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IL-2 deprivation triggers apoptosis which is mediated by c-Jun N-terminal kinase 1 activation and prevented by Bcl-2

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Abstract

A variety of environmental stresses, as well as inflammatory cytokines, induce activation of c-Jun N-terminal kinases. We describe here that IL-2 deprivation-induced apoptosis in TS1$\alpha\beta$ cells does not modify c-Jun protein levels and correlates Bcl-2 downregulation and an increase in JNK1, but not JNK2, activity directly related to the induction of apoptosis. Indeed, downregulation of JNK1 expression using antisense oligonucleotides inhibits apoptosis induced by IL-2 withdrawal. Overexpression of Bcl-2 promotes cell survival and blocks JNK1 activation as well as apoptosis caused by IL-2 deprivation. This suggests that inhibition of the JNK1 signaling pathway may be a mechanism through which Bcl-2 promotes cell survival and prevents apoptosis triggered by growth factor withdrawal.

Keywords: Bcl-2; IL-2 receptor; JNK; apoptosis

Abbreviations: IL-2, interleukin-2; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; SAP, stress activated protein kinase; TNF, tumor necrosis factor; NGF, nerve growth factor; ICE, interleukin-1β converting enzyme

Introduction

Interleukin-2 (IL-2) is a regulator of cell cycle progression in T lymphocytes that binds to a specific cell surface receptor composed of three distinct subunits, α, β, and γ or p55, p70 and p64, respectively.[1–3] IL-2-dependent cell survival is triggered by the suppression of an active cell death program induced by growth factor deprivation.[4–7]

Extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinase/stress activated protein kinases (JNK/SAPK) are both critical in transducing extracellular signals into intracellular responses. These responses include activation of kinase cascades, leading to expression and activation of many transcription factors including c-Fos and c-Jun, and expression of genes such as c-*fos* and c-*jun* that play a role in regulating cell growth and differentiation.[8–11]

JNK kinases are activated by stimuli such as UV-light,[12] γ-irradiation,[13,14] protein synthesis inhibitors,[15] ceramide,[16] DNA-damaging drugs,[17,18] TNF and IL-1.[19,20] JNK activity is also induced by mitogenic signals, including growth factors,[21,22] oncogenic Ras,[12] CD-40 ligation[23,24] and T cell activation signaling.[14,25] JNKs phosphorylate transcription factors such as c-Jun, ATF-2 and Elk-1 and strongly augment their transcription activity.[11,26,27]

Among JNK kinases are included the 46 kDa JNK1 and the 55 kDa JNK2 isoforms.[15,21] JNK activation requires phosphorylation at Thr and Tyr residues[12] by a dual-specific kinase, SEK1 or MKK4, which is also activated following phosphorylation by other protein kinases such as MEKK1 and MELK. Although the precise regulatory mechanism of this kinase cascade is still obscure, involvement of Ras, Cdc42 and Rac GTP-binding proteins in JNK pathway regulation has recently been demonstrated.[22,28,29]

One potential function of JNK may be the initiation of programmed cell death, although activation of the JNK pathway does not necessarily lead to apoptosis; IL-3, erythropoietin and thrombopoietin activate the Ras/MEKK1/SEK1/JNK pathway without induction of apoptosis.

Apoptosis is involved not only in normal physiological processes, but also in pathogenesis resulting from an imbalance between positive and negative regulators of cell survival.[30,31] Numerous studies have demonstrated that Bcl-2 is a positive regulator of cell survival.[32] This 26 kDa intracellular protein protects various cell types from cell death induced by growth factor deprivation, heat-shock and viral agents.[33–36]

In this paper, we describe the activation of JNK1 following IL-2 deprivation of TS1$\alpha\beta$ cells. JNK1 activation induced by lymphokine withdrawal correlates with apoptosis induction, since downregulation of JNK1 expression inhibits apoptosis. In addition, JNK1 activation can be prevented by Bcl-2 expression in IL-2-deprived cells. The relevance of these findings in the context of apoptosis is discussed.

Results

Apoptosis in IL-2-deprived TS1$\alpha\beta$ cells correlates with Bcl-2 downregulation and JNK expression

TS1$\alpha\beta$ cells proliferate independently in IL-2, IL-4 or IL-9. When no lymphokine is added to the medium, cells undergo apoptosis (Figure 1A); as soon as 2 h after IL-2 withdrawal, 6% of cells are apoptotic, reaching approximately 35% at 24 h, whereas control cells maintained in the presence of IL-2 show no apoptosis. It was of interest to determine whether

these events could modify expression of Bcl-2 and the JNK protein kinases, molecules known to be related to apoptosis.

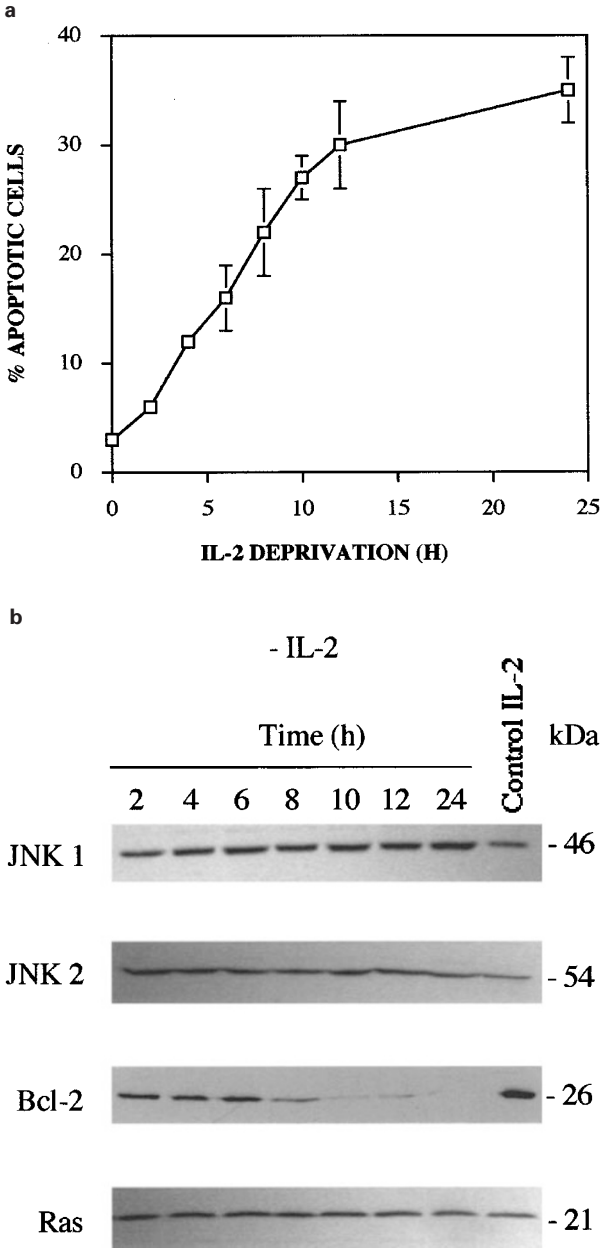

Figure 1 Time course of apoptosis and JNK1 and Bcl-2 expression in IL-2 deprived TS1$\alpha\beta$ cells (**A**) Cells (2×10^5) were cultured in the presence or absence of IL-2 (5 ng/ml) for 24 h and cell cycle was analyzed at different times for apoptosis using propidium iodide. Cells were permeabilized, stained with propidium iodide, and analyzed for DNA staining using fluorescence flow cytometry. The zero time point corresponds to control cells cultured in IL-2. Error bars represent $\pm$S.D. from three independent experiments. (**B**) Control or IL-2-deprived cells (2×10^6 cells) were harvested at different time points and lysed as described in Materials and Methods. Protein extracts were separated in SDS–PAGE, blotted to nitrocellulose and probed sequentially with anti-JNK1, -JNK2, -Bcl-2 and -Ras antibody, the last as an internal protein loading control. Protein bands were detected using the ECL system. Similar results were obtained in three independent experiments. Molecular weight markers are indicated

Figure 1B shows the protein level of JNK1, JNK2 and Bcl-2 after IL-2 starvation of TS1$\alpha\beta$ cells. Control IL-2-cultured cells show JNK1 expression, which increases slightly after IL-2 deprivation. JNK2 expression shows no difference throughout the starvation period analyzed. Downregulation of Bcl-2 expression is time dependent (Figure 1B); 8 h after IL-2 deprivation, inhibition of Bcl-2 expression is observed and its expression disappears completely 24 h after IL-2 deprivation. Unaltered Ras expression is shown for all conditions as an internal protein loading control. It is important to note that gradual increase in apoptosis correlates with downregulation of Bcl-2 expression. These results suggest that, in TS1$\alpha\beta$ cells, IL-2 deprivation modifies Bcl-2 expression, whereas JNK2 and JNK1 expression are unaffected or only slightly increased, respectively.

IL-2 deprivation does not block c-*jun* expression

In the majority of cell types, c-*jun* is expressed at low levels and is induced rapidly and transiently by extracellular signals. Induction of c-*jun*, the substrate of JNKs, appears to be mediated via Ras activation and plays an important role in controlling cell growth and apoptosis. We next examined whether IL-2 deprivation results in modification of the c-*jun* expression level.

Figure 2A shows Western blot analysis of c-*jun* expression in TS1$\alpha\beta$ cells throughout the IL-2 deprivation time course. Control cells cultured in IL-2 express c-*jun* and, similarly, c-*jun* levels were not altered during the starvation period analyzed. This suggests that IL-2 withdrawal-induced apoptosis proceeds along pathways that do not involve changes in c-*jun* expression. The blot was stripped and reprobed for detection of Ras as an internal control of protein loading.

JNK1, but not JNK2, is activated in IL-2 deprivation-induced apoptosis

To examine the possible involvement of JNK in IL-2 deprivation-induced apoptosis, we measured JNK1 and JNK2 activity in IL-2-stimulated and in IL-2-deprived TS1$\alpha\beta$ cells. The JNKs were immunoprecipitated with anti-JNK1- or anti-JNK2-specific antibodies at various time points after IL-2 deprivation. The protein kinase activity in the immunoprecipitates was measured using [γ-^{32}P]ATP and purified c-jun-GST fusion protein. JNK1 activity was rarely seen in IL-2 stimulated cells, but a marked increase in activity was detected as soon as 2 h after IL-2 deprivation, reaching maximal activity after 4 h of IL-2 deprivation (Figure 2B). The kinase activity decreases and basal activity was detected 12 h after IL-2 deprivation. Conversely, JNK2 activity was almost undetectable and remained constant throughout the IL-2-deprivation time course studied.

As an internal protein loading control, the kinase reaction product detected in anti-JNK1 immunoprecipitates measured in the presence of [γ-^{32}P]ATP and c-jun-GST was separated in SDS–PAGE, transferred to nitrocellulose and probed with anti-c-Jun mAb. A similar level of c-Jun was

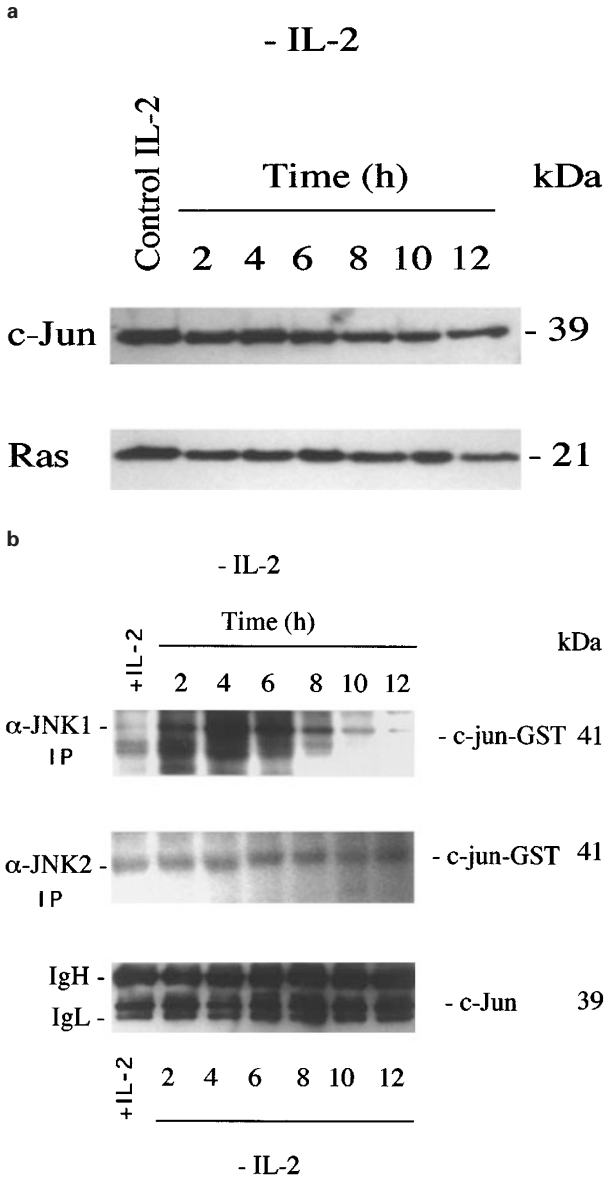

Figure 2 IL-2 deprivation induces JNK1 activation and c-*jun* expression. (**A**) Control IL-2-stimulated (5 ng/ml) or IL-2-deprived TS1$\alpha\beta$ cells (1×10^6) were harvested at different times (from 2–12 h) and lysed as described in Materials and Methods. Total protein extracts were separated in SDS–PAGE, transferred to nitrocellulose and probed with anti-c-Jun antibody. The blot was stripped and reprobed with anti-Ras antibody as an internal control of protein loading. Protein bands were detected using the ECL system. Similar results were obtained in three independent experiments. Molecular weight markers are shown. (**B**) Cell lysates from control IL-2-stimulated or IL-2-deprived cells (1×10^7) (from 2–12 h) were immunoprecipitated with anti-JNK1 or -JNK2 antibodies. JNK1 and JNK2 immune complexes were assayed by phosphorylating the c-jun-GST substrate in the presence of [γ-^{32}P]ATP. Phosphorylated proteins were separated in SDS–PAGE and visualized by autoradiography. As an internal control of c-jun-GST protein loading in the reaction, JNK1 kinase activity, measured in the presence of c-jun-GST and [γ-^{32}P]ATP, was separated by SDS–PAGE, transferred to nitrocellulose and probed with anti-c-Jun antibody as described in Materials and Methods. Similar results were obtained in three independent experiments. Molecular weight markers are shown

detected in the kinase reaction at all time points analyzed (Figure 2B), indicating that JNK1 is activated in response to IL-2 deprivation, and the activation precedes induction of apoptosis. This result also suggests that JNK1 activation may contribute to cell death.

Downregulation of JNK1 expression inhibits apoptosis

To test the hypothesis that JNK1 activation contributes to the induction of apoptosis, we inhibited JNK1 expression using antisense oligonucleotides. Sense or antisense oligonucleotide-treated TS1$\alpha\beta$ cells were cultured with or without IL-2. Cells treated with sense or antisense oligonucleotides in the presence of IL-2 do not show apoptosis (Figure 3A). A similar result was observed in untreated, IL-2-stimulated control cells. When cells are treated with antisense oligonucleotides and deprived of IL-2, the fraction of apoptotic cells is reduced to approximately 50% of that observed in sense oligonucleotide-treated cells maintained in the absence of IL-2, strongly suggesting that stimulation of the JNK1 signaling pathway contributes to apoptosis, and indicating a direct correlation between JNK1 activation and apoptosis.

The effect of sense or antisense oligonucleotide addition at the protein expression level is shown in Figure 3B. Control or sense-treated cells express JNK1 in the presence or absence of IL-2; however, JNK1 expression is almost undetectable in antisense-treated cells. It is interesting to note that 23% of apoptosis detected in IL-2-deprived cells treated with antisense oligonucleotide is probably due to residual JNK1 expression. This suggests that the JNK1 protein detected after antisense treatment in the absence of IL-2 is minimal but active, since it is able to induce apoptosis. The membrane was stripped and reprobed with anti-Ras antibody as an internal protein loading control.

Bcl-2 prevents JNK1 activation in IL-2-deprived cells

Since Bcl-2 expression blocks IL-2 deprivation-induced apoptosis, it was of interest to determine whether Bcl-2 could inhibit JNK activation induced by IL-2 deprivation. TS1$\alpha\beta$ cells were transiently transfected with Bcl-2 and cultured with or without lymphokine for 24 h. Bcl-2 expression greatly reduces the level of apoptosis in IL-2-deprived cells compared to mock transfectants (Figure 4A), indicating that Bcl-2 prevents apoptosis induced by IL-2 deprivation. No differences are observed in mock or Bcl-2-transfected cells maintained in the presence of IL-2.

To determine the point at which Bcl-2 prevents apoptosis in IL-2-deprived cells in relation to JNK1 activation, we examined the level of JNK1 activity in Bcl-2-transfected, IL-2-deprived cells. IL-2 deprivation induces JNK1 activation, whereas Bcl-2 overexpression in IL-2-deprived cells inhibits JNK1 activity, reaching the basal level of activation observed in control IL-2-treated cells (Figures 4B and 2B). IL-2 cultured cells express Bcl-2 while Bcl-2 expression was highly increased in transfected cells. IL-2 deprived cells show almost undetectable levels of Bcl-2

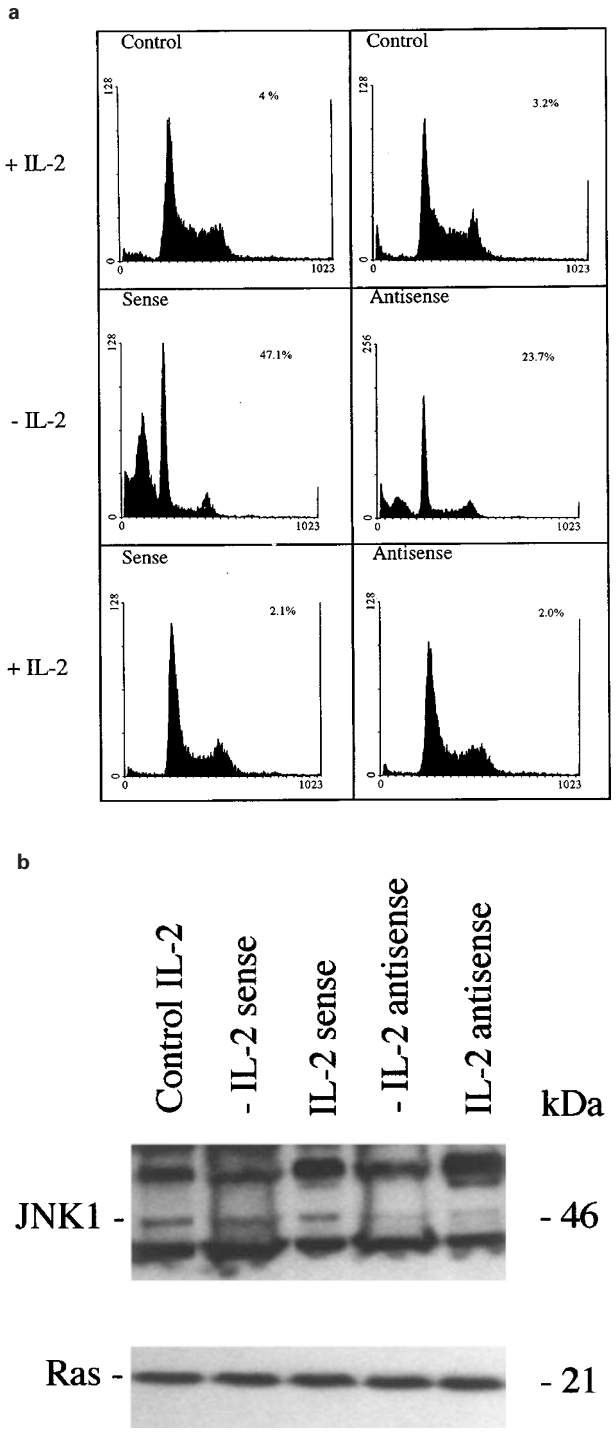

Figure 3 Effect of JNK1 sense or antisense oligonucleotides in TS1$\alpha\beta$ cells. (**A**) Cell cycle analysis of JNK1 sense or antisense oligonucleotide-treated cells. Cells were cultured with or without IL-2 (5 ng/ml) in the presence or absence of 15 μM of sense or antisense oligonucleotide for 36 h. Fresh oligonucleotide was added at 24 h. Cells were then collected, washed, permeabilized and stained with propidium iodide. Samples were analyzed by flow cytometry. The most proximal region of the fluorescence scale represents the sub-G_1 region. The percentages of apoptotic cells in each sample are superimposed. IL-2-cultured cells were used as control. Similar results were obtained in three independent experiments. (**B**) Effect of sense or antisense oligonucleotides in JNK1 expression. Cells were treated as in (**A**) and samples analyzed by Western blot as described in Materials and Methods. Protein extracts were separated by SDS–PAGE, blotted to nitrocellulose and probed sequentially with anti-JNK1 and anti-Ras, the latter as an internal protein loading control. Protein bands were detected using the ECL system. Similar results were obtained in three independent experiments. Molecular weight markers are shown

expression (Figure 4C). This suggests that Bcl-2 overexpression blocks the action of the JNK1 signaling pathway.

Discussion

We have analyzed the role of JNKs in the process of cell death promoted by IL-2 deprivation in the lymphokine-dependent T cell line TS1$\alpha\beta$. IL-2 deprivation elicits downregulation of Bcl-2 expression. IL-2 deprivation does not modify c-Jun protein levels, but induces JNK1 activation which parallels triggering of apoptosis, since downregulation of its expression inhibits apoptosis. Finally, activation of JNK1 caused by IL-2 deprivation is blocked by Bcl-2 expression.

Lymphokine deprivation of TS1$\alpha\beta$ cells induces a transient increase in Raf phosphorylation, but not in ERK1 or ERK2, suggesting that the downstream effectors of the Ras death pathway are different from those of the MAPK pathway.[36] It can be speculated that, in TS1$\alpha\beta$ cells, the JNK pathway may be responsible for cellular responses distinct from those regulated by ERK/MAPK. This concurs with the report that c-Jun phosphorylation in 3T3 fibroblast correlates with JNK activation but not that of ERK, suggesting that ERKs are probably involved in the induction of c-*fos* expression. Transcription factors Elk-1 and SAP-1 bind together with SRF to the serum response element present in the c-fos promoter. ERK, JNK and p38 phosphorylate and activate Elk-1 while SAP-1 is activated by ERK and p38 but not by JNK. The differential utilization of MAPK signaling pathway may represent a potential mechanism for the determination of specific responses to extracellular stimuli.[37]

In our cellular model, JNK1 is activated by apoptosis-inducing treatments, such as IL-2 deprivation. Inhibition of JNK1 expression by antisense oligonucleotides greatly reduces the level of apoptosis detected in IL-2-deprived TS1$\alpha\beta$, suggesting that activation of the JNK signaling pathway is required for apoptosis induction in TS1$\alpha\beta$ cells. Nerve growth factor (NGF) withdrawal from PC12 pheochromocytoma cells leads to JNK and p38 activation, which is critical for apoptosis induction in these cells. These data suggest that the balance between ERK and JNK activation pathways may be important in determining whether a cell survives or undergoes apoptosis.[38] JNK activation does not necessarily lead to apoptosis: IL-1 activates the JNK signaling pathway and does not induce apoptosis, suggesting that under these conditions, JNK activation may be necessary but not sufficient to induce apoptosis.[12,39] IL-3, erythropoietin and thrombopoietin also activate JNK1 and JNK2, and do not induce apoptosis.[40] Interestingly, no activation of SEK1/MKK4, the classical kinase activated in the JNK signaling pathway, was

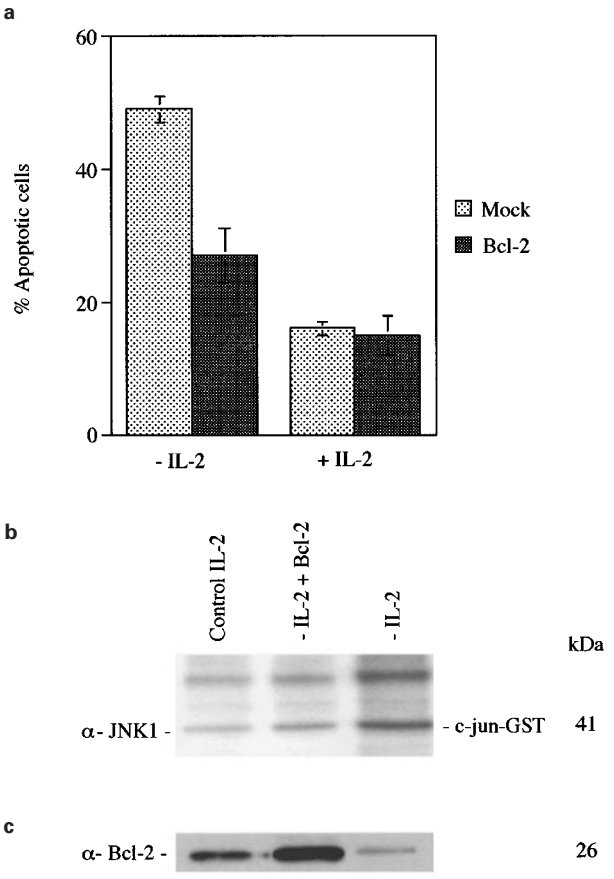

Figure 4 Overexpression of Bcl-2 suppresses apoptosis and JNK1 activation induced by IL-2 deprivation. (**A**) TS1$\alpha\beta$ cells (1×10^7) were transiently transfected with Bcl-2 and pHook-3 and cultured with or without IL-2. Twenty-four hours after transfection, cells were washed, selected using the Capture-Tec pHook-3 kit, permeabilized and stained with propidium iodide. Samples were analyzed by flow cytometry and the proportion of apoptotic cells determined. Two independent experiments were performed with similar results. Error bars represent $\pm$ S.D. (**B**) Similarly, TS1$\alpha\beta$ cells were transiently transfected with Bcl-2 and pHook-3. After transfection, cells were washed, selected using the Capture-Tec pHook-3 kit, and subjected to kinase assay as shown in Figure 3B, using c-jun-GST agarose as substrate and [γ-^{32}P]ATP. Proteins were separated in SDS–PAGE and visualized by autoradiography. Similar results were obtained in three independent experiments. Molecular weight markers are shown. (**C**) Control IL-2-stimulated (5 ng/ml) or IL-2-deprived or IL-2-deprived Bcl-2 transfected cells were lysed as described in Materials and Methods. Protein extracts were separated in SDS–PAGE, transferred to nitrocellulose and probed with anti-Bcl-2 antibody. Protein bands were detected using the ECL system. Similar results were obtained in three independent experiments. Molecular weight markers are indicated

detected, implying that hematopoietic cytokines activate JNKs through a kinase other than SEK1/MKK4.[15,41,42] It has also been shown that JNK activity alone is not sufficient to block cell proliferation.[43,44]

The mechanisms by which JNK activation participates in cell death are unknown. One possibility could be by activating the transcription of apoptotic signaling genes; consistent with this, JNK phosphorylates and enhances the transcriptional activity of c-Jun.[45] An alternative is that activated JNK phosphorylates c-Jun, which in turn sequesters other transcription factors whose activities are required for cell survival. A third hypothesis is that JNK phosphorylates and modulates the activity of other components involved in the cell death pathway. Among such targets may be molecules that regulate the cell cycle and/or ICE family members.

It has been shown that Bcl-2 rescues from cell death induced by growth factor depletion, viral agents and heat shock. In TS1$\alpha\beta$ cells, IL-2 deprivation results in a gradual disappearance of Bcl-2 protein levels, and cells are committed to die by apoptosis. Under these conditions, Bcl-2 expression itself constitutes a rescue pathway for IL-2 deprivation-mediated apoptosis of TS1$\alpha\beta$ cells.[36] In addition, Bcl-2 expression is able to prevent JNK1 activation and cell death in IL-2-deprived cells, suggesting that Bcl-2 might disrupt the JNK signaling cascade initiated by IL-2 deprivation. Our results also indicate that there is no JNK stimulation in Bcl-2-expressing cells, and that the JNK signaling pathway may be downstream of the targets of Bcl-2 action. An alternative is that Bcl-2 inhibits one or more apoptotic signaling pathways. It is intriguing, however, to suppose that Bcl-2 may influence upstream signaling pathways which prevent JNK activation and apoptosis. Four hours after IL-2 deprivation in TS1$\alpha\beta$ cells, we detect maximum JNK1 kinase activity, Bcl-2 expression and approximately 6–8% apoptosis. It is tempting to speculate that the JNK1 signaling pathway has been triggered, inducing Bcl-2 downregulation, and that only Bcl-2 overexpression can prevent apoptosis triggered by IL-2 deprivation-induced JNK1 activity. We cannot exclude the hypothesis of cross talk between Bcl-2 and JNK1. According with our results, it has been shown that Bcl-2 and crmA attenuate the activation of JNKs following cell suspension. Moreover, inhibition of JNK pathway blocks ICE activation. These results suggest a signaling pathway flowing from integrins to Bcl-2, triggering ICE/JNKS and, finally, apoptosis.[46] It appears also that suppression of JNK pathway may be critical for the antiapoptotic function of Bcl-2 and Bcl-X_L.[47]

Although the detailed mechanism by which Bcl-2 blocks JNK activation is not clear, it has been shown that Bcl-2 disrupt the activation of MEKK1, an upstream protein kinase in the JNK signaling pathway.[48] Interestingly, JNK1 can also be regulated through MEKK1-independent pathways.[49]

Several reports indicate that activation of JNKs is necessary for apoptosis of trophic factor-deprived PC12 cells. In this system, death is suppressed by Bcl-2 and by inhibition of ICE family proteases. Recently, it has been proposed the order in which these agents block apoptosis relative to JNK activation. Experimental results indicate that stimulation of the JNK signaling cascade in response to NGF deprivation does not require ICE family proteases. This implies either that ICE family proteases actions lie downstream of the JNK signaling cascade or that the two lie on independent pathways, both required for cell death.[50] It has been reported that Bcl-2 counteracts the apoptotic effects of ICE.[51] Accordingly, Park *et al*,[50] have shown that Bcl-2 is upstream of ICE proteases and, JNK and ICE pathways are subject to regulation by Bcl-2.

Death of trophic factor-dependent PC12 cells and neurons appears to require transcription.[52–54] In contra-

diction, it has been reported that activation of JNK in response to NGF deprivation occurs even in the absence of transcriptional activity.[55] This implies that JNK activation occurs by post-translational mechanism and is not a consequence of genes regulated in response to NGF deprivation. The findings presented here provide new and original insights into the mechanism by which JNK and Bcl-2 regulate cell survival and death in TS1$\alpha\beta$ cells.

Materials and Methods

Cells and cultures

TS1$\alpha\beta$ is a murine T cell line stably transfected with the α and β chains of the human IL-2 receptor,[56] which can be maintained independently in the presence of IL-2, IL-4 or IL-9. Cells were cultured in RPMI 1640 (BioWhittaker Walkersville, MD, USA) supplemented with 5% heat-inactivated fetal calf serum (Gibco, Gaithersburg, MD, USA), 2 mM glutamine, 10 mM HEPES, 0.55 mM arginine, 0.24 mM asparagine, 50 μM 2-mercaptoethanol and 5 ng/ml rIL-2 or 60 U/ml of IL-4.

Lymphokines, antibodies and reagents

Human recombinant IL-2 was provided by Roussel Uclaf (Paris, France). Murine rIL-4 or cultured supernatant of a HeLa subline (H28) transfected with the pKCRIL-4neo plasmid was used as a source of mouse IL-4. Mouse anti-pan Ras mAb (Ab-3) and polyclonal anti-c-Jun Ab were obtained from Oncogene Science (Cambridge, MA, USA), anti-mouse Bcl-2 mAb was from Pharmingen (San Diego, CA, USA) and mouse monoclonal anti-p-JNK1 and rabbit polyclonal anti-JNK1 and anti-JNK2 were from Santa Cruz (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-rabbit or anti-mouse Ig antibody was from Dako (Glostrup, Denmark). Enhanced chemiluminiscence (ECL) reagent and [γ-^{32}P]ATP were from Amersham (Amersham, UK). Agarose-conjugated human c-jun-GST was from UBI (Lake Placid, NY, USA), NP-40 was from Boehringer Mannheim (Mannheim, Germany) and the Capture-Tec pHook-3 kit was from Invitrogen (San Diego, CA, USA).

Sense and antisense oligonucleotides

The phosphothioate analogs of the oligonucleotides to the 5′ end of the JNK1 kinase, including the ATG initiation codon (15mer), were synthesized in an automated synthesizer (Applied Biosystems, Foster City, CA, USA) according to the published protocol.[57] The oligomers were purified as described previously.[58] The oligonucleotide sequence for JNK1 sense (5′–3′): ATG TGC ATC GGT GAC and for JNK1 antisense (5′–3′): GTC ACC GAT GCA CAT.

Cell cycle analysis

A total of 2.5×10^5 cells were washed and resuspended in PBS, permeabilized with 0.1% NP-40 and stained with 50 μg/ml of propidium iodide (PI) immediately before analysis. Samples were analyzed using an Epics XL flow cytometer (Coulter Corp. Miami, FL, USA). Cells were gated by forward and side light scatters and only the viable cell population was selected.

In vitro kinase assay

Cells (10×10^6) were lysed in RIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% Na deoxycholate, 150 mM NaCl, 1 mM EGTA and protease inhibitors). Supernatants were immunoprecipitated with anti-JNK1 or anti-JNK2 antibody, followed by incubation with protein-A Sepharose beads. Immunoprecipitates were washed and mixed with 3 μg of agarose-conjugated purified c-Jun-GST and 20 μM [γ-^{32}P]ATP in 30 μl of kinase buffer (20 mM HEPES pH 7.6, 20 mM $MgCl_2$, 100 μM sodium orthovanadate and 20 mM β-glycerophosphate) and incubated at 30°C for 20 min. The reaction was mixed with SDS–PAGE sample buffer and boiled for 5 min before loading onto a 10% acrylamide gel. The gel was dried and autoradiographed.

Western blot

Cells (2×10^6) were lysed in 50 mM Tris-HCl pH 7.5, 1% NP-40, 150 mM NaCl, 5 mM EDTA with protease inhibitors for 15 min at 4°C, and centrifuged at 13 000 $\times$ *g*. Protein concentrations in supernatants were determined using the Bio-Rad DC Protein Assay (BioRad, Hercules, CA, USA). Protein extracts were separated by SDS–PAGE, transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk in 20 mM Tris-HCl pH 7.5, 150 mM NaCl (TBS) and incubated with primary antibody in TBS+0.5% non-fat dry milk. Membranes were washed with 0.05% Tween 20 in TBS and incubated with peroxidase-conjugated second antibody or protein G. After washing, labeled proteins were developed using the ECL system. When stripping of the blots was required, membranes were incubated with 6.25 mM Tris-HCl pH 6.8, 2% SDS, 0.1 M 2-mercaptoethanol for 1 h and washed extensively with TBS before reblocking and probing.

Transient transfection

Transfections were performed using the DEAE-dextran method. Cells in exponential growth (10×10^6) were washed in TS buffer (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4) and resuspended in 0.5 mg/ml DEAE-dextran (Pharmacia, Uppsala, Sweden) in TS buffer containing 2.5 μg of Bcl-2 and pHook-3 plasmids. After 20 min incubation at room temperature, 6.75 ml RPMI with 5% FCS were added. Cells were incubated for 1 h at 37°C, centrifuged and resuspended in 10 ml RPMI with 5% FCS alone or supplemented with 5 ng/ml IL-2. The Capture-Tec pHook-3 kit (Invitrogen) was used for isolation of transiently transfected cells from a mixed population of transfected and untransfected cells. This system utilizes a specially designed vector (pHook-3) that expresses and displays a single-chain antibody (sFv) against a specific hapten on the surface of transfected cells. Cells expressing the sFv can then be isolated from the culture by binding to hapten-coated magnetic beads.

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References

1. Leonard WJ, Depper JM, Crabtree GR, Rudikoff S, Punprey J, Robb RJ, Kronke M, Svetlik PB, Pfeffer NJ, Waldmann TA and Green WC (1995) Molecular cloning and expression of cDNAs for the human UL-2 receptors. Nature 311: 626–631
2. Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M and Taniguchi T (1989) IL-2 receptor beta chain: generation of three receptor forms by cloned human alpha and beta chain cDNAs. Science 244: 551–556

3. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, Munakata H, Nakamura M and Sugamura K (1992) Cloning of the gamma chain of the human IL-2 receptor. Science 275: 379 – 382
4. Gómez J, Martínez-A C, Fernández B, García A and Rebollo A (1996) Critical role of Ras in the proliferation and prevention of apoptosis mediated by IL-2. J. Immunol. 157: 2772 – 2781
5. Minami Y and Taniguchi T (1995) IL-2 signaling: recruitment and activation of multiple protein tyrosine kinases by the components of the IL-2 receptor. Curr. Opin. Cell. Biol. 7: 156 – 162
6. Miyazaki T, Liu ZJ, Kawahara A, Minami Y, Yamada K, Tsujimoto Y, Barsoumian L, Pelmutter RM and Taniguchi T (1995) Three distinct IL-2 signaling pathways mediated by Bcl-2, c-Myc and Lck cooperate in hematopoietic cell proliferation. Cell 81: 223 – 231
7. Broome HE, Dargan CM, Bressent EF, Krajewski S and Reed JC (1995) Apoptosis and Bcl-2 expression in cultured murine splenic T cells. Immunol. 84: 375 – 381
8. Englaro W, Rezzonico R, Durand-Clement M, Lallemand D, Ortonne JP and Balloti R (1995) MAPK pathway and AP-1 are activated during cAMP-induced melanogenesis in B-16. J. Biol. Chem. 270: 24315 – 24320
9. Gille H, Strahl T and Shaw PE (1995) Activation of ternary complex factor Elk-1 by SAPK. Curr. Biol. 5: 1191 – 1200
10. Zink R, Cahill MA, Kracht M, Sachsenmaier C, Hipskind RA and Nordheim A (1995) Protein synthesis inhibitors reveal differential regulation of MAPK and SAPK pathways that converge on Elk-1. Mol. Cell. Biol. 15: 4930 – 4938
11. Whitmarch AJ, Shore P, Sharroks AD and Davis RJ (1995) Integration of MAP kinase signal transduction pathway at the serum response elements. Science 269: 403 – 407
12. Dérijard B, Hibi M, Wu YH, Barrett T, Su B, Deng T, Karin M and Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and H-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76: 1025 – 1037
13. Kharbanda S, Ran R, Pandey P, Shafman TD, Feller SM, Weichselbaum RR and Kufe DW (1995) Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. Nature 376: 785 – 788
14. Chen YR, Meyer CF and Tan TH (1996) Persistent activation of JNK1 in gamma radiation-induced apoptosis. J. Biol. Chem. 27: 631 – 643
15. Kiryakis JM, Banerjee P, Nikolakaki E, Dai T, Rubi EA, Ahmad MF, Avruch J and Woodgett RJ (1994) The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369: 156 – 160
16. Westwick JK, Bielawska AE, Dbaibo G, Hannun YA and Brenner DA (1995) Ceramide activates the SAPK. J. Biol. Chem. 270: 22689 – 22692
17. Yu R, Shtil AA, Tan TH, Roninson IB and Kong AN (1996) Adriamycin activates JNK in humlan leukemia cells: a relevance to apoptosis. Cancer Lett. 107: 73 – 81
18. Van Dam H, Wilhem D, Herr I, Steffen A, Herlich P and Angel P (1995) ATF-2 is preferentially activated by SAPK to genotoxic agents. EMBO J. 14: 1789 – 1811
19. Sluss HK, Barrett T, Dérigard H and Davis RJ (1994) Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Mol. Cell. Biol., 14: 8376 – 8384
20. Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ and Davis RJ (1995) Pro-inflammatory cytokines and environmental stress cause p38 MAPK activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. 270: 7420 – 7426
21. Hibi M, Lin A, Smeal T, Minden A and Karin M (1993) Identification of an oncoprotein and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. 7: 2135 – 2148
22. Minden A, Lin A, McMahon M, Lange-Carter C, Dérijard B, Davis RJ, Johnson GL and Karin M (1994) Differential activation of ERK and JNK mitogen-activated protein kinases by Raf1 and MEKK. Science 266: 1719 – 1723
23. Sakata N, Patel HR, Terada N, Aruffo A, Johnson GL and Gelfond EW (1995) Selective activation of c-Jun kinase mitogen-activated protein kinase by CD40 on human B cells. J. Biol. Chem. 270: 30823 – 30828
24. Berberich I, Shu G, Siebelt F, Woodgett R, Kyriakis JM and Clark EA (1996) Cross-linking CD40 on b cells preferentially induces SAPJ rather than MAPK. EMBO J. 15: 92 – 101
25. Su B, Jacinto E, Hibi M, Kallunki T, Karin M and Ben-Neriah Y (1994) JNK is involved in signal integration during costimulation of T lymphocytes. Cell. 77: 727 – 736
26. Gupta S, Campbell D, Dérijard B and Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science 267: 389 – 393
27. Karin M (1995) The regulation of AP-1 activity by MAP kinases. J. Biol. Chem. 270: 16483 – 16486
28. Coso OA, Teramoto H, Simonds WF and Gutkinds JS (1996) Signaling from G. protein-coupled receptors to c-Jun kinase involves beta gamma subunits of heterotrimeric G. proteins acting on Ras and Rac-dependent pathways. J. Biol. Chem. 27: 3963 – 3966
29. Olson MF, Ashworth A and Hall A (1995) An essential role for Rho, Rac, and Cdc42 GTPase in cell cycle progression through G1. Science 269: 1270 – 1272
30. Wyllie AH (1993) Apoptosis. Br. J. Cancer 67: 205 – 208
31. Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. Science 267: 1456 – 1462
32. White E (1996) Life, death and the pursuit of apoptosis. Gene Dev. 10: 1 – 15
33. Dubois-Dauphin M, Frankowski H, Tsujimoto Y, Hnarte J and Martinou JC (1994) Neonatal motoneurons overexpressing the Bcl-2 protooncogene in transgenic mice are protected from axotomy-induced cell death. Proc. Natl. Acad. Sci. U.S.A. 91: 3309 – 3313
34. Reed JC (1994) Bcl-2 and the regulation of programmed cell death. J. Cell. Biol., 124: 1 – 6
35. Park JR and Hockenbery DM (1996) Bcl-2, a novel regulator of apoptosis. J. Cell. Biochem. 60: 12 – 17
36. Gómez J, Martínez-A C, Fernández B, García A and Rebollo A (1997) Ras activation leads to cell proliferation or apoptotic cell death upon IL-2 stimulation or lymphokine deprivation respectively. Eur. J. Immunol. 27: 1610 – 1618
37. Lopez-llaseca M, Li W, Uren A, Yu JC, Kazlauskas A, Gutkin JS and Heidaran MA (1997) Requirement of PI3 kinase for activation of JNK/SAPK by PDGF. Biochem. Biophys. Res. Commun. 232: 273 – 282
38. Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270: 1326 – 1331
39. Atfi A, Djelloul S, Chastre E, Davis R and Gespach C (1997) Evidence for a role of Rho-like GTPase and SAP/JNK in TGFβ-mediated signaling. J. Biol. Chem. 272: 1429 – 1433
40. Nagata Y, Nishida E and Todokoro K (1997) Activation of JNK signaling pathway by erythropoietin, thrombopoietin and IL-3. Blood 89: 2664 – 2669
41. Whitmarsh AJ, Yang SH, Su SM, Sharrocks AD and Davis RJ (1997) Role of p38 and JNK in the activation of ternary complex factors. Mol. Cell. Biol. 17: 2360 – 2366
42. Moriguchi T, Kawasaki H, Matsuda S, Gotoh Y and Nishida E (1995) Evidence for multiple activators for SAPK/JNK. Existence of novel activators. J. Biol. Chem. 270: 12969 – 12973
43. Dinarello CA (1994) The biological properties of IL-1. Eur. Cytokine Network 5: 517 – 522
44. Shu J, Masahiro H and Stacey D (1996) Activation of JNK/SAPK pathway is not directly inhibitory for cell cycle progression in NIH3T3 cells. Oncogene 13: 2421 – 2430
45. Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, Johnson GL and Karin M (1995) Identification of a dual specificity kinase that activates the Jun kinase and p38-Mpk2. Science 268: 286 – 290
46. Frisch SM, Vuori K, Kelaita D and Sicks S (1996) A role of JNK in anoikis; suppression by Bcl-2 and crmA. J. Cell Biol. 135: 1377 – 1382
47. Park J, Kim I, Oh YJ. Lee K, Han P and Choi EJ (1997) Activation of JNK antagonizes an anti-apoptotic action of Bcl-2. J. Biol. Chem. 272: 16725 – 16728
48. Yan M, Doi T, Deak JC, Kyriakis JM, Zou LI, Woodgett JR and Templeton DJ (1994) MAPK: new JNK expands the group. Nature 372: 798 – 800
49. Ichijo H, Nishida E, Irie K, Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K and Gotoh Y (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathway. Science 275: 90 – 94
50. Park DS, Stefanis L, Irene CY, Farinelli SE and Greene LA (1996) Ordering the cell death pathway. J. Biol. Chem.. 271: 21898 – 21905
51. Miura M, Zhu H, Rotello R, Harweig EA and Yuan J (1993) Induction of apoptosis by IL-1 beta converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell. 75: 653 – 660
52. Martin D, Schmidt R, DiStefano P, Lowry O, Carter J and Johnson EM (1988) Inhibition of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J. Cell. Biol. 106: 829 – 844
53. Oppenheim RW, Prevette D, Tylell M and Homm S (1990) Naturally occurring and induced neuronal death in the chick embryo in vivo requires protein and RNA synthesis: evidence for the role of cell death genes. Dev. Biol. 138: 104 – 113

54. Mesner PW, Winters TR and Green SH (1992) Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. J. Cell Biol. 119: 1669 – 1680
55. Burnstein DE and Greene LA (1978) Evidence for RNA synthesis-dependent and -independent pathways in stimulation of meurite outgrowth by nerve growth factor. Proc. Natl. Acad. Sci. USA. 75: 6059 – 6063
56. Pitton C, Rebollo A, Van Snick J, Theze J and García A (1993) High affinity and intermediate affinity forms of the human IL-2 receptor, expressed in an IL-9-dependent murine T cell line, deliver proliferative signals via differences in their transduction pathway. Cytokine 5: 362 – 371
57. Matsukura M, Shinozuka K, Zon G, Mitsuya H, Reitz M, Cohen JS and Boder S (1987) Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA 84: 7708 – 7710
58. Narayanan R, Lawlor KG, Schaapueld QJ, Cho KR, Vogelstein B, Bui-Vinch P, Osborne MP and Telong NT (1990) Antisense RNA to the putative tumor-suppressor gene DCC transforms Rat-1 fibroblasts. Oncogene 7: 553 – 561