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Cytokine-dependent activation of distinct signaling pathways is a common scheme thought to be required for the subsequent programmation into cell proliferation and survival. The PI 3-kinase/Akt, Ras/MAP kinase, Ras/ NFIL3 and JAK/STAT pathways have been shown to participate in cytokine mediated suppression of apoptosis in various cell types. However the relative importance of these signaling pathways seems to depend on the cellular context. In several cases, individual inhibition of each pathway is not sufficient to completely abrogate cytokine mediated cell survival suggesting that cooperation between these pathways is required. Here we showed that individual inhibition of STAT5, PI 3-kinase or MEK activities did not or weakly affected the IL-3 dependent survival of the bone marrow derived Ba/F3 cell line. However, the simultaneous inhibition of STAT5 and PI 3-kinase activities but not that of STAT5 and MEK reduced the IL-3 dependent survival of Ba/F3. Analysis of the expression of the Bcl-2 members indicated that phosphorylation of Bad and Bcl-x expression which are respectively regulated by the PI 3-kinase/Akt pathway and STAT5 probably explain this cooperation. Furthermore, we showed by co-immunopre-cipitation studies and pull down experiments with fusion proteins encoding the GST-SH2 domains of p85 that STAT5 in its phosphorylated form interacts with the p85 subunit of the PI 3-kinase. These results indicate that the activations of STAT5 and the PI 3-kinase by IL-3 in Ba/F3 cells are tightly connected and cooperate to mediate IL-3-dependent suppression of apoptosis by modulating Bad phosphorylation and Bcl-x expression. *Oncogene* (2000) **19,** 1164–1172.

Keywords: apoptosis; STAT5; PI3-kinase; Bad; Bcl-x

Introduction

Cytokine binding to their cognate receptors triggers proliferation and survival of hematopoietic cells through activation of distinct signaling pathways (Ihle, 1996). The use of cytokine receptor mutants or dominant negative/constitutively active forms of components of these signaling pathways identifies the phosphatidylini-sitol 3-kinase (PI 3-kinase)/Akt, Ras/NFIL3, Ras/Raf/ MAP kinase (MAPK) and JAK/STAT pathways as crucial regulators of survival and/or proliferation of

hematopoietic cells (Songyang et al., 1997; Kuribara et al., 1999; Kinoshita et al., 1997; Liu et al., 1997; Dumon et al., 1999). Coordinated activation of these signaling pathways is commonly induced by most cytokines. The JAK protein tyrosine kinases play a central role in this process. Activated JAK proteins phosphorylate tyrosine residues present in the cytoplasmic region of the cytokine receptors which serve as docking sites for different SH2 containing molecules like the p85 subunit of the PI 3-kinase, STAT, SHC and SHP-1 (Watanabe and Arai, 1996; Itoh et al., 1998; Onishi et al., 1998a). Recent data also indicated that JAK proteins directly interact with p85 and activate PI 3-kinase (Migone et al., 1998; Al-Shami and Naccache, 1999). Interactions between STAT5 and JAK2 and JAK and Raf have been reported (Fujitani et al., 1997; Sakatsume et al., 1998). Relation-ships between Ras and PI 3-kinase have been also described indicating that these pathways are connected and probably regulate each other (Franke et al., 1997). Activation of these signaling pathways promotes cell survival by regulating downstream the expression or the phosphorylation of the Bcl-2 family members (Lotem and Sachs, 1996; Park, 1996; del Peso et al., 1997). The PI 3-kinase activates the protein kinase Akt which phosphorylates Bad, thereby inhibiting its pro-apoptotic activity (del Peso et al., 1997; Zha et al., 1996). Ras/ MAPK and JAK/STAT pathways have also been shown to be required for the regulation of Bcl-2 and Bcl-x expression (Kinoshita et al., 1995; Dumon et al., 1999; Sakai et al., 1997; Leverrier et al., 1997; Fujio et al., 1997).

Among the seven mammalian STAT members that have been characterized, STAT5A and STAT5B are two highly related transcription factors that are activated by various stimuli including cytokines, hormones, growth factors or different oncogenes underlying their potential importance in cell prolifera-tion (Liu et al., 1995; Mui et al., 1995; Gouilleux et al., 1995; Wakao et al., 1995; Pallard et al., 1995; Lacronique et al., 1997). Proliferation of macrophages in response to GM-CSF in STAT5A−/− mice and of NK cells in STAT5B−/− mice was shown to be affected (Feldman et al., 1997; Imada et al., 1998). Simultaneous inactivation of STAT5A and STAT5B genes results in a total defect in peripheric T cell proliferation due to a block in cell cycle progression (Moriggl et al., 1999). In hematopoietic cell lines, the use of dominant negative or constitutively active STAT5 mutants showed that this factor is required for cell growth (Mui et al., 1996; Onishi et al., 1998b; Matsumura et al., 1999). Similarly, hematopoietic cell lines expressing cytokine receptor mutants that are

unable to activate STAT5, have a reduced proliferation rate (Gobert *et al.*, 1996; Friedmann *et al.*, 1996). The role of STAT5 in the cytokine mediated suppression of apoptosis has been also documented. STAT5 promotes the IL-3 and IL-2 dependent survival of different hematopoietic cell lines and constitutive STAT5 activity has been observed in a T Lymphoma cell line treated with an anti-apoptotic reagent (Dumon *et al.*, 1999; Zamorano *et al.*, 1998; Rui *et al.*, 1998). We recently found that expression of a dominant negative form of STAT5 (STAT5AΔ749) increased the susceptibility of Ba/F3 cells to undergo apoptosis after IL-3 withdrawal. We also showed that regulation of Bcl-x gene expression by IL-3 in Ba/F3 cells is dependent on STAT5 (Dumon *et al.*, 1999). In the present report, we provided evidence that individual inhibition of STAT5 transcriptional activity, PI 3-kinase/Akt and MEK/MAPK pathways did not or weakly affected the survival of Ba/F3 cells in presence of IL-3. The use of the PI 3-kinase specific inhibitor LY294002 showed that PI 3-kinase is essential for the proliferation of Ba/F3 cells but not for their survival. However, LY294002 has a more pronounced effect on the viability of Ba/F3 cells expressing STAT5AΔ749 indicating that STAT5 and PI 3-kinase act in concert to protect Ba/F3 cells from apoptosis. Inhibition of MEK activity by the compound PD98059 neither affected proliferation nor survival of the different Ba/F3 cell lines used. The cooperative effect of the PI 3-kinase and STAT5 on the survival of Ba/F3 cells might be explained by the levels of phosphorylated Bad and Bcl-xL which are regulated by the PI 3-kinase and STAT5 respectively. We also found by co-immunoprecipitations studies an interaction between STAT5 and the p85, the regulatory subunit of PI 3-kinase. Pull down experiments with GST fusion proteins showed that STAT5 in its phosphorylated form interact with the SH2 domains of p85. This study indicates that the activations of the PI 3-kinase and STAT5 by IL-3 in Ba/F3 are connected and needed to protect the cells from apoptosis by regulating the balance between pro versus anti-apoptotic molecules.

Results

STAT5AΔ749 does not inhibit the IL-3 dependent proliferation and survival of Ba/F3 cells

We recently established Ba/F3 cell lines expressing a dominant negative form of STAT5 deleted of its last 45 amino acids (STAT5AΔ749). This mutant is tyrosine phosphorylated in response to IL-3, binds to DNA but is unable to transactivate a STAT5 regulated promoter and inhibits the transcriptional activity of endogenous STAT5 proteins (Moriggl *et al.*, 1996). Expression of this STAT5 truncated form increases apoptosis of Ba/F3 cells after IL-3 withdrawal (Dumon *et al.*, 1999). We examined the effect of STAT5AΔ749 on the IL-3 dependent Ba/F3 cell proliferation and viability. Proliferation was determined by measuring the total number of living cells or by 3H thymidine incorporation experiments (data not shown) and apoptosis was evaluated by annexinV/propidium iodide staining of Ba/F3neo and Ba/F3Δ749 cells (Figure 1a,b). Cells were stimulated with IL-3 for the indicated times and

total viable cell number was counted. Results showed that parental Ba/F3, Ba/F3neo or Ba/F3Δ749 (clone 31) cells exhibited similar growth curves (Figure 1a). Similar growth curves were obtained with the second cell clone 32. Apoptotic cells (AnnexinV positive) were next quantified in cell cultures in absence or presence of IL-3 by flow cytometry. Cells were first deprived of IL-3 for 16 h and IL-3 was added back for different lengths of time (Figure 1b). Just before the addition of IL-3, 35% of apoptotic cells were already observed in Ba/F3Δ749 cells and 5% in parental or Ba/F3neo cells. Addition of IL-3 induced a complete recovery of Ba/F3Δ749 cell viability after 48 h while in absence of IL-3, 20% of surviving cells were present. Thus, in presence of IL-3, STAT5AΔ749 did not inhibit the proliferation or affect the viability of Ba/F3 cells. Similar results were obtained with Ba/F3Δ749 cells from clone 32 (data not shown).

PI 3-kinase and STAT5 cooperate to regulate survival of Ba/F3 cells

In search of other pathways that could promote the survival of Ba/F3 cells, we studied the contribution of
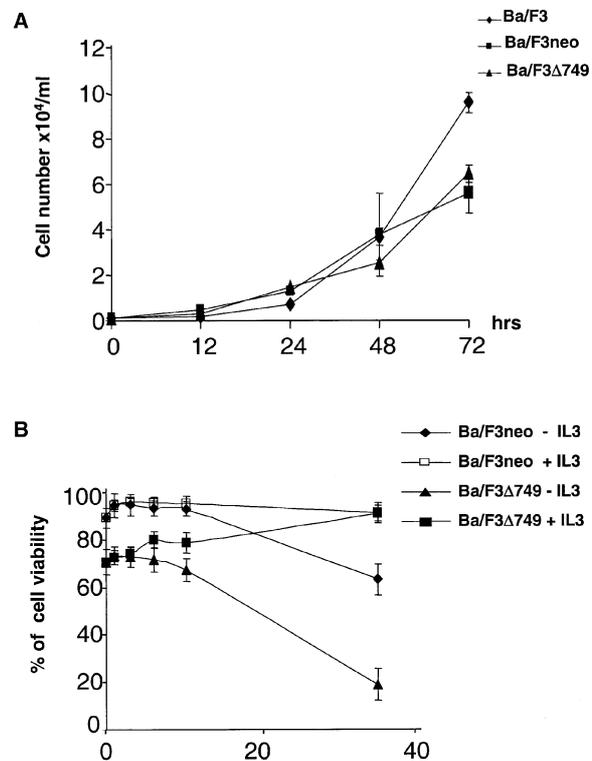

Figure 1 IL-3 dependent survival and proliferation of Ba/F3 cells expressing STAT5AΔ749. (**a**) Growth curve analyses were performed on parental Ba/F3, Ba/F3neo and Ba/F3Δ749 cells. Cells were cultured without IL-3 for 16 h and then stimulated with 10 ng/ml of recombinant IL-3 for the indicated times. The total number of viable cells was determined by trypan blue exclusion assays. Results are representative of three independent experiments. (**b**) Apoptosis was assessed by staining Ba/F3neo and Ba/F3Δ749 cells with AnnexinV-fluos and propidium iodide. The percentage of early apoptotic cells (AnnexinV positive; propidium iodide negative) as well as late apoptotic cells (AnnexinV positive; propidium iodide positive) were determined by flow cytometry analysis. Values were presented as the percentage of total viable cells (annexinV negative cells) present in the cultures and are the mean of three independent experiments (mean of individual percentages)

the PI 3-kinase/Akt and MEK/MAPK pathways in this process. We used LY294002 and PD98059, two specific inhibitors of PI 3-kinase and MEK respectively and analysed their effects on the viability of Ba/F3neo and Ba/F3Δ749 cells. Apoptosis of Ba/F3Δ749 and Ba/F3neo cells in presence of LY294002 was first evaluated by annexinV/propidium iodide staining in absence of IL-3. Cells were incubated with or without LY294002 (50 μm) and IL-3 was removed from the medium for 16 h (Figure 2a). LY294002 had a weak effect on the viability of Ba/F3neo cells since 80% of viable cells were observed in cultures treated with the inhibitor versus 90% in the untreated cell cultures.
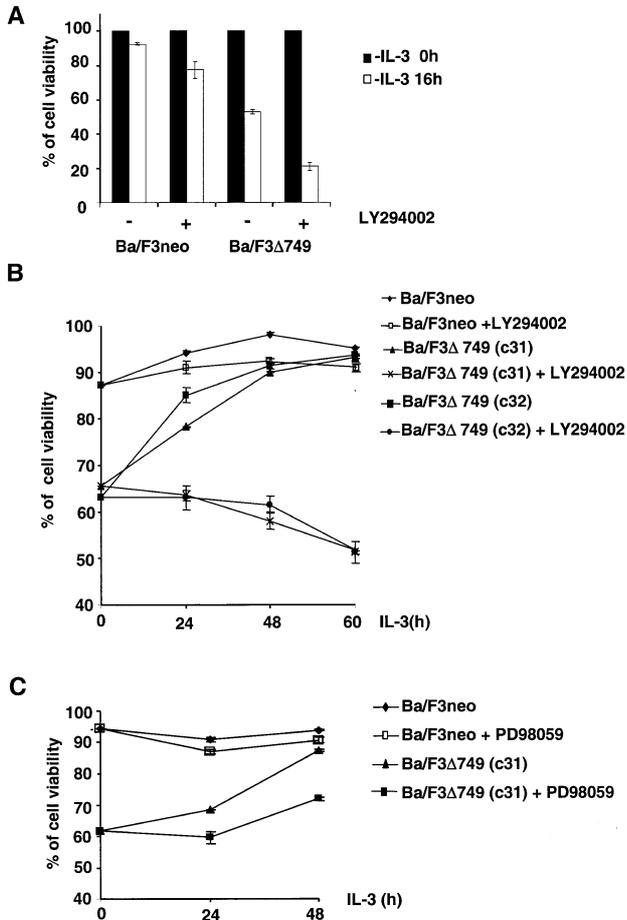

Figure 2 Effect of LY294002 and PD98059 on the viability of Ba/F3neo and Ba/F3Δ749 cells. (**a**) Ba/F3neo and Ba/F3Δ749 (clone 31) cells were cultured without IL-3 during 16 h (open bars) in absence or presence of LY294002 (50 μm). Equal number of viable cells were used at the time of starvation (solid bars). Cells were stained with annexinV-fluos and propidium iodide and apoptotic cells were determined by flow cytometry. Values are given as the percentages of viable cells (AnnexinV negative cells) present in the culture and are the mean of three independent experiments (mean of individual percentages). (**b**) Similar experiments were performed in presence of IL-3 (10 ng/ml). Ba/F3neo, and Ba/F3Δ749 cells (clones 31 and 32) were cultured without IL-3 for 16 h. Fifteen minutes before IL-3 stimulation, cells were incubated with LY294002 (50 μm) and then treated with IL-3 for the indicated times. 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 and are the mean of four different experiments (mean of individual percentages). (**c**) Similar experiments were performed with the MEK inhibitor, PD98059 (50 μm) excepted that cells were incubated 1 h with the inhibitor before IL-3 stimulation

Although 50% of apoptotic cells were already observed in Ba/F3Δ749 cell cultures in absence of the inhibitor, addition of LY294002 reduced about twofold the percentage of viable Ba/F3Δ749 cells. A similar experiment was done in presence of IL-3 (Figure 2b). Cells were deprived of IL-3 for 16 h and incubated with LY294002 for 30 min before the addition of IL-3. The percentage of apoptotic cells was next determined after different times of exposure to IL-3. The effect of LY294002 on the Ba/F3neo cell viability was almost negligible during the time course of IL-3 stimulation while a slow increase in the percentage of apoptotic cells was observed in two different clones of Ba/F3Δ749 cells (clones 31 and 32). Thus, inhibition of PI 3-kinase differentially affected the viability of Ba/F3 cells expressing or not the dominant negative form of STAT5. Similar experiments were performed with PD98059. Cells were deprived of IL-3 as above and incubated 1 h with PD98059 (50 μM) before IL-3 addition. Cells were stained with AnnexinV/propidium iodide and the percentage of apoptotic cells was determined in both cell cultures by FACS analysis (Figure 2c). PD98059 had no effect on the viability of Ba/F3neo and Ba/F3Δ749 cells. However, the recovery of Ba/F3Δ749 cell viability in presence of the inhibitor was slightly delayed. As a control experiment, we analysed the effect of both inhibitors on the phosphorylation of Akt and Erk2 (Figure 3a,b). IL-3 induced the phosphorylation of Erk2 and Akt in Ba/F3neo and Ba/F3Δ749 cells as judged by immunoblots with an anti-Erk2 antibody (Figure 3a, lanes 3, 7) and an anti-P-Akt antibody (Figure 3a, lane 2). These phosphorylations were efficiently inhibited by PD98059 (Figure 3a, lanes 4, 8) and LY294002 (Figure 3b, lane 4). Moreover, LY294002 inhibited the IL-3 induced Akt phosphorylation in a dose dependent manner. Thus at the concentrations used in our experiments, PD98059 and LY294002 were effective in the inhibition of MEK/MAPK and PI 3-kinase pathways. Altogether, these data suggested that the PI 3-kinase and STAT5 were required to promote BaF/3 cell survival while activation of the MEK/MAPK was not essential for this effect.

PI 3-kinase activation is required for Ba/F3 cell proliferation and cell cycle progression

Activation of PI 3-kinase has been shown to promote cytokine-dependent cell proliferation (Craddock *et al.*, 1999). We therefore examined the effect of LY294002 on Ba/F3neo and Ba/F3Δ749 cell proliferation. Total viable cell number was determined by trypan blue dye exclusion during the time course of IL-3 stimulation in Ba/F3neo and Ba/F3Δ749 cells in absence or presence of the inhibitor. We found that LY294002 inhibited cell proliferation in both cell cultures (data not shown). To examine the effect of LY294002 on the cell cycle progression, Ba/F3neo and Ba/F3Δ749 were left unstimulated or were stimulated with IL-3 for 24 h in absence or presence of LY294002 and stained with propidium iodide. Cell cycle was next analysed by using flow cytometry (Figure 4). In absence of LY294002, IL-3 induced the G1/S transition in Ba/F3neo and Ba/F3Δ749 cells. Addition of LY294002 to the cell culture completely blocked this progression.

Thus, PI 3-kinase is required for proliferation and cell cycle progression of Ba/F3neo and Ba/F3Δ749 cells in response to IL-3.
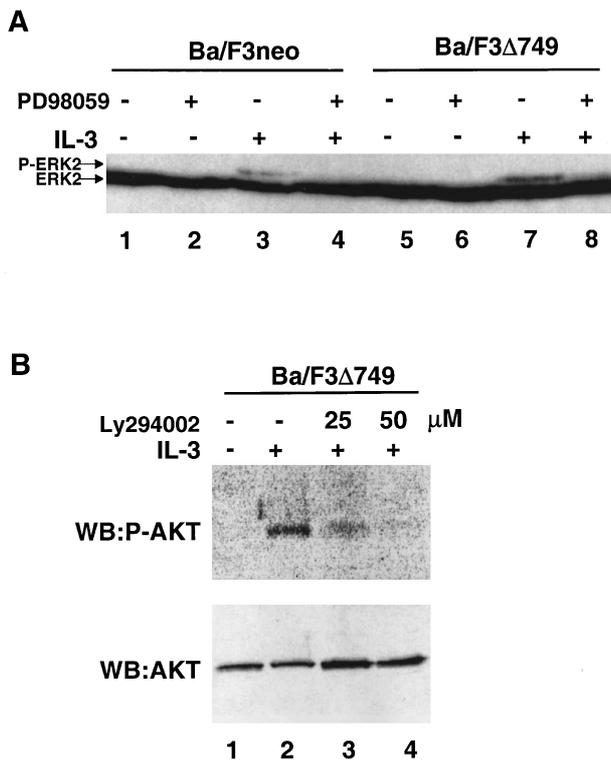

Figure 3 Effect of PD98059 and LY294002 on the phosphorylation of Akt and Erk2. (**a**) Cells were cultured without IL-3 for 16 h and left unstimulated (lanes 1, 2, 5, 6) or stimulated with 10 ng/ml IL-3 for 8 h (lanes 3, 4, 7, 8) in absence (lanes 1, 3, 5, 7) or presence of PD98059 (lanes 2, 4, 6, 8). Cell extracts were analysed by immunoblot for the detection of phosphorylated Erk2 expression. (**b**) Cells were cultured without IL-3 for 16 h and left unstimulated (lane 1) or stimulated with 10 ng/ml IL-3 for 2 h (lanes 2–4) in absence (lanes 1, 2) or presence of LY294002 at the indicated concentrations (lanes 3, 4). Cell extracts were prepared and analysed by Western blot with an anti-P-Akt antibody (upper panel) or with an anti-Akt antibody (lower panel)

Regulation of Bcl-xL expression by STAT5, PI 3-kinase and MEK

Cytokines suppress apoptosis by regulating expression or phosphorylation of Bcl-2 family members. STAT5 regulates the expression of the anti-apoptotic protein Bcl-xL (Dumon *et al.*, 1999; del Peso *et al.*, 1997). We analysed the levels of Bcl-xL and Bad expression by immunoblot in Ba/F3neo and Ba/F3Δ749 (clone 31) cells in absence or presence of LY294002 or PD98059 (Figure 5). We first examined the effect of LY294002 on the expression of Bcl-xL (Figure 5a). IL-3 induced expression of Bcl-xL in Ba/F3 neo cells and addition of LY294002 did not inhibit the basal or the IL-3-induced Bcl-xL protein levels (Figure 5a, lanes 1–4). In Ba/F3Δ749 cells, as expected, no induction of Bcl-xL protein expression by IL-3 was observed and the Bcl-x expression was not affected by LY294002 (Figure 5a, lanes 5–8). A similar experiment was done in presence of PD98059 (Figure 5d). Inhibition of MEK did not change the basal or the IL-3-induced Bcl-xL expression in both cell types. Similar results were obtained with Ba/F3Δ749 cells from clone 32 (data not shown). We analysed the effect of these two inhibitors on the expression of Bcl-2 but we failed to detect any change (data not shown). Thus PI 3-kinase and MEK are not involved in the IL-3 dependent regulation of Bcl-xL and Bcl-2 expression in Ba/F3neo and Ba/F3Δ749 cells.

Regulation of Bad and Akt phosphorylation by PI 3-kinase and MEK

The PI 3-kinase activates the serine/threonine kinase Akt which phosphorylates Bad and inhibits its pro-apoptotic function. We next analysed the expression and phosphorylation of Bad and Akt in Ba/F3neo and Ba/F3Δ749 (clone 31) cells in a time course of IL-3 stimulation (Figure 6). Bad expression and phosphorylation were measured by immunoblot (Figure 6a). After 30 min of stimulation, IL-3 induced in both cell lines the appearance of two forms of Bad protein that we referred to BadP1 and BaP2. These two
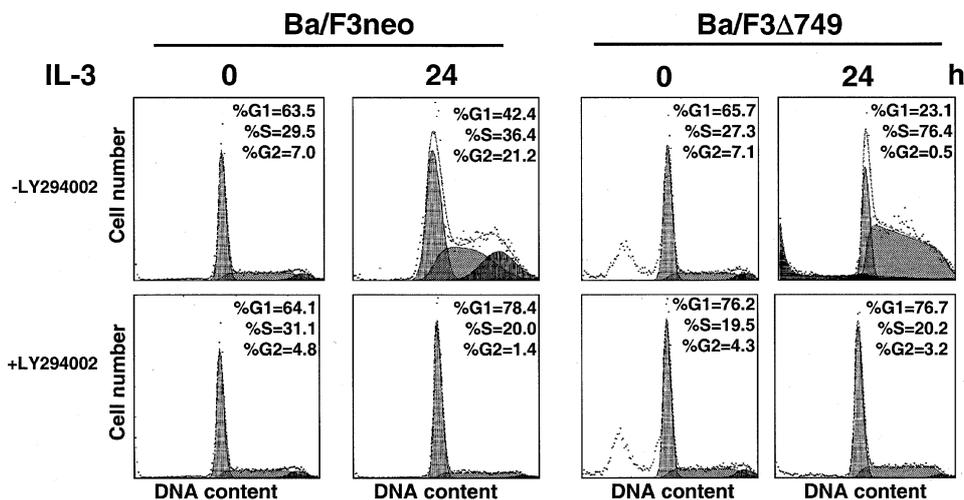

Figure 4 Effect of LY294002 on the cell cycle progression of Ba/F3neo and Ba/F3Δ749 cells. Cells were cultured in absence of IL-3 for 16 h and then stimulated with 10 ng/ml of IL-3 for the indicated time in absence (upper panel) or presence (lower panel) of LY294002 (50 μM). Cells were stained with propidium iodide and analysed by flow cytometry. Only viable cells having a DNA content ≥ 2 N were used and DNA histograms were obtained by using the multicycle software. Percentages of the cells present in the different phases of the cell cycle are indicated
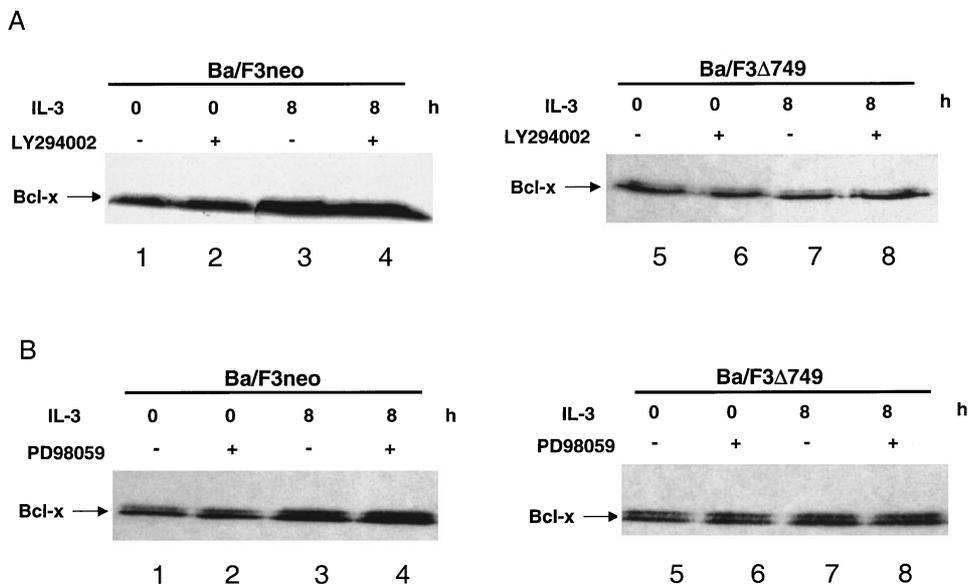
A

B

Figure 5 Effect of LY294002 and PD98059 on the regulation of Bcl-x protein expression in Ba/F3neo and Ba/F3Δ749 cells. Cells were cultured without IL-3 for 16 h and left unstimulated (lanes 1, 2, 5, 6) or stimulated with 10 ng/ml IL-3 (lanes 3, 4, 7, 8) in absence (lanes 1, 3, 5, 7) or presence (lanes 2, 4, 6, 8) of 50 μm LY294002 (**a**) or 50 μM PD98059 (**b**). Cellular extracts were prepared and the levels of Bcl-x protein expression were determined by immunoblot with a specific anti-Bcl-x antibody
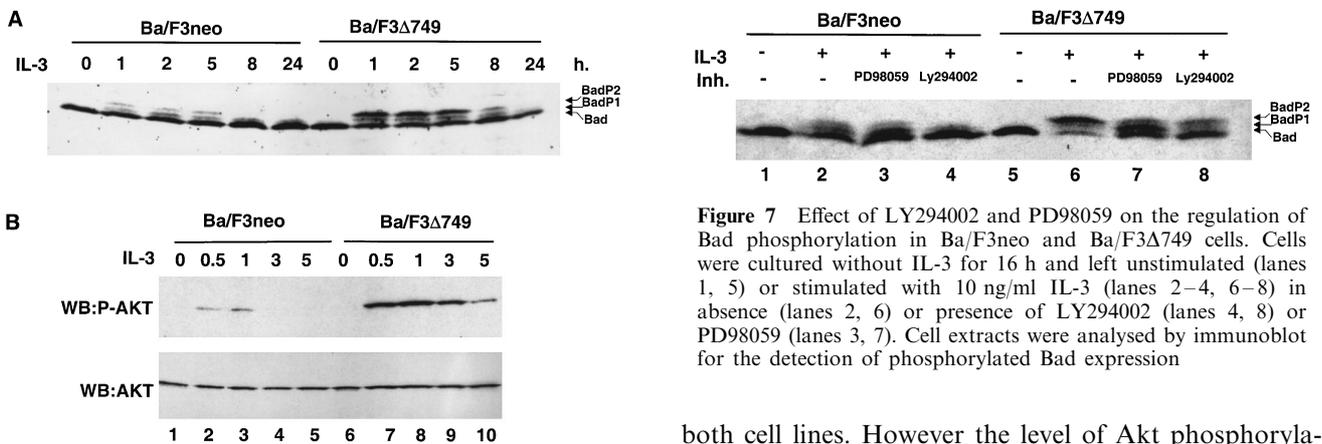
A

B

Figure 6 IL-3 dependent regulation of Bad expression and AKT phosphorylation in Ba/F3neo and Ba/F3Δ749 cells. (**a**) Ba/F3neo and Ba/F3Δ749 cells (clone 31) were treated with IL-3 for the indicated times. Cell extracts were analysed by Western blot with an anti-Bad antibody. Positions of unphosphorylated Bad, BadP1 and BadP2 are indicated. (**b**) Ba/F3neo and Ba/F3Δ749 cells (clone 31) were cultured without IL-3 for 16 h and were stimulated with 10 ng/ml IL-3 for the indicated times (in h). Cellular extracts were prepared and analysed by Western blot with an anti-P-Akt antibody (upper panel) or with an anti-Akt antibody (lower panel)

Figure 7 Effect of LY294002 and PD98059 on the regulation of Bad phosphorylation in Ba/F3neo and Ba/F3Δ749 cells. Cells were cultured without IL-3 for 16 h and left unstimulated (lanes 1, 5) or stimulated with 10 ng/ml IL-3 (lanes 2–4, 6–8) in absence (lanes 2, 6) or presence of LY294002 (lanes 4, 8) or PD98059 (lanes 3, 7). Cell extracts were analysed by immunoblot for the detection of phosphorylated Bad expression

proteins disappeared after treatment of cell extracts with different phosphatases indicating that they represented different phosphorylated forms of Bad (data not shown). BadP1 and BadP2 were observed up to 8 h after IL-3 addition and levels of BadP1 and BadP2 started to decrease after this period. Expression of BadP2 was stronger in Ba/F3Δ749 cells than in Ba/F3neo cells. In a similar experiment, the expression and phosphorylation of Akt were examined by immunoblot with anti P-Akt and anti-Akt antibodies (Figure 6b). In absence or presence of IL-3, the levels of Akt expression were similar in Ba/F3neo cells and Ba/F3Δ749 cells (Figure 6b, lower panel). After stimulation with IL-3, Akt was phosphorylated in both cell lines. However the level of Akt phosphorylation was stronger in Ba/F3Δ749 cells. Furthermore phosphorylated Akt disappeared 1 h after IL-3 stimulation in Ba/F3neo cells while phosphorylated Akt was still detectable 5 h after IL-3 stimulation in Ba/F3Δ749 cells (Figure 6b, upper panel). The effects of LY294002 and PD98059 on the levels of BadP1 and BadP2 were next examined (Figure 7). Cells were stimulated with IL-3 in absence (Figure 7, lanes 2, 6) or presence of LY294002 (Figure 7, lanes 4, 8) or PD98059 (Figure 7, lanes 3, 7). BadP2 protein expression was strongly reduced in presence of LY294002 in Ba/F3Δ749 cells and completely inhibited in Ba/F3neo cells. Consequently, level of unphosphorylated Bad was increased. In contrast, level of BadP1 protein was weakly reduced (Figure 7, lanes 2–4, 7 and 8). Thus activation of the PI 3-kinase induced the expression of BadP2 and to a lesser extent, the BadP1 form in Ba/F3neo and in Ba/F3Δ749 cells. In presence of PD98059, decrease of BadP2 in both cell types was associated with an increase in BadP1 and in the unphosphorylated form of Bad. Similar results were also obtained with the clone 32 (data not shown). These results indicate that PI 3-kinase and MEK differentially regulate the phosphorylation of Bad.

Interaction between STAT5 and the p85 subunit of PI 3-kinase

Cross talk between the PI 3-kinase and the JAK/STAT pathways has been reported. Interactions of JAK1, JAK2 or STAT3 with the p85 subunit of PI 3-kinase have been observed in different cell types (Migone *et al.*, 1998; Al-Shami *et al.*, 1999; Pfeffer *et al.*, 1997). We examined the possibility of an *in situ* association between the p85 subunit of PI 3-kinase and STAT5. Ba/F3neo and Ba/F3Δ749 (clone 31) cells were stimulated with IL-3 for different lengths of time. Cell extracts were prepared and immunoprecipitation of the p85 subunit with a specific antibody was performed. Immunoprecipitates were separated on SDS–PAGE and blotted with an anti-STAT5 specific antibody that recognizes STAT5A, STAT5B and the truncated form STAT5AΔ749 (Figure 8a). STAT5 was present in the p85 immunoprecipitates from Ba/F3neo and Ba/F3Δ749 cells only after stimulation with IL-3 (Figure 8a, upper panel). In Ba/F3Δ749 cells, STAT5AΔ749 was also detected. Immunoprecipitation of STAT5 from parental Ba/F3, Ba/F3neo and Ba/F3Δ749 cell extracts was then realized and the presence of p85 in the STAT5 immunoprecipitates was determined (Figure 8b). p85 was detected in STAT5 immunoprecipitates from untreated cells but the level of associated p85 increased after stimulation with IL-3 (Figure 8b, upper panel) and tyrosine phosphorylation of STAT5 (Figure 8b, middle panel). A lysate from Ba/F3 neo cells was also loaded on the gel as a control (lys). These results indicated that STAT5 interacts with the p85 subunit. This association was increased after IL-3 stimulation. We next determined if tyrosine phosphorylated STAT5 interacted with p85 through the SH2 domains of this protein (Figure 9). Cellular lysates from Ba/F3 cells stimulated or not with IL-3 were incubated with GST fusion proteins containing the SH2 domains of p85 (Figure 9, lanes 3–6) or with GST protein alone (Figure 9, lanes 1, 2). Immunoprobing these precipitates with an anti-STAT5 antibody showed that STAT5 was associated with the COOH-terminal SH2 domain (Figure 9, lane 5) and weakly with the NH2-terminal SH2 domain of p85 (Figure 9, lane 6) but not with the GST protein. This association was strictly dependent on the IL-3 stimulation of the cells. Thus tyrosine phosphorylated STAT5 interacts *in vitro* with the SH2 domains of the p85 subunit of the PI 3-kinase.

Discussion

Our results showed that (1) PI 3-kinase and STAT5 cooperate to promote IL-3 mediated suppression of apoptosis in Ba/F3 cells and that (2) STAT5 interacts with the p85 subunit of the PI-3 kinase after IL-3 stimulation through the SH2 domains of p85. To demonstrate this cooperation, we used Ba/F3 cells expressing a COOH terminal truncated form of STAT5 that did not inhibit the tyrosine phosphorylation of this factor but blocked its transactivation capacity. These cells are more sensitive to undergo apoptosis after IL-3 withdrawal and we recently reported that regulation of the anti-apoptotic protein Bcl-xL expression by IL-3 is STAT5 dependent
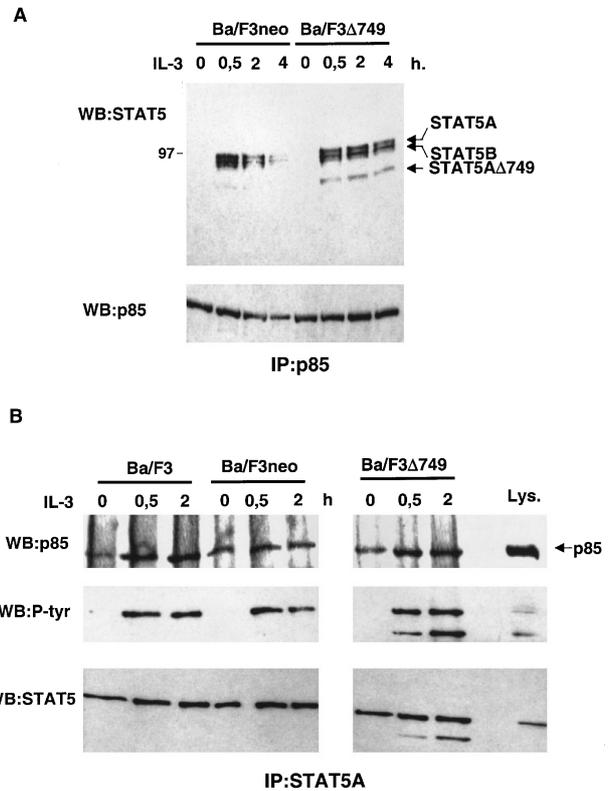

Figure 8 *In vivo* interaction between STAT5 and the p85 subunit of PI 3-kinase. Ba/F3neo and Ba/F3Δ749 cells were stimulated with IL-3 for the indicated times. Cells were harvested at each time point and cellular lysates were prepared as described in Materials and methods. (**a**) Immunoprecipitation was conducted with an anti-p85 antibody and immunoblotted with anti-STAT5 antibody (upper panel). Membrane was reprobed with an anti-p85 antibody (lower panel). Positions of STAT5A, STAT5B and STAT5AΔ749 cells are indicated. (**b**) The same experiments were performed on parental Ba/F3, Ba/F3neo and Ba/F3Δ749 cells but immunoprecipitation was conducted with an anti-STAT5A antibody and immunoblotting with anti-p85 antibodies and anti-ptyr antibodies respectively. Position of p85 is indicated. A control cellular lysate was also included in the experiment (Lys)
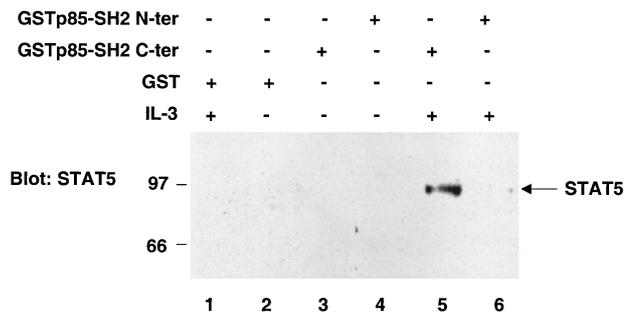

Figure 9 Tyrosine phosphorylated STAT5 interacts with the SH2 domains of p85 *in vitro*. Ba/F3 cells were untreated or treated with IL-3 for 30 min. Cellular lysates were prepared and incubated with GST alone (lanes 1, 2) or GST carrying the carboxyl-terminal (lanes 3, 5) or the amino-terminal (lanes 4, 6) SH2 domain of the p85 subunit. Membrane was immunoblotted with an anti-STAT5 antibody

(Dumon *et al.*, 1999). However, in presence of IL-3, expression of STAT5AΔ749 failed to inhibit cell proliferation and recovery of cell viability was observed after 48 h of cell culture. This is in contrast to previous report in Ba/F3 cells showing that expression of a truncated form of STAT5 inhibited

proliferation in response to IL-3 (Mui *et al.*, 1996). In these experiments, the mutant used exhibited a larger deletion of the COOH terminal region that removes the tyrosine residue 694 crucial for STAT5 activation. This mutant was no longer activated and is supposed to act by blocking endogenous STAT5 activation. Hematopoietic cell lines expressing cytokine receptor mutants unable to activate STAT5 displayed also a lower rate of proliferation and conversely, expression of a constitutively active form of STAT5 induces Ba/F3 cell proliferation independently of IL-3 (Onishi *et al.*, 1998b; Gobert *et al.*, 1996; Friedmann *et al.*, 1996). Altogether, these different data and ours would indicate that inhibition of STAT5 transcriptional activity may not be sufficient to affect Ba/F3 cell proliferation and suggest that additional events which are not directly related to STAT5 function as a transcription factor may be involved. The interaction of STAT5 with the adapter molecule Crkl, with the tyrosine kinase JAK2 or with the phosphatase SHP-1 has been reported (Fujitani *et al.*, 1997; Ozaki *et al.*, 1998; Ram and Waxman, 1997). In the present case, we provided evidence that STAT5 interacts with the p85 subunit of the PI 3-kinase *in vivo*. Distinct mechanisms of PI 3-kinase activation have been reported. PI 3-kinase is activated by recruitment of the p85 subunit to the phosphorylated receptor, by direct binding to the JAK tyrosine kinases or indirectly by binding to IRS-1/IRS-2 or GAB-1 (Migone *et al.*, 1998; Miura *et al.*, 1994; Verdier *et al.*, 1997; Lecoq-Lafon *et al.*, 1999). Previous report indicated also that STAT3 acts as an adapter molecule that couples the PI 3-kinase to the interferon receptor (Pfeffer *et al.*, 1997). This interaction requires STAT3 phosphorylation at Y656 present in a motif YXXM, a known consensus binding site for the SH2 domain of p85. We demonstrated that tyrosine phosphorylated STAT5 interacts with p85 through its SH2 domains. 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, interaction between STAT5 and p85 *in vivo* has been detected in human UT-7 cells stimulated with EPO (P Mayeux, unpublished data) or in human neutrophils stimulated with GM-CSF (Al-Shami and Naccache, 1999). Thus, interaction of STAT5 with p85 is not specific to Ba/F3 cells and is also observed in different cell types in response to distinct cytokines. Whether or not this interaction is direct or requires an intermediate protein remains to be elucidated.

The functional meaning of this interaction will be important to determine because we found that LY294002 inhibits the proliferation of Ba/F3 cells by preventing progression into the cell cycle. Similar results were recently obtained in Ba/F3 cells expressing a dominant negative form of p85 and the inducible activation of an active version of p110, the catalytic subunit of PI 3-kinase, has been shown to be sufficient to promote G1/S transition (Craddock *et al.*, 1999; Klippel *et al.*, 1998). The downstream effectors of the PI 3-kinase involved in the G1-S progression in Ba/F3 cells are not known. One candidate could be the cell cycle regulator E2F which has been shown to be a target of PI 3-kinase in T-cells (Brennan *et al.*, 1997). Expression of proteins involved in G1-S transition like cyclins D, cyclin dependent kinases and inhibitors of

cycline dependent kinases will require also careful analysis.

The use of LY294002 showed that the PI 3-kinase did not dramatically affect the survival of Ba/F3neo cells while it has a more pronounced effect in Ba/F3Δ749 in absence or presence of IL-3. These data would indicate that coordinated activation of STAT5 and PI 3-kinase are required to promote survival of Ba/F3 cells. This is in agreement with a recent report showing that constitutive STAT5 activity and PI 3-kinase/Akt pathway act in concert to protect the EPO independent HCD57-SREI cells from apoptosis (Bao *et al.*, 1999). Moreover, it was observed that inhibition of Akt activation alone by LY294002 was not sufficient to induce apoptosis in the EPO-dependent HCD57 cells (Bao *et al.*, 1999). Recently, it has been shown that LY294002 inhibits the survival of Ba/F3 cells when stimulated with IGF-1 but not with IL-3, indicating that IL-3 provides additional survival signals (Leverrier *et al.*, 1999). Similar results were obtained in Ba/F3 cells expressing a dominant negative form of p85 (Craddock *et al.*, 1999). Our data showing that inhibition of STAT5 transcriptional activity alone is not sufficient to induce apoptosis in presence of IL-3 support the hypothesis that activation of both STAT5 and PI 3-kinase by IL-3 is required to protect Ba/F3 cells from apoptosis.

We showed that PI 3-kinase is not involved in the regulation of Bcl-xL expression and that the IL-3-dependent Bad phosphorylation is not inhibited in Ba/F3 cells expressing STAT5AΔ749. In contrast, we observed a stronger expression of phosphorylated Bad in Ba/F3Δ749 cells as well as a higher level of phosphorylated Akt, suggesting that in these cells, the activation of the PI 3-kinase/Akt pathway by IL-3 is more pronounced than in control cells. The reason for this discrepancy remains to be determined. Previous reports indicated that Akt activation and Bad phosphorylation are required to prevent apoptosis (Songyang *et al.*, 1997; del Peso *et al.*, 1997; Datta *et al.*, 1997). However, our data showed that this seems not to be true in Ba/F3neo cells. Conflicting results on the role of the PI 3-kinase/Akt pathway and Bad phosphorylation in cell survival have emerged recently with the findings that Bad phosphorylation and Akt activation by PI 3-kinase do not automatically confer a survival signal in hematopoietic cells (Hinton and Welham, 1999). Thus, the importance of Bad phosphorylation may be dependent on the cell type and/or on the ratio of pro- and anti-apoptotic Bcl-2 members. Ba/F3neo and Ba/F3Δ749 cells have different levels of Bcl-xL protein and it has been reported that unphosphorylated Bad proteins heterodimerize with Bcl-xL thereby inhibiting its anti-apoptotic function (Kelekar *et al.*, 1997). Thus, the difference in the ratio of unphosphorylated Bad/Bcl-xL in Ba/F3neo and Ba/F3Δ749 cells might explain the differential effect of LY294002 on the survival of these cell lines. However, the inhibition of PI 3-kinase and STAT5 activities on the induction of apoptosis in Ba/F3 cells is more potent in absence than in presence of IL-3 indicating that the cooperative effect of STAT5 and PI 3-kinase on the survival is not probably the whole story. It is possible that the higher expression of the BadP2 form in Ba/F3Δ749 cells and the uncomplete inhibition of Bad phosphorylation after addition of LY294002 may

partially compensate the low level of Bcl-xL protein found in these cells. Alternatively, it is also possible that IL-3 induces an additional survival pathway in Ba/F3 cells. Recently, the IL-3 inducible Ras/NFIL3 pathway has been shown to partially protect Ba/F3 cells from apoptosis. The downstream survival effectors are still not known but are clearly distinct from Bcl-xL (Kuribara *et al.*, 1999).

Conflicting results regarding the role of MEK/MAPK in preventing apoptosis have emerged these last years (Kinoshita *et al.*, 1995, 1997; Miike *et al.*, 1999; Scheid and Duronio, 1998). Recently, it has been shown that the MEK inhibitor PD98059 did not prevent the IL-3- or GM-CSF-mediated survival of MC/9 cells but reduced Bad phosphorylation (Scheid and Duronio, 1998). Our results also support these conclusions. However, we showed that PD98059 actually decreased the expression of only one phosphorylated form of Bad and increased expression of the other one. This is consistent with the fact that Bad may be phosphorylated on different sites and by different protein kinases (Zha *et al.*, 1996; Scheid and Duronio, 1998; Gajewski and Thompson, 1996). The role of these different phosphorylated forms remains however to be elucidated.

In summary, IL-3 induces the coordinated activation of distinct signaling pathways that are involved in cell survival by regulating the proper balance between pro- versus anti-apoptotic molecules. We found that the PI 3-kinase/Akt pathway regulates Bad phosphorylation and STAT5 regulates Bcl-xL expression and that at least both pathways cooperate to promote cell survival. In addition, we found an interaction between the PI 3-kinase and STAT5. Inhibition of STAT5 activation will therefore help us to elucidate the functional meaning of this interaction in the IL-3-dependent proliferation and -cell cycle progression of Ba/F3 cells.

Materials and methods

Cell culture and reagents

IL-3-dependent Ba/F3 cells were grown in RPMI 1640 medium (Life Technologies) containing 4% Wehi-3B supernatant as a source of IL-3, 10% fetal calf serum (Biological Industries), L-glutamine 2 mM (Life Technologies) and penicillin-streptomycin (10 U/ml and 10 μg/ml respectively; Life Technologies) at 37°C with 5% CO_2. LY294002 and PD98059 were purchased from Sigma and New England Biolabs respectively.

Electroporation and cell cloning

The pRSV-STAT5AΔ749 or pRSV-neo plasmids were electroporated in Ba/F3 cells (250 V, 960 μF). Electroporated cells were expanded for 24 h before G418 selection (1 mg/ml). Pools of G418 resistant cells were cloned by limited dilution.

Apoptosis studies

Ba/F3neo and Ba/F3Δ749 cells were cultured in RPMI 10% FCS and 4% of Wehi-3B conditioned medium as a source of IL-3. 5×10^5 cells were harvested for 16 h in the absence of Wehi-3B supernatant. Stimulations were performed with 10 ng/ml recombinant murine IL-3 (Valbiotech). Cells were washed in the incubation buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 5 mM $CaCl_2$) and were resuspended in the incubation buffer with propidium iodide (0.5 μg/ml, Interchim), and Annexin-V-Fluos (Boehringer Mannheim) and incubated 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 on an EPICS Elite cytometer (Coulter Corporation, Florida, USA). Cell survival was also determined by counting viable cells using the trypan blue dye exclusion method.

Cell cycle analysis

Ba/F3 cells were treated as before and then washed in cold PBS 5 mM EDTA, resuspended in PBS, 5 mM EDTA with RNase A (0.2 mg/ml) and incubated at room temperature for 30 min. Propidium iodide solution (50 μg/ml, Interchim) was added and cell cycle was analysed by flow cytometry. DNA histograms were obtained by using the Multicycle Software.

Western blotting and immunoprecipitation studies

NP40 lysates were obtained by resuspending the cells in the following buffer: 1% NP40, 50 mM Tris pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 100 μM Na3VO4, 0.5 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin. They 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 P-Akt (Biolabs), Bad, ERK-2 (Santa Cruz), Bcl-x, STAT5 (Transduction laboratories), Akt, p85 or with a monoclonal p-Tyr antibody (4G10). Immunoprecipitation studies were performed with a rabbit polyclonal anti-STAT5A antibody raised against the COOH terminal region of STAT5A or a rabbit polyclonal anti-p85 antibody raised against the SH2 domains of p85. NP40 lysates from 5×10^6 cells were used to immunoprecipitate p85 or STAT5A. Lysates were centrifuged 10 min at 14 000 *g* and precleared with 50 μl of protein G-agarose beads. Supernatants were incubated with the specific antibodies 1 h at 4°C and incubated with protein G agarose beads for an additional hour at 4°C. Beads were washed once in a lysis buffer containing 500 mM NaCl and twice in a lysis buffer containing 150 mM NaCl. Beads were resuspended in SDS loading buffer before electrophoresis.

In vitro *association with p85*

NP40 lysates were used and incubated with GST fusion proteins carrying the COOH terminal or the NH2 terminal SH2 domains of p85. Preclearing was first carried out with agarose conjugated GST alone at 4°C during 1 h. Supernatants were then incubated with the different agarose conjugated SH2 domains of p85 for 1 h at 4°C. Agarose beads were extensively washed in lysis buffer (6 times). Samples were electrophoresed on SDS–PAGE and blotted onto cellulose membrane.

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