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Structural and Signaling Requirements for BCR-ABL-Mediated Transformation and Inhibition of Apoptosis

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BCR-ABL is a deregulated tyrosine kinase expressed in Philadelphia chromosome-positive human leukemias. Prolongation of hematopoietic cell survival by inhibition of apoptosis has been proposed to be an integral component of BCR-ABL-induced chronic myelogenous leukemia. BCR-ABL elicits transformation of both fibroblast and hematopoietic cells and blocks apoptosis following cytokine deprivation in various factor-dependent cells. To elucidate the mechanisms whereby BCR-ABL induces transformation and blocks apoptosis in hematopoietic cells, we examined the biological effects of expression of a series of BCR-ABL mutants. Single amino acid substitutions in the GRB2 binding site (Y177F), Src homology 2 domain (R552L), or an autophosphorylation site in the tyrosine kinase domain (Y793F) do not diminish the antiapoptotic and transforming properties of BCR-ABL in hematopoietic cells, although these mutations were previously shown to drastically reduce the transforming activity of BCR-ABL in fibroblasts. A BCR-ABL molecule containing all three mutations (Y177F/R552L/Y793F) exhibits a severe decrease in transforming and antiapoptotic activities compared with the wild-type BCR-ABL protein in 32D myeloid progenitor cells. Ras is activated, the SHC adapter protein is tyrosine phosphorylated and binds GRB2, and *myc* mRNA levels are increased following expression of all kinase active BCR-ABL proteins with the exception of the Y177F/R552L/Y793F BCR-ABL mutant in 32D cells. We propose that BCR-ABL uses multiple pathways to activate Ras in hematopoietic cells and that this activation is necessary for the transforming and antiapoptotic activities of BCR-ABL. However, Ras activation is not sufficient for BCR-ABL-mediated transformation. A BCR-ABL deletion mutant (Δ176-427) that activates Ras and blocks apoptosis but has severely impaired transforming ability in 32D cells has been identified. These data suggest that BCR-ABL requires additional signaling components to elicit tumorigenic growth which are distinct from those required to block apoptosis.

Activating mutations within mitogenic signaling pathways have long been associated with cancer. More recently, cellular abnormalities involving apoptotic, differentiative, and negative growth control pathways have been shown to contribute to the process of multistep oncogenesis. Specific oncogenes may affect one or multiple cellular regulatory pathways that normally safeguard against tumorigenesis.

bcr-abl is a chimeric oncogene formed by a chromosomal translocation that unites sequences from the *bcr* gene upstream of the second exon of c-*abl*. The two naturally occurring forms of BCR-ABL, p185 and p210, differ in the amount of the BCR protein fused to ABL. p185 is primarily associated with acute lymphocytic leukemia (ALL) and p210 is found in most cases of chronic myelogenous leukemia (CML) (43). BCR-ABL chimeric proteins exhibit deregulated tyrosine kinase activity and transform both fibroblast and hematopoietic cells in culture (1, 10, 39). More recently, BCR-ABL has been shown to inhibit apoptosis (22, 39, 52). BCR-ABL blocks cell death in response to cytokine removal in interleukin 3 (IL-3)-dependent BaF3 cells (10). The $p210^{bcr\text{-}abl}$-expressing K562 cells are resistant to apoptosis induced by actinomycin D, camptothecin, etoposide, cycloheximide, or serum withdrawal. Antisense *bcr-abl* abolishes resistance to apoptosis in K562 cells after treatment with these agents (29).

Apoptosis is programmed cell death characterized by DNA degradation, chromatin condensation, and nuclear fragmentation (56) and is a key regulatory mechanism to achieve and maintain homeostasis within multicellular organisms. Apoptosis can be induced by multiple stimuli, including growth factor deprivation and DNA damage, and is likely regulated by many intracellular signaling pathways which may converge on a common apoptotic machinery (8, 12, 46). Disruption of normal apoptotic pathways in tumor cells can give these cells a selective advantage (56). Many proteins within mitogenic signaling pathways interact with apoptotic pathways. The *myc* proto-oncogene is critical for cell cycle progression and contributes to transformation in some cell contexts (13), but it also induces apoptosis when expressed inappropriately (3, 4, 14). Deregulation of apoptosis is important in the initiation and progression of many cancers (15, 21, 53, 56).

The clinical progression of CML and ALL suggests that BCR-ABL-initiated signals may impinge on apoptotic, differentiative, and proliferative pathways (6). CML is characterized by a chronic phase consisting of an abnormal expansion of the myeloid compartment followed by an acute blast crisis (6). Philadelphia chromosome (Ph^1)-positive ALL is characterized by an excessive accumulation of lymphoblasts primarily within the B-cell lineage and is particularly refractory to treatment compared with Ph^1-negative ALL (43). The mechanisms by which BCR-ABL interacts with the apoptotic, differentiative, and proliferative pathways remain to be elucidated.

BCR-ABL contains many structural motifs and can potentially activate multiple signaling cascades. Recently a connection between BCR-ABL and $p21^{ras}$ activation was identified (39, 42). The Src homology 2 (SH2) domain of the GRB2 adapter protein binds to an autophosphorylated tyrosine at position 177 within the BCR portion of the molecule. Mutation

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of this phosphorylation site to a phenylalanine abrogates binding to GRB2, blocks Ras activation, and severely impairs transforming activity in Rat1 fibroblasts (1, 39). The importance of a Ras-dependent signaling pathway to the antiapoptotic activity of BCR-ABL has yet to be established.

The relationship between the transforming and antiapoptotic effects of BCR-ABL is unknown. Signaling pathways required for suppression of apoptosis may be distinct from mitogenic pathways as shown for various cytokines (20, 24, 25). In this study, we sought to identify structural motifs within the BCR-ABL protein that activate specific signaling pathways required for transducing its antiapoptotic and transforming activities in hematopoietic cells.

MATERIALS AND METHODS

Production of BCR-ABL mutants. The SH2 domain point mutation (R552L) was produced by PCR, using the mutagenic primer 5′TATCCGCTGAGCAGC GGGATCAATGGCAGCTTCTTGGTGCTCGAGAGTGAGAGCAGTCC-3′. The Y793F mutation was transferred from the corresponding mutation in p210 (Y1294F). A *Kpn*I fragment from pSRαp210 Y1294F was inserted into the corresponding site in pSRαp185. The Y177F/R552L/Y793F mutation was constructed by first joining the Y177F/Y793F mutations, using *Kpn*I fragments of the single mutants in the pSRα vector, followed by swapping the *Kpn*I-*Bsr*G1 fragment from the p185 R552L molecule into p185 Y177F/Y793F. All mutations were confirmed by DNA sequence analysis.

Cell culture. BaF3 cells were obtained from Joseph Schlessinger (New York University), and 32D cells were obtained from James Ihle (St. Jude Children's Research Hospital). Both cell types were maintained in RPMI containing 10% fetal calf serum and 10% WEHI conditioned media (WEHI-CM) as a source of IL-3. Stable mass population cell lines expressing *bcr-abl* transgenes were produced using retroviral infection. The pSRαMSVTkneo vector was used to produce helper-free retroviral stocks (32). Retroviruses were produced in Bosc-23 cells (35). Infections were performed essentially as previously described (37). G418 (0.7 mg/ml) selection was initiated 48 h postinfection and continued until a G418-resistant cell population was obtained (usually within 9 to 15 days following the infection); 10% WEHI-CM was included in the media during the infections and selection procedure. Expression of BCR-ABL was confirmed by immunoblotting.

Western blotting (immunoblotting). An equal number of cells were lysed directly in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol). Lysates were analyzed on SDS-polyacrylamide gels (8%) and transferred to nitrocellulose. Blots were probed with an anti-ABL monoclonal antibody or antiphosphotyrosine antibody (PY20; ICN Flow) as previously described (39). Protein bands were visualized by enhanced chemiluminescence detection (Amersham).

Measurements of factor-independent growth and apoptosis. Apoptosis and factor-independent growth were analyzed immediately after establishment of G418-resistant cell populations. To eliminate variability, cells were adjusted to approximately 5×10^5/ml the day before all experiments. Cells were washed twice and seeded at 2×10^5/ml in RPMI–10% fetal calf serum–G418 (0.7 mg/ml) with or without WEHI-CM as a source of IL-3. Cell viability, growth, and apoptosis were monitored by three methods: viable and dead cells were counted by trypan blue exclusion, cells were examined for DNA fragmentation, and apoptosis was quantified by flow cytometry.

Flow cytometric measurement of apoptosis was performed by propidium iodide (PI)-Hoechst 33342 (HO) double staining as described previously (11, 40). A total of 550,000 cells were pelleted and resuspended in 100 μl of PI (20 μg/ml; Molecular Probes). After incubation on ice for 30 min, 1.9 ml of 25% ethanol in phosphate-buffered saline was added with 50 μl of 0.25 mM HO (Molecular Probes). Cells were stained on ice for a minimum of 2 h and then analyzed on a Becton Dickinson FACStar$^+$ flow cytometer with 488-nm and 351-nm argon lasers. Necrotic cells were excluded on the basis of their intense PI staining. Apoptotic cells have reduced PI and HO staining due to DNA fragmentation. The proportion of live cells in various phases of the cell cycle was also determined from the HO staining intensities.

Gamma irradiation treatment. Cells were washed twice and then resuspended in media with or without IL-3 and subjected to gamma irradiation at increasing doses from 0 to 1,600 rads. At 12 (32D cells) or 20 (BaF3 cells) hours after gamma irradiation, the cells were harvested and assayed for viability and apoptosis as described above.

Tumor challenges. Cells (10^6 BaF3 and 5×10^6 32D) expressing the BCR-ABL proteins were injected into 5- to 7-week-old athymic *nu*/*nu* female mice obtained from the Cancer Center Isolation Facility at Duke University Medical Center. Prior to subcutaneous injection, the cells were washed and resuspended in Hanks balanced salt solution. Tumor formation was monitored every few days.

BCR-ABL in vitro kinase assay. 32D cells (10^7) expressing each of the BCR-ABL mutant forms were harvested and lysed in kinase lysis buffer, consisting of 10 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0), 150 mM NaCl, 1% Triton X-100, 0.05% SDS, 5 mM EDTA, 20 μg of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride (PMSF). BCR-ABL was immunoprecipitated with anti-ABL (pex4) antibody, washed twice in kinase lysis buffer, and then washed twice with 50 mM Tris (pH 6.8). Kinase reactions were performed in 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) containing 20 mM $MnCl_2$, 30 μM unlabeled ATP, 5 μCi of [γ-^{32}P]ATP, and 1 μg of baculovirus-produced glutathione *S*-transferase (GST)–SH2SH1 fusion protein containing the SH2 and SH1 domains of kinase-defective ABL as an exogenous substrate. A small amount of the lysate was also mixed with SDS sample buffer, analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and subjected to Western blotting to assess the level of BCR-ABL protein expression.

Northern (RNA) blot analysis. BCR-ABL-expressing 32D cells were starved for serum and IL-3 for 5 h before RNA extraction. Ten micrograms of total cellular RNA was separated on an agarose gel and blotted onto a nylon membrane. A full-length *myc* cDNA was used as a probe. The blot was stripped and reprobed with *GAPDH* cDNA as a control for equal RNA loading.

Ras-GTP loading assay. The procedure for assaying the percentage of GTP bound to Ras was essentially as described previously (18). Briefly, 10^7 cells were washed twice in RPMI without phosphate and resuspended in 3 ml of RPMI without phosphate and containing 0.5 mCi of carrier-free $^{32}P_i$ per ml. The cells were labeled at 37°C for 3 h. Cells were lysed in 1 ml of a mixture containing 1% Triton X-114, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 1 mg of bovine serum albumin (BSA) per ml, 5 mM $MgCl_2$, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 10 mM benzamidine, 1 mM PMSF, and 0.2 mM sodium orthovanadate. Nuclei were pelleted by centrifugation for 4 min at 12,000 × *g*, 100 μl of 5 M NaCl was added to the supernatant, and the lysate was incubated at 37°C for 1.5 min and then centrifuged at room temperature for 1.5 min. The supernatant was removed, and the lower phase was washed in 50 mM HEPES–0.5 M NaCl–5 mM $MgCl_2$–1.0 mg of BSA per ml. The remaining cell extract was solubilized in 1% Triton X-100–50 mM HEPES (pH 7.4)–0.5% (wt/vol) deoxycholate–1.0 mg of BSA per ml–5 mM $MgCl_2$–10 μg of aprotinin per ml–10 μg of leupeptin per ml–10 mM benzamidine–1 mM PMSF–0.2 mM sodium orthovanadate. Lysates were precleared by using protein A-agarose and rabbit anti-rat immunoglobulin G. Ras was immunoprecipitated with Y 13-259 (Santa Cruz Biotechnology) for 1.5 h and then incubated with protein A-agarose and goat anti-rat immunoglobulin G. Immunocomplexes were washed with a buffer containing 50 mM HEPES (pH 7.4), 500 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100–0.005% SDS, and then GTP and GDP were eluted with 2 mM EDTA–5 mM dithiothreitol–0.2% SDS–1 mM GDP–1 mM GTP at 68°C for 20 min. The eluted sample was separated on a thin-layer chromatography plate, using 0.75 M KH_2PO_4 (pH 3.3), and visualized by autoradiography. The ratio of GDP and GTP was quantified on a Betagen scanner.

Analysis of SHC phosphorylation and GRB2 binding. Equal numbers of cells were lysed in PLC lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 20 μg of leupeptin per ml, 20 μg of aprotinin per ml, 1 mM sodium orthovanadate, 0.2 mM sodium molybdate, 25 mM sodium fluoride). Affinity-purified anti-SHC polyclonal antibody (2 μg; Transduction Laboratories) was incubated with cell lysates for at least 90 min at 4°C. Protein A-Sepharose was added to lysates, and incubation continued for another 90 min. Immunoprecipitates were washed three times with incubation buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 20 μg of leupeptin per ml, 20 μg of aprotinin per ml, 1 mM sodium orthovanadate, 0.2 mM sodium molybdate, 25 mM sodium fluoride) and resuspended in SDS sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. For immunoblotting with anti-SHC and anti-GRB2 antibodies, filters were blocked in Tris-buffered saline (25 mM Tris [pH 7.5], 75 mM NaCl) with 0.1% Tween and 1% BSA for 2 h at room temperature. For blotting with antiphosphotyrosine antibody, filters were blocked in Tris-buffered saline with 5% BSA overnight. Anti-GRB2 and anti-SHC monoclonal antibodies (Transduction Laboratories) and antiphosphotyrosine antibody PY20 (Santa Cruz Biotechnology) were used to probe the filters, followed by horseradish peroxidase-conjugated goat anti-mouse (Bio-Rad) secondary antibody. Proteins were detected by enhanced chemiluminescence (Amersham).

RESULTS

Production of BCR-ABL-expressing myeloid and lymphoid cell lines. BCR-ABL is known to block apoptosis, stimulate growth factor-independent proliferation, and induce tumor growth when expressed in factor-dependent hematopoietic cell types (10, 37). To determine whether the antiapoptotic and tumorigenic effects of BCR-ABL require the same or distinct structural features, wild-type and mutant p185$^{bcr\text{-}abl}$ proteins were expressed in hematopoietic cells. BCR-ABL selectively induces disorders within hematopoietic compartments (17). Therefore, we chose two hematopoietic cell types, 32D and BaF3 cells, as model systems. 32D cells are multipotent my-

eloid progenitor cells, and BaF3 are pro-B cells (33, 58). Both cell types are dependent on IL-3 for growth and survival.

Various mutant forms of p185$^{bcr\text{-}abl}$ have been previously shown to exhibit impaired transforming properties (1, 31, 37, 39). These mutants were chosen to study the structural requirements for the antiapoptotic effects of BCR-ABL. The mutants include a kinase-defective (K671R) mutant; a BCR-ABL protein that lacks the SH2 domain and is significantly impaired in its ability to transform fibroblasts (1, 31); an autophosphorylation mutant (Y793F) which has been shown previously to uncouple growth factor-independent proliferation from transformation in clone H pre-B cells expressing p210$^{bcr\text{-}abl}$ (39); a tyrosine phosphorylation site mutant (Y177F) that is defective in binding to GRB2 and activating Ras in fibroblasts (39); and a deletion mutant (Δ176-427) that lacks GRB2 and 14-3-3 protein binding sites, BCR serine kinase activity, and a serine/threonine-rich region (26, 38, 44). Additional BCR-ABL mutants were created that contained combinations of the two autophosphorylation mutations Y177F and Y793F and an SH2 domain point mutation (R552L) that abolishes binding to phosphotyrosine-containing sequences.

Wild-type and mutant forms of p185$^{bcr\text{-}abl}$ were cloned into the pSRαMSVTkneo retroviral vector, which contains the neomycin resistance gene. Retroviruses were prepared as previously described (32, 35) and used to infect 32D and BaF3 cells. To minimize the acquisition of secondary genetic alterations which might lead to growth factor-independent proliferation, we selected for expression of BCR-ABL proteins, using G418 in the presence of 10% WEHI-CM as a source of IL-3. Mass populations were isolated to minimize the time between infection and the biological assays on the G418-resistant populations. Typically G418-resistant cell populations were ready for biological and biochemical characterization within 9 to 15 days after infection. Multiple cell lines were derived for each BCR-ABL protein. All the mutants were expressed at approximately the same levels in 32D cells (Fig. 1A) and BaF3 cells (data not shown).

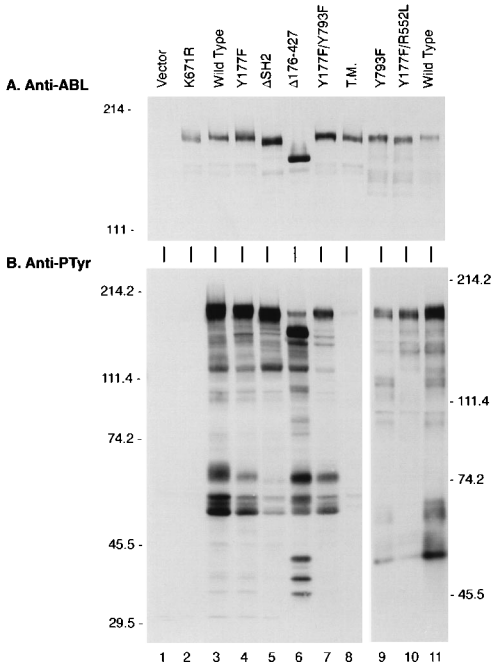


FIG. 1. Expression of BCR-ABL proteins in 32D cells. Retroviral expression vectors containing the indicated cDNAs were used to infect 32D cells. G418-resistant mass populations expressing the constructs indicated above the lanes (T.M. denotes the Y177F/R552L/Y793F triple mutant) were lysed directly in SDS sample buffer. Anti-ABL (A) or antiphosphotyrosine (B) Western blotting was performed after separation by SDS-PAGE. Sizes are indicated in kilodaltons.

BCR-ABL expression blocks apoptosis following IL-3 deprivation. To assess the ability of BCR-ABL expression to block apoptosis induced by cytokine deprivation, G418-selected mass population cells were deprived of IL-3 and analyzed for changes in the percentage of apoptotic cells by flow cytometric analysis. The wild-type, Y177F, Y793F, ΔSH2, Y177F/Y793F, and Y177F/R552L BCR-ABL-expressing 32D cells were resistant to apoptosis after IL-3 withdrawal and underwent factor-independent proliferation (Fig. 2A). In contrast, cells expressing the kinase-defective K671R BCR-ABL mutant and the Y177F/R552L/Y793F BCR-ABL triple mutant underwent apoptosis with similar kinetics as cells expressing vector alone (Fig. 2A). 32D cells expressing the Δ176-427 BCR-ABL mutant were partially resistant to apoptosis but did not proliferate. Approximately 20% of the cells remained viable 7 days after removal of IL-3. Thus, expression of p185 Δ176-427 partially protects 32D cells from cell death but does not cause immediate factor-independent proliferation.

Interestingly, expression of the Y177F/R552L/Y793F and Δ176-427 BCR-ABL mutants had different effects in BaF3 cells compared with 32D cells. BaF3 cells expressing these mutants were resistant to apoptosis after IL-3 removal, similar to BaF3 cells expressing the wild-type BCR-ABL protein (Fig. 2B). This result indicates that the structural requirements for the inhibition of apoptosis by BCR-ABL following IL-3 deprivation are dependent on the cell context.

BCR-ABL prevents apoptosis following DNA damage. Next we investigated whether BCR-ABL could inhibit apoptosis induced by stimuli other than IL-3 deprivation. DNA damage induces apoptosis; however, IL-3 can protect BaF3 and 32D cells from undergoing cell death after exposure to DNA-damaging agents such as gamma irradiation (reference 9 and Fig. 3). We tested the abilities of wild-type and mutant forms of BCR-ABL to block gamma irradiation-induced cell death. Gamma irradiation-induced apoptosis was measured by flow cytometric analysis of DNA integrity. In the absence of IL-3, all forms of BCR-ABL tested inhibited gamma irradiation-induced apoptosis in BaF3 cells with the exception of the tyrosine kinase-defective BCR-ABL mutant (K671R) (Fig. 3A). The data for the wild-type, Δ176-427 deletion mutant, and Y177F/R552L/Y793F triple-mutant BCR-ABL proteins in addition to the K671R mutant are shown in Fig. 3A. Similar antiapoptotic effects by BCR-ABL were observed following treatment of these cells with the chemotherapeutic agent cisplatin at concentrations of up to 10 μg/ml (data not shown). These data suggest that BCR-ABL tyrosine kinase activity is necessary to suppress apoptosis induced by DNA damage in BaF3 cells.

In contrast to the results for BaF3 cells, 32D cells expressing the Y177F/R552L/Y793F BCR-ABL mutant were not resistant to gamma irradiation-induced apoptosis in the absence of IL-3 (Fig. 3B). The Δ176-427 BCR-ABL mutant conferred resistance to gamma irradiation to 32D cells but was not as effective as wild-type BCR-ABL (Fig. 3B). These results are consistent with the ability of these mutant BCR-ABL proteins to inhibit apoptosis following cytokine deprivation in 32D cells.

In vivo and in vitro tyrosine kinase activities of BCR-ABL proteins. The tyrosine phosphorylation patterns of intracellular proteins were examined to determine whether structural changes in BCR-ABL resulted in specific changes in tyrosine-phosphorylated proteins that correlated with the biological

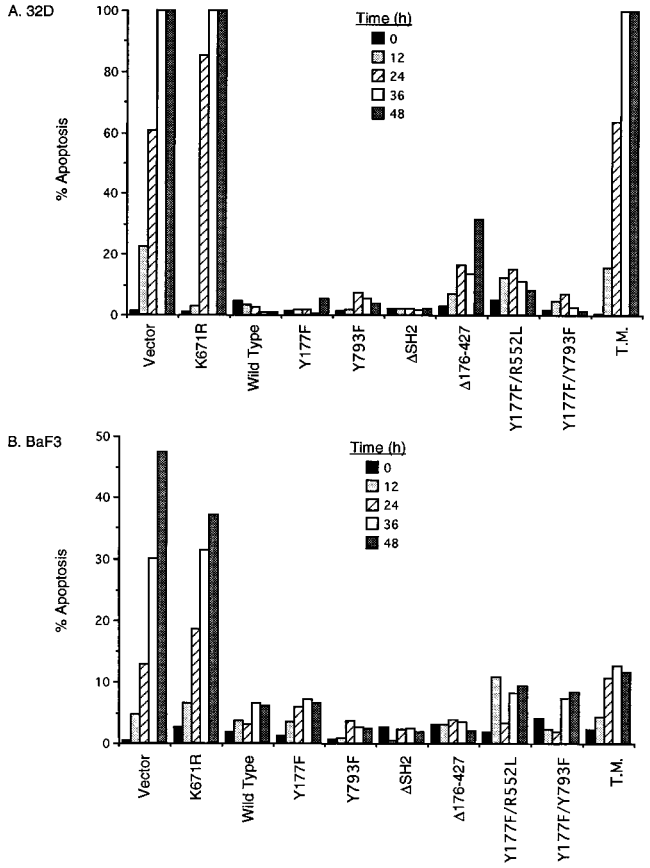


FIG. 2. Effects of wild-type and mutant forms of BCR-ABL on apoptosis induced by IL-3 deprivation. 32D (A) and BaF3 (B) cells expressing the indicated BCR-ABL proteins were washed twice in IL-3-free medium and then seeded at a density of 2×10^5 cells per ml in the absence of IL-3. Apoptosis was quantified at the indicated times by flow cytometric analysis of DNA content.

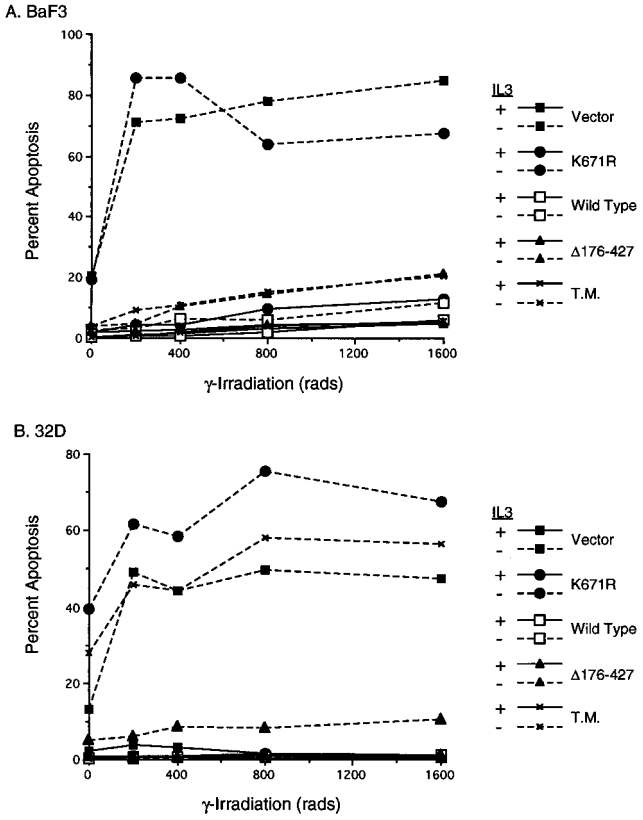


FIG. 3. Gamma irradiation-induced apoptosis in BaF3 and 32D cells expressing wild-type and mutant forms of BCR-ABL. BaF3 (A) or 32D (B) cells expressing p185 K671R, p185 wt (wild type), p185 Δ176-427, and p185 Y177F/R552L/Y793F (triple mutant [T.M.]) were analyzed for their apoptotic responses to increasing doses of gamma irradiation. Cells were washed twice and seeded at 2×10^5/ml in 9 ml of medium with (solid lines) or without (dashed lines) IL-3. The cells were then exposed to increasing doses (0 to 1,600 rads) of gamma irradiation. At 20 (A) or 12 (B) h later, 550,000 cells were harvested, and apoptosis was quantified by flow cytometry.

effects of the BCR-ABL mutants. Wild-type BCR-ABL expression results in elevated tyrosine phosphorylation of intracellular proteins in 32D cells (Fig. 1B). Qualitatively similar results are obtained with the BaF3 cells (data not shown). Deletion of the SH2 domain in BCR-ABL results in the loss of tyrosine-phosphorylated proteins in the range of 62 to 70 kDa (Fig. 1B, lane 5). 32D cells expressing p185 Δ176-427 display high levels of tyrosine-phosphorylated proteins similar to that in wild-type $p185^{bcr\text{-}abl}$-expressing 32D cells. However, in addition to the common tyrosine-phosphorylated proteins, three protein bands with apparent molecular masses of 30 to 40 kDa are observed in cells expressing the p185 Δ176-427 mutant but not in any of the other cells expressing the various $p185^{bcr\text{-}abl}$ proteins. Interestingly, 32D cells expressing the Y177F/R552L/Y793F BCR-ABL mutant have almost no increase in tyrosine-phosphorylated proteins over vector control 32D cells (Fig. 1B). A slight increase over control cells in tyrosine phosphorylation is observed in BaF3 cells expressing the Y177F/R552L/Y793F BCR-ABL protein (data not shown).

The reduction in tyrosine phosphorylation of cellular proteins in 32D and BaF3 cells expressing the Y177F/R552L/Y793F BCR-ABL mutant suggested that this protein may exhibit lower intrinsic tyrosine kinase activity than wild-type BCR-ABL. To examine this possibility, we used an in vitro kinase assay with baculovirus-produced GST-SH2SH1 fusion protein containing the SH2 and SH1 domains of kinase-defective ABL as a substrate. Transphosphorylation was examined rather than autophosphorylation since several of the mutant BCR-ABL proteins lacked specific tyrosine residues previously shown to be autophosphorylation sites (37, 39). Following immunoprecipitation from the infected and G418-selected 32D cells, all of the BCR-ABL proteins exhibited similar high levels of kinase activity except for the tyrosine kinase-defective BCR-ABL K671R mutant (Fig. 4A). Similar amounts of BCR-ABL protein were present in all kinase reactions (Fig. 4B). Thus, all of the BCR-ABL mutants with the exception of the tyrosine kinase-defective BCR-ABL protein exhibited similar tyrosine kinase activities in vitro.

Analysis of the transforming abilities of BCR-ABL proteins. Previous studies demonstrated that alterations in the SH2 domain, the GRB2 binding site (Y177F), or a tyrosine phosphorylation site in the SH1 domain impair the ability of BCR-ABL to transform fibroblasts (1, 31, 37, 39). Interestingly, we observe that BCR-ABL proteins with mutations in those regions (ΔSH2, Y177F, and Y793F) can efficiently block apoptosis in hematopoietic cells. These results suggest that different structural motifs may be required for the antiapoptotic activities versus the transforming activities of BCR-ABL. Alternatively, these BCR-ABL mutant proteins may have different biological effects in fibroblasts and hematopoietic cells. To distinguish between these possibilities, the transformation potentials of

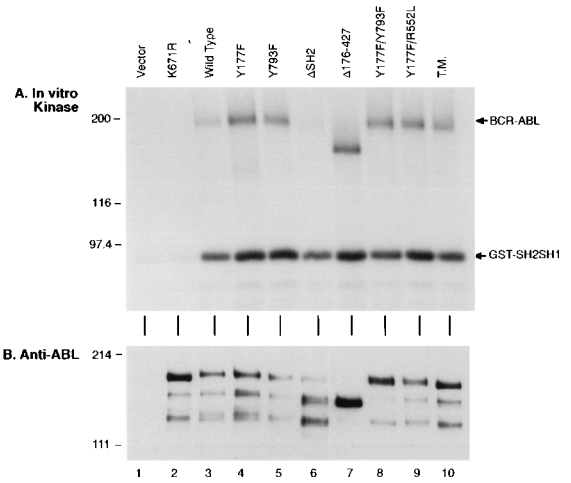


FIG. 4. In vitro kinase activities of BCR-ABL mutant proteins expressed in 32D cells. Equal numbers of G418-resistant 32D cells expressing the constructs indicated above the lanes (T.M. denotes the Y177F/R552L/Y793F triple mutant) were washed and lysed. (A) The BCR-ABL proteins were immunoprecipitated from the lysates and subjected to an in vitro kinase reaction with GST-SH2SH1 fusion protein containing the SH2 and SH1 domains of kinase-defective ABL as a substrate. (B) A small amount of the lysate was mixed with SDS sample buffer and subjected to SDS-PAGE followed by Western blotting with an anti-ABL monoclonal antibody. Sizes are indicated in kilodaltons.

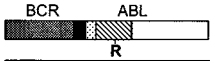
wild-type and mutant BCR-ABL proteins in BaF3 and 32D cells were assessed by determination of IL-3-independent growth and tumorigenicity in nude mice.

All of the BCR-ABL proteins with the exception of the K671R tyrosine kinase-defective, Δ176-427, and Y177F/R552L/Y793F BCR-ABL mutants elicited IL-3-independent growth of 32D cells (Table 1). The Δ176-427 BCR-ABL mutant protein did not deliver an immediate proliferative signal to the 32D cells grown in the absence of IL-3. 32D cells expressing this BCR-ABL deletion mutant required 3 to 4 weeks before the emergence of IL-3-independent cell populations. In addition, 32D cells expressing the Y177F/R552L/Y793F triple mutant did not proliferate without IL-3 and died rapidly after IL-3 deprivation (Table 1 and Fig. 2A). In contrast, both the Δ176-427 and Y177F/R552L/Y793F BCR-ABL mutants elicited IL-3-independent growth of BaF3 cells (Table 1). This property correlates with their ability to block apoptosis in BaF3 cells (Fig. 2B) and illustrates the cell context dependence of the biological activities elicited by BCR-ABL.

To determine whether IL-3-independent growth resulted from autocrine stimulation of proliferation, the medium from IL-3-independent cells was tested for the presence of factors that promote cell growth or viability. No evidence was found for the presence of secreted growth factors in the media (data not shown). The lack of detectable autocrine stimulation is consistent with results of other studies using various BCR-ABL growth factor-independent cell lines (10, 37, 59) and contrasts with the ability of an activated Src tyrosine kinase to cause factor-independent proliferation in 32D cells by eliciting an autocrine stimulation (2).

To assess the tumorigenic potential of the various BCR-ABL mutants, BCR-ABL-expressing cells were injected subcutaneously into nude mice. Mice were observed for signs of palpable or visible tumor at the injection site. The kinase-defective BCR-ABL-expressing BaF3 cells did not form tumors in nude mice (Table 1). The remainder of the BaF3 cell lines expressing wild-type or mutant forms of BCR-ABL formed tumors that progressed rapidly until the mice were sacrificed when the tumors reached a size of 1 cm^3 (Fig. 5B). The BaF3 cells expressing Y177F/R552L/Y793F BCR-ABL required approximately three times as long to form visible tumors compared with the wild-type BCR-ABL-expressing BaF3 cells (Table 1). BaF3 cells expressing either Y177F/Y793F BCR-ABL or Y177F/R552L/Y793F BCR-ABL formed tumors that progressed significantly more slowly than those elicited by the wild-type BCR-ABL-expressing BaF3 cells (Fig. 5B).

TABLE 1. Tumorigenicity and factor-independent growth of hematopoietic cells expressing wild-type and mutant forms of BCR-ABL

p185 BCR-ABL Construct[a]		IL-3 Independent Growth and Tumorigenicity					
		32D			BaF3		
		IL-3 Ind.[b]	Incidence[c]	Latency[d]	IL-3 Ind.	Incidence	Latency
	K671R	-	0/5	-	-	0/3	-
	Wild Type	+	6/6	6-8	+	4/4	6-8
	Y177F	+	6/6	8	+	3/3	6-8
	Δ SH2	+	3/3	6-8	+	3/3	8
	Δ176-427	-/+[e]	3/6	41-48	+	5/5	7-12
	Y793F	+	4/4	12	+	5/5	5-11
	Y177F/Y793F	+	3/3	14-20	+	4/4	8-11
	Y177F/R552L	+	4/4	5-12	+	4/4	5-8
	Y177F/Y793F/R552L	-	6/6	27-35	+	6/6	5-20

[a] Names and schematic diagrams of the $p185^{bcr\text{-}abl}$ proteins expressed in 32D and BaF3 cells.
[b] Ind., induction.
[c] Number of mice with tumors/total number of mice injected subcutaneously with the indicated cells.
[d] Time (days) postinjection when tumors were first observed.
[e] These cells proliferated without growth factor only after prolonged periods (>3 weeks) in culture.

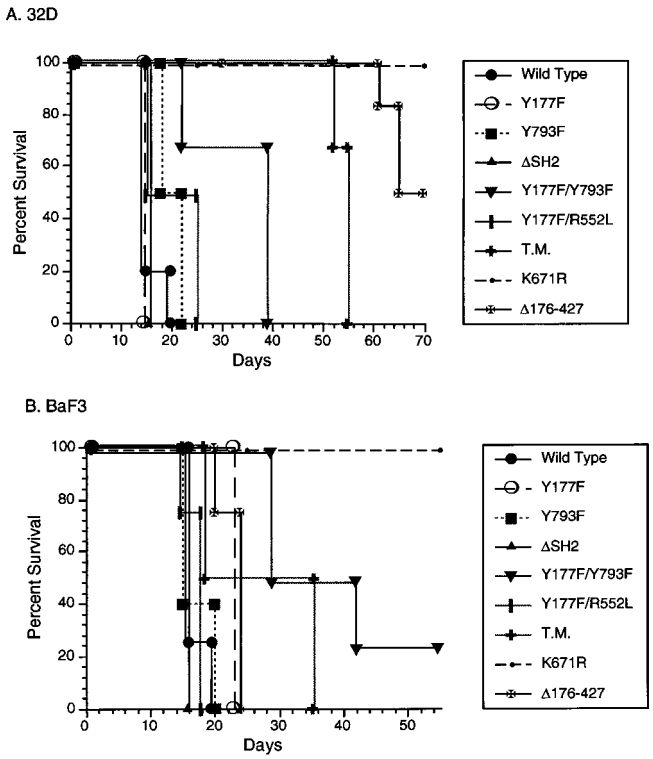


FIG. 5. Survival curves of mice implanted with BCR-ABL-expressing 32D and BaF3 cells. A total of 5×10^6 32D cells (A) or 10^6 BaF3 cells (B) expressing the indicated BCR-ABL mutant proteins were injected subcutaneously into nude mice. The mice were monitored for tumor progression and sacrificed when the tumors reached a size of 1 cm^3. T.M., Y177F/R552L/Y793F triple mutant.

In contrast to the results obtained in BaF3 cells, 32D cells expressing the Δ176-427 BCR-ABL mutant did not cause tumors in three of six nude mice (Table 1). Three of the six mice developed tumors that did not appear until 7 weeks postinjection, compared with a 1-week latency for mice injected with 32D cells expressing wild-type BCR-ABL and the majority of the other BCR-ABL mutants. While the mice injected with 32D cells expressing the wild-type, Y177F, Y793F, ΔSH2, and Y177F/R552L BCR-ABL proteins developed tumors that reached a size of 1 cm^3 at approximately 20 days postinjection, mice injected with 32D cells expressing the Y177F/R552L/Y793F triple-mutant BCR-ABL protein developed tumors of the same size at approximately 52 to 54 days postinjection (Fig. 5A). Also, tumors in mice injected with 32D cells expressing the Y177F/Y793F BCR-ABL protein progressed at approximately one-half the rate of the tumors produced by the wild-type BCR-ABL-expressing 32D cells (Fig. 5A). These results demonstrate that the structural requirements for BCR-ABL-induced transformation are cell context dependent. Combining mutations in the GRB2 binding site (Y177F), SH2 domain (R552L), and kinase domain autophosphorylation site (Y793F) within the same BCR-ABL molecule severely reduces its tumorigenic potential. Furthermore, the viability and transforming signals elicited by BCR-ABL are separable, as demonstrated by the ability of the Δ176-427 BCR-ABL mutant expressed in 32D cells to inhibit apoptosis without causing tumorigenic growth in some of the mice injected with these cells.

***myc* mRNA is elevated by BCR-ABL expression.** Potential downstream effectors of BCR-ABL were examined to correlate the biological activities of the BCR-ABL mutant proteins with disruption of specific signaling pathways. We focused on 32D cells because the Δ176-427 and Y177F/R552L/Y793F proteins exhibited markedly distinct biological activities compared with wild-type BCR-ABL in this cell type. Activating *abl* oncogenes have previously been shown to cause growth factor independence and transformation via pathways involving the nuclear *myc* proto-oncogene (7, 49). Furthermore, activation of a Ras-dependent pathway and the induction of *myc* are both required for the proliferative effects of many cytokines (47, 50). Therefore, we examined whether the Δ176-427 and Y177F/R552L/Y793F BCR-ABL proteins were capable of elevating the level of *myc* expression in 32D cells. Northern analysis indicates that *myc* is induced by most of the BCR-ABL mutant proteins, including p185 Δ176-427. Only the kinase-defective and Y177F/R552L/Y793F BCR-ABL molecules did not elevate *myc* mRNA (Fig. 6). These results indicate that the lack of tumorigenicity of the 32D Δ176-427 BCR-ABL-expressing cells cannot be explained by the lack of *myc* induction. The region between amino acids 176 and 427 has a number of identified functions, including BCR-serine kinase activity (26), 14-3-3 protein binding (44), and GRB2 binding (39). Further mutational analysis of this region will be needed to identify the specific sequences which are required for BCR-ABL-induced proliferation and transformation.

Ras activation correlates with the antiapoptotic activity of BCR-ABL. Ras activation is a central component of many mitogenic signaling pathways, and abnormal Ras activation is frequently observed in tumor cells. We have shown that Ras activation is a critical component of BCR-ABL transformation of Rat1 fibroblasts (16, 39). In fibroblasts, the interaction between phosphorylated tyrosine 177 of BCR-ABL and the GRB2 SH2 domain appears to be necessary for Ras activation, since mutation of this tyrosine to a phenylalanine blocks Ras-dependent transcriptional activation (39). However, expression of Y177F BCR-ABL in 32D and BaF3 cells blocked apoptosis and induced tumorigenic growth in mice, similar to findings for the wild-type BCR-ABL protein. Therefore, we sought to determine whether these and other BCR-ABL mutant proteins were capable of activating Ras in hematopoietic cell lines and to correlate this ability with the biological properties of the mutant proteins. Ras activation was analyzed by direct exam-

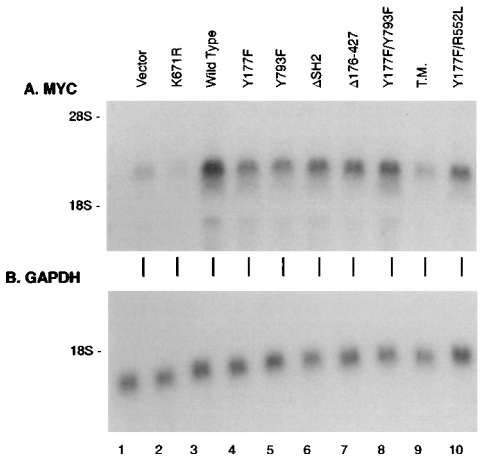


FIG. 6. Induction of *myc* RNA expression in BCR-ABL-expressing 32D cells. Cells were starved for serum and IL-3 for 5 h before RNA extraction. Ten micrograms of total cellular RNA was isolated from 32D cells expressing the constructs indicated above the lanes (T.M. denotes the Y177F/R552L/Y793 triple mutant). Following electrophoresis, the blot was probed for *myc* RNA (A) and then stripped and reprobed for *GAPDH* RNA (B).

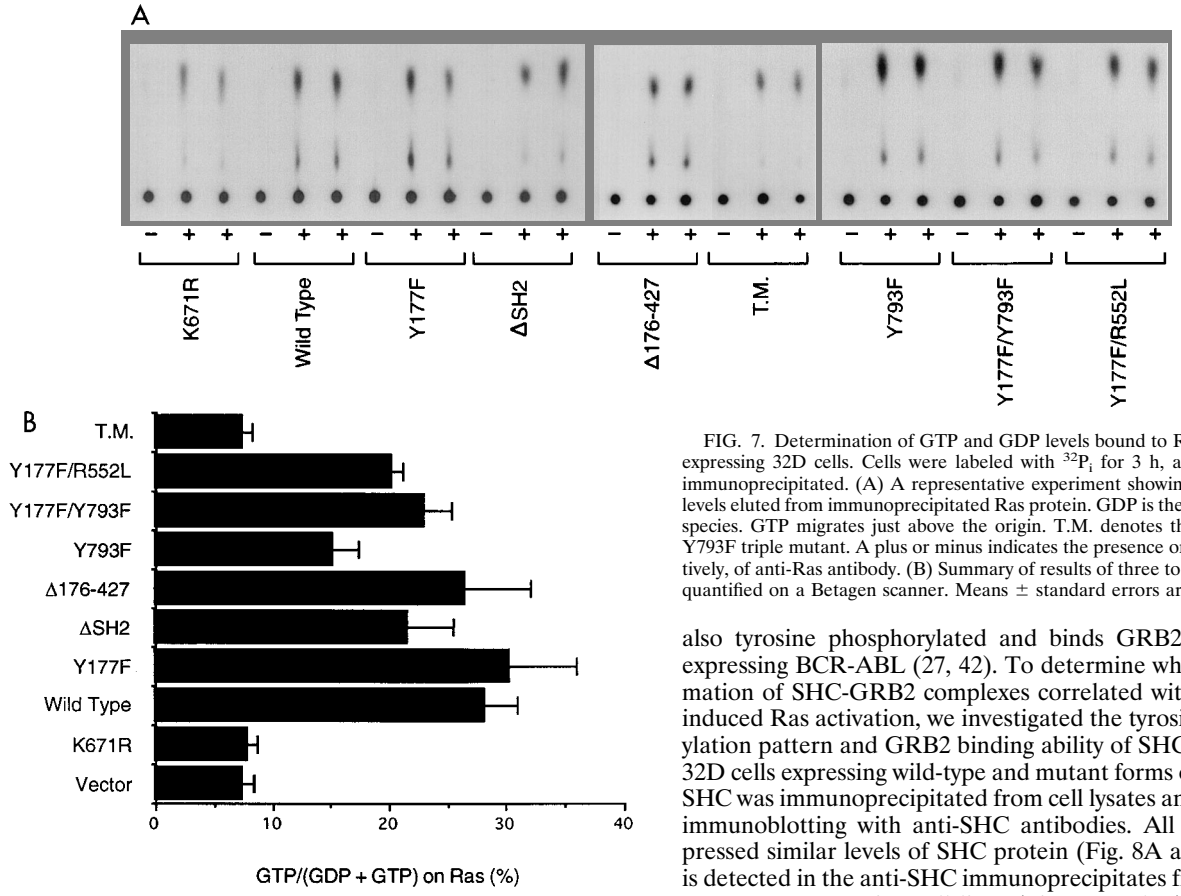


FIG. 7. Determination of GTP and GDP levels bound to Ras in BCR-ABL-expressing 32D cells. Cells were labeled with $^{32}P_i$ for 3 h, and then Ras was immunoprecipitated. (A) A representative experiment showing GTP and GDP levels eluted from immunoprecipitated Ras protein. GDP is the fastest-migrating species. GTP migrates just above the origin. T.M. denotes the Y177F/R552L/Y793F triple mutant. A plus or minus indicates the presence or absence, respectively, of anti-Ras antibody. (B) Summary of results of three to four experiments quantified on a Betagen scanner. Means ± standard errors are shown.

ination of Ras-GTP levels in 32D cells stably expressing various BCR-ABL mutants. We found that expression of the majority of the mutant BCR-ABL molecules, including the Y177F and Δ176-427 proteins, led to significant increases in GTP bound to Ras (Fig. 7). Ras activation was assessed in cells expressing Δ176-427 BCR-ABL prior to acquisition of IL-3-independent growth and at the same time as the tumorigenicity assays were performed. Only the kinase-defective K671R BCR-ABL mutant and the Y177F/R552L/Y793F BCR-ABL mutant did not activate Ras in the 32D cells (Fig. 7).

Interestingly, Ras is activated in Y177F/R552L/Y793F BCR-ABL-expressing BaF3 cells (data not shown), which correlates with the ability of this mutant to block apoptosis in these cells (Fig. 2B and 3A). Our results show that all of the tumorigenic cells had constitutively activated Ras, but activated Ras is not sufficient to fully transform the 32D Δ176-427 BCR-ABL cells. However, Ras activation by the various BCR-ABL forms correlates with their ability to inhibit apoptosis in both 32D and BaF3 cells.

SHC-GRB2 association correlates with Ras activation. The BCR-ABL Y177F mutant protein, deficient in GRB2 binding, activates Ras in 32D and BaF3 cells. This finding suggests that in addition to direct interaction with GRB2, the activated BCR-ABL tyrosine kinase may recruit multiple signaling molecules to induce Ras activation in these hematopoietic cell lines. Recently, the existence of many routes from activated tyrosine kinases to Ras has been proposed (34). Several proteins, including the SHC adapter, have been implicated in Ras activation (34). SHC is tyrosine phosphorylated in cells stimulated by epidermal growth factor and insulin (36, 51). SHC is also tyrosine phosphorylated and binds GRB2 in cell lines expressing BCR-ABL (27, 42). To determine whether the formation of SHC-GRB2 complexes correlated with BCR-ABL-induced Ras activation, we investigated the tyrosine phosphorylation pattern and GRB2 binding ability of SHC in BaF3 and 32D cells expressing wild-type and mutant forms of BCR-ABL. SHC was immunoprecipitated from cell lysates and detected by immunoblotting with anti-SHC antibodies. All cell lines expressed similar levels of SHC protein (Fig. 8A and C). GRB2 is detected in the anti-SHC immunoprecipitates from wild-type BCR-ABL-expressing cell lines (Fig. 8B and D), and SHC is tyrosine phosphorylated (data not shown). Similar results were obtained for both BaF3 and 32D cells expressing the following BCR-ABL mutants: Y177F, ΔSH2, Y793F, Δ176-427, Y177F/Y793F, and Y177F/R552L. In contrast, expression of the kinase-defective K671R BCR-ABL mutant failed to elicit the formation of GRB2-SHC complexes and cause SHC tyrosine phosphorylation in either 32D or BaF3 cells. The Y177F/R552L/Y793F mutant behaved differently in the two cell types. In BaF3 cells, expression of Y177F/R552L/Y793F BCR-ABL caused formation of a SHC-GRB2 complex, but SHC-GRB2 complexes were not detected in 32D cells expressing this mutant (Fig. 8B and 8D, lanes 6). Thus, the formation of SHC-GRB2 complexes correlates with BCR-ABL-induced Ras activation in both 32D and BaF3 cells.

DISCUSSION

Prolongation of hematopoietic cell survival has been proposed to be an important component of BCR-ABL-induced leukemias (6). To elucidate the mechanisms whereby BCR-ABL elicits cell survival, we compared the structural and signaling requirements for the antiapoptotic and transforming activities of BCR-ABL in hematopoietic cells. We have demonstrated that BCR-ABL's antiapoptotic and transforming activities result from overlapping and distinct signals which include Ras activation and *myc* RNA induction.

The structural requirements for BCR-ABL-induced Ras activation and transformation are different in hematopoietic and fibroblast cells. Disruption of the SH2 domain severely impairs transformation in Rat1 fibroblasts but has no effect in either BaF3 or 32D cells (reference 31 and Table 1). We have pre-

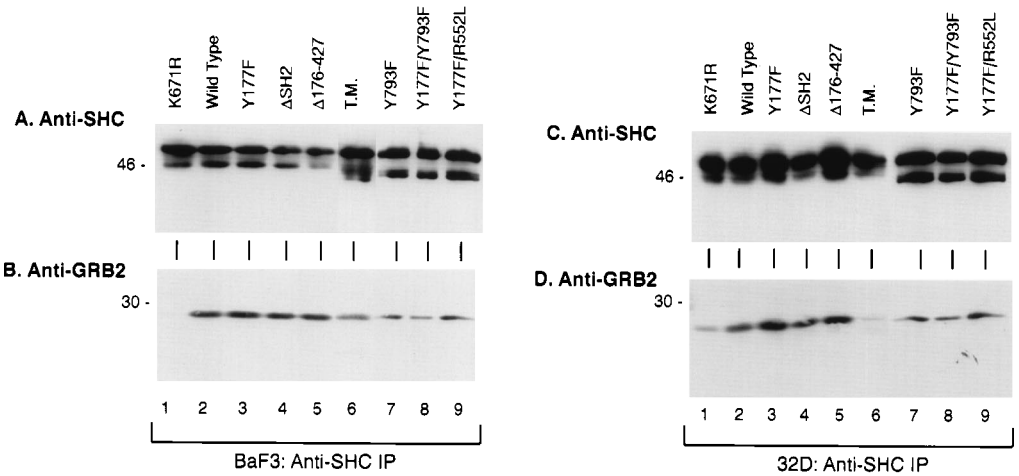


FIG. 8. SHC is complexed with GRB2 following expression of mutant and wild-type BCR-ABL. SHC was immunoprecipitated (IP) from lysates of equal numbers of BaF3 (A and B) and 32D (C and D) cells expressing the constructs indicated above the lanes (T.M. denotes the Y177F/R552L/Y793F triple mutant). Following washing, the immunocomplexes were solubilized in SDS sample buffer, subjected to SDS-PAGE, and blotted with the indicated antibodies.

viously shown that a phosphorylated tyrosine at position 177 in BCR-ABL binds to GRB2 and is essential for transformation and Ras activation in fibroblasts (39). However, expression of the Y177F BCR-ABL mutant in 32D and BaF3 cells elicits Ras activation and transformation. These data suggest that BCR-ABL utilizes multiple pathways to activate Ras, depending on the cell context. Similarly, the platelet-derived growth factor (PDGF) receptor autophosphorylation sites required for Ras activation have been shown to be cell context dependent. PDGF receptor mutations that abolish PDGF-stimulated Ras activation in fibroblasts do not impair PDGF-stimulated Ras activation in BaF3 cells (48).

Potential mediators of BCR-ABL-induced Ras activation in hematopoietic cells include the SHC adapter protein and the SYP tyrosine phosphatase (54, 55). SHC phosphorylation and SHC-GRB2 complex formation correlate with Ras activation in both 32D and BaF3 cells expressing wild-type and mutant BCR-ABL proteins. SYP is also tyrosine phosphorylated and complexed with GRB2 in BCR-ABL-expressing 32D and BaF3 cells (36a). Both SHC-GRB2 and SYP-GRB2 complexes have been implicated in epidermal growth factor- or PDGF-stimulated Ras activation (23, 28, 36, 41, 45). Our data suggest that this network of protein interactions which normally provides fine-tuning control over Ras activation can be recruited by BCR-ABL to participate in transformation of hematopoietic cells. Interestingly, SHC has been reported to be tyrosine phosphorylated and associated with GRB2 in fibroblasts expressing BCR-ABL proteins (42). However, SHC does not appear to complement the defect in Ras activation observed in fibroblasts expressing the $p185^{bcr\text{-}abl}$ Y177F mutant (39).

There appear to be differences in the structural requirements for the antiapoptotic and transforming properties of BCR-ABL among different hematopoietic cell lines. Expression of the Y177F/R552L/Y793F and the Δ176-427 BCR-ABL mutants produced different effects in BaF3 lymphoid cells and 32D myeloid cells. The differences are not a result of differences in the retroviruses used, since the same retroviral stocks of these mutant molecules yielded different biological consequences in the two cell types. The disparate results may reflect normal differences in the signaling components of the cell types or inherent differences acquired by mutation during cell culture.

The biological activity of the BCR-ABL Δ176-427 deletion mutant illustrates the requirements for multiple signaling pathways for the proliferative actions of BCR-ABL. This mutant inhibits apoptosis in 32D cells without causing tumorigenic growth (Table 2). The viability signals initiated by BCR-ABL constitute a subset of the signals required for full malignant transformation. Expression of BCR-ABL Δ176-427 activates Ras. Ras activation is not sufficient for BCR-ABL Δ176-427 to induce transformation or proliferation, but it correlates with inhibition of apoptosis by this BCR-ABL mutant. Recent work on the common β_c subunit of the IL-3 and granulocyte-macrophage colony-stimulating factor receptors in BaF3 cells suggests that proliferative and antiapoptotic signals from these receptors are distinct (20). Receptor mutants, which were defective in Ras activation, were capable of inducing DNA synthesis. However, these mutants failed to prevent apoptosis. Furthermore, expression of an activated Ras allele blocked apoptosis following IL-3 deprivation (20). Thus, Ras activation appears to be involved in the antiapoptotic effects of both IL-3 and BCR-ABL in BaF3 cells. Furthermore, our data indicate that Ras activation is a necessary but not sufficient component of BCR-ABL-induced transformation.

Introduction of a deregulated tyrosine kinase in cultured hematopoietic cells is not sufficient to cause abnormal proliferation or tumorigenicity in animals. The Δ176-427 BCR-ABL mutant exhibits elevated tyrosine kinase activity both in vitro and in vivo but has severely impaired transforming activity in 32D cells. Thus, tyrosine kinase activity is necessary but not sufficient for BCR-ABL-induced transformation.

The biological activity of the Y177F/R552L/Y793F BCR-ABL mutant provides insights into the structural requirements for in vivo tyrosine kinase activity and the signaling properties of BCR-ABL. Mutation of the autophosphorylation sites Y177 and Y793 or deletion of the ABL SH2 domain alone does not diminish BCR-ABL-induced transformation of 32D or BaF3 cells or the ability of BCR-ABL to block apoptosis in these cells. Two double-mutant BCR-ABL proteins, Y177F/Y793F and Y177F/R552L, retain the ability to block apoptosis, cause factor-independent growth, and form tumors in animals. In contrast, expression of the Y177F/R552L/Y793F BCR-ABL triple mutant in 32D cells did not protect the cells from undergoing apoptosis following cytokine removal, and this mutant exhibited decreased transforming properties. These results may indicate that a single signaling mechanism dependent

TABLE 2. Summary of biological and biochemical properties of wild-type and mutant BCR-ABL proteins in 32D cells

$p185^{bcr\text{-}abl}$ construct	Block of apoptosis	IL-3 independence	Tumorigenicity	Ras activation	*myc* induction	SHC-GRB2 complex
K671R	−	−	−	−	−	−
Wild type	+	+	+	+	+	+
Y177F	+	+	+	+	+	+
ΔSH2	+	+	+	+	+	+
Δ176-427	+	−/+[a]	−/+[b]	+	+	+
Y793F	+	+	+	+	+	+
Y177F/Y793F	+	+	+	+	+	+
Y177F/R552L	+	+	+	+	+	+
Y177F/R552L/Y793F	−	−	−/+[c]	−	−	−

[a] These cells proliferated without IL-3 only after prolonged culture, in contrast with the immediate IL-3-independent proliferation induced by the majority of the other BCR-ABL mutant proteins.

[b] Three of six mice did not form tumors. The tumors in the remaining three mice were not observable until 7 weeks postinjection, compared with a 1-week latency of tumor formation induced by the majority of the other BCR-ABL mutants.

[c] Six of six mice developed tumors. The latency of tumor formation was fivefold longer than in mice injected with the wild-type BCR-ABL-expressing 32D cells, and the tumors progressed significantly more slowly.

on the integrity of all three structural motifs is impaired in the triple mutant. Alternatively, multiple redundant signaling pathways may exist within these cells. The marked reduction in the tyrosine phosphorylation of intracellular proteins and in autophosphorylation in cells expressing the Y177F/R552L/Y793F BCR-ABL mutant suggested initially that combining these three mutations had impaired the tyrosine kinase activity of the Y177F/R552L/Y793F protein. However, we showed that this protein exhibited autophosphorylation and substrate phosphorylation activities that are similar to those of the wild-type protein in vitro (Fig. 4). It is possible that the presence of the three mutations in one molecule prevents functional dimerization of BCR-ABL proteins which is required for transphosphorylation in vivo (30). This mutant may be defective in binding to a critical protein which is normally associated with BCR-ABL and acts to transduce specific intracellular signals. The decrease in general intracellular tyrosine phosphorylation levels may indicate that these three sites are important for recruitment of substrates or protection of phosphorylated sites from phosphatase action. Further experimentation is required to distinguish between these possibilities. Regardless of the mechanism(s) that leads to inactivation of the Y177F/R552L/Y793F BCR-ABL protein, the diminished biological activities of this mutant in 32D cells may be explained by its inability to activate Ras and induce *myc* RNA expression.

In BaF3 cells, the Y177F/R552L/Y793F BCR-ABL protein is capable of slightly increased auto- and transphosphorylation over that observed in BaF3 cells expressing vector alone (data not shown). In BaF3 cells, this mutant elicits Ras activation and SHC phosphorylation (data not shown). The ability of the Y177F/R552L/Y793F BCR-ABL mutant to cause transformation and block apoptosis in BaF3 cells correlates with its ability to activate Ras in these cells. It is possible that the slightly higher level of tyrosine phosphorylation observed in BaF3 cells expressing this mutant than in 32D cells may account for these differences. Alternatively, BaF3 cells may have acquired mutations that complement the defective signaling pathway for the Y177F/R552L/Y793F BCR-ABL protein.

Our results indicate that BCR-ABL can function to activate both a Ras-dependent signaling pathway and a pathway leading to *myc* RNA induction. Many oncogenes are incapable of causing transformation of IL-3-dependent cells without abnormal Myc expression. v-Raf and activated Ras alleles delay apoptosis in hematopoietic cells, and expression of v-Raf and expression of v-Myc cooperate to abrogate IL-3 dependence of 32D cells, but expression of these oncogenes alone does not elicit IL-3-independent growth (20, 57). Activated *abl* oncogenes appear to cause IL-3-independent growth partly because they can activate both Ras and Myc signaling pathways which are required for full BCR-ABL-induced transformation (7, 16, 39, 49). Interestingly, Myc expression in the absence of a Ras-dependent signal leads to apoptosis (3, 4). It will be interesting to determine whether BCR-ABL mutants which retain the ability to induce *myc* RNA but do not activate Ras can be found. These BCR-ABL proteins may be expected to induce, rather than to inhibit, apoptosis.

A recent report suggests that *myc* induction may be mediated through the ABL SH2 domain since hyperexpression of Myc restored fibroblast transforming activity to a BCR-ABL SH2 point mutant (1). Our data indicate that a BCR-ABL molecule lacking the SH2 domain can increase *myc* RNA in 32D cells to levels which are comparable to those obtained with wild-type BCR-ABL. Thus, the BCR-ABL-induced pathway that leads to *myc* RNA induction in hematopoietic cells has yet to be elucidated.

Controversy exists regarding the contribution of prolonged cell survival and mitogenic signaling pathways to the oncogenicity of BCR-ABL. It has been suggested that antiapoptotic but not mitogenic signaling is the primary consequence of BCR-ABL expression in BaF3 cells, as expression of a temperature-sensitive $p210^{bcr\text{-}abl}$ protein did not lead to immediate IL-3-independent proliferation in these cells (19). In contrast, another report indicated that a distinct temperature-sensitive $p210^{bcr\text{-}abl}$ mutant protein produced immediate IL-3-independent proliferation after shift to the permissive temperature in 32D cells (5). Our data are based on stable transfectants of 32D and BaF3 cells; therefore, we cannot directly assess whether the biological and signaling effects that we have observed are a primary or secondary consequence of BCR-ABL expression. However, we favor a model in which BCR-ABL is directly responsible for initiating signaling pathways which cause Ras activation and *myc* mRNA induction in cultured hematopoietic cells. The short time between infection and selection of a stable BCR-ABL-expressing mass population in the absence of selective pressure (since IL-3 is maintained in the media), combined with the observation that usually more than 90% of the G418-selected cells proliferate in the absence of growth factor, argues against a model in which secondary mutations gives rise to the growth factor-independent proliferating cells. Further support for Ras activation by BCR-ABL expression comes from the observation that wild-type BCR-ABL causes transcriptional activation from an *ets*/AP1 pro-

moter in a transient-transfection assay (39). Whether Ras activation and growth factor-independent proliferation are primary or secondary consequences of BCR-ABL expression, our data are consistent with the hypothesis that BCR-ABL expression is the initiating event for these biochemical and biological changes.

The antiapoptotic effect of BCR-ABL combined with deregulated mitogenic signals may cause the myeloid expansion observed during the chronic phase of CML. Abnormal cell survival can facilitate the accumulation of secondary mutations that lead to blast crisis. By analyzing the structural and signaling components required for BCR-ABL's antiapoptotic and transforming effects, we have demonstrated that they have overlapping but also distinct signaling requirements. BCR-ABL initiates multiple signaling pathways which are different in hematopoietic and fibroblast cells, and as a result, the structural requirements for BCR-ABL transformation are cell context dependent. Our results suggest that the antiapoptotic properties of BCR-ABL are an important component of its tumorigenic potential in hematopoietic cells. Understanding the mechanisms whereby BCR-ABL elicits a block of apoptosis may allow for the development of specific therapeutic strategies for patients with Ph^1-positive leukemias.

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