

Role of Akt and c-Jun N-terminal Kinase 2 in Apoptosis Induced by Interleukin-4 Deprivation

Ana Cerezo,* Carlos Martínez-A,* Diego Lanzarot,* Siegmund Fischer,[†] Thomas F. Franke,[‡] and Angelita Rebollo*[§]

*Department of Immunology and Oncology, Centro Nacional de Biotecnología, Universidad Autónoma, Campus de Cantoblanco, E-28049 Madrid, Spain; [†]Laboratoire d'Oncologie Cellulaire et Moléculaire, Institut National de la Santé et de la Recherche Médicale-363, Hôpital Cochin, F-75014 Paris, France; and [‡]Department of Pharmacology, Columbia University, New York, New York 10032

Submitted April 6, 1998; Accepted August 31, 1998
Monitoring Editor: Martin Raff

We have shown previously that interleukin-4 (IL-4) protects TS1 $\alpha\beta$ cells from apoptosis, but very little is known about the mechanism by which IL-4 exerts this effect. We found that Akt activity, which is dependent on phosphatidylinositol 3 kinase, is reduced in IL-4-deprived TS1 $\alpha\beta$ cells. Overexpression of wild-type Akt or a constitutively active Akt mutant protects cells from IL-4 deprivation-induced apoptosis. Readdition of IL-4 before the commitment point is able to restore Akt activity. We also show expression and c-Jun N-terminal kinase 2 activation after IL-4 deprivation. Overexpression of the constitutively activated Akt mutant in IL-4-deprived cells correlates with inhibition of c-Jun N-terminal kinase 2 activity. Finally, TS1 $\alpha\beta$ survival is independent of Bcl-2, Bcl-x, or Bax.

INTRODUCTION

Interleukin-4 (IL-4) is a pleiotropic cytokine, produced predominantly by T cells, mast cells, and basophils, that stimulates proliferation of T cells, B cells, and mast cells (Seder *et al.*, 1981; Howard *et al.*, 1982; Brown *et al.*, 1987; Paul, 1991). The biological functions of IL-4 are mediated via its binding to a specific cell surface receptor. This widely distributed receptor consists of two chains that are members of the type I cytokine receptor superfamily (Cosman, 1993), a ligand-binding chain (IL-4R α), and the common γ chain, which is shared with the IL-2, IL-7, IL-9, and IL-15 receptors (Noguchi *et al.*, 1993; Kondo *et al.*, 1993, 1994; Russell *et al.*, 1993, 1994; Giri *et al.*, 1994; Kimura *et al.*, 1995). Treatment of cells with IL-4 induces various biological responses, including an increase in cell proliferation and gene transcription. Some of these responses are unique to IL-4, and some are also induced by other cytokines. Although the receptors for IL-4 and IL-2 have several features in common, because of the γ chain receptor component, IL-4 evokes responses that IL-2 does not (Morla *et al.*, 1988; Lin *et al.*, 1995; Quelle *et al.*, 1995).

Apoptotic cell death is a process that ultimately leads to activation of endogenous nucleases that promote internucleosomal DNA degradation (Wyllie, 1980). Apoptosis can be induced by growth factor deprivation (Duke and Cohen, 1986; Nuñez *et al.*, 1990; Williams *et al.*, 1990), signaling via surface receptors (Smith *et al.*, 1989; Benhamou *et al.*, 1990; Hasbold and Klaus 1990), and exposure to drugs (Zubiaga *et al.*, 1992) or DNA-damaging agents (Waters, 1992). Cell surface receptor-mediated mechanisms that control apoptosis often act through a signal transduction system involving the stimulation of the receptor, the activation of protein kinase and phosphatase cascades, and the release of second messengers to up-regulate or suppress the transcription of specific genes.

Many components of the machinery that regulate and execute programmed cell death have been identified (Nagata *et al.*, 1997). In addition to the central role of the caspase family of proteases and the Bcl-2 family of apoptosis regulators, recent reports have suggested that c-Jun N-terminal kinases (JNK) may be involved in controlling apoptosis (Kyriakis and Avruch, 1996).

JNKs are activated by stimuli such as UV light (Dèrijard *et al.*, 1994), γ -irradiation (Chen *et al.*, 1995; Kharbanda *et al.*, 1995), protein synthesis inhibitors (Kyri-

[§] Corresponding author. E-mail address: arebollo@cnb.uam.es.

akis *et al.*, 1994), ceramide (Westwick *et al.*, 1995), and DNA-damaging drugs (Van Dam *et al.*, 1995; Yu *et al.*, 1996), TNF and IL-1 (Sluss *et al.*, 1994; Raingeaud *et al.*, 1995). JNK activity is also induced by mitogenic signals, including growth factors (Hibi *et al.*, 1993; Minden *et al.*, 1994), oncogenic Ras (Dèrijard *et al.*, 1994), CD40 ligation (Sakata *et al.*, 1995; Berberich *et al.*, 1996), and T cell activation signaling (Chen *et al.*, 1996; Su *et al.*, 1994). Included among JNKs are the 46-kDa JNK1 and the 55-kDa JNK2 isoforms (Hibi *et al.*, 1993; Kyriakis *et al.*, 1994), which phosphorylate transcription factors such as c-Jun, ATF-2, and Elk-1 (Gupta *et al.*, 1995; Karin, 1995; Whitmarsch *et al.*, 1995). JNK activation requires phosphorylation at the Thr and Tyr residues by the dual-specificity kinases SEK1 or MKK4, which are activated following phosphorylation. One potential function of JNKs appears to be the initiation of programmed cell death, although JNK pathway activation does not necessarily lead to apoptosis; IL-3, erythropoietin, and thrombopoietin activate the Ras/MEKK1/SEK1/JNK pathway without induction of apoptosis (Nagata *et al.*, 1997).

The serine and threonine kinase c-Akt, also known as Rac α or protein kinase B α (Bellacosa *et al.*, 1991; Coffey and Woodgett, 1991; Jones *et al.*, 1991), is the cellular homologue of the v-Akt oncogene. Akt is composed of an N-terminal pleckstrin homology domain, followed by a catalytic domain and a short C-terminal tail. Akt is regulated by both phosphorylation and the direct binding of PI3 kinase (PI3K) lipid products to the pleckstrin homology domain of Akt (Konishi *et al.*, 1996). In addition, other PI3K-independent mechanisms of Akt activation have been identified (Didichenko *et al.*, 1996; Franke *et al.*, 1997; Klippel *et al.*, 1997). Akt is a mediator of growth factor-induced survival and suppresses the apoptotic death of a number of cell types induced by a variety of stimuli, including IL-2 and IL-3 deprivation, cell-cycle discordance, loss of cell adhesion, and DNA damage (Ahmed *et al.*, 1997; Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Khwaja *et al.*, 1997; Kulik *et al.*, 1997; Songyang *et al.*, 1997).

In this study, we examined the role of Akt and JNK2 in apoptosis mediated by IL-4 deprivation of TS1 $\alpha\beta$ cells, and the relevance of these findings is discussed in the context of apoptosis.

MATERIALS AND METHODS

Cells and Cultures

TS1 $\alpha\beta$ is a lymphokine-dependent mouse T cell line stably transfected with the α and β chains of the human IL-2R (Pitton *et al.*, 1993). The TS1 $\alpha\beta$ double transfectant responds independently to IL-2, IL-4, or IL-9. Cells were cultured in RPMI 1640 (Bio-Whittaker, Verviers, Belgium) supplemented with 5% heat-inactivated FCS (Life Technologies, Paisley, UK), 2 mM glutamine, 10 mM HEPES, 0.55 mM arginine, 0.24 mM asparagine, 50 μ M 2-mercaptoethanol, and 60 U/ml IL-4 or 5 ng/ml recombinant IL-2.

Lymphokines, Antibodies, Reagents, and Plasmids

Human recombinant IL-2 was provided by Roussel Uclaf (Paris, France). Murine recombinant IL-4 or cultured supernatant of a HeLa subline (H28) transfected with the pKCRIL-4.neo plasmid was used as a source of murine IL-4. Mouse antipan Ras monoclonal antibody (Ab3) was obtained from Oncogene Science (Cambridge, MA). Rabbit polyclonal anti-JNK1 and -JNK2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Akt has been previously described (Franke *et al.*, 1995). Monoclonal anti-p85 PI3K, -Bcl-2, -Bax, and -Bcl-x antibodies were from Transduction Laboratories (Lexington, KY). Peroxidase (PO)-conjugated goat anti-rabbit or -mouse immunoglobulin antibody was provided by Dako (Glostrup, Denmark). Wortmannin and histone H2B were from Sigma Chemical Co. (St. Louis, MO), agarose-conjugated human c-Jun-GST was obtained from Upstate Biotechnology (Lake Placid, NY) and DEAE-dextran was from Pharmacia (Uppsala, Sweden). Enhanced chemiluminescence (ECL) reagent and γ^{32} P were provided by Amersham (Buckinghamshire, UK), and Capture-Tec pHook-3 kit was obtained from Invitrogen (San Diego, CA). The annexin-FITC kit was from Immunotech (Marseille, France).

Generation of dominant negative Akt (Akt K179 M), constitutively active Akt (Myr-Akt), and wild-type (wt) Akt have been previously described (Franke *et al.*, 1995). In brief, constitutively activated myr-Akt.HA was generated by addition of a N-terminal src myristoylation signal to a construct encoding Akt.HA.

Transient Transfection

Transfections were performed using the DEAE-dextran method. Cells in exponential growth (1×10^7) were washed in TS buffer (25 mM Tris HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.5 mM MgCl₂, and 0.6 mM Na₂HPO₄) and resuspended in 0.5 mg/ml DEAE-dextran in TS buffer containing 5 μ g of the corresponding plasmid and pHook-3. A total of 6.75 ml RPMI with 5% FCS were added after 20 min incubation at room temperature. Cells were incubated for 1 h at 37°C, centrifuged, and resuspended in 10 ml RPMI with 5% FCS alone or supplemented with 60 U/ml IL-4. The capture-Tec pHook-3 kit was used for isolation of transiently transfected cells from a mixed population of transfected and nontransfected cells.

Cell Cycle Analysis

Propidium Iodide Staining. A total of 2×10^5 cells were washed and resuspended in PBS, permeabilized with 0.1% NP-40, and stained with 50 μ g/ml propidium iodide immediately before analysis. Samples were analyzed using an Epics XL flow cytometer (Coulter, Miami, FL). Apoptosis was measured as the percentage of cells present in the subG1 region of the fluorescence scale having a hypodiploid DNA content.

Annexin Staining. A total of 4×10^5 cells were washed with ice-cold PBS, diluted in ice-cold binding buffer, and stained with annexin and propidium iodide. Samples were maintained on ice for 10 min in the dark and then analyzed by flow cytometry.

In Vitro JNK Assay

Cells (1×10^7) were lysed in radioimmunoprecipitation assay buffer (50 mM Tris HCl, 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 -JNK2 antibody, followed by incubation with protein-A Sepharose beads. Immunoprecipitates were washed, mixed with 3 μ g agarose-conjugated purified c-Jun-GST and 20 μ M [γ -³²P]ATP in 30 μ l kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl₂, 100 μ M sodium orthovanadate, and 20 mM β -glycerophosphate), and incubated at 30°C for 20 min. The reaction was stopped with SDS-PAGE sample buffer and boiled for 5 min before loading onto a 10% acrylamide gel. The gel was dried and exposed to x-ray film.

In Vitro Akt Kinase Assay

Cells (1×10^7) were lysed in lysis buffer (50 mM Tris HCl, pH 7.5, 1% NP-40, 150 mM NaCl, and 5 mM EDTA) with protease inhibitors for 20 min at 4°C and centrifuged (13,000 rpm for 15 min at 4°C). Supernatants were immunoprecipitated with anti-Akt antibody and protein-A Sepharose beads were then added. Immunoprecipitates were washed and the kinase assay carried in 30 μ l Akt kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂, and 10 mM MnCl₂) containing 10 μ Ci [γ -³²P]ATP, 5 μ M unlabeled ATP, and 1 mM DTT. Histone H2B was added as exogenous substrate at a final concentration of 0.01 mg/ml. After 20 min at room temperature, the kinase assay was terminated by the addition of SDS-PAGE sample buffer and boiled for 5 min before loading onto a 12.5% gel. The gel was dried and exposed to x-ray film.

In Vitro PI3K Assay

Cells (1×10^7) were lysed in lysis buffer (137 mM NaCl, 20 mM Tris HCl, pH 8, 1 mM MgCl₂, 1 mM CaCl₂, 1% NP-40, and 10% glycerol) with protease and phosphatase inhibitors for 20 min at 4°C and centrifuged (13,000 rpm for 10 min at 4°C). Supernatants were precleared with protein-A Sepharose beads and immunoprecipitated with anti-p85 PI3K antibody. Protein-A Sepharose was added for 1 h at 4°C. Immunoprecipitates were then washed once with PBS; once with 0.5 M LiCl and Tris HCl, pH 7.4; once with H₂O; and once with 10 mM Tris HCl, pH 7.4, 100 mM NaCl, and 1 mM EDTA. The kinase assay was performed in a final volume of 50 μ l with 15 μ Ci [γ -³²P]ATP, S-adenosine, and 20 μ g L- α -phosphatidylinositol for 20 min at 24°C and terminated with 100 μ l 1 M HCl. The lipids were extracted with 200 μ l chloroform/methanol (1:1), washed with 80 μ l methanol/1 M HCl (1:1), and separated by thin-layer chromatography on silica gel 60 plates coated with 1% potassium oxalate. Plates were developed in chloroform/methanol/4 M NH₄OH (9:7:2) and exposed to x-ray film.

Western Blot

Cells (2×10^6) were lysed in Laemmli sample buffer and protein extracts separated by SDS-PAGE, transferred to nitrocellulose membrane, blocked with 5% nonfat dry milk in TBS (20 mM Tris HCl, pH 7.5, 150 mM NaCl), and incubated with the primary antibody in TBS/0.5% nonfat dry milk. Membranes were washed with 0.05% Tween-20 in TBS and incubated with a PO-coupled second antibody. After washing, labeled proteins were developed using the ECL system.

When stripping of blots was required, membranes were incubated with 62.5 mM Tris HCl, pH 6.8, 2% SDS, and 0.1 M 2-mercaptoethanol for 1 h at 56°C and washed extensively with TBS before reblocking and probing.

RESULTS

Apoptosis in IL-4-deprived TS1 α β Cells Correlates with Up-regulation of JNK2 Expression

TS1 α β cells proliferate in the presence of IL-2, IL-4, or IL-9. When IL-4-maintained cells are deprived of lymphokine, they undergo apoptosis as estimated by flow cytometry (Figure 1A). As soon as 2 h after IL-4 withdrawal, 6% of cells were apoptotic, reaching ~32% at 24 h, whereas control cells maintained in the presence of IL-4 showed no apoptosis. Similar results were obtained when the percentage of apoptotic cells was estimated by annexin staining (Figure 1B). The commitment point for apoptosis was 4–6 h (our unpublished results). It was thus of interest to determine

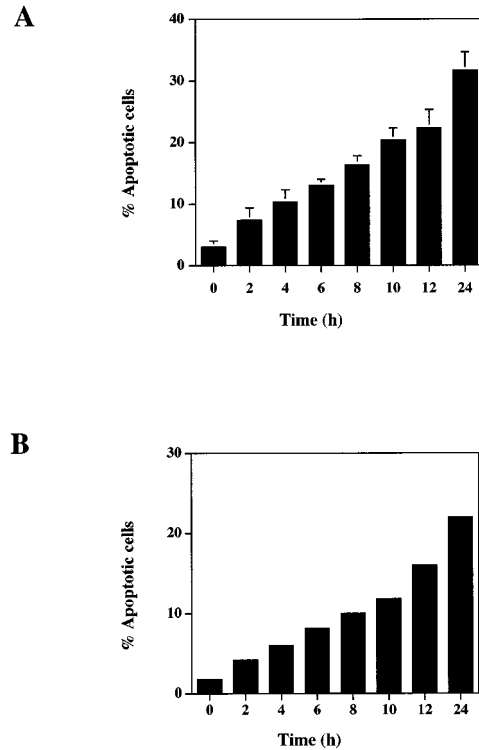


Figure 1. IL-4 deprivation induces apoptosis of TS1 α β cells. (A) Cells (2×10^5) were cultured in the presence or absence of IL-4 (60 U/ml) for 24 h. At different times, cells were harvested and analyzed for the percentage of apoptosis. Cells were permeabilized, stained with propidium iodide, and analyzed for DNA staining using fluorescence flow cytometry. The zero time point corresponds to control cells cultured in IL-4. Error bars represent \pm SD from three independent experiments. Apoptosis was measured as cells present in the subG1 region of the fluorescence scale and having a hypodiploid DNA content. (B) Cells were treated as in A, diluted in ice-cold binding buffer, stained with annexin and propidium iodide, and maintained on ice for 10 min in the dark. Samples were analyzed by flow cytometry.

whether induction of apoptosis by IL-4 deprivation could modify the expression of JNKs, apoptosis-related molecules, and Akt kinase, a molecule that is involved in apoptosis prevention as well as to Bcl-2.

The protein level of JNK1, JNK2, and Akt after IL-4 deprivation of TS1 α β cells is shown in Figure 2A. JNK1 expression showed no difference throughout the starvation period analyzed compared with IL-4-stimulated control cells. Similarly, control IL-4-cultured or -deprived cells showed constant Akt expression throughout the starvation period analyzed. Interestingly, JNK2 expression was up-regulated after IL-4 deprivation; maximum expression was observed 4–8 h after IL-4 withdrawal, and disappeared almost completely 12 h after deprivation. Unaltered Ras expression was shown for all conditions as an internal protein loading control. These results show that in TS1 α β

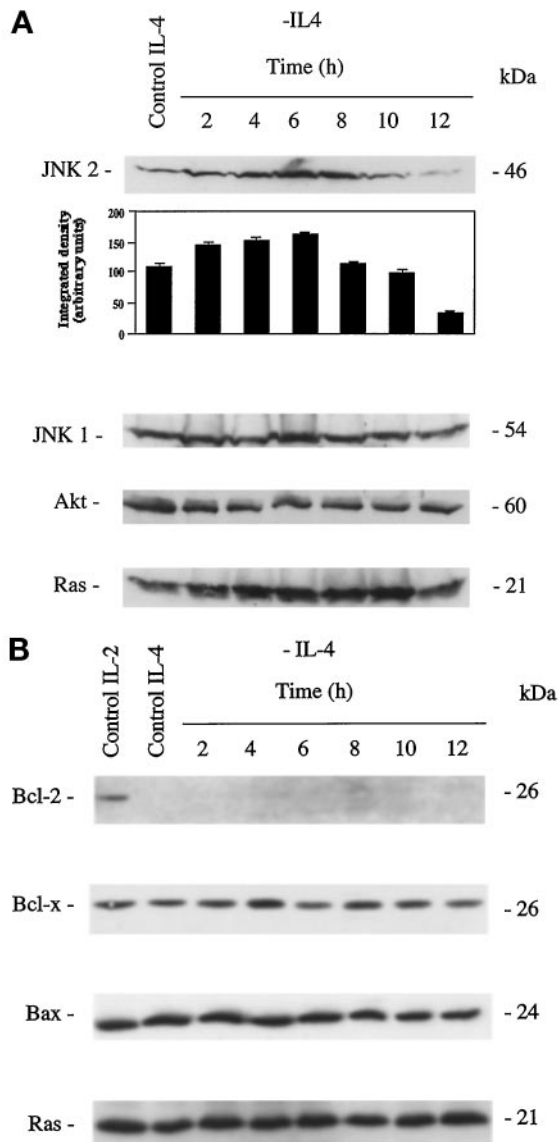


Figure 2. Time course of JNK1, JNK2, Akt, Ras, Bcl-2, Bcl-x, and Bax expression in IL-4-deprived cells. (A) Control or IL-4-deprived cells (2×10^6) were harvested at different time points, lysed, and protein extracts separated in SDS-PAGE. Proteins were transferred to nitrocellulose and probed sequentially with anti-JNK1, -JNK2, -Akt, 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 mass markers are indicated. Densitometric analysis of JNK2 expression is shown below the blot. (B) TS1 α β cells were stimulated by IL-4 or deprived of lymphokine. At different time points, cells (2×10^6) were lysed and protein extracts separated, transferred to nitrocellulose, and probed with anti-Bcl-2, -Bax, and -Bcl-x. Cells maintained in IL-2 were used as a positive control. Protein bands were detected using the ECL system. An internal control of homogeneous Ras expression in all samples is shown. Similar results were obtained in three independent experiments.

cells, IL-4 deprivation modifies JNK2 expression, whereas JNK1 and Akt expression are unaffected.

The TS1 α β cell line expressed the antiapoptotic protein Bcl-2 upon IL-2 stimulation, whereas IL-4-mediated growth proceeds in the absence of Bcl-2 (Figure 2B) and Bax expression (our unpublished results). Bcl-x and Bax levels were unaltered throughout the IL-4 starvation period analyzed. This suggests that IL-4 deprivation-induced apoptosis proceeds along pathways that do not involve changes in Bcl-x or Bax expression.

IL-4 Deprivation-induced Apoptosis Correlates with Reduction of Akt and PI3K and Induction of JNK2 Activity

To examine the possible involvement of Akt, PI3K, and JNKs in IL-4 deprivation-induced apoptosis, we measured JNK1, JNK2, PI3K, and Akt kinase activity in IL-4-stimulated and IL-4-deprived TS1 α β cells. Akt was immunoprecipitated with a specific antibody at various time points after IL-4 deprivation, and the kinase activity in the immunoprecipitates was measured using [γ - 32 P]ATP and H2B as substrate. Akt activity was detected in IL-4-cultured control cells. Down-regulation of Akt activity was time dependent; 2 h after lymphokine deprivation, Akt activity was inhibited and was almost undetectable 12 h after IL-4 withdrawal (Figure 3A). To ensure equal protein loading, Akt activity was separated in SDS-PAGE, transferred to nitrocellulose, and probed with anti-Akt antibody.

PI3K activity was determined in α -p85 PI3K immunoprecipitates of control or IL-4-deprived cells (Figure 3A). PI3K activity was detected in IL-4-maintained cells, and decreased gradually throughout the IL-4 starvation period analyzed. The lowest level of activity was detected 12 h after deprivation.

Because Akt activity was reduced 2 h after IL-4 deprivation, it was of interest to know whether readdition of IL-4 after Akt activity down-regulation and before the commitment point would be able to restore Akt activity. Ts1 α β cells were deprived of IL-4 for 3 h and then IL-4 was added to the culture medium. Akt activity was immunoprecipitated before and after IL-4 readdition and the kinase activity measured using [γ - 32 P]ATP and H2B as substrate (Figure 3B). IL-4-cultured control cells showed Akt activity. Two hours after lymphokine deprivation, Akt activity was decreased. Interestingly, readdition of IL-4 after 3 h of starving restored Akt activity. As internal control of protein loading, Akt activity was separated in SDS-PAGE, transferred to nitrocellulose, and probed with anti-Akt antibody.

JNK was immunoprecipitated with anti-JNK1 or -JNK2-specific antibodies at various time points after IL-4 deprivation. The kinase activity in the immunoprecipitates was measured using [γ - 32 P]ATP and purified c-jun-GST fusion protein. JNK1 activity was al-

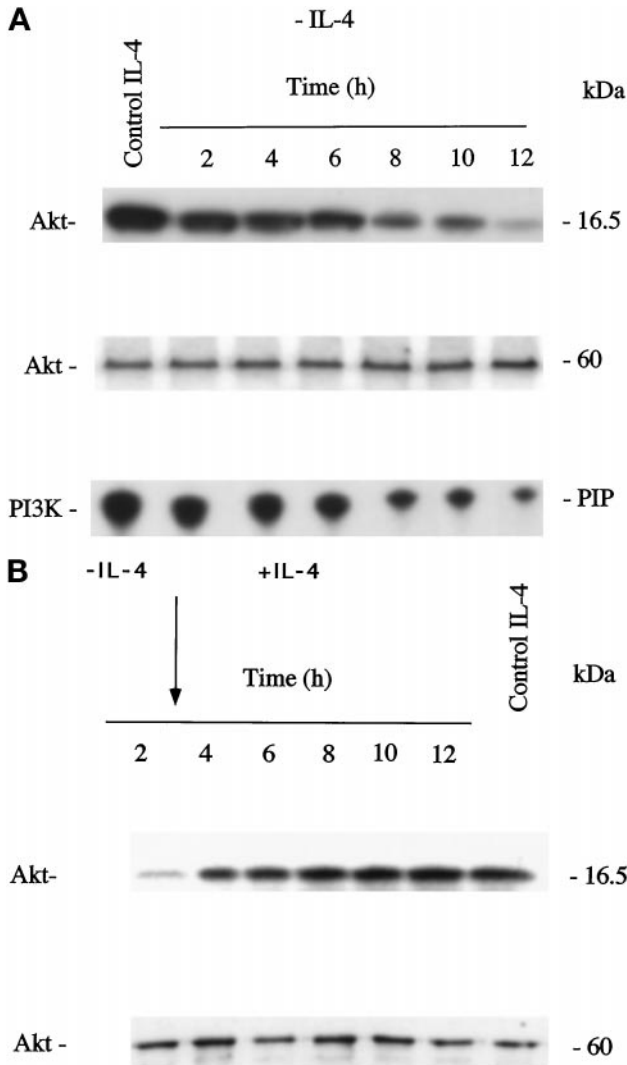


Figure 3. IL-4 deprivation down-regulates Akt and PI3K activity. (A) Cell lysates from control or IL-4-deprived cells (1×10^7) were immunoprecipitated with anti-Akt antibody, and the Akt immunocomplex assayed by phosphorylating H2B substrate in the presence of [γ - 32 P]ATP. Phosphorylated proteins were separated in SDS-PAGE and visualized by autoradiography. As an internal control of protein loading, Akt was separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Akt antibody. Similar results were obtained in three independent experiments. Molecular mass markers are shown. PI3K activity was immunoprecipitated with anti-p85 PI3K antibody and determined by incorporation of [γ - 32 P]ATP into propidium iodide. Radiolabeled phosphatidylinositol phosphate (PIP) was analyzed by thin-layer chromatography on silica gel 60 plates and developed in chloroform/methanol/4 M NH_4OH (9:7:2). Dried plates were exposed to x-ray film. Migration of PIP is indicated in the margin. Similar results were obtained in three independent experiments. (B) Cells were IL-4 deprived for 3 h and then IL-4 was added to the culture medium. Cell lysates from control, IL-4-deprived, or IL-4-restimulated cells (1×10^7) were immunoprecipitated with anti-Akt antibody and assayed as in A. Akt was separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Akt antibody as internal control of protein loading. Molecular mass markers are shown.

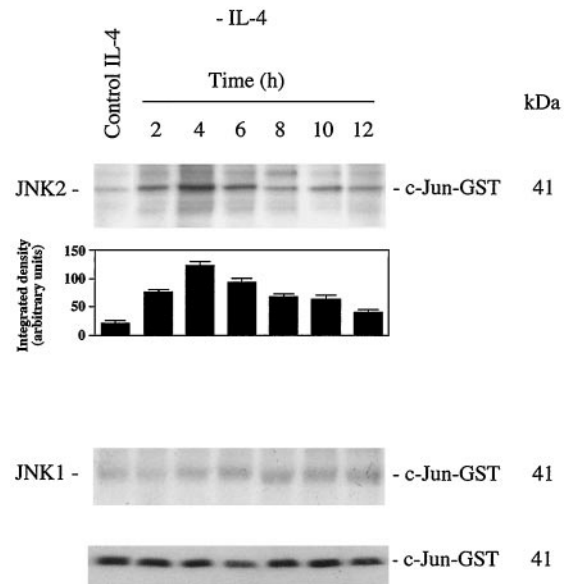


Figure 4. IL-4 deprivation induces JNK2 activation. Cell lysates from control or IL-4-deprived cells (1×10^7) were immunoprecipitated with anti-JNK1 or -JNK2 antibody. JNK1 and JNK2 immune complexes were assayed by phosphorylating the c-Jun-GST substrate in the presence of [γ - 32 P]ATP. Phosphorylated proteins were separated in SDS-PAGE and visualized by autoradiography. Densitometric analysis of JNK2 activity is shown below the autoradiography. As an internal control of c-Jun-GST protein loading in the reaction, JNK2 kinase activity measured in the presence of [γ - 32 P]ATP was separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-c-Jun antibody. Similar results were obtained in three independent experiments. Molecular mass markers are shown.

most undetectable and remained constant throughout the IL-4 deprivation time course analyzed (Figure 4). A marked increase in JNK2 activity was detected as soon as 2 h after IL-4 deprivation, reaching a plateau at 4 h after starvation (Figure 4); kinase activity then decreases and basal activity was detected 12 h after IL-4 deprivation, as well as in control cells. As an internal protein loading control, JNK2 kinase activity was separated in SDS-PAGE, transferred to nitrocellulose, and probed with anti-c-Jun antibody. In addition, c-jun levels were not altered during the starvation period analyzed (our unpublished results).

Transient Expression of Constitutively Activated Akt Prevents IL-4 Withdrawal-mediated Apoptosis

Because IL-4 deprivation in TS1 α B cells correlates with inhibition of Akt activity and apoptosis, we hypothesized that transfection with a myr-Akt mutant or wt Akt may activate a rescue pathway for apoptosis mediated by IL-4 deprivation.

Cells transfected with myr-Akt, a constitutively activated mutant, and deprived of IL-4 showed a reduction of ~45% in the fraction of apoptotic cells com-

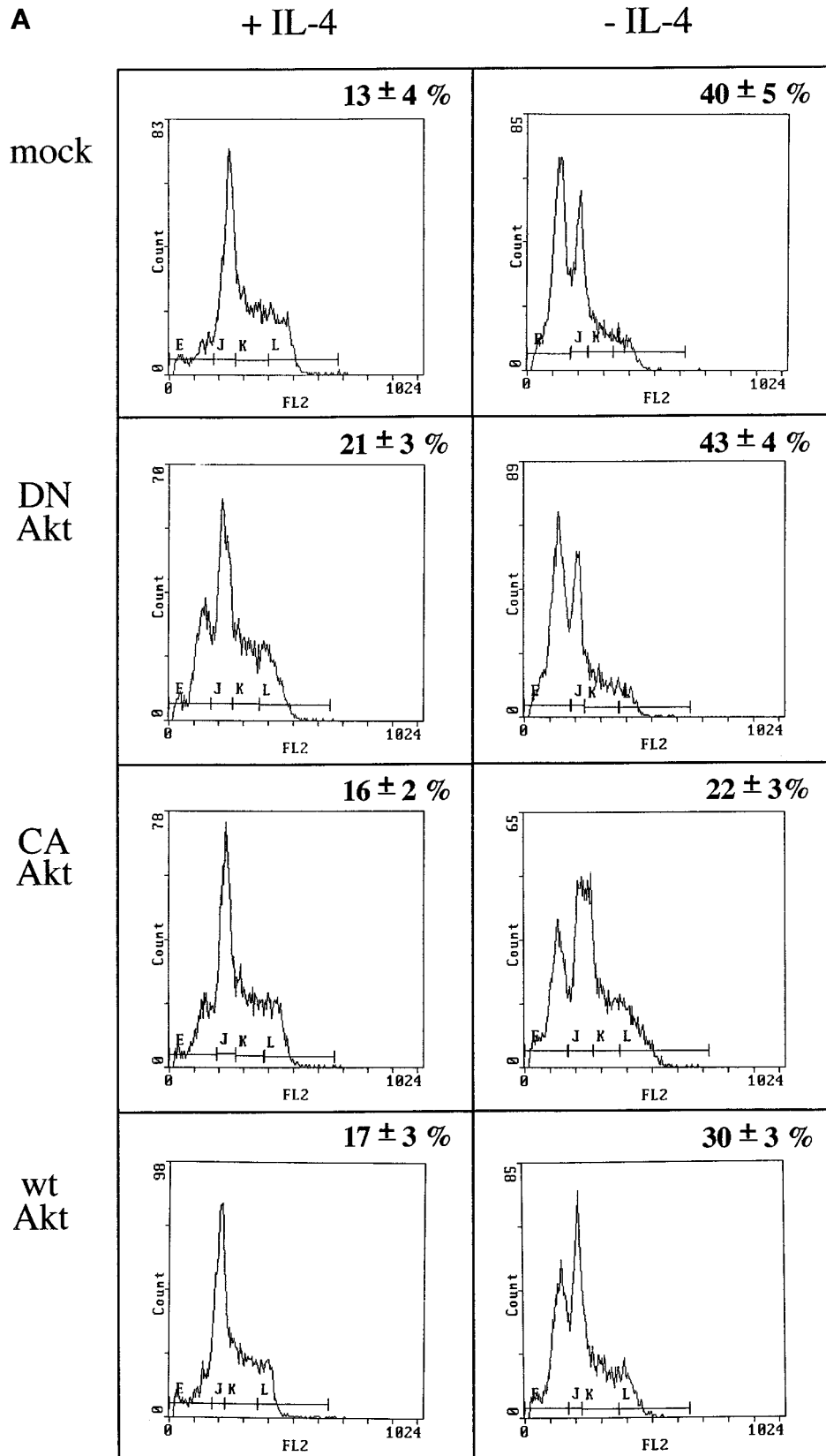


Figure 5.

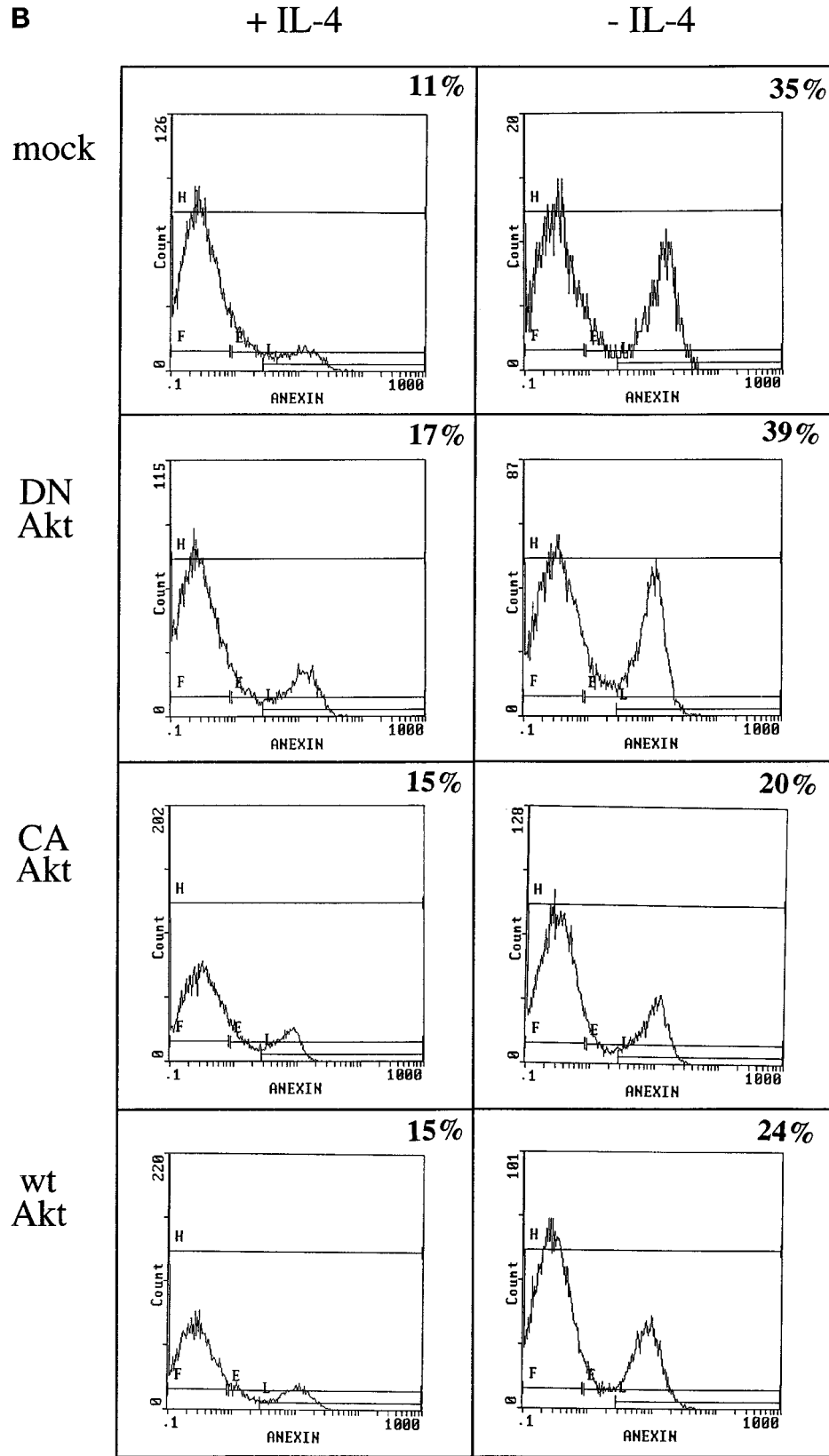


Figure 5 (cont).

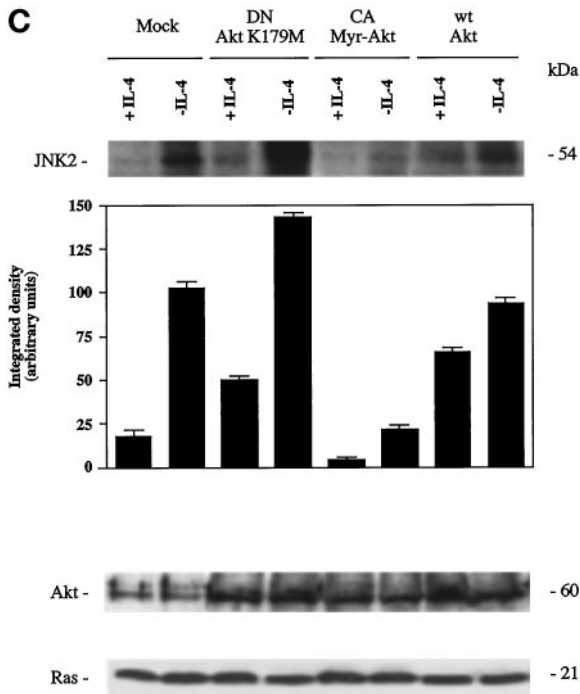


Figure 5. Overexpression of Akt induces JNK2 inhibition and prevents apoptosis induced by IL-4 deprivation in TS1 $\alpha\beta$ cells. (A) Cells were transfected with the indicated constructs by the DEAE-dextran method; maintained for 24 h in the presence or absence of IL-4; and then selected, washed, permeabilized, and stained with propidium iodide. Samples were analyzed by flow cytometry. The most proximal region of the fluorescence scale (region E) represents the subG1 region and corresponds to cells having a hypodiploid DNA content (apoptotic cells). The percentages of apoptotic cells in each sample are superimposed. Similar results were obtained in three independent experiments. (B) Cells were treated as in A. After selection, samples were diluted in ice-cold binding buffer, stained with annexin and propidium iodide, and analyzed by flow cytometry. Percentage of apoptotic cells is superimposed. (C) JNK2 activation and expression of wt Akt, Myr-Akt, or Akt K179 M in cells maintained in the presence or absence of IL-4. Cells were transiently transfected using the DEAE-dextran procedure and maintained 24 h after transfection without lymphokine or in the presence of IL-4. Cells were lysed and protein extracts immunoprecipitated with anti-JNK2 antibody and assayed for JNK2 activity as in Figure 4, or separated by SDS-PAGE, transferred to nitrocellulose, probed with anti-Akt antibody and developed using the ECL system. Densitometric analysis of JNK2 activity is shown below the autoradiography. The blot was stripped and probed with anti-Ras antibody as an internal control of protein loading. Similar results were obtained in three independent experiments. Molecular mass markers are shown.

pared with IL-4-deprived mock-transfected control cells (Figure 5A). Similarly, when wt Akt-transfected cells were deprived of IL-4, the fraction of apoptotic cells was reduced to ~25% of those observed in growth factor-deprived, mock-transfected cells. The apoptosis detected in cells transfected with the dominant negative Akt K179 M mutant in the absence of IL-4 was comparable with that seen in mock-transfected cells under the same culture conditions. The

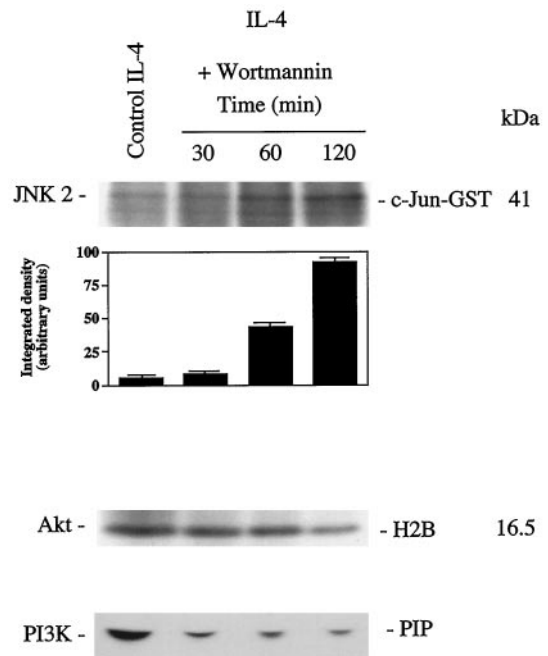


Figure 6. Effect of wortmannin on PI3K, Akt, and JNK2 activity. Cell lysates from control or 100 nM wortmannin-treated cells were immunoprecipitated with anti-Akt or -JNK2 antibody. Akt or JNK2 immunocomplexes were assayed as described in Figures 3 and 4. Densitometric analysis of JNK2 activity is shown below the autoradiography. PI3K activity was determined as described in Figure 3. Migration of the PIP and molecular mass markers are indicated. Similar results were obtained in three independent experiments.

frequency of apoptotic cells remained ~13–17% in all transfected cells in the presence of IL-4, except for Akt K179 M transfectants, which showed a slightly higher level of apoptosis, even in the presence of IL-4. Similar results were obtained when the percentage of apoptotic cells was estimated by annexin staining (Figure 5B).

It was of interest to determine whether constitutive expression of Akt could inhibit JNK2 activation shown after IL-4 deprivation. For this purpose, IL-4-deprived cells were transiently transfected with the constitutively activated myr-Akt mutant. Myr-Akt expression in IL-4-deprived cells strongly blocked JNK2 activation (Figure 5C); similarly, overexpression of wt Akt also significantly blocked JNK2 activation.

Expression of the transiently transfected Akt mutants in TS1 $\alpha\beta$ cells was confirmed by direct comparison of Akt protein levels in transfected cells and in mock-transfected controls (Figure 5C). Twenty-four h posttransfection, Akt expression was increased in IL-4-stimulated or IL-4-deprived cells transfected with myr-Akt, wt Akt, and Akt K179 M compared with mock transfectants. Unaltered Ras expression is shown as an internal control of protein loading.

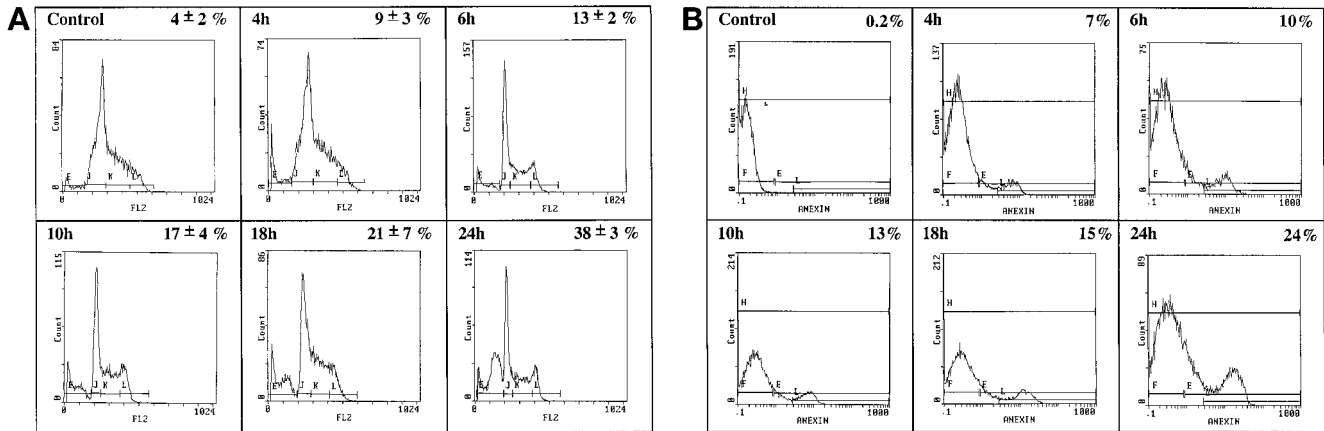


Figure 7. Effect of the PI3K inhibitor wortmannin on the cell survival. (A) Cells cultured in the presence of IL-4 were supplemented with 100 nM wortmannin for different periods of time. After treatment, the percentage of apoptotic cells was analyzed. Cells were permeabilized, stained with propidium iodide, and analyzed for DNA staining by flow cytometry. The most proximal region of fluorescence, region E, represents the subG1 region, which corresponds to cells having a hypodiploid DNA content (apoptotic cells). Similar results were obtained in three independent experiments. (B) Cells were treated as in A. After washing, samples were diluted in ice-cold binding buffer, stained with annexin and propidium iodide, and analyzed by flow cytometry. Percentage of apoptotic cells is superimposed.

Inhibition of PI3K Correlates with Reduction of Akt Activity and JNK2 Activation

To test the role of PI3K in Akt activation and prevention of apoptosis, we inhibited PI3K activity using the inhibitor wortmannin. TS1 α β cells maintained in the presence of IL-4 were treated for different time periods with wortmannin. Treatment of cells induced a remarkable decrease in PI3K and Akt activity as compared with untreated control cells, and wortmannin treatment of IL-4 cultured cells increased JNK2 activity (Figure 6). Given that wortmannin induced reduction of Akt activity, it was of interest to analyze whether PI3K activity inhibition induced apoptosis. IL-4-maintained cells were treated with wortmannin for different time periods and apoptosis was analyzed. Apoptosis increased gradually with time, reaching 38% at 24 h (Figure 7A), which is similar to levels observed after IL-4 deprivation for a similar period of time (Figure 1). The percentage of apoptotic cells estimated by annexin staining was similar to that observed using propidium iodide staining (Figure 7B).

DISCUSSION

Using the lymphokine-dependent T cell line TS1 α β as a model, we analyzed the role of Akt and JNK in the process of cell death promoted by IL-4 deprivation. PI3K-dependent Akt activation is reduced in IL-4-deprived cells and readdition of IL-4 before the commitment point is able to restore Akt activity. Apoptosis induced by lymphokine withdrawal can be prevented by overexpression of Akt. In addition, IL-4 starvation correlates with induction of JNK2 activity.

Recent studies show that Akt activation by growth factors depends on D3 phosphoinositides (Ahmed *et al.*, 1993; Burgering and Coffey 1995; Cross *et al.*, 1995; Downward, 1995; Franke *et al.*, 1995; Alessi *et al.*, 1997; Stokoe *et al.*, 1997), whose synthesis is catalyzed by PI3K (Datta *et al.*, 1996; Klippel *et al.*, 1996; Carpenter and Cantley, 1997). The PI3K inhibitor wortmannin inhibited IL-4-mediated protection from apoptosis in TS1 α β cells. Our results suggest that PI3K and Akt play a role in IL-4-mediated protection from apoptosis, because overexpression of a dominant negative Akt mutant triggers apoptosis, even in the presence of IL-4.

IL-3-dependent activation of Akt is mediated by PI3K activation; consistent with results showing that Akt promotes survival after IL-3 withdrawal (Songyang *et al.*, 1997), Akt also promotes survival after IL-4 deprivation in TS1 α β cells. Myr Akt, which is constitutively activated independently of PI3K by localizing to the plasma membrane, reduces the level of IL-4 deprivation-induced apoptosis by ~45%. wt aKT, which is activated by PI3K, is less effective in preventing apoptosis mediated by IL-4-deprivation, because PI3K activity is down-regulated in the absence of IL-4. In addition, IL-4 is able to restore Akt activity after 3 h of lymphokine deprivation. Akt is also important for IGF-I-dependent survival of primary cerebellar neurons (Dudek *et al.*, 1997) and fibroblasts (Kulik *et al.*, 1997).

Cells dependent on growth factor usually undergo apoptosis upon growth factor deprivation (Downward, 1995). Two major components play a role in the control of the cell death pathway: the Bcl-2 family members, which block apoptosis, and the interleukin

convertin enzyme-like protease family, which executes the apoptotic pathway. In the case of IL-2, a signal transduced by Akt regulates Bcl-2 and c-Myc expression; as a result, these molecules inhibit apoptosis and stimulate proliferation (Ahmed *et al.*, 1997). In TS1 $\alpha\beta$ cells, IL-4-mediated growth and inhibition of apoptosis proceed in the absence of Bcl-2 expression. Consistent with our results, IL-3-dependent Akt activation uses a mechanism for cell survival that does not involve Bcl-2 (Songyang *et al.*, 1997). In addition, we detected no significant change in the steady-state levels of Bcl-x or Bax, either in IL-4-cultured cells or following lymphokine deprivation. IL-4 also induces protection from apoptosis in the myeloid progenitor cell line 32D and in murine spleen B cells via the insulin receptor substrate pathway (Zamorano *et al.*, 1996). Finally, activation of the antiapoptotic signaling pathway PI3K/Akt protects fibroblasts from apoptosis induced by UV-B light and promotes survival of superior cervical neurons (Kulik *et al.*, 1997; Philpott *et al.*, 1997).

JNK and stress-activated protein kinase have been previously associated with the induction of apoptosis (Chen *et al.*, 1995; Dèrijard *et al.*, 1995; Van Dam *et al.*, 1995; Yu *et al.*, 1996). In our cell model, IL-4 deprivation-induced apoptosis correlates with JNK2 activation and up-regulation at the protein level. JNK activation does not necessarily lead to apoptosis; however, IL-1, IL-3, erythropoietin, and thrombopoietin also activate JNK and do not induce apoptosis (Nagata *et al.*, 1997).

Akt activation by growth factors is at least partially dependent on Ras activation in some cell types (Franke *et al.*, 1995), and coexpression of activated mutants of Ras stimulates Akt activity (Klippel *et al.*, 1996; Kauffmann-Zeh *et al.*, 1997). The ability of Ras to stimulate Akt is dependent on PI3K activation, placing this enzyme downstream of Ras in this signaling pathway. In IL-4-dependent T cell lines such as TS1 $\alpha\beta$ -Ras is not activated via the IL-4 receptor (Gómez *et al.*, 1997).

Our results suggest that PI3K and Akt activity correlates with cell survival through the IL-4 receptor. PI3K and Akt inhibition caused by IL-4 deprivation or wortmannin treatment also correlates with JNK2 activation and the ability of Myr-Akt to mediate survival correlates with the inhibition of JNK2 activity.

Although many factor-dependent cell lines respond to IL-4 with increased [³H]thymidine incorporation into DNA, only a few cell lines have been successfully adapted to growth in IL-4 alone. Among them, TS1 $\alpha\beta$ and LD8, two murine T cell lines described by our group, can be propagated indefinitely in IL-4. The findings presented here using an IL-4-dependent murine T cell line as a cellular model, provide new and original insights into the mechanism by which IL-4 could regulate cell survival.

ACKNOWLEDGMENTS

We thank C. Mark for editorial assistance. The Department of Immunology and Oncology was founded and is supported by the Consejo Superior de Investigaciones Científicas and Pharmacia and Upjohn.

REFERENCES

- Ahmed, N.N., Franke, F., Bellacosa, A., Datta, K., Gonzalez-Portal, M.E., Taguchi, T., Testa, J.R., and Tschlis, P.N. (1993). The proteins encoded by c-akt and v-akt differ in post-translational modifications, subcellular localization and oncogenic potential. *Oncogene* 8, 1957–1963.
- Ahmed, N.N., Grimes, H.L., Bellacosa, A., Chan, T.O., and Tschlis, P.N. (1997). Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proc. Natl. Acad. Sci. USA* 94, 3627–3632.
- Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., and Cohen, P. (1997). 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p7056 kinase in vivo and in vitro. *Curr. Biol.* 7, 261–269.
- Bellacosa, A., Testa, J.R., Staal, S.P., and Tschlis, P.N. (1991). A retroviral oncogene, AKT, encoding a serine-threonine kinase containing an SH2-like region. *Science* 254, 244–247.
- Benhamou, L.E., Cazenave, P.A., and Sarthou, P. (1990). Anti-immunoglobulins induce death by apoptosis in WEHI-231 B lymphoma cells. *Eur. J. Immunol.* 20, 1405–1407.
- Berberich, I., Shu, G., Siebelt, F., Woodgett, J.R., Kyriakis, J.M., and Clark, E.A. (1996). Cross-linking CD40 on B cells preferentially induces stress-activated protein kinases rather than mitogen activated protein kinases. *EMBO J.* 15, 92–101.
- Brown, M.A., Pierce, J.H., Watson, C.J., Falcon, J., Ihle, J.N., and Paul, W.E. (1987). B cell stimulatory factor-1/IL-4 mRNA is expressed by normal and transformed mas cells. *Cell* 50, 809–818.
- Burgering, B.M., and Coffey, P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376, 599–602.
- Carpenter, C.L., and Cantley, L.C. (1997). Phosphoinositide kinases. *Curr. Opin. Cell Biol.* 8, 153–158.
- Chen, Y.-R., Meyer, C.F., and Tan, T.H. (1996). Persistent activation of c-Jun N-terminal kinase (JNK1) in gamma radiation-induced apoptosis. *J. Biol. Chem.* 271, 631–634.
- Chen, Y.R., Meyer, C.F., and Tan T.H. (1995). Persistent activation of JNK1 in gamma irradiation-induced apoptosis. *J. Biol. Chem.* 27, 631–643.
- Coffey, P.J., and Woodgett, J.R. (1991). Molecular-cloning and characterization of a novel putative protein-serine kinase related to the cAMP-dependent and protein-kinase-C families. *Eur. J. Biochem.* 201, 475–481.
- Cosman, D. (1993). The hematopoietic receptor superfamily. *Cytokine* 5, 95–106.
- Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by PKB. *Nature* 378, 785–789.
- Datta, K., Bellacosa, A., Chan, T.O., and Tschlis, P.N. (1996). Akt is a direct target of the phosphatidylinositol 3 kinase. Activation by growth factor, v-src and v-Ha-ras in Sf9 and mammalian cells. *J. Biol. Chem.* 271, 30835–30839.
- Dèrijard, B., Hibi, M., Wu, Y.H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. (1994). JNK1: a protein kinase stimulated by UV

- light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 76, 1025–1037.
- Didichenko, S.A., Tilton, B., Hemmings, B.A., Ballmer-Hofer, K., and Thelen, M. (1996). Constitutive activation of the protein kinase B and phosphorylation of the p47phox by a membrane-targeted phosphoinositide 3-kinase. *Curr. Biol.* 6, 1271–1278.
- Downward, J. (1995). Signal transduction. A target for PI(3) kinase. *Nature* 376, 553–554.
- Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.S., Kaplan, D.R., and Greenberg, M.E. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 275, 661–665.
- Duke, R.C., and Cohen, J.J. (1986). IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent T cells. *Lymphokine Res.* 5, 289–299.
- Franke, T.F., Kaplan, D.R., Cantley, L.C., and Toker, A. (1997). Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 275, 665–668.
- Franke, T.F., Yang, S.Y., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., and Tsichlis, P.N. (1995). The protein kinase encoded by the akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81, 727–736.
- Giri, J.G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumanki, S., Namen, A., Park, L.S., Cosman, D., and Anderson, D. (1994). Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* 13, 2822–2830.
- Gómez, J., Martínez-A., C., Fernandez, B., Garcia, 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.
- Gupta, S., Campbell, D., Dèrijard, B., and Davis, R.J. (1995). Transcription factor ATF2 regulation by JNK signal transduction pathway. *Science* 267, 389–393.
- Hasbold, J., and Klaus, G.G.B. (1990). Anti-immunoglobulins antibodies induce apoptosis in immature B cell Lymphomas. *Eur. J. Immunol.* 20, 1685–1690.
- 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.
- Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hanaoka, T., and Paul, W.E. (1982). Identification of a T cell derived beta cell growth factor distinct from IL-2. *J. Exp. Med.* 155, 914–923.
- Jones, P.F., Jakubowicz, T., Pitossi, F.J., Maurer, F., and Hemmings, B.A. (1991). Molecular-cloning and identification of a serine threonine protein-kinase of the 2nd-messenger subfamily. *Proc. Natl. Acad. Sci. USA* 88, 4171–4175.
- Karin, M. (1995). The regulation of AP1 activity by MAP kinases. *J. Biol. Chem.* 270, 16483–16486.
- Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffey, P., Downward, J., and Evan, G. (1997). Suppression of the c-Myc-induced apoptosis by Ras signaling through PI(3)K and PKB. *Nature* 385, 544–548.
- Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordan, J., Bellacosa, A., Tsichlis, P.N., and Hay, N. (1997). The PI3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev.* 11, 701–713.
- Kharbanda, S., Ran, R., Pandey, P., Shafman, T.D., Feller, S.M., Weichselbaum, R.R., and Kufe, D.W. (1995). Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. *Nature* 376, 785–788.
- Khawaja, A., Rodriguez-Viciana, P., Wennstorn, S., Warne, P.H., and Downward, J. (1997). Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* 16, 2783–2793.
- Kimura, K., Leonard, W.J., Noguchi, M., and Russell, S.M. (1995). Sharing of a common gamma chain by the IL-4, IL-2 and IL-7 receptor. *Adv. Exp. Med. Biol.* 385, 225–232.
- Klippel, A., Kavanaugh, W.M., Pot, D., and Williams, L.T. (1997). A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol. Cell. Biol.* 17, 338–344.
- Klippel, A., Reinhard, C., Kavanaugh, W.M., Apell, G., Escobedo, M.A., and Williams, L.T. (1996). Membrane localization of phosphatidylinositol 3 kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol. Cell. Biol.* 16, 4117–4127.
- Kondo, M., Takeshita, H., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S., and Sugamura, K. (1994). Functional participation of the IL-2R gamma chain in IL-7 receptor complexes. *Science* 263, 1453–1454.
- Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K., and Sugamura, K. (1993). Sharing the IL-2 receptor gamma chain between receptors for IL-2 and IL-4. *Science* 262, 1874–1877.
- Konishi, K., Matsuzaki, H., Tanaka, M., Ono, Y., Tokunaga, C., Kuroda, S., and Kikkawa, U. (1996). Activation of Rac-protein kinase by heat shock and hyperosmolarity stress through a pathway independent of PI3 kinase. *Proc. Natl. Acad. Sci. USA* 93, 7639–7644.
- Kulik, G., Klippel, A., and Weber, M.J. (1997). Antiapoptotic signaling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Mol. Cell. Biol.* 17, 1595–1606.
- Kyriakis, J.M., and Avruch, J. (1996). Protein kinase cascades activated by stress and inflammatory cytokines. *Bioessays* 18, 567–577.
- Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubi, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994). The stress-activated protein kinase subfamily of the c-Jun kinases. *Nature* 369, 156–160.
- Lin, J.-X., et al. (1995). The role of pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13 and IL-15. *Immunity* 2, 331–339.
- Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Dèrijard, B., Davis, R.J., Johnson, G.L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* 266, 1719–1723.
- Morla, A.O., Schreurs, J., Miyajima, A., and Wang, J.Y.J. (1988). Hematopoietic growth factors activate the tyrosine phosphorylation of distinct sets of proteins in interleukin-3-dependent murine cell lines. *Mol. Cell. Biol.* 8, 2214–2218.
- Nagata, Y., Nishida, E., and Todokono, K. (1997). Activation of JNK signaling pathway by erythropoietin, thrombopoietin and IL-3. *Blood* 89, 2664–2669.
- Noguchi, M., Nakamura, Y., Russell, S.M., Ziegler, S.F., Tsang, M., Cao, X., and Leonard, W.J. (1993). IL-2 receptor gamma chain: a functional component of the IL-7 receptor. *Science* 262, 1877–1880.
- Núñez, G., London, L., Hockenbery, D., Alexander, M., McKeown, J.P., and Korsmeyer, S.J. (1990). Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell line. *J. Immunol.* 144, 3602–3610.
- Paul, W.E. (1991). IL-4: a multifunctional regulator of immunity and inflammation. *J. Cancer Res.* 82, 1458–1459.
- Philpott, K.L., McCarthy, M.J., Klippel, A., and Rubin, L.L. (1997). Activated PI3 Kinase and Akt kinase promote survival of superior cervical neurons. *J. Cell. Biol.* 139, 809–815.

- Pitton, C., Rebollo, A., Van Snick, J., Theze, J., and García, A. (1993). High-affinity and intermediate-affinity forms of the human interleukin 2 receptor, expressed in an interleukin 9-dependent murine T cell line, deliver proliferative signals via differences in their transduction pathways. *Cytokine* 5, 362–371.
- Quelle, F.W., *et al.* (1995). Cloning of murine Stat6, Stat proteins that are tyrosine phosphorylated in response to IL-4 and IL-3 but are not required for mitogenesis. *Mol. Cell. Biol.* 15, 3336–3343.
- Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J., and Davis, R.J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* 270, 7420–7426.
- Russell, S.M., Johnston, J.A., Noguchi, M., Kawamura, M., Bacon, C.M., Friedman, M., Berg, M., McVicar, D.W., Witthunn, B.A., and Silvennoinen, O. (1994). Interaction of IL-2R beta and gamma chains with Jak1 and Jak3. implications for XSCID and XCID. *Science* 266, 1042–1045.
- Russell, S.M., Keegan, A.D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M.C., Miyajima, A., Puri, R.K., and Paul, W.E. (1993). IL-2R gamma chain, a functional component of the IL-4 receptor. *Science* 262, 1880–1883.
- Sakata, N., Patel, H.R., Terada, N., Aruffo, A., Johnson, G.L., and Gelfond, E.W. (1995). Selective activation of c-Jun kinase mitogen-activated protein kinase by CD-40 on human B cells. *J. Biol. Chem.* 270, 30823–30828.
- Seder, R.A., Paul, W.E., Ben-Sasson, S.Z., LeGross, G.S., Kagey-Sobotka, A., Finkelman, F.D., Pierce, J.H., and Plant, M. (1981). Production of IL-4 and other cytokines following stimulation of mast cell lines and in vivo mast cells/basophils. *Int. Arch. Allergy. Appl. Immunol.* 94, 137–140.
- Sluss, H.K., Barrett, T., Dèrijard, B., and Davis, R.J. (1994). Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol. Cell. Biol.* 14, 8376–8384.
- Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J., and Owen, J.J.T. (1989). Antibodies to CD3/T cell receptor complex induce cell death by apoptosis in immature T cell in thymic culture. *Nature* 337, 181–184.
- Songyang, Z., Baltimore, D., Cantley, L.C., Kaplan, D.R., and Franke, T.F. (1997). Interleukin 3-dependent survival by the Akt protein kinase. *Proc. Natl. Acad. Sci. USA* 94, 11345–11350.
- Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reese, C.B., Painter, G.F., Holmes, A.B., McCormick, F., and Hawkins, P.T. (1997). Dual role of phosphatidylinositol 3,4,5-triphosphate in the activation of protein kinase B. *Science* 277, 567–570.
- Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994). JNK is involved in signal integration during co-stimulation of T lymphocytes. *Cell* 77, 727–736.
- Van Dam, H., Wilhem, D., Herr, I., Steffen, A., Herlich, P., and Angel, P. (1995). ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J.* 14, 1798–1811.
- Waters, R.L. (1992). Radiation-induced apoptosis in a murine T-cell hybridoma. *Cancer Res.* 52, 883–890.
- Westwick, J.K., Bielawska, A.E., Dbaibo, G., Hannun, Y.A., and Brenner, D.A. (1995). Ceramide activates the stress-activated protein kinases. *J. Biol. Chem.* 270, 22689–22692.
- Whitmarsch, A.J., Shore, P., Sharroks, A.D., and Davis, R.J. (1995). Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 269, 403–407.
- Williams, G.T., Smith, C.A., Spooncer, E., Drexler, T.M., and Taylor, D.R. (1990). Haemopoietic colony-stimulating factors promote cell survival by suppressing apoptosis. *Nature* 343, 76–79.
- Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous nuclease activation. *Nature* 284, 555–556.
- Yu, R., Shtil, A.A., Tan, T.H., Roninson, I.B., and Kong, A.N.T. (1996). Adriamycin activates c-jun N-terminal kinase in human leukemia cells. A relevance to apoptosis. *Cancer Lett.* 107, 73–81.
- Zamorano, J., Wang, H.Y., Wang, L.M., Pierce, J.H., and Keegan, A.D. (1996). IL-4 protects cells from apoptosis via the insulin receptor substrate pathway and a second independent signaling pathway. *J. Immunol.* 157, 4926–4934.
- Zubiaga, A.M., Muñoz, E., and Huber, B.T. (1992). IL-4 and IL-2 selectively rescue Th cell subsets from glucocorticoid-induced apoptosis. *J. Immunol.* 149, 107–112.