TEL-JAK2 MEDIATES CONSTITUTIVE ACTIVATION OF THE PHOSPHATIDYLINOSITOL 3’ KINASE/PROTEIN KINASE B SIGNALING PATHWAY.

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Running Title: TEL-JAK2 Activates the PI 3’ Kinase/PKB Signaling Pathway.

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SUMMARY

A subset of chromosomal translocations that participate in leukemia involve activated tyrosine kinases. The ets transcription factor, TEL, undergoes translocations with several distinct tyrosine kinases including JAK2. TEL-JAK2 transforms cell lines to factor-independence and constitutive tyrosine kinase activity results in the phosphorylation of several substrates including STAT1, STAT3 and STAT5.

In this study we have shown that TEL-JAK2 can constitutively activate the phosphatidylinositol 3’ kinase (PI 3’ kinase) signaling pathway. The regulatory subunit of PI 3’ kinase, p85, associates with TEL-JAK2 in immunoprecipitations and this was shown to be mediated by the amino terminal SH2 domain of p85 but independent of a putative p85 binding motif within TEL-JAK2. The scaffolding protein Gab2 can also mediate the association of p85. TEL-JAK2 constitutively phosphorylates the downstream substrate Protein Kinase B/AKT. Importantly, the pharmacologic PI 3’ kinase inhibitor, LY294002, blocked TEL-JAK2 factor-independent growth and phosphorylation of PKB. However, LY294002 did not alter STAT5 tyrosine phosphorylation, indicating that STAT5 and PKB activation mediated by TEL-JAK2 are independent signaling pathways. Therefore, activation of the PI 3’ kinase signaling pathway is an important event mediated by TEL-JAK2 chromosomal translocations.
INTRODUCTION

Chromosomal translocations play a central role in the development of leukemia. The participating genes generally fall into three groups involving tyrosine kinases, transcription factors or factors that modify transcriptional activation (1). The prototypical tyrosine kinase is the BCR-ABL translocation which is the causative agent in chronic myelogenous leukemia (2). The ets transcription factor, TEL, is a frequent participant in chromosomal translocations, and a subset of these fusions involve tyrosine kinases including PDGFβR (3), ABL (4,5), ARG (6), TRKC (7,8) and JAK2 (9,10). We are particularly interested in characterizing the properties of the TEL-JAK2 translocation that mediate leukemogenesis.

TEL-JAK2 translocations have been described in three patients to date. Two patients, each harboring primary translocations were diagnosed with acute lymphoblastic leukemia (ALL). One patient expressed a fusion of TEL exon 4 to JAK2 exon 17 (t(9;12)(p24;p13); TEL-JAK2 (4-17)) (9), whereas the other had a TEL exon5 to JAK2 exon 19 translocation (t(9;12)(p24;p13); TEL-JAK2 (5-19)) (10). The third isolated TEL-JAK2 product arose from a compound t(9;12;15)(p24;q15;p13) translocation in which one allele of TEL was fused to JAK2 (TEL-JAK2 (5-12)) (9) and the other TEL allele was fused to EVI1 (11). All three fusions have been shown to convert IL-3 dependent hematopoietic cells to factor-independence (10,12).

Many studies have focused on the mechanism of constitutive activation mediated by BCR-ABL. Substrates that are activated downstream of BCR-ABL include STAT1 (13-15), STAT3 (15), STAT5 (13-16) and STAT6 (15). Grb2-Sos can be recruited to BCR-ABL either directly or indirectly through other adaptor proteins including Ship1 (17), Shp2 (18), Shc (19-23) and Cbl (24-28). BCR-ABL has also been shown to stimulate activation of Ras (29) and the related family member Rac (30).
BCR-ABL also has been shown to participate in pathways that are involved in the prevention of apoptosis. For example, BCR-ABL activates the PI 3’ kinase signaling pathway (31,32). Recent studies have shown that the PI 3’ kinase inhibitor, LY294002, blocks growth of BCR-ABL expressing hematopoietic cells (32).

PI 3’ kinases are important modulators of cell survival, mitogenesis, cytoskeletal remodeling, metabolic control and vesicular trafficking (reviewed in (33)). There are 3 classes of these enzymes. Class I PI 3’ kinases are heterodimers consisting of a 110 kDa catalytic subunit and a 85 kDa regulatory subunit. Binding of the p85 subunit to phosphotyrosines stimulates activity of the associated p110 subunit (34-36). The two SH2 domains of p85 can interact with phosphorylated tyrosines on activated receptor tyrosine kinases or on adaptor proteins such as Gab2 (37) and IRS-2 (38). The activation of PI 3’ kinase catalyzes the phosphorylation of phosphatidylinositol (PtdIns) lipids on the D3-hydroxy group generating products such as PtdIns(3,4)P2 and PtdIns(3,4,5)P3. These lipids can modulate the subcellular localization and activation of a number of proteins. The serine/threonine kinase, Akt/PKB, is one well studied target of PI 3’ kinase activation implicated in mediating signals for cell survival and growth (reviewed in (39)).

TEL-JAK2 has been shown to transform cell lines to factor-independence through constitutive tyrosine kinase activity (10,12,40). Importantly, TEL-JAK2 does not activate endogenous JAK kinases but does result in constitutive tyrosine phosphorylation and DNA binding of STAT1 (12,40), STAT3 (40) and STAT5 (12,40). Bone marrow transplant studies demonstrate that TEL-JAK2 (5-19) gives rise to a biphenotypic disease with elements of myeloid and lymphoproliferation (12). TEL-JAK2 (5-19) transgenic mice develop a fatal T cell leukemia (41). The importance of STAT5 in TEL-JAK2 mediated leukemogenesis was recently
demonstrated as TEL-JAK2 transduced bone marrow cells failed to induce neoplasia when introduced into a genetic background devoid of STAT5a/b (42). However, a constitutively activated form of STAT5 resulted in only a myeloproliferative disease (42). In summation, these elegant studies have shown that signaling pathways distinct from STAT5a/b activation play a role in leukemogenesis mediated by TEL-JAK2. The goal of this study is to characterize PI 3’ kinase-dependent signaling mitigated by TEL-JAK2.
EXPERIMENTAL PROCEDURES

Generation of TEL-JAK2 constructs

Constructs were generated as described (43). The Quick-Change site directed mutagenesis kit (Stratagene) was used to introduce the Y624F mutation into TEL-JAK2 (5-19) with the following primers: 5’-GCCAGATGAGATCTTTATG-3’ and 5’-GCATTCTGTCATGATCATAAAGATCTCATCTGGGC-3’.

Cell lines and culture

Murine Ba/F3 cells were maintained in complete media (RPMI-1640 medium with antibiotics, 10% (vol/vol) fetal bovine serum (FBS) (Sigma), 50 µM β-mercaptoethanol (FisherBiotech) containing 100 pg/mL of recombinant murine IL-3 (IL-3) (R&D Systems) in a 5% CO₂ incubator at 37°C. The same conditions using G418 selection media (complete media containing 100 pg/mL IL-3 with 1 mg/mL Geneticin (GibcoBRL)) maintained subclones of Ba/F3 cells expressing TEL-JAK2 (4-17), TEL-JAK2 (5-19), TEL-JAK2 (5-19) Y624F, TEL-JAK2 (5-12), BCR-ABL p210, or pcDNA3 vector alone.

Electroporations were performed as described (40,44), using 20 µg of DNA for the various constructs, vector alone, or no vector (350 mV and 950 µF) into Ba/F3 cells (GenePulser, BioRad). G418 resistant populations were selected and subclones were isolated by limiting dilution. The expression of TEL-JAK2 and BCR-ABL was confirmed by immunoblotting and the IL-3 dependent growth characteristics of each subclone was confirmed by performing an XTT assay.
**XTT assay**

XTT assays were performed as described (40,44). Cytokine-depleted cells (2000/well) were added to a 96-well plate in a final volume of 100 μL containing complete media with varying concentrations of LY294002 and a constant concentration of IL-3. Plates were incubated at 37°C for 48 hours prior to addition of sodium 3,3’-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) (2 mg/mL) (Diagnostic Chemicals) and phenazine methosulfate (PMS) (3 μM) (Sigma) (final volume of 125 μL). Cells were incubated for an additional 4 hours at 37°C prior to measuring the absorption of the soluble formazan reduction product at 450 nm.

**Preparation of cellular protein lysates**

Cells were depleted of cytokine by washing three times with Hanks BSS containing 10mM Hepes (pH 7.4) and incubating at 37°C for 18 hours in complete media. Cells were then stimulated in the presence or absence of 10 ng/mL IL-3 in complete media for 10 minutes at 37°C. Cells were washed once in cold Hanks BSS containing 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 10 mM EDTA and 1 mM sodium orthovanadate. Lysates were prepared in ice cold lysis buffer, containing 50 mM TrisHCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10 mM Na₂PO₄, 10 mM NaF, 10 mM EDTA, 1 mM Na₃VO₄, 1 μM phenylmethysulfonyl fluoride (PMSF), 1 μM aprotinin, 1 μM leupeptin, and 2 μM pepstatin A, incubated for 10 minutes on ice, and centrifuged at 10000g for 5 minutes at 4°C. Lysate concentrations were quantified by the Bradford colorimetric method (BioRad). For immunoblot analyses of lysates, 100 μg of lysate was boiled for 5 minutes in Laemmli sample buffer with 100 μM dithiothreitol (DTT). Samples
were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF transfer membrane (NEN Life Science).

**Inhibitors**

LY249002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) (Calbiochem) was resuspended in dimethyl sulfoxide (DMSO) (FisherBiotech) to a final concentration of 65.06 mM. Preparation of cellular protein lysates for inhibitor studies is exactly as described for the preparation of cellular protein lysates with an additional incubation step: prior to murine IL-3 stimulation, cells were incubated with 10 or 20 µM LY294002 or carrier alone for 30 minutes.

**Antibodies**

The anti-phosphotyrosine antibody, 4G10, was generously provided by Dr Brian Druker, Oregon Health Sciences University, Portland, OR. Rabbit anti-myc was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Mouse anti-p85 was obtained from Transduction Laboratories, Lexington, KY. Rabbit anti-IRS-2, rabbit anti-Gab2, and rabbit anti-p85 were purchased from Upstate Biotechnology, Lake Placid, NY. Anti-phospho-PKB (Ser473) and anti-PKB antibodies were purchased from New England BioLabs, Beverly, MA. Phospho-STAT5 antibody was purchased from Zymed, South San Francisco, CA and anti-STAT5 antibody was generously provided by Dr James Ihle, St Jude’s Childrens Hospital, Memphis, TN. A peptide-specific anti-TEL antibody was generated using a KLH-coupled peptide corresponding to amino acids 138–154 of TEL. Immunoblotting secondary reagents used were Horseradish peroxidase (HRP) conjugated protein A or HRP-sheep anti-mouse immunoglobulin obtained from Amersham Life Science.
**Immunoprecipitations**

Immunoprecipitations were performed with 1.5 mg of protein lysates. Primary antibody was added for 1 hr, followed by 1 hr incubation with Protein A-Sepharose (Amersham Pharmacia Biotech). Alternatively, primary antibody and Protein A-Sepharose was added together and incubations were performed overnight. Bead-bound immune complexes were washed 3 times with ice cold lysis buffer, eluted by boiling for 5 minutes in Laemmli sample buffer containing 100 μM DTT, and separated by SDS-PAGE and transferred to PVDF transfer membrane for immunoblotting.

**In vitro Mixes – GST fusion protein binding experiments**

GST fusion proteins (2.5 μg) expressing the amino, carboxy, or amino and carboxy terminal SH2 domains of p85 (generously provided by Dr Ben Margolis, U of M, Ann Arbor, MI) or GST alone immobilized to Glutathione sepharose 4B beads (Amersham Pharmacia Biotech), were incubated with 1.5 mg of protein lysates. After a 1 hour incubation at 4°C, the precipitate was washed 3 times with ice cold lysis buffer. Samples were boiled for 5 minutes in Laemmli sample buffer with 100 μM DTT to elute proteins before separation on SDS-PAGE gels and transfer to PVDF transfer membrane.

**Immunoblotting**

For most immunoblotting experiments, membranes were blocked at room temperature with 2.5% BSA in Tris-buffered saline (TBS; 50 mM Tris (pH8.0), 150 mM NaCl) for 1 hour. Following two washes in TBST (TBS, 0.1% Tween-20), membranes were incubated with the
appropriate dilution of primary antibody solution for 1 hour at room temperature. Membranes were then washed four times in TBST and incubated with the relevant HRP-conjugated secondary antibody (1:5000 dilution in TBST) for 30 minutes. Following four washes in TBST, reactive proteins were visualized by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) with autoradiographic film (Amersham Life Science).

PVDF membranes for phospho-PKB and PKB immunoblots were blocked in 5% skim milk in TBST for 1 hour at room temperature, washed once in primary antibody dilution buffer and incubated with primary antibody (1:1000 dilution in 1% BSA in TBST) overnight at 4°C. After 6 washes in TBST, the membrane was incubated with HRP-Protein A (1:2000 dilution in 2.5% skim milk in TBST) for 1 hour at room temperature. The membrane was washed 6 times in TBST and visualized by ECL.

Membranes for phospho-STAT5 immunoblots were blocked in 5% milk in TBST for 1 hour at room temperature, washed 2 times in TBST and incubated with primary antibody (1:1000 dilution in 3% BSA in TBST) for 3 hours at room temperature. After 4 washes in TBST, the membrane was incubated with HRP-Protein A (1:5000 dilution in 2.5% BSA in TBST) for 1 hour at room temperature. The membrane was washed 4 times in TBST prior to visualization by ECL. For reprobing, membranes were stripped in 62.5 mM Tris-HCl (pH6.8), 2% SDS and 0.1M β-mercaptoethanol for 30 minutes at 50°C, and rinsed twice in TBST.

**Apoptosis assays**

Annexin V, 7AAD and 10X binding buffer were purchased from PharMingen. Briefly, at distinct time points, untreated and treated cells were washed in 1X binding buffer (10 mM Hepes (pH7.4), 140 mM NaCl, 2.5 mM CaCl₂). 1x10⁶ cells were then resuspended in 50 μL 1X binding
buffer and incubated with 2 µL Annexin V antibody conjugated to PE for 10 minutes at room temperature. Samples were adjusted to a final volume of 1 mL prior to FACS analysis (Becton Dickinson). Acquisition and analysis were performed using the CellQuest software.
**RESULTS**

TEL-JAK2 isoforms have been constructed with breakpoints as described from patient samples (9,10) (Figure 1). These constructs were introduced into the murine IL-3 dependent myeloid cell line Ba/F3 via electroporation. TEL-JAK2 (4-17), TEL-JAK2 (5-19) and TEL-JAK2 (5-12) subclones were isolated by limiting dilution and those displaying similar expression were selected for further characterization. Expression of TEL-JAK2 in Ba/F3 cells resulted in factor-independent proliferation and constitutive tyrosine phosphorylation of each fusion protein in all subclones, consistent with previous reports (10,12).

*TEL-JAK2 fusion proteins constitutively activate Protein Kinase B(PKB)/Akt.*

Growth factors and cytokines, including IL-3, induce the activity of PI 3’ kinases. In addition, oncogenic tyrosine kinase fusions have been shown to activate PI 3’ kinase. The transforming ability of BCR-ABL has been shown to require the PI 3’ kinase signaling pathway and activation of the serine-threonine kinase PKB (45). PKB is one downstream component of the PI 3’ kinase signaling pathway important in influencing cell survival. We were interested in determining whether TEL-JAK2 mediated PKB activation (Figure 2). Since the phosphorylation of PKB is associated with its activation (46), activation-specific antibodies have been developed that detect PKB phosphorylated at ser-473. IL-3 stimulation of Ba/F3 cells led to a strong activation of PKB phosphorylation (lane 2). TEL-JAK2 (4-17) stimulated a level of PKB phosphorylation in the absence of IL-3 stimulation (lane 3) which was higher than that in unstimulated Ba/F3 cells (lane 1). Expression of TEL-JAK2 (5-12) and TEL-JAK2 (5-19) in Ba/F3 cells resulted in higher levels of constitutive PKB phosphorylation (lanes 5 and 7, respectively). Ba/F3 cells expressing BCR-ABL also stimulated PKB phosphorylation in the
absence of IL-3 (lane 9). Upon IL-3 stimulation, all cell lines exhibited comparable levels of PKB phosphorylation (even lanes). Equal loading was confirmed by reprobing the blot with a total PKB antibody (lower panel). This experiment demonstrated that TEL-JAK2 expression constitutively activates PKB phosphorylation.

**PI 3’ Kinase Activation is Required for TEL-JAK2 Mediated Cell Proliferation/Survival**

To determine whether TEL-JAK2 mediated factor-independent growth was dependent on PI 3’ kinase, we performed XTT assays in the absence or presence of IL-3 (100 pg/mL) and increasing concentrations of the PI 3’ kinase inhibitor, LY294002 (47) (Figure 3). A reduction in the number of Ba/F3 and all TEL-JAK2 expressing Ba/F3 cells was observed with increasing LY294002 concentrations (upper panel), even in the presence of IL-3 (lower panel). Subclones of Ba/F3 cells expressing vector alone had identical kinetics as untransfected Ba/F3 cells (data not shown). Moreover, this decrease in cell number was not seen in the presence of the carrier, DMSO (data not shown). These results suggest that TEL-JAK2, and IL-3 (48-50), signal for cell survival and proliferation through a PI 3’ kinase dependent pathway.

To test whether TEL-JAK2 phosphorylation of PKB is dependent on PI 3’ kinase, IL-3 depleted Ba/F3 cells and Ba/F3 cells expressing TEL-JAK2 (5-19) were pretreated with the PI 3’ kinase inhibitor, LY294002 (Figure 4). As illustrated above, TEL-JAK2 (5-19) induced the constitutive phosphorylation of PKB (lane 5). Treatment of these cells with 20 μM LY294002 for 30 minutes diminished PKB phosphorylation to basal levels (lane 7). LY294002 treatment also significantly impaired IL-3 stimulated PKB phosphorylation in Ba/F3 cells (lane 4) and Ba/F3 cells expressing TEL-JAK2 (5-19) (lane 8). This experiment suggests that TEL-JAK2 signaling through PKB is dependent upon PI 3’ kinase activity.
The p85 Regulatory Subunit of PI 3’ Kinase Associates with TEL-JAK2 Fusion Proteins

Activation of the PI 3’ kinase catalytic subunit p110 is dependent on association of the regulatory p85 subunit with activated tyrosine kinases (36), reviewed in (51)). For example, the oncogenic kinase BCR-ABL has been shown to activate PI 3’ kinase by association with p85 (26,45,52). We wished to analyze whether TEL-JAK2 associated and/or phosphorylated p85 and to determine the complexes that are constitutively bound to p85 (Figure 5). IL-3 stimulated the association of IRS-2, p115, Gab2, Shp2 and Shc with p85 (lane 2). In addition, tyrosine phosphorylated (first panel) TEL-JAK2 (4-17) (lanes 3 and 4), TEL-JAK2 (5-12) (lanes 5 and 6) and TEL-JAK2 (5-19) (lanes 7 and 8) all co-immunoprecipitated with p85 in both the absence and presence of IL-3. As observed in IL-3 stimulated Ba/F3 cells, several additional phosphoproteins associate with p85 in TEL-JAK2 expressing cells. Increased association of Gab2 with p85 was observed in TEL-JAK2 (5-12) (lane 5) and TEL-JAK2 (5-19) (lane 7), but not TEL-JAK2 (4-17) transfectants. This is also observed on reprobing the membrane with an anti-Gab2 antibody (third panel). Gab2 belongs to a family of adaptor proteins that link receptor tyrosine kinases to downstream signaling molecules. The 170 kDa tyrosine phosphorylated substrate was identified to be IRS-2 from reprobing the membrane. The 68 kDa and 52 kDa bands co-immunoprecipitating with p85 (pTyr immunoblot) represent Shp2 and Shc, as revealed by reprobing. In addition, there was an unidentified band of approximately 115 kDa co-immunoprecipitating with p85 (pTyr immunoblot). In all cell lines, in both the absence and presence of IL-3, p85 was not tyrosine phosphorylated (p85 immunoblot). Preimmune serum did not immunoprecipitate any tyrosine phosphorylated proteins or appreciable amounts of IRS-2, p85, Shp2, Shc or Gab2 (lanes 9 to 12). These data indicate that TEL-JAK2 interacts with p85.
The p85 subunit of PI 3’ kinase contains two SH2 domains and one SH3 domain. We next determined whether the TEL-JAK2-p85 interaction was SH2 dependent. An in vitro mixing experiment was performed using cell lysates from Ba/F3 and TEL-JAK2 (5-19) expressing Ba/F3 cells (Figure 6A). In TEL-JAK2 (5-19) cell lysates, a GST fusion protein containing both the amino and carboxy terminal SH2 domains of p85 constitutively bound TEL-JAK2 (5-19) (lanes 15 and 16) as determined by anti-phosphotyrosine immunoblotting. A GST fusion protein containing only the amino terminal SH2 domain of p85 (lanes 9 to 12) resulted in the same interactions. In contrast, no interactions were observed with a GST fusion protein containing only the carboxy terminal SH2 domain of p85 (lanes 5 to 8). In vitro mixing experiments with Ba/F3 cells expressing TEL-JAK2 (4-17) or TEL-JAK2 (5-12) confirmed that these two isoforms associate with the p85 SH2 domains in the same manner as TEL-JAK2 (5-19) (data not shown). From these experiments, we concluded that the amino terminal SH2 domain of p85 is sufficient for the association of p85 with TEL-JAK2 (5-19).

In order to further identify TEL-JAK2 (5-19) in the p85 in vitro mixing experiments, an immunodepletion experiment was performed prior to capture of proteins on GST-p85 N+C SH2 domains (Figure 6B). A peptide-specific TEL antibody was raised against amino acids 138-154 of TEL. This antibody is capable of immunoprecipitating TEL and TEL-JAK2 (Kim, H., Nguyen M. H.-H., and Barber, D.L., Manuscript in preparation). Ba/F3 and Ba/F3 TEL-JAK2 (5-19) lysates were immunoprecipitated with preimmune IgG or anti-TEL (lanes 1 to 8). The remaining supernatant was then incubated with GST or GST-p85 N+C SH2 fusion proteins (lanes 9 to 19). TEL-JAK2 (5-19) was observed in the TEL immunoprecipitations (lanes 7 and 8). Following immunodepletion with preimmune IgG, TEL-JAK2 (5-19) associated with GST-p85 N+C SH2 domains (lanes 18 and 19). However, prior incubation with a peptide-specific TEL antibody
removed TEL-JAK2 (5-19) from the lysate as TEL-JAK2 (5-19) was absent upon incubation with GST-p85 N+C SH2 domains (lanes 11 and 12). GST failed to bind any tyrosine phosphorylated proteins in Ba/F3 or Ba/F3 TEL-JAK2 (5-19) cells (lanes 13 to 15). This experiment demonstrates that the 73 and 77 kDa proteins that associate in a SH2-dependent manner with p85 are the two isoforms of TEL-JAK2 (5-19).

A Potential p85 Binding Motif in TEL-JAK2 is Dispensable for TEL-JAK2 Mediated Proliferation, PI 3’ Kinase Activation and p85 Association with TEL-JAK2 (5-19)

The SH2 domains of p85 have been shown to preferentially interact with the linear amino acid motif pYXXM (53). All three TEL-JAK2 fusion proteins contain a YMIM motif at the carboxy terminus that may serve as a p85 docking site in a region conserved with the FGF Receptor1 tyrosine kinase domain. This site has been shown to be important in PI 3’ kinase activation downstream of FGF receptor activation (54). Site directed mutagenesis was used to mutate this tyrosine, amino acid 624, to phenylalanine in TEL-JAK2 (5-19). The effect of this mutation on p85 binding and PI 3’ kinase activation was examined (Figure 7). The phosphorylation of PKB at serine 473 (Figure 7A) in the absence of IL-3 by the mutant TEL-JAK2 (5-19) Y624F (lanes 5 and 7) was higher than Ba/F3 cells (lane 1), but similar to TEL-JAK2 (5-19) (lane 3). PKB phosphorylation in all cell lines was comparable upon IL-3 stimulation (even lanes).

Furthermore, LY294002 reduced both constitutive and IL-3-induced PKB phosphorylation (data not shown). Upon treatment with LY294002 (Figure 7B), the growth/survival of wild type and mutated TEL-JAK2 (5-19) declined in a similar manner, both in the absence (upper panel) and presence of 100 pg/mL IL-3 (lower panel). Ba/F3 TEL-JAK2
(5-19) Y264F cells displayed IL-3 independence and constitutive tyrosine phosphorylation of this mutant fusion protein was also comparable to that of wild type TEL-JAK2 (5-19) (data not shown).

Immunoprecipitations using anti-p85 antibodies revealed similar interactions as described above (Figure 7C). Interestingly, tyrosine phosphorylated TEL-JAK2 (5-19) Y624F was capable of co-immunoprecipitating with p85 in both the absence and presence of IL-3 (lanes 5 to 8) with similar intensity to TEL-JAK2 (5-19) (lanes 3 and 4). No proteins were observed to immunoprecipitate with preimmune IgG (lanes 9 to 12). In vitro mixing experiments (Figure 7D) confirmed that GST-N+C SH2 p85 could mediate the interaction between TEL-JAK2 (5-19) Y624F and p85 (lanes 9 to 12). Further analysis revealed that the amino terminal SH2 domain of p85 was sufficient for this interaction (data not shown). These results indicate that PI 3’ kinase can still actively mediate PKB phosphorylation and cell proliferation induced by TEL-JAK2 (5-19) Y624F. The mutation of tyrosine 624 does not abolish TEL-JAK2-p85 association although a putative p85 binding site has been disrupted.

The Scaffolding Protein Gab2 is a Possible Mediator of PI 3’ Kinase Association with TEL-JAK2

The involvement of adaptor molecules is widespread in linking signaling pathways. With the suggestion that direct recruitment of p85 to TEL-JAK2 via the YMIM motif is not significant, we next investigated the possibility of adaptor molecules mediating the association between TEL-JAK2 and p85. Gab2 (37) has been shown to contain multiple consensus p85 binding sites thereby mediating PI 3’ kinase signaling downstream of cytokine, growth factor and antigen receptor activation (37,38).
To address the possibility of Gab2 association with TEL-JAK2 and with p85, immunoprecipitations were performed with a peptide-specific Gab2 antibody (Figure 8). A basal level of Gab2 tyrosine phosphorylation was observed in cytokine-depleted Ba/F3 cells (lane 1). Stimulation with IL-3 (even lanes) results in the association of Shp2 (68 kDa) and Shc (52 kDa). The association of TEL-JAK2 (5-12) (lanes 5 and 6), TEL-JAK2 (5-19) (lanes 7 and 8) and TEL-JAK2 (5-19) Y624F (lanes 9 to 12) with Gab2 were independent of IL-3 stimulation. Interestingly, co-immunoprecipitation of TEL-JAK2 (4-17) with Gab2 was not detected (lanes 3 and 4). Gab2 is constitutively tyrosine phosphorylated under basal conditions, TEL-JAK2 (5-12) and TEL-JAK2 (5-19) promote increased tyrosine phosphorylation, while IL-3 stimulation produces the slowest migrating Gab2 in these experiments. In addition, reprobing the membrane with a peptide-specific p85 antibody revealed constitutive binding of the regulatory subunit of PI 3’ kinase. IL-3 stimulates the association of Shp2 and Shc with Gab2 in Ba/F3 cells. Shp2 is constitutively associated with TEL-JAK2 (5-12) (lane 5), TEL-JAK2 (5-19) (lane 7) and TEL-JAK2 (5-19) Y524F (lanes 9 and 11) but not in TEL-JAK2 (4-17) cells (lane 3) (Shp2 reprobe). All TEL-JAK2 isoforms stimulate the binding of Shc to Gab2 (Shc reprobe). Preimmune IgG did not immunoprecipitate any tyrosine phosphorylated proteins or p85, Shp2, Shc or Gab2 from Ba/F3 or Ba/F3 TEL-JAK2 (5-19) cell lysates (lanes 13 to 16). Reprobing the membrane with a Gab2 antibody showed differential mobility of Gab2. The decreased migration after stimulation with IL-3 is likely a product of post-translational modification. These interactions indicate a role for Gab2 linking p85 to TEL-JAK2.
Expression of TEL-JAK2 Protects Ba/F3 Cells from Apoptosis

We have demonstrated an association between TEL-JAK2 and PI 3’ kinase and the significance of this pathway in factor-independent growth. However, PI 3’ kinase and PKB have also been implicated in modulating protection from programmed cell death (55-59). We were interested in assessing whether TEL-JAK2 expression can lead to decreased apoptosis of cells, thereby contributing to an increase in cell number. To compare the number of cells undergoing apoptosis when depleted of IL-3, we performed Annexin V and 7AAD staining (Figure 9). Annexin V serves as a marker for apoptosis in the early phase while 7AAD stains the DNA of late apoptotic or necrotic cells whose membrane integrity has been compromised (60,61). The percentage of early apoptotic Ba/F3 cells which stain for only Annexin V, peaked after approximately 12 hours of IL-3 withdrawal. This was followed by a dual Annexin V- and 7AAD-positive population (bottom panel), representing Ba/F3 cells in a later apoptotic stage. After 48 hr, 90% of the apoptotic cells were double stained for Annexin V and 7AAD (bottom panel). In contrast, TEL-JAK2 (5-19) expressing cells cultured in the absence of IL-3 exhibited Annexin V and 7AAD staining at levels comparable to untransfected and TEL-JAK2 (5-19) expressing cells growing in the presence of IL-3 (top and bottom panels). In accordance with apoptosis and not necrosis, staining for only 7AAD in both cell lines was very low (< 1%) (data not shown). These results would suggest that Ba/F3 cells undergo apoptosis upon withdrawal of IL-3, however, expression of TEL-JAK2 conferred resistance to apoptosis. One possible mechanism may be mediated through the activation of PI 3’ kinase and PKB.
Activation of STAT5a/b and PKB Are Independent Signaling Pathways Downstream of TEL-JAK2

The importance of STAT5 in TEL-JAK2 signaling and leukemogenesis has been demonstrated in vitro (10,12,40) and in vivo (12,42). However, bone marrow transplants performed with a constitutively active STAT5 or a STAT5 target gene, failed to recapitulate the phenotype of TEL-JAK2 transplanted mice (42). This suggests that TEL-JAK2 activates signaling targets distinct from STAT5. Therefore, we examined whether pretreatment of TEL-JAK2 expressing cells with LY294002 would affect STAT5 tyrosine phosphorylation (Figure 10). IL-3 (lanes 5-8) and TEL-JAK2 (5-19) (lanes 13 to 16) mediated PKB phosphorylation was decreased by LY294002 pretreatment. However, there was no diminution of constitutive or IL-3 induced STAT5 phosphorylation as determined by immunoblotting with a phospho-specific STAT5 antibody in the presence of either 10 or 20 µM LY294002 (third panel). Our results indicate that PI 3’ kinase is involved in PKB phosphorylation, and that this signaling pathway is distinct from STAT5 phosphorylation and activation.
DISCUSSION

The pathways of hematopoietic cell transformation mediated by TEL-JAK2 have not been extensively characterized. TEL-JAK2 has been shown to be oncogenic in vivo (12) and capable of transforming cells to factor independence in vitro (10,12,40). The constitutive activation of STAT1, STAT3 and STAT5 is observed downstream of TEL-JAK2 activation, however, it is known that pathways distinct from STAT5 are required in vivo for leukemic progression (42). PI 3’ kinase and PKB have been implicated in signaling cell proliferation and survival upon activation by normal and oncogenic tyrosine kinases. In this study we have demonstrated that TEL-JAK2 can interact with PI 3’ kinase, mediating the phosphorylation of PKB and signals for cell survival and proliferation.

Activation of PI 3’ kinase requires the association of the regulatory p85 subunit with tyrosine kinases leading to activation of the PI 3’ kinase catalytic subunit p110 kinases ((36), reviewed in (51)). Chromosomal translocations involving BCR-ABL (31) and NPM-ALK (62) have been shown to constitutively activate the PI 3’ kinase signaling pathway. p85 associates with tyrosine phosphorylated TEL-JAK2 in immunoprecipitations, and in vitro mixing experiments confirm that the amino terminal SH2 domain of p85 can mediate this interaction. p85 may be binding directly to TEL-JAK2 through a phosphotyrosine motif, or indirectly by association with adaptor proteins. Any direct interaction between p85 and TEL-JAK2 does not occur solely via the putative p85 binding site on JAK2. We have shown that disruption of the optimal p85 binding motif, YMIM, by mutation of tyrosine 624 in TEL-JAK2 (5-19) did not affect p85 interaction, PKB phosphorylation or factor independent growth. There are no other YXXM motifs in any of the TEL-JAK2 isoforms that may mediate this interaction, however, it is
possible that direct recruitment of p85 to TEL-JAK2 occurs through a phosphotyrosine motif other than YXXM as has been shown for the erythropoietin receptor (63).

Adaptor proteins including Gab1, Gab2, IRS-1 and IRS-2 have been shown to play a critical role in coupling tyrosine kinase activation to PI 3’ kinase recruitment (37,64,65). In Ba/F3 cells, Gab1 and IRS-1 are not expressed (data not shown), so our efforts focused on Gab2 and IRS-2. These adaptor proteins contain YXXM motifs, in addition to multiple tyrosine phosphorylation sites and motifs for binding SH2 and SH3 domains (37,38). Our studies suggest that the association of Gab2 with p85 in Ba/F3 cells is constitutive. The tyrosine phosphorylation of Gab2 was higher in TEL-JAK2 expressing cells in the absence of IL-3, particularly with TEL-JAK2 (5-19) and TEL-JAK2 (5-12) expression, and increased upon IL-3 stimulation. This increase in tyrosine phosphorylation of Gab2, however, did not influence the amount of p85 associated with Gab2, as determined by immunoblotting. It is possible that a steady state complex between p85 and Gab2 exists and PI 3’ kinase is activated only upon specific tyrosine phosphorylation of the adaptor protein. As such, TEL-JAK2 expression may activate PI 3’ kinase by inducing tyrosine phosphorylation of Gab2. In addition, we have shown that IRS-2 can associate with p85 in cell lines expressing TEL-JAK2 isoforms.

Expression of TEL-JAK2 results in constitutive activation of the PI 3 kinase signaling pathway through indirect recruitment of adaptor proteins including Gab2 and IRS-2. The mechanism of recruitment of these adaptor proteins to TEL-JAK2 is unknown and a topic for further investigation. Interestingly our experiments show that TEL-JAK2 (4-17) stimulates PKB phosphorylation and Gab2 association and tyrosine phosphorylation to a lesser extent than TEL-JAK2 (5-12) and TEL-JAK2 (5-19). TEL-JAK2 isoforms containing exon 5 of TEL contain a consensus Grb2 binding site (Y314MN). A recent report suggested that Grb2 couples to the PI 3
kinase signaling pathway through recruitment of a Gab2-p85 complex (66). Recent studies in our laboratory suggest that mutation of tyrosine 314 in exon 5 of TEL can interfere with Gab2–TEL-JAK2 (5-19) association, and the constitutive tyrosine phosphorylation of Gab2 (data not shown). The absence of this tyrosine in TEL-JAK2 (4-17) may account for the weak constitutive phosphorylation of Gab2 in TEL-JAK2 (4-17) expressing cells, the undetectable association of Gab2 with TEL-JAK2 (4-17) in immunoprecipitations and weak PKB phosphorylation.

One consequence of PI 3’ kinase activity is the activation of PDKs which in turn activates PKB by phosphorylation at serine 473 and threonine 308. PKB is an important downstream target of PI 3’ kinase modulating cell survival. It has been shown that the overexpression of PKB partially protects cells from apoptosis induced by stresses such as growth factor withdrawal or PI 3’ kinase inhibition, and perhaps induce oncogenic transformation (57,58,67-69). TEL-JAK2 expression in Ba/F3 cells leads to the constitutive phosphorylation of PKB, and this event is PI 3’ kinase dependent. Furthermore, the decrease in PKB phosphorylation with PI 3’ kinase inhibition correlates with a decrease in cell number upon PI 3’ kinase inhibition. These results would suggest that the PI 3’ kinase/PKB pathway is important in TEL-JAK2 transformation to factor independence. Many studies have shown that PKB prevents cell death by inactivating proapoptotic factors such as BAD (32,70,71) and caspase 9 (72). PKB may exert a wider affect on cell survival and apoptosis by regulation of gene transcription, directly acting on the forkhead family of transcription factors (73), or indirectly regulating factors such as GSK3 (74), CREB (75), E2F (76) and NF-kB (77) transcription factors.

PKB can also be regulated by inositol 5’ phosphatases, since PIP$_3$ has been shown to activate PKB. PTEN-deficient murine embryonic fibroblasts were shown to have elevated levels of basal PKB phosphorylation (78), whereas mast cells from Ship1-deficient mice had elevated
PKB phosphorylation (79). BCR-ABL appears to down-regulate Ship1 expression in Ba/F3-BCR-ABL transfectants (80), however, TEL-JAK2 does not alter Ship1 protein levels. Deciphering the role of the lipid phosphatases, PTEN, Ship1 and the related gene, Ship2, in PKB activation will be a subject of particular interest. Future studies will be required to determine what targets upstream and downstream of PKB are regulated by TEL-JAK2 expression, potentially contributing to its transforming ability.

The expression of TEL-JAK2 confers IL-3 independent growth in vitro (10,12,40). Use of the PI 3’ kinase inhibitor, LY294002, has allowed us to examine the importance of PI 3’ kinase activity in TEL-JAK2-mediated factor-independent cell growth/survival. In addition, expression of the fusion protein protects cells from apoptosis in the absence of IL-3. This would suggest that TEL-JAK2 is capable of inducing both proliferation and survival. However, it remains to be determined if protection from programmed cell death is PI 3’ kinase dependent. Recent studies indicate that in the context of IL-3 signaling, the class I PI 3’ kinases are required for cell proliferation and the phosphorylation of PKB and BAD but not for protection from apoptosis (48). If signals activated by TEL-JAK2 are a subset of those activated by IL-3, particularly with respect to signals downstream of PI 3’ kinase, then it may also be true that the PI 3’ kinase/PKB pathway activated by TEL-JAK2 predominantly targets cell proliferation. In support of this, it has been shown using LY294002 that PI 3’ kinase is not absolutely required for the protection of cells expressing BCR-ABL from apoptosis (81). A recent study of PI 3’ kinase and Raf pathways in BCR-ABL signaling alludes to the importance of both pathways acting independently but overlapping in their anti-apoptotic activity (32). Future studies examining cell cycle and utilizing dominant negative mutants of p85, PKB as well as downstream substrates
including BAD may help distinguish the cell proliferation and survival signals activated by TEL-JAK2.

The leukemogenic potential of TEL-JAK2 has been demonstrated in mice. Bone marrow transplants have illustrated that TEL-JAK2 induces a fatal myelo- and lymphoproliferative disease (12). Interestingly, using STAT5a/b deficient mice, it was revealed that there was no onset of disease (42). However, only a myeloproliferative disease results when bone marrow cells are transduced with constitutively active STAT5a (42). This transplantation model and studies of other oncogenic tyrosine kinases, such as BCR-ABL, would imply that activation of multiple signaling pathways is necessary for cellular transformation and disease induction. Our results indicate that TEL-JAK2 activates the PI 3’ kinase pathway and two additional pathways: the Ras/MEK/MAPK (43) pathway and STAT5 (42). We have shown that the tyrosine phosphorylation status of STAT5 is unaffected by the PI 3’ kinase inhibitor, suggesting that the PI 3’ kinase/PKB pathway can act in parallel with STAT5 activation downstream of TEL-JAK2. The MEK/MAPK pathway has also been implicated in regulating apoptosis (82,83). However, the relevance of PI 3’ kinase in TEL-JAK2 leukemogenesis will remain to be determined using murine bone marrow transplant models.

In summary, this study demonstrates the importance of PI 3’ kinase in TEL-JAK2 mediated factor independent cell proliferation and phosphorylation of PKB. The association of TEL-JAK2 and the p85 subunit of PI 3’ kinase is most likely mediated by adaptor proteins such as Gab2 and IRS-2. The putative p85 binding site in JAK2 is dispensable for this interaction. Activation of the PI 3’ kinase/PKB pathway is a common element observed in oncogenic progression. The requirement of this pathway for the onset of disease by TEL-JAK2 will be a subject of future investigation.
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FIGURE LEGENDS

Figure 1. Schematic diagram of TEL-JAK2 fusion proteins. Illustration of the characterized TEL-JAK2 fusions and the wild type forms of TEL and JAK2. The breakpoints involved in the TEL-JAK2 chromosomal translocations are indicated by arrows. The TEL-JAK2 (4-17) translocation fused nucleotide (nt) 463 of Tel to nt 2126 of Jak2. Whereas TEL-JAK2 (5-19) and TEL-JAK2 (5-12) resulted in the fusion of Tel nt 1009 to Jak2 nt 2426, and Tel nt 1009 and Jak2 nt 1506, respectively. The three fusion proteins also contain a myc-tag at the carboxy (C-) terminus.

Figure 2. TEL-JAK2 constitutively activates PKB phosphorylation.
Ba/F3 (lanes 1 and 2), Ba/F3 TEL-JAK2 (4-17) (lanes 3 and 4), Ba/F3 TEL-JAK2 (5-12) (lanes 5 and 6), Ba/F3 TEL-JAK2 (5-19) (lanes 7 and 8) and Ba/F3 BCR-ABL (lanes 9 and 10) cells were depleted of cytokine and stimulated in the presence (+) or absence (-) of IL-3. Phosphorylated PKB proteins were detected in lysates using an anti-phospho-PKB antibody specific for the phosphorylation of serine 473 (upper panel). A total anti-PKB antibody was used for reprobing the membrane (lower panel).

Figure 3. IL-3 independent growth/survival mediated by TEL-JAK2 is PI 3’ kinase dependent.
Ba/F3, Ba/F3 TEL-JAK2 (4-17), Ba/F3 TEL-JAK2 (5-12) and Ba/F3 TEL-JAK2 (5-19) cells were treated as described in Materials and Methods, in the presence of increasing concentrations of LY294002. Cells were incubated in the absence of IL-3 (upper panel), or in the presence of
100 pg/mL IL-3 (lower panel). XTT assays were performed in triplicate and mean absorption values with standard deviation were graphed.

**Figure 4. Phosphorylation of PKB by TEL-JAK2 and IL-3 are PI 3’ kinase dependent.**

Ba/F3 (lanes 1 to 4) and Ba/F3 TEL-JAK2 (5-19) (lanes 5 to 8) cells were depleted of cytokine, pretreated with (+) or without (-) 20µM LY294004 for 30 minutes and stimulated in the presence (+) or absence (-) of IL-3. Phosphorylated PKB proteins were detected in lysates using an anti-phospho-PKB antibody specific for the phosphorylation of serine 473 (upper panel). A total anti-PKB antibody was used for reprobing the membrane (lower panel).

**Figure 5. TEL-JAK2 co-immunoprecipitates with the p85 subunit of PI 3’ kinase.**

Ba/F3 (lanes 1, 2, 9 and 10), Ba/F3 TEL-JAK2 (4-17) (lanes 3 and 4), Ba/F3 TEL-JAK2 (5-12) (lanes 5 and 6) and Ba/F3 TEL-JAK2 (5-19) (lanes 7, 8, 11 and 12) cells were depleted of cytokine and stimulated in the presence (+) or absence (-) of IL-3. Immunoprecipitations were performed using an anti-p85 antibody (lanes 1 to 8) or preimmune IgG (lanes 9 to 12) and tyrosine phosphorylated proteins were detected by phosphotyrosine (pTyr) immunoblotting (first panel). IRS-2 (second panel), Gab2 (third panel), Shp2 (fourth panel), Shc (fifth panel) and p85 (sixth panel) proteins were detected upon reprobing with the appropriate peptide-specific antibodies.
Figure 6. p85 Amino terminal SH2 domain associates with TEL-JAK2.

(A) Ba/F3 (lanes 1, 2, 5, 6, 9, 10, 13, 14, 17 and 18) and Ba/F3 TEL-JAK2 (5-19) (lanes 3, 4, 7, 8, 11, 12, 15 and 16) cells were depleted of cytokine and then stimulated in the presence (+) or absence (-) of IL-3. In vitro mixes were performed using Glutathione sepharose beads bound to GST alone (lanes 1 to 4) or GST fusion proteins containing the carboxy (C SH2) (lanes 5 to 8), amino (N SH2) (lanes 9 to 12) or both amino and carboxy terminal SH2 domains of p85 (N+C SH2) (lanes 13 to 16). Tyrosine phosphorylated proteins were detected by phosphotyrosine (pTyr) immunoblotting (upper panel). Controls with GST alone were included in lanes 1 to 4. An immunoblot reprobe with an anti-GST antibody confirmed levels of fusion protein (lower panel).

(B) Ba/F3 (lanes 1, 2, 5, 6, 9, 10, 13, 14, 16 and 17) and Ba/F3 TEL-JAK2 (5-19) (lanes 3, 4, 7, 8, 11, 12, 15, 18 and 19) cells were depleted of cytokine and then stimulated in the presence (+) or absence (-) of IL-3. An immunoprecipitation was performed with a preimmune IgG (lanes 1 to 4) or an anti-TEL antibody (lanes 5 to 8). The respective supernatant fractions, pre-cleared with preimmune IgG or an anti-TEL antibody, were then incubated with GST (lanes 13 to 15) or a GST N+C SH2 p85 fusion protein (lanes 9 to 12 and 16 to 19). Tyrosine phosphorylated proteins were detected by phosphotyrosine (pTyr) immunoblotting (upper panel). An immunoblot reprobe with an anti-GST antibody confirmed equivalent levels of fusion protein (lower panel).
Figure 7. PI 3’ kinase association with TEL-JAK2 is independent of a consensus YXXM motif found on JAK2.

A) Ba/F3 (lanes 1 and 2), Ba/F3 TEL-JAK2 (5-19) (lanes 3 and 4) and two subclones of Ba/F3 TEL-JAK2 (5-19) Y624F (lanes 5 to 8) cells were depleted of cytokine and stimulated in the presence (+) or absence (-) of IL-3. Phosphorylated PKB proteins were detected in lysates using an anti-phospho-PKB antibody specific for the phosphorylation of serine 473 (upper panel). A total anti-PKB antibody was used for reprobing the membrane (lower panel).

B) Ba/F3, Ba/F3 TEL-JAK2 (5-19) and two subclones of Ba/F3 TEL-JAK2 (5-19) Y624F cells were treated as described in Materials and Methods, in the presence of increasing concentrations of LY294002. Cells were incubated in the absence of IL-3 (upper panel), or in the presence of 100 pg/mL IL-3 (lower panel). XTT assays were performed in triplicate and mean absorption values with standard deviation were graphed.

C) Ba/F3 (lanes 1, 2, 9 and 10), Ba/F3 TEL-JAK2 (5-19) (lanes 3, 4, 11 and 12) and two subclones of Ba/F3 TEL-JAK2 (5-19) Y624F (lanes 5 to 8) cells were depleted of cytokine and stimulated in the presence (+) or absence (-) of IL-3. Immunoprecipitations were performed using an anti-p85 antibody (lanes 1 to 8) or preimmune IgG (lanes 9 to 12). Tyrosine phosphorylated proteins were detected by phosphotyrosine (pTyr) immunoblotting (upper panel) and p85 was detected by reprobing the membrane with anti-p85 antibody (lower panel).

D) Ba/F3 (lanes 1, 2, 5 and 6) Ba/F3 TEL-JAK2 (5-19) (lanes 3, 4, 7 and 8) and two subclones of Ba/F3 TEL-JAK2 (5-19) Y624F (lanes 9 to 12) cells were depleted of cytokine and then stimulated in the presence (+) or absence (-) of IL-3. In vitro mixing experiments were performed using Glutathione sepharose beads bound to GST alone (lanes 1 to 4) or GST fusion protein containing both amino and carboxy terminal SH2 domains of p85 (N+C SH2) (lanes 5 to...
Tyrosine phosphorylated proteins were detected by phosphotyrosine (pTyr) immunoblotting (upper panel). In vitro mixing experiments with GST alone were included in lanes 1 to 4. An immunoblot reprobe with an anti-GST antibody confirmed levels of fusion protein (lower panel).

**Figure 8. Gab2 constitutively associates with TEL-JAK2 and p85**

Ba/F3 (lanes 1, 2, 13 and 14), Ba/F3 TEL-JAK2 (4-17) (lanes 3 and 4), Ba/F3 TEL-JAK2 (5-12) (lanes 5 and 6), Ba/F3 TEL-JAK2 (5-19) (lanes 7, 8, 15 and 16) and two subclones of Ba/F3 TEL-JAK2 (5-19) Y624F (lanes 9 to 12) cells were depleted of cytokine and then stimulated in the presence (+) or absence (-) of IL-3. Immunoprecipitations were performed using an anti-Gab2 antibody and tyrosine phosphorylated proteins were detected by phosphotyrosine (pTyr) immunoblotting (first panel). Total p85 (second panel), Shp2 (third panel), Shc (fourth panel) and Gab2 (fifth panel) were detected upon reprobing with the appropriate peptide-specific antibodies.

**Figure 9. TEL-JAK2 confers protection from apoptosis.**

Ba/F3 and Ba/F3 TEL-JAK2 (5-19) were washed and grown in complete media in the absence or presence of 100 pg/mL IL-3. Aliquots of cells were removed at the indicated time points and stained with Annexin V (FL2) and 7AAD (FL3). Cells were analyzed by flow cytometry and 10000 events were recorded for each sample. These results are the average of three independent experiments. The top panel indicates the percentage of early apoptotic cells, positive for only Annexin V over time. The bottom panel indicates the percentage of late apoptotic cells, positive for both Annexin V and 7AAD over time.
Figure 10. Activation of PKB is independent of STAT5 tyrosine phosphorylation.

Ba/F3 (lanes 1 to 8) and Ba/F3 TEL-JAK2 (5-19) (lanes 9 to 16) cells were depleted of cytokine, pretreated with medium (-), DMSO vehicle (D), 10 or 20 µM LY294002 for 30 minutes and stimulated in the presence (+) or absence (-) of IL-3. Phosphorylated PKB proteins were detected in lysates using an anti-phospho-PKB antibody specific for the phosphorylation of serine 473 (first panel). A total anti-PKB antibody was used for reprobing the membrane (second panel). Phosphorylated STAT5 proteins were detected in lysates using a phospho-specific STAT5 antibody (third panel). The membrane was reprobed with a total anti-STAT5 antibody (fourth panel).
Figure 1
Figure 2

Lysates

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|----|
| IL-3: | - | + | - | + | - | + | - | + | - | + |

IB: P-PKB (S473)

- P-PKB

IB: PKB

- PKB

Ba/F3  | TEL-JAK2 (4-17)  | TEL-JAK2 (5-12)  | TEL-JAK2 (5-19)  | BCR-ABL

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Figure 3
Figure 4

| Lysates | Ba/F3 | TEL-JAK2 (6-19) |
|---------|------|----------------|

| LY294002: | -    | +    | +    | -    | +    | +    | +    | -    |
| IL-3:     | -    | +    | +    | -    | +    | -    | +    | -    |

IB: PKB

IB: P-PKB (S473)

IB: P-PKB
Figure 5

**IB: pTyr**
- IRS-2
- Gab2
- TEL-JAK2 (5-12)
- TEL-JAK2 (5-19)
- Shp2
- TEL-JAK2 (4-17)
- Shc

**IB: IRS-2**
- IRS-2

**IB: Gab2**
- Gab2

**IB: Shp2**
- Shp2

**IB: Shc**
- Shc

**IB: p85**
- p85

**IP:**
- p85
- PI
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10

| LYS294002: | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 |
|------------|------------|
| IL-3:      | - + - - + - + - + - + - + - - + |

| IB: P-PKB (S473) | |
| IB: PKB          | |
| IB: P-STAT5      | |
| IB: STAT5        | |

LY294002: 10 20 20

Ba/F3

TEL-JAK2 (5-19) 10 20 10

Lysates

P-PKB

P-KB

P-STAT5

STAT5
TEL-JAK2 mediates constitutive activation of the phosphatidylinositol 3' kinase/protein kinase B signaling pathway
Melody H.-H. Nguyen, Jenny M.-Y. Ho, Bryan K. Beattie and Dwayne L. Barber

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