IL-2-Mediated Cell Cycle Progression and Inhibition of Apoptosis Does Not Require NF-κB or Activating Protein-1 Activation in Primary Human T Cells[1]

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The IL-2 growth hormone is the major growth factor of activated T lymphocytes during a developing immune response. IL-2 is required not only for cell cycle progression but also to protect Ag-activated T cells from programmed cell death. In several cell types, activation of NF-κB and/or activating protein-1 (AP-1) has been demonstrated to be extremely important in blocking apoptosis. To determine whether either or both of these transcription factors are involved in cell survival or cell cycle progression in response to IL-2, primary human T cells responsive to the growth factor were analyzed for NF-κB and AP-1 activation. The current study clearly demonstrates that IL-2 does not induce IκBα degradation or NF-κB activation in primary human T cells that respond to IL-2 by entering the cell cycle and avoiding apoptosis. Similarly, IL-2 neither activates JNK nor increases AP-1 binding activity to a consensus o-tetradecanoylphorbol 13-acetate (TPA) response element. On the other hand, the growth factor does induce the activation of STAT3 and STAT5 in these cells, as has been previously demonstrated. These data show that neither NF-κB nor AP-1 activation is required for IL-2-mediated survival or cell cycle progression in activated primary human T cells. ***The Journal of Immunology,*** **1999, 162: 3308–3315.**

The IL-2 growth factor is produced upon antigenic stimulation of T cells and is vital to the cellular expansion required for a productive immune response (for review, see Refs. 1–3). While a lack of IL-2 production results in the development of an unresponsive state in Ag-stimulated T cells (for review, see Ref. 4), IL-2 stimulation both induces cell cycle progression and inhibits programmed cell death in these cells. Many studies have been performed to delineate the signal transduction pathways and later events that are responsible for the biological consequences of IL-2 stimulation on cell cycle progression and relief from apoptosis, but these events in primary human T cells are still incompletely understood. It has been clearly demonstrated that the activation of NF-κB can protect several cell types from apoptotic death in response to various signals (Refs. 5–9; for review, see Refs. 10 and 11). IL-2, like other growth factors, inhibits apoptosis (Ref. 12; for review, see Ref. 13) and has been shown to activate NF-κB (14, 15). Therefore, it is logical to propose that the inhibition of cell death by IL-2 is mediated by NF-κB but it is not known whether this mechanism protects primary human T cells from the death that is a consequence of growth factor deprivation.

The AP-1[5] transcription factor has also been demonstrated to protect cells from apoptotic death (16, 17). On the other hand, activation of the Jun N-terminal kinases, JNK1/2, and AP-1 activity have also been shown to correlate with apoptotic cell death in many circumstances (Refs. 18–22; for review, see Refs. 23 and 24). Both AP-1 binding activity (25) and JNK activation (26, 27) have been reported to be the result of IL-2 stimulation in responsive cell lines, but it is unclear whether activation of AP-1 or JNK correlates with either the induction of apoptosis in IL-2-deprived primary human T cells or, conversely, the inhibition of apoptosis in IL-2-stimulated primary human T cells.

The current study was conducted to determine whether NF-κB and/or AP-1 activation is correlated with cell cycle progression or survival in primary human T cells responding to IL-2. As demonstrated here, neither of these transcription factors is activated by IL-2 in the primary human T cell populations analyzed. In contrast, STAT proteins, previously demonstrated to be activated by IL-2 in responsive T cells (28–33), are activated by IL-2 in these cells. These data strongly suggest that neither AP-1 nor NF-κB activation is involved in cell cycle progression or the inhibition of apoptosis observed in primary human T cells in response to IL-2.

Materials and Methods

Cell culture

Thymic blast cells were prepared as described previously (34). Briefly, thymocytes were isolated from human tissue by mincing and passage over a Ficoll gradient. The isolated thymocytes were then stimulated with 5 μg/ml PHA (Sigma, St. Louis, MO) and 10 ng/ml PMA (Sigma) for 48 h. After stimulation, the cells were resuspended in complete medium (RPMI 1640 (Sigma), 10% FBS (HyClone, Logan, UT), L-glutamate, and antibiotics) with 10 U/ml IL-2 (Chiron, Emeryville, CA) and fed each day for 3–6 days. These cells are referred to as thymic blast cells (TBCs). Cells were deprived of IL-2 by washing twice and culturing in complete medium without IL-2 for the indicated times. The cells were then restimulated for

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Received for publication August 13, 1998. Accepted for publication December 16, 1998.

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[1] This work was supported by National Cancer Institute Grant CA53382 and National Institutes of Health/Minority Biomedical Research Support Grant GM45765 (to K.L.M.) and an Achievement Rewards for College Scientists scholarship (to F.R.).

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[5] Abbreviations used in this paper: AP-1, activating protein-1; JNK, Jun N-terminal kinase; TBC, thymic blast cell; PB, peripheral blood; EMSA, electrophoretic mobility shift assay; TPA, o-tetradecanoylphorbol 13-acetate; TRE, TPA response element; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

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the indicated times with 100 U/ml IL-2, 10 ng/ml PMA, 1.5 μM ionomycin, and/or 25 ng/ml anisomycin. All TBC preparations were checked for responses to IL-2 using [^{3}H]thymidine uptake, cell cycle progression, or STAT3 phosphorylation analysis.

PBMC were isolated by Ficoll density gradient centrifugation, cultured with PHA and PMA for 48 h, grown in IL-2 for 4 days, and then deprived of IL-2 for approximately 44 h, followed by restimulation for 4 h with 100 U/ml IL-2, 10 ng/ml PMA, or PMA and 1.5 μM ionomycin. The response of this population to IL-2 was verified by cell cycle analysis using propidium iodine staining as described below, and cell surface staining revealed that the cells were 97% T cells ($CD3^+$) and approximately 50% $CD4^+$ and 50% $CD8^+$, and essentially 100% expressed the IL-2R α-chain. These cells are referred to in the text as peripheral blood (PB) T cells.

Cell cycle analysis

TBCs analyzed for cell cycle progression were deprived of IL-2 for 32 h or for 8 h followed by a 24-h restimulation with 100 U/ml IL-2. PB T cells were deprived of IL-2 for 44 h or for 16 h followed by a 28-h restimulation with IL-2. Cells were washed once with PBS and fixed by resuspension in PBS/ethanol (1/1). Before analysis, the fixed cells were resuspended into 100 μg/ml RNase A in PBS and incubated for 30–60 min at room temperature. Propidium iodine (50 μg/ml) was added, and the cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA). Analysis of the FACS data was performed using WINMIDI, version 2.1.4. The percentage of cells in G_0/G_1, S, or G_2+M was determined using the MultiCycle AV program from Phoenix Flow (San Diego, CA).

TUNEL assay

TBCs analyzed for apoptosis using the TUNEL technique were deprived of IL-2 for 32 h or for 8 h followed by a 24-h restimulation with IL-2 or PMA. Cells were then harvested into polystyrene tubes (previously precoated overnight at 4°C with 2% FBS and BSA in PBS) and fixed in 1% paraformaldehyde in PBS. The samples were washed once in PBS, resuspended in 70% ethanol, and stored at −20°C. Before FACS analysis, samples were washed with PBS and resuspended into elongation buffer (1× TdT buffer, 2.5 mM $CoCl_2$, 5 μM biotin-16-dUTP, and 10 U TdT; Boehringer Mannheim, Indianapolis, IN) and incubated at 37°C for 30 min. After elongation the cells were washed with PBS and resuspended into staining solution (5× SSC, 5% milk, 0.1% Triton X-100, and a 1/2000 dilution of FITC-avidin (Sigma)) and incubated for 30 min at room temperature. After staining, the cells were washed and resuspended in 0.5% formaldehyde/PBS before analysis by FACS.

Western blot analysis

Cells treated as indicated (~5 × 10^7/condition) were harvested into 300 mM NaCl, 100 mM Tris-Cl (pH 7.0), 2% Triton X-100, 2% sodium deoxycholate, and 0.2% SDS containing phosphatase and protease inhibitors (NaF and sodium orthovanadate at 25 μg/ml; soybean trypsin inhibitor, leupeptin, aprotonin, and pepstatin A at 2 μg/ml; and PMSF at 1 mM). Protein concentrations were determined using the Bio-Rad protein assay system according to the manufacturer's directions (Bio-Rad, Hercules, CA), and proteins were subjected to SDS-PAGE and transferred to nitrocellulose for Western blot analysis. Equal loading was confirmed by Ponceau S staining of the filter after transfer. Antisera recognizing IκB-α (C-15, Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated and total JNK1/2 (PhosphoPlus SAPK/JNK (Thr^{183}/Tyr^{185}) Ab Kit, New England Biolabs, Beverley, MA), phosphorylated and total STAT3 (PhosphoPlus Stat3 (Tyr^{705}) Ab Kit, New England Biolabs), and phosphorylated (phospho-specific STAT5A (Tyr^{694}), New England Biolabs) and total (Transduction Laboratories, Lexington, KY) STAT5 were used according to the manufacturer's recommendations. Proteins were detected using horseradish peroxidase-conjugated anti-rabbit antisera (or anti-mouse antisera for total STAT5) and chemiluminescence according to the manufacturer's directions (New England Biolabs). Stripping of blots for reprobing was performed in 62.5 mM Tris (pH 6.7), 2% SDS, and 100 mM 2-ME for 30 min at 65°C.

Nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were isolated as previously described (35). The protein concentration of the nuclear extracts was determined as described above. EMSAs were also performed essentially as previously described (35). Briefly, 5–10 μg of nuclear extract was incubated with 0.5–2 μg poly(dI-dC) in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol, and 0.5 mM DTT at room temperature for 15 min. Similarly, if applicable, 1 μg of antiserum specific for Rel/NF-κB (0.6 μg for Bcl-3) or AP-1 family member proteins was added during this preincubation step. Antisera for p50 (no. 1141), RelA (no. 1207), and c-Rel (no. 1136) were provided by Nancy Rice, and Bcl-3- and c-Fos-specific antisera were obtained from Santa Cruz Biotechnology. ^{32}P-labeled consensus TRE, NF-κB, or Oct probe (20,000 cpm) was added, and the reaction was incubated for an additional 15 min at room temperature. The reactions were then electrophoresed through 6.5% (Figs. 3 and 4) or 4% (Fig. 6) native acrylamide gels in 0.5× TBE running buffer. The gels were dried and exposed to XAR5 film. The sequences of the probes used in this analysis were: NF-κB, 5′-gatcCAACGGCAGGGGAATCTCCCTCTCCTT; TRE, 5′-gatcCGGCTGACTCATCA; and Oct, 5′-gatcTGTCGAATGCAAATCACTAGAA.

Results

Growth factors are known to induce cell cycle progression and inhibit apoptosis in cells expressing the appropriate receptors. To evaluate the activation of transcription factors that may be responsible for these biological responses in primary human T cells responding to IL-2, cells were isolated from thymus tissue obtained from patients undergoing corrective cardiac surgery at Children's Hospital in San Diego. The cells were stimulated to express the high affinity IL-2R and then cultured in IL-2-containing medium for several days, resulting in a population of blast cells called TBCs. The phenotype and IL-2 responsiveness of TBCs have previously been described in detail (34). These cells express high levels of CD3, approximately two-thirds of the cells are single positive for CD4 or CD8, one-third of the cells are double positive for both markers, and all cells express the IL-2R α-chain. The high TCR/CD3 expression levels and the response of these cells to IL-2 strongly suggest that they represent the more mature cells found in the thymus rather than immature or developing T cells, which usually express low levels of the TCR/CD3 complex. Culturing the cells in this manner allows for IL-2-dependent events to be studied independently of activation events that occur via the TCR. The advantage of TBCs over PB T cells is that large numbers of cells can be obtained from a single thymus to study both cellular and molecular events in a single population. Upon IL-2 withdrawal, about 90% of TBCs synchronize in the G_0/G_1 phase of the cell cycle within 32 h, and an average of 22% are apoptotic (Fig. 1). In contrast, when stimulated with IL-2 for 24 h, only about 65% of TBCs are in G_1, with 30% in S phase, and only 11% of the cells, on the average, are apoptotic.

As indicated above, the activation of NF-κB has been correlated with the inhibition of cell death in many cell types. The activation of NF-κB is normally accomplished by the phosphorylation and degradation of IκBα, a protein that holds in the cytoplasm the p50/RelA heterodimers that make up the active NF-κB transcription factor (for review, see Refs. 36–38). Upon degradation of IκBα, NF-κB is rapidly transported to the nucleus, where it is transcriptionally active. To determine whether IL-2 activates NF-κB in TBCs, the fate of IκBα was analyzed in cells stimulated with IL-2. As can be seen in Fig. 2, no significant degradation of IκBα was detected with IL-2 stimulation at any time point analyzed, even several hours after stimulation. In contrast, PMA induced a significant and sustained (up to 4 h) degradation of IκBα at concentrations well within those used normally in experimentation on primary human T cells.

IκB-α is only one of several IκB proteins (36–38), and mechanisms other than IκB degradation have been reported for the activation of NF-κB. For instance, tyrosine phosphorylation of IκB-α leads to NF-κB release without degradation of the protein (39). To definitively determine whether NF-κB is present in nuclei of TBCs stimulated with IL-2, NF-κB binding activity was analyzed. TBCs were deprived of IL-2 to synchronize them and stimulated with IL-2 or PMA, and nuclear extracts were then isolated. NF-κB binding activity was accessed by EMSA analysis using the

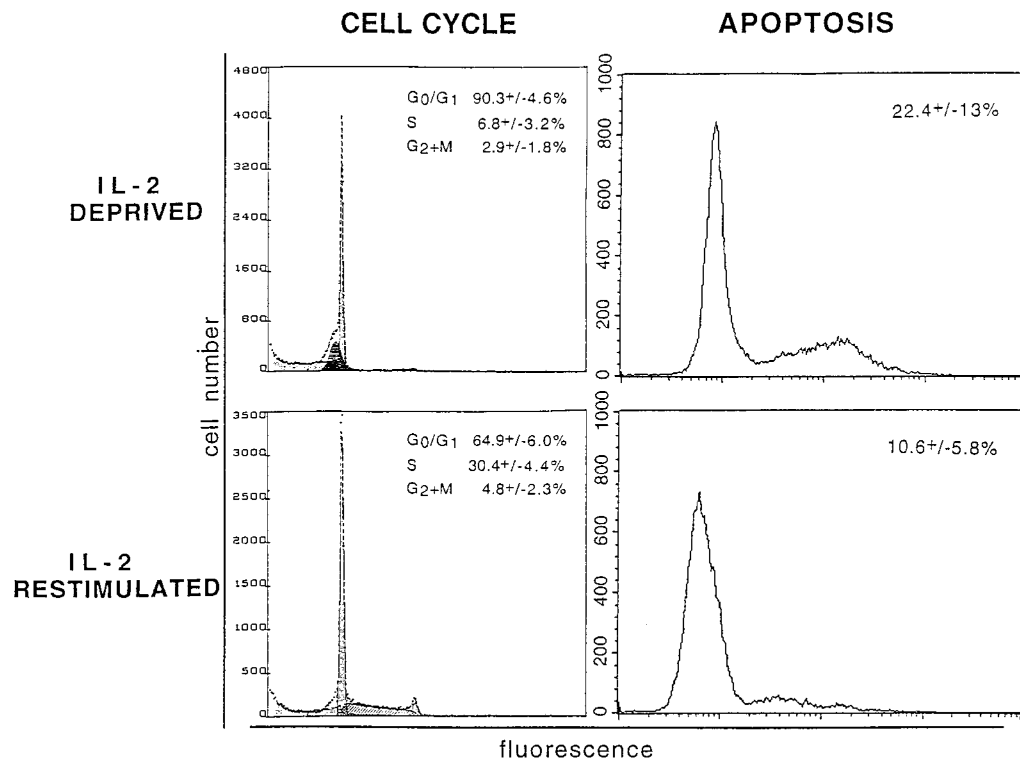

FIGURE 1. IL-2 induces cell cycle progression and inhibits apoptosis in TBCs. TBCs were prepared as described in the text and analyzed for DNA content using propidium iodide (*left*) and apoptosis using TUNEL (*right*). The graphs shown are representative examples, and the numbers given are the average of 10 independent experiments with SDs indicated. The rather large SDs obtained in the TUNEL assay are indicative of a variability between TBC preparations in the number of cells entering apoptosis 32 h after deprivation and not of the ability of IL-2 to induce cell survival. The presence of IL-2 routinely (10/10 experiments) reduces the level of apoptotic cell death observed.

consensus NF-κB binding site from the human IL-2R α-chain promoter. As shown in Fig. 3*A*, TBCs deprived of IL-2 contained NF-κB binding activity, which consisted of p50 homodimers, but little RelA or c-Rel binding was observed. IL-2 stimulation did not induce the formation of a new complex, nor did it appreciably increase RelA or c-Rel binding to this sequence over that observed in deprived cells. Because a significant level of p50 homodimer binding was evident in both IL-2-deprived and -restimulated cells, we also analyzed these complexes for increases in Bcl-3 binding. While Bcl-3 has been demonstrated to interact with and enhance the transcriptional activity of p50 homodimers (40–42), no Bcl-3 binding was detected in IL-2-stimulated cells using this assay. PMA, on the other hand, decreased p50 homodimer formation and increased the formation of the two complexes containing p50 and RelA or c-Rel. The PMA data indicate that NF-κB can be activated in TBCs. Fig. 3*B* demonstrates that NF-κB (RelA or c-Rel) binding is not increased in TBCs stimulated from 30 min to 24 h with IL-2. Collectively, the data demonstrate that IL-2 does not activate NF-κB in cells that respond to the growth factor by progressing through the cell cycle and avoiding apoptosis, suggesting that NF-κB activation does not play a role in these biological consequences of IL-2 stimulation in TBCs.

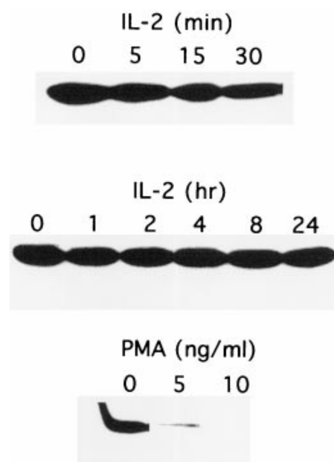

FIGURE 2. IκB-α is not degraded in IL-2-stimulated TBCs. TBCs were deprived of IL-2 for 24 h and restimulated with IL-2 for the times indicated or with PMA at the concentrations indicated for 4 h, and total protein was analyzed for IκB-α levels using Western blot analysis. The results shown are representative of two independent TBC preparations.

Previous studies have indicated that NF-κB is activated by IL-2 in freshly isolated PB T cells that have been treated with very high concentrations of IL-2 (14). Because TBCs are derived from thymic tissue, they may not be equivalent to mature PB T cells in their response to IL-2. Therefore, to determine whether the activation of mature T cells by IL-2 is similar to the activation of TBCs, PB T cells were isolated after culture in IL-2 for 4 days. These culture conditions resulted in a population of pure (>95%) mature T cells that have been treated identically to TBCs. These cells were washed, deprived, and then restimulated with IL-2 or PMA, and nuclear extracts isolated from these cells were evaluated for NF-κB binding activity. As can be seen in Fig. 4, PB T cells also did not activate novel NF-κB binding activity when responding to IL-2 despite the fact that they did respond to the growth factor by progressing through the cell cycle (46% of cells are in S, G_2, and M phases in the stimulated population 24 h after the addition of

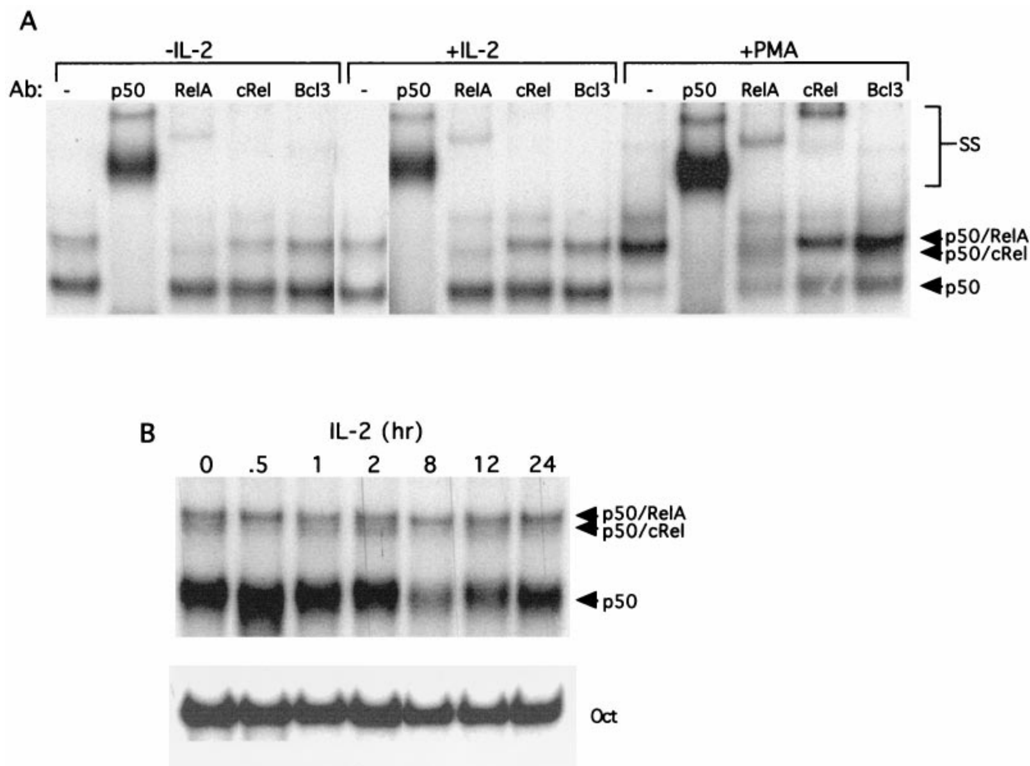

FIGURE 3. IL-2 does not induce NF-κB binding activity in TBCs. Arrows indicate complexes that contain the p50, RelA, and c-Rel proteins and SS denotes supershifted species. The results shown are representative of three independent TBC preparations. *A*, EMSA analysis using nuclear extracts from TBCs deprived of IL-2 for 28 h or for 24 h followed by IL-2 or PMA restimulation for 4 h. *B*, An extensive time-course analysis of NF-κB binding post-IL-2 restimulation (*upper panel*). Protein levels in the extracts were verified by analyzing binding activity to an Oct-1 probe (*lower panel*).

IL-2 vs 14% of the IL-2-deprived cells). In contrast to what was seen with IL-2, PMA stimulation enhanced the formation of complexes that contained p50, RelA, and c-Rel (Fig. 4) just as it did in TBCs. These data suggest that mature T cells, like TBCs, do not activate NF-κB when responding to IL-2.

The data shown above demonstrate that while PMA activates NF-κB in both TBCs and PB T cells, IL-2 does not. To determine whether NF-κB activation can be correlated with an inhibition of apoptosis in these cells, the effect of PMA stimulation on the apoptosis seen in IL-2-deprived TBCs was analyzed. As shown in Table I, TBCs deprived of IL-2 contained approximately 23% apoptotic cells, and IL-2 stimulation decreased this number to approximately 10% (similar but independent results are shown in Fig. 1). Only 11% apoptotic cells were observed when the cells were stimulated with PMA instead of IL-2, suggesting that PMA is almost as effective as the growth factor at blocking apoptotic death. Not surprisingly, PMA was not efficient at inducing cell cycle progression in these cells (Table I). Taken together, these data indicate that while the activation of NF-κB may correlate with an inhibition of apoptosis in these cells, it does not appear to be the mechanism employed by IL-2.

The AP-1 transcription factor, i.e., a Fos/Jun heterodimer, has also been proposed to save many cell types from apoptotic death, although in contrast to NF-κB, activation of the Jun N-terminal kinases, JNKs, and c-Jun has also been proposed to induce apoptotic death in some cell types (16–22). Therefore, it is unclear whether the activation of AP-1 can be correlated with the inhibition of apoptosis or cell cycle progression in IL-2-stimulated cells. To determine whether IL-2 activates the JNK pathway that can lead to transcriptionally active c-Jun, JNK phosphorylation on

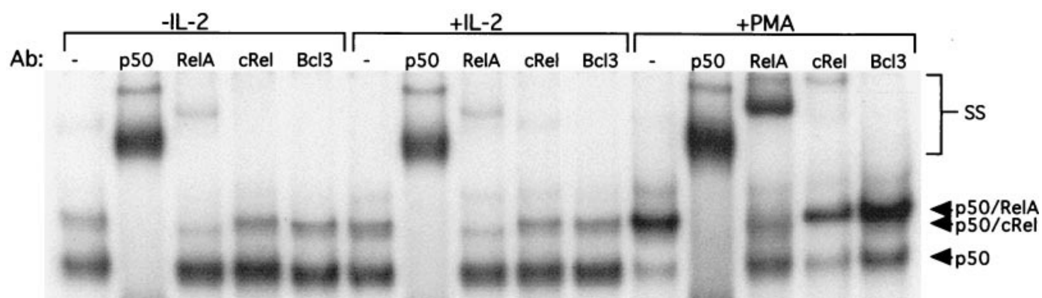

FIGURE 4. IL-2 does not induce NF-κB binding activity in PB T cells. A confirmatory preparation of PB T cells was isolated as described in the text to verify the results observed in TBCs. The cells were deprived of IL-2 for 47 h (−IL-2) or for 43 h followed by IL-2 or PMA restimulation as indicated for 4 h before nuclear extracts were isolated. Arrows indicate complexes that contain the p50, RelA, and c-Rel proteins, and SS denotes supershifted species.

Table I. *PMA effeciently relieves apoptosis but not cell cycle synchronization in TBCs deprived of IL-2*

Treatment	G_0/G_1[a]	S	G_2+M	TUNEL+[a]
IL-2 deprived	92.7 ± 0.9	4.5 ± 0.6	2.8 ± 0.6	23.2 ± 10
IL-2 restimulated	65.2 ± 8.4	29.6 ± 7.5	5.2 ± 1.5	9.9 ± 4.6
PMA restimulated	83.3 ± 13	10.1 ± 6.7	6.6 ± 6.6	10.8 ± 4.6

[a] Numbers given are the average of three (cell cycle) or five (apoptosis) independent experiments, and standard deviations are shown.

threonine 183/tyrosine 185 was analyzed in TBCs stimulated with IL-2. As shown in Fig. 5, significant phosphorylation of JNK in response to IL-2 was not observed in TBCs, suggesting that AP-1 cannot be activated by IL-2. Anisomysin, a known activator of the SEK/JNK pathway, did induce JNK phosphorylation in TBCs, demonstrating that JNK can be activated in these cells. In contrast, phosphorylation of STAT3 and STAT5, transcription factors known to be activated by IL-2 in both primary T cells and IL-2-responsive cell lines (28–33), was readily observed in IL-2-stimulated TBCs.

To confirm that AP-1 is not activated by IL-2 in TBCs, nuclear extracts were isolated from cells restimulated with IL-2 or PMA and ionomycin for the indicated times. These extracts were analyzed for AP-1 binding activity to a consensus TRE sequence. As shown in Fig. 6*A*, although a slight increase in TRE binding was observed with restimulation (1.5-fold when corrected for Oct binding activity), IL-2 did not induce in TBCs the formation of novel TRE-specific binding activity over that observed in cells deprived of IL-2. In addition, c-Fos was not present in the TRE-specific complex(es) observed, demonstrating that AP-1 was not present.

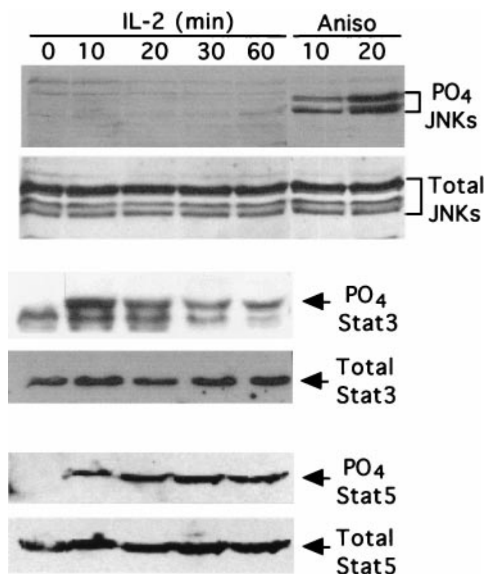

FIGURE 5. IL-2 stimulation of TBCs results in phosphorylation of STAT3 and STAT5, but not JNK1/2. Total protein was isolated from TBCs deprived of IL-2 for 24 h and then restimulated with IL-2 or anisomysin for the times indicated. Protein (50–75 μg/lane) was loaded and probed for either phosphorylated JNK1/2 or phosphorylated STAT3 and STAT5 using specific antisera. Total protein levels were determined for all molecules by stripping and reprobing the filters with antisera recognizing both phosphorylated and nonphosphorylated forms of JNK1/2, STAT3, and STAT5 as applicable. The results shown are representative of three independent TBC preparations.

PMA and ionomycin, on the other hand, induced strong TRE binding, which was due to AP-1, as evidenced by the presence of c-Fos. Similar results were obtained with PB T cells, in that PMA and ionomycin strongly induced TRE-specific binding activity, which includes c-Fos as a component of the complex (Fig. 6*B*). IL-2, on the other hand, did not activate TRE binding in PB T cells over that observed in IL-2-deprived cells and did not induce c-Fos binding (Fig. 6*B*). These data along with the evidence that JNK is not activated strongly suggest that IL-2 does not use the AP-1 transcription factor to promote cell cycle progression or to inhibit apoptosis in primary human T cells.

Discussion

Several studies have demonstrated that the NF-κB transcription factor is involved in the inhibition of apoptosis in many cell types (5–9; for review, see Refs. 10 and 11). Because IL-2 inhibits apoptosis in activated human T cells in addition to inducing cell cycle progression, it is logical to propose that activation of NF-κB occurs after IL-2 stimulation and that this mechanism is important to the inhibition of cell death. In fact, previous studies have found that IL-2 does activate NF-κB in cell lines and in freshly isolated primary human T cells stimulated with very high levels of the factor (14, 15). In addition, IL-2 is known to transcriptionally up-regulate the expression of the IL-2R α-chain gene, and it is known that the activation of this promoter by TCR/CD3 stimulation requires NF-κB. However, it was recently discovered that the IL-2 response element in the IL-2R α-chain promoter is a STAT5 binding site instead of the previously described NF-κB enhancer (43, 44). Therefore, IL-2 does not need to activate NF-κB to up-regulate this gene, and the data presented here demonstrate that IL-2 does not induce NF-κB binding activity in primary human T cells. It is important to note that resting T cells, previously shown to activate NF-κB in response to high concentrations of IL-2, are probably not the T cells responding to IL-2 in vivo, because they do not express the high affinity receptor. In previously activated T cells that already express the high affinity receptor, activation of NF-κB by IL-2 does not appear to occur.

The data presented here clearly demonstrate that NF-κB activation is not obligatory in primary T cells that respond to IL-2 by both progressing through the cell cycle and avoiding apoptosis. The observation that PMA both activates NF-κB and inhibits apoptosis suggests that this transcription factor may block death in T cells. In fact, several lines of evidence have supported the observation that NF-κB is activated when human T cells are stimulated through the Ag receptor, and it may be particularly important to survival signals in these cells in the thymus during development (45, 46). These processes, however, do not appear to involve IL-2 and are probably not relevant to mature T cells proliferating solely in response to the growth factor. It does appear that protection from apoptosis via the activation of NF-κB may be important in some cell lines that grow in response to IL-2. Because IL-2 does not inhibit apoptosis or induce cell cycle progression in primary human T cells via the activation of NF-κB, these observations suggest that IL-2R signaling pathways may be subverted in some immortalized cell lines. These unnatural signaling pathways may be very important to the immortalization and/or transformation of the cells, but this subversion may provide misleading results in the study of IL-2R signaling when applied to the events that occur in normal T cells responding to the growth factor.

Previous studies have demonstrated that both c-*fos* and c-*jun* are up-regulated by IL-2, leading to the obvious conclusion that AP-1 activity may be involved in the biologic consequences of IL-2 stimulation (for review, see Ref. 3). Because the Ca^{2+}-dependent

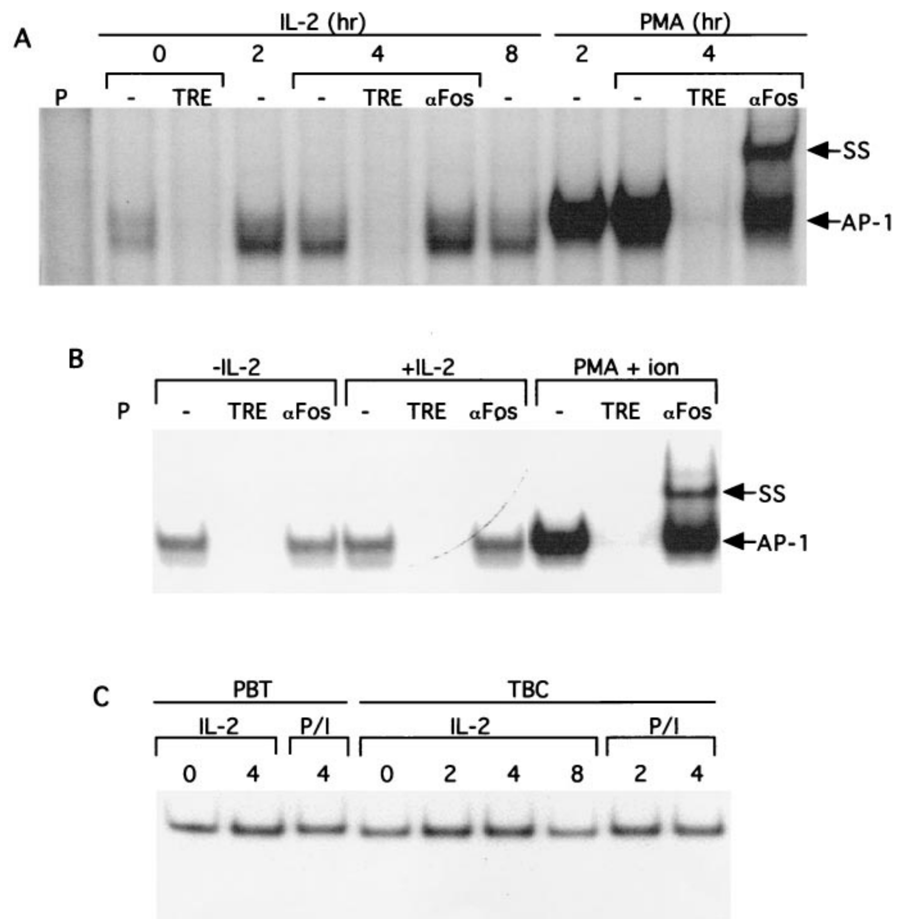

FIGURE 6. PMA and ionomycin, but not IL-2, activate AP-1 binding in TBCs and PB T cells. EMSA analysis using nuclear extracts isolated from TBCs (*A*) or PB T cells (*B*) deprived of IL-2 (−IL-2) or restimulated with IL-2 or with PMA and ionomycin for the times noted (*A*) or for 4 h (*B*). The arrow indicates the TRE-specific complex that contains c-Fos. SS denotes the supershifted species. In lanes denoted TRE and αFos, a 200-fold excess of unlabeled TRE DNA or c-Fos-specific antiserum was added, respectively, to the binding reaction to demonstrate the specificity of the complexes and the presence of the c-Fos protein. *C*, The levels of protein in each nuclear extract were controlled for loading by Oct binding. Differences in protein levels, as evidenced by differences in Oct binding, are responsible for much, but not all, of the increased TRE binding observed in TBC extracts. The experiments shown are representative of four independent T cell preparations (three thymic and one PB).

phosphatase calcineurin is required for the activation of JNK in T cells (47–49), and IL-2 does not induce a Ca^{2+} flux (3), it is logical to predict that JNK activation is not a consequence of IL-2 stimulation. Indeed, it is shown here that JNK activation is not the result of IL-2 stimulation in primary human T cells, nor is AP-1 binding activity observed up to 8 h poststimulation. However, JNK activation has been demonstrated to be the result of IL-2 stimulation in some factor-dependent T cell lines (27). Another study demonstrated that HTLV-1-transformed cells contain constitutively active JNK, but that IL-2-dependent cell lines immortalized by HTLV-1 infection do not contain active JNK in the absence of IL-2 (26). It was concluded from this latter study that IL-2 activates JNK in T cells and that the constitutive activation of JNK is an essential step in the development of the IL-2 independence required for transformation. Because IL-2 does not activate JNK in primary cells, JNK activation by IL-2 may somehow be a consequence of the immortalization process these cell lines have undergone, related to either HTLV-1 infection or the expression of the viral Tax protein in these cells. Again, the data suggest that IL-2R signaling may be subverted in at least some cell lines responding to the growth factor. This observation requires that caution be used when inferring that results obtained with these cells can be applied to nontransformed human T cells.

It is still unclear what mechanisms are responsible for cell cycle progression and the inhibition of apoptosis in human T cells responding to IL-2. Previous studies, such as those discussed above, demonstrating the activation of transcription factors to be protective against apoptosis strongly suggest that transcription is required for the inhibition of cell death in response to IL-2. By ruling out the involvement of NF-κB and AP-1 in the biological consequences of IL-2 stimulation, other transcription factors may now become the focus of investigation. It has been shown that the E2F transcription factor is activated by IL-2 and that this is mediated by phosphoinositol 3-kinase (PI-3K) (50), a kinase well established to be activated by the growth factor (3). PI-3K is known to activate the MEK/ERK (mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase) pathway in T cells responding to IL-2 (51), but it also activates AKT/PKB (protein kinase B) in many cell types tested (for review, see Refs. 52 and 53), and this kinase has been implicated in both cell cycle progression and the inhibition of apoptosis in cells responding to IL-2 (54). E2F activation requires AKT, but not ERK, and correlates well with cell cycle progression in cells responding to IL-2, although it is not sufficient in and of itself to induce proliferation (50). Whether E2F or, alternatively, the activation of other transcription factors that might be induced by AKT are involved in the inhibition of apoptosis mediated by IL-2 is not yet known.

As noted above, the JAK/STAT pathway is also stimulated by IL-2 (28, 30–33, 55–58). Both STAT3 and STAT5 are known to be activated by IL-2, and indeed, the activation of these STATs is readily observable in TBCs (Fig. 5). Zamorano et al. (59) recently reported that STAT5 is required for protection from apoptosis but not for cell cycle progression in 32D cells expressing the IL-2R. Additional studies have suggested that STATs are not required for proliferation induced by IL-2 (31, 60), but T cells from STAT-5A-deficient mice have a proliferative defect (44). STAT5 is required for IL-2-mediated IL-2Rα expression (43, 44), but decreased high affinity receptor expression may not be entirely

responsible for the decrease in proliferation observed in STAT5A-deficient mice (61). These data demonstrate that studies in primary mouse T cells may not always agree with what has been observed in immortalized murine cell lines, and it will be interesting to determine what else STAT-deficient mice will reveal about IL-2R signaling mechanisms. Further studies are required to determine whether STAT proteins are included among the transcription factors that are important for IL-2-mediated cell cycle progression or inhibition of apoptosis in primary human T cells. Either way, NF-κB and AP-1 are not activated by IL-2 in these cells, suggesting that other transcription factors must be involved in these events in primary human T cells responding to IL-2.

Acknowledgments

We thank Chiron Corp. for rIL-2, New England Biolabs for antisera specific for phosphorylated STAT5, Dr. Constantine Tsoukas for helpful discussion, Dennis Young of the Stem Cell Laboratory at University of California at San Diego for assistance with FACS analysis, and Carol Worden and the staff of the operating room at Children's Hospital of San Diego for thymic specimens.

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