Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase

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ABSTRACT The interleukin-2 (IL-2) receptor (IL-2R) is composed of three subunits. Of these, IL-2Rα is required for high-affinity IL-2 binding, while IL-2Rβ and IL-2Rγc are required for the transduction of IL-2-generated signals. Signals transduced via the S region of the IL-2Rβ (amino acids 267–322) in BAF/3 cells activate the phosphatidylinositol 3-kinase 3-kinase and induce the expression of Bcl-2 and c-myc. Through the induction of Bcl-2, IL-2 inhibits apoptosis and through the combination of Bcl-2 and c-myc it stimulates progression through the cell cycle. Here we show that the protein kinase encoded by the Akt proto-oncogene is activated by IL-2. Akt activation by IL-2 depends on PI3-kinase signals transduced via the S region of the IL-2Rβ and is linked to the translocation of Akt to the cell membrane. Expression of catalytically active Akt mutants in BAF/3 cells expressing IL-2Rβ[ΔS] promotes the expression of Bcl-2 and c-myc, inhibits apoptosis induced by IL-3 deprivation or staurosporine, and stimulates cell cycle progression. The same mutants also stimulate cell cycle progression in 2780a, an IL-2-dependent cell line that undergoes G1 arrest rather than apoptosis after IL-2 deprivation. The activation of Akt by IL-2 via the PI3-kinase and the rescue of the PI3-kinase-mediated antiapoptotic and proliferative IL-2 signals by catalytically active Akt indicate that these signals are transduced by Akt. The serine-threonine protein kinase encoded by the Akt proto-oncogene is activated by a variety of growth factors and intracellular signaling molecules via signals transduced by the PI3-kinase (12–19). Because both the activation of the PI3-kinase and the induction of Bcl-2 and c-myc depend on signals originating in the S region of the IL-2Rβ (10, 11), we questioned whether Akt is activated by IL-2 and whether, after activation, it induces expression of Bcl-2 and c-myc.

The data presented in this report show that Akt is activated by IL-2. Akt activation by IL-2 depends on PI3-K-mediated signals originating in the S region of the IL-2Rβ and is linked to its translocation to the cell membrane. Expression of catalytically active Akt mutants in BAF/3 cells expressing the wild-type IL-2Rβ, but not its ΔS (amino acids 267–322) mutant, promotes the expression of Bcl-2 and c-myc, inhibits apoptosis induced by growth factor deprivation or staurosporine, and stimulates cell cycle progression. The same mutants also stimulate cell cycle progression in 2780a, an IL-2-dependent cell line, which undergoes G1 arrest rather than apoptosis after IL-2 withdrawal. These data indicate that the IL-2 antiapoptotic and proliferative signals that induce Bcl-2 and c-myc originate in the S region of the IL-2Rβ and are transduced via the PI3-kinase/Akt pathway.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. EL4-IL-2 (ATCC TIB 181) cells were purchased from the American Type Culture Collection, and BAF/3 cells were kindly provided by G. A. Evans (National Cancer Institute, Frederick, MD). 2780a is an IL-2-dependent rat T cell lymphoma cell line, which was established in this laboratory and undergoes G1 arrest rather than apoptosis after IL-2 withdrawal (20, 21). EL4-IL-2 cells were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% (vol/vol) horse serum and penicillin (50 units/ml), streptomycin (50 μg/ml), and kanamycin (100 μg/ml) (PSK). BAF/3 and 2780a cells were cultured at 37°C and 5% CO₂ in RPMI medium 1640 supplemented with 10% fetal bovine serum, PSK, and 10% WEHI cell supernatant (IL-3 source), or IL-2 (100 units/ml), respectively.

Expression Constructs and Transfections. Expression constructs of IL-2Rβ (pDKCR-IL-2Rβ) and the IL-2RβΔΔS mutant (pLCR-B-S) (10) were kindly provided by Zhao-Jun Liu and T. Taniguchi (University of Tokyo, Tokyo, Japan). Wild-type Akt and a myristylated Akt mutant, fused to a C-terminal hemagglutinin epitope tag (AktHA and Myr-AktHA), were cloned in the cytomegalovirus (CMV)-based expression vector CMV-6 and the retrovirus vector 5Rα. Finally, a PH domain Akt mutant (E40K) that exhibits enhanced basal kinase activity, but responds to physiological stimuli, (A.B., unpublished work) was cloned in CMV-6. BAF/3 cells were transfected by electroporation with the IL-2Rβ or IL-2RβΔΔS constructs, and

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 Abbreviations: IL, interleukin; IL-2R, IL-2 receptor; PI3-kinase, phosphatidylinositol 3-kinase; CMV, cytomegalovirus.

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they were selected for hygromycin resistance. The cells selected after transfection of these constructs were supertransfected also by electroporation with CMV-6 or the CMV-6-based expression constructs of Akt along with a plasmid encoding the Neo<sup>e</sup> gene, and they were selected for G418 resistance. Finally, 2780α cells were infected with SRα or the SRα-based myristylated Akt constructs.

Transient transfections of Akt-HA and Myr-Akt-HA in EL4 cells were carried out by electroporation as previously described (21).

**In Vitro Kinase Assays.** Five × 10<sup>6</sup> EL4-IL-2 cells or BAF/3 cells stably transfected with the IL-2Rβ or the IL-2RβΔS mutant were cultured at a concentration of 0.5 × 10<sup>6</sup> cells per ml. Subsequently, the EL4-IL-2 cells were serum-starved, and the BAF/3 cells were IL-3- and IL-2-starved for 16 h. Some of the starved cultures then were stimulated with IL-2 (100 units/ml) for 10 min. Cells were lysed using an Nonidet P-40 lysis buffer (1% Nonidet P-40/10% glycerol/137 mM NaCl/20 mM Tris-HCl, pH 7.4) containing 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Akt was immunoprecipitated from the cell lysates with the anti-Akt-CT antibody (12, 17, 22). Kinase assays were performed as described previously using histone H2B as the exogenous substrate (12, 17, 22). The products of the in vitro kinase reaction were analyzed by SDS/PAGE. The same protocol also was used to carry out *in vitro* kinase assays of Akt[α0]HA and Myr-Akt-HA immunoprecipitated with the anti-HA monoclonal antibody 12CA5 from transiently transfected EL4-IL-2 cells.

**Immunofluorescence.** 10<sup>6</sup> EL4-IL-2 cells were transiently transfected with C-terminally tagged Akt (Akt-HA) or myristylated Akt (Myr-Akt-HA) expression constructs and were cultured at the concentration of 0.5 × 10<sup>6</sup> cells per ml. Twenty-four hours after transfection the cells were serum-starved for 16 h, and then were stimulated with IL-2 (100 units/ml). Cells were harvested before, and 10 min after, stimulation with IL-2 and were stained with the anti-hemagglutinin monoclonal antibody 12CA5 and an anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Sigma). Immunofluorescence staining was carried out as follows. 10<sup>6</sup> cells were resuspended in 100 μl of complete medium and were evenly spread on microscope slides using a cytocentrifuge. The cells then were fixed in 3.5% fresh paraformaldehyde for 15 min and washed three times with PBS plus 0.1% Tween 20 (wash solution). Subsequently, they were incubated with the primary antibody 12CA5 (1:250 dilution) at room temperature for 1 h and then washed three times with wash solution before incubation for 45 min with the secondary antibody (1:100 dilution). After three additional washes, cell nuclei were stained with Hoechst 33258 (0.5 μg/ml) for 1 min and were washed three more additional times. Coverslips were mounted on the microscope slides with Vectorshield (Vector Laboratories).

**Cell Death and Cell Cycle Analyses.** BAF/3-IL-2RβΔS cells, stably transfected with a CMV-6 construct of the carboxyl-terminally tagged myristylated Akt (Myr-Akt-HA) or the CMV-6 vector alone, were cultured in IL-2- and IL-3-deficient media at the concentration of 0.5 × 10<sup>6</sup> cells per ml. Live and dead cells were counted daily by trypan blue exclusion. The numbers of live and dead cells then were used to calculate the ratio of dead/live cells. The viability was calculated as the percentage of dead cells at sequential time points after growth factor withdrawal. The same methods were used to calculate the percentage of dead cells in BAF/3 cells expressing IL-2RΔS with or without myristylated Akt and treated with staurosporine (2 μM). BAF/3 cell lines expressing IL-2RβΔS and myristylated Akt (BAF/3ΔSMA1 and MA2) and 2780α T cells infected with SRα or SRα-based myristylated Akt constructs were incubated with ethidium bromide in FACS buffer (3.4 mM sodium citrate/10 mM NaCl/0.1% Nonidet P-40/75 μM ethidium bromide) at the 72-h (3 days) time point after IL-3 withdrawal, and they were analyzed for DNA content by flow cytometry as described (23).

**Western Blotting.** 10<sup>6</sup> cells (BAF/3, EL4-IL-2 or 2780α) transfected transiently or stably with a variety of expression constructs were lysed after serum- and growth-factor starvation and treated with various growth factors as described in Results. Lysis was carried out using the Nonidet P-40 lysis buffer described earlier. SDS/PAGE of the cell lysates or immunoprecipitates were probed with the following antibodies: anti-Akt-CT (12) or anti-HA (12), anti-Bcl-2 (Santa Cruz Biotechnology), and anti-c-myc (Upstate Biotechnology, Lake Placid, NY). Western blots were developed using enhanced chemiluminescence (Amersham) as previously described (12).

**RESULTS**

To determine the involvement of Akt in the transduction of IL-2 antiapoptotic and proliferative signals we first examined whether IL-2 activates Akt in serum-starved EL4[α0]IL-2 cells and in IL-2- and IL-3-starved BAF/3 cells engineered to stably express the IL-2Rβ [3]. The results (Fig. 1A and B) showed that Akt is indeed activated by IL-2 as well as by IL-3. Akt activation by IL-2 and IL-3 similarly to its activation by other growth factors (6–9) was inhibited by wortmannin (6), a PI3-kinase inhibitor, and d<sub>0</sub>p<sub>85</sub>α, a dominant negative mutant of the PI3-kinase (24) (data not shown). In addition, IL-2, but not IL-3, failed to activate Akt in BAF/3 cells expressing an S region deletion mutant of the IL-2Rβ (ΔS), which does not activate the PI3-kinase in response to IL-2 stimulation (11) (Fig. 1C).

The PI3-kinase and the phosphoinositides it generates are localized at the cell membrane (25). We hypothesized therefore that membrane translocation could be a necessary step for the activation of Akt by growth factors. To address this hypothesis, a carboxyl-terminally tagged HA construct of wild-type Akt (Akt-HA) was transiently transfected into EL4 cells. After 16 h of serum starvation, starting at 24 h after the transfection, half of the transfected cultures were stimulated with IL-2. Ten minutes later, cytospins of both the unstimulated and IL-2-stimulated cultures were fixed with paraformaldehyde and stained with the anti-HA monoclonal antibody 12CA5. The results showed that Akt was localized in the cytosol, in the majority of the transiently transfected unstimulated cells. After IL-2 stimulation, however, the majority of Akt was detected on the cell membrane (Fig. 2A). Therefore, membrane translocation, perhaps due to the interaction of the Akt PH domain with PI3-kinase-generated phosphoinositides, may play an important role in the activation of Akt by IL-2. Strong support to this hypothesis was provided by the observation that an Akt hybrid molecule containing a c-src-derived myristylation signal (MGSSKSKPKP) (26) at its N terminus is attached to the cell membrane (Fig. 2A) and is constitutively active (Fig. 2B).

Like the parental BAF/3 cells, BAF/3-IL-2RβΔS cells undergo apoptosis within 48–72 h after IL-3 withdrawal, both in the presence and in the absence of IL-2 (10). Because IL-2 does not activate Akt in these cells (Fig. 1C), we hypothesized that its failure to rescue them from apoptosis induced by IL-3 withdrawal was due to its inability to activate Akt. To test this hypothesis, BAF/3 cells expressing IL-2RβΔS were transfected with CMV-6 or a CMV-6-based expression construct of myristylated Akt. Stably transfected cells were cultured in IL-3 deficient media in the presence or absence of IL-2. Live and dead cells then were counted daily for 3 days, and their numbers were used to calculate the ratio of dead/live cells and the percentage of dead cells for each time point. Fig. 3A shows the mean values of the percentage of dead cells ±
continue to cycle, cells were harvested at 72 h after IL-3 deprivation, and they were analyzed for DNA content by flow cytometry. The results (Fig. 3 A2) showed that, at 72 h, surviving cells are distributed throughout the cell cycle. Cycling of these cells was confirmed by experiments demonstrating BrdUrd incorporation in the cell DNA (data not shown). Because BAF/3 cells engineered to express Bcl-2 also survive IL-3 withdrawal, but arrest in G1 (27), this finding suggests that Akt not only inhibits apoptosis but stimulates cell cycle progression. Exposure of BAF/3 cells expressing myristylated Akt and IL-2RβΔS to IL-2 further enhanced progression through the cell cycle (Fig. 3A2), suggesting that Akt complements the signaling defect of the IL-2RβΔS mutant and that IL-2 proliferative signals are transduced via both Akt-dependent and independent pathways. The effects of Akt on the cell cycle were confirmed in an IL-2-dependent T cell line (2780a), which undergoes G1 arrest rather than apoptosis after IL-2 withdrawal (20). Expression of myristylated Akt in these cells allowed them to escape G1 arrest induced by IL-2 deprivation (Fig. 3B).

The myristylated Akt mutant is constitutively localized at the cell membrane (Fig. 2A). To address the physiological nature of the biological effects of this mutant, we examined whether a PH domain Akt mutant (E40K) that exhibits enhanced basal kinase activity, but responds to physiological stimuli (A.B., N.N.A., T.O.C., and P.N.T., unpublished work), produces effects similar to those produced by myristylated Akt in BAF/3-IL-2RβΔS cells. The results (Table 1) showed that the Akt E40K mutant also inhibits apoptosis induced by IL-3 withdrawal. Its antiapoptotic activity, however, similarly to its action on myristylated Akt, was more effective in IL-2-stimulated cells than in unstimulated cells.
via the S region of the IL-2Rβ. Because these IL-2 signals induce the expression of Bcl-2 and c-myc (10), we hypothesized that if Akt is a mediator of such signals it also would induce Bcl-2 and c-myc expression. To address this question Western blots of lysates of IL-2- and IL-3-starved BAF/3-IL-2Rβ-ΔS cells expressing myristylated Akt were probed with anti-Bcl-2 and anti-c-myc antibodies. Lysates of BAF/3-IL-2Rβ and BAF/3-IL-2Rβ-ΔS cells before and after IL-2 stimulation were used as controls. The results (Fig. 4 A1 and B) confirmed that IL-2 induces expression of Bcl-2 and c-myc via signals transduced through the S region of the IL-2Rβ. Moreover, they showed that Akt induces the expression of both oncoproteins.

Earlier studies had shown that overexpression of Bcl-2 is sufficient to inhibit apoptosis in BAF/3 cells cultured in the absence of IL-3 (10, 27). The antiapoptotic role of Bcl-2 in Akt expressing BAF/3-IL-2Rβ-ΔS cells, suggested by these earlier studies, was further supported by experiments showing that these cells exhibit a delay in staurosporine-induced apoptosis (Fig. 4A2). Because apoptosis induced by staurosporine is delayed in cells overexpressing Bcl-2, but not in cells protected from apoptosis by other antiapoptotic signals (28), these data show that the induction of Bcl-2 in myristylated Akt-expressing cells is antiapoptotic. The Akt-mediated stimulation of the cell cycle, described in this report, appears to be mediated by the combined induction of Bcl-2 and c-myc as suggested by earlier studies, which had shown that overexpression of both Bcl-2 and c-myc in BAF/3 cells is sufficient to induce progression through the cell cycle (10).

**DISCUSSION**

The data in this report addressed several important questions regarding both the mechanism of activation of Akt by growth factors and the biological effects of the activated Akt. Our earlier studies and studies by others (12–16) had shown that Akt activation by growth factors depends on the PI3-kinase. More recent studies (17–19) provided support to the earlier suggestion that Akt is a direct target of the D3 phosphoinositides whose synthesis is catalyzed by the PI3-kinase (12). In this report we provide a link that was so far missing from the emerging scheme of Akt activation. Specifically, we show that, after growth factor stimulation, Akt translocates to the cell membrane. Therefore, the binding of the D3 phosphoinositides to the PH domain of Akt, suggested by the earlier studies, is likely to be responsible for membrane translocation. Moreover, because the myristylated Akt mutant, which is constitutively associated with the cell membrane is constitutively active, membrane translocation is likely to play an important role in Akt activation.

### Table 1. Rate of apoptosis of BAF/3-IL-2Rβ-ΔS cells stably transfected with CMV-6 or CMV-6-based expression constructs of catalytically active Akt mutants

<table>
<thead>
<tr>
<th>Cells</th>
<th>Apoptosis, % dead cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>BAF/3-ΔS CMV-6</td>
<td>74 ± 7.5</td>
</tr>
<tr>
<td>BAF/3-ΔS E40K-Akt</td>
<td>52 ± 3.5</td>
</tr>
<tr>
<td>BAF/3-ΔS Myr-Akt</td>
<td>45 ± 4.1</td>
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Six independent cultures of BAF/3-IL-2Rβ-ΔS cells stably transfected with CMV-6 or CMV-6-based expression constructs of Akt E40K or myristylated Akt were cultured in the absence of IL-2 and IL-3 at the concentration of 0.5 × 10⁶ cells per ml. Live and dead cells were counted daily and the percentage of dead cells (mean ± SD) at each time point was calculated as in Fig. 3A1. Surviving Akt E40K and myristylated Akt-expressing cells from all the cultures gave rise to growth factor-independent cell lines. Visible evidence of factor independent growth was observed within a week after growth factor deprivation.
Fig. 4. Myristylated Akt replaces IL-2 signals transduced via the S region of the IL-2Rβ and induces Bcl-2 and c-myc expression in BAF/3-IL-2RβΔS cells. (A1) 10^6 BAF/3-IL-2Rβ or BAF/3-IL-2RβΔS cells were cultured at the concentration of 0.5 × 10^6 cells per ml in the absence of IL-2 or IL-3. Twenty-four hours later, the indicated cultures (lanes 2 and 4) were stimulated with IL-2 (100 units/ml) for 10 min. Unstimulated (lanes 1 and 3) and IL-2-stimulated (lanes 2 and 4) cells were lysed in the Nonidet P-40 lysis buffer. One × 10^6 cells from four independent BAF/3-IL-2RβΔS lines expressing a hemagglutinin-tagged myristylated Akt (MA1, MA2, MA3, and MA4) were cultured in parallel with the preceding cells also for 24 h in the absence of IL-2 and IL-3, and they were lysed in the same buffer. Western blots of all the lysates were probed with an anti-Bcl-2 rabbit polyclonal antiserum or with the anti-hemagglutinin tag antibody (12CA5). (A2) Five × 10^6 cells from four independent cultures of myristylated Akt-transfected, MA1, MA2, MA3, and MA4 and two of CMV-α-transfected BAF/3-IL-2RβΔS cells as1 and as2 were cultured in the presence of IL-2 (100 units/ml). At time 0, all cells were treated with staurosporine (2 μM). Cells were harvested at sequential time points as indicated, and live and dead cells were counted. The percentage of dead cells was calculated as described in Materials and Methods. (B) Western blots of the cell lysates, analyzed in A1, were probed with an anti-c-myc rabbit polyclonal antibody (Upstate Biotechnology) (dilution 1/2,000).

Hematopoietic growth factors promote cell viability and cellular proliferation (21, 29). Cells dependent on a given growth factor usually undergo apoptosis upon growth factor deprivation (16). In the case of IL-2, signals transduced by the S region of the IL-2Rβ via the PI3-kinase induce the expression of Bcl-2 and c-myc, and as a result they inhibit apoptosis and stimulate cellular proliferation (3, 30). In this report we have shown that the inability of a deletion mutant of the IL-2Rβ (ΔS) to induce the expression of Bcl-2 and c-myc in response to IL-2, correlates with its inability to activate Akt. Moreover, we have shown that catalytically active mutants of Akt induce expression of both Bcl-2 and c-myc. As a result, they inhibit apoptosis induced by growth factor withdrawal or staurosporine, stimulate progression through the cell cycle, and give rise to growth factor independent cell lines. Collectively, these data indicate that Akt transduces the IL-2 signals originating from the S region of the IL-2Rβ, which lead to Bcl-2 and c-myc expression, thus inhibiting apoptosis and stimulating cellular proliferation (Fig. 5).

Our earlier studies on Akt were centered primarily on the mechanism(s) of its activation (12, 17, 22). These studies identified Akt as a direct target of the PI3-kinase and suggested that it may contribute to the transduction of signals involved in a great variety of biological processes known to be PI3-kinase-dependent (31). The results presented here extend our earlier observations on Akt activation by showing that it is linked to the translocation of Akt to the cell membrane. In addition, they place Akt in a clearly defined pathway of IL-2 signaling. IL-2 signals transduced by Akt along this pathway regulate the expression of Bcl-2 and c-myc, and as a result they inhibit apoptosis and stimulate cellular proliferation.

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