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# Involvement of the NF- $\kappa$ B pathway in the transforming properties of the TEL–Jak2 leukemogenic fusion protein

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**Abstract** Constitutively active tyrosine kinases are frequently expressed in various types of human leukemias as the result of chromosomal translocations. The TEL–Jak2 fusion oncoprotein possesses transforming properties in both animal and cellular models, that are tightly dependent on Stat5 activation. In the IL-3-independent TEL–Jak2-transformed Ba/F3 cells, activation of the PI-3K/Akt pathway appears essential to cell proliferation. Here we report a sustained activation of NF- $\kappa$ B factors in Ba/F3 cells, which inhibition dramatically impairs cell viability, indicating that NF- $\kappa$ B signaling exerts a major role in the anti-apoptotic activities of TEL–Jak2 oncoprotein. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** NF- $\kappa$ B; TEL–Jak2 fusion protein; Leukemogenesis

## 1. Introduction

NF- $\kappa$ B transcription factors act as important mediators in acute and inducible cellular responses to a variety of extracellular stimuli, in particular in the immune system (reviewed in [1]). Mammalian NF- $\kappa$ B includes the RelA/p65, RelB, c-Rel, p50/p105 and p52/p100 proteins, structurally related through a Rel homology (RH) domain implicated in their DNA binding, dimerization and interaction with I $\kappa$ B inhibitor proteins. NF- $\kappa$ B transcriptional activity is primarily controlled by cytoplasm retention of the complexes by any of several I $\kappa$ B family members (reviewed in [2]). The extracellular signals leading to the activation of NF- $\kappa$ B focus on the IKK complex responsible for the phosphorylation of I $\kappa$ B. These phosphorylations lead to I $\kappa$ B proteasome-mediated degradation and allow in turn translocation of NF- $\kappa$ B complexes into the nucleus where they can modulate specific gene expression.

As the NF- $\kappa$ B signaling pathway participates in the control of apoptosis and cell proliferation, it is a target for transforming activities of viral and cellular oncogenes. Dysregulated NF- $\kappa$ B activity has been reported in human hematopoietic and solid tumors, due to direct alterations of genes encoding

components of the NF- $\kappa$ B pathway or by yet uncharacterized mechanisms (reviewed in [3]). Constitutively activated tyrosine kinase oncoproteins, BCR-ABL [4] and TEL-PDGFR [5] have been shown to activate the NF- $\kappa$ B pathway. The TEL–Jak2 fusion protein, observed in various human hematopoietic malignancies [6,7] represented the first example of the direct involvement of the Jak-Stat pathway in human oncogenesis. In this report, NF- $\kappa$ B activity was evaluated in murine lymphoid Ba/F3 cells transformed by TEL–Jak2 and compared with that of TEL–ABL expressing cells [8].

## 2. Materials and methods

### 2.1. Cell culture and reagents

The parental Ba/F3 cells were grown in RPMI 1640 medium with 10% fetal calf serum and 4% WEHI conditioned medium (WCM) as a source of IL-3. Stimulation experiments were performed with recombinant murine IL-3 (Valbiotech). The IL-3-independent Ba/F3 cells stably expressing the TEL–Jak2 and TEL–ABL fusions have been described [9]. The Ba/F3 $\Delta$ 31 cell line expresses a dominant negative form of the Stat5A protein [10].

### 2.2. Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Deprivation was for 16 h in the absence of WCM for parental Ba/F3 cells. Nuclear extracts were performed as described [11]. For EMSAs, 10  $\mu$ g of nuclear extracts (determined with the BCA protein assay reagent (Pierce)) were incubated in a buffer containing 20 mM HEPES, 4% Ficoll, 70 mM NaCl, 2 mM dithiothreitol, 100  $\mu$ g/ml bovine serum albumin and 0.01% NP40 with <sup>32</sup>P-labeled NF- $\kappa$ B probe (AGTTGAGGGGACTTCCCAGG). Rabbit polyclonal antibodies raised against the p50 and p65 were kindly provided by Nancy Rice (NCI-FCRDC, Frederick, MD, USA). For experiments using the specific inhibitor of I $\kappa$ B $\alpha$  phosphorylation BAY 11-7082 (Calbiochem), cells were incubated 1 h in the presence of 5 or 10  $\mu$ M of the compound prior to nuclear protein extraction.

### 2.3. Transient transfection reporter assays

For NF- $\kappa$ B luciferase reporter assay,  $0.35 \times 10^6$  cells/ml of the different cell lines were transfected with the indicated amounts of luciferase reporter pNF- $\kappa$ B-Luc plasmid (PathDetect NF- $\kappa$ B *cis*-reporting system, Stratagene) using the Lipofectin reagent (Gibco BRL). Cells were collected 48 h post transfection and lysed in the reporter lysis buffer (Promega). Luciferase assays were performed with 15–20  $\mu$ g of lysates using the kit Luciferase assay system (Promega) in a Lumat LB9501 (Berthold). Luciferase activities are expressed as fold induction relative to values obtained from normally growing parental Ba/F3 cells cultured in WCM.

### 2.4. Apoptosis study

Parental Ba/F3 and Ba/F3 $\Delta$ 31 cells were incubated for 16 h in the absence of WCM then cultured one h with or without 10  $\mu$ M of BAY 11-7082, 10  $\mu$ M of the MEKK kinase inhibitor UO126 (Calbiochem) or 20  $\mu$ M of the p38 inhibitor SB 203580 (Calbiochem) and then

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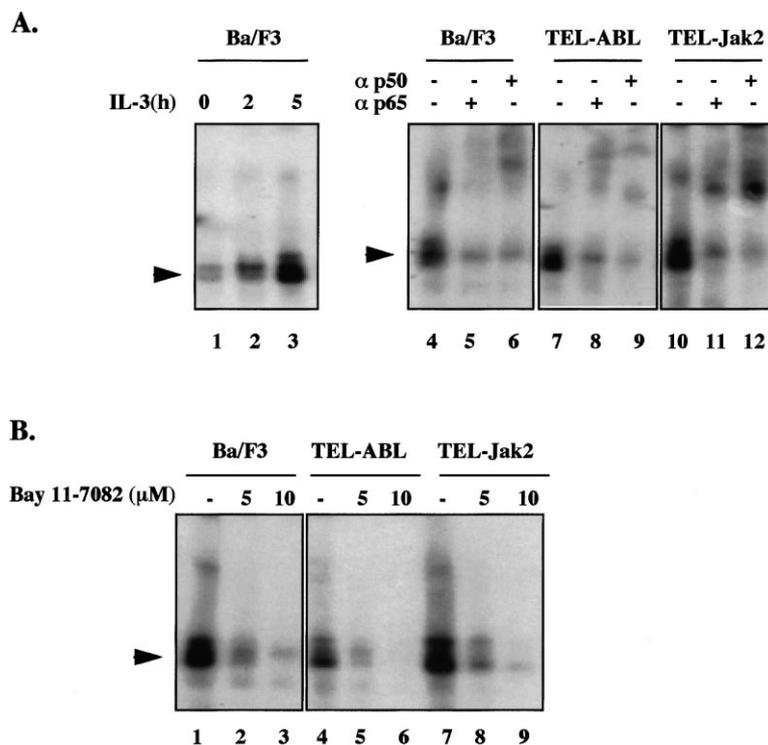


Fig. 1. Constitutive activation of NF- $\kappa$ B complexes in Ba/F3 cells transformed by the TEL-Jak2 and TEL-ABL fusions. A: Analyses of NF- $\kappa$ B DNA-binding activities were performed by EMSA with nuclear extracts from the IL-3-independent TEL-Jak2 and TEL-ABL-expressing Ba/F3 cells (lanes 7–12) and an oligonucleotide probe containing an NF- $\kappa$ B-binding site. As a control, nuclear extracts from parental Ba/F3 cells growing in WCM (lanes 4–6) or starved for 16 h and left unstimulated (lane 1) or stimulated with recombinant IL-3 for the indicated times (lanes 2, 3) were loaded. Supershift experiments were performed with specific anti-p50 and anti-p65 antibodies. B: To test for the inhibitory impact of BAY 11-7082 on the NF- $\kappa$ B DNA-binding activity, the above cell lines were treated with 5  $\mu$ M or 10  $\mu$ M of inhibitor prior to extraction of nuclear proteins.

stimulated with 10 ng/ml of recombinant IL-3 for the indicated times. The TEL-Jak2- and TEL-ABL-expressing Ba/F3 cells were cultured with or without identical concentrations of the inhibitors. Cells were washed once in the incubation buffer (10 mM HEPES-NaOH, pH 7.4; 140 mM NaCl; 5 mM CaCl<sub>2</sub>) and resuspended in the incubation buffer with 0.5  $\mu$ g/ml of propidium iodide (Interchim) and Annexin V-fluos (Boehringer Mannheim) at room temperature for 30 min. The percentages of early apoptotic cells (Annexin V-fluos positive, propidium iodide negative) and late apoptotic cells (Annexin V-fluos positive, propidium iodide positive) were determined by using an EPICS Elite cytometer (Coulter Corporation, FL, USA).

### 2.5. Cell extracts and Western blotting

Cells were incubated 1 h in the presence of BAY 11-7082 (10  $\mu$ M), UO126 (10  $\mu$ M) or SB 203580 (20  $\mu$ M) and extraction (nuclear (NE) and cytoplasmic (CE)) performed as reported [9]. For Western blotting, 100  $\mu$ g of extracts were separated by electrophoresis on 10% SDS-PAGE, transferred to cellulose membrane (Hybond-C super membrane, Amersham Life Science) which were incubated with the indicated antibodies. The anti-I $\kappa$ B $\alpha$ , anti-phospho(Thr180/Tyr182)-p38 and anti-p38 antibodies were purchased from New England Biolabs. Immune complexes were detected using the ECL detection kit (Amersham).

## 3. Results

### 3.1. Expression of the TEL-Jak2 and TEL-ABL fusion proteins in Ba/F3 cells leads to the activation of NF- $\kappa$ B complexes

We recently reported a constitutive activation of the PI-3K/Akt pathway in TEL-Jak2- and TEL-ABL-transformed Ba/F3 cells that appears essential for the mitogenic properties but dispensable for the anti-apoptotic activities of these oncopro-

teins [12]. To investigate the activation of NF- $\kappa$ B factors in TEL-ABL- and TEL-Jak2-expressing Ba/F3 cells, we first examined their DNA-binding activity by EMSA (Fig. 1A). As controls, nuclear extracts from parental cells growing in WCM (lanes 4–6) or deprived for 16 h and left unstimulated

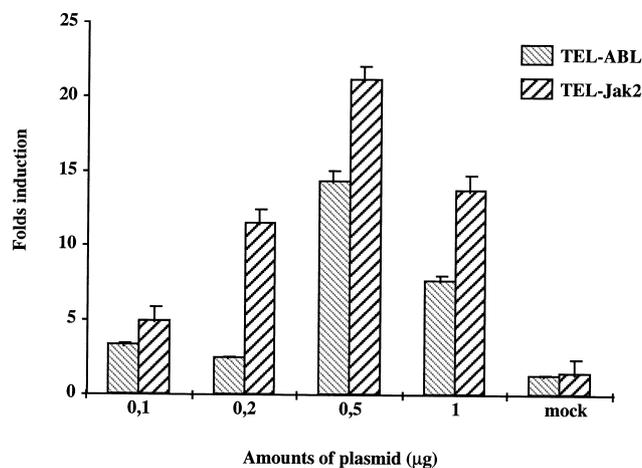


Fig. 2. NF- $\kappa$ B complexes are transcriptionally active in the TEL-Jak2 and TEL-ABL-expressing Ba/F3 cells. Luciferase reporter assays were performed on TEL-Jak2- and TEL-ABL-expressing cells transiently transfected with the indicated amounts of a NF- $\kappa$ B luciferase reporter plasmid. Results are expressed as fold induction relative to luciferase activities obtained in parental Ba/F3 cells growing in WCM medium. Data shown are from representative experiments where transfections were done in duplicate.

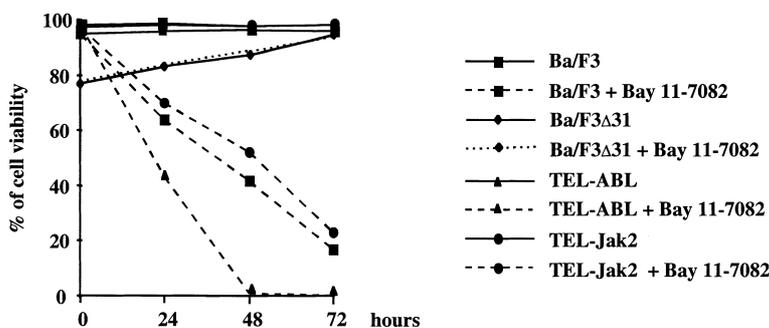


Fig. 3. Inhibition of the NF- $\kappa$ B pathway totally impairs the viability of the TEL-Jak2 and TEL-ABL-transformed Ba/F3 cells. Apoptosis analysis was performed on TEL-Jak2- and TEL-ABL-expressing cells cultured in the absence or presence of BAY 11-7082 (10  $\mu$ M), as reported in Section 2. Values are given as the percentage of total viable cells remaining in the culture. As controls, the same experiments were performed using parental Ba/F3 cells and Ba/F3 $\Delta$ 31 cells [10] which were deprived of WCM for 16 h, then treated or not with BAY 11-7082 (10  $\mu$ M) before being replaced in the presence of WCM for the remaining period.

(lane 1) or stimulated with recombinant IL-3 for 2 h (lane 2) or 5 h (lane 3) were loaded. Extracts from TEL-Jak2-expressing cells showed roughly equivalent amounts of bound NF- $\kappa$ B complexes to growing parental cell controls (compare lanes 4 and 10) indicating that TEL-Jak2 induces the activation of NF- $\kappa$ B. Similarly, cells stably expressing the TEL-ABL fusion showed a constitutive NF- $\kappa$ B DNA-binding activity (lane 7), although repeatedly weaker than in the TEL-Jak2 context. Supershift experiments using antibodies to p50 (lanes 6, 9, 12) or to p65 (lanes 5, 8, 11) indicated the presence of p50 and of p65/RelA in the complexes. C-Rel might also be

present in the complexes detected in the three cell line extracts, as suggested by the use of anti-c-Rel antibodies (data not shown).

Phosphorylation of the I $\kappa$ B inhibitors, that interact with and sequester NF- $\kappa$ B subunits in the cytoplasm, leads to their degradation and to the nuclear translocation of NF- $\kappa$ B factors. In order to confirm the activated status of NF- $\kappa$ B complexes in our Ba/F3 cell lines, we used the BAY 11-7082 compound described as a potent inhibitor of I $\kappa$ B $\alpha$  phosphorylation [13]. EMSA experiments using nuclear extracts from BAY 11-7082-treated cells showed a clear dose-

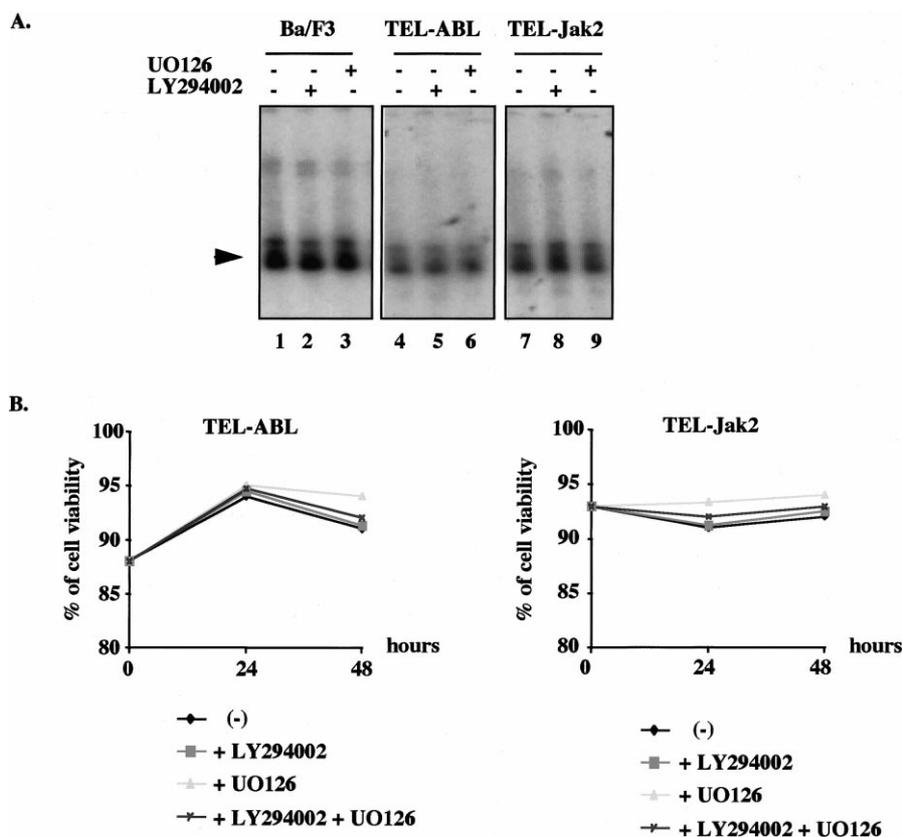


Fig. 4. Activation of NF- $\kappa$ B signaling in TEL-Jak2- and TEL-ABL-expressing Ba/F3 cells is not mediated by the PI-3K or MAP kinase pathways. A: Nuclear extracts from LY294002- or UO126-treated cells were analyzed by EMSA using an oligonucleotide probe containing an NF- $\kappa$ B-binding site. B: TEL-Jak2- and TEL-ABL-expressing cells were left untreated (-) or treated with LY294004 (50  $\mu$ M) or UO129 (10  $\mu$ M) inhibitors or with a combination of both. The percentages of viable cells were determined as described in Section 2.

dependent decrease in the NF- $\kappa$ B DNA-binding activity in TEL-ABL (compare lanes 4–6), TEL-Jak2 (compare lanes 7–9) and IL-3 stimulated Ba/F3 cells (compare lanes 1–3) (Fig. 1B). As shown by Western blot analyses of the same extracts, p38 MAP kinase (see Fig. 5A) or nuclear p65 (data not shown) proteins remained expressed at similar level in BAY 11-7082-treated cells, ruling out a non-specific effect of the inhibitor. Taken together, our results show the constitutive activation of NF- $\kappa$ B in the two transformed Ba/F3 cell lines.

To ensure the transcriptional activity of the activated NF- $\kappa$ B complexes, we performed luciferase reporter assays. The parental, TEL-ABL- and TEL-Jak2-expressing cells were transiently transfected with increasing amounts of a luciferase reporter gene containing multimers of the consensus Ik-binding site. Fig. 2 shows the ratio of the measured luciferase activities with respect to the parental Ba/F3 cells. Both TEL-Jak2- and TEL-ABL-expressing cells demonstrated a marked increase in NF- $\kappa$ B-dependent transcriptional activation, with respect to parental cell controls. This activity was repeatedly found higher in the TEL-Jak2- than in the TEL-ABL-expressing cells, in keeping with the differences in NF- $\kappa$ B DNA-binding activities observed between TEL-Jak2 and TEL-ABL cell lines (see Fig. 1A).

3.2. Functional inhibition of NF- $\kappa$ B totally impairs the viability of TEL-Jak2- and TEL-ABL-expressing Ba/F3 cells

We next examined the biological effect of the BAY 11-7082 compound on the cell viability. Cell lines were cultured in the presence or absence of BAY 11-7082 at a concentration (10  $\mu$ M) that was previously shown to completely inhibit NF- $\kappa$ B DNA-binding activity (cf. Fig. 1B). Cell viability was esti-

mated daily by Annexin V-propidium iodide flow cytometry (Fig. 3). When TEL-Jak2- and TEL-ABL-transformed Ba/F3 cells were treated, a significant decrease in cell viability was observed after 24 h. Maximum rates of apoptotic cells were achieved after 72 h treatment for both TEL-Jak2- and TEL-ABL-transformed Ba/F3 cells. A lower dose of the compound (5  $\mu$ M) led to similar results although with a delayed kinetics (data not shown). The parental Ba/F3 behaved similarly to TEL-Jak2- and TEL-ABL-transformed cells. This was not due to a toxic effect of the drug on Ba/F3 cells since the viability of another IL-3-dependent Ba/F3 cell line, referred to as the Ba/F3 $\Delta$ 31 [10], was not affected by this compound even after 72 h treatment (Fig. 3).

3.3. Activation of NF- $\kappa$ B complexes in TEL-Jak2- and TEL-ABL-expressing cells is independent of the PI-3K and MAP kinase pathways

Both PI-3K and MAP kinase pathways have been reported to functionally activate NF- $\kappa$ B in various hematopoietic cell types [1]. To address the implication of these pathways in NF- $\kappa$ B activation in our Ba/F3 cell lines, we examined the biological effects of the specific PI-3K inhibitor LY294002 and MEK1/2 kinase inhibitor UO126. As shown in Fig. 4A, EMSA experiments performed with nuclear extracts did not show obvious differences in NF- $\kappa$ B DNA-binding activity of cells treated with either one or both inhibitors (Fig. 4A). As previously reported [12], treatment of TEL-Jak2- and TEL-ABL-expressing cells with LY294002 impaired cell cycle progression but not cell viability. Similarly, the cell viability of the two cell lines was not affected upon treatment with LY294002 or UO126 or with both inhibitors. A compilation of these experiments is shown in Fig. 4B.

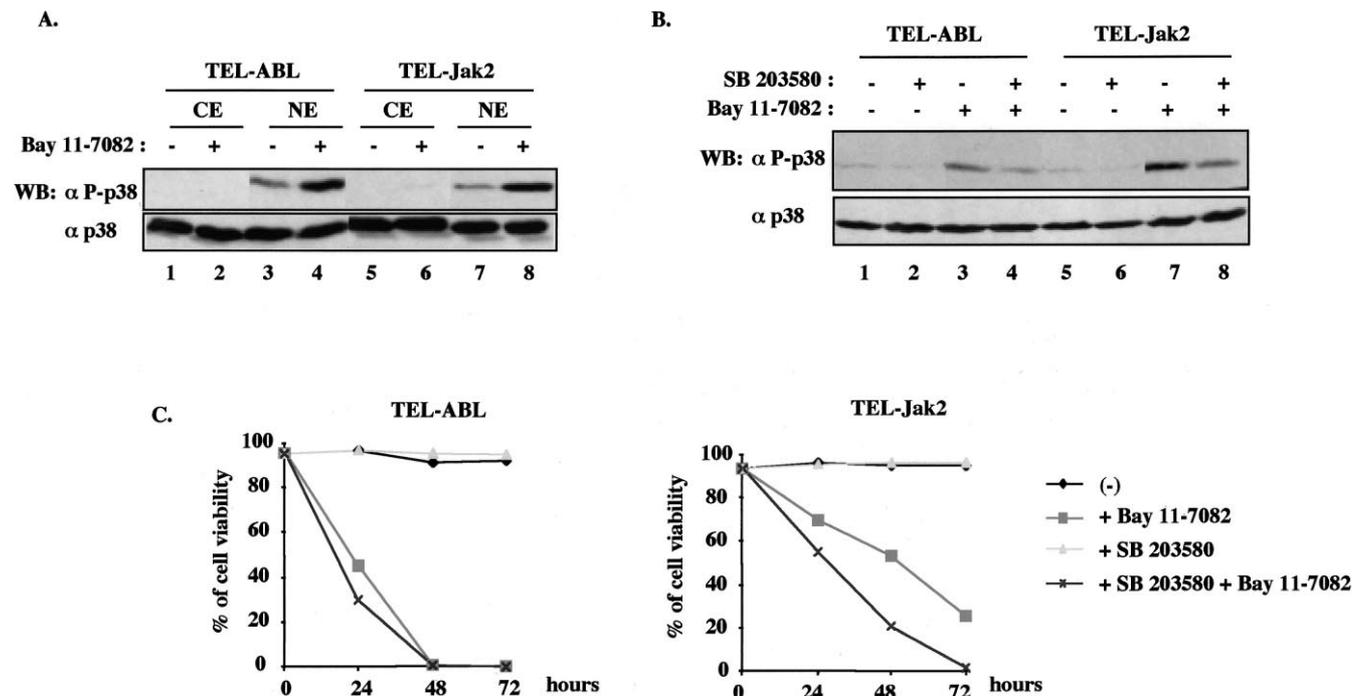


Fig. 5. Inhibition of the p38 MAP kinase does not alter the viability of TEL-Jak2- and TEL-ABL-transformed Ba/F3 cells. A: Nuclear (NE) and cytoplasmic (CE) extracts from BAY 11-7082-treated TEL-Jak2- and TEL-ABL-expressing cells were analyzed by Western blot by using anti-phospho p38 (upper panel) and anti-p38 (lower panel) antibodies. B: Western blot analysis of nuclear extracts from TEL-Jak2- and TEL-ABL-expressing cells treated or not with the indicated inhibitors. C: Apoptosis analysis was performed on TEL-Jak2- and TEL-ABL-expressing cells cultured in the presence or not of the indicated inhibitors. Cellular viability was estimated as reported in Section 2.

### 3.4. Inhibition of the p38 MAP kinase does not alter the viability of TEL–Jak2- and TEL–ABL-expressing cells

In addition to inhibition of I $\kappa$ B $\alpha$  phosphorylation, treatment with BAY 11-7082 has been reported to stimulate the activation of the p38 MAP kinase. Accordingly, Western blotting experiments showed an increase in phosphorylated p38 following BAY 11-7082 treatment of TEL–Jak2- and TEL–ABL–Ba/F3 cells (Fig. 5A). As the MKK3/4/6 kinases known to activate p38, are not inhibited by the UO126 compound used in experiments shown in Fig. 4, we analyzed the effect of SB 203580, a specific p38 inhibitor. EMSA analysis did not show any variations in the NF- $\kappa$ B DNA-binding activity from nuclear extracts of SB 203580-treated cells (data not shown), as observed for other hematopoietic cell lines [14]. Levels of phosphorylated p38 significantly decreased when cells were incubated in 20  $\mu$ M of SB 203580 prior to BAY 11-7082 treatment (Fig. 5B) without returning to a basal level (compare lanes 1–4 and lanes 5–8). Annexin V-propidium iodide flow cytometry experiments did not reveal modification of cell behavior in response to SB 203580 (Fig. 5C). The BAY 11-7082-mediated apoptotic process was however more pronounced in the presence of SB 203580, suggesting that a cooperation between the NF- $\kappa$ B and p38 pathways might occur. Collectively, our data support a major contribution of the NF- $\kappa$ B signaling pathway in the survival mediated by the TEL–Jak2 and TEL–ABL fusion proteins.

## 4. Discussion

Human hematological malignancies widely illustrate the critical role of a dysregulated NF- $\kappa$ B signaling in oncogenesis. Constitutively activated NF- $\kappa$ B complexes are recurrently observed in childhood acute lymphoblastic leukemias [15], although the underlying molecular mechanisms are still largely unknown. In some instances, aberrant NF- $\kappa$ B activity results from the rearrangements of genes encoding the NF- $\kappa$ B/I $\kappa$ B family members or of alterations in regulatory proteins that control this signaling pathway. In turn, NF- $\kappa$ B activates a large number of anti-apoptotic mediator genes [16] and several cell cycle genes such as cyclin D1 that is required for G1/S cell cycle progression [17].

In this report, we established the direct implication of this pathway in the anti-apoptotic properties of two leukemogenic fusion proteins TEL–Jak2 and TEL–ABL. This was not unexpected since activation of the NF- $\kappa$ B pathway has also been implicated in the transforming properties of other constitutively activated tyrosine kinases associated with human hematopoietic tumors [4,5]. In the BCR–ABL-expressing 32D murine cell line, NF- $\kappa$ B activity is dispensable for apoptosis inhibition upon cytokine withdrawal, but is essential for its tumorigenicity in nude mice [4]. Similarly to BCR–ABL, TEL–ABL is expected to activate a large number of signaling pathways [18–20] and several of them are likely to redundantly focus on NF- $\kappa$ B activation.

Many signal transduction pathways, such as Ras/MAP ki-

nases or PI-3K/Akt, exhibit extensive cross-talk with components of the IKK-I $\kappa$ B/NF- $\kappa$ B pathway. As shown here and in our previous work [12], the specific PI-3K inhibitor (LY294002) and MEK inhibitor (UO126) do not alter the survival process mediated by TEL–Jak2 in Ba/F3 cells. Thus, the sustained activity of NF- $\kappa$ B is unlikely to rely on the Ras/MAP kinase or PI-3K/Akt pathways but rather implies dysregulation of components of the IKK complex or defective I $\kappa$ B-mediated inhibition. In this regard, phosphorylations of I $\kappa$ B $\alpha$  proteins mediated by tyrosine kinases have been reported to activate NF- $\kappa$ B complexes without proteolytic degradation of I $\kappa$ B $\alpha$  [21].

The TEL–Jak2 oncoprotein appears to activate substrates very similar to those activated by the normal Jak2 tyrosine kinase. Among them, the Stat5 transcription factors are essential for its transforming activity in cellular and animals models [9,22]. Our results suggest that Stat5 activation could be mainly responsible for cell proliferation whereas NF- $\kappa$ B activation would be responsible for cell survival. Links between the Jak2- or Stat5-mediated signaling pathways and NF- $\kappa$ B activity are now to be investigated.

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