

Mimicry of a constitutively active pre-B cell receptor in acute lymphoblastic leukemia cells

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Pre-B cells undergo apoptosis unless they are rescued by pre-B cell receptor-dependent survival signals. We previously showed that the BCR-ABL1 kinase that is expressed in pre-B lymphoblastic leukemia bypasses selection for pre-B cell receptor-dependent survival signals. Investigating possible interference of BCR-ABL1 with pre-B cell receptor signaling, we found that neither SYK nor SLP65 can be phosphorylated in response to pre-B cell receptor engagement. Instead, Bruton's tyrosine kinase (BTK) is constitutively phosphorylated by BCR-ABL1. Activated BTK is essential for survival signals that otherwise would arise from the pre-B cell receptor, including activation of PLC γ 1, autonomous Ca²⁺ signaling, STAT5-phosphorylation, and up-regulation of *BCLX_L*. Inhibition of BTK activity specifically induces apoptosis in *BCR-ABL1*⁺ leukemia cells to a similar extent as inhibition of BCR-ABL1 kinase activity itself. However, BCR-ABL1 cannot directly bind to full-length BTK. Instead, BCR-ABL1 induces the expression of a truncated splice variant of BTK that acts as a linker between the two kinases. As opposed to full-length BTK, truncated BTK lacks kinase activity yet can bind to BCR-ABL1 through its SRC-homology domain 3. Acting as a linker, truncated BTK enables BCR-ABL1-dependent activation of full-length BTK, which initiates downstream survival signals and mimics a constitutively active pre-B cell receptor.

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Abbreviations used: BTK, Bruton's tyrosine kinase; PLC γ , phospholipase C γ ; siRNA, small interfering RNA; SAGE, serial analysis of gene expression; SH3, SRC-homology domain 3; SYK, spleen tyrosine kinase; WCL, whole cell lysate.

The *BCR-ABL1* gene rearrangement encodes a constitutively active tyrosine kinase and drives malignant transformation of pre-B cells in humans (1). In a B cell-specific mouse model, expression of *BCR-ABL1* is sufficient to induce pre-B lymphoblastic leukemia and is required for maintenance of leukemic transformation (2).

During their development, human B cells have to pass multiple checkpoints at which they are selected for the expression of a functional pre-B or B cell receptor (3). However, signals from the pre-B cell receptor also may initiate apoptosis (4). Therefore, the expression of a functional pre-B cell receptor constitutes an effective means to regulate tightly the survival or elimination of individual B cell clones. In this regard, it is not surprising that in several

B cell-derived malignancies, pre-B or B cell receptor function is compromised (5). For instance, Hodgkin and Reed-Sternberg cells in classic Hodgkin's disease carry "crippled" Ig variable region gene rearrangements as a result of the acquisition of deleterious somatic mutations (6). Likewise, transformation of human B cells by the EBV mimics antigen receptor-dependent survival signals through the viral oncoprotein LMP2A, and prevents the B cell receptor from signaling by excluding it from glycolipid-enriched membrane domains (7). Autonomous growth and survival of transformed B cells seem to favor a situation in which the B cell receptor is not active. In accordance with this notion, we recently observed that pre-B lymphoblastic leukemia clones harboring a *BCR-ABL1* fusion gene carry only nonproductively rearranged Ig variable region

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genes in 9 of 12 cases (8). Even the few leukemia clones that do express a pre-B cell receptor were not responsive to pre-B cell receptor engagement (8). Analogous to the EBV-encoded LMP2A protein, the expression of the oncogenic BCR-ABL1 kinase may confer autonomous survival signals, and interfere with antigen receptor signaling.

Among others, Bruton's tyrosine kinase (BTK) represents a crucial component of the (pre)-B cell receptor signaling chain and links the (pre)-B cell receptor to Ca^{2+} signals in B lymphoid cells (9). In humans, deficiency of BTK leads to X-linked agammaglobulinemia which results from a differentiation block at the pre-B cell-stage (10). Of note, BTK promotes developmental progression of pre-B cells (11), and acts as a tumor suppressor to prevent pre-B lymphoblastic leukemia in mice (12, 13). To elucidate how BCR-ABL1 can bypass selection for pre-B cell receptor-dependent survival signals, the current study investigates possible interference of BCR-ABL1 with pre-B cell receptor-related signaling molecules, including spleen tyrosine kinase (SYK), SLP65, phospholipase C γ (PLC γ)2, and BTK.

RESULTS

BCR-ABL1 kinase activity induces tyrosine-phosphorylation of BTK

Normal pre-B cells can survive in human bone marrow only if they receive survival signals through their pre-B cell receptor (14). In a previous study, we observed that in the majority of cases of pre-B lymphoblastic leukemia expressing the oncogenic BCR-ABL1 kinase, the leukemia cells do not harbor a functionally rearranged *IGH* allele, and hence, cannot express a pre-B cell receptor on their surface (8). Therefore, we wondered how a pre-B cell-derived leukemia cell can survive and even clonally expand in the absence of pre-B cell receptor-dependent survival signals.

To determine if BCR-ABL1 interferes with signal transduction of the pre-B cell receptor, we examined whether the pre-B cell receptor-related signaling molecules SYK, SLP65, and BTK are expressed and if they can be phosphorylated upon pre-B cell receptor engagement in the leukemia cells.

Western blot using antibodies against phosphorylated SYK^{Y323}, SLP65^{Y96}, and BTK^{Y223} showed that neither SYK nor SLP65 was phosphorylated in response to cross-linking of the pre-B cell receptor (unpublished data). However, BTK was constitutively phosphorylated in the leukemia cells (at 77 kD; Fig. 1 A).

Using a specific inhibitor of BCR-ABL1, termed STI571 (15), we found that phosphorylation of BTK was sensitive to inhibition of BCR-ABL1 kinase activity (77kD; Fig. 1 A). To determine whether constitutive phosphorylation of 77-kD BTK is specific for leukemia cells that carry a *BCR-ABL1* gene rearrangement, BTK phosphorylation also was studied in pre-B lymphoblastic leukemias that harbored an *E2A-PBX1*, *MLL-AF4*, *TEL-AML1*, or *TEL-PDGFR β* gene rearrangement (Fig. 1 B, Table I). Although the BTK^{Y223}-specific antibody detected smaller tyrosine-phosphorylated proteins also in

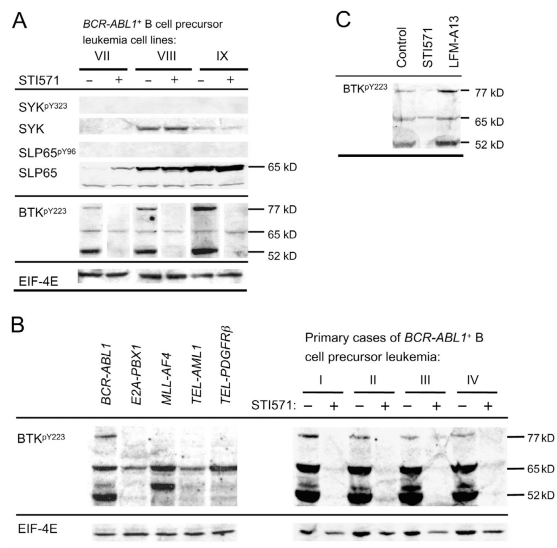


Figure 1. BCR-ABL1 induces phosphorylation of BTK in pre-B lymphoblastic leukemia cells. Protein expression and tyrosine phosphorylation of the pre-B cell receptor downstream signaling molecules SYK, SLP65, and BTK was studied by Western blot in three *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines (VII, VIII, and IX in Table I) in the presence or absence of STI571. The BTK^{Y223} antibody detected full-length BTK (77 kD) as well as two additional tyrosine phosphorylated proteins of 65-kD and 52-kD size (A-C). To confirm that constitutive phosphorylation of 77-kD full-length BTK is specific for BCR-ABL1 kinase activity, BTK^{Y223}-phosphorylation was studied in four BCR-ABL1-negative B cell precursor cell lines (B, left panel; see XI, XII, XIII, and XV in Table I). Dependence of BTK on BCR-ABL1 kinase activity also was confirmed in four primary cases of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia, from which matched sample pairs before and during therapy with STI571 were available (B, right panel; cases I to IV in Table I). EIF-4E was used as a loading control. To determine whether Y223 phosphorylation of BTK is mediated by transphosphorylation through BCR-ABL1 or autophosphorylation, *BCR-ABL1*⁺ leukemia cells (IX; Table I) were treated for 24 h with the BCR-ABL1 kinase inhibitor, STI571, or the BTK inhibitor, LFM-A13 (C).

other leukemias, constitutive Y223 phosphorylation of BTK (77 kD) was specific for *BCR-ABL1*⁺ pre-B cell lymphoblastic leukemia cell lines (Fig. 1 B, left).

To confirm that BTK phosphorylation is dependent on BCR-ABL1 kinase activity, we induced BCR-ABL1 expression in a murine B lymphoid cell line TONB210 (16) that carries an inducible *BCR-ABL1* transgene. As opposed to *BCR-ABL1*⁺ leukemia cells, this cell line grows independently from BCR-ABL1 in the presence of IL-3. In the presence, but not in the absence, of doxycycline-induced BCR-ABL1 expression, BTK was heavily phosphorylated (see Fig. 5 C). We conclude that BTK is constitutively phosphorylated by BCR-ABL1, but not by the pre-B cell receptor in the leukemia cells.

BTK phosphorylation depends on BCR-ABL1 kinase activity in vivo

To confirm that phosphorylation of BTK also depends on BCR-ABL1 kinase activity in primary leukemia cells, expres-

Table I. Description of leukemia and lymphoma cases and cell lines studied

Case	Age	Sex	Translocation		Igκ/λ or SLC	IGH configuration		Clinical course
			Fusion gene	IgH		Alleles	Coding capacity	
I ^a	39	m	<i>BCR-ABL1</i> ; p210	-	-	(1) V _H 4.34-D _H 6.13-J _H 5 (2) D _H 5-J _H	no; out-of-frame	ALL, relapse after STI571 treatment
II ^a	54	f	<i>BCR-ABL1</i> ; p190	-	-	(1) V _H 4.61-D _H 2.2-J _H 4 (2) D _H 2.4-J _H 4	no; out-of-frame, stop in junction	ALL, relapse after STI571 treatment
III ^a	61	f	<i>BCR-ABL1</i> ; p190	ND	ND	ND	-	ALL, relapse after STI571 treatment
IV ^a	47	f	<i>BCR-ABL1</i> ; p190	ND	ND	ND	-	ALL, relapse after STI571 treatment
V ^a	34	m	<i>BCR-ABL1</i> ; p190	-	-	(1) V _H 4.34-D _H 6.13-J _H 5 (2) D _H 5-J _H	out-of-frame	ALL, complete remission after BMT
VI ^a	32	m	<i>BCR-ABL1</i> ; p190	-	-	(1) V _H 4.4-D _H 2.2-J _H 4 (2) D _H 2.4-J _H 4	out-of-frame, stop codons in junction	ALL, died after BMT
VII ^a	45	m	<i>BCR-ABL1</i> ; p210	-	-	(1) V _H 3.48-D _H 2.15-J _H 3 (2) D _H 2-J _H	no; out-of-frame, stop in junction	ALL, died after relapse (BV173 cell line)
VIII ^a	3	f	<i>BCR-ABL1</i> ; p210	μ chain	SLC	(1) D _H 4-J _H (2) V _H 3.9-D _H 2.21-J _H 3	no yes	ALL, died after relapse (NALM1 cell line)
IX ^a	9	m	<i>BCR-ABL1</i> ; p190	μ chain	SLC	(1) V _H 3.53-D _H 2.8-J _H 6	yes ^b	ALL, died after relapse (SUP-B15 cell line)
X	32	f	<i>MLL-AF4</i>	-	-	(1) V _H 3.20-D _H 2.8-J _H 5 (2) V _H 6.1-D _H 1.7-J _H 4	no; out-of-frame no; stop in junction	ALL, relapse after chemotherapy (RS4;11 cell line)
XI	5	f	<i>MLL-AF4</i>	-	-	(1) D _H 3-J _H (2) <i>IGH</i> in germline	no	ALL, relapse after chemotherapy (SEM cell line)
XII	15	f	<i>TEL-AML1</i>	-	-	(1) V _H 3.15-D _H 3.10-J _H 6 (2) <i>IGH</i> in germline	no; out-of-frame	ALL, relapse after chemotherapy (REH cell line)
XIII	12	m	<i>E2A-PBX1</i>	μ chain	SLC	(1) V _H 4.34-D _H 3.22-J _H 2 (2) V _H 2.26-D _H 2.2-J _H 4	no; out-of-frame, stop in junction yes	ALL, relapse after chemotherapy (697 cell line)
XIV	15	m	<i>E2A-PBX1</i>	μ chain	SLC	(1) V _H 3.7-D _H 3.10-J _H 4	yes ^b	ALL, cell line (KASUMI-2) established at diagnosis
XV	19	m	<i>TEL-PDGFRβ</i>	μ chain	SLC	(1) V _H 1.69-D _H 3.10-J _H 6	yes ^b	ALL, relapse after chemotherapy (NALM6 cell line)
XVI	5	m	<i>IGH-MYC</i>	IgM	Igλ	(1) V _H 3.53-D _H 3.3-J _H 6 (2) V _H 4.34-D _H 2.15-J _H 5	no; stop in junction yes	Burkitt's lymphoma cell line (MHH-PREB1) established at diagnosis
XVII	73	f	<i>IGH-BCL2</i>	IgG	Igκ	(1) V _H 4.39-D _H 3.10-J _H 6 ^c (2) V _H 3.9-D _H 3.22-J _H 6 ^c (3) V _H 3.23-D _H 6.13-J _H 5 ^c	yes no; out-of-frame no; out-of-frame	Diffuse large B cell lymphoma cell line (KARPAS-422) established at diagnosis

^aThese cases were described in Klein et al. (reference 8).

^bThe second *IGH* allele was not subjected to sequence analysis if a potentially functional allele was already amplified.

^cThree *IGHVDJ* gene rearrangements are compatible with three chromosome 14s in these B cell lymphoma cells. Alternatively, the cell line may comprise subclones with different *IGHVDJ* gene rearrangements.

ALL, acute lymphoblastic leukemia; n.d., not done; SLC, surrogate light chain.

sion of tyrosine-phosphorylated BTK was compared in matched leukemia sample pairs from four patients before and during continued therapy with STI571 (cases I to IV, Fig. 1 B, right panel). The content of *BCR-ABL1*⁺ leukemia cells was similar in the samples taken before and during STI571

treatment as assessed by semiquantitative amplification of *BCR-ABL1* fusion transcripts (see Fig. 2 A). As with inhibition of BCR-ABL1 kinase by STI571 in vitro, in vivo ablation of BCR-ABL1 kinase activity resulted in the loss of BTK phosphorylation in all four patients (Fig. 1 B, right panel).

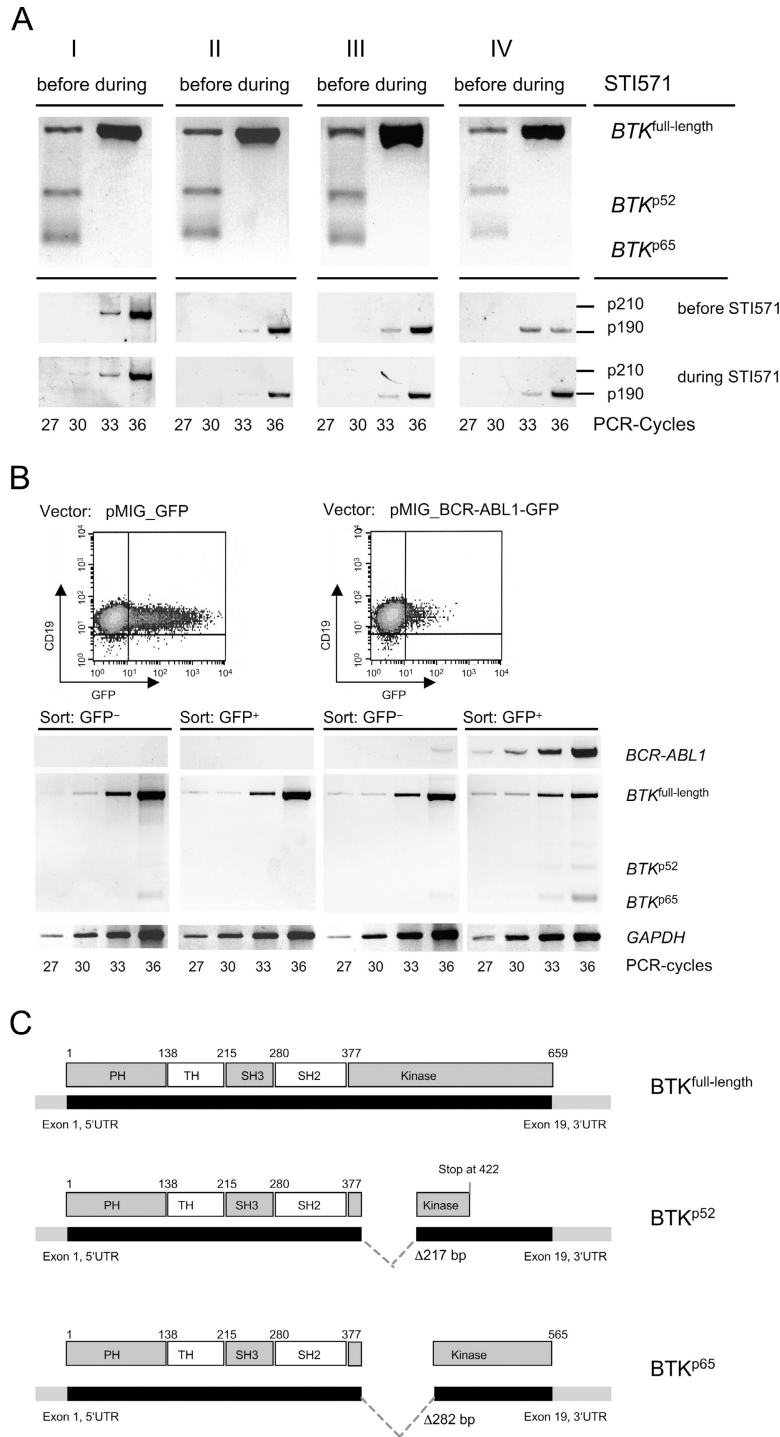


Figure 2. BCR-ABL1 induces expression of kinase-deficient BTK isoforms. Comparing matched leukemia sample pairs taken before and during continued therapy with the BCR-ABL1 kinase-inhibitor, STI571, BTK transcripts, including splice variants (*BTK*^{p52} and *BTK*^{p65}), were amplified (A, top). cDNA samples were normalized for equal content of leukemia cells by amplification of *BCR-ABL1* (A, bottom). To determine whether aberrant splicing of BTK is induced by BCR-ABL1, pre-B lymphoblastic leukemia cells carrying an *E2A-PBX1*—but no *BCR-ABL1* rearrangement—were transiently transfected with a retroviral expression vector which encoded BCR-ABL1 and GFP, or GFP alone (B). GFP⁻ and GFP⁺ cells were

sorted after 24 h (B, top) and subjected to RT-PCR analysis. cDNA amounts were normalized by amplification of a *GAPDH* fragment (B, bottom). In addition to full-length BTK, sequence analysis of BTK transcripts revealed six isoforms (Table S2). Among these isoforms, two were recurrently amplified: *BTK*^{p52} and *BTK*^{p65} were detected in all nine cases of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia studied (C). Both isoforms carry a deletion within their kinase domain. Skipping of exon 15 (*BTK*^{p52}) leads to the loss of the reading frame and COOH-terminal truncation as the result of a premature translation stop at codon 442 (C, middle). *BTK*^{p65} lacks exons 15 and 16 and leads to an in-frame deletion of 282 bp (*BTK*^{p65}).

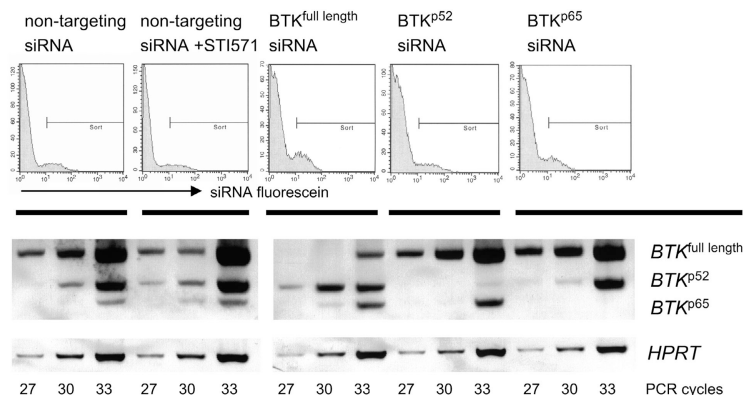


Figure 3. Specific silencing of full-length BTK or BTK splice variants by RNA interference. *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells were transfected with a nontargeting siRNA duplex in the presence or absence of STI571 (for 12 h), as well as with a mixture of siRNAs against full-length BTK, BTK^{p52}, or BTK^{p65}. All siRNA duplexes were labeled with fluorescein before transfection. Transfection was repeated after 24 h, and

10⁵ cells carrying fluorescein-labeled siRNAs (between 3–5% of all viable cells) were sorted by FACS 48 h after the first transfection (upper panel). Total RNA was isolated and subjected to semiquantitative RT-PCR analysis of BTK isoform expression (lower panel). mRNA levels for *HPRT* and *COX6B* (see Fig. 8 C) were stable under all conditions.

BTKY²²³ can be transphosphorylated by BCR-ABL1

To clarify further BCR-ABL1–dependent phosphorylation of BTKY²²³, we investigated whether phosphorylation at Y223 depends only on BCR-ABL1 or also on endogenous BTK activity. In normal B cells, B cell receptor engagement leads first to LYN-mediated transphosphorylation of COOH-terminal BTKY⁵⁵¹ (17). BTKY²²³ phosphorylation occurs as a secondary event by autophosphorylation through endogenous BTK activity (17). Therefore, the effect of the BTK inhibitor, LFM-A13, on BTKY²²³ phosphorylation in *BCR-ABL1*⁺ pre-B cell lymphoblastic leukemia cells was examined. BTKY²²³ phosphorylation was sensitive to BCR-ABL1 kinase inhibition by STI571, but not to BTK inhibition by LFM-A13 (Fig. 1 C). We conclude that BTKY²²³ is accessible to transphosphorylation by BCR-ABL1 in the absence of endogenous BTK activity.

Expression of truncated BTK splice variants in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia

In the BTK Western blot analysis of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines, we observed that the antibody that recognized phosphorylated BTKY²²³ at the expected size of 77 kD also detected two smaller tyrosine-phosphorylated proteins of ~65 kD and 52 kD in size (Fig. 1, A–C). This prompted us to investigate splice variants of BTK at the mRNA level.

By amplifying specific BTK cDNA fragments that cover the entire coding region of BTK, we detected shorter PCR products in all three *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines (not depicted) and also in six primary cases of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia (cases I to VI, Table I; Fig. 2 A).

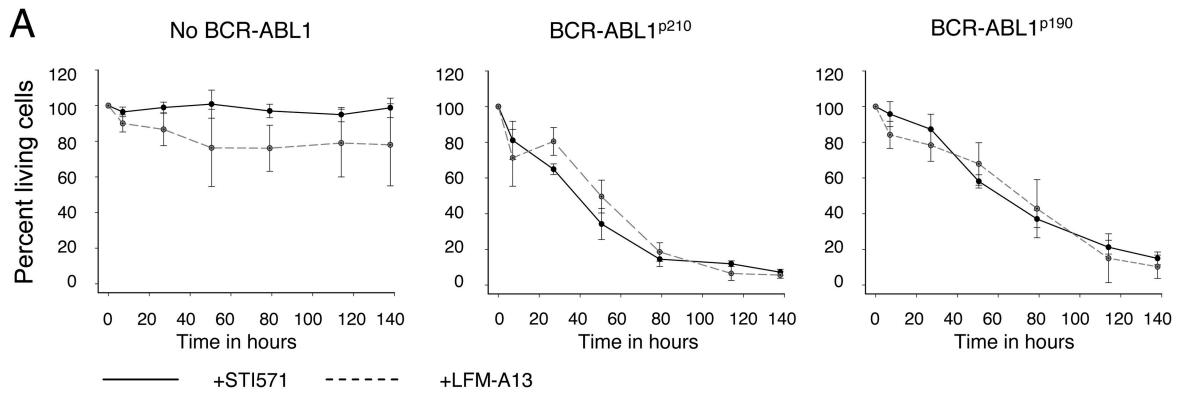
In total, sequence analysis revealed six different BTK isoforms in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (Table S2, available at <http://www.jem.org/cgi/content/>

full/jem.20042101/DC1; sequence data available from GenBank/EMBL/DDBJ under accession nos. AJ888376–AJ888381). All of these splice variants carry large deletions within the kinase domain (Table S2). Four of these isoforms were unique, were generated by usage of cryptic splice sites, and only were amplified from individual cases of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia. However, two BTK isoforms were identified recurrently.

In addition to a previously described BTK isoform which lacks exons 15 and 16 (BTK^{p65} in Fig. 2 A; reference 18), another truncated splice variant that lacks only exon 15 was amplified from all nine cases of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia (Table S2). Joint skipping of exons 15 (217 bp) and 16 (65 bp) leads to an in-frame 282-bp deletion (BTK^{p65}; Fig. 2 C). The predicted size of the protein encoded by BTK^{p65} matches the 65-kD protein recognized by the BTKY²²³ antibody (Fig. 1, A–C). Skipping of exon 15 alone leads to loss of the reading frame and generates a premature stop codon which results in the expression of a truncated protein of 442 amino acids instead of 659 amino acids (BTK^{p52}; Fig. 2 C). The predicted truncated BTK^{p52} (442 amino acids) exactly matches the size of the heavily phosphorylated 52-kD protein that we detected with the phospho-specific anti-BTKY²²³ antibody (Fig. 1, A–C).

By comparing matched sample pairs of leukemia cells before and during continued therapy with the BCR-ABL1 kinase inhibitor, STI571, we amplified BTK^{p52} (lacking exon 15 alone) or BTK^{p65} (lacking exons 15 and 16) in the presence (but not in the absence) of BCR-ABL1 kinase activity (Fig. 2 A). These findings suggest that aberrant splicing of BTK may be linked to BCR-ABL1 kinase activity.

BCR-ABL1 was implicated previously in altered splice-site selection and exon skipping through up-regulation of RNA-binding proteins, including FUS, hnRNP A1, and hnRNP E2 (19). In addition, a recent study shows that



B

Survival [%]	Cell line	Cell type	Translocation	Genes involved
93.6 ± 0.6	RS4;11	pro-B cell leukemia	t(4;11)(q21;q23)	<i>MLL-AF4</i>
97 ± 5.1	SEM	pro-B cell leukemia	t(4;11)(q21;q23)	<i>MLL-AF4</i>
49.4 ± 7.6	REH	pre-B cell leukemia	t(12;21)(p12;q22)	<i>TEL-AML1</i>
66.8 ± 8.4	697	pre-B cell leukemia	t(1;19)(q23;p13)	<i>E2A-PBX1</i>
88.4 ± 3.4	KASUMI-2	pre-B cell leukemia	t(1;19)(q23;p13)	<i>E2A-PBX1</i>
89.2 ± 1.3	LCL	polyclonal EBV ⁺ mature B cells	no chromosomal aberration	
85.2 ± 9.6	MHH-PREB-1	Burkitt's Lymphoma	t(8;14)(q24;q32)	<i>MYC-IGH</i>
76.2 ± 7.0	KARPAS-422	Diffuse Large B Cell Lymphoma	t(14;18)(q32;q21)	<i>BCL2-IGH</i>
7.3 ± 4.1	BV173	pre-B cell leukemia	t(9;22)(q34;q11)	<i>BCR-ABL1</i>
19.3 ± 15.5	SUP-B15	pre-B cell leukemia	t(9;22)(q34;q11)	<i>BCR-ABL1</i>

Relative survival [%] average: 80.7 ± 5.4 in non-*BCR-ABL1*⁺ B cell lineage cells
13.3 ± 9.8 in *BCR-ABL1*⁺ pre-B cell leukemia

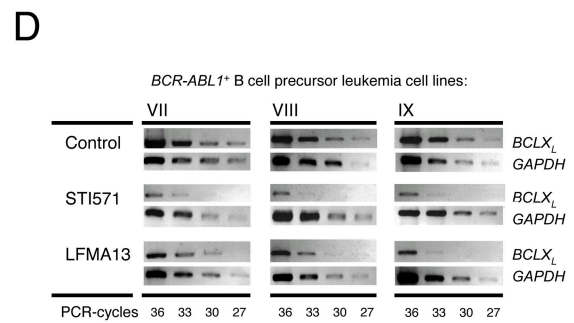
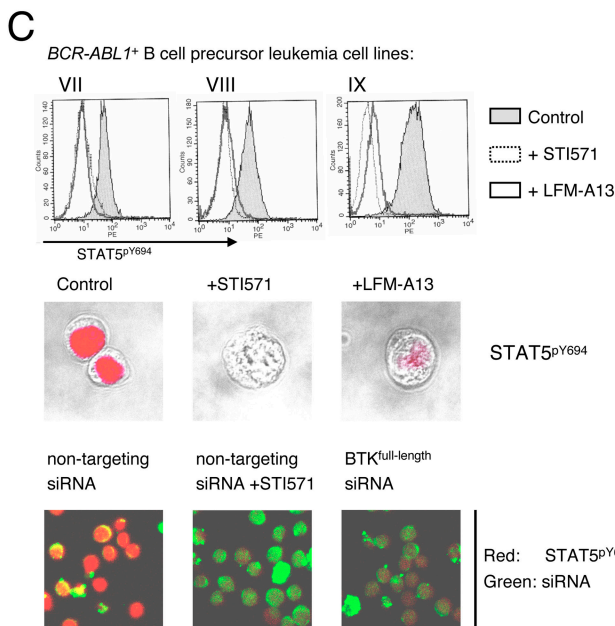


Figure 4. Inhibition of BTK induces apoptosis specifically in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells. *BCR-ABL1*-negative B lymphoid leukemia and lymphoma cell lines (A, left panel) and two *BCR-ABL1*⁺ leukemia cell lines (A, middle and right panel) were incubated for the times indicated under control conditions, with STI571 (solid line) or with the BTK inhibitor, LFM-A13 (dashed line; A). Relative viability (percent living cells)

was calculated as the ratio of viability in the presence of STI571 or LFM-A13 and viability in untreated cells. Two *BCR-ABL1*⁺ and eight *BCR-ABL1*-negative B lymphoid cell lines were incubated in the presence of LFM-A13 or under control conditions for 114 h (B). Relative survival of the treated cells as compared with control conditions is given as the mean of three independent experiments ± SD. To analyze *BCR-ABL1*-mediated and BTK-dependent

BCR-ABL1 induces aberrant splicing of PYK2 premRNA in human hematopoietic progenitor cells, which can be reverted by STI571 (20). These findings exemplify that BCR-ABL1 frequently interferes with normal regulation of premRNA splicing (e.g., by reduction of stringency of splice-site selection).

To assess directly whether BCR-ABL1 can interfere with splicing of BTK premRNA, we transiently transfected pre-B lymphoblastic leukemia cells that carry an *E2A-PBX1*, but no *BCR-ABL1*, gene rearrangement with an expression vector which encodes GFP only or GFP and BCR-ABL1. For both transfections, GFP⁺ and GFP⁻ cells were sorted and analyzed separately for expression of BTK splice variants (Fig. 2 B). Although *E2A-PBX1*⁺ pre-B lymphoblastic leukemia cells under control conditions express BTK^{P65} at low levels, induced expression of BCR-ABL1 leads to the expression of multiple BTK splice variants (Table S2), including BTK^{P52} (Fig. 2 B). We conclude that BCR-ABL1 induces aberrant splicing of BTK and expression of BTK^{P52}.

By comparing 19 genome-wide gene expression profiles by serial analysis of gene expression (SAGE), we identified three SAGE-tags that distinguish between full-length BTK, BTK^{P65}, and BTK^{P52} (Table S3, available at <http://www.jem.org/cgi/content/full/jem.20042101/DC1>). A SAGE-tag represents a 14-bp cDNA sequence which immediately flanks the extreme 3' restriction site (CATG) of the tagging enzyme (NlaIII) that is used as a unique identifier of any processed transcript. Although full-length BTK is expressed widely in normal B cell populations and BTK^{P65} also is expressed in non-*BCR-ABL1* B lymphoid malignancies, BTK^{P52} seems to be expressed specifically in *BCR-ABL1*⁺ leukemia cells (Table S3).

Specific silencing of BTK splice variants by RNA interference

To investigate the function of full-length BTK and its splice variants in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells, we established an RNA interference approach to silence full-length BTK and BTK isoforms specifically. Among the six BTK splice variants (Table S2), only BTK^{P52} and BTK^{P65} were amplified recurrently. Therefore, we focused our analysis on BTK^{P52} and BTK^{P65}. To clarify the specific function of BTK^{P52} (lack of exon 15, loss of reading frame) and BTK^{P65} (in-frame deletion of exons 15 and 16), we designed three small interfering RNA (siRNA) duplexes against the junction of BTK exons 14 and 16 (corresponding to BTK^{P52}) and the junction of BTK exons 14 and 17 (corre-

sponding to BTK^{P65}). For each target, the three siRNA duplexes were mixed and fluorescein labeled. Also for full-length BTK, three siRNA duplexes were synthesized (specifically binding to BTK exon 15), mixed, and fluorescein labeled. As a control, a fluorescein-labeled nontargeting siRNA duplex was used. After two transfections, the proportion of siRNA-carrying cells ranged between 3% and 5% (Fig. 3). For each condition, 10⁵ fluorescein⁺ cells were sorted and subjected to RT-PCR analysis for BTK splice variants. mRNA levels of the reference genes *HPRT* and *COX6B* remained unchanged (Fig. 3; see Fig. 8). Sorted cells exhibited specific silencing of full-length BTK, BTK^{P52}, or BTK^{P65}, and no cross-inhibition of BTK isoform expression was observed (Fig. 3). Because lymphoid cells typically are difficult to transfect, we conclude that double transfection of a mixture of three siRNA duplexes and cell sorting of siRNA-carrying cells represents an effective strategy to silence specifically target mRNA sequences in B lymphoid leukemia cells.

From in vitro kinase assays of BTK mutants that were derived from *BTK*-deficient patients who had X-linked agammaglobulinemia, it is known that even replacement mutations in the distal portion of the kinase domain result in a complete loss of BTK activity (<http://bioinf.uta.fi/BTKbase/BTKbasebrowser.html>). Hence, only full-length BTK—but none of the BTK splice variants identified here—should exhibit BTK activity. Therefore, we investigated the relevance of BTK activity for BCR-ABL1-mediated survival signaling in pre-B lymphoblastic leukemia cells.

Inhibition of BTK activity induces apoptosis specifically in human *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells

To clarify the role of full-length BTK (as compared with kinase-deficient truncated BTK) in pre-B lymphoblastic leukemia cells, we analyzed the effect of BTK inhibition on the survival of *BCR-ABL1*⁺ leukemia cells in comparison with inhibition of BCR-ABL1 by STI571. As a control we used several B lymphoid cell lines that do not carry a *BCR-ABL1* gene rearrangement. Neither inhibition of endogenous c-ABL1 (by STI571) nor BTK (by LFM-A13) kinase activity alone significantly reduced viability of *BCR-ABL1*-negative B lymphoid cells (Fig. 4 A, B). Conversely, inhibition of BCR-ABL1 rendered almost all *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells apoptotic within 5 d (Fig. 4 A). In both *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines that were tested, but not in *BCR-ABL1*-negative cell lines,

tyrosine phosphorylation of STAT5 (C), three *BCR-ABL1*⁺ B cell precursor cell lines (VII, VIII, and IX in Table I) were treated with the BCR-ABL1 kinase inhibitor, STI571 (dotted line), with the BTK inhibitor, LFM-A13 (solid line), or incubated under control conditions (shaded areas; C). Nuclear localization of activated STAT5 in treated and untreated leukemia cells was visualized by confocal laser microscopy (C; red stain, middle). *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells were transfected with a mixture of fluorescein-labeled siRNAs against full-length BTK as shown in Fig. 3. Fluorescein⁺

cells carrying siRNA molecules (green) were stained for tyrosine-phosphorylated STAT5 (red) and analyzed by confocal laser microscopy (C, bottom panel). As a negative control, nontargeting siRNA duplexes were used. Next, mRNA levels of *BCLX_L* were studied in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells in the presence or absence of STI571 or LFM-A13. Total RNA was isolated from the leukemia cells and subjected to semi-quantitative RT-PCR analysis for mRNA levels of *BCLX_L* (D). cDNA amounts were normalized by amplification of a *GAPDH* cDNA fragment.

inhibition of BTK had a very similar effect as inhibition of BCR-ABL1, and also induced apoptosis in ~90 percent of the leukemia cells within 5 d (Fig. 4 A, B). These findings are in agreement with an RNA interference experiment that showed that silencing of full-length BTK expression (Fig. 3) induced apoptosis in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (see Fig. 8 A). Thus, BTK activity is required for sustained growth and survival of BCR-ABL1-transformed human pre-B lymphoblastic leukemia cells.

BCR-ABL1-induced tyrosine phosphorylation and nuclear localization of STAT5 requires BTK activity

Besides other survival signals, STAT5 activation is critical for BCR-ABL1-mediated transformation (21, 22). Given that BTK activity can activate STAT5 through phosphorylation at tyrosine 694 (23), we investigated the impact of BCR-ABL1 or BTK inhibition on STAT5^{Y694} phosphorylation in three *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines by intracellular staining and flow cytometry. STAT5^{Y694} is

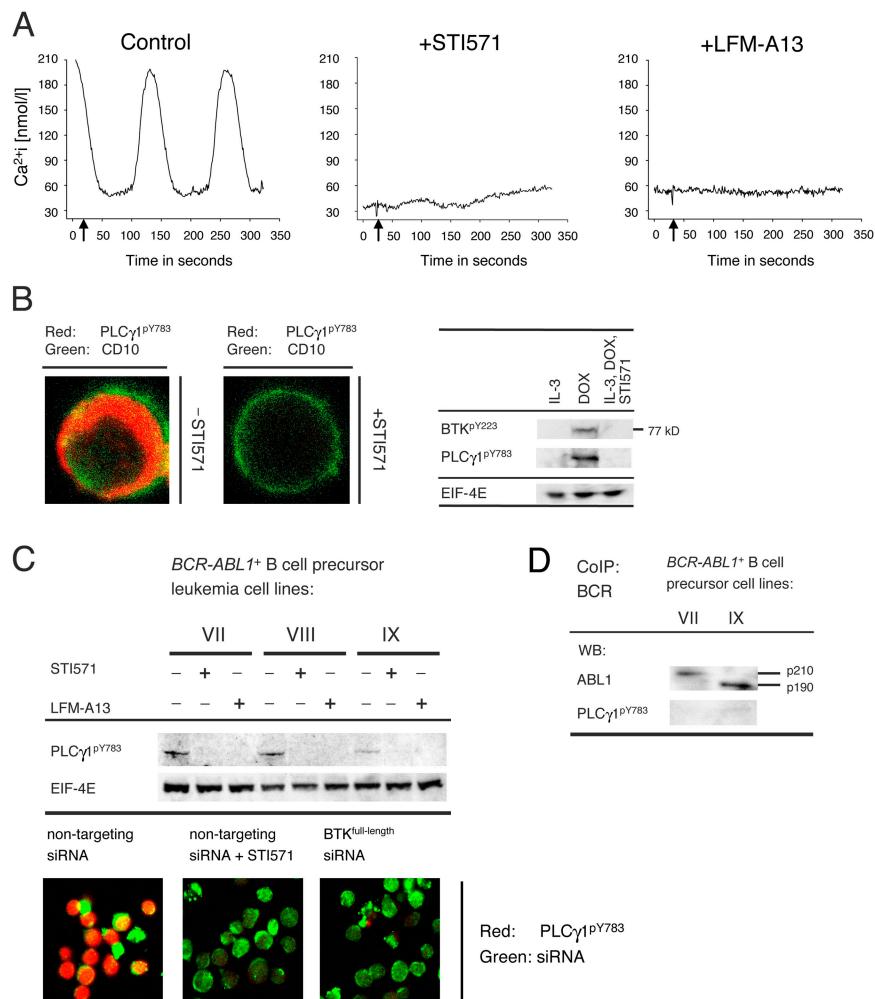


Figure 5. BTK links BCR-ABL1 to PLCγ1-mediated Ca²⁺ signals in pre-B lymphoblastic leukemia cells. Cytoplasmic Ca²⁺ levels were measured in single leukemia cells by confocal laser microscopy. Before Ca²⁺ measurement, cells were kept under control conditions or treated for 24 h with STI571 or LFM-A13 (A). Pre-B cell receptor engagement by addition of μ -heavy chain-specific antibodies (arrows) did not change cytoplasmic Ca²⁺ concentrations. Although PLCγ1^{Y783} is phosphorylated in the presence—but not in the absence (+ STI571)—of BCR-ABL1 kinase activity (B, left panels), PLCγ2 was not tyrosine phosphorylated by BCR-ABL1 (not depicted). As a control for nonspecific side effects of STI571, murine B lymphoid cells carrying a doxycycline-inducible *BCR-ABL1* transgene were analyzed in the presence or absence of 1 μ g/ml doxycycline (DOX). These cells remain viable in the absence of BCR-ABL1 expression if the cell culture

fluid is supplemented with 2 ng/ml murine recombinant IL-3. Protein extracts from these cells were subjected to Western blot using human- and mouse-reactive antibodies (B, right). Tyrosine phosphorylation of PLCγ1 was studied in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells in the presence or absence of STI571 or LFM-A13. EIF-4E was used as a loading control in both Western blots (B, C). Expression of full-length BTK was silenced by RNA interference in a *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell line (IX in Table I). Sorted cells carrying fluorescein-labeled siRNAs (green) were permeabilized, stained for PLCγ1^{pY783} (red), and analyzed by confocal laser microscopy (C, bottom). As a negative control, nontargeting siRNA duplicates were used. BCR-ABL1-binding proteins were coimmunoprecipitated using an antibody against BCR. The immunoprecipitation was controlled by a Western blot using an ABL1-specific antibody (D).

constitutively phosphorylated in all three leukemia cell lines and exhibits nuclear localization (Fig. 4 C), which is required for STAT5 activity (21, 22). However, ablation of BCR-ABL1 kinase activity by STI571 and BTK inhibition by LFM-A13 largely reduced phosphorylation and nuclear localization of STAT5^{Y694} (Fig. 4 C).

In addition, three siRNA duplexes against full-length BTK were mixed, fluorescein labeled, and transfected into *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (IX in Table I) using fluorescein-labeled, nontargeting siRNAs as a control. After two transfections, fluorescein⁺ leukemia cells were sorted and analyzed for STAT5 phosphorylation (Fig. 4 C, lower panel). Efficiency and specificity of BTK full-length silencing was controlled by RT-PCR (Fig. 3). In agreement with BTK inhibition by LFM-A13, silencing of full-length BTK by RNA interference largely attenuated STAT5^{Y694} phosphorylation as shown by confocal laser microscopy (Fig. 4 C) and intracellular FACS staining (see Fig. 8 B).

BTK-mediated transcriptional activation of BCLX_L in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells

In *BCR-ABL1*⁺ leukemia cells, STAT5 mainly accomplishes its antiapoptotic effect by transcriptional activation of BCLX_L (24). By comparing the frequency of SAGE-tags that distinguish between antiapoptotic BCLX_L and its proapoptotic isoform, BCLX_S, in 19 genome-wide SAGE-libraries, SAGE-tags specific for BCLX_L were particularly frequent in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (Table S3).

By analyzing mRNA levels for BCLX_L in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells in the presence or absence of STI571 or LFM-A13, we found that inhibition of BCR-ABL1 or BTK leads to a considerable reduction of BCLX_L expression in the leukemia cells (Fig. 4 D). This is consistent with the effect of siRNA-mediated silencing of full-length BTK on BCLX_L mRNA levels (see Fig. 8 C).

A recent study identified SRC kinase activity as a requirement for BCR-ABL1-mediated transformation of murine pre-B cells (25). The findings presented here indicate that BTK activity also is required specifically for the BCR-ABL1-dependent survival signals, and thus, may represent an important target for the therapy of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia.

BTK activity is required for autonomous Ca²⁺ signal activity in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells

In normal pre-B cells, BTK activity induces Ca²⁺ release upon pre-B cell receptor engagement through BTK-dependent phosphorylation of PLCγ2. Constitutive phosphorylation of BTK by BCR-ABL1 and specific requirement of BTK activity for the survival of *BCR-ABL1*⁺ leukemia cells suggest that BCR-ABL1 also can trigger BTK-dependent Ca²⁺ release from cytoplasmic stores. In *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells that express a pre-B cell receptor on the surface (Table I; reference 8), BCR-ABL1 kinase activity was linked to an autonomous oscillatory Ca²⁺ signal, which was not changed by pre-B cell receptor engagement (arrows, left panel of Fig. 5

A). Analysis of Ca²⁺ release in multiple pre-B lymphoblastic leukemia cells at the single-cell level showed that inhibition of BCR-ABL1 terminated the autonomous Ca²⁺ signaling activity (Fig. 5 A, middle panel). Autonomous Ca²⁺ signals required BCR-ABL1 kinase activity and were abrogated by inhibition of BTK activity through treatment with LFM-A13 (Fig. 5 A, right panel). Comparing sorted fluorescein⁺ leukemia cells which were transfected with full-length BTK-specific or nontargeting siRNAs, autonomous oscillatory Ca²⁺ signals were

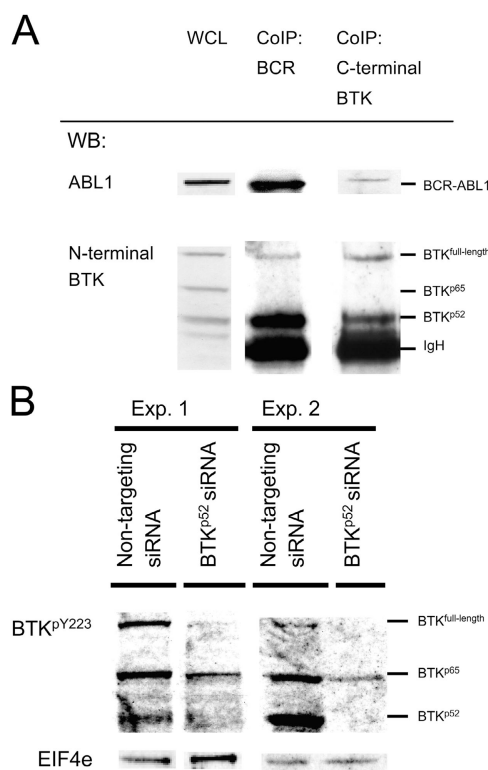


Figure 6. COOH-terminally truncated BTK functions as a linker between full-length BTK and BCR-ABL1. Proteins binding to BCR-ABL1 were coimmunoprecipitated with an anti-BCR antibody (A). Immunoprecipitation was controlled by an anti-ABL1-specific Western blot (WB) showing the characteristic BCR-ABL1 fusion protein expressed by the leukemia cell line. Proteins binding to full-length BTK were coimmunoprecipitated with an antibody against COOH-terminal BTK. Immunoprecipitation was controlled by Western blot using an antibody against NH₂-terminal BTK. Full-length BTK and BTK^{p52} proteins coimmunoprecipitating with BCR-ABL1 or full-length BTK were visualized by Western blot using an antibody against NH₂-terminal BTK (A). As a control for quantitative distribution of full-length BTK and BTK^{p52} before coimmunoprecipitation with BCR-ABL1 or full-length BTK, WCLs were used (A). To analyze the effect of BTK^{p52} on BCR-ABL1-dependent phosphorylation of full-length BTK, BTK^{p52} expression was inhibited by RNA interference as described in Fig. 3. As a control, nontargeting siRNA duplexes were used. siRNAs were fluorescein-labeled and fluorescein⁺ cells were sorted and subjected to Western blot using antibodies specific for tyrosine-phosphorylated BTK^{Y223} (B). Because patterns of tyrosine phosphorylation varied between replicate experiments, Western blots of two representative experiments (Exp. 1 and 2) are shown. EIF4e was used as a loading control.

diminished greatly in the leukemia cells that carried full-length BTK-specific siRNAs (see Fig. 9 B).

Pre-B cell receptor-dependent Ca²⁺ signals typically are initiated by BTK-mediated phosphorylation of PLCγ2 (9).

Assuming that BCR-ABL1 targets downstream molecules of the pre-B cell receptor signal cascade in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells, we investigated whether BCR-ABL1-dependent autonomous Ca²⁺ signals also are trans-

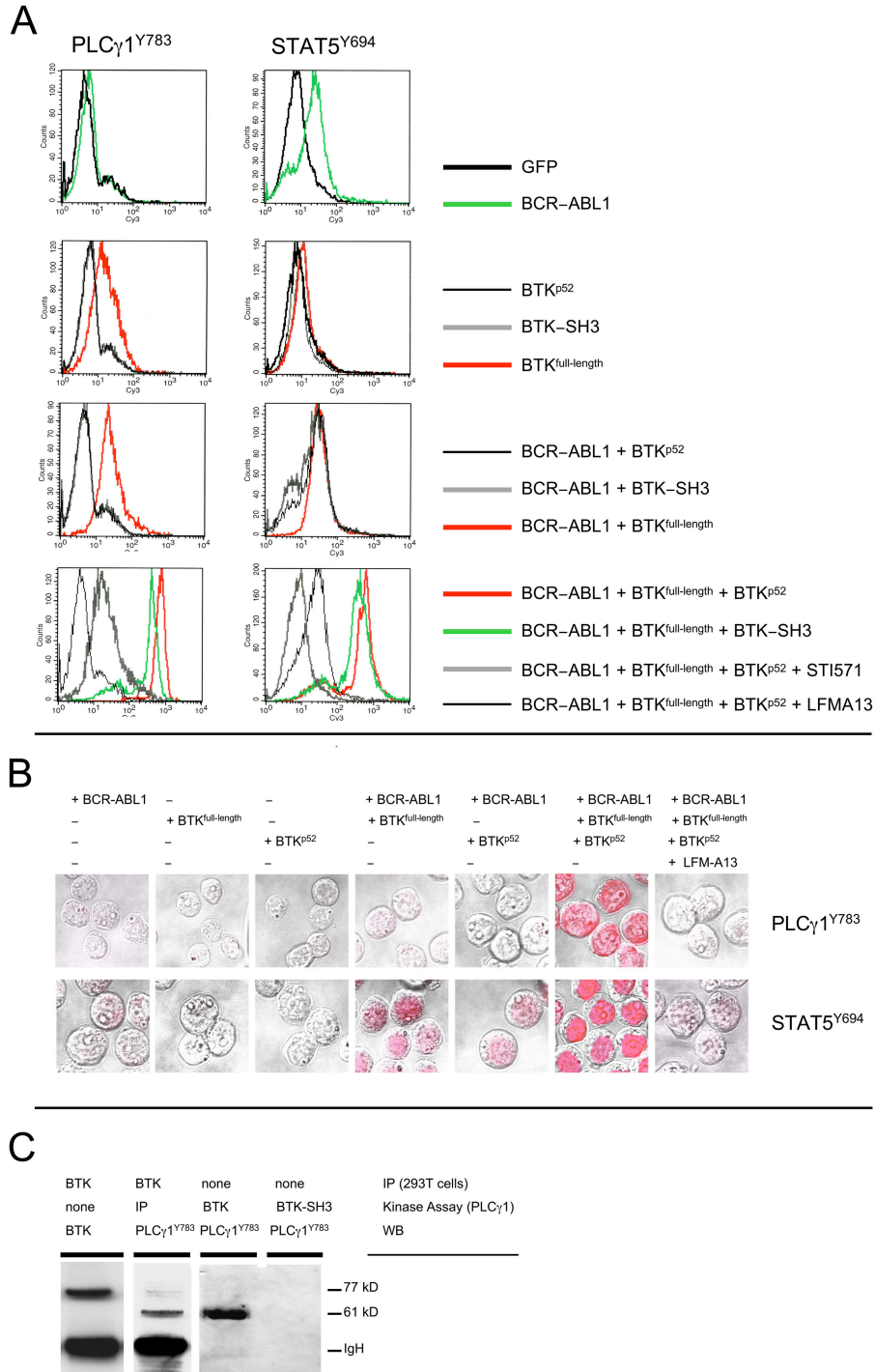


Figure 7. BTK^{p52} facilitates BTK- and BCR-ABL1-dependent activation of PLCγ1 and STAT5 through its SH3 domain. BCR-ABL1, full-length BTK, BTK^{p52}, and the SH3-domain of BTK were expressed in 293T embryonic

kidney cells alone or in various combinations in the presence or absence of STI571 or LFM-A13. As a readout, cells were harvested, subjected to intracellular staining for tyrosine-phosphorylated PLCγ1 or STAT5, and analyzed by

duced by BTK and PLC γ 2. Unexpectedly, PLC γ 1—but not PLC γ 2 (not depicted)—is phosphorylated by BCR-ABL1 in human leukemia cells (Fig. 5, B and C) and in mouse B lymphoid cells that carry an inducible BCR-ABL1 transgene (Fig. 5 B). As shown by coimmunoprecipitation, BCR-ABL1 does not bind directly to PLC γ 1 (Fig. 5 D). Phosphorylation of PLC γ 1 was sensitive to inhibition of BTK by LFM-A13 (Fig. 5 C) which indicates that PLC γ 1 is activated through BTK as in normal lymphocytes and not directly by BCR-ABL1. Silencing of full-length BTK by RNA interference using fluorescein-labeled full-length BTK-specific siRNAs also results in a marked reduction of PLC γ 1^{Y783} phosphorylation as assessed by confocal laser microscopy (Fig. 5 C) and intracellular FACS staining (see Fig. 9 A).

PLC γ 1 contributes to survival signals in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells

Whereas the role of STAT5 in BCR-ABL1-mediated survival signaling is well-established (21, 22, 24), a possible role of PLC γ 1 in the promotion of survival of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells has not been investigated. To determine whether BCR-ABL1- and BTK-dependent activation of PLC γ 1 and PLC γ 1-mediated Ca²⁺ release contributes to BCR-ABL1-mediated survival signals, we silenced PLC γ 1 mRNA expression by RNA interference in three pre-B lymphoblastic leukemia cell lines that carry a *BCR-ABL1* gene rearrangement (VII to IX, Table I) and four cell lines that carry other chromosomal translocations (*MLL-AF4*, X; *TEL-AML1*, XII; *E2A-PBX1*, XIII; *TEL-PDGFRB*, XV; Table I). As a control, nontargeting siRNAs were used. Cells were transfected twice with fluorescein-labeled siRNAs. To assess viability, fluorescein⁺ cells were analyzed for propidium iodide uptake and annexin V staining after 48 h. Among the three *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines, viability (mean of three experiments \pm SD) was 90.3% \pm 2.7% with nontargeting siRNAs and 18.6% \pm 13.7% with PLC γ 1-specific siRNAs. Among four leukemia cell lines that carry other gene rearrangements, viability was 84.3% \pm 12.2% with nontargeting siRNAs and 67.6% \pm 16.9% with PLC γ 1-specific siRNAs. Unlike *BCR-ABL1*-negative leukemia cells, silencing of PLC γ 1 in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells results in a fivefold reduction of viability. Thus, PLC γ 1 contributes to survival signaling specifically in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines.

COOH-terminally truncated BTK functions as a link between full-length BTK and BCR-ABL1

BCR-ABL1-dependent phosphorylation of BTK suggests that BTK represents a substrate of the BCR-ABL1 kinase.

However, previous work demonstrated that BCR-ABL1 cannot phosphorylate full-length BTK directly, but the NH₂-terminal BTK-SH3 domain fused to GST (26).

To determine how BCR-ABL1 can induce phosphorylation of full-length BTK, we studied whether full-length BTK or BTK splice variants are part of the BCR-ABL1-signalosome. Using protein extracts of two *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines, BCR-ABL1 signaling complexes were immunoprecipitated with a BCR-specific antibody. Immunoprecipitation was controlled using an anti-ABL1 antibody. Western blot showed that low amounts of full-length BTK coimmunoprecipitates with BCR-ABL1 (Fig. 6 A). As a control for quantitative distribution of full-length BTK, BTK^{P65}, and BTK^{P52} before coimmunoprecipitation with BCR-ABL1, whole cell lysates (WCLs) from two *BCR-ABL1*⁺ leukemia cell lines were subjected to Western blot (Fig. 6 A). Although full-length BTK, BTK^{P65}, and BTK^{P52} are expressed at similar levels in the leukemia cells (WCL, Fig. 6 A), the amount of full-length BTK that coimmunoprecipitated with BCR-ABL1 was \sim 20-fold less than the amount of BTK^{P52} that coimmunoprecipitated with BCR-ABL1. Although BTK^{P65} is expressed clearly in the leukemia cells (WCL, Fig. 6 A), coimmunoprecipitation of BTK^{P65} with BCR-ABL1 could not be detected (Fig. 6 A).

Preferential binding of BCR-ABL1 to BTK^{P52} that lacked the COOH terminus but retained the NH₂-terminal BTK-SH3 domain is consistent with previous findings that demonstrated that BCR-ABL1 cannot directly phosphorylate full-length BTK but can phosphorylate a BTK-SH3 domain-GST fusion molecule (26). However, binding of BTK^{P52} to BCR-ABL1 also may be indirect in this case. That the NH₂-terminal BTK SH3 domain, but not full-length BTK, can be phosphorylated readily by BCR-ABL1 suggests that the COOH terminus of BTK may interfere with binding of BCR-ABL1 to BTK. Likewise, BTK^{P65} molecules that harbor an in-frame deletion but retain the BTK COOH terminus do not immunoprecipitate with BCR-ABL1. In this regard, the expression of truncated BTK splice variants that lack the COOH terminus of BTK may represent a mechanism to facilitate phosphorylation of full-length BTK by BCR-ABL1. Because BTK molecules also can self-associate through NH₂-terminal SH3 domains (27), BTK^{P52} may bind to BCR-ABL1 as well as to full-length BTK and acts as link between the two kinases.

We next investigated whether full-length BTK can bind to BTK^{P52}. Using an antibody specific for COOH-terminal BTK (only recognizes full-length BTK) for immunoprecipitation and an NH₂-terminal BTK antibody (recognizes full-

flow cytometry (A). To visualize cytoplasmic localization of tyrosine-phosphorylated PLC γ 1 and nuclear localization of activated STAT5, the stained cells also were subjected to analysis by confocal laser microscopy (B). 10⁶ 293T cells were transfected transiently with full-length BTK for 24 h and subjected to immunoprecipitation of full-length BTK. Immunoprecipitation (IP)

was controlled by a BTK-specific Western blot (C). Kinase activity of immunoprecipitated BTK was analyzed in an in vitro kinase assay using 150 ng of a PLC γ 1 fragment (amino acids 530 to 850) as substrate. In parallel, 25 ng of recombinant active BTK and 100 ng of kinase-deficient SH3-domain of BTK were used in kinase assays as positive and negative controls, respectively.

length BTK and BTK^{p52}) for subsequent Western blotting, we observed that BTK^{p52} coimmunoprecipitates with full-length BTK (Fig. 6 A). These findings suggest a role for BTK^{p52} as a link between BCR-ABL1 and full-length BTK.

In this case, BTK^{p52} also would facilitate tyrosine phosphorylation of full-length BTK by BCR-ABL1. Therefore, we tested whether silencing of BTK^{p52} in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells results in decreased tyrosine phosphorylation of full-length BTK. *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells were transfected with nontargeting or BTK^{p52}-specific fluorescein-labeled siRNAs (Fig. 3). Fluorescein⁺ cells were sorted and analyzed for expression of tyrosine-phosphorylated BTK by Western blot (Fig. 6 B). Silencing of BTK^{p52} mRNA expression by siRNAs did not interfere with mRNA expression of full-length BTK (Fig. 3), yet tyrosine phosphorylation of full-length BTK was decreased by >90% (Fig. 6 B).

Given that BCR-ABL1 and full-length BTK cannot bind directly to each other; BTK^{p52} can bind to BCR-ABL1 and full-length BTK; and expression of BTK^{p52} is required for efficient tyrosine phosphorylation of full-length BTK, we

conclude that BTK^{p52} can act as a link between BCR-ABL1 and full-length BTK.

This is in agreement with previous work which suggested that kinase-deficient BTK may act as a linker molecule between upstream kinases (e.g., SYK) and downstream effector molecules (e.g., PLC γ 2; reference 11).

COOH-terminally truncated BTK can link full-length BTK to BCR-ABL1 through its SH3 domain

Based on this assumption, one would predict that the coexpression of BTK^{p52} with full-length BTK can facilitate the interaction between BTK and BCR-ABL1, and hence, the phosphorylation of downstream signaling molecules. To test this possibility, BCR-ABL1, BTK, and its truncated variant, BTK^{p52} were expressed in 293T embryonic kidney cells alone, in various combinations, or in the presence of BTK (LFM-A13) or BCR-ABL1 (STI571) kinase inhibitors. As a readout of this experiment, tyrosine phosphorylation of PLC γ 1 and STAT5 was measured. According to a previous study, 293T cells do not express BTK but do express PLC γ 1 and STAT5 (28).

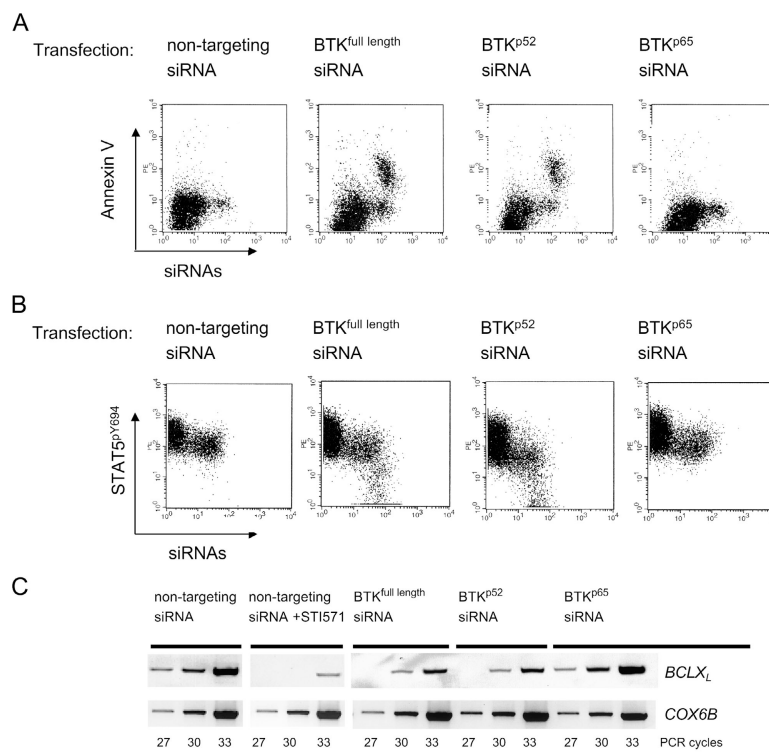


Figure 8. BTK^{p52}, but not BTK^{p65}, promotes cell survival, tyrosine phosphorylation of STAT5, and up-regulation of BCLX_L. *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (IX, Table I) were transfected with fluorescein-labeled siRNA duplexes against full-length BTK, BTK^{p52}, or BTK^{p65} as described above (Fig. 3), and were analyzed by flow cytometry for annexin V expression (A). As a negative control, nontargeting siRNA duplexes were used. Likewise, siRNA-treated leukemia cells were subjected to intracellular staining for tyrosine-phosphorylated STAT5 and analyzed by flow cytometry

(B). For the analysis of mRNA levels of BCLX_L, *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells were transfected with a nontargeting siRNA duplex in the presence or absence of STI571 (for 12 h), as well as with siRNAs against full-length BTK, BTK^{p52}, or BTK^{p65}. 10⁵ leukemia cells carrying fluorescein-labeled siRNAs sorted by FACS and subjected to semiquantitative RT-PCR analysis for mRNA levels of BCLX_L (C). mRNA levels for *COX6B* (C) and *HPRT* (Fig. 3) remained stable in all experiments.

The expression of full-length BTK, BTK^{p52}, or BCR-ABL1 alone had no effect on tyrosine phosphorylation of downstream molecules in general. However, to some extent, single transfection with full-length BTK induced activation of PLC γ 1. Likewise, to some extent, BCR-ABL1 alone induced tyrosine phosphorylation of STAT5 (Fig. 7, A and B); this indicates that BCR-ABL1 can activate STAT5 partially in the absence of BTK. Coexpression of BTK^{p52} which lacked a functional kinase domain and BCR-ABL1 did not increase tyrosine phosphorylation as compared with expression of BCR-ABL1 alone. However, coexpression of BTK^{p52} with full-length BTK and BCR-ABL1 drastically up-regulated PLC γ 1 and STAT5 phosphorylation (Fig. 7 A). As assessed by intracellular staining, phosphorylated PLC γ 1 and STAT5 were distributed in the cytoplasm and the nucleus, respectively (Fig. 7 B).

Activation of PLC γ 1 and STAT5 upon triple transfection with full-length BTK, BTK^{p52}, and BCR-ABL1 largely was sensitive to BTK (LFM-A13) or BCR-ABL1 (STI571) kinase inhibition. Hence, enzymatic activity of both kinases is required for activation of PLC γ 1 and STAT5. As a control, the enzymatic activity of BTK expressed in transfected 293T cells was confirmed in an in vitro kinase assay as described in the Materials and methods section. The kinase activity of BTK which was immunoprecipitated from 10⁵ transfected 293T cells roughly was comparable to that of 25-ng (0.008 U) recombinant BTK (Fig. 7 C).

Because BTK-SH3 domains can bind directly to BCR-ABL1 (26) and also bind to proline-rich regions of other BTK molecules (27), we tested whether the BTK-SH3 domain is sufficient to link full-length BTK to BCR-ABL1. In fact, transfection of 293T cells with a vector encoding only the BTK-SH3 domain had a similar effect compared with transfection with BTK^{p52} comprising PH, TH, SH3, SH2 and a truncated kinase domain (Fig. 2 C). The SH3 domain was sufficient to enable BCR-ABL1-driven activation of PLC γ 1 and STAT5 in the presence of active full-length BTK (Fig. 7 A). We conclude that BTK^{p52}, mainly through its SH3 domain, can act as a linker between full-length BTK and BCR-ABL1.

BTK^{p52} but not BTK^{p65} cooperates with full-length BTK to transduce BCR-ABL1-dependent survival signals

To clarify the specific contribution of BTK^{p52} and BTK^{p65} to BCR-ABL1 downstream survival signaling, annexin V expression, STAT5 phosphorylation, and BCLX_L mRNA levels were measured in leukemia cells after transfection with fluorescein-labeled siRNAs against BTK^{p52} and BTK^{p65} and nontargeting siRNAs (Fig. 8, A–C). Transfection of leukemia cells with nontargeting siRNAs or siRNAs against BTK^{p65} had no effect on viability as assessed by annexin V staining (Fig. 8 A), tyrosine phosphorylation of STAT5 (Fig. 8 B), and BCLX_L mRNA levels (Fig. 8 C). In contrast, siRNA-mediated silencing of BTK^{p52} induced apoptosis in BCR-ABL1⁺ pre-B lymphoblastic leukemia

cells to a similar extent as full-length BTK (Fig. 8 A). Likewise, silencing of full-length BTK or BTK^{p52} decreased tyrosine phosphorylation of STAT5 (Fig. 8 B) and reduced mRNA levels of BCLX_L in the leukemia cells (Fig. 8 C). Collectively, these findings indicate that survival signals in BCR-ABL1⁺ pre-B lymphoblastic leukemia cells require BTK activity, and BTK^{p52} to act as a link between full-length BTK and BCR-ABL1.

Requirement of BTK^{p52} for BCR-ABL1-driven autonomous Ca²⁺ signaling activity

Autonomous oscillatory Ca²⁺ signals in BCR-ABL1⁺ pre-B lymphoblastic leukemia cells require kinase activity of BCR-ABL1 and BTK (Fig. 5 A), as well as PLC γ activity (9). Therefore, we investigated the impact of specific silencing of full-length BTK, BTK^{p52}, and BTK^{p65} on PLC γ 1 activation (Fig. 9 A). siRNA-mediated knockdown of full-length BTK and BTK^{p52} expression similarly reduced tyrosine phosphorylation of PLC γ 1, whereas siRNAs against BTK^{p65} had no effect (Fig. 9 A). In agreement with this, siRNA-mediated inhibition of full-length BTK or BTK^{p52} expression—but not expression of BTK^{p65}—largely reduced

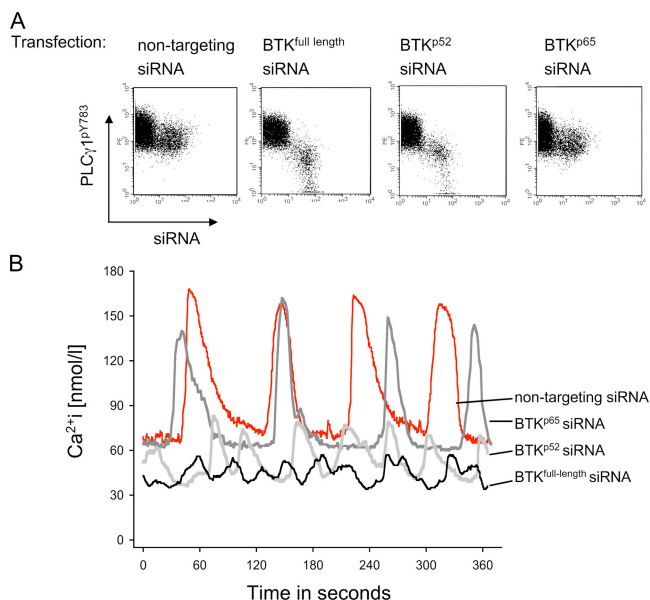


Figure 9. Specific silencing of BTK full-length or BTK^{p52} reduces PLC γ 1 phosphorylation and autonomous Ca²⁺ oscillations in BCR-ABL1⁺ pre-B lymphoblastic leukemia cells. BCR-ABL1⁺ pre-B lymphoblastic leukemia cells were transfected with nontargeting siRNAs or siRNAs against full-length BTK, BTK^{p52}, or BTK^{p65}, then subjected to intracellular staining for tyrosine-phosphorylated PLC γ 1 and analyzed by flow-cytometry (A). Cytoplasmic Ca²⁺ levels [nmol/l] were measured in single siRNA-containing cells by confocal laser-scanning microscopy by continuous scanning for 6 min (B). Before analysis of Ca²⁺ levels, leukemia cells carrying fluorescein-labeled siRNAs were sorted by FACS. For each condition, ~50 individual cells were recorded. As a control, cytoplasmic Ca²⁺ levels were measured in cells with nontargeting siRNAs (red line).

the amplitude of autonomous Ca^{2+} oscillations in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (Fig. 9 B). We could not identify a specific function of BTK^{p65} with respect to BCR-ABL1-mediated transformation in pre-B lymphoblastic leukemia cells.

DISCUSSION

The leukemogenic BCR-ABL1 kinase mimics a constitutively active pre-B cell receptor in pre-B lymphoblastic leukemia cells. Although the leukemia cells frequently carry only nonfunctional *IGH*-alleles (8), pre-B cell receptor signaling is compromised even in the few cases in which the leukemia cells express a pre-B cell receptor on their surface. Important components of the pre-B cell receptor signaling cascade, including SYK, SLP65, and BTK, are not phosphorylated in response to pre-B cell receptor engagement, and BTK is constitutively phosphorylated by BCR-ABL1. BCR-ABL1-dependent activation of BTK is critical for autonomous survival signals that otherwise would arise from the pre-B cell receptor. We conclude that BTK activity is no longer responsive to pre-B cell receptor-dependent signals, but contributes to multiple aspects of BCR-ABL1-driven survival signaling in pre-B lymphoblastic leukemia cells.

Although BCR-ABL1 cannot interact with full-length BTK directly (26), BCR-ABL1-induced aberrant splicing of BTK premRNA leads to the expression of a truncated splice variant of BTK that acts as a linker molecule between the two kinases. Acting as a linker molecule, not only can truncated BTK^{p52} facilitate BCR-ABL1-dependent activation of full-length BTK but it can also facilitate BCR-ABL1-dependent activation of downstream molecules, including PLC γ 1, STAT5, and BCLX_L.

The interaction between BCR-ABL1 and BTK is critical because inhibition of BCR-ABL1 by STI571 and interference with BTK activity specifically induces apoptosis in *BCR-ABL1*⁺ B lymphoid leukemia cells. Because resistance to STI571 is frequent in the therapy of this leukemia entity (15), inhibition of BTK or its truncated splice variant potentially represents a therapeutic approach to circumvent STI571 resistance of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells.

MATERIALS AND METHODS

Patient samples and cell lines. Clinical data on patient samples and cytogenetic data on cell lines used are summarized in Table I and are described in detail in supplemental Materials and methods (available at <http://www.jem.org/cgi/content/full/jem.20042101/DC1>). All studies on human materials were performed on samples provided in compliance with Institutional Review Board regulations (Department of Hematology, University of Frankfurt).

Sequence analysis of BTK and semiquantitative RT-PCR. In a search for BTK isoforms, fragments of the BTK cDNA were amplified covering the entire coding region using the PCR primer pairs listed in Table S1 (available at <http://www.jem.org/cgi/content/full/jem.20042101/DC1>). BTK amplification products were sequenced as described previously (29). Primers used for semiquantitative RT-PCR analysis of human *BCR-ABL1*, *BCLX_L*, *GAPDH*, *HPRT*, and *COX6B* transcripts are listed in Table S1.

Transient expression of BCR-ABL1 in pre-B lymphoblastic leukemia cells. Pre-B lymphoblastic leukemia cells (697 cell line) that carry an *E2A-PBX1*, but no *BCR-ABL1*, gene rearrangement were transfected transiently by electroporation (250 V and 950 μF) with pMIG-GFP or pMIG-GFP BCR-ABL1 vectors that encoded GFP or GFP plus BCR-ABL1 as described previously (30). For both transfections, GFP⁺ and GFP⁻ cells were sorted after 24 h and subjected to mRNA isolation.

RNA interference with BTK and PLC γ 1 expression. For each target, three different siRNA duplexes were synthesized (MWG Biotech). Sequences of siRNA duplexes are listed in Table S1. For knockdown of PLC γ 1 mRNA expression, a pool of three validated siRNAs which target different PLC γ 1 exons was used (Upstate). As a control, a nontargeting siRNA duplex was used that does not match a known mRNA sequence. All siRNA duplexes were labeled with fluorescein using an siRNA labeling kit (Ambion) according to the manufacturer's protocol. *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells were transfected with a mixture of three fluorescein-labeled siRNAs for each target sequence at a concentration of 100 nmol/l using oligofectamine in Opti-MEM1 medium (Invitrogen). After 24 h, leukemia cells were retransfected with labeled siRNAs and incubated for an additional 24 h. Transfection efficiency was controlled by fluorescence microscopy and by FACS. The silencing effect of siRNAs for specific BTK isoforms was controlled by RT-PCR analysis of BTK splice variants in sorted of fluorescein⁺ cells. siRNA-containing fluorescein⁺ cells were sorted using a FACStar 440 cell sorter. For each condition, 5×10^5 cells were sorted and subjected to RNA isolation and cDNA synthesis.

Western blotting. For the detection of signaling molecules by Western blot, antibodies against BTK, PLC γ 1, PLC γ 2, SLP65, and SYK (Cell Signaling Technology), and phosphotyrosine-specific antibodies against BTK^{Y223}, PLC γ 1^{Y783}, PLC γ 2^{Y1217}, SLP65^{Y96}, and SYK^{Y323} (Cell Signaling Technology) were used with the WesternBreeze immunodetection system (Invitrogen). Mouse monoclonal antibodies against tyrosine-phosphorylated BTK^{Y223} and BTK^{Y551} were provided by O.N. Witte (University of California, Los Angeles, CA).

Coimmunoprecipitation. For immunoprecipitation of BCR-ABL1 or BTK, 1.7 mg protein lysate in a volume of 800 μl was incubated with 30 μl rProtein G Agarose beads (Invitrogen) for 2 h at 4°C on a shaker. The supernatant was separated from the agarose beads and incubated with 6 μg of an antibody against BCR (Santa Cruz Biotechnology) or with 15 μg of an antibody against COOH-terminal BTK (provided by Owen N. Witte). After 8 h, 30 μl of agarose beads were added and incubation was continued overnight. The agarose beads were washed twice with lysis buffer, resuspended in 35 μl of lysis buffer, and subjected to Western blotting.

Flow cytometry. For FACS analysis of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells, antibodies against tyrosine-phosphorylated BTK^{Y223}, tyrosine-phosphorylated PLC γ 1^{Y783}, and tyrosine-phosphorylated STAT5^{Y694} (Cell Signaling Technologies) were used. Apoptotic or dead cells were identified by propidium iodide or PE-labeled annexin V (BD Biosciences). As secondary antibody, anti-rabbit or anti-mouse IgG-Cy3 (Jackson ImmunoResearch Laboratories) was used.

Immunofluorescence and confocal laser microscopy. Nuclear, cytoplasmic, or membrane localization of phosphorylated BTK^{Y223}, PLC γ 1^{Y783}, and STAT5^{Y694} was analyzed using primary antibodies from Cell Signaling Technology together with anti-rabbit IgG-Cy5 (Jackson ImmunoResearch Laboratories) as secondary antibody. Cells were fixed with 0.4% paraformaldehyde and incubated for 10 min in 90% methanol on ice and subjected to confocal laser-scanning microscopy as described previously (14).

Expression of BCR-ABL1, full-length BTK, BTK^{p52}, and the BTK-SH3 domain in 293T cells. 10^6 293T embryonic kidney cells were plated on a 24-well plate 24 h before transfection. Different combinations

of expression vectors were prepared as indicated and 5 μ g of each vector was incubated with 8 μ l of FuGENE6 (Roche) and 50 μ l of serum- and antibiotic-free RPMI 1640 medium for 15 min at room temperature. The expression vector *pMIG-bcr/abl* for *BCR-ABL1*^{P210} (provided by D. Baltimore, California Institute of Technology, La Jolla, CA) was used as described previously (30). The expression vector *pMIG-flagBtk* for human full-length *BTK* was generated by cloning of the human *BTK* cDNA into the *pMIG-R* vector (30). For expression of *BTK*^{P52} (GenBank/EMBL/DBJ accession no. AJ888378), the expression vector *pcDNA3.1* was used with the directional TOPO Expression Kit (Invitrogen). For expression of the *BTK-SH3* domain, the *pEBG BTK-SH3* vector (a gift from M. Bäckesjö, Karolinska Institute, Huddinge, Sweden) was used as described previously (26).

Measurement of Ca²⁺ signals in BCR-ABL1⁺ pre-B lymphoblastic leukemia cells. Pre-B lymphoblastic leukemia cells that carry a *BCR-ABL1* gene rearrangement were cultured in the presence or absence of the *BCR-ABL1* inhibitor, ST1571 (Novartis), or the *BTK* inhibitor, LFM-A13 (Calbiochem), for the times indicated. In a different set of experiments, cells were transfected with fluorescein-labeled siRNA duplexes and fluorescein⁺ cells were sorted and conditioned in RPMI medium at 37°C before measurement of cytoplasmic Ca²⁺ concentrations. Cells were washed and stained with Fluo-3 dye (Calbiochem) for 30 min. Changes of cytosolic Ca²⁺ were measured by confocal laser microscopy (31). For each condition, Ca²⁺ release of ~50 individual cells was recorded.

In vitro kinase assay. *BTK* that was immunoprecipitated from transfected 293T cells was used in a kinase assay, including a Mg²⁺/ATP cocktail (100 μ mol/l) and a 61-kD fragment of PLC γ 1 (Santa Cruz Biotechnology) as a substrate of *BTK*. Equal amounts of PLC γ 1 peptide (150 ng) and ATP were incubated for 10 min at 30°C with *BTK* immunoprecipitates from 10⁵ transfected 293T cells; 25-ng active human recombinant *BTK* (Upstate) was used as a positive control or 100 ng of a peptide corresponding to the *BTK-SH3* domain (Labvision; lacking kinase activity) was used as a negative control. The kinase activity of *BTK* that was immunoprecipitated from 5 \times 10⁵ transfected 293T cells was roughly comparable to that of 25-ng (0.008 U) recombinant *BTK* (Fig. 7 C).

Online supplemental material. A detailed description of patient samples and cell lines that were used in this paper is available as supplemental Materials and methods. Table S1 lists all PCR primers and siRNA duplexes used. Table S2 describes all aberrant splice variants of *BTK* amplified from *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells. Table S3 depicts SAGE-data on mRNA levels of *BCLX* and *BTK* isoforms in normal B cell subsets and *BCR-ABL1*⁺ leukemia cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20042101/DC1>.

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