with dithiothreitol (DTT) (1 mM), phenylmethylsulfonyl fluoride (1 mM), aprotinin (0.15 unit/ml), and sodium orthovanadate (1 mM). Lysates were stored at −80°C. Immunoprecipitations were done as described (*23*), except that $LiCl_2$ washes were omitted.
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20. The anti-Raf immunoprecipitates were prepared as described (*16*) and washed one more time with kinase buffer [25 mM Hepes (pH 7.4), 1 mM DTT, 10 mM $MgCl_2$, and 10 mM $MnCl_2$] before assay. For kinase reactions, washed immunoprecipitates were incubated in 40 μl of kinase buffer containing 15 μM nonradioactive adenosine triphosphate (ATP), 10 μCi (370 kilobecquerels) of [γ-^{32}P]ATP (3000 Ci/mmol), and 0.2 μg of 5′-*p*-fluorosulfonyl-bezoyladenosine–treated MEK-1 at room temperature for 30 min in the presence or absence of purified 14-3-3 protein or 14-3-3 protein that had been boiled in kinase buffer for 4 min. The assays were terminated by addition of Laemmli sample buffer and resolved by SDS–polyacrylamide gel electrophoresis. The phosphorylation of MEK-1 was quantitated by Phosphorimager, Molecular Dynamics, Sunnyvale, CA, and the amount of Raf-1 protein in each lane was quantitated by a protein immunoblot and Fluroimager, Molecular Dynamics, Sunnyvale, CA.
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Association of the Protein Kinases c-Bcr and Bcr-Abl with Proteins of the 14-3-3 Family

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In this study, a protein that interacts with sequences encoded by the first exon of the protein kinase Bcr was cloned. The Bcr-associated protein 1 (Bap-1) is a member of the 14-3-3 family of proteins. Bap-1 interacts with full-length c-Bcr and with the chimeric Bcr-Abl tyrosine kinase of Philadelphia chromosome (Ph^1)–positive human leukemias. Bap-1 is a substrate for the Bcr serine-threonine kinase and is also phosphorylated on tyrosine by Bcr-Abl but not by c-Abl. Bap-1 may function in the regulation of c-Bcr and may contribute to the transforming activity of Bcr-Abl in vivo. 14-3-3 proteins are essential for cell proliferation and have a role in determining the timing of mitosis in yeast. Through direct binding to sequences present in Bcr and in other proteins implicated in signaling, the mammalian 14-3-3 proteins may link specific signaling protein components to mitogenic and cell-cycle control pathways.

The product of the *bcr* gene is a 160-kD protein with multiple functional and structural domains. Among the functional domains ascribed to c-Bcr are a serine-threonine kinase encoded by the NH_2-terminal first exon sequences (*1*) and a COOH-terminal domain-encoded guanosine triphosphatase (GTPase)–activating function for the Rac GTP-binding protein (*2*). Several structural domains have been identified in c-Bcr, including an oligomerization domain (*3*), a region that binds Src homology 2 (SH2) domains in a phosphotyrosine-independent manner (*4*), a region of sequence similarity to guanine nucleotide exchange factors for the Rho family of GTP-binding proteins (*5*), a calcium-dependent lipid binding (Calb) domain, and a pleckstrin homology domain (*6*). Though the presence of these distinct biochemical and structural properties in c-Bcr suggests that this protein may function as a point of cross-talk among multiple intracellular signaling pathways, little is known about its biological mechanism of action.

The *bcr* gene was first discovered because of its involvement in Ph^1-positive leukemias. Ph^1 is produced by a reciprocal translocation event between chromosomes 9 and 22. The translocation fuses the *bcr* gene upstream of the second exon of the *c-abl* proto-oncogene (*7*). Two alternative Bcr-Abl chimeric proteins are produced, P210 and P185, which are associated with chronic myelogenous and acute lymphocytic leukemias, respectively (*8*). Sequences within the first exon of Bcr are essential for the transforming activity of Bcr-Abl (*9*). A tyrosine (Tyr^{177}) within the Bcr first exon becomes phosphorylated in the activated Bcr-Abl oncoproteins and serves as a binding site for the SH2 domain of the Grb2 adaptor protein (*10*). A mutant of Bcr-Abl that lacks Tyr^{177} is defective in transformation (*10*). The first exon sequences of Bcr have the potential to interact with cellular proteins in phosphotyrosine-dependent and phosphotyrosine-independent manners. Together, these interactions may contribute to the activation of the tyrosine kinase and the transforming activities of Abl in the Bcr-Abl chimera (*4*, *10*).

To understand the mechanism or mechanisms whereby the first exon of Bcr activates the transforming activity of Abl in the Bcr-Abl chimera and to gain insight into the normal functions of c-Bcr, we sought to identify cellular proteins that bind directly to this region of Bcr in a phosphotyrosine-independent manner. A λEXlox mouse embryo library was screened with amino acid sequences encoded by the first exon of Bcr (amino acids 1 to 413). The Bcr sequences were fused downstream of glutathione-S-transferase (GST) and the GST-Bcr (1–413) fusion was then cloned into a baculovirus vector for expression in insect cells. Sf9 insect cells were infected with the purified recombinant GST-Bcr (1–413) baculovirus. After cell lysis, the fusion protein was purified on glutathione Sepharose beads. The GST-Bcr (1–413) protein was labeled with ^{32}P by its intrinsic kinase activity (*1*). A single phosphorylated protein band was detected after gel electrophoresis and autoradiography. The phosphorylated protein was recognized by antibodies to Bcr. The labeled GST-Bcr protein was used as a probe to screen a complementary DNA (cDNA) library from a 16-day mouse embryo (*11*). Seven independent phage clones were isolated. There were two sets of identical clones, of five and two clones, respectively, that overlapped in sequence. Northern (RNA) blot analysis revealed the presence of two closely migrating transcripts of about 1.9 and 2.2 kb in all murine and human tissues examined (*12*). Analysis of the DNA sequence corresponding to the longest insert (1.9 kb) revealed that the cDNA encoded a member of a large family of proteins. The protein was named Bcr-associated protein 1 (Bap-1) and is identical in sequence (except for a single aspartic acid to glutamic acid substitution) to a member of the 14-3-3 family of proteins isolated from human T cells (*13*). The 14-

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3-3 proteins are expressed in all mammalian tissues that have been examined and are widely conserved in other eukaryotic organisms, including plants, insects, amphibians, and yeast (*14*). Multiple activities have been ascribed to various isoforms of the 14-3-3 family of proteins (*14*, *15*). A 14-3-3 protein is required as a cofactor for the adenosine 5′-diphosphate (ADP)–ribosyl transferase activity of the *Pseudomonas aeruginosa* toxin exoenzyme S (*16*). Thus, the 14-3-3 proteins may function in the regulation of multiple signal transduction processes that are important in growth regulation, development, and cell-cycle control.

To examine whether full-length c-Bcr could interact with Bap-1 in solution, we did in vitro binding assays with the full-length Bap-1 protein fused downstream of GST (GST–Bap-1) (*17*). The fusion protein was expressed in bacteria and affinity-purified on glutathione Sepharose beads. The c-Bcr protein produced in baculovirus-infected Sf9 insect cells bound to GST–Bap-1 but not to GST alone (Fig. 1A). The Bcr-Abl tyrosine kinase also bound to GST–Bap-1 in this assay (Fig. 1A). We examined whether c-Bcr and Bcr-Abl could be found in a complex with Bap-1 in mammalian cell lysates. After transfection of *c-bcr* and *bcr-abl* cDNAs into human 293 cells, lysates were prepared and incubated with control antibodies or with antibodies to Bap-1 (*18*) (Fig. 1B). The c-Bcr and Bcr-Abl proteins were immunoprecipitated with the endogenous Bap-1 protein (Fig. 1B). Co-immunoprecipitation of Bcr-Abl with endogenous Bap-1 was also observed in Rat1 cells expressing Bcr-Abl (*19*). Thus, full-length c-Bcr and Bcr-Abl proteins interact effectively with Bap-1.

To determine whether Bap-1 could serve as a substrate for the c-Bcr and Bcr-Abl kinases, we purified Bap-1 from bacterial cell lysates by affinity chromatography of the GST–Bap-1 fusion protein on glutathione Sepharose beads. The GST–Bap-1 protein was then subjected to proteolytic cleavage with Factor Xa to release Bap-1 from GST. The purified soluble Bap-1 protein was then incubated with c-Bcr, c-Abl, or P185 Bcr-Abl that had been immunoprecipitated with specific antisera from lysates of baculovirus-infected Sf9 cells. Purified recombinant Bap-1 was phosphorylated by c-Bcr (Fig. 2A). Phosphoamino acid analysis revealed that c-Bcr phosphorylated Bap-1 exclusively on serine (Fig. 2B). Bap-1 was also phosphorylated by Bcr-Abl but not by c-Abl (Fig. 2A). The Bcr-Abl chimera phosphorylated Bap-1 on both serine and tyrosine residues (Fig. 2B). To further examine whether Bap-1 could be phosphorylated by Bcr-Abl after immunoprecipitation of the proteins from mammalian cells, Bap-1 was introduced into Rat1 cells that stably expressed the P210 Bcr-Abl protein tyrosine kinase. An influenza hemagglutinin (HA) tag was placed in frame at the NH_2-terminus of Bap-1 to facilitate detection (*17*). Protein immunoblotting with an antibody to HA confirmed the presence of the HA–Bap-1 fusion protein in several independent clonal cell lines (*19*). Lysates were prepared and incubated with normal rabbit serum or with antibodies to Bap-1 or Abl (Fig. 2C). The immunoprecipitates were washed and subjected to in vitro kinase assays. Analysis of the phosphorylated proteins by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the P210 Bcr-Abl tyrosine kinase co-immunoprecipitated with the antibodies to Bap-1 and that, similarly, the tagged Bap-1 protein was found in the Abl immunoprecipitates (Fig. 2C). Furthermore, the tagged Bap-1 was phosphorylated in the immunoprecipitates after incubation of the lysates with antibodies to Bap-1 (anti–Bap-1) and antibodies to Abl (anti-Abl) (Fig. 2C). To confirm that the 30-kD protein observed in the immunoprecipitates was HA–Bap-1, we re-immunoprecipitated the phosphorylated proteins with antibodies to Bap-1 or with a control antiserum. HA–Bap-1 was recognized by the antibodies to Bap-1 but not by the control antiserum (*19*). These data indicate that Bap-1 forms a stable complex with Bcr-Abl and is effectively phosphorylated in the complex.

To determine whether Bap-1 is an in vivo as well as in vitro substrate of c-Bcr and Bcr-Abl, we subjected the phosphorylated Bap-1 protein to two-dimensional tryptic phosphopeptide analysis. Phosphopeptide maps of Bap-1 phosphorylated by c-Bcr in vitro revealed the presence of four major sites of phosphorylation (Fig. 2D). Bcr-Abl phosphorylated Bap-1 at these same four sites and at three additional major sites (phosphopeptides 5, 6, and 7) and at two minor sites (phosphopeptides 8 and 9) (Fig. 2D). To distinguish phosphorylated Bap-1 from other phosphorylated 14-3-3 isoforms in intact cells, we derived several Rat1 and Rat1–Bcr-Abl cell lines that stably expressed an HA-tagged Bap-1 protein. Cell lines were labeled with ^{32}P-orthophosphate, lysates were prepared, and the HA–Bap-1 protein was immunoprecipitated with an antibody to the HA epitope tag. Tryptic phosphopeptide maps of Bap-1 phosphorylated in vivo in Rat1 cells revealed the presence of five major phosphopeptides (Fig. 2D). Two-dimensional analysis of a mix of tryptic phosphopeptides of Bap-1 phosphorylated in vitro by c-Bcr and phosphorylated in vivo in Rat1 cells indicated that four of the five phosphopeptides co-migrated with those phosphorylated by the Bcr kinase (Fig. 2D). Bap-1 was phosphorylated in Rat1 cells expressing P210 Bcr-Abl at five major sites (Fig. 2D). Phosphopeptides 1 to 4 are those phosphorylated by the Bcr kinase. The fifth phosphopeptide co-migrated with one of the additional sites obtained after in vitro phosphorylation with Bcr-Abl and was not phosphorylated by the Bcr kinase. This site may be phosphorylated by the tyrosine kinase activity of Bcr-Abl. Because this phosphopeptide is observed in metabolically labeled Bap-1 isolated from both Rat1 and Rat1–Bcr-Abl cells, it is likely that a tyrosine kinase distinct from Bcr-Abl may phosphorylate this site in vivo. A low level

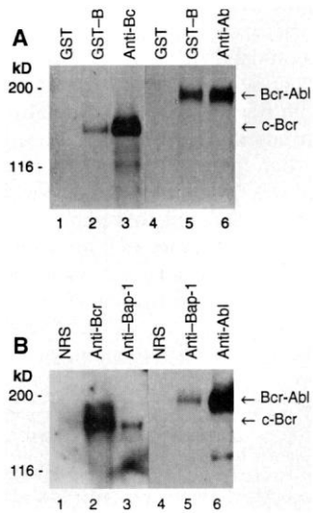

Fig. 1. Interaction of c-Bcr and Bcr-Abl with Bap-1. (**A**) c-Bcr and Bcr-Abl were expressed in Sf9 insect cells (*4*). Three days after infection, the cells were labeled with ^{35}S-labeled amino acids (*4*). Lysates from cells expressing c-Bcr (lanes 1 to 3) and Bcr-Abl (lanes 4 to 6) proteins were incubated with GST (lanes 1 and 4), GST–Bap-1 (lanes 2 and 5), and antibodies to Bcr (lane 3) or to Abl (lane 6). The GST and GST–Bap-1 proteins were bound to glutathione Sepharose beads. Antibody complexes were collected on protein A–Sepharose beads. After a 2-hour incubation period, protein complexes bound to beads were washed extensively with buffer containing SDS and deoxycholate (*4*). Bound proteins were eluted in sample buffer and analyzed by SDS-PAGE and fluorography. (**B**) Immunoprecipitation of c-Bcr and Bcr-Abl with endogenous Bap-1. The *c-bcr* gene cloned into pCMV-5 (lanes 1 to 3) and the *bcr-abl* gene cloned into the pSRα vector (lanes 4 to 5) were transfected into human 293 cells. Two days after transfection, the cells were lysed and the lysates were incubated with normal rabbit sera (NRS) (lanes 1 and 4) and with antibodies to Bap-1 (lanes 3 and 5), Bcr (lane 2), or Abl (lane 6). Immunoprecipitates were washed, and the bound proteins were eluted in sample buffer and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose filters, and the filters were incubated with rabbit polyclonal antibodies to Bcr (lanes 1 to 3) or mouse monoclonal antibody to Abl (lanes 4 to 6). The c-Bcr protein was detected by incubation with ^{125}I-labeled protein A and autoradiography. The Bcr-Abl protein was visualized by incubation of the filters with goat antibodies to mouse immunoglobin G conjugated to horseradish peroxidase, followed by enhanced chemiluminescence detection (Amersham) and autoradiography.

of tyrosine phosphorylation is detected on Bap-1 isolated from Rat1 and Rat1–Bcr-Abl cells by immunoblotting with antibodies to phosphotyrosine (*19*). These results identify Bap-1 as the first in vivo substrate of the unique Bcr serine kinase. Bcr phosphorylates Bap-1 at four major sites in the context of full-length c-Bcr and in the Bcr-Abl chimera. Our data suggest that Bcr is the principal protein kinase that phosphorylates Bap-1 in Rat1 fibroblasts.

Polyomavirus middle tumor antigen (MT) binds to several subspecies of the 14-3-3 family of proteins (*20*). 14-3-3 proteins bind to the Raf-1 protein kinase (*21*). Raf-1 functions as an essential component in signaling pathways that are critical for the transmission of mitogenic and developmental stimuli (*22*). The 14-3-3 proteins that associate with MT and Raf-1 serve as cofactors for the ADP-ribosylation activity of the toxin exoenzyme S (*20*, *21*). The amino acid sequence of the 14-3-3 ζ isoform that binds to Raf-1 and MT is 80% identical to that of Bap-1. We compared the biochemical properties of Bap-1 with those of 14-3-3 ζ. Bap-1 could effectively replace 14-3-3 ζ as an essential cofactor for the ADP-ribosylation activity of exoenzyme S (Fig. 3A). To examine whether 14-3-3 ζ could also bind to c-Bcr and Bcr-Abl, we conducted an in vitro binding assay with GST fusion proteins of Bap-1 and 14-3-3 ζ. The 14-3-3 ζ isoform bound to both c-Bcr and Bcr-Abl in amounts that were comparable to those obtained with Bap-1 (Fig. 3B). The 14-3-3 ζ protein was a poor substrate for the c-Bcr and Bcr-Abl kinases, and the amount of ^{32}P incorporated into 14-3-3 ζ was one-fifteenth of that incorporated into Bap-1 (Fig. 3C). There are four serines in Bap-1 that are not present in 14-3-3 ζ. One or more of these serines in Bap-1 may be phosphorylated by the c-Bcr kinase. These results indicate that, despite their similarities, there may be functional differences among isoforms of the 14-3-3 family of proteins.

To address the biological role of 14-3-3 binding to c-Bcr and Bcr-Abl, it was important to localize the region in Bcr that was implicated in binding to Bap-1 and to other 14-3-3 family members. We used in vitro transcription and translation to generate a number of deletion mutants of Bcr that were tested for binding to Bap-1. A Bcr deletion that removes the sequences downstream of amino acid 297 showed a decrease in binding to GST–Bap-1 (Fig. 4). Sequences downstream of amino acid 297 in the first exon of Bcr contain a cysteine-rich region and are very rich in serine and threonine residues (*1*, *4*). The paired cysteine residues in this region are essential for the Bcr phosphotransferase activity (*1*). The serine- and threonine-rich sequences deleted in Bcr (Δ298–1271) constitute one of two regions that have been implicated in binding to the SH2 domain of Abl (*4*). The sequences encoded by the serine- and threonine-rich region in Bcr are 32% identical over an 87–amino acid stretch to the conserved region 2 (CR2) of the Raf-1 protein kinase (*21*, *22*). There are 17 serine and threonine residues that are conserved between Bcr and Raf-1 in this region. Moreover, a cysteine-rich region similar to that found in the first exon of Bcr is also present

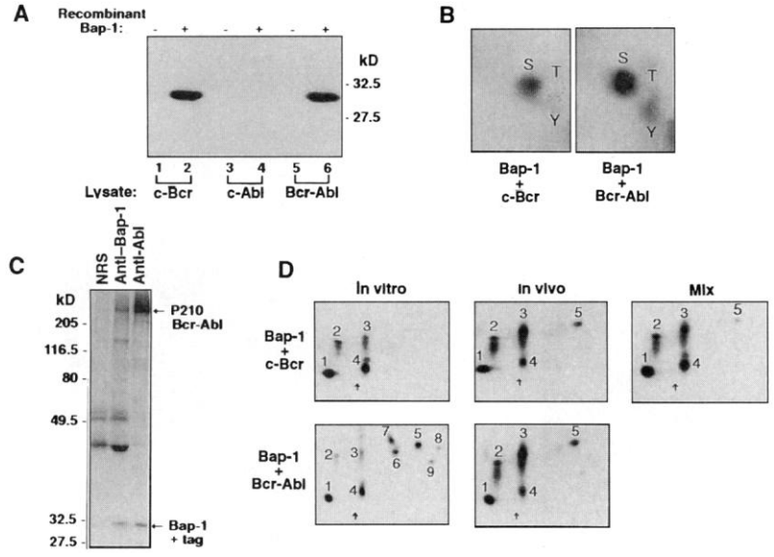

Fig. 2. Phosphorylation of Bap-1 by c-Bcr and Bcr-Abl. (**A**) Phosphorylation of purified Bap-1 by c-Bcr and Bcr-Abl. Lysates of Sf9 cells expressing c-Bcr, c-Abl, or P185 Bcr-Abl were incubated with specific antisera. The immunoprecipitates were washed extensively and processed for in vitro kinase reactions in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of purified recombinant Bap-1. Phosphorylation reactions were done in the presence of [γ-^{32}P]ATP and $MnCl_2$ for 30 min at 30°C. At the completion of the reactions, proteins from a portion of each sample were immunoprecipitated with antiserum to Bap-1 and analyzed by SDS-PAGE followed by autoradiography. (**B**) Phosphoamino acid analysis of the phosphorylated proteins in (A) was done after excision of the proteins from the gel. The positions of unlabeled phosphoamino acid standards are indicated: S, serine; T, threonine; and Y, tyrosine. (**C**) Phosphorylation of HA-tagged Bap-1 immunoprecipitated with antibodies to Bap-1 or Abl. Rat1 cells stably expressing P210 Bcr-Abl were transfected with *bap-1* cDNA cloned into the pCGN expression vector (*17*). An HA tag was fused in frame at the NH_2-terminus of the *bap-1* gene. The pCGN vector contains the gene that confers resistance to hygromycin. After hygromycin selection, several lines were derived that expressed both P210 Bcr-Abl and Bap-1. Cells were lysed, and the lysate was incubated with antibodies to Bap-1, antibodies to Abl, or NRS. The immunoprecipitates were washed and subjected to in vitro kinase reactions. Phosphorylated proteins were eluted in sample buffer and analyzed by SDS-PAGE and autoradiography. (**D**) Tryptic phosphopeptide maps of Bap-1 phosphorylated in vitro and in vivo. In vitro phosphorylated Bap-1 was prepared as described in (A). In vivo phosphorylated Bap-1 was prepared by labeling Rat1 or Rat1–P210 Bcr-Abl cells that stably expressed HA-tagged Bap-1 with ^{32}P-orthophosphate (1 mCi/ml) for 6 hours. Cell lysates were prepared, and the HA-tagged Bap-1 was immunoprecipitated with antibody to HA (Boehringer Mannheim). Phosphorylated Bap-1 protein was subjected to two-dimensional mapping (*30*). Tryptic phosphopeptide maps of Bap-1 phosphorylated by c-Bcr in vitro and metabolically labeled Bap-1 immunoprecipitated from Rat1/HA–Bap-1 cells are shown in the upper panels. Equal numbers of counts of ^{32}P-labeled tryptic phosphopeptides from in vitro– and in vivo–labeled Bap-1 were mixed and analyzed by two-dimensional mapping (right upper panel). Tryptic phosphopeptide maps of Bap-1 phosphorylated in vitro by Bcr-Abl and in vivo in Rat1–P210 Bcr-Abl plus HA–Bap-1 cells are shown in the lower panels. The origin is indicated by the arrow. Phosphopeptides are numbered.

Table 1. Differential rescue of P185 Bcr-Abl mutants with c-Myc. Rat1 cells or Rat1 cells stably expressing c-Myc (Rat1-Myc) were infected with helper-free retroviruses encoding for wild-type or mutant forms of P185 Bcr-Abl (*10*, *23*). Equivalence of viral stock titers after fibroblast infection was determined by immunoblot analysis of total lysates. Soft agar assays were done as previously described (*10*, *25*). Cells were plated in agar 3 days after infection with the helper-free virus stocks. The data represent the average number of colonies observed in three independent experiments. Samples were plated in duplicate in each experiment. Macroscopic colonies (>0.4 mm) were counted 14 days after plating of the cells in agar.

Retroviruses	Rat1 (no. of colonies per 10^5 cells)	Rat1-Myc (no. of colonies per 10^5 cells)
P185 wild-type	37	570
P185 (Y177F)	0	170
P185 (Δ176–426)	0	0
Vector	0	0

in the conserved region 1 (CR1) of the Raf-1 kinase (*21*, *22*). Like Bcr and Raf-1, polyoma virus MT contains cysteine- and serine-rich regions (*20*). The cysteine- and serine-rich regions in Bcr are primary sites for interaction with 14-3-3 proteins. However, additional sequences upstream of amino acid 297 in Bcr are likely to be important in binding to Bap-1 and other 14-3-3 proteins. Longer exposure of the gel shown in Fig. 4 revealed lower but detectable amounts of binding to Bap-1 by the Bcr fragments. A gradual decrease in binding activity was observed with processive truncations of Bcr. Among the Bcr deletion mutants examined, the least amount of Bap-1 binding was associated with Bcr (Δ160–1271) (*19*).

We examined the potential contribution of 14-3-3 binding to the oncogenicity of Bcr-Abl. Sequences downstream of amino acid 160 in the first exon of Bcr have been implicated in the phosphotyrosine-independent binding to SH2 domains in vitro (*4*) and in the binding to the Grb2 adaptor protein by means of the phosphorylated Tyr^{177} in vitro and in vivo (*10*). Deletion of amino acids 176 to 426 or mutation of Tyr^{177} to phenylalanine greatly diminish the transforming activity of Bcr-Abl (*4*, *10*). To examine whether differences existed in the oncogenicity of the Bcr-Abl (Δ176–426) and the Bcr-Abl $Tyr^{177} \rightarrow Phe^{177}$ (Y177F) mutant proteins, we used a Myc complementation assay. Overexpression of c-Myc protein has been shown to restore the transforming activity of several Bcr-Abl mutants to varying degrees (*23*, *24*). These studies, together with the finding that the c-Myc protein synergizes with wild-type Bcr-Abl in the transformation of fibroblasts and hematopoietic cells (*25*), suggest that c-Myc is a downstream component in the Bcr-Abl signaling pathway. Expression of P185 (Y177F) in Rat1 fibroblasts that stably overexpress c-Myc resulted in partial rescue of the transforming activity of this Bcr-Abl mutant (Table 1). The P185 (Y177F) mutant is approximately one-third as transforming as the wild-type P185 in this assay. This result was reproducibly observed with several Rat1-Myc cell lines. In contrast, the transforming activity of the P185 (Δ176–426) deletion mutant was not rescued by Myc overexpression (Table 1), which is in agreement with previous results (9). The P185 (Δ176–426) mutant lacks the Grb2 binding site and the two serine-rich regions and the cysteine-rich re-

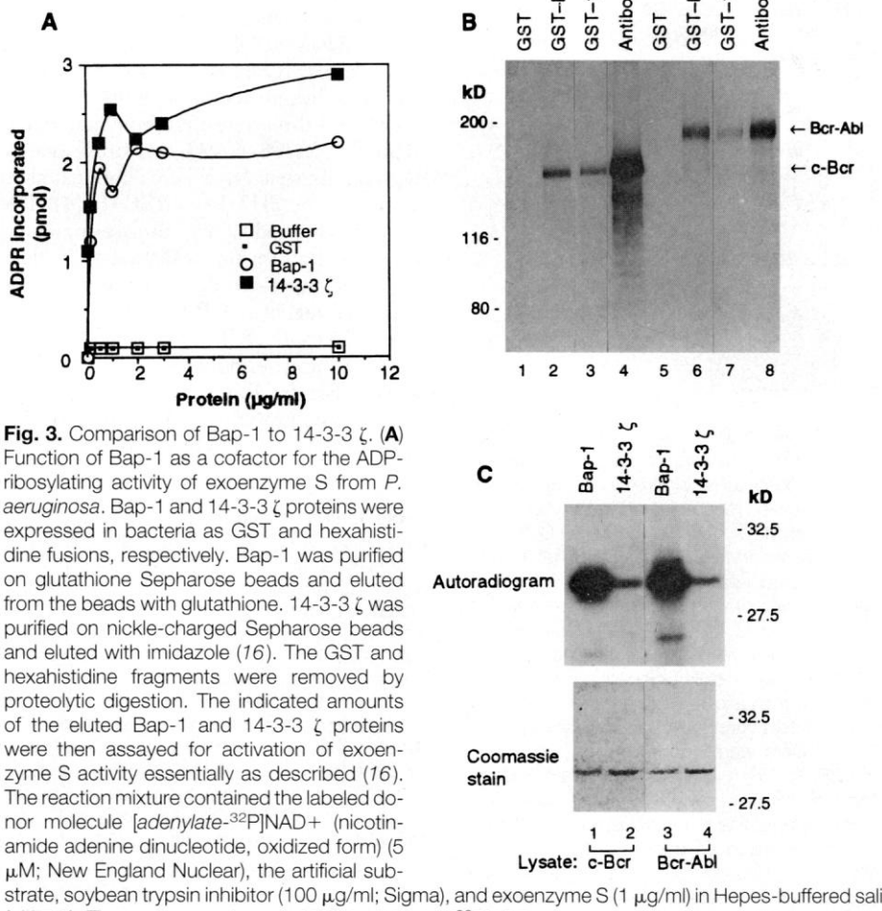

Fig. 3. Comparison of Bap-1 to 14-3-3 ζ. (**A**) Function of Bap-1 as a cofactor for the ADP-ribosylating activity of exoenzyme S from *P. aeruginosa*. Bap-1 and 14-3-3 ζ proteins were expressed in bacteria as GST and hexahistidine fusions, respectively. Bap-1 was purified on glutathione Sepharose beads and eluted from the beads with glutathione. 14-3-3 ζ was purified on nickle-charged Sepharose beads and eluted with imidazole (*16*). The GST and hexahistidine fragments were removed by proteolytic digestion. The indicated amounts of the eluted Bap-1 and 14-3-3 ζ proteins were then assayed for activation of exoenzyme S activity essentially as described (*16*). The reaction mixture contained the labeled donor molecule [*adenylate*-^{32}P]NAD+ (nicotinamide adenine dinucleotide, oxidized form) (5 μM; New England Nuclear), the artificial substrate, soybean trypsin inhibitor (100 μg/ml; Sigma), and exoenzyme S (1 μg/ml) in Hepes-buffered saline (pH 7.3). The assay was done at 23°C for 30 min. [^{32}P]ADP-ribose incorporation into TCA-precipitable material was quantified in a liquid scintillation counter after extensive washing. The specific incorporation of [^{32}P]ADP-ribose into substrate was verified by SDS-PAGE and autoradiography. (**B**) Binding of GST–14-3-3 ζ fusion protein (*21*) to c-Bcr and Bcr-Abl proteins produced in insect cells. The GST–Bap-1 fusion was included for comparison in the assay. Binding reactions were done as described (Fig. 1A). (**C**) Phosphorylation of purified Bap-1 and 14-3-3 ζ proteins by immunoprecipitated c-Bcr or Bcr-Abl was done as described (Fig. 2A). Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography (top panel). The corresponding Coomassie-stained gel is shown (bottom panel).

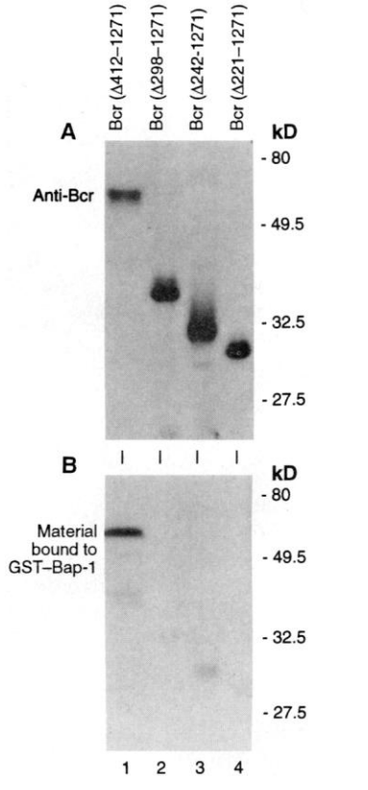

Fig. 4. Mapping of the Bap-1 binding site in the first exon of Bcr. Transcripts were generated from linearized *bcr* DNA, which was digested with various restriction enzymes to generate a number of processive truncations. Transcripts were generated from the SP6 promoter in the pGEM4 vector (Promega) as previously described (*4*). Bcr fragments were synthesized in vitro in a rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-labeled amino acids (Amersham). A portion of the labeled Bcr fragments was subjected to immunoprecipitation with antibodies to an NH_2-terminal portion of Bcr (**A**). Portions of the lysates were diluted with buffer (*4*) and incubated with GST–Bap-1 bound to beads (**B**). After incubation for 2 hours at 4°C, the beads were washed and the bound proteins were eluted with sample buffer and analyzed by SDS-PAGE and fluorography.

gion that are the primary sites for interaction with Bap-1. These results suggest that the binding of Bap-1 to the first exon sequences of Bcr may be required for the full transforming activity of Bcr-Abl.

A role for 14-3-3 proteins in cell proliferation was provided recently by the identification of two 14-3-3 protein homologs in fission yeast that are required for the DNA damage checkpoint and affect the time of entry into mitosis (*15*). Double null mutants for the two 14-3-3 genes are inviable, which suggests that together the 14-3-3 protein homologs provide a function that is essential for cell proliferation in fission yeast (*15*). 14-3-3 proteins may have similarly conserved functions in mammalian cells. Some of the properties ascribed to 14-3-3 proteins provide potential functional mechanisms for their involvement in intracellular signaling cascades. The 14-3-3 proteins have a short 16– to 18–amino acid sequence stretch that is similar to a conserved region present in the annexins, a family of Ca^{2+}- and lipid-binding proteins (*26*). Annexins have been implicated in the mediation of cytoskeletal-membrane interactions, and some members of this protein family may serve as receptors for protein kinase C (PKC) (*26*, *27*). Translocation of PKC from the cytosol to the particulate fraction requires the binding of PKC not only to proteins and lipids in the plasma membrane but also to cytoskeletal-associated proteins (*27*). Similarly, the Raf-1 protein kinase (*28*) and Bcr-Abl (*3*) associate with cytoskeletal components. Binding of 14-3-3 proteins to c-Bcr and Bcr-Abl may allow for proper localization of these proteins to the membrane cytoskeleton and facilitate interactions with other cellular proteins implicated in signaling. 14-3-3 proteins have a high degree of α-helical structure with an amphipathic nature and readily form dimers (*29*). These properties may provide a mechanism whereby the 14-3-3 proteins could serve as bridges between distinct signaling protein complexes.

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11. A 16-day mouse embryo library (λEXlox, Novagen) was screened with the first exon of Bcr. Approximately 500,000 recombinant phages were screened by standard in situ nitrocellulose filter assays. Seven independent phage clones were isolated that corresponded to two overlapping sets of clones. The longest insert was 1.9 kb.
12. Northern blot analysis of total cytoplasmic RNA from adult (liver, lung, brain, and kidney) and embryo mouse tissues demonstrated the presence of two closely migrating transcripts of 1.9 and 2.2 kb. Similarly sized transcripts were observed after probing a multiple tissue blot containing human RNAs (Clontech). The mRNA was ubiquitously expressed, with the greatest amounts observed in heart, brain, placenta, and kidney.
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17. The 5′ untranslated region of the *bap-1* cDNA was deleted by cutting with Nco I, which cuts just 5′ of the *bap-1* ATG start codon, and the ends were filled with Klenow polymerase. A Bam HI linker was ligated to permit in-frame fusion with GST in the pGEX-3X vector (Pharmacia). The Pst I site in the 3′ untranslated sequence was changed to an Eco RI site by linker addition. The *bap-1* cDNA was cloned in the pGEX-3X vector as a Bam HI–Eco RI fragment to generate a GST–Bap-1 fusion protein. The *bap-1* cDNA was also cloned into the Bam HI site of the pCGN expression vector [M. Tanaka and W. Herr, *Cell* **60**, 375 (1990)]. To maintain the correct reading frame, a Bam HI linker was added to the 5′ end of the *bap-1* cDNA.
18. Antibodies to Bap-1 were generated in rabbits with the GST–Bap-1 fusion protein containing full-length Bap-1.
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30. Phosphorylated Bap-1 was separated by SDS-PAGE, transferred to nitrocellulose filters, autoradiographed, and excised from the filters. After digestion with trypsin and treatment with performic acid, the phosphopeptides were spotted onto thin layer cellulose plates [B. J. Boyle, P. Van Der Geer, T. Hunter, *Methods Enzymol.* **201**, 110 (1991)]. Thin layer electrophoresis was carried out for 20 min at 1 kV in pH 1.9 buffer. Thin layer chromatography in the second dimension was done in isobutyric acid buffer for 4.5 hours.

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