Activation of the Phosphatidylinositol 3-Kinase/Akt Pathway Protects against Interleukin-3 Starvation but Not DNA Damage-induced Apoptosis*

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Baf-3 cells are dependent on interleukin-3 (IL-3) for their survival and proliferation in culture. To identify anti-apoptotic pathways, we performed a retroviral-insertion mutagenesis on Baf-3 cells and selected mutants that have acquired a long term survival capacity. The phenotype of one mutant, which does not overexpress bcl-x and proliferates in the absence of IL-3, is described. We show that, in this mutant, Akt is constitutively activated leading to FKHRL1 phosphorylation and constitutive glycolytic activity. This pathway is necessary for the mutant to survive following IL-3 starvation but is not sufficient or necessary to protect cells from DNA damage-induced cell death. Indeed, inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in Baf-3 cells does not prevent the ability of IL-3 to protect cells against γ-irradiation-induced DNA damage. This protective effect of IL-3 rather correlates with the expression of the anti-apoptotic Bcl-x protein. Taken together, these data demonstrate that the PI3K/Akt pathway is sufficient to protect cells from growth factor starvation-induced apoptosis but is not required for IL-3 inhibition of DNA damage-induced cell death.

Growth factors are necessary to inhibit the intrinsic apoptotic machinery, which is constitutively expressed in all cells. Baf-3 cells are dependent on IL-3 for their proliferation and survival (1). A number of signaling pathways that are activated by IL-3 play a role in the inhibition of apoptosis (2–4). One major signaling pathway involved in the control of cell death by growth factors is the PI3K/Akt pathway. Activation of PI3K leads to the generation of 3′-phosphorylated phosphatidylinositides that act by multiple mechanisms to activate Akt (5). Akt will in turn phosphorylate proteins that play a key role in the control of apoptosis. These proteins include Bad, a pro-apoptotic bcl-2 family member, which when phosphorylated by Akt, releases Bcl-x allowing it to perform its anti-apoptotic function (6, 7). Caspase 9, another effector protein of the intrinsic cell-death machinery can also be inactivated following phosphorylation by Akt (8). Finally, the transcription factor FKHRL1 that regulates the expression of genes encoding pro-apoptotic proteins such as Bax-ligand is located in the cytoplasm following its phosphorylation by Akt on serine 253 and threonine 32 (9, 10).

Growth factors can also delay DNA damage-induced death leading in some systems to an increased clonogen survival (11, 12). It has previously been shown that IL-3 protects Baf-3 cells from DNA damage-induced apoptosis (13, 14). Indeed, in the absence of IL-3, the kinetics of cell death is accelerated following DNA damage. In contrast, in the presence of IL-3, cells are resistant to high doses of DNA damage-inducing agents. The increased rate of death observed when cells are irradiated in the absence of IL-3 is dependent on functional p53, indicating that IL-3 acts by inhibiting a p53-dependent apoptotic pathway (15). There are multiple pathways down-stream of p53 that are potentially involved in the induction of apoptosis (16–18). p53 regulates the transcription of pro-apoptotic genes such as Bax, Fas, or PERP (19–21), which could play a role in this process, although some of them such as Bax could have a redundant function because p53-dependent death is not affected by its absence (22). Recently it was shown that, in irradiation or myc-induced p53-dependent death, APAF-1 and Caspase 9 were essential down-stream targets of p53 (23).

The signaling pathways involved in the inhibition of p53-dependent apoptosis by growth factors have been studied in a number of systems. In erythropoietin-dependent myeloid cell lines, Jak2 kinase activation by erythropoietin receptor mutants was shown to be necessary and sufficient to inhibit p53-dependent apoptosis induced by γ-irradiation (24). In the same system, activation of other signaling pathways, including PI3K, STATs, and Ras was not required to inhibit death. In contrast, it has recently been shown that death induced by p53 expression could be inhibited by activating the PI3K/Akt pathway (25). Finally, it has been suggested that IL-3 could delay p53-dependent apoptosis induced by γ-irradiation by regulating the levels of p21 and Rb, two proteins involved in the regulation of the G1/S transition (14, 26). Although in one report, the expression of v-Src or activated c-Raf could mimic the effect of IL-3 on p21 levels, the signaling pathway activated by IL-3, involved in the control of G1 arrest or apoptosis following DNA damage, has not been identified.

We have performed a retroviral insertion mutagenesis to
obtain mutants that are resistant to apoptosis following growth factors starvation. We describe the characterization of one mutant that proliferates in the absence of growth factors and that, in contrast to previously described mutants, does not overexpress bcl-x (27). This mutant (the S4 mutant) is able to survive for prolonged periods of time in the absence of IL-3 but shows no resistance to γ-irradiation-induced cell death. This mutant shows a constitutive IL-3-independent, 3'-phosphorylated phosphatidylinositol-dependent, Akt kinase activation. In this mutant, we could demonstrate that Akt activation leads to FKHR1 phosphorylation and constitutive glycolytic activity. In Baf-3 cells, glycolysis is regulated by IL-3 and is the main ATP-generating source (28). The activation of Akt was necessary for the survival observed in the absence of IL-3. In contrast we show in the S4 mutant and in Baf-3 cells that Akt activation is neither sufficient nor necessary to inhibit p53-dependent DNA damage-induced cell death. These results indicate that IL-3 activates multiple signaling pathways, which can inhibit growth factor starvation-induced apoptosis (called intrinsic apoptosis hereafter); however, only some of these can delay DNA damage-induced p53-dependent apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—The bone marrow-derived IL-3-dependent Baf-3 cells were maintained in DMEM containing 6% fetal calf serum (Roche Molecular Biochemicals), 2 mM l-glutamine (Life Technologies, Inc.), and 5% WEHI 3B cell-conditioned medium as a source of IL-3. Cells were grown at a density of 5 × 10^4 to 8 × 10^4 per ml. To remove IL-3, cells were washed twice with warm DMEM/fetal calf serum 6%/glutamine 2 mM and cultured in this medium. Baf-3 cells overexpressing bcl-x (Baf-bcl-x) and bcl-2 (Baf-bcl-2) have been described elsewhere (27, 29). Recombinant murine IL-3 was obtained from PeproTech. Ly294002 (20 mM, Calbiochem) was resuspended in Me2SO (Sigma Chemical Co.). 2-Deoxy-glucose was obtained from Sigma.

**Retroviral Infection of Baf-3 Cells**—Baf-3 cells (1) (6–7 × 10^7) were infected by co-incubation with M3Pneo-sup-producing cells (PAPM3 cells) (30). Baf-3 cells were co-cultivated for 48 h with semi-confluent PAPM3 cells. Polybrene at the concentration of 8 μg/ml (Sigma) and 5% newborn bovine serum (Life Technologies) added to the Baf-3 cell culture and cultured for another 2 days to allow retrovirus-induced expression of neighboring cellular genes. Thereafter, cells resistant to IL-3 starvation-induced apoptosis were selected.

Individual clones were obtained by limiting dilutions. After a first IL-3 starvation resistance screen, mutants were further characterized for their survival capacity to other apoptotic pathways.

**Measurement of Apoptosis and Proliferation**—Apoptotic and dead cells were detected by propidium iodide staining. 5 × 10^4 to 10^5 cells were incubated a few minutes with propidium iodide (Sigma) at a concentration of 5 μg/ml. For each sample, 5000 cells were counted on a FACScan (Becton-Dickinson) and analyzed on a FSC/FL2 dot plot. This staining protocol allows the distinction between dead (FL-2 bright, FSC high), apoptotic (FL-2 dull, FSC intermediate), and live cells (FL-2-negative, FSC high). The percentage of viable cells corresponds to the percentage of FL-2-negative cells.

Annexin V staining was realized according to the manufacturer’s instructions (PharMingen). For proliferation assay, life cells were plated at 10^5 cells per well and pulsed for 16 h with 0.5 μCi of [γ32P]thymidine/well (2.0 Ci/mmol, Amersham Pharmacia Biotech). 

**Glucose Metabolism Evaluation**—1.5 × 10^5 cells were incubated in 4 ml of Krebs-Henseleit buffer (31), pH 7.4, containing 0.1 unit/ml recombinant IL-3, 12.5 mM [14C]glucose uniformly labeled (3.14 Bq/ml) in 25-ml stoppered conical flasks with an isolated central well and filled with a 95%/5%O2/CO2 mixture. Incubations were performed for 2 h in a shaking water bath at 37 °C. Reactions were stopped by adding perchloric acid (2% final concentration) in each flask. 14CO2 collection was performed by addition of NaOH 5% in the central well in isopropyl alcohol used to quench the reaction. 14CO2 release was measured by liquid scintillation counting as described previously (32). The Krebs medium from each flask was centrifuged to remove denatured proteins, and the supernatant was neutralized using a 20% KOH/1% phosphoric acid solution before the measurement of metabolite concentrations. Glucose consumption and pyruvate and lactate accumulation were evaluated as previously described (32, 33). Substrate utilization or metabolites production were calculated as the difference between metabolites content in the flask before incubation and after 2-h incubation.

**Lactate Production Measurement**—Cells were washed twice in pre-warmed DMEM containing 1% fetal calf serum and 2 mM glutamine and resuspended in 1 ml of DMEM containing no lactate or no IL-3 and 1 mM 2-deoxy-glucose. Lactate was measured using a lactate measurement kit (Sigma Diagnostics) according to the manufacturer’s instructions.

**Western Blot**—Cells were washed in cold PBS and pellets were lysed for 10 min at 4 °C in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 20 μg/ml aprotinin, 10 μg/ml pepstatin, 20 μg/ml leupeptin, 200 μg/ml PMSF, and 1 mM orthovanadate). Cells were then centrifuged 15 min at 4 °C to eliminate cellular debris. Protein concentration was determined by the Bradford procedure using the Bio-Rad protein assay. Proteins were separated on 12.5%, 10%, or 7.5% polyacrylamide SDS-polyacrylamide gel electrophoresis gels. Gels were electroblotted onto polyvinylidene difluoride membranes (Millipore) using a liquid transfer apparatus (Bio-Rad).

**Retrovirus Production**—The pseudotyped retrovirus was isolated using the RNA Now method (Ozyme) according to the manufacturer’s instructions. The bcl-x mRNA level was measured by RNase protection assays using the Riboquant kit (Becton-Dickinson) following the instructions of the supplier. Briefly, 5 μg of RNA was hybridized overnight to the 32P-labeled RNA probe, which had previously been synthesized from the supplied template (m-npo). Single-stranded RNA and free probe were digested by RNase A and T1. Subsequently, protected RNA was purified and analyzed on a 6% denaturing polyacrylamide gel. The quantity of protected RNAs was determined using a PhosphorImager and ImageQuant (both from Molecular Dynamics, Sunnyvale, CA).

**Lactate Production**—Lactate production was measured using a lactate measurement kit (Sigma Diagnostics) according to the manufacturer’s instructions. The bcl-x mRNA level was measured by RNase protection assays using the Riboquant kit (Becton-Dickinson) following the instructions of the supplier. Briefly, 5 μg of RNA was hybridized overnight to the 32P-labeled RNA probe, which had previously been synthesized from the supplied template (m-npo). Single-stranded RNA and free probe were digested by RNase A and T1. Subsequently, protected RNA was purified and analyzed on a 6% denaturing polyacrylamide gel. The quantity of protected RNAs was determined using a PhosphorImager and ImageQuant (both from Molecular Dynamics, Sunnyvale, CA).

**IL-3 Starvation**—IL-3 was withdrawn from the culture medium of parental Baf-3 cells for 0, 1, 2, 3, or 4 days and then assayed by limiting dilution.

**RESULTS**

The S4 Mutant Survives and Proliferates in the Absence of IL-3—The IL-3 starvation-resistant Baf-3 mutant S4 was obtained by retroviral insertion mutagenesis as described under “Experimental Procedures.” The survival capacity of this mutant was compared with Baf-3 cells overexpressing bcl-2 or bcl-x. Results in Fig. 1A show that the S4 mutant survives to IL-3 starvation for prolonged periods of time, more than 70% of the cells being viable after 5 days in culture in the absence of IL-3. In contrast, nearly all parental Baf-3 control cells were dead after 1-day culture in the absence of IL-3. Overexpression of bcl-2 or bcl-x leads to a marked survival advantage of growth factor but was unable to confer long-term survival, because most cells were dead by 4 days of culture. The proliferative capacity of this mutant in the absence of IL-3 was also assessed. As previously described, the Baf-3 cell lines overexpressing bcl-2 or bcl-x did not proliferate in the absence of IL-3 (Fig. 1B) (27, 29). In contrast, 48 h following IL-3 removal, the...
The S4 mutant were irradiated with full diamond
age-induced apoptosis. Cells overexpressing the S4 mutant, we have measured its resistance to DNA dam-

To further characterize the survival capacity of culture experiments (data not shown).

Indeed we have been unable to induce the survival of parental Baf-3 cells with the supernatant of the S4 mutant or by co-

Finally, we have excluded the possibility that this mutant is
table involved in anaerobic glycolysis. The glycolytic pathway is
regulated by IL-3 and is the main ATP-generating source in
IL-3-dependent cell lines (35, 36). To characterize the glucose
metabolism in Baf-3 cells and in the S4 mutant, we have

FIG.1. S4 mutant survival and proliferation in the absence of IL-3. A, cells were grown in the absence of IL-3 and the percentage of viable cells was measured by propidium iodide exclusion at indicated time. One representative experiment out of three is presented. Full circle, S4 cells; open square, Baf-bcl-x cells; open circle, Baf-bcl-2 cells; and full diamond, Baf-3 cells. B, cells were deprived of IL-3 for 48 h. Proliferation in the absence of IL-3 was assessed on 1 × 10⁶ viable cells by a 16-h [³H]thymidine incorporation pulse. Data are presented as mean values ± S.D. of three independent experiments. C, Baf-3 and S4, Baf-bcl-x cells were cultured in the presence or in the absence of IL-3 during 8 and 24 h, respectively. Total RNA was purified as described under “Experimental Procedures,” and 10 μg was used to perform a Northern blot analysis. Expression of bcl-x and GAPDH was detected using the probes described under “Experimental Procedures.”

The Glycolytic Activity Is IL-3-independent in the S4 Mu-

The S4 Mutant Is Not Resistant to DNA Damage-induced Cell Death—To further characterize the survival capacity of the S4 mutant, we have measured its resistance to DNA damage-induced apoptosis. Cells overexpressing bcl-2 or bcl-x and the S4 mutant were irradiated with γ-rays. Their survival was monitored after 24-h culture in the absence of IL-3 (Fig. 2A). As previously described, bcl-2- or bcl-x-overexpressing cells sur-
vived to irradiation doses up to 12.5 Gy, confirming that over-
expression of these proteins confers a resistance to growth factor starvation and DNA damage-induced cell death (13, 34). In contrast, the S4 mutant, which showed more than 70% viable cells after 24 h culture in the absence of IL-3, was unable to survive irradiation doses as low as 1.5 Gy. Indeed, following

irradiation with 1.5 Gy and culture in the absence of IL-3, this mutant showed a death kinetic similar to parental Baf-3 cells (Fig. 2B). Results obtained with the S4 mutant suggest that the signaling pathway activated by IL-3, which protects against DNA damage-induced death, differs from the signaling pathway leading to the inhibition of the intrinsic death program triggered in the absence of growth factors. Indeed the S4 mu-
tant can be maintained in culture in the absence of IL-3 for more than 5 days, but shows the same susceptibility as Baf-3 cells to apoptosis induced by irradiation in the absence of growth factors. The absence of protection was not due to an increased sensitivity of S4 mutant to DNA damage that could not be inhibited by IL-3, because this mutant showed the same death kinetic as parental Baf-3 cells when irradiated in the presence of IL-3 (data not shown). This indicates that, in this mutant, the survival pathway that protects cells from DNA damages has not been activated, whereas one survival pathway leading to the inhibition of the intrinsic death program is preserved.
Baf-3 cells were cultured in the presence of 2-deoxy-glucose, a glucose analogue, which is a potent inhibitor of glycolysis acting as a competitive inhibitor of glucose transport. Cell viability was then measured at different time points. After 16 h in these conditions, more than 30% of Baf-3 cells are apoptotic as measured by propidium iodide and Annexin V staining (Fig. 3D). Similarly, 48% of control Baf-3 cells are apoptotic 16 h following IL-3 starvation. These results show that regulation of glucose metabolism by IL-3 in Baf-3 cells is essential for their survival.

**Akt and FKHR1 Are Constitutively Phosphorylated in the Absence of IL-3 in the S4 Mutant—Insulin regulates glycolysis through activation of the Phosphatidylinositol 3-kinase/PKB pathway. Phosphorylation by Akt/PKB leads to the activation of phosphofructo-2-kinase and the inhibition of glycogen synthase kinase-3, which are key regulators of the glycolytic pathway (37, 38). Akt has also been involved in glucose transporters translocation, mainly GLUT4 and GLUT1, leading to an increase in glucose uptake (39–41). To test if glycosylation, as mirrored by lactate production, is regulated by IL-3 through a PI3K-dependent pathway, we have measured the lactate produced by Baf-3 and S4 cells in the presence or absence of the PI3K inhibitor Ly294002. As shown in Fig. 4A, the lactate concentration produced by Baf-3 and S4 cells in the presence of IL-3 was reduced by more than 70% in the presence of the PI3K inhibitor Ly294002. This inhibition was independent of cell death, because cells were more than 80% viable at that time point. These results indicate that PI3K activation by IL-3 regulates anaerobic glycolysis in Baf-3 cells and in the S4 mutant. Similarly, the lactate production, which is observed in S4 cells in the absence of IL-3, was inhibited by the addition of the PI3K inhibitor Ly294002 (Fig. 4B). This indicates that a PI3K-dependent pathway is activated in the absence of IL-3 in the S4 mutant.

To confirm these results, the activation status of the serine/threonine kinase Akt/PKB was measured. PI3K activation following growth factor stimulation leads to the activation and phosphorylation of Akt on serine 473 and threonine 308 (42). The phosphorylation status of Akt in the S4 mutant was thus monitored using a phospho-Akt (Ser-473)-specific antibody. In IL-3-starved Baf-3 and S4 cells, Akt phosphorylation was strongly induced by addition of IL-3 (Fig. 5A), confirming Akt regulation by this growth factor. In Baf-3 cells, no phosphorylation of Akt could be detected following 8 h culture in the absence of IL-3. In contrast, in S4 cells deprived of IL-3 for 16 h we detected a level of Akt phosphorylation similar to the physiological level, which is observed in Baf-3 or S4 cells continuously maintained in the presence of IL-3. This phosphorylation could be inhibited by the addition of the PI3K inhibitor LY294002 (Fig. 5B), indicating that in the S4 mutant constitutive Akt activation was dependent on the generation of 3'-phosphorylated phosphatidylinositides.

To test if the constitutive phosphorylation of Akt on Ser-473 observed in the S4 mutant in the absence of IL-3 leads to an *in vivo* Akt activation, we have monitored the level of phosphorylation of different known Akt substrates. In Baf-3 cells we have been unable to detect the phosphorylation of the Bad protein in response to IL-3. In contrast, an IL-3-dependent phosphorylation of FKHRL1 on threonine 32 was found in Baf-3 and S4 cells (Fig. 5C). FKHRL1 phosphorylation on serine 253 was also found in both cell lines but was independent of IL-3. In the S4 mutant an IL-3-independent, 3'-phosphorylated phosphatidylinositides-dependent FKHRL1 phosphorylation was found (Fig. 5D), confirming that the constitutive Akt phosphorylation, which is observed in the S4 mutant in absence of IL-3, leads to an *in vivo* Akt activation.
Akt Activation Is Required for the Survival of the S4 Mutant in the Absence of IL-3—

To determine whether activation of Akt was necessary for the survival of the S4 mutant in the absence of IL-3, we have measured the survival of these cells when grown in the presence of the PI3K inhibitor Ly294002. The survival of the S4 mutant is strongly decreased by using 10 μM Ly294002 (Fig. 6A). This concentration inhibits the constitutive Akt activation (Fig. 5B) and the glycolytic activity (Fig. 4B) observed in the S4 mutant in the absence of IL-3. In contrast, using similar doses of the PI3K inhibitor Ly294002, IL-3-induced survival was maintained confirming that a PI3K-independent survival pathway is activated by IL-3 in Baf-3-derived cells (43). These results confirm that the constitutive Akt activation observed in the S4 mutant is necessary for the survival of these cells in the absence of IL-3. The constitutive Akt activation did not allow the maintenance of bcl-x mRNA or protein levels (Fig. 6, B and C) normally found in the presence of IL-3, indicating that inhibition of the intrinsic death pathway can take place in the absence of high Bcl-x levels.

Inhibition of DNA Damage-induced Apoptosis by IL-3 Is Independent of Akt—Although Akt activation in S4 cells is necessary and sufficient for survival in the absence of growth factor, it is not able to delay DNA damage-induced apoptosis. The acceleration of death that is observed in the absence of growth factor following DNA damage is dependent on functional p53 (15). Hence our results would indicate that the Akt pathway is not involved in the inhibition of p53-dependent apoptosis by growth factors. To confirm this hypothesis we tested the ability of IL-3 to inhibit DNA damage-induced death following PI3K inhibition by Ly294002 in Baf-3 cells. Results in Fig. 7A show that following irradiation the death rate of Baf-3 cells was increased in the absence of IL-3 but not in the presence of growth factors. In both conditions 1.5-Gy irradiation was associated with a p53 up-regulation within 1 h (Fig. 7B), which was maintained up to 10 h (Fig. 7C), at a time where, in the absence of IL-3, more than 50% of irradiated Baf-3 cells had already undergone apoptosis. The relative increase in p53 protein level induced by irradiation was not affected by the concentration of Ly294002 used, indicating that the p53-dependent apoptotic pathway was also activated in these conditions. Inhibition of PI3K by Ly294002 did inhibit the Akt phosphorylation observed in response to IL-3 (Fig. 7B) but did not abrogate the ability of IL-3 to protect against DNA damage-induced death (Fig. 7A). We reproducibly found that the level of total Akt protein was reduced in Baf-3 cells irradiated and cultured in the absence of IL-3. This could reflect a selective degradation of that protein in apoptotic cells that represents more than 50% of the pellet. These results confirm that Akt activation by IL-3 is not necessary for the protection against DNA damage-induced apoptosis. In contrast, there was a

**Fig. 3.** Glucose transport and glycolytic activity are independent of IL-3 in the S4 mutant. A, cells were maintained in the absence or in the presence of IL-3 as indicated, at a concentration of 1 × 10⁵ cells per ml. Viability (open bars) and lactate concentration (full bars) for 1 × 10⁵ viable cells were measured after 9 h. Data are presented as mean values ± S.D. of three independent experiments. B, the S4 mutant was cultured in the presence (full circle) or in the absence (full square) of IL-3 at a concentration of 1 × 10⁵ cells per ml, and the lactate concentration in the supernatant was measured. Values correspond to the lactate concentration per 1 × 10⁵ viable cells at the indicated times. One representative experiment out of three is presented. C, Baf-3, Baf-bcl-x, and S4 cells were cultured in the presence or absence of IL-3 during 8 h. 2-Deoxy[³H]glucose uptake was measured as indicated under “Experimental Procedures.” Data are presented as mean values ± S.D. of four experiments. D, Baf-3 cells were cultured in the presence of IL-3 (+IL-3), in the absence of IL-3 (−IL-3), or in the presence of IL-3 in a low glucose Dulbecco’s modified Eagle’s medium containing 6 mM 2-deoxyglucose (+IL-3+2DG). Viability was measured after 16 h by staining cells with Annexin V (FL1) and propidium iodide (FL2) and analysis by flow cytometry. Percentages indicated correspond to percent apoptotic cells (propidium iodide-positive and Annexin V-positive) contained in the R1 gate.
strong correlation between expression of high levels of Bcl-x protein and resistance to DNA damage-induced cell death (Fig. 7, A and C).

DISCUSSION

In this study we report that, in a Baf-3 mutant cell line, the S4 mutant, which was obtained after retroviral-insertion mutagenesis, shows an IL-3 independent Akt phosphorylation on serine 473, which leads to FKHR1 phosphorylation on threonine 32 and constitutive glycolysis. Akt activation was necessary for the inhibition of the intrinsic death pathway and long term survival of these cells in the absence of growth factor. The gene modified upstream of Akt responsible for the constitutive activation of Akt has not yet been identified. Several pathways downstream of Akt could be responsible for the survival of the S4 mutant in the absence of growth factor. In neurons, inactivation of the FKHR1 transcription factor following phosphorylation by Akt is potentially involved in the inhibition of apoptosis by growth factors (5, 9). Indeed, FKHR1 mutants where the three residues phosphorylated by Akt have been converted to alanine are strong transactivators and trigger apoptosis when overexpressed in a number of cell types. Conversely, replacement of these residues by aspartic acid, which mimics the presence of a phosphate group, disrupts the transactivation function of FKHR1 (44). FKHR1-mediated death could result from the expression of death genes, such as Fas-ligand, which expression is regulated by FKHR1 and which are strongly up-regulated following growth factor withdrawal in neurons (9). In Baf-3 cells we have found that FKHR1 threonine 32 phosphorylation is controlled by IL-3. In contrast, FKHR1 serine 253 remains phosphorylated in Baf-3 cells grown in the absence of IL-3 up to a stage when cells are starting to enter apoptosis. Because FKHR1 phosphorylation on serine 253 should maintain FKHR1 in the cytoplasm (9), these results suggest that IL-3 starvation-induced apoptosis is not mediated by FKHR1-dependent gene transactivation. This is in agreement with data showing that IL-3 starvation-induced apoptosis proceeds with similar kinetics in the presence of protein or RNA synthesis inhibitors (45). In the Baf-3 cells or the S4 mutant we have been unable to detect a phosphorylation of the Bad protein. Even if we cannot completely exclude a role for Bad in the inhibition of death, we have shown that in the S4 mutant the Bcl-x mRNA and protein are downregulated following IL-3 starvation, suggesting that the Bad-Bcl-x pathway is not involved in the survival of these cells in the absence of IL-3. In contrast, there is a strong correlation between expression of Bcl-x and protection against DNA dam-
age-induced death. Hence, pathways such as Jak/Stat5 or MAPK, which are involved in the up-regulation of Bcl-x by IL-3 could play a role in the inhibition of DNA damage-induced death (45-47). The PI3K/Akt pathway has also been involved in the regulation of bcl-x expression (43, 48). Indeed, PI3K inhibition delays bcl-x mRNA content in 5 μg of total RNA was measured by a RNase protection assay as described under “Experimental Procedures.” C, Baf-3 and S4 cells were cultured in the presence (+) of IL-3 or in the absence (-) of IL-3 during 8 and 24 h, respectively. Cells were then lysed, and 80 μg of total proteins from each sample was loaded on a 12.5% acrylamide gel. Detection of Bcl-x protein was done as described under “Experimental Procedures.”

The maintenance of a glycolytic activity in the absence of IL-3 could contribute to the survival of the S4 mutant in these conditions. Indeed, glucose deprivation or inhibition of glycolysis by 2-deoxyglucose can lead to growth arrest or to apoptosis, suggesting that some as yet undefined checkpoint able to induce apoptosis in these conditions exists (49). In IC.DP IL-3-dependent cells, survival in the absence of IL-3 following v-abl transfection has been shown to be dependent on glucose transport activation. Indeed v-ABL activation in these cells induced an increased survival in the absence of growth factor which correlated with a stimulation of glucose uptake. Hence the control of glycolytic activity by growth factors could be an essential step in the inhibition of the intrinsic apoptotic pathway (50). Acquisition of a growth factor-independent glycolytic activity could delay the onset of apoptosis and contribute to the process of transformation. Indeed, tumor cells frequently exhibit a high rate of anaerobic glycolysis even under aerobic conditions (51). In Baf-3 and S4 cells, we confirmed that anaerobic glycolysis is the main glucose-derived ATP-generating source, because these cells do not undergo aerobic glycolysis. The S4 mutant shows constitutive glucose transport and glycolytic metabolism in the absence of IL-3 as measured by glucose uptake and lactate production. In the presence or absence of IL-3, glycolytic activity was dependent on the PI3K/Akt pathway. In the absence of IL-3, inhibition of the glycolytic pathway using PI3K inhibitors, glucose deprivation, or 2-deoxy-glucose all resulted in rapid apoptotic death of the S4 mutant but not of Bcl-x-overexpressing cells (data not shown). These results indicate that the maintenance of the glycolytic activity of these cells was essential for their survival in the absence of growth factor.

We also show that the inhibition of p53-dependent DNA damage-induced death is not inhibited by Akt activation. Indeed, the activation of Akt in the S4 mutant in the absence of growth factor is unable to protect these cells against DNA damage-induced death. Similarly, in parental Baf-3 cells inhibition of the PI3K/Akt pathway does not abrogate the protection conferred by IL-3. These results are in contradiction with data showing that overexpression of PI3K or activated Akt can
protect against apoptosis following p53 transfection (25). This discrepancy could be due to difference in the level of Akt activation obtained by overexpressing an activated form of Akt compared with the level of Akt activation obtained in the S4 mutant. Indeed, the level of Akt phosphorylation in these cell in the absence of growth factors is lower than the level obtained when growth factor-starved cells are synchronously restimulated with IL-3. However, it is similar to the physiological level observed in Baf-3 or S4 cells continuously maintained in the presence of IL-3 and might mimic more accurately the functions fulfilled by Akt in IL-3-dependent cell lines. Alternatively, one could imagine that the apoptotic pathway induced by p53 following DNA damage-induced death is different from the pathway induced by p53 overexpression. p53 has multiple functions that are regulated by post-translational modifications such as protein phosphorylation or protein/protein interaction. A better understanding of the apoptotic pathways activated by p53 in these different experimental conditions might help to resolve this issue.

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