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A Direct Binding Site for Grb2 Contributes to Transformation and Leukemogenesis by the Tel-Abl (ETV6-Abl) Tyrosine Kinase

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A direct binding site for the Grb2 adapter protein is required for the induction of fatal chronic myeloid leukemia (CML)-like disease in mice by Bcr-Abl. Here, we demonstrate direct binding of Grb2 to the Tel-Abl (ETV6-Abl) fusion protein, the product of complex (9;12) chromosomal translocations in human leukemia, via tyrosine 314 encoded by *TEL* exon 5. A Tel-Abl point mutant (Y314F) and a splice variant without *TEL* exon 5 sequences (Δe5) lacked Grb2 interaction and exhibited decreased binding and phosphorylation of the scaffolding protein Gab2 and impaired activation of phosphatidylinositol 3-kinase, Akt, and extracellular signal-regulated kinase/mitogen-activated protein kinase in hematopoietic cells. Tel-Abl Y314F and Δe5 were unable to transform fibroblasts to anchorage-independent growth and were defective for B-lymphoid transformation in vitro and lymphoid leukemogenesis in vivo. Previously, we demonstrated that full-length Tel-Abl induced two distinct myeloproliferative diseases in mice: CML-like leukemia similar to that induced by Bcr-Abl and a novel syndrome of small-bowel myeloid infiltration endotoxemia and hepatic and renal failure. Lack of the Grb2 binding site had no effect on development of small bowel syndrome but significantly attenuated the induction of CML-like disease by Tel-Abl. These results suggest that direct binding of Grb2 is a common mechanism contributing to leukemogenesis by oncogenic Abl fusion proteins.

The *BCR-ABL* oncogene, the product of the t(9;22) Philadelphia (Ph) chromosome translocation, encodes a dysregulated cytoplasmic protein-tyrosine kinase, Bcr-Abl, that is the direct cause of the myeloproliferative disease chronic myeloid leukemia (CML) and Ph$^+$ acute B-lymphoblastic leukemia (B-ALL). Bcr-Abl activates multiple intracellular signaling pathways including Ras, mitogen-activated protein kinase (MAPK), Jun N-terminal kinase (JNK), STAT5, and phosphatidylinositol 3-kinase (PI 3-kinase) (52) and transforms fibroblasts (33), cytokine-dependent hematopoietic cell lines (6,18), and primary bone marrow B-lymphoid cells (36) in vitro. Retroviral transduction of the *BCR-ABL* gene into murine bone marrow followed by transplantation into irradiated recipient mice results in the development of either CML-like myeloproliferative disease (30, 44, 62) or B-ALL (50) in all recipients, depending on the transduction conditions. The mouse retroviral bone marrow transduction/transplantation system provides accurate and quantitative models of human CML and Ph$^+$ B-ALL (58) that have proven useful for analyzing the molecular pathophysiology of these diseases (15, 29, 30, 39, 50, 63).

Fusion of the *ABL* gene to a different partner, the *TEL* (*ETV6*) gene on chromosome 12p13, has been reported to occur in a small number of patients with leukemia, some who had acute leukemia of B-lymphoid (43), T-lymphoid (60), or myeloid (13, 40) origin and some who presented with atypical (3, 28) or typical (1, 60) CML. *TEL* encodes a ubiquitously expressed 452-amino-acid protein with homology to the Ets family of transcription factors (12). Two different *TEL-ABL* fusions have been observed. In two patients (one with B-ALL and one with atypical CML), the first four exons of *TEL* were fused to *ABL* exon 2, while the other patients had *TEL* exons 1 to 5 fused to *ABL* exon 2. The resulting chimeric Tel-Abl proteins contain Tel amino acids 1 to 154 or 1 to 336, respectively, fused to the same 1,104 COOH-terminal amino acids of c-Abl that are found in the Bcr-Abl fusion proteins. Both Tel-Abl fusion proteins have an NH$_2$-terminal region of Tel (the PNT homology domain) that mediates homo-oligomerization (13, 26), exhibit increased tyrosine kinase activity (13, 43), and transform cytokine-dependent Ba/F3 hematopoietic cells to cytokine independence (13, 17). Recently, we tested the ability of the larger Tel-Abl fusion protein to induce myeloid leukemia in mice by using the retroviral bone marrow transduction/transplantation model. Under conditions where p210 Bcr-Abl induces fatal CML-like myeloproliferative disease in all recipients within 4 weeks, Tel-Abl induced two distinct diseases, CML-like leukemia that was very similar to that induced by Bcr-Abl and a novel fatal syndrome characterized by small-bowel myeloid cell infiltration and necrosis, increased levels of circulating endotoxin and tumor necrosis factor alpha, and fulminant hepatic and renal failure (38). Disease induction required both the Tel PNT oligomerization domain and Abl tyrosine kinase activity. These results demonstrate that Tel-Abl has different leukemogenic properties from Bcr-Abl.

Bcr-Abl binds directly to the SH2 domain of the Grb2

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adapter protein through phosphorylated tyrosine 177 of Bcr (46, 48). The importance of direct binding of Grb2 by Bcr-Abl has been controversial; although the initial report suggested that a Bcr-Abl Y177F mutant, which cannot bind Grb2, was completely defective for transformation of fibroblasts and primary bone marrow B-lymphoid cells (46), subsequent studies demonstrated that Bcr-Abl Y177F could transform both primary B cells (11) and cytokine-dependent hematopoietic cell lines in vitro (5, 11). While transactivation of a Ras-responsive reporter gene by Bcr-Abl in fibroblasts is impaired by the Y177F mutation (46), there is no defect in Ras activation in 32D myeloid cells expressing this mutant (5). However, a critical role for the Grb2 binding site in leukemogenesis by Bcr-Abl was conclusively established by the recent demonstration that the p210 Bcr-Abl Y177F mutant is completely defective for induction of fatal CML-like myeloproliferative disease in mice by using the retroviral bone marrow transduction-transplantation model (21, 39, 63). The product of the transforming gene of Abelson murine leukemia virus, p160 v-Abl, lacks direct binding to Grb2 (39) and is also incapable of inducing CML-like leukemia in mice (14, 39). These results suggest that direct binding of Grb2 contributes to the induction of myeloproliferative disease by activated Abl proteins.

To extend the generality of this conclusion, we searched for evidence of direct binding of Grb2 to the Tel-Abl fusion proteins. Here, we confirm that Tel-Abl contains a direct binding site for the Grb2 SH2 domain (41) and identify tyrosine 314 in the *TEL* exon 5-encoded sequence as the only Grb2 binding site in this fusion protein. The Grb2 binding site in Tel-Abl contributes to activation of the Gab2, PI 3-kinase, and extracellular signal-regulated kinase (ERK/MAPK) signaling pathways and is required for transformation of fibroblasts and B-lymphoid cells and for efficient induction of CML-like myeloproliferative disease by Tel-Abl in mice.

MATERIALS AND METHODS

DNA constructs. For induction of leukemia, the MSCV *neo* vector (19) was employed. *TEL-ABL* (*TEL* exon 5-*ABL* exon 2 fusion) (13) and *TEL-ABL* K581R kinase-inactive mutant cDNAs in MSCV *neo* were described previously (38). The *TEL-ABL* Y314F and Δe5 mutants were generated by enzymatic inverse PCR (23); the mutations were confirmed by DNA sequencing and subcloned into the parental MSCV *neo-TEL-ABL* vector. For signaling and transformation studies with NIH 3T3, Ba/F3, and primary bone marrow myeloid cells, the p210 *BCR-ABL*, p210 *BCR-ABL* Y177F (39, 46), *TEL-ABL*, *TEL-ABL* Y314F, *TEL-ABL* Δe5, and *TEL-ABL* K581R cDNAs were introduced into the retroviral expression vector MINV *neo* (20) at a position 5′ to the internal ribosomal entry site followed by a neomycin resistance gene.

Virus stocks. High-titer, helper virus-free retroviral stocks were prepared by transient transfection of 293T cells, using the *kat* ecotropic packaging system (7), as described previously (30). All viral stocks had titers of 3×10^6 to 5×10^6 neomycin-resistant CFU per ml in NIH 3T3 cells and gave equivalent proviral copy number in transduced NIH 3T3 or primary bone marrow cells as determined by Southern blotting.

In vitro kinase assay. Tel-Abl and Bcr-Abl proteins were expressed in 293 cells, immunoprecipitated with anti-Abl antisera, and subjected to an immune complex kinase assay utilizing a glutathione *S*-transferase (GST)-Crk substrate as described previously (30, 50). To normalize for the amount of Abl proteins, cells were labeled with L-[^{35}S]methionine before being harvested. The amount of ^{35}S and ^{32}P incorporation was quantitated by PhosphorImager analysis (STORM 850; Molecular Dynamics), and the relative protein levels were corrected for methionine content and used to calculate the relative kinase activity.

Grb2 interaction assays. Analysis of Grb2 binding to Bcr-Abl and Tel-Abl proteins by far-Western blotting with a GST-SH2 fusion protein (41) or coimmunoprecipitation was performed as described previously (34, 39).

Signal transduction analysis. For analysis of cell signaling, Bcr-Abl and Tel-Abl proteins were expressed in Ba/F3 cells and NIH 3T3 fibroblasts. To avoid the cytostatic effect of Abl in fibroblasts that interferes with propagation of transduced cells, the permissive 4A2+ subclone (49) of NIH 3T3 cells (the kind gift of Jean Wang, University of California, San Diego, Calif.) was employed. Both cell types were transduced with *ABL* oncogenes in the MINV *neo* vector and selected for resistance to neomycin. Neomycin-resistant Ba/F3 cells (except those transduced with Tel-Abl K581R) were further selected for interleukin-3 (IL-3)-independent growth, which occurred with approximately equal efficiency, and cell lysates were prepared within 1 week of transduction following 4 h of starvation for serum (and IL-3, where appropriate). Activation of ERK, SAPK/JNK, and Akt was analyzed by Western blotting with phosphospecific antibodies (Cell Signaling Technology, Beverly, Mass.) as described previously (50). Anti-Gab2 antibodies were the kind gift of Haihua Gu (Beth Israel-Deaconess Medical Center, Boston, Mass.). PI 3-kinase activity was assessed in antiphosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, N.Y.) and anti-Abl (polyclonal anti-GEX4 antisera [59]) immunoprecipitates as described previously (25, 50).

Fibroblast transformation and Ba/F3 cell transformation. NIH 3T3 and Ba/F3 cells were transduced with retroviral stocks expressing p210 Bcr-Abl, Tel-Abl, Tel-Abl Y314F, Tel-Abl Δe5, or Tel-Abl K581R or with empty MINV *neo* vector. For fibroblast transformation, the cells were replated in soft agar 48 h posttransduction as described previously (54). Anchorage-independent colonies were counted 21 days later, and the results were expressed as colonies per 10^5 cells plated, normalized to one provial copy per cell as determined by Southern blotting of genomic DNA. For Ba/F3 transformation, cells were selected immediately posttransduction for resistance to G418 in the presence of IL-3, washed, and plated in soft agar in the absence of IL-3, as described previously (2). Colonies were counted 10 days later.

B-lymphoid transformation and leukemogenesis. For analysis of transformation of primary bone marrow B-lymphoid progenitors and induction of B-ALL by *TEL-ABL*, bone marrow from donors not pretreated with 5-fluorouracil (5-FU) was used as described previously (50) after lysis of erythrocytes with NH$_4$Cl solution. Prestimulation was omitted, and the cells were subjected to a single round of transduction and cosedimentation with retroviral stock matched for titer (3×10^6 to 5×10^6 neomycin-resistant CFU/ml) in Dulbecco minimal essential medium containing 5% WEHI-3B cell-conditioned medium. The different retroviral stocks demonstrated equivalent transduction of primary bone marrow by Southern blot determination of the proviral DNA content in genomic DNA (data not shown). Immediately after transduction, cells were plated for in vitro growth in Whitlock/Witte-style cultures as described previously (55) or transplanted into irradiated (2×450 cGy) syngeneic female recipient mice (10^6 cells each). Whitlock/Witte cultures were performed in triplicate in 24-well plates at 1×10^6, 3×10^5, 1×10^5, 3×10^4, 1×10^4, 3×10^3, 1×10^5, or 3×10^2 transduced cells per well in RPMI 1640 medium supplemented with 10% fetal calf serum, 200 μM L-glutamine, 50 μM 2-mercaptoethanol, and penicillin-streptomycin. All wells except the 1×10^6-cell well had untransduced female BALB/c bone marrow cells added to make the total number of cells equal at 1×10^6. Cells were fed twice weekly by careful removal of 0.5 ml of medium from each well and replacement with an equal volume of fresh medium without agitation. Beginning 5 days post-plating, nonadherent cells were counted daily in wells exhibiting evidence of growth, and a cell density of $\geq 10^6$ cells/well was designated as positive growth.

Induction of myeloproliferative disease. Myeloid leukemogenesis by *TEL-ABL* was assessed in the retroviral bone marrow transduction-transplantation model as described previously (38, 39). Briefly, male BALB/c mice (Taconic Farms, Germantown, Md) 6 to 12 weeks of age were primed by intravenous injection with 5-FU (200 mg/kg) 4 days before harvest. Bone marrow cells were harvested, prestimulated for 24 h in medium containing IL-3, IL-6, and stem cell factor (Peprotech, Rocky Hill, N.J.), and subjected to two rounds of retroviral transduction by cosedimentation followed by transfer of 5×10^5 bone marrow cells by lateral tail vein injection into syngeneic female mice prepared with two doses of 450 cGy of gamma irradiation. Diseased recipient mice were subjected to histopathological and biochemical analysis as described previously (30, 38). For analysis of signaling in primary myeloid cells, marrow from normal donors was transduced twice with MINV *neo* retroviruses without prestimulation and then selected for resistance to G418 (1.0 mg/ml) for 3 days in medium supplemented with IL-3, IL-6, and stem cell factor. Mononuclear cells were purified by sedimentation through Ficoll-Hypaque and deprived of serum and cytokines for 4 h, and protein lysates were prepared by direct boiling in sample buffer as described previously (38).
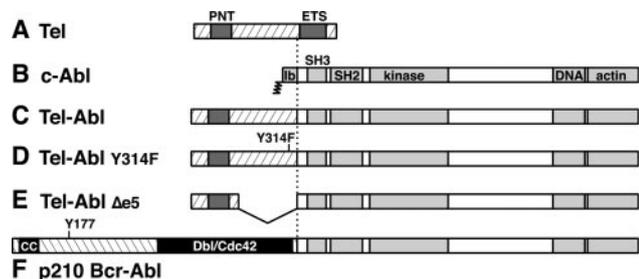
FIG. 1. Structure of Tel, Abl, and chimeric Tel-Abl and Bcr-Abl proteins. (A) Full-length Tel (ETV6) protein of 452 amino acids, with the positions of the PNT homology domain and ETS DNA-binding domain indicated by shaded boxes. (B) Full-length type Ib c-Abl protein of 1,142 amino acids, with the NH_2-terminal myristoylation site, SH3 and SH2 domains, tyrosine kinase catalytic domain, and COOH-terminal DNA and actin binding domains indicated by shaded boxes. (C) Tel-Abl fusion protein, consisting of sequences encoded by *TEL* exons 1 to 5 (amino acids 1 to 336) fused to the 1,104 COOH-terminal amino acids of c-Abl. (D) Tel-Abl Y314F mutant, containing a point mutation of Tyr314 to Phe. (E) Tel-Abl Δexon5 (Δe5), consisting of sequences encoded by *TEL* exons 1 to 4 (amino acids 1 to 154) fused to the 1,104 COOH-terminal amino acids of c-Abl (43). (F) p210 Bcr-Abl fusion protein, consisting of Bcr amino acids 1 to 927 fused to the 1,104 COOH-terminal amino acids of c-Abl, with the NH_2-terminal coiled-coil (CC) domain, Tyr177 Grb2 binding site, and region of homology to Db1/Cdc42 indicated.

RESULTS

Tyrosine 314 of Tel-Abl is a direct binding site for the Grb2 SH2 domain. In vitro binding-site selection experiments indicate that the optimal Grb2 SH2 binding sequence has an asparagine at the +2 position relative to the tyrosine (56). Previous studies with Ba/F3 cells indicated that Tel-Abl, like Bcr-Abl, could also directly bind the SH2 domain of Grb2 (41). This Grb2 binding site is likely to be in the Tel sequence because the only SH2-dependent interaction Grb2 has with Bcr-Abl is at Tyr177 of Bcr (46). Inspection of the first five exons of Tel that are fused with Abl revealed only one candidate tyrosine for direct binding of Grb2 at position 314, with a primary sequence of $Y_{314}MNH$. This residue has an asparagine at the +2 position, and the hydrophobic methionine at +1 also provides a favored interaction with Grb2 (56). Interestingly, the *TEL-ABL* fusion identified in the two patients with B-ALL B-lymphoid leukemia (43) and atypical CML (3) contains only exons 1 to 4 of *TEL* (designated here as *TEL-ABL* Δe5) and excludes Tyr314. To test whether Grb2 has a direct binding interaction with Tyr314 of Tel-Abl, we made a tyrosine-to-phenylalanine point mutation, Y314F, to disrupt any potential phosphotyrosine-SH2 interaction (Fig. 1).

A Grb2 SH2 domain-GST fusion protein was used as a probe in a far-Western blot against the full-length (exons 1 to 5) Tel-Abl fusion protein (referred to hereafter simply as Tel-Abl), the Δe5 Tel-Abl variant (Tel-Abl Δe5), and the Tel-Abl Y314F point mutant (Tel-Abl Y314F). This probe specifically bound full-length Tel-Abl but not the Δe5 or Y314F proteins, mapping the site of the Grb2 interaction to Tyr314 (Fig. 2A). In addition, Grb2 was found to coimmunoprecipitate with full-length Tel-Abl, while the Δe5 and Y314F versions of Tel-Abl lacked this interaction (Fig. 2B). As expected, Grb2 bound to p210 Bcr-Abl but not the Bcr-Abl Y177F mutant (39, 46).
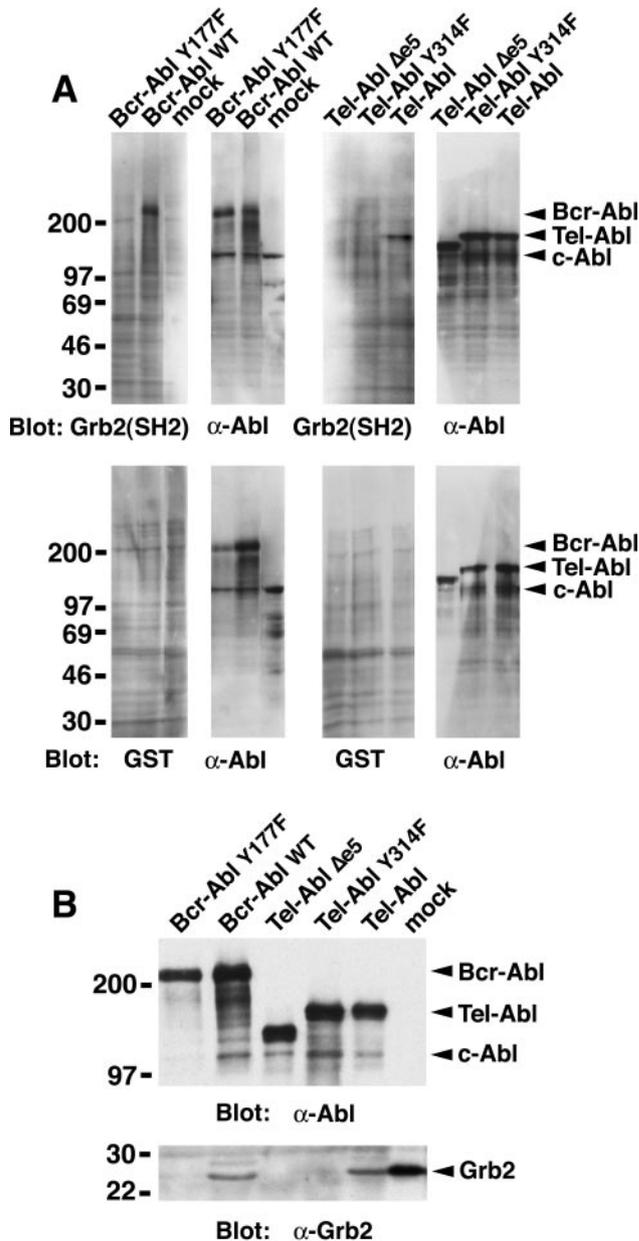

FIG. 2. Tyrosine 314 of Tel is a direct binding site for the SH2 domain of Grb2. (A) Far-Western blot. The indicated Abl proteins were expressed by transient transfection in 293T cells, and whole-cell extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose filters, and hybridized with a GST-Grb2(SH2) fusion protein (first and third panels, top row) or with GST alone (first and third panels, bottom row). Bound GST protein was detected by anti-GST antibodies and enhanced chemiluminescence. Filters were subsequently stripped and rehybridized with anti-Abl antibody (second and fourth panels of each row). Molecular mass standards are on the left, and the positions of the Bcr-Abl, Tel-Abl, and c-Abl proteins are indicated by arrowheads on the right. (B) Coimmunoprecipitation. The indicated Abl proteins were expressed by transient transfection of 293T cells, immunoprecipitated with anti-Grb2 (mock-transfected cells only [right lane]) or anti-Abl (all other samples) antibody, fractionated by SDS-PAGE, transferred to nitrocellulose, and hybridized with anti-Abl (top panel) or anti-Grb2 (bottom panel) antibody. Molecular mass standards are on the left, and the positions of the Bcr-Abl, Tel-Abl, c-Abl, and Grb2 proteins are indicated by arrowheads on the right.
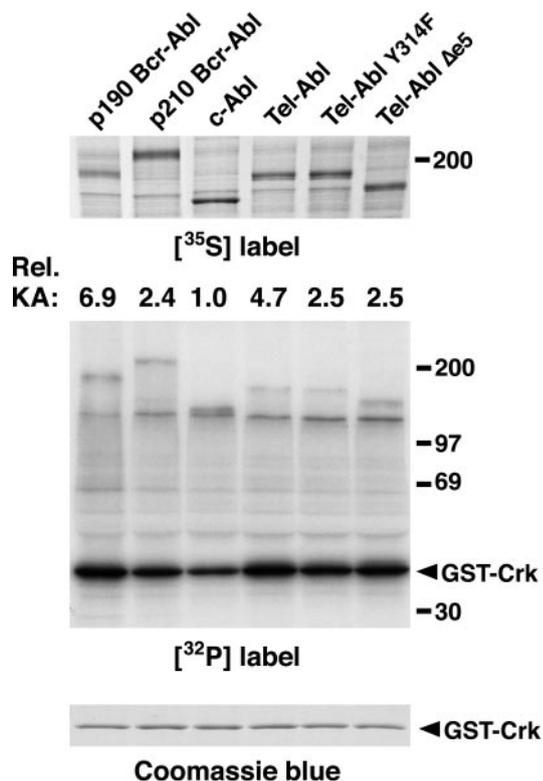
FIG. 3. In vitro tyrosine kinase activity of Tel-Abl proteins. The indicated Abl proteins were expressed by transfection of 293T cells and labeled in vivo with L-^{35}[S]methionine. The lysates were immunoprecipitated with anti-Abl antibodies, and immune complexes were incubated with [γ-^{32}P]ATP and GST-Crk substrate. Results are representative of two independent experiments. (Top) ^{35}S label, indicating relative levels of expression of the different Abl proteins. (Middle) ^{32}P label. The position of the GST-Crk substrate is indicated by the arrowhead. The kinase activity (KA) of the different Abl proteins relative to c-Abl after correction for levels of expression is shown at the top. (Bottom) Coomassie blue stain demonstrating equal amounts of the GST-Crk substrate in all reaction mixtures.

These results demonstrate that Tyr314 of Tel is phosphorylated in the Tel-Abl fusion and is the only direct binding site for Grb2.
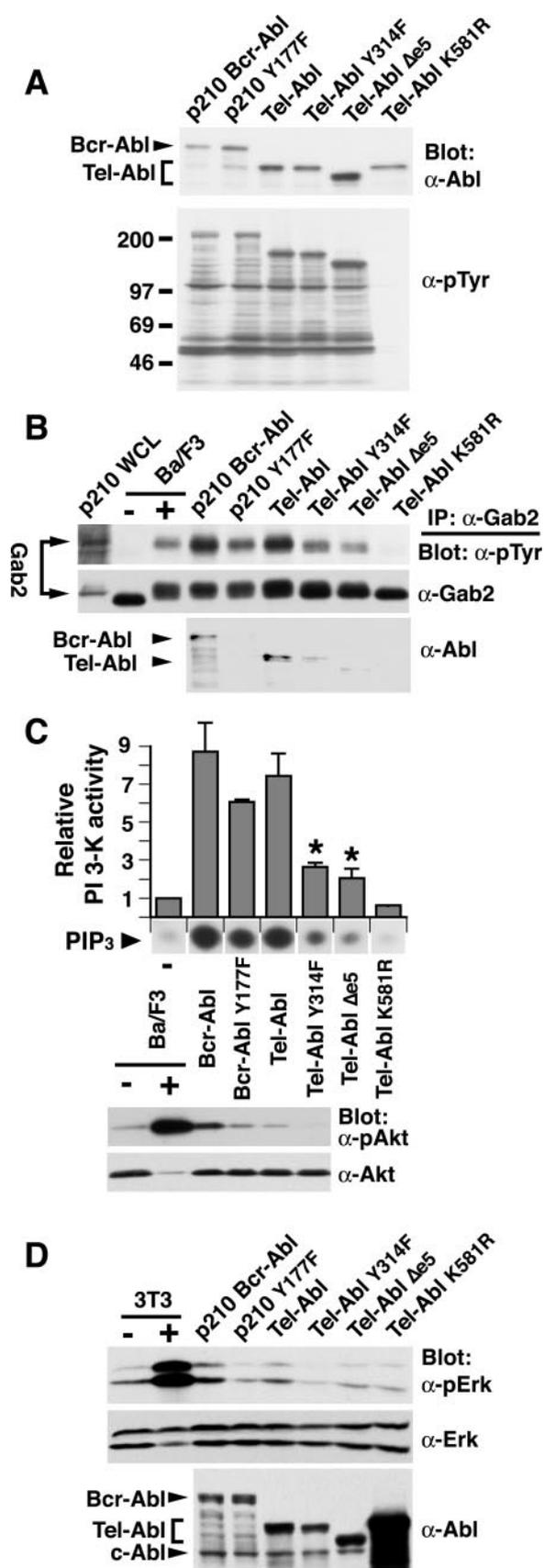
Tel-Abl fusion proteins have elevated in vitro kinase activity. We assessed the effect of Grb2 binding-site mutations on the catalytic activity of Tel-Abl in a quantitative immunoprecipitation kinase assay (Fig. 3). Relative to c-Abl, p190 and p210 Bcr-Abl exhibited increased kinase activity, in agreement with previous observations (24, 30, 32). The tyrosine kinase activity of full-length Tel-Abl was also increased and was significantly higher than that of p210 Bcr-Abl, as previously observed (38). The two Tel-Abl Grb2 binding-deficient proteins, Y314F and Δe5, had slightly reduced in vitro kinase activity, about twofold less than that of Tel-Abl and about equal to that of p210 Bcr-Abl. The reason for the reduced in vitro kinase activity of the Tel-Abl Y314F and Δe5 proteins is unclear, but it is possible that binding of Grb2 or phosphorylation at Tyr314 may stimulate Abl kinase activity in this assay.

Direct binding of Grb2 enhances activation of PI 3-kinase and ERK/MAPK by Tel-Abl and Ber-Abl. To assess signaling by the Grb2 binding mutants, the Bcr-Abl wild-type and Y177F

and Tel-Abl full-length, Y314F, Δe5, and K581R mutant proteins were stably expressed in the IL-3-dependent murine pro-B lymphoid cell line Ba/F3 (42) by retroviral transduction. The overall level and pattern of tyrosine-phosphorylated proteins were similar in Ba/F3 cells expressing the different catalytically active Abl proteins (Fig. 4A), and there were no significant differences in the constitutive activation of MAPK/ERK, SAPK/JNK, or STAT5, as assessed by Western blotting with phosphorylation-specific antibodies (data not shown). However, further analysis revealed that phosphorylation of the scaffolding/adapter protein p97 Gab2 (16) was significantly decreased in cells expressing Bcr-Abl Y177F and Tel-Abl Y314F and Δe5, and coprecipitation of these Abl fusion proteins with Gab2 was impaired (Fig. 4B). Because phospho-Gab2 is a major binding partner for activated PI 3-kinase in cytokine-stimulated hematopoietic cells (9), we assessed PI 3-kinase activity in antiphosphotyrosine immunoprecipitates from the Ba/F3 cells and observed a 35% decrease in lipid kinase activity in Bcr-Abl Y177F-expressing cells mutant and larger decreases with the Tel-Abl Y314F and Δe5 mutants (Fig. 4C). Similar results were obtained with anti-Abl immunoprecipitates (data not shown). Consistent with these observations, constitutive activation of protein kinase B/Akt, a downstream effector of PI 3-kinase, was also diminished in cells expressing the Bcr-Abl Y177F and Tel-Abl Y314F mutants (Fig. 4C). These results suggest that Grb2 mediates the binding of Gab2 to Abl fusion proteins and enhances the tyrosine phosphorylation of Gab2, contributing to the activation of PI 3-kinase and Akt in hematopoietic cells.

Another important binding partner of Grb2 is the guanine nucleotide exchange factor Sos, which activates Ras and the MAPK pathway. The Bcr-Abl Y177F mutant was reported to be defective for activation of a Ras-responsive promoter on transient transfection in fibroblasts (46), but the same mutant induced high levels of GTP-associated Ras in 32D hematopoietic cells (5). In agreement, we observed constitutive activation of Ras in Ba/F3 cells by both Bcr-Abl and Tel-Abl that was independent of a direct Grb2 binding site (data not shown). Because the mechanism of Ras activation by Bcr-Abl appears to be different between hematopoietic cell lines and fibroblasts, we also compared Ras activation by Bcr-Abl, Tel-Abl, and the Grb2 binding-site mutants in a subclone of NIH 3T3 fibroblasts (4A2) that is "permissive" for transformation by activated Abl kinases (49) and allows the propagation of cells stably expressing the various fusion proteins. Surprisingly, we were unable to demonstrate significant constitutive activation of Ras in fibroblasts by wild-type Bcr-Abl or Tel-Abl (data not shown) when using either anti-Ras immunoprecipitation of cells labeled with radioactive phosphate or a pull-down assay involving a fusion protein of GST with the activation domain of Raf (57). However, we readily detected constitutive activation of ERK/MAPK by Bcr-Abl and Tel-Abl in the same cells which was completely abolished on the loss of the respective Grb2 binding site in these proteins (Fig. 4D). These results demonstrate that the Grb2 binding site is also required for constitutive activation of the ERK/MAPK pathway by Tel-Abl, at least in some cell types.

Direct binding of Grb2 contributes to transformation of Ba/F3 and 3T3 cells by Tel-Abl. With the exception of the Tel-Abl K581R kinase-inactive mutant, all the Abl fusion pro-

FIG. 4. The Grb2 binding site contributes to activation of PI 3-kinase and MAPK by Tel-Abl. (A) Western blot of whole-cell lysates of Ba/F3 cells expressing the indicated Abl fusion proteins. (Top) Anti-Abl antibody; (bottom) antiphosphotyrosine antibody (α-pTyr). The positions of the Bcr-Abl, Tel-Abl, and c-Abl proteins and molecular mass markers are indicated. (B) Gab2 phosphorylation and complex formation. Lysates from Ba/F3 cells expressing the indicated Abl fusion proteins were immunoprecipitated (IP) with anti-Gab2 antibodies and blotted with antiphosphotyrosine (top), anti-Gab2 (middle), or anti-Abl (bottom) antibodies. As a control, lysates from parental Ba/F3 cells that were starved of IL-3 and serum ($-$) and then stimulated for 5 min with IL-3 ($+$) were included. Whole-cell lysate from p210-expressing Ba/F3 cells was included to demonstrate the position of Gab2 (arrows on the left). (C) PI 3-kinase and Akt activation. (Top). The PI 3-kinase activity in antiphosphotyrosine immunoprecipitates from Ba/F3 cells expressing the indicated Abl fusion proteins was determined and is expressed as mean fold increased activity relative to serum- and IL-3-starved parental Ba/F3 cells; error bars indicate standard error. The relative levels of incorporation of ^{32}P into PIP$_3$ product in a representative experiment are shown below the histogram. The difference in PI 3-kinase activity induced by Tel-Abl WT and either Tel-Abl Y314F or Tel-Abl Δe5 (asterisks) was statistically significant ($P = 0.05$; unpaired t test), while the difference between PI 3-kinase activity induced by p210 Bcr-Abl and p210 Y177F was only of borderline significance ($P = 0.10$; unpaired t test). (Bottom) Whole-cell lysates from the indicated cells were blotted with phospho-Akt-specific antibodies (pAkt) and subsequently with pan-Akt antibodies (Akt). (D) ERK/MAPK activation. (Top and middle) 4A2+ fibroblasts expressing the indicated Bcr-Abl and Tel-Abl proteins after retroviral transduction were deprived of serum for 4 h, and lysates were prepared, fractionated by SDS-PAGE, and blotted with anti-phospho-ERK (top) or anti-ERK (middle) antibodies. As a control, lysates from parental 4A2+ cells without ($-$) or with ($+$) stimulation with 10% serum and 20 ng of platelet-derived growth factor per ml were included. (Bottom) Expression of Abl fusion proteins in these cells, with the positions of Bcr-Abl, Tel-Abl, and c-Abl indicated by the arrowheads. The kinase-defective Tel-Abl K581R protein was expressed at significantly higher levels than the other proteins, consistent with a lack of cytostatic or toxic effect.

teins transformed Ba/F3 cells to IL-3 independence in liquid culture (data not shown), in agreement with previous observations (13, 17). However, in a very stringent assay that measures IL-3-independent colony formation (2), Tel-Abl Y314F had significantly decreased transforming activity compared with full-length Tel-Abl (Fig. 5A). p210 Bcr-Abl Y177F also demonstrated decreased transformation of Ba/F3 cells relative to wild-type Bcr-Abl, consistent with previous findings (51).

Transformation of fibroblasts to anchorage-independent growth by Bcr-Abl often correlates better with leukemogenesis than does transformation of cytokine-dependent hematopoietic cell lines. For example, the Bcr-Abl coiled-coil (55) and SH2 (50) domains are required for transformation of fibroblasts and induction of CML-like disease but not for Ba/F3 transformation (24, 55). Grb2 binding is essential for p210 Bcr-Abl to transform NIH 3T3 and Rat-1 fibroblasts (11, 46), while Tel-Abl (exons 1 to 5) has been shown previously to transform Rat-1 cells with slightly less efficiency than p210 Bcr-Abl does (13). In a quantitative transformation assay, we confirmed that full-length Tel-Abl was able to confer anchorage-independent growth in NIH 3T3 fibroblasts but did so about 10-fold less efficiently than p210 Bcr-Abl did (Fig. 5B). The discrepancy with the previous study might be explained by the difference in cell type. In contrast, Tel-Abl Δe5 and Y314F
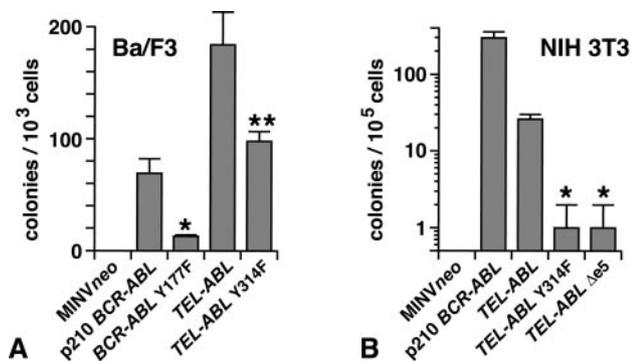
FIG. 5. The Grb2 binding site contributes to transformation of Ba/F3 cells and fibroblasts by Tel-Abl. Ba/F3 (A) and NIH 3T3 (B) cells were transduced with retrovirus lacking any insert (MINV *neo*) or containing p210 *BCR-ABL*, *TEL-ABL*, *TEL-ABL* Y314F, or *TEL-ABL* Δexon 5 (Δe5), and colony formation in soft agar was assessed as described in Materials and Methods. Error bars indicate standard deviation. (A) The difference between *BCR-ABL* WT and *BCR-ABL* Y177F (∗) and between *TEL-ABL* and *TEL-ABL* Y314F (∗∗) is statistically significant ($P = 0.005$ or $P = 0.02$, respectively; unpaired *t* test). (B) The difference between *TEL-ABL* and either *TEL-ABL* Y314F or *TEL-ABL* Δe5 (asterisks) is statistically significant ($P = 0.01$; unpaired *t* test).

Tel-Abl induced very small numbers of anchorage-independent colonies that were not significantly different from the background of vector-transduced cells. These results demonstrate that direct binding of Grb2 contributes to the transformation of fibroblasts and cytokine-dependent hematopoietic cells by Tel-Abl as well as Bcr-Abl.

In vitro transformation of primary bone marrow B-lymphoid cells by Tel-Abl requires the Grb2 binding site. We compared the ability of the different *ABL* fusion oncogenes to stimulate the growth of immature B-lymphoid cells in Whitlock/Witte-style cultures after retroviral transduction of primary murine bone marrow from donors not pretreated with 5-FU (36). The pre-B lymphoid cells that initially accumulate in such cultures are not fully transformed because they require stroma and are not fully leukemogenic in syngeneic mice. As originally reported, the assay allowed a semiquantitative comparison of the B-lymphoid transforming activity of different forms of *BCR-ABL*, because p190-transduced marrow cultures reached maximal density sooner (2 to 3 weeks) and more frequently (>90%) than did p210-transduced cultures, where only about 50% of cultures reached maximal density and this required 4 to 5 weeks (35). However, our experience has shown that *BCR-ABL*-transduced marrow cultures initiated under these conditions reach maximal density within 8 days, with p210-transduced cultures delayed only 1 or 2 days relative to p190-transduced cultures, which probably reflects the much higher retroviral titers we currently employ. We have modified this procedure to include serial dilutions of transduced marrow to restore the semiquantitative nature of the assay (55) (see Materials and Methods). Under these conditions, p190 *BCR-ABL* was again observed to be more efficient than p210, since all cultures initiated with 3×10^3 p190-transduced marrow cells grew to confluence while all p210-transduced cultures at the same density failed to grow (Fig. 6). *TEL-ABL* was relatively inefficient in B-lymphoid transformation relative to p210
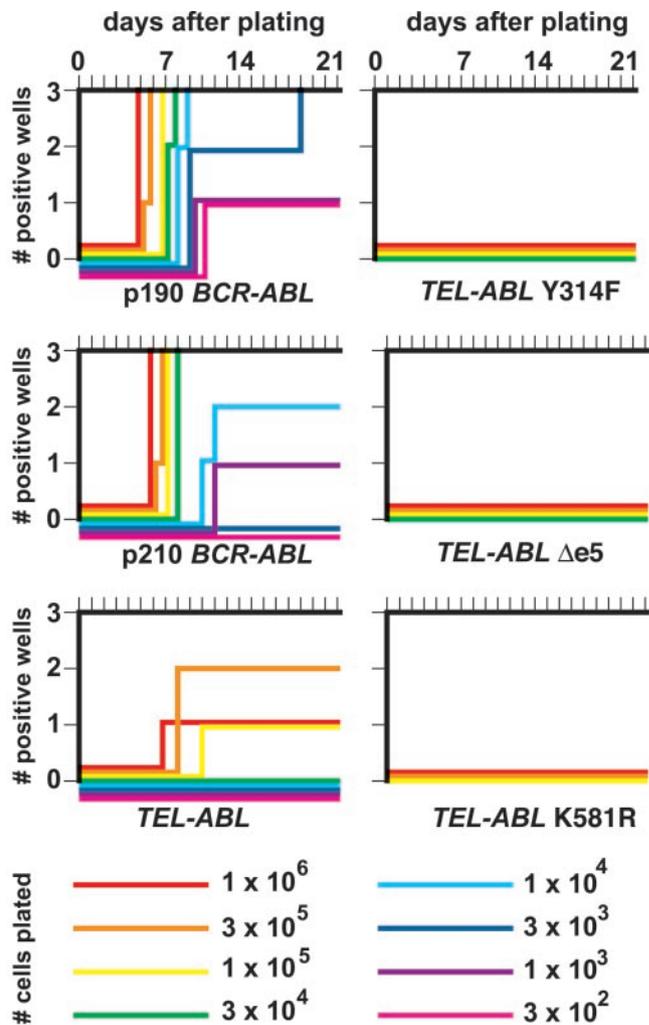

FIG. 6. The Grb2 binding site is required for transformation of primary bone marrow B-lymphoid cells by Tel-Abl in vitro. Primary bone marrow from non-5-FU-treated BALB/c donor mice was transduced with MSCV *neo* retrovirus expressing p190 or p210 *BCR-ABL*, *TEL-ABL*, *TEL-ABL* Y314F, or *TEL-ABL* Δexon5 (Δe5); transduction efficiency was equivalent based on determination of the proviral copy number in bone marrow cells by Southern blotting (data not shown). Serial dilutions of transduced marrow were plated in triplicate on syngeneic stromal layers derived from untransduced marrow and cultured for 3 weeks as described in Materials and Methods. The plating density is indicated by the line color, the number of cultures that reached confluence (defined as $>10^6$ nonadherent cells) is indicated on the ordinate, and the time to reach confluence is shown on the abscissa.

BCR-ABL, since only some of the cultures initiated with $\geq 10^5$ transduced cells demonstrated any growth. In contrast, the *TEL-ABL* Y314F, Δe5, and K581R mutants were completely defective for transformation of primary marrow B-lymphoid cells at all plating densities tested (Fig. 6), demonstrating that the Grb2 binding site is required for in vitro transformation of primary bone marrow B-lymphoid cells by Tel-Abl.

Low efficiency of induction of B-lymphoid leukemia in mice by Tel-Abl requires the direct binding site for Grb2. We have developed an assay for B-lymphoid leukemogenesis by *BCR-ABL*, where bone marrow from non-5-FU-treated donors is
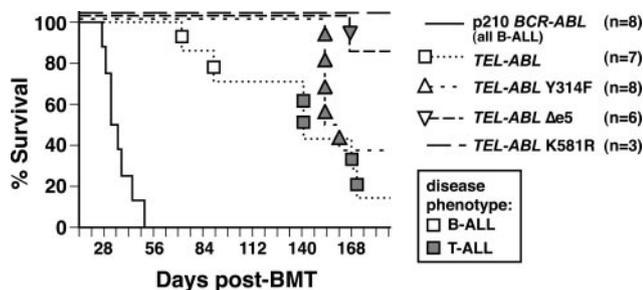
FIG. 7. Induction of B-lymphoid leukemia in mice by Tel-Abl requires the Grb2 binding site. A Kaplan-Meier-style survival curve for recipients of bone marrow from non-5-FU-treated donors transduced with MSCV *neo* retrovirus expressing p210 *BCR-ABL*, *TEL-ABL*, *TEL-ABL* Y314F, and *TEL-ABL* Δexon5 (Δe5) is shown. The number of recipients in each arm is shown in parentheses; all recipients of p210 *BCR-ABL*-transduced marrow developed B-ALL. The symbols indicate individual *TEL-ABL* recipient mice, with the disease phenotype of each designated by the shading (open, B-ALL; shaded, T-ALL). The difference in the survival of recipients of p210 *BCR-ABL*-transduced marrow and any of the recipients of *TEL-ABL*-transduced marrow was highly significant ($P < 0.001$; Mantel-Cox test). The difference in survival between recipients of marrow transduced with full-length *TEL-ABL* and *TEL-ABL* Δe5 was significant ($P = 0.016$; Mantel-Cox test), while the difference between *TEL-ABL* and *TEL-ABL* Y314F or between the two *TEL-ABL* mutants was not significant. BMT, bone marrow transplantation.

transduced and immediately transplanted into lethally irradiated syngeneic recipient mice (50). Under these conditions, p210 *BCR-ABL* induces B-ALL in all recipients within 7 weeks posttransplantation. In this assay, *TEL-ABL* induced B-ALL in only two of seven recipients with a significant delay (10 to 12 weeks posttransplantation), while no B-ALL was observed in recipients of *TEL-ABL* Y314F- or *TEL-ABL* Δe5-transduced marrow (Fig. 7). Consistent with the in vitro transformation assay (Fig. 6), these results demonstrate that *TEL-ABL* is a weak B-lymphoid oncogene relative to *BCR-ABL* and that this minimal activity absolutely requires the Grb2 binding site. Interestingly, the majority of recipients of *TEL-ABL*- and *TEL-ABL* Y314F-transduced marrow developed T-lymphoid leukemia/lymphoma at around 6 months post-transplantation (Fig. 7, dotted symbols). These mice had thymic and/or abdominal masses of Thy 1^+ CD4/8$^+$ blasts that were mono- to oligoclonal by proviral integration (data not shown) and were very similar to the late T lymphomas that develop in recipients of *BCR-ABL* Y177F-transduced marrow (39). This suggests that direct binding of Grb2 is not required for T lymphomagenesis by *ABL* fusion oncogenes.

Direct binding of Grb2 is required for efficient induction of CML-like disease by Tel-Abl but does not contribute to the development of small-bowel syndrome. In previous studies, we demonstrated that the full-length form of Tel-Abl induced two distinct myeloproliferative diseases in recipient mice: CML-like leukemia, which was very similar to that induced by Bcr-Abl, and a novel fatal syndrome (termed small-bowel syndrome), characterized by small-bowel myeloid cell infiltration and necrosis, increased levels of circulating endotoxin and TNF-α, and fulminant hepatic and renal failure (38).

To test the contribution of Grb2 binding to Tel-Abl-induced CML-like disease and small-bowel syndrome, marrow from donors pretreated with 5-FU was transduced with retrovirus

expressing full-length *TEL-ABL*, *TEL-ABL* Y314F, and *TEL-ABL* Δe5 and then transplanted into irradiated syngeneic recipients. Animals receiving marrow transduced with either *TEL-ABL* Δe5 or Y314F exhibited a very similar biphasic survival (Fig. 8A); the overall difference in survival between recipients of either *TEL-ABL* Δe5- or *TEL-ABL* Y314F-transduced marrow and that of *TEL-ABL* recipients was highly significant ($P < 0.001$, Mantel-Cox test). About half of the recipients of Δe5- and Y314F-transduced marrow died of typical small-bowel syndrome, including infiltration of small-bowel villi with neutrophils, acute fatty liver, and acute tubular necrosis and renal failure, within 5 weeks posttransplantation. There was moderate elevation in the number of peripheral blood leukocytes and minimal splenomegaly. The remaining animals developed classical CML-like myeloproliferative disease, but their survival was significantly prolonged relative to recipients of *TEL-ABL*-transduced bone marrow. Southern blot analysis demonstrated that the myeloproliferative disease induced by the *TEL-ABL* mutants was polyclonal (data not shown) and indistinguishable from that induced by full-length *TEL-ABL* (38). The histopathology of the CML-like disease induced by *TEL-ABL* Δe5 and Y314F was identical to that observed in *TEL-ABL* recipients, except for a few animals that survived longer than 200 days. These mice had prominent splenic and bone marrow fibrosis (data not shown), evidence of an attenuated and chronic myeloproliferative disorder. These results are similar to those obtained with Bcr-Abl Y177F, where the induction of CML-like disease is greatly attenuated but not completely abolished by lack of direct Grb2 binding (39, 63). The defect in leukemogenesis correlated with decreased activation of ERK/MAPK and Akt in primary myeloid progenitor cells by Tel-Abl Y314F (Fig. 8B). These results suggest that direct interaction of Grb2 with Tel-Abl is specifically required for efficient induction of CML-like myeloproliferative disease but not small-bowel syndrome.

DISCUSSION

Although Tel-Abl has been observed in only a small number of leukemia patients, comparative analysis of the signaling and leukemogenic properties of Tel-Abl should improve our understanding of the pathophysiology of Bcr-Abl-induced leukemia. The portion of Bcr fused to Abl contains a coiled-coil oligomerization domain (37) and the Tyr177 Grb2 binding site (45, 48), both of which are required for transformation and induction of CML-like disease by Bcr-Abl. The amino acid sequence of Tel bears no homology to Bcr, but the Tel PNT domain mediates oligomerization (13, 26) and is required for dysregulated in vivo kinase activity (13) and induction of myeloproliferative disease in mice (38) by Tel-Abl. This suggests that oligomerization of Abl is a critical step in the dysregulation of Abl kinase activity leading to leukemia (54, 55). However, oligomerization alone appears to be insufficient for full oncogenic activation of Abl, because fusion of just the Bcr oligomerization domain to c-Abl yields proteins that are defective for transformation (37) and leukemogenesis (21, 63). Here, we have identified a second shared function of Bcr and Tel: direct binding of the Grb2 adapter protein. We identified Tyr314 in the *TEL* exon 5-encoded sequence as the principal Grb2 binding site in Tel-Abl and demonstrated that a Tel-Abl
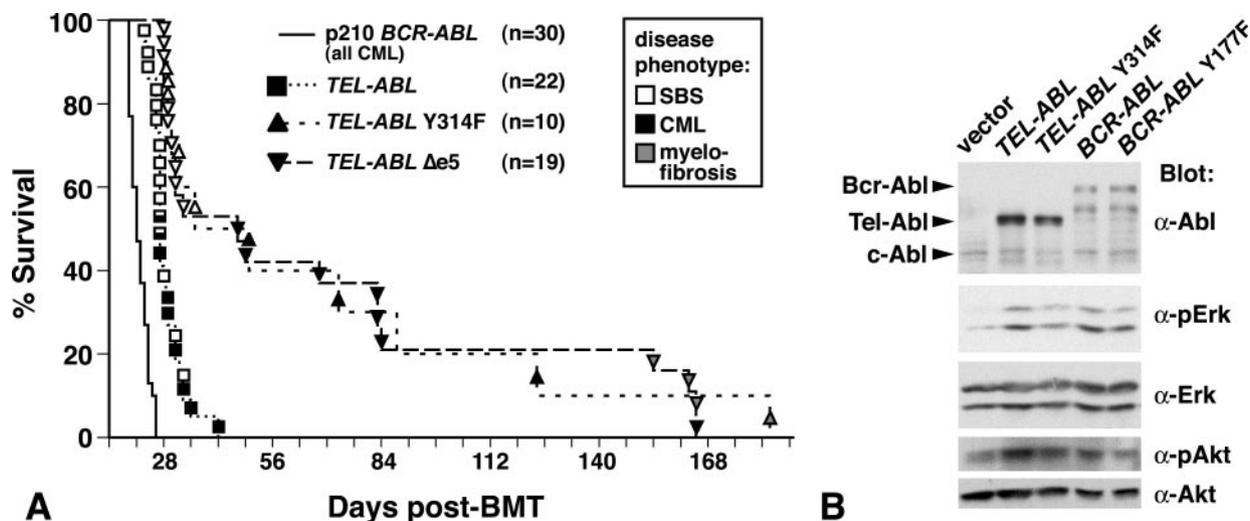
FIG. 8. Decreased myeloproliferative disease and activation of MAPK and Akt by Tel-Abl Grb2 binding mutants. (A) Loss of the Grb2 binding site attenuates the induction of CML-like disease by Tel-Abl but has no effect on development of small-bowel syndrome. A Kaplan-Meier-style survival curve for recipients of bone marrow from 5-FU-treated donors transduced with MSCV *neo* retrovirus expressing p210 *BCR-ABL*, *TEL-ABL*, *TEL-ABL* Y314F, or *TEL-ABL* Δexon5 (Δe5) is shown. The number of recipients in each arm is shown in parentheses. The survival curve for *BCR-ABL* is a composite of curves for previously reported mice (29) and additional mice transplanted concurrently with the *TEL-ABL* recipients; all recipients in this arm developed CML-like disease. The symbols indicate individual *TEL-ABL* recipient mice, with the disease phenotype of each designated by the shading (solid, CML-like disease; open, small-bowel necrosis with acute fatty liver [SBS] [38]); shaded, splenic and marrow fibrosis. Mice with prominent histopathological features of both disease processes are indicated by dually shaded symbols. The *TEL-ABL* (exons 1 to 5) recipients were from transplants conducted concurrently with the *TEL-ABL* mutants and have been reported previously (38). The difference in survival between recipients of *TEL-ABL*-transduced marrow and recipients of marrow transduced with either *TEL-ABL* mutant was highly statistically significant ($P < 0.001$; Mantel-Cox test), while there was no significant difference between the survival of recipients of marrow transduced by the two *TEL-ABL* mutants. BMT, bone marrow transplantation. (B) Impaired ERK/MAPK and Akt activation in primary myeloid progenitors by Tel-Abl Y314F. Lysates from primary bone marrow mononuclear cells transduced with empty MINV*neo* vector or MINV*neo* expressing the indicated Abl fusion protein were blotted with anti-Abl, anti-phospho-ERK (α-pErk), anti-pan-ERK (α-Erk), anti-phospho-Akt (α-pAkt), or anti-pan-Akt (α-Akt) antibodies, as indicated. The results are representative of two independent experiments.

point mutant (Y314F) and a variant Tel-Abl fusion found in some leukemia patients that lacks Tel exon 5 sequences (Δe5) fail to bind Grb2. The Tel-Abl Y314F and Δe5 fusions are defective for in vitro transformation of fibroblasts and primary B-lymphoid cells and for induction of B-ALL and impaired for the induction of CML-like myeloproliferative disease. Although Tel-Abl Y314F and Δe5 exhibited slightly lower in vitro tyrosine kinase activity than did full-length Tel-Abl, it is unlikely that this explains their lower oncogenic activity, since both kinases are as catalytically active as Bcr-Abl.

We found the full-length Tel-Abl protein to be relatively inefficient for transformation of primary bone marrow B-lymphoid cells in vitro and for induction of B-ALL in vivo, relative to p210 Bcr-Abl. However, Tel-Abl Y314F and Δe5, lacking the Grb2 binding site, were completely defective in both assays. These results were somewhat unexpected, because the full-length Tel-Abl protein was previously found to induce the growth of primary pre-B-lymphoid cells in 100% of Whitlock/Witte cultures (12 of 12) (13), while the Tel-Abl Δe5 protein was originally described in a patient with B-ALL (43). The discrepancy with the previously published in vitro B-lymphoid transformation data very probably reflects a quantitative defect in transformation of primary B-lymphoid cells by full-length Tel-Abl relative to Bcr-Abl, which may not have been observed previously because of the large number (5×10^6) of cells plated per well in the earlier study. It is difficult to be precise about the relative decrease in B-lymphoid transformation by

full-length Tel-Abl, but our data argue that it may be as much as 1 order of magnitude, which could easily account for the decreased incidence of B-ALL observed in the bone marrow transplantation assay, where the B-ALL induced by Bcr-Abl is mono- to oligoclonal under optimal conditions (50). The association of Tel-Abl Δe5 with a patient with B-ALL is more difficult to explain, but there was no karyotype reported for this patient and it is possible that the Tel-Abl fusion was not the principal cause of this leukemia. It would have been interesting to see if this patient would have responded clinically to the Abl kinase inhibitor imatinib (40), which has also been shown to inhibit Tel-Abl (4). Further comparative studies of Tel-Abl-expressing human and murine B-lymphoid leukemias will be necessary to determine the pathogenic role of Grb2 binding in human B-ALL.

Our previous studies demonstrated that the full-length form of Tel-Abl induced two distinct myeloproliferative-like diseases in recipients of transduced bone marrow, typical CML-like disease and a novel small-bowel syndrome (39). Disruption of the Grb2 binding site in Tel had no effect on the frequency or latency of development of the small-bowel syndrome but significantly attenuated the development of fatal CML-like myeloproliferative disease in mice. The precise relationship of the small-bowel syndrome to the CML-like disease is not known, but these results offer additional evidence that the two illnesses are distinct processes. The attenuation of CML-like disease by mutation of the Grb2 binding site in

Tel-Abl appears somewhat less profound than with mutation of Tyr177 in Bcr-Abl, where no recipient died of myeloproliferative disease (39, 63). However, most recipients of *BCR-ABL* Y177F-transduced marrow do develop clinical myeloproliferation but succumb to acute lymphoid leukemia beginning around 70 days posttransplantation (39, 63). If these recipients survived longer, it is possible that myeloproliferative disease or myelofibrosis would be a cause of morbidity or death, as it is in *TEL-ABL* recipients. Hence, the difference in survival and disease phenotype between *BCR-ABL* Y177F and *TEL-ABL* Y314F recipients may chiefly reflect the decreased induction of B- and T-lymphoid leukemia by *TEL-ABL* in recipients of 5-FU-primed marrow. Overall, our results argue strongly that direct binding of Grb2 to leukemogenic Abl fusion proteins is a shared and important mechanism for induction of myeloproliferative disease.

What signaling pathways lie downstream of Tyr314 in Tel-Abl and Tyr177 in Bcr-Abl? While there is no direct evidence that Grb2 is the critical SH2-containing protein that binds to these sites in vivo, this is suggested by the observations that fibroblast transformation by Bcr-Abl is inhibited by dominant-negative forms of Grb2 (10) and that proliferation of Bcr-Abl-expressing hematopoietic cells is inhibited by cell-shuttling peptides that disrupt ligand binding by the N-terminal SH3 domain of Grb2 (27). Because Bcr-Abl transformation is also inhibited by dominant-negative Ras and constitutively active RasGAP (53), it has been assumed that the Tyr177-Grb2 pathway involves the activation of Ras via the Sos guanine nucleotide exchange factor, which binds to Grb2. We found constitutive activation of Ras in hematopoietic cell lines by both Bcr-Abl and Tel-Abl that was independent of Grb2 binding, but, surprisingly, we did not observe significant constitutive activation of the total Ras pool in fibroblasts even by wild-type Bcr-Abl and Tel-Abl. However, activation of ERK/MAPK by Bcr-Abl and Tel-Abl and transformation of fibroblasts to anchorage-independent growth absolutely required the Grb2 binding site. It is possible that activation of a minor fraction of cellular Ras in the immediate vicinity of Bcr-Abl and Tel-Abl through Grb2-Sos binding may be critical for these events. Interestingly, there are several isoforms of the Tel-Jak2 fusion protein that also differ by the presence or absence of *TEL* exon 5-encoded sequences. Two recent studies demonstrated coimmunoprecipitation of Grb2 with a Tel (exons 1 to 5)-Jak2 fusion protein but not with Tel (exons 1 to 4)-Jak2 (8) and implicated Tel Tyr314 as a major Grb2 binding site (22) in Tel-Jak2. In contrast to our findings with Tel-Abl, a Tel-Jak2 Y314F mutant had impaired activation of Ras in Ba/F3 cells but no decrease in ERK/MAPK activation (22). While leukemogenesis by these Tel-Jak2 variants has not been compared directly, it is clear that Grb2-dependent signals induced by oncogenic tyrosine kinases differ depending on the cell type and the particular kinase involved.

It is likely that there are effector molecules of Grb2 other than Sos that contribute to leukemogenesis by Bcr-Abl and Tel-Abl. Here, we have identified the scaffolding/adapter protein Gab2 as a component of a second important signaling pathway downstream of Grb2. Phosphorylation of Gab2 is significantly diminished in hematopoietic cells expressing the Bcr-Abl and Tel-Abl Grb2 binding-site mutants, as is activation of PI 3-kinase and Akt. Gab2 is one of the major physiological binding partners of the p85 regulatory subunit of PI-3K (47) and also binds one of the SH3 domains of Grb2 (31). Thus, a physical interaction of Bcr-Abl and Tel-Abl with a Grb2-Gab2 complex through Tyr177 or Tyr314, respectively, promotes the phosphorylation of Gab2 and the activation of PI 3-kinase and Akt. Tyrosine phosphorylation of the SHP-2 tyrosine phosphatase, another major binding partner of Gab2 (16), is also decreased in cells expressing the Bcr-Abl and Tel-Abl Grb2 binding-site mutants (data not shown). SHP-2 and its *Drosophila* homolog *corkscrew* have been implicated in positive regulation of the Ras-MAPK pathway (61) and represent another Grb2-dependent mechanism leading to MAPK activation.

In primary myeloid progenitors, activation of both ERK and Akt was decreased but not abolished by the Tel-Abl Y314F mutation, which correlated with attenuated induction of myeloproliferative disease. This is consistent with the residual tyrosine phosphorylation of Gab2 in cells expressing the Tel-Abl and Bcr-Abl Grb2 binding mutants, suggesting that Gab2 can also be activated to some extent by a mechanism that is independent of a Grb2-Abl interaction. A critical role for Gab2 in Bcr-Abl transformation is supported by the finding that bone marrow from Gab2-deficient mice is completely resistant to transformation by Bcr-Abl in vitro (51). The role of Gab2 in leukemogenesis by Bcr-Abl and Tel-Abl is currently being addressed by utilizing bone marrow from Gab2-deficient mice in the retroviral transduction-transplantation model system. In conclusion, our results demonstrate that direct binding to Grb2 is a common and important feature of leukemogenic Abl tyrosine kinase fusion proteins. The data further validate the Grb2-Sos-Ras-MAPK and Grb2–Gab2–PI 3-kinase pathways as targets for the development of rational therapeutics for Abl-induced lymphoid and myeloid leukemias, although the widespread activation of MAPK by physiological stimuli suggests that inhibition of the Gab2 pathway may be less toxic to normal cells.

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