Interleukin-7 induces apoptosis of 697 pre-B cells expressing dominant-negative forms of STAT5: evidence for caspase-dependent and -independent mechanisms

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ORIGINAL PAPER

Interleukin-7 induces apoptosis of 697 pre-B cells expressing dominant-negative forms of STAT5: evidence for caspase-dependent and -independent mechanisms

Olivia Lanvin[1], Fabrice Gouilleux[1], Catherine Mullié[1], Cécile Mazière[2], Vincent Fuentes[1], Eliane Bissac[1], Françoise Dantin[1], Jean-Claude Mazière[2], Aline Régnier[1], Kaiss Lassoued[1] and Valérie Gouilleux-Gruart*[,1]

[1]*Laboratoire d'Immunologie, INSERM, EMI 0351, 3 rue des Louvels, 80036 Amiens, France;* [2]*Laboratoire de Biochimie, CHU Amiens, Place Victor Pauchet, 80054 Amiens, France*

The transcription factors STAT5A and STAT5B (STAT: signal transducer and activator of transcription) play a major role in the signaling events elicited by a number of growth factor and cytokine receptors. In this work, we aimed to investigate the role of STAT5 in human precursor B cell survival by introducing dominant-negative (DN) forms of STAT5A or STAT5B in the 697 pre-B cell line. All clones expressing DN forms of either transcription factor exhibited a higher spontaneous apoptotic rate that was massively enhanced upon interleukin-7 (IL-7) stimulation. This was associated with caspase 8 cleavage, mitochondrial transmembrane potential disruption and caspase 3 activation. However, the DN forms of STAT5 did not alter the expression of Bcl-2, Bax, Bcl-x, Bim, A1 and Mcl1 proteins in IL-7-stimulated cells. The pancaspase inhibitor Z-Val-Ala-Asp-fluoromylmethyl ketone partially suppressed IL-7-mediated mitochondrial transmembrane potential disruption and cell death, suggesting that IL-7 induced the death of DN STAT5 expressing 697 cells through caspase-dependent and -independent mechanisms that both require mitochondrial activation.
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Introduction

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway plays a major role in cytokine and growth factor signaling. Among the seven STAT family members, STAT5A and STAT5B, two highly related transcription factors, are significant regulators of cell proliferation and survival (Zamorano *et al*., 1998; Battle and Frank, 2002; Kisseleva *et al*., 2002). Both regulate expression of genes involved in cell cycle progression like cyclin D1, cyclin D2 and $p21^{Waf1}$ and cell survival like Bcl-2, Bcl-x and A1 (Feldman *et al*., 1997; Matsumura *et al*., 1997; Dumon *et al*., 1999; Matsumura *et al*., 1999; Moriggl *et al*., 1999; Lord *et al*., 2000). The *stat5a*/*stat5b* double knockout mice point to the important role of STAT5 in hematopoietic cell development (Teglund *et al*., 1998). These mice lack NK and mast cells, exhibit impaired response of myeloid and erythroid progenitors to granulocyte–macrophage colony-stimulating factor and erythropoietin, respectively, and altered T cell response to interleukin-2 (IL-2) (Moriggl *et al*., 1999; Socolovsky *et al*., 1999; Kieslinger *et al*., 2000; Shelburne *et al*., 2002). Finally, precursor B cell compartment is substantially reduced in these animals presumably due to an inability of progenitor B cells to proliferate in response to interleukin-7 (IL-7) (Sexl *et al*., 2000). However, differentiation toward mature B cells seems unaffected in *stat5a*/*stat5b* gene deleted mice.

IL-7 signals through the common cytokine gamma chain (γc) and also uses a second component, the IL-7 receptor (IL-7R) α chain. Mice in which the IL-7 or IL-7Rα chain or γc subunit encoding genes have been deleted exhibit a severe impairment in B (and T) cell development (Peschon *et al*., 1994; Cao *et al*., 1995; DiSanto *et al*., 1995; von Freeden-Jeffry *et al*., 1995; Fry and Mackall, 2002). IL-7 promotes the survival and proliferation of mouse precursor B cells, and also renders these cells more sensitive to certain apoptotic signals (LeBien, 2000). Although not essential for human B cell development, IL-7 exerts a number of effects on human precursor B cells, inducing proliferation of $CD19^+/CD34^+$ progenitors (Dittel and LeBien, 1995), enhancement of CD19 surface expression and downmodulation of *rag1* and *rag2* genes as well as terminal deoxynucleotidyl transferase transcripts (Wolf *et al*., 1993; Billips *et al*., 1995).

IL-7 signaling involves a number of nonreceptor tyrosine kinase pathways, some of which require the JAK/STAT proteins, mainly the STAT5A and STAT5B transcription factors, and to a lesser extent STAT1 and STAT3 (van der Plas *et al*., 1996; Qin *et al*., 2001; Fry

*Correspondence: V Gouilleux-Gruart;
E-mail: Valerie.Gouilleux@sa.u-picardie.fr
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and Mackall, 2002). STAT5 is also involved in the signaling pathway of the thymic stromal lymphopoietin that shares with IL-7 the IL-7Rα chain and many biological properties (Isaksen *et al*., 1999). The role of STAT5A/5B in B cell development may not be restricted to its implication in cytokine signaling. Recent data have shown their possible involvement in B cell receptor (BCR) transduction signal (Karras *et al*., 1996). Moreover, Bruton's tyrosine kinase, a tyrosine kinase that is activated upon BCR and pre-BCR ligation, was shown to interact with and to activate STAT5 *in vitro*. In keeping with these findings, XID mice exhibit impaired activation of STAT5 following BCR stimulation (Mahajan *et al*., 2001). STAT5 also functionally interacts with the phosphatidylinositol 3-kinase/Akt pathway, which is implicated in the signaling cascades of some major receptors expressed by immature and mature B cells (Santos *et al*., 2001). However, the role of STAT5 in human B lymphopoiesis remains unknown and the STAT5 target genes in the B cell lineage remain to be identified.

Besides their role in normal B cell development, there is a body of evidence suggesting that STAT5 activation is also incriminated in the malignant transformation of leukemic cells, including acute lymphoblastic leukemia cells of either pro-B or pre-B phenotype (Gouilleux-Gruart *et al*., 1996). To evaluate the role of STAT5 in the proliferation and survival of human leukemic B cell precursors, we introduced dominant-negative (DN) forms of STAT5 (STAT5AΔ749 or STAT5BΔ754) in the human 697 pre-B cell line. We show herein that the expression of these STAT5 truncated forms induces apoptosis of IL-7-stimulated 697 cells. The potential mechanisms of this effect are presented and discussed.

Results

Expression of STAT5AΔ749 in the 697 pre-B cell line

The DN form of STAT5A, for example STAT5AΔ749 was introduced by electroporation in the 697 pre-B cells. This mutant was deleted in its carboxyl-terminal transactivation domain and as a consequence still binds to DNA following activation but is unable to induce the transcription of target genes (Moriggl *et al*., 1996). As control, the 697 cell line was electroporated with the empty vector and selected with G418 (697neo cells). Cell screening was performed by Western blot using an antibody (Ab) raised against the SH2 domain of STAT5 recognizing the endogenous and the DN forms of STAT5 (Figure 1a). Four subclones 697Δ5A1, 697Δ5A2, 697Δ5A3 and 697Δ5A4 were obtained from two separate transfections. Like parental 697 and 697neo cells, all subclones expressed a 94–95 kDa band corresponding to the known molecular weight of endogenous STAT5. An additional 84 kDa band corresponding to STAT5AΔ749 was only detected in the 697Δ5A1, 697Δ5A2, 697Δ5A3 and 697Δ5A4 cell extracts. Whereas the four different clones expressed comparable levels of endogenous STAT5 protein, expression of the DN STAT5 was lower in the 697Δ5A1 clone.

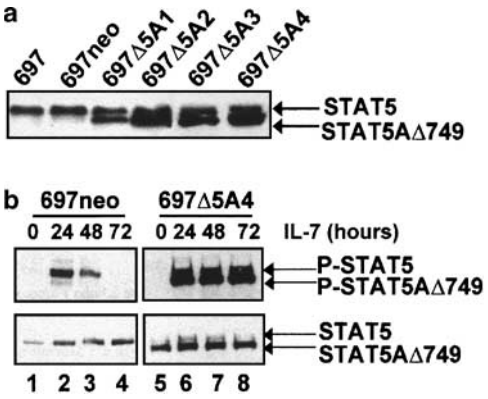

Figure 1 Expression and activation by IL-7 of STAT5AΔ749 in 697 cells. (**a**) Cell extracts were analysed by Western blot using an Ab directed to the SH2 domain of STAT5 and STAT5AΔ749. (**b**) Cells were incubated with IL-7 (10 ng/ml) for up to 72 h and analysed by Western blot for phosphorylated STAT5 (upper panel) and STAT5 protein expression (lower panel)

Both STAT5 and STAT5AΔ749 are phosphorylated following IL-7 activation

The activation of STAT5 and STAT5AΔ749 by IL-7 was next examined by immunoblot. Cell lysates from 697neo and 697Δ5A4 cells stimulated or not with IL-7 (10 ng/ml) for 24, 48 and 72 h were analysed by Western blot using an antiphosphotyrosine-STAT5 Ab. As illustrated in Figure 1b, endogenous STAT5 was phosphorylated upon IL-7 stimulation in control cells (lanes 2–4). This activation was clearly observed at 24 h, decreased at 48 h and was nearly undetectable after 72 h. Phosphorylation of both endogenous and truncated STAT5 in 697Δ5A4 cells was detected after IL-7 stimulation but remained stable for up to 72 h (Figure 1b, lanes 6–8). Similar results were obtained with 697Δ5A1, 697Δ5A2 and 697Δ5A3 clones (data not shown).

IL-7 induces apoptosis of STAT5AΔ749-transfected clones

Although 697 cells are independent of IL-7 for their growth and survival, we examined the biological consequences of DN STAT5 activation in IL-7-stimulated 697 cells. The effects of IL-7 on cell growth and survival were analysed by cell counting and using the trypan blue exclusion method. IL-7 did not induce any significant changes in the proliferation and survival of parental 697 cells and 697neo cells (Figure 2a). However, analysis of DN STAT5A expressing cells revealed an increased spontaneous cell death and induced a time-dependent increase in their death rate (Figure 2). Interestingly, the more the cells expressed STAT5AΔ749, the higher the IL-7-induced death rate (Figure 2b, lanes 2, 4 and 5). In keeping with this finding, loss of the DN of STAT5A in long-term culture of 697Δ5A2 cells was

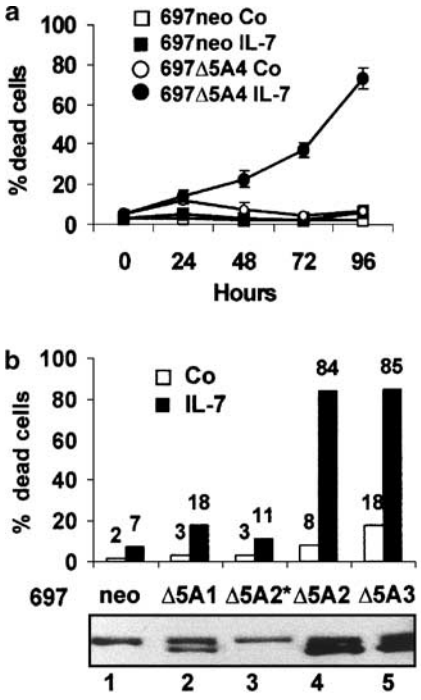

Figure 2 Expression of STAT5AΔ749 increases the mortality of IL-7-stimulated transfected cell clones. (**a**) Cells were incubated in the presence or absence of IL-7 (10 ng/ml) for up to 96 h. Cell death rate was evaluated by trypan blue dye exclusion. The means of three independent experiments are presented. (**b**) Simultaneous analysisofcellmortality(upperpanel)andSTAT5AandSTAT5AΔ749 expression (lower panel) in one representative experiment. Cell death was assessed after 96 h of stimulation with IL-7, using the trypan blue exclusion assay. Protein expression was analysed by Western blot using an anti-STAT5 Ab. After 6 months of culture, the 697ΔA2 clone lost its DN STAT5 and was then renamed 697ΔA2*

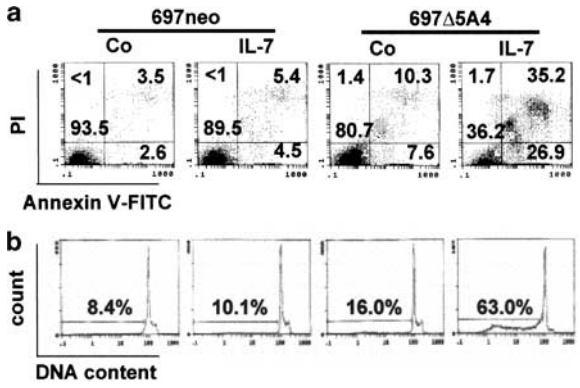

Figure 3 IL-7 induces apoptosis of STAT5AΔ749 expressing cells. (**a**) 697neo and 697Δ5A4 cells were cultured in the presence (48 h) or absence of IL-7. Apoptotic cells were estimated by annexin-V/PI staining and flow cytometry analysis. (**b**) Cells were stimulated for 72 h with IL-7, and then stained with PI. DNA content was evaluated by flow cytometry. One representative experiment is shown in this figure

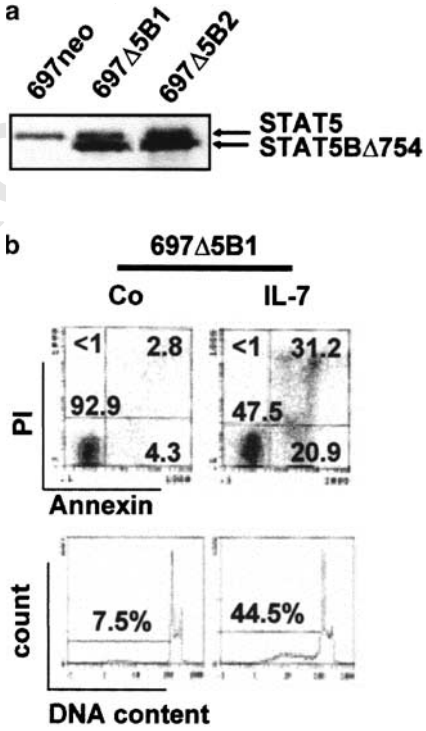

Figure 4 IL-7-induced apoptosis in STAT5BΔ754-transfected 697 cells. (**a**) 697neo and 697Δ5B1/B2 cell extracts were analysed by Western blot using an Ab raised against the SH2 domain of STAT5. (**b**) 697neo and 697Δ5B1 cells were incubated with or without IL-7 for 48 h, and apoptotic cells were estimated by annexin-V/PI staining and flow cytometry analysis. DNA content was examined by flow cytometry using cells stimulated (or not) for 48 h with IL-7, and then stained with PI. One representative experiment is presented

accompanied by a loss of sensitivity to IL-7-induced cell death (Figure 2b, lanes 3 and 4), suggesting that the DN form of STAT5 was directly involved in the IL-7-mediated cell death. Both annexin-V/propidium iodide (PI) labeling (Figure 3a) and DNA content analysis (Figure 3b) of 697Δ5A4 cells demonstrated that pre-B cell death was due to apoptosis. We then checked whether a DN of STAT5B called STAT5BΔ754, which was also deleted in the COOH terminal transactivation domain, might exert the same effects as the STAT5A mutant. Two stable clones (697Δ5B1 and 697Δ5B2) expressing the mutated form of STAT5B were obtained (Figure 4a). As for STAT5AΔ749 expressing cells, IL-7-induced cell death related to an apoptotic process (Figure 4b).

IL-7 triggers mitochondrial transmembrane potential ($\Delta\Psi_m$) disruption, cleavage of caspase 8 and activation of caspase 3 in DN STAT5 expressing cells

To determine whether or not IL-7-mediated apoptosis proceeds through mitochondrial perturbations, we measured the $\Delta\Psi_m$ by staining the 697neo and 697Δ5A4 cells with the sensitive dye $DiOC_6(3)$ before and after IL-7 treatment (Table 1). A decrease in $\Delta\Psi_m$ is considered as an early and irreversible step in the process of mitochondria-dependent apoptosis. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore causing a complete disruption of the $\Delta\Psi_m$, was

used as control in our experiments. As shown in Table 1, a fall in the $\Delta\Psi_m$ characterized by an increase in the percentage of $DiOC_6(3)^{low}$ cells started as early as the 24 h of IL-7 incubation in the 697Δ5A4 cells but not the control cells, suggesting that mitochondria were implied in IL-7-induced cell death. Similar results were obtained with the other transfected clones (data not shown).

We then addressed the issue of whether some members of the Bcl-2 family were implicated in the IL-7-induced apoptosis. Expressions of Bcl-2, Bcl-x, Bax, Bim, Mcl1 and A1 were unaffected by the DN form of STAT5A (not shown). Stimulation of cells with IL-7 for up to 72 h induced an enhancement of Mcl1, Bcl-x and Bax in both 697neo and 697Δ5A4 cells. In addition, there were no significant changes in Bcl-2, A1 and Bim expression in neither 697neo nor 697Δ5A4 cells (not shown).

To determine whether IL-7-mediated apoptosis proceeds through caspase activation, the different 697 subclones were incubated with IL-7 and the cleavage of caspase 8 was analysed by Western blot using a specific mAb. We found that 48 h of stimulation with IL-7-induced cleavage of caspase 8 featured by the appearance of the p18 subunit in 697Δ5A2 and 697Δ5A4 cell extracts (Figure 5a, lanes 4 and 6). No cleavage of caspase 8 was detected in 697neo cells stimulated or not with IL-7, nor in unstimulated 697Δ5A4 cells, whereas unstimulated 697Δ5A2 cells exhibited tiny amounts of p18. Finally, using a fluorimetric assay we showed that the caspase 3 enzymatic activity was sharply enhanced in a time-dependent manner in IL-7-stimulated 697Δ5A2 cells but not in 697 control cells (Figure 5b). Identical results were found with other 697Δ5A and 697Δ5B clones (data not shown).

IL-7 mediates apoptosis through caspase-dependent and -independent pathways

To evaluate the role of caspases in IL-7-induced apoptosis, we examined the effects of the broad-spectrum caspase inhibitor Z-Val-Ala-Asp-fluoromylmethyl ketone (Z-VAD.fmk) on cell viability using the trypan blue exclusion method. Z-VAD.fmk activity was assessed by analysing the Fas-mediated pathway, since 697 cells were previously shown to express Fas receptor and to undergo a caspase-mediated apoptosis upon Fas ligation (Levy *et al*., 1997).

The addition of anti-Fas Ab (1 μg/ml) for 96 h resulted in a higher death rate in DN STAT5-transfected cells (76.9 ± 3.7 and $41.4 \pm 3.2\%$ for 697Δ5A4 and 697Δ5B2, respectively) compared to 697neo cells ($32.1 \pm 3.9\%$, $P < 0.005$) (Figure 6a). This higher sensitivity to Fas-mediated cell death occurred in the absence of modification of CD95 surface expression as assessed by flow cytometry and without variation in procaspase 8 and cFLIP protein expression by Western blot (data not shown) in unstimulated as well as in IL-7-stimulated cells. The addition of Z-VAD.fmk completely suppressed Fas-mediated cell death (Figure 6a and b, lane 6) in 697neo cells as well as in DN STAT5 expressing cells and abolished caspase 3 processing in 697Δ5A4 cells (figure 6b, lane 6). In contrast, Z-VAD.fmk could only partially inhibit IL-7-mediated apoptosis in 697Δ5A4 or 697Δ5B2 cells (64 and 34% of inhibition, respectively). Moreover, IL-7-induced caspase 3 cleavage was abrogated by Z-VAD.fmk as demonstrated by Western blot analysis (Figure 6b) and by fluorimetric assay (not shown) in spite of persisting cell death (42% with IL-7 versus 21% with IL-7 + Z-VAD.fmk at 72 h using the 697Δ5A4 clone). These data suggest that IL-7-induced cell death in DN STAT5 expressing cells involved caspase-dependent and -independent mechanisms.

Finally, we examined whether mitochondria were implicated in these mechanisms, by measuring the $\Delta\Psi_m$

Table 1 $\Delta\Psi_m$ disruption by IL-7[a]

		% $DiOC_6$ (3)^{Low}			
Hours		*24*	*48*	*72*	*96*
697neo	Control	16.2	14.9	15	16.3
	IL-7	12.3	14.2	26.1	15.9
697Δ5A4	Control	20.6	19.1	36	25.7
	IL-7	32	32.7	65.1	74.5
697Δ5A4[b]	CCCP	73.4	60.8	93.2	61.6

[a]Cells were incubated with IL-7 for indicated times and stained with the $\Delta\Psi_m$ sensitive dye $DiOC_6(3)$ and PI. [b]The positive control CCCP was added 1 h before staining with $DiOC_6(3)$ and PI at 24, 48, 72 and 96 h

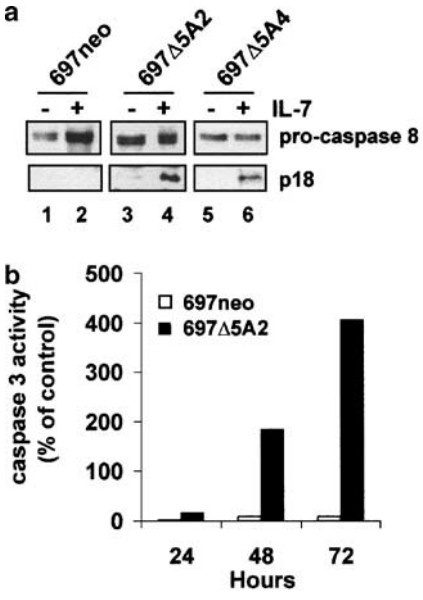

Figure 5 Effects of IL-7 on caspases 8 and 3 activation. 697neo and 697Δ5A2/A4 cells were incubated in the presence or not of IL-7, solubilized in Laemmli's buffer and analysed by Western blot using an anticaspase 8 Ab (**a**). IL-7-induced caspase 3 activation was measured by fluorimetric assay. Results were calculated as follows: [(IL-7-stimulated cells)−(control cells)/(control cells) × 100] (**b**)

in IL-7-stimulated 697neo, 697Δ5A4 and 697Δ5B1 cells, treated or not with Z-VAD.fmk. For this purpose, cells were stained with the sensitive dye $DiOC_6(3)$ and analysed by flow cytometry (Table 2). Z-VAD.fmk could only partially restore the fall in the $\Delta\Psi_m$ observed in IL-7-stimulated 697Δ5A4 and 697Δ5B1 cells, whereas it totally abrogated $\Delta\Psi_m$ disruption in Fas-stimulated cells either in control or in DN STAT5 expressing cells. Altogether, these results suggest the participation of mitochondria in both caspase-dependent and -independent pathway-mediated apoptosis.

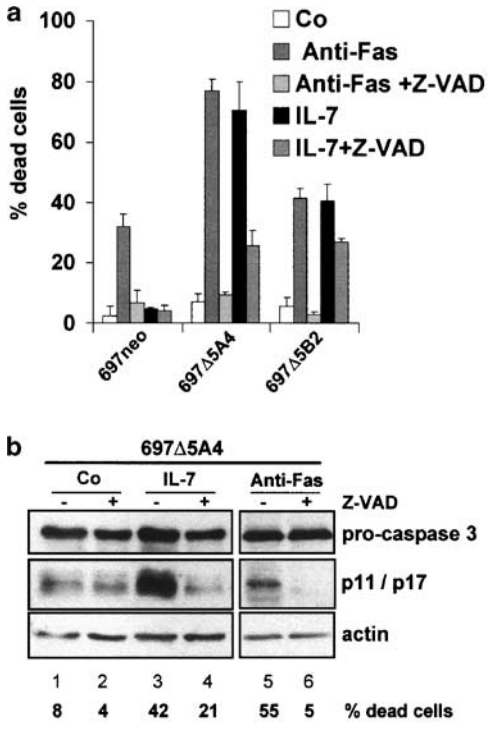

Figure 6 Z-VAD.fmk partially inhibits IL-7-mediated apoptosis. (**a**) 697neo and 697ΔA4/B2 cells were cultured with IL-7 during 96 h in the presence or absence of 50 μM Z-VAD.fmk caspase inhibitor. To assess Z-VAD.fmk activity, anti-Fas Ab (1 μg/ml) was added in some wells in the same conditions of culture as for IL-7. Cell viability was evaluated by trypan blue exclusion assays. The results are expressed as means of three separate experiments. (**b**) Cells were incubated with medium alone, Z-VAD.fmk, IL-7, IL-7 + Z-VAD.fmk, anti-Fas Ab and anti-Fas Ab + Z-VAD.fmk for 72 h. Expression of caspase 3 and of its 11/17 kDa cleavage product was evaluated by Western blot. Actin expression was used as loading control. The percentage of dead cells at 72 h is indicated below each condition

Table 2 $\Delta\Psi_m$ disruption[a]

	% DiOC$_6$ (3)^{Low}		
	697neo	*697Δ5A4*	*697Δ5B1*
Control	8.6	22.5	11.1
Anti-Fas	45.7	74.6	43.7
Anti-Fas + Z-VAD	12.9	14	17.5
IL-7	12.1	70.1	44.1
IL-7 + Z-VAD	10.6	49.3	28

[a]697 pre-B cells were cultured with IL-7 during 96 h in the presence or absence of Z-VAD.fmk (50 μM). Anti-Fas Ab (1 μg/ml) was used as a positive control in the same conditions. Then cells were stained with the $\Delta\Psi_m$ sensitive dye $DiOC_6(3)$ and PI

Discussion

To investigate the role of STAT5A and STAT5B in the survival of leukemic pre-B cell, a DN form of each transcription factor (STAT5AΔ749 and STAT5BΔ754) was introduced in the human 697 pre-B cell line. In this model, IL-7-induced phosphorylation of both truncated and wild-type forms of STAT5A or 5B and promoted apoptosis of cells expressing DN forms of these transcription factors.

The activation of the various forms of STAT5 upon IL-7 incubation was first examined. The kinetics of STAT5 phosphorylation were different between control and transfected cells. In particular, phosphorylation of STAT5AΔ749 and also of endogenous STAT5 was found to be more robust during the time course of IL-7 stimulation in transfected cells. This is in keeping with previous works showing that deletion of the transactivation domain of STAT5 increases the stability of the phosphorylated protein (Moriggl *et al*., 1996; Wang *et al*., 2000). Our data indicate that expression of the DN STAT5 in 697 cells also affects the stability of phosphorylated endogenous STAT5.

The expression of DN forms of STAT5A or STAT5B has previously been shown to affect the survival of various cell types (Mui *et al*., 1996; Dumon *et al*., 1999; Lord *et al*., 2000; Ahonen *et al*., 2003). In addition, deletion of *stat5* genes in mouse can also disturb the survival and maturation of myeloid and erythroid progenitors cultured in the presence of granulocyte–macrophage colony-stimulating-factor and erythropoietin (Socolovsky *et al*., 1999; Kieslinger *et al*., 2000). *Stat5a/b*$^{-/-}$ precursor B cells were also found to have a reduced capacity to proliferate in response to IL-7 (Sexl *et al*., 2000); however, one cannot rule out the possibility that they were also insensitive to the survival signal provided by this cytokine. Indeed, IL-7 was shown to promote both proliferation and survival of normal precursor B cells, mainly those expressing the pre-BCR (LeBien, 2000; Fry and Mackall, 2002) and our present data clearly suggest that STAT5A/5B may be required for the survival signal mediated by IL-7. The mechanisms by which STAT5 promotes cell survival usually involve the antiapoptotic Bcl-2, Bcl-x and A1 proteins (Feldman *et al*., 1997; Dumon *et al*., 1999; Lord *et al*., 2000). However, our results indicate that Bcl-x, Bcl-2 and A1 were not implicated in the IL-7-induced death of 697 cells expressing the DN forms of STAT5. The expression of Mcl1, another antiapoptotic molecule whose gene can be regulated by the JAK/STAT pathway (Puthier *et al*., 1999), was also found to be unchanged in

IL-7-stimulated DN STAT5 expressing 697 cells. Close examination of the expression levels of the proapoptotic protein Bax and Bim did not reveal differences between control and DN STAT5 expressing cells, following IL-7 stimulation. However, and importantly, IL-7-induced apoptosis of DN STAT5 expressing cells was clearly found to involve a mitochondrion-dependent mechanism as demonstrated by disruption of the $\Delta\Psi_m$ following IL-7 stimulation. Taken together, our data indicate that IL-7-induced apoptosis of DN STAT5 expressing cells involves mitochondrial changes but not the major members of the Bcl-2 family.

Caspases, a family of cysteine proteases, constitute potent enzymatic machinery that cleaves crucial proteins of the nucleus and the cytoplasm and may lead to apoptosis (Cohen, 1997). Caspase and Bid cleavage (not shown) was significantly enhanced by IL-7, indicating the implication of a caspase-dependent pathway in IL-7-induced apoptosis of DN expressing cells. This is reminiscent of the recent report of caspases 3 and 9 activation in mast cells derived from the bone marrow of *Stat5*-deficient mice, exposed to IL-3 and SCF (Shelburne *et al*., 2002). These results and ours indicate that loss of STAT5 function triggers caspase activation via so far unidentified mechanisms. In most models of apoptosis, the broad-spectrum caspase inhibitor Z-VAD.fmk blocks cell death by irreversibly inhibiting the activation or processing of caspases (Komoriya *et al*., 2000). We found herein that in 697 cells expressing DN STAT5, Z-VAD.fmk only partially blocked the IL-7-mediated apoptosis, suggesting that both caspase-dependent and -independent mechanisms take place in this process. An increasing number of experimental systems support the notion that programmed cell death can also proceed in a caspase-independent manner, with stereotyped features (Susin *et al*., 2000; Bidere and Senik, 2001). For example, anti-CD2 mAb or staurosporine induced caspase-independent cell death in activated human peripheral T lymphocytes. These apoptosis inducers operate in the presence of broad-spectrum caspase inhibitor (Deas *et al*., 1998). The mitochondrial apoptosis-inducing factor (AIF) family proteins might be involved in this process (Susin *et al*., 1999). Further experiments will be performed to evaluate the participation of such proteins in the apoptosis mediated by DN STAT5 in 697 pre-B cells. Interestingly, DN STAT5 expressing 697 cells were found to be more sensitive to Fas-mediated cell death than controls, suggesting that STAT5 not only promotes survival of leukemic pre-B cells but also negatively regulates certain apoptotic pathways. The mechanisms involved in this enhanced sensitivity to Fas-induced apoptosis are now under investigation.

In summary, we found that 697 pre-B cells expressing DN STAT5 undergo apoptosis upon IL-7 stimulation following a caspase-dependent and -independent pathway. Our findings provide a new insight into the role of STAT5 in the survival process of 697 leukemic pre-B cells and outline the major place taken by STAT5 in the IL-7-mediated pre-B cell survival.

Materials and methods

Cell culture, electroporation and cloning

The IL-7-independent 697 human pre-B cell line (Findley *et al*., 1982) was maintained in RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum at 37°C, 5% CO_2.

The pRSV-STAT5AΔ749, pRSV-STAT5BΔ754 or pRSV-neo plasmids (Moriggl *et al*., 1996) were electroporated at 300 V, 975 μF in the 697 pre-B cell line (20 μg/4 $\times$ 10^6 cells). Electroporated cells were expanded for 24 h before G418 selection (1 mg/ml). Pools of G418 resistant cells expressing STAT5AΔ749 or STAT5BΔ754 by Western blot were cloned by limited dilution.

Antibodies and reagents

The following Abs were used: anti-CD95 (CH11), anticaspase 8 (Coulter/Immunotech, Marseille, France), anticaspase 3 (Becton Dickinson, Pharmingen), anti-cFLIP (calbiochem, CA, USA), anti-SH2 domain of STAT5 (Transduction Laboratories, Lexington, Ky), anti-Bcl-2, anti-Bcl-x, anti-Mcl1, anti-Bax, anti-A1 and antiactin (Santa Cruz Biotechnology, CA, USA), anti-Bim (Affinity BioReagents, CO, USA), antiphosphotyrosine-STAT5 (Biolabs-Cell Signaling, Beverly-New England, USA), antiperoxydase-conjugated Abs specific for rabbit or mouse IgG (Amersham Pharmacia biotech, England) or for goat IgG (Santa Cruz Biotechnology, CA, USA). IL-7 was purchased from Peprotech.

Analysis of cell death

The cell viability was analysed by the trypan blue exclusion assay. Briefly, 2.5 $\times$ 10^4 cells/well were incubated as triplicates in flat bottom plates in culture medium supplemented or not with IL-7 (10 ng/ml) for 24, 48, 72 or 96 h, and the percentage of dead cells was evaluated. In some experiments, the broad-spectrum caspase inhibitor Z-VAD.fmk (50 μM) (Bachem) was added alone or in combination with IL-7. Z-VAD.fmk activity was assessed by its ability to inhibit CD95-induced 697 cell death. CD95 Ab was used in the conditions described above with IL-7.

For measurement of cellular DNA content, 10^6 cells were washed in cold phosphate-buffered saline (PBS)–EDTA 5 mM, and permeabilized with 500 μl 70% ethanol PBS at -20°C for 10 min. After two washes in cold PBS, cells were resuspended in PBS–EDTA 5 mM supplemented with RNase A (1.3 mg/ml, Sigma) and PI (33 μg/ml, Sigma) for 30 min at room temperature. Samples were analysed by flow cytometry (EPICS Elite Cytometer, Beckman, Coulter). Annexin-V-FITC staining was performed according to the manufacturer's instructions (Boehringer, Mannheim, Germany). The percentage of apoptotic cells was analysed by flow cytometry.

The $\Delta\Psi_m$ disruption was evaluated using the cationic lipophilic dye $DiOC_6(3)$. For this purpose, cells were washed in PBS–EDTA 5 mM, incubated for 20 min with 40 nM $DiOC_6(3)$ (Calbiochem) and 5 μg/ml of PI at room temperature and then immediately analysed by flow cytometry. As a positive control, cells were treated in parallel samples with the protonophore uncoupling agent CCCP 250 μM (Calbiochem) 1 h before staining with $DiOC_6(3)$ and PI.

Caspase 3 assay

The enzymatic activity was measured with a fluorogenic substrate as previously described (Cuvillier *et al*., 1998). The

results were calculated as follows: [(IL-7-stimulated cells)−(control cells)/(control cells) × 100].

Western blot analysis

Cells were lysed in Laemmli's buffer (50 μl/10^6 cells) and boiled for 10 min. Proteins from whole lysates (25 μl) were resolved by SDS–polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane (BioRad) and probed with various Abs. The blots were developed using an enhanced chemiluminescence detection system (ECL kit, Amersham) and appropriate specific peroxydase-conjugated Abs.

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