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Constitutively active STAT5 variants induce growth and survival of hematopoietic cells through a PI 3-kinase/Akt dependent pathway

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Signal Transducer and Activator of Transcription (STATs) are important mediators of cytokine and growth factor-induced signal transduction. STAT5A and STAT5B have been shown to play a role in survival and proliferation of hematopoietic cells both *in vitro* and *in vivo* and to contribute to the growth and viability of cells transformed by the TEL-JAK2 oncoprotein. In this study, we investigated the molecular mechanisms by which constitutively active STAT5 proteins induce cell proliferation and survival of Ba/F3 cell lines expressing either dominant positive STAT5A or STAT5B variants or TEL-JAK2 or TEL-ABL fusion proteins. Our results showed that active STAT5 constitutively interacted with p85, the regulatory subunit of the PI 3-kinase. A constitutive activity of the PI 3-kinase/Akt pathway was observed in these cells and required for their cell cycle progression. In contrast, while activity of the PI 3-kinase/Akt pathway was required for survival of Ba/F3 cells expressing the constitutively active forms of STAT5A or STAT5B, it was dispensable for cells transformed by TEL-JAK2 or TEL-ABL fusion proteins, suggesting that additional survival pathways take place in these transformed cells. *Oncogene* (2001) 20, 2080–2090.

Keywords: STAT5; PI 3-kinase; TEL-JAK2; survival

Introduction

Cytokine dependent proliferation and survival of hematopoietic cells require activation of distinct signaling pathways. The JAK protein tyrosine kinases play a central role in these processes (Ihle *et al.*, 1998). Activated JAK proteins phosphorylate tyrosine residues present in the cytoplasmic domain of cytokine receptors which serve as docking sites for SH2 containing molecules such as, STAT, Shc and the p85 subunit of the PI 3-kinase. These events trigger the activation of both the PI 3-kinase/Akt and the Ras/

Raf/MAP kinase pathways as well as the transcriptional activity of STAT.

Class I PI 3-kinases are composed of a p85 regulatory subunit and a p110 protein with catalytic activity (Kapeller and Cantley, 1994). The binding of the SH2 domains of p85 to proteins containing phosphorylated tyrosine residues present in YXXM motifs increases PI 3-kinase activity (Backer *et al.*, 1992). Binding of p85 to activated cytokine receptor, to adaptor molecules like IRS1/2 and the related proteins Gab1 and Gab2, to SHP-2 and STAT3 have been reported indicating that PI 3-kinase can be activated by different mechanisms in response to cytokines or growth factors (Craddock and Welham, 1997; Gu *et al.*, 1998; Lecoq-Lafon *et al.*, 1999; Nishida *et al.*, 1999; Pfeffer *et al.*, 1997; Verdier *et al.*, 1997; Yamauchi *et al.*, 1998). The PI 3-kinase then catalyzes the production of phosphatidylinositol 3,4-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-triphosphate (PIP3), two lipid products needed to activate various isoforms of PKC and Akt proteins (Datta *et al.*, 1995; Franke *et al.*, 1995; Toker *et al.*, 1994). Activated Akt phosphorylates the Bcl2 member Bad thereby inhibiting its pro-apoptotic function and promoting cell survival (del Peso *et al.*, 1997; Zha *et al.*, 1996). On the other hand, the PI 3-kinase/Akt pathway is also able to regulate the activity or expression of proteins like E2-F and cyclin D1 supporting its role in cell cycle progression (Brennan *et al.*, 1997; Gille and Downward, 1999; Muise-Helmericks *et al.*, 1998).

Like PI 3-kinase, the STAT proteins have been shown to play a role in cell survival and proliferation. These latent cytoplasmic transcription factors become tyrosine phosphorylated following cytokine addition. Activated STATs dimerize (homo- or hetero-dimerize), migrate into the nucleus, bind to specific DNA elements and activate the transcription of responsive genes (Ihle *et al.*, 1998). Seven mammalian STAT members have been characterized. Among them, STAT5A and STAT5B are two highly related proteins that are activated by various stimuli including cytokines, hormones, growth factors and oncogenes. In hematopoietic cell lines, expression of mutated cytokine receptors unable to activate STAT5, or of dominant negative forms of STAT5 compromise cell survival and/or proliferation (Dumon

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et al., 1999; Gobert *et al.*, 1996; Matsumura *et al.*, 1999). These data have been supported *in vivo*. In STAT5A/B double knock-out mice, hematopoietic cells from lymphoid and myeloid lineages were affected in their proliferation and/or survival (Moriggl *et al.*, 1999; Socolovsky *et al.*, 1999; Kieslinger *et al.*, 2000). Identification of genes whose expression could be regulated by STAT5 has retained a particular attention and it has been found that STAT5 regulates expression of cyclin D1, D2 and the anti-apoptotic Bcl-2 member, Bcl-x (Moriggl *et al.*, 1999; Matsumura *et al.*, 1999; Dumon *et al.*, 1999; Kieslinger *et al.*, 2000; Socolovsky *et al.*, 1999). All these data underline the importance that constitutive activity of STAT5 might have in cell transformation and leukemogenesis. Indeed, constitutive activities of STAT5 have been observed in cells from leukemic patients as in hematopoietic cell lines transformed by the leukemogenic TEL-JAK2, TEL-ABL, TEL/PDGFR and BCR-ABL fusion proteins (Carlesso *et al.*, 1996; Chai *et al.*, 1997; Gouilleux-Gruart *et al.*, 1996; Lacronique *et al.*, 1997; Shuai *et al.*, 1996). Expression of a dominant negative form of STAT5 in TEL-JAK2 transformed hematopoietic cells pinpointed to the crucial role of STAT5 in cell growth and viability (Lacronique *et al.*, 2000). Furthermore, expression of a constitutively active STAT5 mutant in the pro-B Ba/F3 cell line can relieve the IL-3 dependence of these cells (Onishi *et al.*, 1998). Similarly the PI 3-kinase/Akt pathway is constitutively activated in hematopoietic cells expressing BCR-ABL or TEL-ABL and introduction of dominant negative forms of Akt or p85 or of constitutively active form of Akt have implicated this pathway in growth and survival of these transformed cells (Neshat *et al.*, 2000; Skorski *et al.*, 1997; Voss *et al.*, 2000). We and others have found that activation of PI 3-kinase in Ba/F3 cells is required for IL-3 dependent proliferation (Craddock *et al.*, 1999; Rosa Santos *et al.*, 2000). We also reported that PI 3-kinase interacts with phosphorylated STAT5 through the SH2 domain of p85. Herein we also showed that erythropoietin (EPO) stimulation of Ba/F3 cells expressing EPO receptors and of normal human erythroid progenitors induced an interaction between phosphorylated STAT5 and p85. We then attempted to study the functional role of this interaction in Ba/F3 cells expressing constitutively active variants of STAT5. We provide evidence that this interaction led to the activation of the PI 3-kinase/Akt pathway which is required for cell cycle progression and survival. We also found a constitutive interaction between active STAT5 and PI 3-kinase in Ba/F3 cells transformed by the oncoproteins TEL-JAK2 or TEL-ABL as in the human leukemic cell line K562 expressing the BCR-ABL oncogene. However, while inhibition of the PI 3-kinase activity in TEL-JAK2 and TEL-ABL expressing Ba/F3 cells suppressed cell proliferation, it did not affect their viability indicating that activation of other survival pathways takes place in these transformed cells.

Results

EPO induces interaction between phosphorylated STAT5 and p85 the regulatory subunit of PI 3-kinase

We reported that IL-3 stimulation of Ba/F3 cells induced an interaction between STAT5 and p85 (Rosa Santos *et al.*, 2000). To investigate whether or not this interaction is cell-type or cytokine specific, we used Ba/F3 cells expressing the EPO receptor. Total extracts from cells stimulated with EPO or IL-3 were prepared and the p85 subunit of PI 3-kinase was immunoprecipitated. Western blot analysis with an anti-STAT5 antibody (Figure 1a) clearly showed that STAT5 was present in immunoprecipitates from cells stimulated with IL-3 or EPO (lanes 2, 4, 8) and absent in non-stimulated cells (lanes 1, 3, 7). To confirm the requirement of tyrosine phosphorylated STAT5 in its interaction with p85, Ba/F3 cells expressing a mutated version of EPO receptor (EPOR Zero) unable to activate STAT5 were stimulated with IL-3 or EPO (lanes 5, 6, 9, 10). In these cells EPO did not induce an interaction between STAT5 and p85 while IL-3 did (lanes 6, 10).

To verify the physiological relevance of EPO-induced STAT5/p85 interaction, we used normal erythroid progenitors isolated from human cord blood. As shown in Figure 1b, EPO also induced an interaction between STAT5 and p85 in human primary cells (lane 2).

*Constitutive activation of STAT5 in Ba/F3 cells expressing STAT5A1*6 or STAT5B1*6*

To investigate the functional significance of this interaction, we established Ba/F3 cell lines expressing the dominant positive forms of STAT5A and STAT5B. These mutants referred to as STAT5A1*6 and STAT5B1*6 have been recently described to induce cell growth and survival in the absence of cytokine (Onishi *et al.*, 1998).

pRSVpuro expression vectors containing STAT5A1*6 or STAT5B1*6 mutants were transfected and puromycin resistant cells were selected. Three independent pools of STAT5A1*6 and 10 independent pools of STAT5B1*6 expressing cells were obtained and found to be able to grow without IL-3. As a control, 10 pools of Ba/F3 cells transfected with the parental pRSVpuro expression vector (Ba/F3RSVpuro) were cultured without IL-3 but all of them died within 48 h (data not shown). We next analysed the expression and tyrosine phosphorylation status of the STAT5 variants, by Western blot, using an anti-phospho-STAT5 antibody (P-STAT5) raised against phosphorylated Tyr694 (Figure 2a). Phosphorylated STAT5A and STAT5B were detected under IL-3 stimulation conditions in control Ba/F3RSVpuro cells (lane 2). In extracts from cells expressing STAT5A1*6 (pools B, F), a thick band appearing as a doublet was easily detected in absence of IL-3 (lanes 3, 4). The same antibody recognized also two proteins with distinct electrophoretic mobilities in extracts from STAT5B1*6

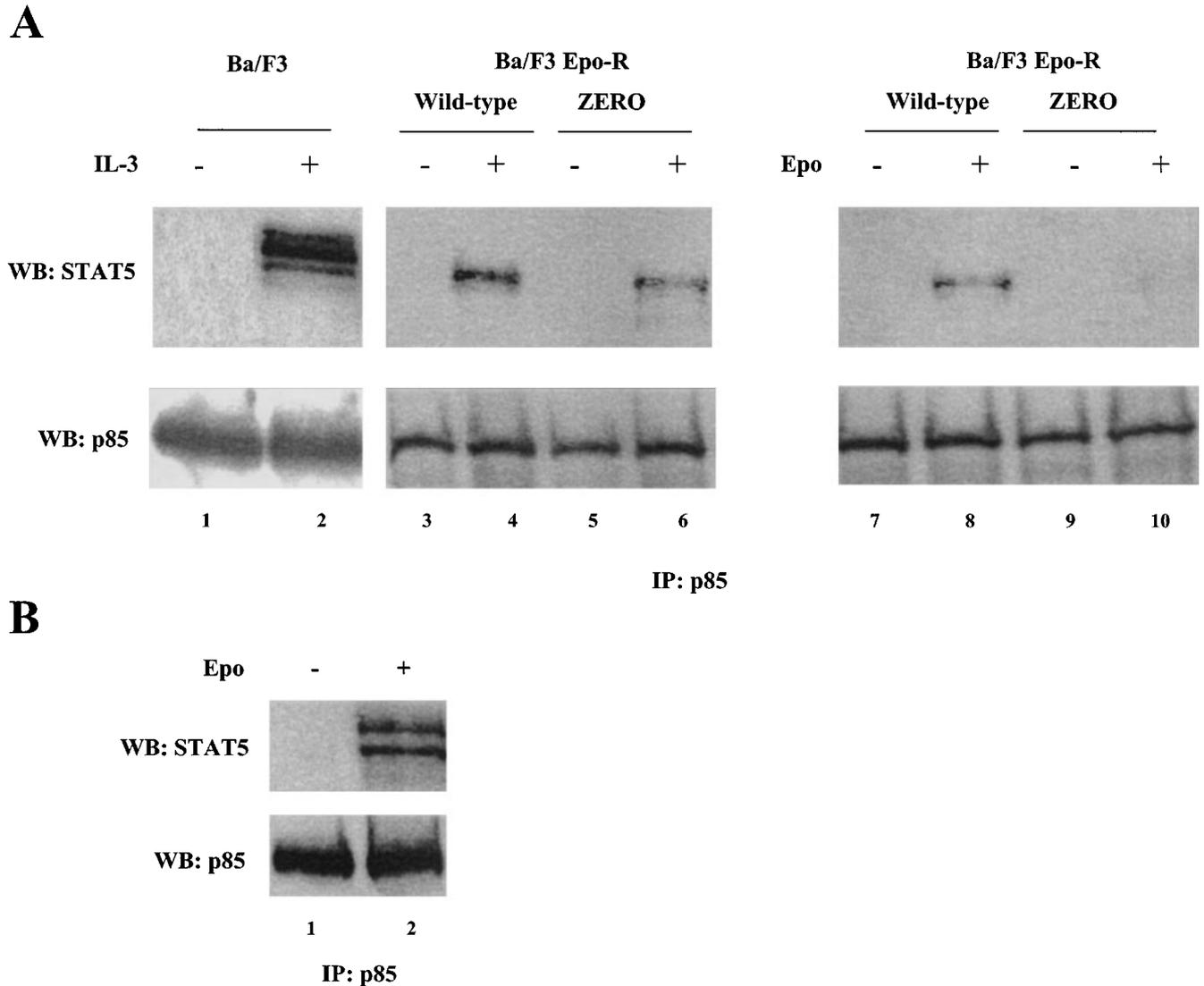


Figure 1 Epo induces interaction between STAT5 and p85 in Ba/F3 cells and in human erythroid progenitors. (a) Ba/F3 cells or Ba/F3 cells expressing the EPO receptor (Wild-type) or its mutated version (ZERO) were left unstimulated during 16 h (lanes 1, 3, 5, 7, 9) or were stimulated with IL-3 (lanes 2, 4, 6) or EPO (2 U/ml) (lanes 8, 10) for 30 min. Immunoprecipitation (IP) of cell lysates was conducted with an anti-p85 antibody followed by immunoblot with anti-STAT5 (upper panel). The membrane was reprobed with the same antibody as in IP (lower panel). (b) A similar experiment was performed on normal human erythroid progenitors left unstimulated (lane 1) or stimulated with EPO (10 U/ml) (lane 2) for 30 min

expressing cells (pools 9, 10 and lanes 5, 6). These two proteins were recognized by an anti-STAT5B antibody indicating that they represented two distinct isoforms of STAT5B1*6 (data not shown). Interestingly, we did not detect any tyrosine phosphorylation of endogenous STAT5 in Ba/F3 cells expressing either STAT5A1*6 or STAT5B1*6 even after long exposure.

The constitutive DNA binding activities of STAT5A1*6 and STAT5B1*6 variants were verified in Electrophoretic Mobility Shift Assay (EMSA) with a β -casein probe (Figure 2b). As a control, extracts from IL-3 stimulated or non-stimulated Ba/F3RSVpuro cells were loaded. Constitutive DNA binding complexes were observed in extracts from pools B, F, 9 and 10 (lanes 5, 8, 11, 14). Retained protein-DNA complexes observed in STAT5A1*6 expressing cell extracts were

fully supershifted with an anti-STAT5A antibody (lanes 6, 9). Interestingly, addition of an anti-STAT5B antibody induced a partial supershift of the complexes (lanes 7, 10). Similarly, complexes detected in Ba/F3STAT5B1*6 cell extracts were supershifted with an anti-STAT5B antibody (lanes 13, 16) while the anti-STAT5A antibody recognized partially these complexes (lanes 12, 15). Thus, STAT5A1*6 and STAT5B1*6 constitutively bind to DNA, homo-dimerized and can also form hetero-dimers with endogenous non-phosphorylated STAT5 proteins.

We next analysed in Ba/F3STAT5A1*6 (pool F, lane 3; pool B, lane 4) and Ba/F3STAT5B1*6 (pool 9, lane 5; pool 10, lane 6) cells, the expression of Bcl-x protein, whose gene is regulated by STAT5 (Figure 2c). In all these IL-3 independent cell lines (lanes 3–6), levels of

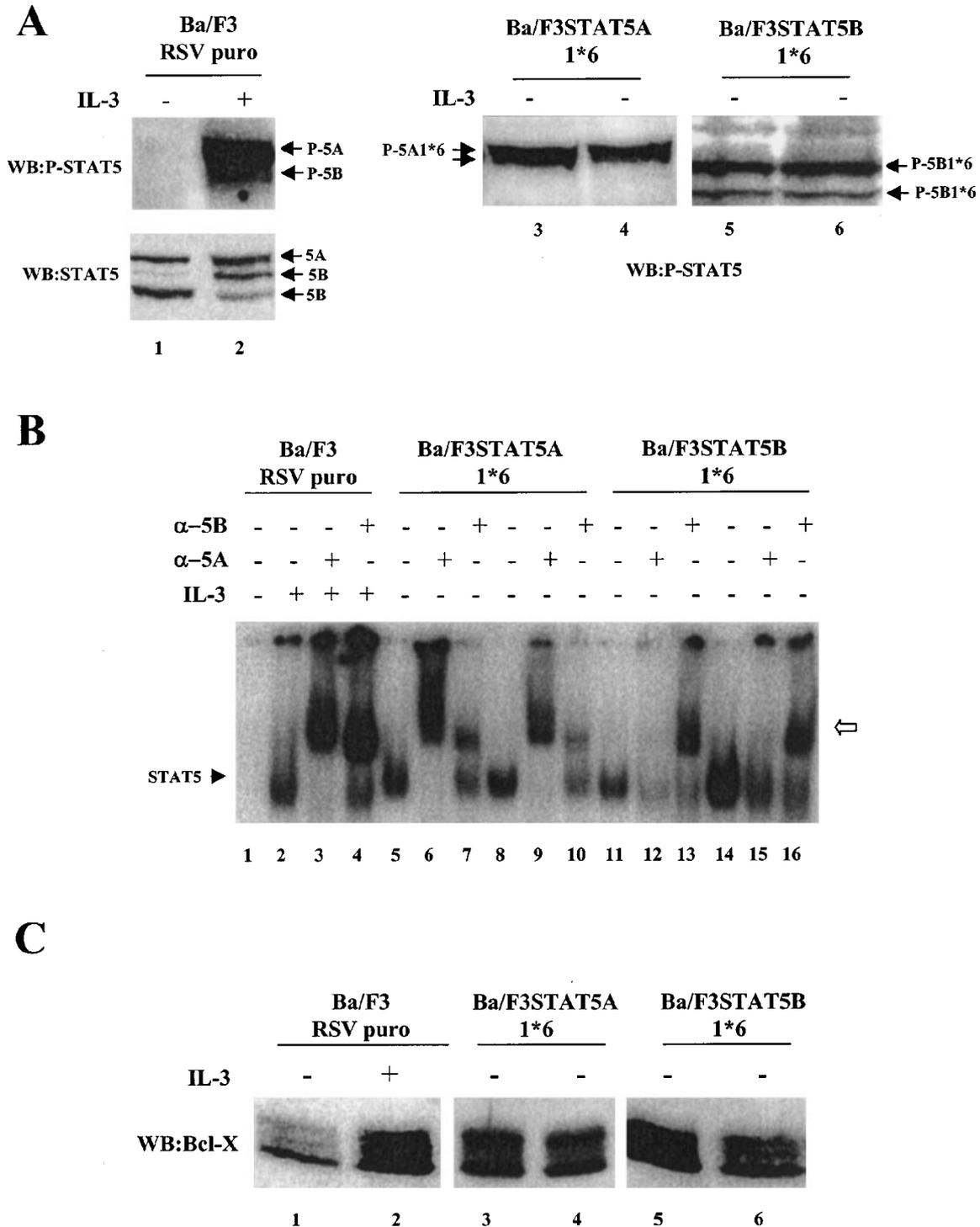


Figure 2 STAT5A1*6 and STAT5B1*6 in Ba/F3 cells: (a) are constitutively phosphorylated on tyrosine. Control Ba/F3RSVpuro cells starved of IL-3 during 16 h and were left unstimulated (lane 1) or stimulated with 10 ng/ml of recombinant IL-3 (lane 2) for 30 min. Ba/F3 cells expressing the mutant STAT5A1*6 (pool B, lane 3; pool F, lane 4) or STAT5B1*6 (pool 9, lane 5; pool 10, lane 6) were cultured without IL-3. Cell extracts were analysed by Western blot with an anti-P-STAT5 (upper panel) or with an anti-STAT5 antibody (lower panel). Positions of endogenous STAT5A or STAT5B and STAT5A1*6 or STAT5B1*6 are indicated. (b) constitutively bind to DNA. Whole cell extracts from Ba/F3 cells expressing STAT5A1*6 (pool B, lane 5; pool F, lane 8) or STAT5B1*6 (pool 9, lane 11; pool 10, lane 14) were analysed in a band shift experiment with a β -casein probe. As a control, whole cell extracts from Ba/F3RSVpuro cells left unstimulated (lane 1) or stimulated with 10 ng/ml of recombinant IL-3 (lane 2) for 30 min were included. Specific anti-STAT5A (lanes 3, 6, 9, 12, 15) or anti-STAT5B (lanes 4, 7, 10, 13, 16) were introduced in the binding reaction to analyse the appearance of a supershifted complex (indicated by an open arrow) (c) regulate expression of Bcl-x protein. Analysis of Bcl-x protein expression was performed in Ba/F3RSVpuro cells treated or not with IL-3 (lanes 1, 2), in Ba/F3STAT5A1*6 (pool F, lane 3; pool B, lane 4) and in Ba/F3STAT5B1*6 (pool 9, lane 5; pool 10, lane 6) with an anti-Bcl-x antibody

Bcl-x protein were found similar to those of IL-3 stimulated control Ba/F3RSVpuro cells (lane 2). Altogether, these data showed that STAT5A1*6 and STAT5B1*6 are constitutively phosphorylated, bind to DNA and induce the expression of Bcl-x.

Constitutive interaction between STAT5 and the p85 subunit of PI 3-kinase in Ba/F3 cells expressing the active STAT5 variants or the oncoproteins TEL-JAK2, TEL-ABL or BCR-ABL

To analyse the possibility of an *in situ* association between p85 and the constitutively active STAT5 mutants, p85 immunoprecipitates from Ba/F3 cells expressing STAT5A1*6 (pools B, F) or STAT5B1*6 (pools 9, 10) were analysed with an anti-STAT5 specific antibody (Figure 3a). By using an antibody allowing the detection of both endogenous and variants STAT5 factors, we observed the presence of STAT5A1*6 (lanes 1, 2) and STAT5B1*6 (lanes 3, 4) as of endogenous STAT5B and STAT5A in the p85 immunoprecipitates (lanes 1, 2 and right panel).

Because constitutive activation of STAT5 has been observed in cell lines transformed by different oncogenes like TEL-JAK2, TEL-ABL and BCR-ABL, we examined the possible interaction of activated STAT5 and PI 3-kinase in Ba/F3 cells expressing TEL-JAK2 or TEL-ABL and in the human leukemic cell line K562 expressing BCR-ABL. We also detected the presence of STAT5A and STAT5B in their phosphorylated and non phosphorylated forms in p85 immunoprecipitates (Figure 3b) from extracts of Ba/F3 cells expressing TEL-JAK2 (lane 1), TEL-ABL (lane 2), as in p85 immunoprecipitates from K562 cell extracts (lane 3). Thus, STAT5 constitutively interacted with p85 in Ba/F3 cells expressing STAT5A1*6 or STAT5B1*6 and in cells transformed by TEL-JAK2, TEL-ABL and BCR-ABL.

*Constitutive phosphorylation of Akt and Bad in Ba/F3STAT5A1*6, Ba/F3STAT5B1*6, Ba/F3TEL-JAK2 and Ba/F3TEL-ABL cells*

The constitutive interaction of active STAT5 variants with p85 prompted us to explore the activation of the PI 3-kinase/Akt pathway. PI 3-kinase induces activation of the serine/threonine kinase Akt which in turn phosphorylates the pro-apoptotic Bad protein. We analysed by Western blot (Figure 4), the expression and phosphorylation of Akt on serine residue 473 in each independent Ba/F3 pools expressing either STAT5A1*6 (pools B, F) or STAT5B1*6 (pools 9, 10) as in Ba/F3 cells transformed by TEL-JAK2 and TEL-ABL fusions (upper panel). Extracts from control Ba/F3RSVpuro cells growing in presence of IL-3 containing medium (CM) or deprived of IL-3 for 16 h and then stimulated with IL-3 were also examined. In these cells IL-3 stimulation induced the phosphorylation of Akt (lanes 8, 9). In contrast, Akt was constitutively phosphorylated in Ba/F3 cells expressing the STAT5 variants (lanes 1–4) or the

oncoproteins (lanes 5, 6) or in Ba/F3 cells grown in CM (lane 7). The membrane was next reprobed with an anti-Akt antibody to demonstrate the presence of equal amounts of Akt protein (middle panel).

Similarly, the expression and phosphorylation of Bad were examined by immunoblot with an anti-Bad antibody (lower panel). We previously reported that IL-3 stimulation of Ba/F3 cells induced the appearance of two phosphorylated forms of Bad referred to as BadP1 and BadP2 and showed that the induction of the BadP2 form is dependent on PI 3-kinase activity (Rosa Santos *et al.*, 2000). In Ba/F3 cells expressing the dominant positive forms of STAT5 and in Ba/F3 cells expressing the oncoproteins TEL-JAK2 or TEL-ABL, BadP1 and BadP2 were observed (lanes 1–6) as in control cells grown in CM (lane 7) or stimulated by IL-3 (lane 9). Collectively, these findings support the hypothesis of an active PI 3-kinase/Akt pathway in Ba/F3 cells expressing the STAT5 variants.

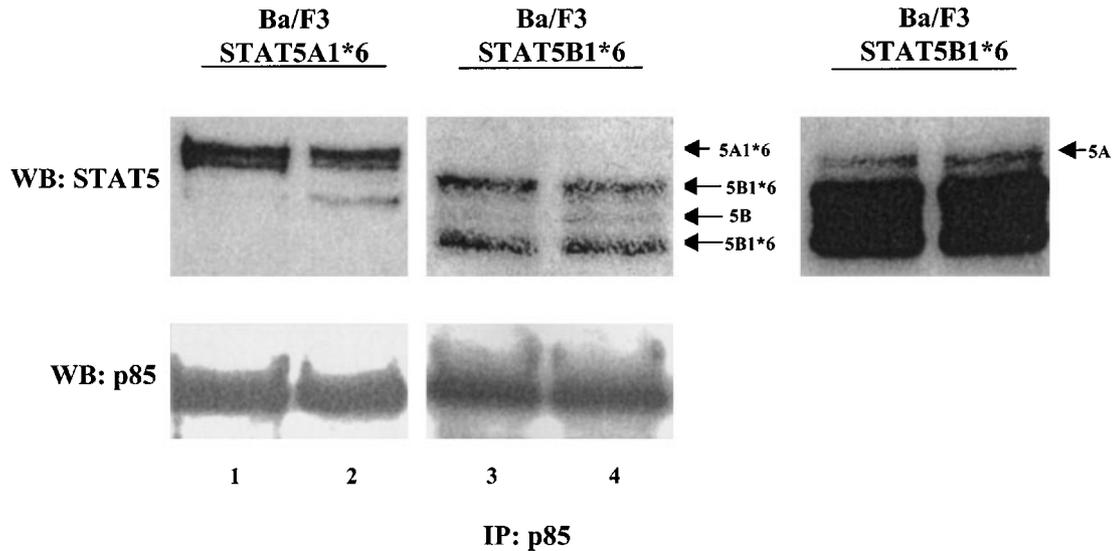
*Activation of the PI 3-Kinase/Akt pathway is required for cell cycle progression in Ba/F3STAT5A1*6, Ba/F3STAT5B1*6, Ba/F3TEL-JAK2 and Ba/F3TEL-ABL cells*

To investigate the role of the PI 3-kinase/Akt pathway in the proliferation of Ba/F3STAT5A1*6, Ba/F3STAT5B1*6, Ba/F3TEL-JAK2 and Ba/F3TEL-ABL cells, cells were incubated with the specific PI 3-kinase inhibitor, LY294002, and their growth capacities were determined. Proliferation of all these cell types was completely blocked by this inhibitor (data not shown). We next analysed their progression in the cell cycle in absence or presence of LY294002 (50 μ M), during 72 h. Cell cycle was analysed after staining with propidium iodide by using flow cytometry. As shown Figure 5, addition of LY294002 induced a G1 arrest in the cell cycle after 24 h of incubation. Thus, activation of PI 3-kinase is crucial for the cell cycle progression of all these cell lines.

PI 3-Kinase activity is crucial for the survival of Ba/F3 cells expressing the active STAT5 variants, while dispensable in TEL-JAK2 or TEL-ABL transformed Ba/F3 cells

To investigate the role of the PI 3-kinase/Akt pathway in the survival of Ba/F3 cells expressing STAT5A1*6, STAT5B1*6, TEL-JAK2 and TEL-ABL, cells were cultured in absence or presence of LY294002. Percentages of apoptotic cells were estimated by AnnexinV-propidium iodide double staining in a time course experiment (Figure 6a). While the effect of LY294002 on the viability of Ba/F3 cells transformed by the TEL-JAK2 or TEL-ABL fusions was negligible even after 72 h of incubation (right panel), a dramatic increase in the percentage of apoptotic cells was observed in Ba/F3STAT5A1*6 and Ba/F3STAT5B1*6 cells (90% of apoptotic cells at the same period) (left panel). These findings lead us to conclude that PI 3-kinase is essential for the survival of Ba/F3 cells

A



B

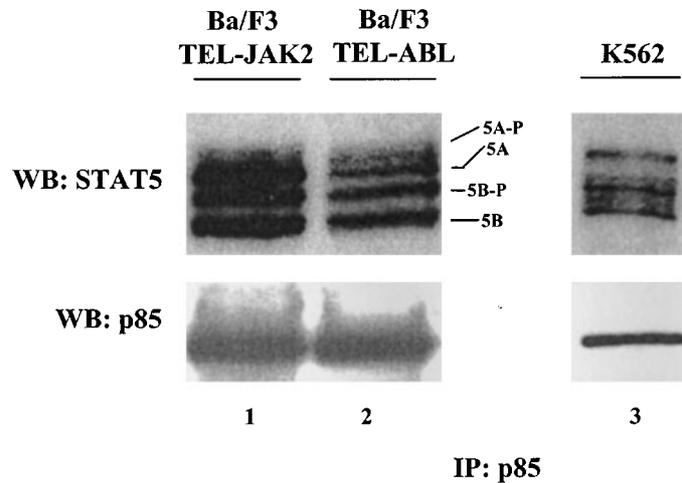


Figure 3 Interaction between STAT5 and the p85 subunit of the PI 3-Kinase in cells expressing. (a) Constitutive STAT5 variants. Ba/F3STAT5A1*6 cells (pool F, lane 1; pool B, lane 2), Ba/F3STAT5B1*6 cells (pool 9, lane 3; pool 10, lane 4) were cultured without IL-3. Immunoprecipitation (IP) of cell lysates was conducted with an anti-p85 antibody followed by immunoblot with anti-STAT5 (upper panel). The positions of STAT5A1*6, STAT5B1*6 and of endogenous STAT5A and STAT5B are indicated. The right panel represents a longer exposure of the immunoblot seen in (a) (lanes 3, 4). The membrane was reprobed with the same antibody as in IP (lower panel). (b) TEL-JAK2 or TEL-ABL and in K562 cells. Anti-p85 immunoprecipitates from Ba/F3 cells expressing TEL-JAK2 (lane 1), TEL-ABL (lane 2) and from K562 cells (lane 3) were analysed by immunoblotting with an anti-STAT5 (upper panel). The membrane was reprobed with an anti-p85 antibody (lower panel)

expressing the active STAT5 variants but is dispensable for the survival of Ba/F3 cells transformed by TEL-JAK2 and TEL-ABL fusions.

To further analyse the differential effect of LY294002 on the survival of Ba/F3 cells expressing the active STAT5 variants or the oncoproteins TEL-

JAK2 and TEL-ABL, we examined by immunoblot the phosphorylation status of Akt and Bad and the expression of Bcl-x after treatment of the various Ba/F3 cell lines with LY294002 (Figure 6b). Inhibition of Akt (ser473) and Bad phosphorylation (as judged by the absence of the BadP2 form) was observed in all

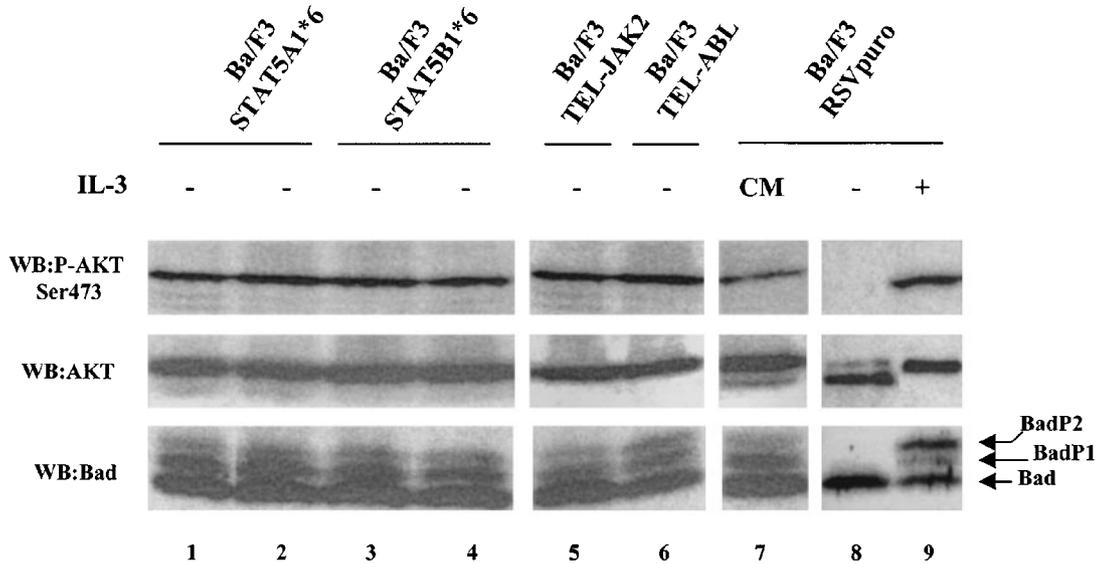


Figure 4 Constitutive phosphorylation of Akt and Bad in Ba/F3STAT5A1*6, Ba/F3STAT5B1*6, Ba/F3TEL-JAK2 and Ba/F3 TEL-ABL cells. Extracts from Ba/F3STAT5A1*6 (pool F, lane 1; pool B, lane 2), Ba/F3STAT5B1*6 (pool 9, lane 3; pool 10, lane 4), Ba/F3TEL-JAK2 (lane 5) and Ba/F3 TEL-ABL (lane 6) cells were analysed by Western blot with an anti-P-Akt antibody (upper panel) or with an anti-Akt antibody (middle panel). As a control Ba/F3RSVpuro cells were cultured in CM (lane 7) or without IL-3 for 16 h and left untreated (lane 8) or treated (lane 9) with IL-3 for 30 min. A similar experiment was performed to analyse Bad phosphorylation with an anti-Bad antibody. Positions of unphosphorylated Bad, BadP1 and BadP2 are indicated

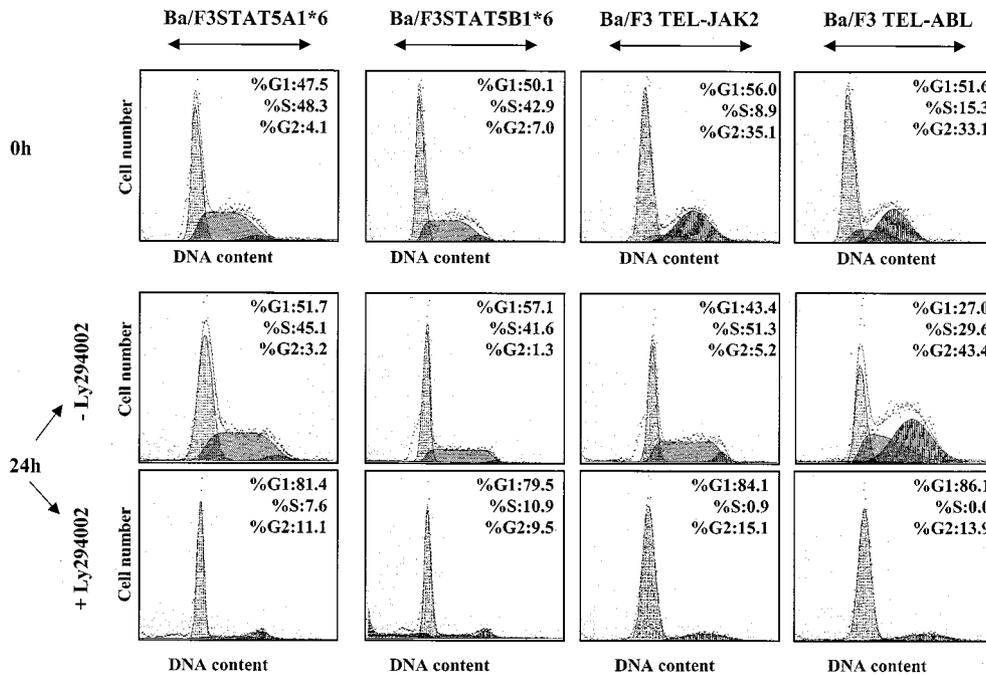


Figure 5 Effect of LY294002 on the cell cycle progression of Ba/F3 cells expressing the STAT5 variants or the oncoproteins TEL-JAK2 or TEL-ABL. Ba/F3STAT5A1*6, Ba/F3STAT5B1*6, Ba/F3TEL-JAK2 and Ba/F3 TEL-ABL cells were cultured without IL-3, in absence or presence of LY294002 (50 μ M). Cells were stained with propidium iodide and analysed by flow cytometry. Only viable cells having a DNA content $\geq 2N$ were used and DNA histograms were obtained by using the multicycle software. Percentages of cells present in the different phases of the cell cycle are indicated

these cells while treatment with LY294002 did not affect Bcl-x expression. Thus LY294002 efficiently

inhibited the PI 3-kinase/Akt pathway in all these Ba/F3 cell lines.

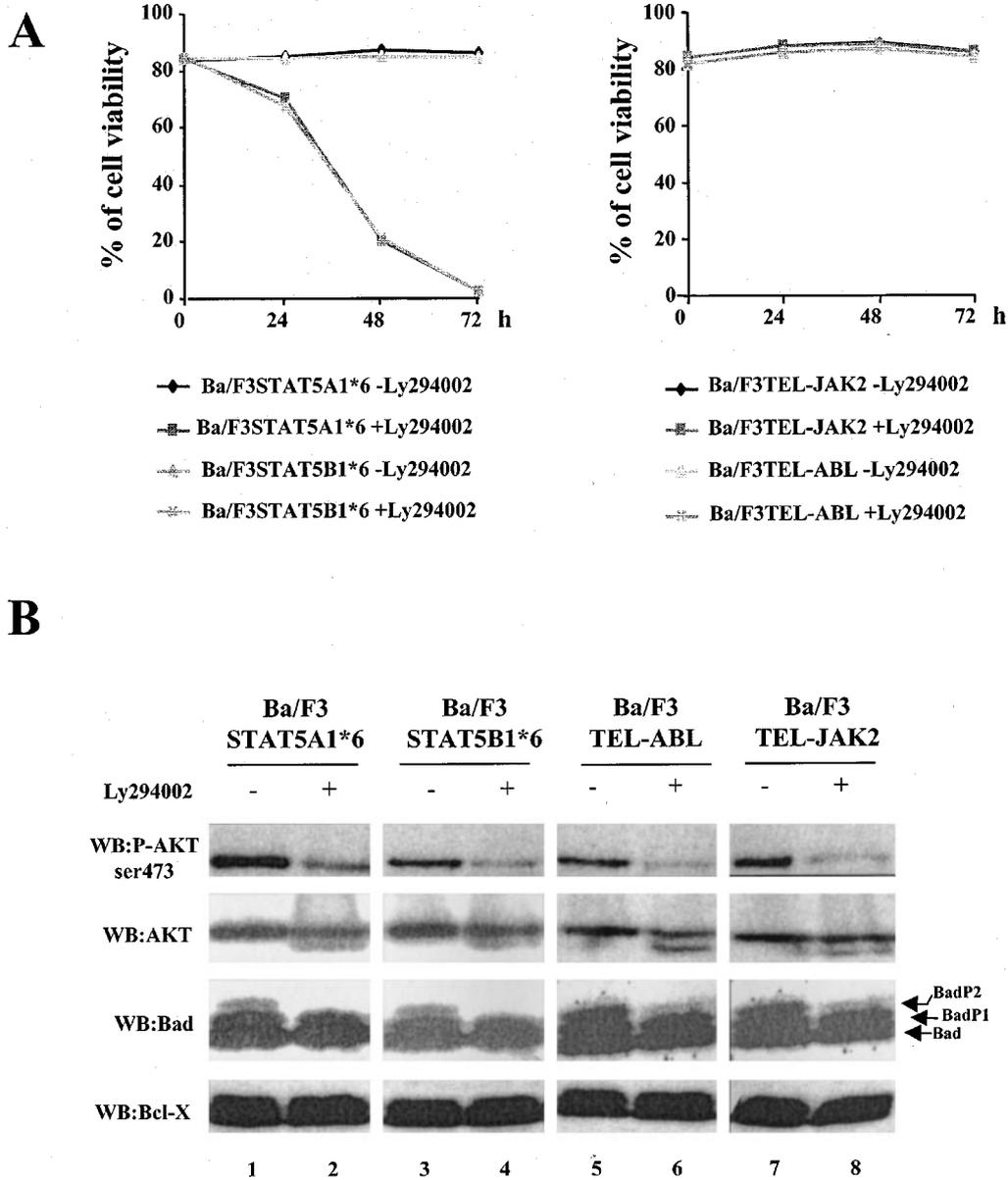


Figure 6 (a) Effect of LY294002 on the viability of Ba/F3 cells expressing STAT5A1*6 or STAT5B1*6 or TEL-JAK2 or TEL-ABL. STAT5A1*6, STAT5B1*6, TEL-JAK2 and TEL-ABL expressing cells were cultured in absence or presence of LY294002 (50 μ M). Cells were stained with annexinV-fluos and propidium iodide and the percentage of apoptotic cells was determined by flow cytometry. Values are given as the percentage of viable cells (AnnexinV negative cells) remaining in the culture. (b) Effect of LY294002 on the phosphorylation of Akt and Bad and expression Bcl-x in Ba/F3 cells expressing STAT5A1*6, STAT5B1*6, TEL-JAK2, TEL-ABL. Cell extracts from Ba/F3 cells expressing STAT5A1*6 or STAT5B1*6 or TEL-JAK2 or TEL-ABL fusions untreated (lanes 1, 3, 5, 7) or treated with LY294002 (lanes 2, 4, 6, 8) were prepared and analysed first with an anti-P-Akt antibody. Membrane was then stripped and reprobred with the indicated antibodies. Positions of unphosphorylated Bad, BadP1 and BadP2 are indicated

Discussion

We previously reported that activated STAT5 proteins interacted with the SH2 domains of the p85 subunit of PI 3-kinase in Ba/F3 cells in an IL-3 dependent fashion (Rosa Santos *et al.*, 2000). These observations have been now extended to other cellular models since we showed here that STAT5 interacted with p85 in Ba/F3 cells expressing EPO receptors as in normal human erythroid progenitors after EPO stimulation. These

results indicate that this interaction occurs in a physiological context and in response to various cytokines. To investigate the functional role of STAT5-p85 interaction, we used Ba/F3 cells expressing the dominant positive STAT5 mutants, STAT5A1*6 or STAT5B1*6. These mutants are constitutively phosphorylated, bind to DNA, regulate expression of STAT5 target genes and are able to relieve the IL-3-dependent growth and survival of Ba/F3 cells (Onishi *et al.*, 1998). Although the precise mechanism of their

constitutive activity is not known, they represent interesting tools for studying the role of STAT5 in cell proliferation and survival as the functional meaning and the consequences of constitutive STAT5 activity often found in leukemic cells. We therefore analysed the involvement of the PI 3-kinase/Akt pathway in the growth and survival of Ba/F3 cells expressing STAT5A1*6 or STAT5B1*6. Our results clearly showed that: (1) a constitutive interaction between STAT5A1*6 or STAT5B1*6 and p85 is observed in these cells; (2) Akt and Bad are phosphorylated indicating that the PI 3-kinase/Akt pathway is constitutively active; and (3) inhibition of the PI 3-kinase activity totally blocks both proliferation and survival of these cells. These observations suggest that IL-3 independent growth induced by the active STAT5 variants requires the PI 3-kinase activity. A constitutive interaction between STAT5 and p85 was also observed in TEL-JAK2 or TEL-ABL transformed Ba/F3 cells as in K562 cells expressing BCR-ABL.

In all the Ba/F3 cell lines studied here, we observed the presence of STAT5A or STAT5B in their non phosphorylated form within the p85 immunoprecipitates, suggesting that hetero as well as homodimers might exist between active and inactive forms of STAT5. Indeed, both endogenous STAT5A and STAT5B were present in the DNA binding complexes observed in Ba/F3STAT5A1*6 and Ba/F3STAT5B1*6 cell extracts despite the fact that these proteins were not tyrosine phosphorylated. These observations argue for a important role of endogenous forms of STAT5 in the properties of STAT5A1*6 and STAT5B1*6 and are supported by the recent findings that the presence of endogenous STAT5A and STAT5B are necessary for the transformation induced by STAT5A1*6 or STAT5B1*6 (G Gilliland (2001), personal communication).

PI 3-kinase activation is triggered by different mechanisms, one involving the interaction of the SH2 domain of p85 with phosphorylated tyrosine in YXXM motifs. Sequence analysis reveals that tyr548 is contained in such consensus binding site in murine STAT5A and STAT5B but not in human STAT5A and STAT5B. Nevertheless, we could detect an interaction between STAT5 and p85 in human erythroid progenitors following Epo stimulation and in the human leukemic cell line K562 indicating that the binding of STAT5 to PI 3-kinase might be realized *via* an adaptor protein. PI 3-kinase has been shown to interact with phosphorylated tyrosine residues of activated receptors, to SHP-2 or to adaptor proteins of the IRS-Gab family (Craddock and Welham, 1997; Verdier *et al.*, 1997; Gu *et al.* 1998; Yamauchi *et al.*, 1998; Lecoq-Lafon *et al.*, 1999; Nishida *et al.*, 1999). These latter proteins contain a Pleckstrin Homology domain thought to be required for their membrane localization and to bring the PI 3-kinase in the vicinity of its substrates. In keeping with this, we found that STAT5 interacted with the adaptor protein Gab2 but not with IRS-2 in Ba/F3 cells stimulated with IL-3 or expressing the active STAT5 variants or TEL-JAK2 or TEL-ABL oncogenes, making likely that STAT5

induced PI 3-kinase activation may involve Gab2 (data not shown). However, we cannot exclude that other unidentified molecules may link STAT5 to PI 3-kinase.

We and others have shown that activation of PI 3-kinase by IL-3 in Ba/F3 cells is required for cell cycle progression and that inhibition of PI 3-kinase activity induced a G1 arrest (Craddock *et al.*, 1999; Rosa Santos *et al.*, 2000). Our results extended these observations to IL-3 independent Ba/F3 cells transformed either by the active STAT5 variants or by the leukemogenic TEL-JAK2 and TEL-ABL fusion proteins. Our preliminary results indicated that cyclin D2, that is required for the G1/S transition is upregulated in STAT5A1*6, STAT5B1*6, TEL-JAK2 and TEL-ABL expressing cell lines as in IL-3 stimulated control Ba/F3 cells. Moreover we also found that cyclin D2 protein expression is dependent on the PI 3-kinase activity (data not shown).

Although the effects of PI 3-Kinase inhibitor on cell growth were similar in all cell lines we examined, contrasting results were observed on cell survival. Our experiments showed that activation of PI 3-kinase is dispensable for the survival of Ba/F3 cells stimulated with IL-3 or transformed by TEL-JAK2 or TEL-ABL fusion proteins while it is absolutely required for the survival of Ba/F3STAT5A1*6 or Ba/F3STAT5B1*6 cells. We previously reported that individual inhibition of PI 3-kinase, MAP kinase or STAT5 activities did not affect the survival of Ba/F3 cells in the presence of IL-3, while the simultaneous inhibition of STAT5 and PI 3-kinase partially induced cell death (Rosa Santos *et al.*, 2000). These data indicate a cooperation between these signaling pathways in the way to protect cells from apoptosis. A similar phenomenon could explain the lack of LY294002 effect on the survival of Ba/F3 cells transformed by TEL-JAK2 and TEL-ABL. However, this is not probably the whole story, because inhibition of PI 3-kinase activity is crucial for the survival of Ba/F3STAT5A1*6 and Ba/F3STAT5B1*6 cells indicating that activation of STAT5 and the strong expression of Bcl-x observed in these cells are not sufficient to overcome the effect of the PI 3-kinase inhibitor. Thus, additional survival pathways independent of PI 3-kinase activity, may be activated by IL-3 and by the TEL-JAK2 and TEL-ABL oncoproteins. Several reports indicated that activations of Ras/Raf/MAP kinase, Ras/NF-IL3 pathways and NF- κ B trigger an anti-apoptotic signals in cytokine stimulated or transformed cells (Besancon *et al.*, 1998; Kuribara *et al.*, 1999; Leverrier *et al.*, 1997; Neshat *et al.*, 2000). From our preliminary results, the Ras/Raf/MAP kinase pathway is dispensable for the survival of cells expressing the STAT5 variants or the oncoproteins TEL-JAK2 or TEL-ABL. The role of NFIL3 and NF- κ B in the survival of these cells is at present under investigation. In summary, our data underline a novel role of STAT5 and suggest that STAT5 might promote cell survival not only as a transcription factor by inducing Bcl-x expression but also as a signaling intermediate in the activation of PI 3-kinase.

Materials and methods

Cell culture and reagents

Murine pro-B Ba/F3 cells were maintained as described (Santos *et al.*, 2000). Stimulation experiments were performed with recombinant murine IL-3 (Valbiotech). The IL-3 independent Ba/F3 cell lines and the cell line K562 were grown in RPMI 1640 medium with 10% FCS.

Ba/F3 cells expressing either the TEL-JAK2 or TEL-ABL fusions have already been described (Lacronique *et al.*, 2000).

The Ba/F3EPOR cell lines (Verdier *et al.*, 1997) were treated with EPO (2 U/ml) (Roche). The propagation and the isolation of human erythroid progenitors have been described elsewhere (Lecoq-Lafon *et al.*, 1999). The PI 3-kinase inhibitor LY294002 was purchased from Sigma.

Plasmids and transfections

The STAT5A1*6 and STAT5B1*6 cDNAs were subcloned into the pRSVpuro vector and electroporated in Ba/F3 cells (250 V, 960 μ F). Electroporated cells were expanded for 24 h in IL-3 containing medium and stable transfectants were selected in the presence of 1 μ g/ml of puromycin. Resistant puromycin cells were then grown in absence of IL-3 to assess IL-3 dependence.

Electrophoresis mobility shift analysis

Whole cell extracts and band shift experiments using the β -casein probe were performed as reported (Dumon *et al.*, 1999). Rabbit polyclonal antibodies raised against STAT5A and STAT5B were used for supershift experiments.

Apoptosis studies

Ba/F3STAT5A1*6, Ba/F3STAT5B1*6, Ba/F3TEL-JAK2 and Ba/F3TEL-ABL cells were cultured in RPMI 10% FCS. Cells were incubated with or without 50 μ M of LY294002 (Sigma).

The percentages of early apoptotic cells (Annexin V-fluorescence positive, propidium iodide negative) and late apoptotic cells

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(Annexin V-fluorescence positive, propidium iodide positive) were determined as described (Santos *et al.*, 2000).

Cell cycle analysis

Ba/F3 cells were treated as before, washed once in cold PBS containing 5 mM EDTA, then incubated in the above buffer supplemented with 0.2 mg/ml RNase A at room temperature for 30 min. Fifty μ g/ml propidium iodide solution was added to the samples and cell cycle was analysed by flow cytometry. DNA histograms were obtained by using the Multicycle Software.

Western blotting and immunoprecipitation studies

Total cell lysates were obtained as described (Santos *et al.*, 2000). Lysates were separated by electrophoresis on SDS-PAGE and blotted onto cellulose membrane (Hybond-C super membrane, Amersham Life Science). Blots were incubated as indicated with antibodies raised against the following murine proteins: Bad (Santa Cruz), Bcl-x, STAT5 (Transduction Laboratories), P-STAT5, P-Akt (Biolabs), Akt (Santa Cruz), a rabbit polyclonal anti-p85 antibody raised against the SH2 domains of p85.

Immunoprecipitation studies were performed as reported (Santos *et al.*, 2000).

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