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Tyrosine Phosphorylation of BCR by FPS/FES Protein-Tyrosine Kinases Induces Association of BCR with GRB-2/SOS

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The human *bcr* gene encodes a protein with serine/threonine kinase activity, CDC24/*dbl* homology, a GAP domain, and an SH2-binding region. However, the precise physiological functions of BCR are unknown. Coexpression of BCR with the cytoplasmic protein-tyrosine kinase encoded by the c-*fes* proto-oncogene in Sf-9 cells resulted in stable BCR-FES protein complex formation and tyrosine phosphorylation of BCR. Association involves the SH2 domain of FES and a novel binding domain localized to the first 347 amino acids of the FES N-terminal region. Deletion of the homologous N-terminal BCR-binding domain from v-*fps*, a *fes*-related transforming oncogene, abolished transforming activity and tyrosine phosphorylation of BCR in vivo. Tyrosine phosphorylation of BCR in v-*fps*-transformed cells induced its association with GRB-2/SOS, the RAS guanine nucleotide exchange factor complex. These data provide evidence that BCR couples the cytoplasmic protein-tyrosine kinase and RAS signaling pathways.

The *bcr* locus was originally defined as the breakpoint of the Philadelphia (Ph) chromosome translocation associated with chronic myelogenous leukemia and acute lymphocytic leukemia. This translocation fuses the c-*abl* proto-oncogene on chromosome 9 with the *bcr* locus on chromosome 22, resulting in the formation of a chimeric *bcr-abl* oncogene (8, 15, 21, 52). The *bcr-abl* locus associated with chronic myelogenous leukemia is expressed as an oncogenically activated protein-tyrosine kinase ($p210^{bcr/abl}$) that is believed to contribute directly to the pathogenesis of the disease (3, 6, 7). In Ph^+ acute lymphocytic leukemia, a larger part of the *bcr* gene is deleted, resulting in the expression of a smaller *bcr/abl* oncoprotein ($p185^{bcr/abl}$ [5, 22]). $p185^{bcr/abl}$ provides a more potent mitogenic stimulus than $p210^{bcr/abl}$ (37), suggesting that BCR sequences retained by $p210^{bcr/abl}$ but not found in $p185^{bcr/abl}$ may influence the kinase and transforming activities of the chimera or permit interactions with other proteins. Activation of RAS may be a common feature of BCR/ABL-mediated transformation, as both forms of BCR/ABL have recently been shown to bind to the SRC homology 2 (SH2) domain of the GRB-2 protein via tyrosine-phosphorylated BCR sequences (46, 47). GRB-2 serves as an adaptor to couple activated tyrosine kinases to the RAS guanine nucleotide exchange factor, SOS (27, 29).

Other studies have established that a functional gene product of 160 kDa is encoded by the *bcr* gene (56, 57). $p160^{bcr}$ is expressed in many cell types and encodes several functional domains. The first of these is a unique serine/threonine kinase which has been localized to exon 1 of *bcr* (33). This domain is critical to the transforming activity of BCR/ABL fusion proteins and may activate the ABL tyrosine kinase by binding to its SH2 domain (40, 41, 45). In addition, *bcr* exon 1 encodes an oligomerization domain that may be essential to BCR/ABL transforming function (38, 39). BCR also exhibits homology to regulators of RAS-related GTP-binding proteins. A domain homologous to several known or putative RAS guanine nucleotide exchange factors, including the yeast CDC24 protein, the rat guanine nucleotide-releasing factor protein, and the *vav* and *dbl* proto-oncogene products, is found in the central portion of BCR (16, 49, 51). The C-terminal domain of BCR exhibits GTPase-activating function (GAP activity) toward the RAS-related proteins RAC1 (10) and CDC42Hs (20). Thus, BCR may link the RAS and RAC subfamilies of small G proteins. Some RAC-related functions of BCR may be important to normal myeloid cell development. RAC is a regulator of the NADPH oxidase complex in mature phagocytes, and *rac* expression is increased during terminal granulocytic differentiation (1, 9).

The human c-*fes* locus encodes a 93-kDa protein-tyrosine kinase ($p93^{c\text{-}fes}$) predominantly expressed in myeloid cells of the granulocytic and monocytic lineages (12, 32, 55). Recent studies have linked $p93^{c\text{-}fes}$ to signal transduction by several hematopoietic cytokines, suggesting that it functions in some aspect of myeloid cell growth regulation (17, 18). This hypothesis is also supported by the finding that transfection of the myeloid leukemia cell line K-562 with the human c-*fes* gene results in growth suppression and terminal differentiation (58). K-562 cells are chronic myelogenous leukemia derived and carry the Ph chromosome (30), indicating that the *fes*-induced signal for differentiation is able to suppress $p210^{bcr/abl}$ transforming activity. Similarly, a virally activated homolog of $p93^{c\text{-}fes}$ encoded by the Fujinami sarcoma virus ($p130^{gag\text{-}fps}$) has been shown to induce the differentiation of chicken myeloid stem cells in vitro (4), despite its ability to transform rodent fibroblasts. Thus, FPS/FES protein kinases appear to promote terminal differentiation when expressed in the appropriate cellular context (myeloid cells) but exhibit transforming activity when ectopically expressed in fibroblasts.

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Both normal and transforming FPS/FES proteins contain three distinct structural domains: a C-terminal kinase domain, an adjacent SH2 domain, and a unique N-terminal domain. The SH2 domains of both $p93^{c\text{-}fes}$ and $p130^{gag\text{-}fps}$ are positive regulators of kinase activity and have been proposed to provide a binding site for effectors or regulatory proteins (23, 26). Unlike the SRC and ABL families of cytoplasmic tyrosine kinases, FPS/FES kinases lack SH3 domains, which are often observed in the context of SH2 domains and also serve regulatory and protein-protein interaction functions (reviewed in references 25 and 43). However, unique N-terminal sequences are shared by $p93^{c\text{-}fes}$ and $p130^{gag\text{-}fps}$. This N-terminal region is critical to the transforming activity of the FPS kinase (2), and is presumably important to the physiological function of FES as well. In this report, we provide evidence for a functional interaction between FPS/FES proteins and BCR that involves the FPS/FES SH2 and unique N-terminal domains. Tyrosine phosphorylation of BCR by FPS leads to association with the GRB-2/SOS guanine nucleotide exchange complex (29), implicating BCR as an intermediate between cytoplasmic protein-tyrosine kinase and RAS signal transduction pathways.

MATERIALS AND METHODS

Expression of recombinant FES and BCR proteins in Sf-9 cells and analysis of BCR-FES protein complex formation. Construction of recombinant baculoviruses for the expression of BCR and ABL proteins is described elsewhere (33). Recombinant baculoviruses were constructed to express human $p93^{c\text{-}fes}$ and the mutants shown in Fig. 2 with the 8-amino-acid FLAG epitope fused to their C termini, thus permitting specific recognition with the M2 anti-FLAG monoclonal antibody resin (IBI/Kodak, Rochester, N.Y.). Details of the construction of the FLAG-*fes* baculoviruses will be published elsewhere (54a). Sf-9 insect cells were obtained from the American Type Culture Collection and maintained in Grace's insect medium supplemented with yeastolate, lactalbumin hydrolysate, 10% fetal bovine serum, and 50 μg of gentamicin per ml. For coinfection experiments, subconfluent monolayers of Sf-9 cells were infected with BCR or FES baculoviruses or the two in combination for 1 h at room temperature. At 48 h postinfection, the cells were lysed by sonication in 0.5 ml of lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.0] containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1.0 mM Na_3VO_4, 0.05 mM Na_2MoO_4, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 50 μg of aprotinin per ml, and 25 μg of leupeptin per ml). Cell lysates were clarified by microcentrifugation for 10 min at 4°C, and 0.2-ml aliquots were diluted with 1.0 ml of incubation buffer (20 mM HEPES [pH 7.0] containing 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 25 μg of leupeptin per ml). Monoclonal antibodies to BCR (Oncogene Science) and protein G-Sepharose (Pharmacia/LKB) or the anti-FLAG monoclonal antibody resin were added, and the reaction mixtures were incubated for 2 h at 4°C. Following incubation, the immunoprecipitates were washed with three 1.0-ml aliquots of incubation buffer, and bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride or nitrocellulose. BCR and FES were visualized by immunoblotting with monoclonal antibodies to phosphotyrosine (PY20; ICN) or polyclonal antisera raised against human BCR.

Expression of GST fusion proteins and BCR-binding assay. Construction of pGEX vectors containing the ABL and FES SH2 domains has been described previously (23, 45). The pGEX-GRB-2/SH2 construct was provided by T. Takenawa, Institute of Medical Science, University of Tokyo (35). The coding sequence of the FES N-terminal domain (amino acids 1 to 450) was amplified by PCR and cloned into the *Bam*HI and *Sma*I sites of pGEX-2T. Two smaller FES N-terminal pGEX constructs were derived by digesting this initial construct with *Sma*I and *Eco*RI or with *Sst*I and *Eco*RI, blunting, and religating. These constructs were used to express fusion proteins containing the N-terminal 347 or 126 amino acids of $p93^{c\text{-}fes}$. To construct BCR exon 1 fusion proteins, the *Bam*HI and *Bgl*II sites in the human BCR sequence were converted to *Eco*RI sites by blunting and *Eco*RI linker addition. The resulting *Eco*RI-*Eco*RI (N-terminal coding sequence) and *Bam*HI-*Eco*RI (C-terminal coding sequence) fragments were cloned into the multiple-cloning site of pGEX/3X. These constructs were used to express BCR exon 1 glutathione *S*-transferase (GST) fusion proteins containing BCR amino acids 1 to 161 or 162 to 413. Procedures for bacterial expression and glutathione-agarose affinity purification of GST fusion proteins from pGEX constructs are described in detail elsewhere (23, 45) and are based on the original procedure of Smith and Johnson (53). Recombinant $p160^{bcr}$ binding to immobilized SH2 and N-terminal domain fusion proteins was analyzed as described previously (45).

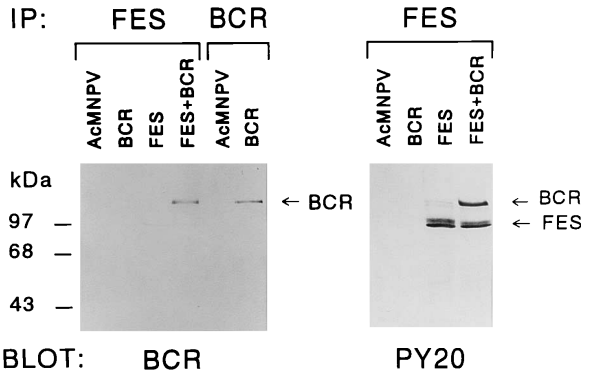

FIG. 1. Formation of FES-BCR protein complexes and phosphorylation of BCR by FES in Sf-9 insect cells. Sf-9 insect cells were infected with either wild-type baculovirus (lanes AcMNPV), recombinant viruses containing either the FES or BCR cDNA, or the two recombinant viruses in combination. Following infection, the cells were lysed in the presence of phosphatase inhibitors and immunoprecipitates (IP) were prepared with antibodies specific for either the FES or BCR proteins. The FES protein had the 8-amino-acid FLAG epitope fused to its C terminus and was precipitated with a FLAG-specific monoclonal antibody (see Materials and Methods). Precipitated proteins were washed, separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to BCR or phosphotyrosine (PY20).

Expression of FPS and BCR proteins in 3Y1 fibroblasts. FPS expression vectors used in this study included p4L-FSV, which contains the genome of the Fujinami sarcoma virus, and pP1, which contains a Fujinami sarcoma virus deletion mutant lacking amino acids 413 to 718 (2). 3Y1 cells were transfected with these expression vectors plus pSV2neo by the calcium phosphate method and selected in the presence of G418 for 2 weeks. Drug-resistant colonies were isolated and expanded to examine the expression and protein-tyrosine kinase activity of FPS or FPS Δ413–718 proteins. Human BCR and BCR Δ162–413 proteins were retrovirally expressed in 3Y1/FPS transformants with the pSRαMSVtkneo vector as described elsewhere (41).

Antibodies, immunoprecipitation, and Western blotting. Preparation and properties of the anti-FPS, anti-BCR, and anti-ABL antibodies are described elsewhere (2, 41, 45, 57). The anti-GRB-2 antibody was a gift from T. Takenawa (34). Antibodies to SOS1 were purchased from Upstate Biotechnology Inc. For Western immunoblotting, nitrocellulose membranes were blocked with either 4% nonfat dry milk or 0.05% Tween 20 and were developed by either enhanced chemiluminescence (Amersham) or alkaline phosphatase (Promega) methods. For immunoprecipitation, 3Y1 cells were lysed in 50 mM HEPES (pH 7.4)–150 mM NaCl–1% Triton X-100–2 mM Na_3VO_4–10 mM NaF–10 mM PP–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride–20 μg of leupeptin per ml–0.02% SDS. BCR and FPS were precipitated from the lysates with antibodies as described in the figure legends. For GRB-2/BCR and SOS/BCR coimmunoprecipitation experiments, both EDTA and SDS were omitted from the lysis buffer. Autophosphorylation of $p130^{gag\text{-}fps}$ and $p95^{gag\text{-}fps\Delta 413\text{–}718}$ was assessed by incubating washed immunoprecipitates with 1 mM cold ATP followed by immunoblotting with PY20.

RESULTS

BCR interacts with FES in a baculovirus/Sf-9 cell expression system. Previous studies have shown that the FES protein-tyrosine kinase can induce the differentiation of the Ph^+ myeloid leukemia cell line K-562, indicating that $p93^{c\text{-}fes}$ can suppress the transfoming activity of $p210^{bcr/abl}$ in these cells (58). This observation suggested that FES may interact with BCR or BCR/ABL proteins in vivo. To test this hypothesis, we developed a baculovirus system for the coexpression of the FES and BCR proteins in Sf-9 insect cells. Sf-9 cells were infected with the FES or BCR baculoviruses alone or in combination. Immunoprecipitates were prepared from cell lysates with antibodies specific for the FES protein and analyzed by immunoblotting with antibodies to either BCR or phosphotyrosine (PY20). As shown in Fig. 1, anti-FES immunoprecipitates prepared from cells expressing both the FES and BCR proteins contained BCR. Note that the FES antibody did not react directly with BCR, indicating that it must be precipitated

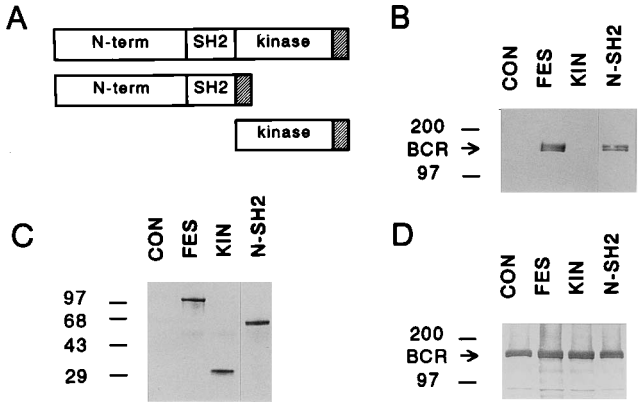

FIG. 2. The N-terminal and SH2 domains of FES associate with BCR in Sf-9 insect cells. Full-length FES, the FES N-terminal and SH2 domains (N-SH2; amino acids 1 to 540), and the kinase domain (KIN; amino acids 541 to 822) were coexpressed with BCR in Sf-9 cells. (A) Structures of the proteins. The position of the C-terminal FLAG epitope is indicated by the hatched box. As a negative control, BCR was expressed alone (lane CON in panels B to D). (B) Immunoprecipitates were prepared from infected-cell lysates with the anti-FLAG monoclonal antibody (see Materials and Methods). Immunoprecipitated protein complexes were washed, and associated BCR was visualized by immunoblotting with anti-BCR antiserum. (C) To establish that equivalent amounts of each FES protein were present in the immunoprecipitates, aliquots were immunoblotted with the anti-FLAG monoclonal antibody. (D) To verify that BCR was expressed at approximately the same level in each culture, aliquots of the clarified lysates were immunoblotted with the anti-BCR antiserum, Rb-1.

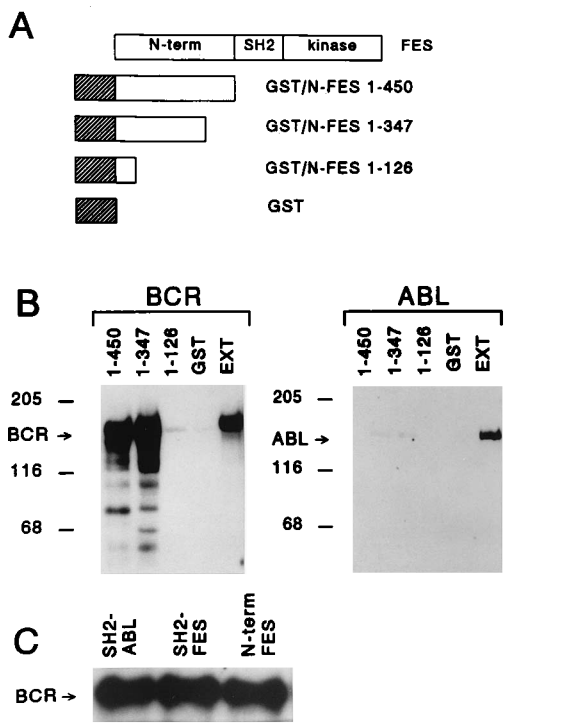

FIG. 3. Interaction of BCR with the FES unique N-terminal and SH2 domains in vitro. (A) Structures of the human FES protein and the GST fusion proteins derived from its unique N-terminal domain. (B) FES N-terminal domain fusion proteins bind to BCR but not to ABL. Lysates were prepared from Sf-9 insect cells infected with either BCR or ABL baculovirus and incubated with immobilized fusion proteins shown in panel A or with immobilized GST. Following incubation and washing, bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. BCR was visualized by immunoblotting with anti-trpE/BCR antibody, while ABL was immunoblotted with the αpEX-5 anti-ABL monoclonal antibody as described in Materials and Methods. Aliquots of infected cell lysates were run as positive controls (lanes EXT). (C) FES N-terminal and SH2 domain GST fusion proteins bind BCR as efficiently as does an ABL SH2 domain fusion protein. Lysates of Sf-9 cells infected with the recombinant BCR baculovirus and labeled with [^{35}S]methionine were incubated with immobilized GST fusion proteins containing either the FES SH2, ABL SH2, or FES N-terminal domain. Following incubation and washing, associated BCR was visualized by SDS-PAGE and fluorography.

as a complex with FES. Identical immunoblots probed with antibodies to phosphotyrosine revealed both FES and BCR, indicating not only that these proteins form a complex but also that BCR is a substrate for FES. Control experiments showed that BCR is not phosphorylated on tyrosine when expressed in the absence of FES (data not shown).

To determine the stoichiometry of the FES-BCR interaction, anti-FES immunoprecipitates from Sf-9 cells coexpressing the two proteins were resolved by SDS-PAGE and stained with Coomassie blue. Densitometry of the resulting gel suggested that the ratio of FES to BCR in the anti-FES immune complex (when corrected for the molecular masses of the two proteins) was approximately 9:1 (data not shown).

To map the FES sequences responsible for interaction with BCR, the regulatory region (N-terminal and SH2 domains; amino acids 1 to 540) and the kinase domain (amino acids 541 to 822) were expressed as FLAG fusion proteins in the baculovirus system (Fig. 2A). These two proteins were compared with full-length FES in terms of their ability to complex with BCR in coimmunoprecipitation experiments. As shown in Fig. 2, the regulatory region of FES containing the unique N-terminal and SH2 domains associated with BCR almost as strongly as did full-length FES. On the other hand, the isolated kinase domain of FES interacted very weakly with BCR. These results suggest that FES-BCR complex formation is mediated primarily via the noncatalytic region of the FES protein.

Association of BCR and FES is mediated by the FES SH2 and unique N-terminal domains. Experiments whose results are shown in Fig. 2 indicate that the noncatalytic N-terminal and SH2 domains of FES are largely responsible for association with BCR. Previous studies of these domains suggest that both may be important for substrate recognition in vivo. For example, deletion of v-*fps* sequences within its unique N-terminal coding region reduces transforming potential without abolishing kinase activity (2), suggesting that N-terminal sequences may be important for effector binding. More-recent studies have shown that BCR exon 1 sequences are able to bind to a select group of SH2 domains in a phosphotyrosine-independent manner (40). To investigate whether both these FES domains are important for interaction with BCR, we conducted in vitro binding assays with recombinant GST fusion proteins containing either the FES SH2 domain (amino acids 451 to 540) or the N-terminal domain (amino acids 1 to 450). The ABL SH2 domain was tested as a positive control, because it has previously been shown to bind to exon 1-encoded sequences of BCR (40). The fusion proteins were immobilized on glutathione-agarose and incubated with lysates from Sf-9 cells expressing BCR, and binding was assessed by SDS-PAGE and either immunoblotting or autoradiography. As shown in Fig. 3C, BCR bound to the FES N-terminal and SH2 domains as efficiently as to the ABL SH2 domain. To map the N-terminal region responsible for BCR binding, two smaller FES N-terminal GST fusion proteins were tested for binding to BCR. As shown in Fig. 3B, an N-terminal fusion protein containing FES amino acids 1 to 347 bound BCR as effectively as did the entire N-terminal domain. However, a smaller fusion protein containing the N-terminal 126 amino acids of the FES protein exhibited no binding activity. These results show that a unique domain responsible for BCR binding is localized to the

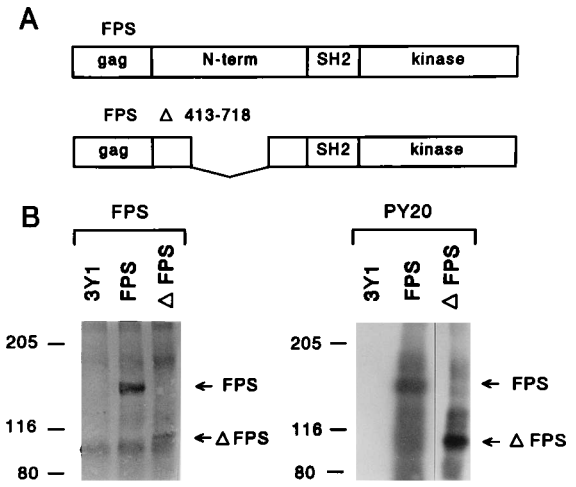

FIG. 4. Deletion of FPS N-terminal amino acids 413 to 718 does not abolish protein-tyrosine kinase activity in vitro. (A) Structures of full-length FPS and FPS Δ413–718 mutant. (B) FPS or FPS Δ413–718 (ΔFPS) proteins were immunoprecipitated from cloned 3Y1 transfectants with anti-FPS antibodies. Immune complexes were incubated with 1 mM ATP and analyzed by immunoblotting with either anti-FPS antibodies (left) or anti-phosphotyrosine antibodies (PY20, right).

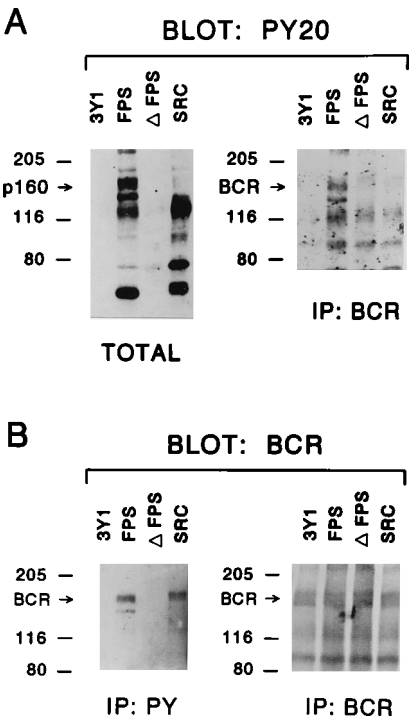

FIG. 5. Deletion of FPS N-terminal amino acids 413 to 718 abolishes phosphorylation of BCR and other cellular proteins in vivo. (A) Total cellular lysates (left) or anti-BCR immunoprecipitates (IP) (right) of 3Y1 cells or 3Y1 transfectants expressing FPS, FPS Δ413–718 (ΔFPS), or SRC were blotted with antiphosphotyrosine antibodies (PY20). (B) As in panel A, except antiphosphotyrosine (left) or anti-BCR (right) immunoprecipitates were immunoblotted with anti-BCR antibodies.

first 347 amino acids of the FES N-terminal region. None of the FES N-terminal fusion proteins bound to the baculovirus-expressed ABL protein, consistent with the idea that a unique BCR interaction domain is found in the N-terminal domain of $p93^{c\text{-}fes}$. No BCR binding activity was detected with immobilized GST (Fig. 3B).

A transformation-defective v-*fps* mutant which lacks the BCR binding region retains protein-tyrosine kinase activity in vitro but does not phosphorylate $p160^{bcr}$ in vivo. To assess the biological significance of the novel BCR interaction domain found in the FES N-terminal region, we examined the effect of deletion of this domain on the tyrosine kinase and transforming activities of the *fes*-related oncogene v-*fps* (14). A deletion mutant of v-*fps* that lacks amino acids 413 to 718 was used (2); these residues correspond to FES amino acids 55 to 370 and thus encompass the BCR-binding domain defined above (Fig. 4A). Rat 3Y1 fibroblasts transfected with this deletion mutant showed no morphological evidence of transformation or colony-forming activity (data not shown), even though the deletion did not abolish autophosphorylation capacity in vitro (Fig. 4B). These results are consistent with previous studies of this mutant (2) and suggest that the unique N-terminal domain of $p130^{gag\text{-}fps}$ is important for the recognition of transformation-related substrates.

Antiphosphotyrosine immunoblots of total cellular proteins from 3Y1 fibroblasts expressing either FPS or the FPS Δ413–718 mutant were performed to determine whether N-terminal FPS/FES sequences are important for the recognition of BCR and other substrates in vivo. As shown in Fig. 5, antiphosphotyrosine Western blots of FPS transformants revealed several strong bands, including a 160-kDa protein. By contrast, cells expressing the FPS deletion mutant showed fewer phosphotyrosine-containing proteins and the 160-kDa protein was completely absent. To establish the identity of the 160-kDa protein as endogenous BCR, antiphosphotyrosine immunoprecipitates were blotted with anti-BCR antibodies and anti-BCR immunoprecipitates were blotted with antiphosphotyrosine antibodies. In both cases, the 160-kDa band was observed, indicating that $p160^{bcr}$ is a likely substrate for $p130^{gag\text{-}fps}$ in vivo. This result is consistent with the results obtained when FES was coexpressed with BCR in the baculovirus system (see above). Interestingly, tyrosine-phosphorylated BCR was also observed in antiphosphotyrosine immunoprecipitates from *src*-transformed 3Y1 cells (Fig. 5B), indicating that BCR phosphorylation may occur during *src*-mediated transformation as well. Recognition of BCR by the SRC kinase may be mediated by its SH2 domain, which binds tightly to BCR in vitro (40). However, little phosphotyrosine was present in BCR immunoprecipitates from SRC transformants (Fig. 5A), suggesting that the stoichiometry of BCR phosphorylation by FPS may be greater than that catalyzed by SRC.

Although we readily observed BCR/FES complexes in Sf-9 cells that overexpress these two proteins, we did not observe BCR/FPS complexes in 3Y1/FPS transformants. This difference may be due to several factors, including the lower level of FPS and BCR proteins present in 3Y1 fibroblasts relative to Sf-9 cells, the inability of the precipitating antibodies to recognize the BCR/FPS complex, and structural differences between the FES and FPS proteins.

The SH2-binding domain of BCR is tyrosine phosphorylated both in vivo and in vitro. To localize the site of BCR that is phosphorylated by the FPS kinase, the BCR mutant lacking the C-terminal half of exon 1 (BCR Δ162–413) was expressed in FPS transformants by retrovirus-mediated gene transfer (33). This mutant lacks tyrosine 177 and eight other tyrosine residues in the SH2-binding domain encoded by BCR exon 1. Phosphorylation of tyrosine 177 of $p210^{bcr/abl}$ leads to association with the GRB-2 adaptor protein and activation of $p21^{ras}$ (46, 47), presumably by recruitment of mSOS (27). As shown

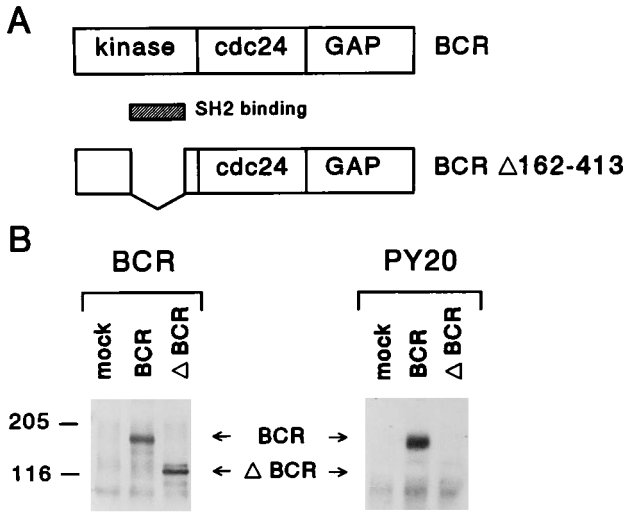

FIG. 6. The site of FPS-catalyzed BCR phosphorylation in vivo maps to the BCR SH2-binding domain within exon 1. (A) Structures of wild-type human BCR and the Δ162–413 deletion mutant. (B) BCR and BCR Δ162–413 (ΔBCR) were expressed in 3Y1/FPS transformants and immunoprecipitated with anti-BCR antibodies. Precipitated BCR proteins were immunoblotted with either anti-BCR (left) or antiphosphotyrosine (PY20, right) antibodies.

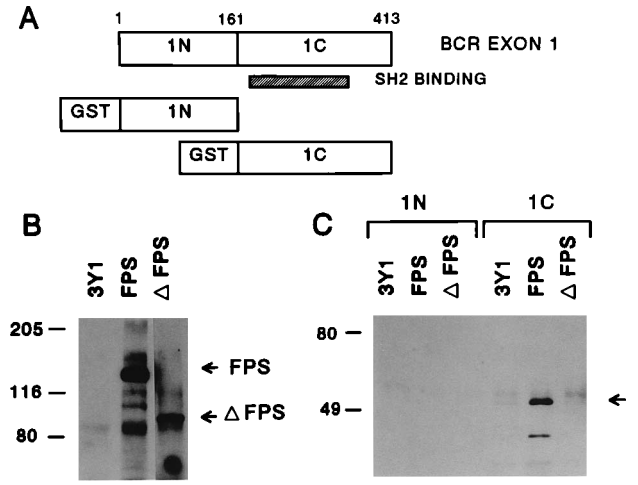

FIG. 7. The site of FPS-catalyzed BCR phosphorylation in vitro maps to the C-terminal half of exon 1 containing tyrosine 177. (A) BCR exon 1-encoded sequences 1 to 161 (1N) or 162 to 413 (1C) were expressed in *E. coli* as GST fusion proteins. Their relationship to the BCR SH2-binding domain is shown. (B and C) BCR exon 1 fusion proteins were added to FPS immunoprecipitates from parental 3Y1 cells or 3Y1 cells expressing either FPS or FPS Δ413–718 (ΔFPS) in the presence of 1 mM ATP. Following incubation, the FPS immune-complex pellets (B) and supernatants containing the GST-BCR exon 1 fusion proteins (C) were run on separate gels and immunoblotted with antiphosphotyrosine antibodies.

in Fig. 6, no detectable phosphorylation of BCR Δ162–413 by $p130^{gag\text{-}fps}$ was observed, suggesting that FPS-catalyzed tyrosine phosphorylation of $p160^{bcr}$ in vivo occurs within *bcr* exon 1-encoded amino acids 162 to 413. Control experiments show that full-length BCR was readily phosphorylated on tyrosine residues following overexpression in FPS-transformed 3Y1 cells (Fig. 6). Similarly, no detectable phosphorylation of BCR Δ162–413 was observed following coexpression of this BCR mutant with FES or SRC in Sf-9 cells (data not shown).

To confirm that BCR amino acids 162 to 413 contain a FPS tyrosine phosphorylation site, *bcr* exon 1 sequences were expressed as two GST fusion proteins in *Escherichia coli* to test as substrates for FPS in vitro (Fig. 7). The N-terminal fusion protein contains amino acids 1 to 161, while the C-terminal fusion protein contains amino acids 162 to 413 and corresponds to the BCR mutant described above. The GST-BCR exon 1 fusion proteins were added to immune-complex kinase assays containing $p130^{gag\text{-}fps}$ or the deletion mutant lacking the BCR interaction domain (FPS Δ413–718). As shown in Fig. 7, $p130^{gag\text{-}fps}$ phosphorylated the C-terminal fusion protein which contains tyrosine 177 and the SH2-binding domain but not the N-terminal fusion protein, even though it contains two tyrosine residues. Interestingly, the FPS deletion mutant lacking the BCR-binding region did not phosphorylate the C-terminal fusion protein, despite its active kinase domain. This result is consistent with the observation that this mutant is also unable to phosphorylate endogenous BCR in vivo (Fig. 5).

GRB-2 and SOS are associated with BCR in FPS transformants. The GST fusion protein containing the C-terminal half of *bcr* exon 1-encoded sequences (amino acids 162 to 413) contains nine tyrosine residues which represent potential phosphorylation sites for $p130^{gag\text{-}fps}$. Among these sites is Tyr-177, which forms a high-affinity binding site for GRB-2 in activated BCR/ABL (46, 47). To determine whether phosphorylation of BCR by FPS in this region also leads to GRB-2 recruitment, anti-BCR immunoprecipitates from 3Y1/FPS transformants were probed with antibodies to the GRB-2 protein. As shown in Fig. 8A, anti-BCR immunoprecipitates from transformed cells contained GRB-2, whereas anti-BCR immunoprecipitates from untransformed cells did not. To investigate the mechanism of FPS-induced BCR–GRB-2 complex formation, binding assays were performed with an immobilized GST-GRB-2/SH2 fusion protein. As shown in Fig. 8B, BCR from 3Y1/FPS cells specifically bound to the GRB-2 SH2 domain. BCR-SH2 interaction was also observed with 3Y1/SRC transformants, although to a lesser extent. No binding was observed with untransformed cell lysates or with immobilized GST without the SH2-derived sequence. To determine whether tyrosine phosphorylation of BCR leads to association with the RAS guanine nucleotide exchange factor SOS via the GRB-2 adaptor, SOS immunoprecipitates from 3Y1/FPS cells were tested for the presence of BCR by immunoblotting. Figure 8C shows that BCR is coprecipitated with SOS in the FPS transformants but not in untransformed 3Y1 cells. Control immunoprecipitation experiments show that BCR is expressed in parental 3Y1 cells. To assess whether FES-catalyzed BCR phosphorylation also induces GRB-2 binding, lysates from Sf-9 cells expressing BCR alone or in combination with FES were incubated with an immobilized fusion protein containing the entire GRB-2 sequence fused to GST. As shown in Fig. 8D, phosphorylation of BCR by FES enhanced GRB-2 binding to BCR more than eightfold. Similar results were obtained with the GRB-2 SH2 domain (data not shown). Taken together, these data suggest that BCR may serve as a biochemical link between FPS/FES cytoplasmic tyrosine kinases and RAS.

DISCUSSION

In this report we provide evidence that BCR is a specific substrate and potential effector for FPS/FES cytoplasmic protein-tyrosine kinases. Tyrosine phosphorylation of BCR occurred within N-terminal exon 1-encoded sequences and led to recruitment of the GRB-2 protein, an SH2-containing adaptor that couples tyrosine-phosphorylated proteins to the RAS guanine nucleotide exchange factor, mSOS (27, 29). Recent stud-

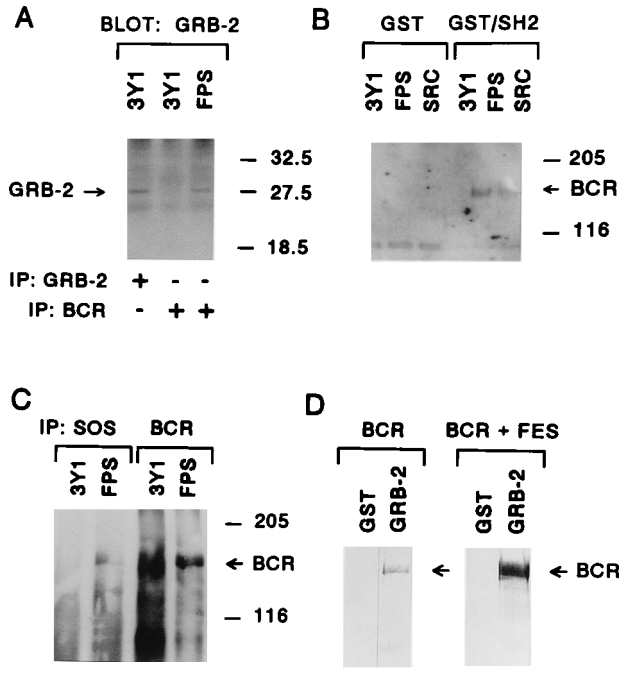

FIG. 8. Phosphorylation of BCR by FPS induces binding to GRB-2/SOS via the GRB-2 SH2 domain. (A) Anti-GRB-2 or anti-BCR immunoprecipitates were prepared from lysates of parental 3Y1 cells or 3Y1/FPS transformants and subjected to immunoblotting with anti-GRB antibodies as described in Materials and Methods. (B) Lysates from 3Y1, 3Y1/FPS, and 3Y1/SRC cells were incubated with immobilized GST or GST-GRB-2/SH2 domain fusion proteins. Following association and washing, BCR was visualized by Western blot analysis (see Materials and Methods). (C) Anti-SOS immunoprecipitates of 3Y1 or 3Y1/FPS cells were immunoblotted with anti-BCR antibodies. To control for the levels of BCR in 3Y1 and 3Y1/FPS cells, anti-BCR immunoprecipitates were prepared in parallel and immunoblotted with anti-BCR antibodies. (D) Lysates from Sf-9 cells expressing BCR alone or in combination with FES were incubated with an immobilized GST/GRB-2 fusion protein (Oncogene Science). Following incubation and washing, associated BCR was visualized by immunoblotting.

ies show that phosphorylation of tyrosine 177 of BCR exon 1-derived sequences within BCR/ABL leads to GRB-2/SOS binding and RAS activation (46, 47). FPS-induced binding of GRB-2/SOS to normal BCR may activate RAS by a similar mechanism. A dominant-negative form of RAS has been shown to block v-*fes*-induced fibroblast transformation (42), indicating that RAS is a critical component of FPS/FES signal transduction.

The observation that FPS/FES-mediated tyrosine phosphorylation of BCR induces binding to GRB-2 suggests that this phosphorylation event may enhance the association of BCR with other effectors containing SH2 domains. Using the baculovirus system, we have recently observed that FES-catalyzed tyrosine phosphorylation of BCR enhances its association with recombinant SH2 domains from *ras* GAP, phospholipase Cγ, and phosphatidylinositol 3′-kinase (46a). Thus, tyrosine phosphorylation of BCR by FES is likely to occur at more than one site and may serve to couple BCR to diverse growth-regulatory signaling pathways.

BCR may represent an effector for other protein-tyrosine kinases of the cytoplasmic class, including SRC (Fig. 5 and 8) and BCR/ABL (28, 31). Phosphorylation of BCR by these kinases may also induce GRB-2/SOS association as a novel mechanism activating RAS signaling, which is critical to protein-tyrosine kinase transforming activity (13, 54). BCR may also serve as a substrate for FER, another cytoplasmic tyrosine kinase closely related to FES but with wider tissue distribution (19, 44). Since BCR expression is also widespread, interaction of FES or FER with BCR may represent part of a general signal transduction pathway in many cell types. Tyrosine kinases involved in BCR phosphorylation may be limited to the cytoplasmic class, because very little BCR phosphorylation has been observed in response to growth factor treatment (32a).

In addition to BCR, FPS/FES protein kinases phosphorylate and interact with *ras* GAP (11, 24) and SHC (36), suggesting that regulators and effectors of small GTP-binding proteins may represent a common set of targets for the FPS/FES protein-tyrosine kinase family. SHC is a major tyrosine phosphoprotein in FPS- and SRC-transformed cells and has been linked to RAS activation via complex formation with GRB-2/SOS (50). In an attempt to determine whether the SHC pathway is the exclusive transformation-linked pathway in 3Y1/FPS cells, the SHC SH2 domain was overexpressed in one of our transformed clones. Overexpression of the SHC SH2 domain might be expected to specifically block FPS-induced phosphorylation of endogenous SHC via a dominant-negative effect. However, high-level expression of the SHC SH2 domain failed to reverse morphological transformation of 3Y1/FPS cells (data not shown), suggesting that multiple pathways may contribute to RAS activation in FPS-transformed cells.

In addition to catalyzing BCR phosphorylation, FES formed a stable complex with BCR. Data presented in Fig. 2 and 3 show that two FES domains mediate this association. The first is the SH2 domain, which binds to BCR as efficiently as does the ABL SH2 domain. Previous studies with the ABL SH2 show that interaction occurs via a serine- and threonine-rich sequence localized to exon 1 of BCR and is independent of phosphotyrosine (40). It is possible that the FES SH2 domain binds to BCR through a similar mechanism. In addition to SH2-mediated binding, we observed the presence of a unique region localized to the FES N-terminal domain that bound BCR as efficiently as did the SH2 domain. Deletion of the corresponding domain from FPS rendered it unable to phosphorylate BCR in vivo. This deletion mutant is also transformation defective (2), indicating that the FPS/FES BCR-interaction domain is essential for biological activity. Although we have not mapped the precise region of BCR responsible for interaction with FES, full-length FES can associate with a BCR deletion mutant containing only the exon 1-encoded Ser/Thr kinase domain (amino acids 1 to 413; data not shown). In addition, the Tyr phosphorylation sites for FPS, FES, and SRC are localized to BCR exon 1 (Fig. 6 and 7) (46a). These data suggest that this BCR N-terminal region is important for tyrosine kinase interaction but do not exclude a role for the other BCR domains.

Previous studies have shown that p93$^{c\text{-}fes}$ suppresses the growth of the Ph$^+$ myeloid leukemia cell line, K-562 (58). Our finding that FES can physically interact with BCR raises the possibility that FES interacts with BCR/ABL as well and alters its transforming activity. Recent studies have established that oligomerization of BCR/ABL induces association with the actin cytoskeleton, a process that may be critical to transformation (38, 39). BCR sequences encoded by exon 1 are responsible for oligomerization (38). Binding of FES to BCR exon 1 sequences within BCR/ABL, possibly via the unique BCR-binding domain described here, may suppress BCR/ABL oligomerization and interfere with transformation. Alternatively, binding of FES to this region of BCR/ABL could prevent association with GRB/SOS or other BCR/ABL effectors that bind to this region, such as the 14-3-3 proteins (48).

In summary, tyrosine phosphorylation of BCR by FPS/FES and other protein-tyrosine kinases may add a new level of

complexity to the regulation of RAS and other small GTP-binding proteins by BCR. Phosphotyrosine-dependent association of GRB-2/SOS or other SH2-containing effectors with BCR may lead to novel changes in their catalytic activities, subcellular localization, or both. Future experiments will focus on the identification of other members of the cytoplasmic class of protein-tyrosine kinases that phosphorylate and bind to BCR and on determination of whether these phosphorylation events lead to association of BCR with downstream effectors containing SH2 domains.

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