due to steric inhibition resulting from attachment of the GST moiety to an SH3-binding protein. We conclude that the novel gene product AAP1 can specifically inhibit c-ABL tyrosine kinase and that this inhibition is depen-

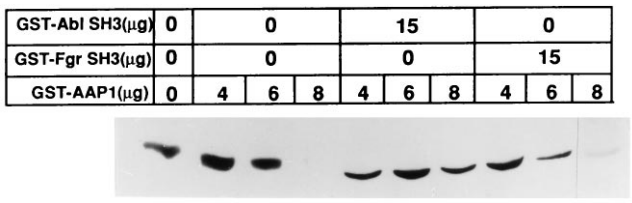

FIG. 6. Endogenous c-ABL protein from 5×10^6 human 293 cells was immunoprecipitated with anti-ABL 8E9 and immobilized on protein G-Sepharose. Increasing amounts of GST-AAP1 were added as indicated. In addition, either 15 μg of GST-ABL SH3 or 15 μg of GST-FGR SH3 was included in the kinase reaction as an excess inhibitor, where indicated. One microgram of GST-CRK(120-225) was used in all kinase reactions as the substrate. The kinase reactions proceeded for 20 min at 30°C, and the products were then separated by SDS–13% PAGE.

dent upon the association between AAP1 and the c-ABL SH3 domain.

DISCUSSION

In this study, a novel cellular protein, AAP1, was identified on the basis of its ability to associate with c-ABL SH3SH2 domains upon expression in *S. cerevisiae*. Accumulated evidence suggests that the SH3 domain of c-ABL negatively regulates c-ABL kinase function. Despite this, there has not been any cellular factor identified that fulfills such a function. Because AAP1 specifically associated with c-ABL but not the oncogenic isoforms, BCRABL and v-ABL, we tested the effect of AAP1 on c-ABL kinase activity. We found that c-ABL kinase activity and to a lesser degree BCRABL, but not v-ABL, are sensitive to down-regulation by an interaction between the c-ABL SH3 domain and AAP1. The ability of AAP1 to inhibit c-ABL substrate phosphorylation was not limited to CRK but also was effective when enolase served as the substrate. We also observed that autophosphorylation of c-ABL was inhibited by the binding of AAP1. Kinase inhibition occurred with c-ABL immunopurified with antibodies directed against two divergent domains, indicating that the presence of bound antibody on c-ABL did not influence the activity of AAP1.

Activation of ABL transforming activity has been shown to correlate not only with alterations in the SH3 domain but also with the cytoplasmic localization of the oncoprotein (43, 45). Our observation that both AAP1 and c-ABL are present within the nucleus suggests that both altered structure and localization contribute to oncogenicity by preventing regulation by AAP1.

There have been several genes identified by screening for proteins that associate with c-ABL. Ciccheti et al. screened bacteriophage expression libraries with isolated c-ABL SH3 domains and cloned several novel genes (2). While these gene products have been instrumental in identifying the polyproline-SH3 ligand interaction (37), they have not been found to be functionally related to c-ABL (3). Two novel proteins, ABI-1 (40) and ABI-2 (7), were isolated when either the COOH termini or the SH3 domain of c-ABL, respectively, was used as bait in a yeast two-hybrid screening. Studies with these gene products have provided evidence that (i) ABI-1 associates with v-ABL in vivo, is a substrate for v-ABL kinase, and can inhibit v-ABL-induced transformation of NIH 3T3 cells (40) and (ii) ABI-2 binds to both the c-ABL SH3 and COOH domains, is a substrate for c- and v-ABL, and activates the oncogenic potential of c-ABL when the ABI-2 SH3 domain is deleted (7). AAP1 function is unique compared with those of these previously described ABL-binding proteins. Unlike ABI-1 or ABI-2 (7, 40), AAP1 does not associate with v-ABL. ABI-1 and ABI-2 are substrates for ABL phosphorylation (7, 40), whereas our data indicate that AAP1 is not a substrate for either c-ABL or the oncogenically activated v-ABL or BCRABL tyrosine kinases. Furthermore, consistent with the proposed negative regulatory role of SH3, we demonstrated that AAP1 can inhibit c-ABL kinase activity through its ability to bind to the c-ABL SH3 domain. AAP1 then joins the retinoblastoma protein (46) as the only direct inhibitors of c-ABL tyrosine kinase, although the retinoblastoma protein acts independently of the SH3 domain.

Both c-CRK and v-CRK are complexed with c-ABL via a short polyproline stretch carboxy terminal to the ABL kinase domain (10, 38). The GST-CRK(120-225) fusion protein that we utilized in these studies contains the N-terminal SH3 domain, responsible for binding with the ABL protein, and Y221, the site of ABL phosphorylation (10, 38). The participation of ABL in CRK signal transduction is supported by the finding that ABL phosphorylates CRK in vivo (10, 38). It was recently shown that association between c-CRK and c-ABL enhanced the ability of c-ABL to hyperphosphorylate $p130^{CAS}$ (27). Therefore, one important function of CRK may be complexing additional substrates to c-ABL, facilitating their phosphorylation. We anticipate that AAP1 may inhibit the phosphorylation of additional c-ABL kinase substrates brought in contact by CRK, although this remains to be tested.

The inhibitory mechanism of AAP1 is presently unclear. The mechanism for the inhibition of both protein kinase A and MLCK may provide some insights. Protein kinase inhibitor inhibits protein kinase activity by masking the active site (5, 6, 47). Studies with MLCK similarly indicated that autoinhibitory C-terminal sequences extended over and essentially transverse the entire substrate-binding groove (17). As the polyproline stretch in AAP1 stabilizes its complex with ABL, another structural region of AAP1 could extend over and block substrate binding by c-ABL tyrosine kinase. This model is consistent with mutational experiments showing that the negative regulatory function of the ABL SH3 domain depends on its position relative to the kinase domain (26). One region that could participate in c-ABL kinase inhibition is the basic C terminus of AAP1. It has been proposed that a basic motif within MLCK binds to acidic residues in the catalytic core, resulting in autoinhibition (23). The lack of c-ABL kinase inhibition observed with another newly identified ABL SH3-binding protein having a different C-terminal structure further supports this possibility (data not shown).

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