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*J Immunol* (1998) 160 (7): 3502–3512.

<https://doi.org/10.4049/jimmunol.160.7.3502>

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# Regulation of Cell Growth by IL-2: Role of STAT5 in Protection from Apoptosis But Not in Cell Cycle Progression<sup>1</sup>

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Cytokines play an essential role in the regulation of lymphocyte survival and growth. We have analyzed the pathways activated by IL-2 that lead to protection from apoptosis and cell proliferation. IL-2 can act as a long-term growth factor in 32D cells expressing the wild-type human (hu)IL-2R $\beta$ . By contrast, cells expressing a truncated form of the huIL-2R $\beta$ , which is able to induce Bcl-2 and *c-myc* expression but not STAT5 activation, were not protected from apoptosis by IL-2; consequently, they could not be grown long term in the presence of IL-2. However, IL-2 promoted cell cycle progression in cells bearing the truncated huIL-2R $\beta$  with percentages of *viable* cells in the G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases similar to cells expressing the wild-type huIL-2R $\beta$ . Transplantation of a region from the erythropoietin receptor, which contains a docking site for STAT5 (Y343) to the truncated huIL-2R $\beta$ , restored the ability of IL-2 to signal both activation of STAT5 and protection from apoptosis. By contrast, transplantation of a region from the huIL-4R $\alpha$  containing STAT6 docking sites did not confer protection from apoptosis. These results indicate that the IL-2-induced cell cycle progression can be clearly distinguished from protection from apoptosis and that STAT5 participates in the regulation of apoptosis. *The Journal of Immunology*, 1998, 160: 3502–3512.

IL-2 is a cytokine with a wide spectrum of biologic activities, which include its ability to act as a growth factor for several cell types, including T and B lymphocytes (1–3). Cell growth is a complex mechanism which requires the efficient activation of at least two different but overlapping processes: mitogenesis and anti-apoptosis. The mitogenic process will allow cells to progress through the cell cycle; it is an active process that ultimately leads cells to replicate DNA. The apoptotic process leads to the activation of endonucleases that promote DNA degradation and, consequently, cell death (4). Therefore, the activation of those cellular pathways that mediate both cell proliferation and protection from apoptosis act in concert to achieve successful cell growth.

IL-2 responses are mediated by its receptor complex, which consists of three different subunits, IL-2R $\alpha$ , IL-2R $\beta$ , and IL-2R $\gamma$  (5–7). It is well known that, like other cytokine receptors, the IL-2R complex lacks intrinsic tyrosine kinase activity. However, IL-2 stimulation induces the tyrosine phosphorylation of numerous proteins (8, 9). Several nonreceptor protein tyrosine kinases such as JAK-1 and JAK-3 (10–12), p56<sup>lck</sup> (13), and Syk (14), which interact with the IL-2R complex, have been shown to become activated after IL-2 engagement. The cytoplasmic tail of the human (hu)IL-2R $\beta$ ,<sup>3</sup> which is believed to play the main role in signal transduction, has been divided in three functional domains (15).

The most membrane-proximal domain contains a serine-rich region. This region has been associated with the activation of JAKs (10, 12), *c-myc* (16–18), and Bcl-2 (19). There is an intermediate domain containing an acidic region that includes Y-338, Y-355, Y358, and Y-361. This domain has been shown to be required for the activation of p56<sup>lck</sup> (13), shc (20, 21), *c-fos*, and *c-jun* (16, 22). Finally, there is an additional carboxyl-terminal domain containing Y392 and Y510, which are required for the activation of STAT5 in response to IL-2 (21, 23, 24).

The expression of JAK tyrosine kinases is necessary for the STAT proteins to become phosphorylated after cytokine stimulation, and this JAK/STAT pathway is ubiquitous in cytokine signaling (25–28). After cytokine stimulation, STAT proteins couple to the cytokine receptor through binding to specific tyrosine residues after phosphorylation. As a result, STAT proteins become phosphorylated, and after dimerization, migrate to the nucleus where they act as transcription factors. IL-2 induces the activation of STAT5, and the role that STAT5 plays in IL-2 signaling is now under active investigation (21–24, 29–32). Although it is established that STAT5 regulates the transcription of several genes (33, 34), its role in regulating cell growth is not clearly defined. Several studies have indicated that different IL-2R $\beta$  constructs unable to activate STAT5 could transmit a proliferative response (15, 23). By contrast, other studies have shown that a form of the IL-2R $\beta$  that did not activate STAT5 could not properly signal cell proliferation in response to IL-2 (21, 35, 36). On the other hand, the role that STAT5 plays in the regulation of apoptosis has not yet been addressed.

Therefore, the aim of our study was to investigate the role of STAT5 in regulating the IL-2-induced cell growth; consequently, we have focused on the IL-2-mediated cell proliferation and protection from apoptosis. We found that STAT5 regulates the long-term survival mediated by IL-2, but it is not required for the IL-2-induced cell cycle progression.

## Materials and Methods

### Cells and reagents

The IL-3-dependent myeloid cell line 32D was maintained in RPMI 1640 culture medium supplemented with glutamine, penicillin, streptomycin, 5% FCS, and 5% WEHI-3 conditioned medium. The 32D cells lacking or

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Received for publication September 26, 1997. Accepted for publication December 1, 1997.

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<sup>1</sup> This work was supported by United States Public Health Service Grant AI 38985 and the American Red Cross.

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<sup>3</sup> Abbreviations used in this paper: huIL-2R $\beta$ , human IL-2R $\beta$ ; huIL-4R $\alpha$ , human IL-4R $\alpha$ ; wt, wild-type; GAS,  $\gamma$ -IFN activation site; EPO, erythropoietin; EPOR, erythropoietin receptor; chim, chimera; aa, amino acid; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

expressing the wild-type (wt) and the truncated huIL-2R $\beta$  have been previously described (36). The 32D cells expressing chimIL4R has previously been termed chim2 (36). Murine rIL-4 expressed in baculovirus was affinity purified as described (37). Recombinant huIL-2 was a gift from Dr. Steven Rosenberg (National Institutes of Health, Bethesda, MD).

#### Immunoprecipitation and immunoblotting

Cells were starved from IL-3 for 18 h at 37°C. After washing,  $20 \times 10^6$  cells were resuspended in medium (RPMI 1640 plus 10% FCS) in the presence or absence of IL-2 (200 U/ml) or IL-3 (5% WEHI-3-conditioned medium) for an additional 20 h. Afterward, the cells were washed in ice-cold PBS. To determine Bcl-2 levels, cell pellets were lysed in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 10 mM pyrophosphate, 1 mM PMSF, and protease inhibitor mixture) and clarified. The protein concentration was measured using the Bio-Rad protein assay, and 40  $\mu$ g of total protein were separated on 12% SDS-polyacrylamide gels before transfer to a polyvinylidene difluoride (PVDF) membrane. The membranes were then probed with an anti-Bcl-2 mAb (Transduction Laboratories, Lexington, KY). The bound Ab was detected using enhanced chemiluminescence (Amersham, Arlington, IL). To determine *c-myc* protein, nuclear extracts were analyzed. After washing, cells were resuspended in buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 5 mM NaF, 0.1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, and protease inhibitor mixture) plus 0.5% Nonidet P-40 for 5 min on ice. Nuclei were pelleted and washed twice in buffer A. Later, nuclei were incubated for 30 min in buffer B (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 5 mM NaF, 0.5 mM DTT, 1 mM PMSF, 25% glycerol, and protease inhibitor mixture) and clarified. Nuclear extract was measured using the Bio-Rad assay, and 40  $\mu$ g of total protein were separated on 12% SDS-polyacrylamide gels. *c-myc* protein was detected with a rabbit anti-*c-myc* Ab (Upstate Biotechnology, Lake Placid, NY) as indicated above.

#### Apoptosis and cell cycle assay

The percentage of apoptotic cells was determined by analyzing the nuclear DNA content by flow cytometry as indicated (38). After specific culture conditions, 32D cells were resuspended in 0.25 ml of propidium iodide solution (50  $\mu$ g/ml propidium iodide, 0.1% sodium citrate, 0.1% Nonidet P-40, and 50  $\mu$ g/ml RNase (Sigma Chemical Co., St. Louis, MO) and incubated for 30 min at room temperature. DNA content was then analyzed by flow cytometer (FACScan, Becton Dickinson, Mountain View, CA). The apoptotic cells were defined as those with less than a 2N DNA content. Cell cycle distribution was analyzed using the same procedure, but apoptotic cells were ignored using the appropriate gate.

The TUNEL assay was performed using the Apotag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) following the manufacturers protocol and later analysis by FACScan.

#### Proliferation assays

The cells were incubated in 96-well plates at 1000 cells in 0.2 ml of complete RPMI in the presence or absence of different concentrations of huIL-2 or IL-3 for the time indicated. The cells were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine for the last 4 h of culture before harvesting using a Packard harvester and the Matrix 9600 direct  $\beta$  count system.

#### Construction of chimeric receptors

The truncated huIL-2R $\beta$  containing the aa 1–378 of the huIL-2R $\beta$  and the chimIL4R (named as chim2) containing the aa 1–378 of the huIL-2R $\beta$  plus the aa 558–657 of the huIL-4R  $\alpha$ -chain has been previously described (36). The chimeric receptor huIL-2R $\beta$ /EPOR was made by a similar strategy. Sense oligonucleotide (bchim-1) corresponding to the sequence 5' to the *Aat*II site of the female huIL-2R $\beta$  (960 bp) and antisense oligonucleotide (epor1) containing sequence derived from the mouse EPOR 5' to the Y343 (1062 bp) and sequence derived from the huIL-2R $\beta$  (1343 bp) were used to amplify a 407-bp fragment from the huIL-2R $\beta$  template. In addition, sense oligonucleotide complementary to the epor1 (epor2) and antisense oligonucleotide (epor3) derived from EPOR sequence 3' to the Y343 (1200 bp) and containing translational Stop and *Xba*I sites were used to amplify a fragment from an EPOR cDNA template. The two PCR products were purified, mixed, and used as template for a second PCR reaction with bchim-1 and epor3 as primers. The 572-bp fragment was purified and digested with *Aat*II and *Xba*I and ligated into the huIL-2R $\beta$ -pME18s vector (a generous gift of Dr. Warren Leonard, National Institutes of Health, Bethesda, MD). Sequence was verified by the Sanger dideoxy chain-termination method and then analyzed on an automated sequencing machine.

The chimEPOR includes the aa 1–378 of the huIL-2R $\beta$ -chain and aa 317–367 of the EPOR.

#### Transfections

Cells were washed and resuspended in PBS. For each transfection,  $2 \times 10^7$  cells were mixed with 2  $\mu$ g of vector carrying neomycin resistance and 20  $\mu$ g of receptor cDNA, and subjected to electroporation using a Bio-Rad gene-pulsar set on 200 V and 960  $\mu$ F. After transfection, the cells were cultured overnight in appropriate media before selection with G418 (Life Technologies, Grand Island, NY). Neomycin-resistant lines were tested for expression of huIL-2R $\beta$  by FACS analysis using biotin-anti-huIL-2R $\beta$  (Endogen, Boston, MA) followed by streptavidin-phycoerythrin (Southern Biotechnology, Birmingham, AL) as described (36). The clones used in this study had similar receptor expression levels (see Fig. 3).

#### Electrophoretic mobility shift assay

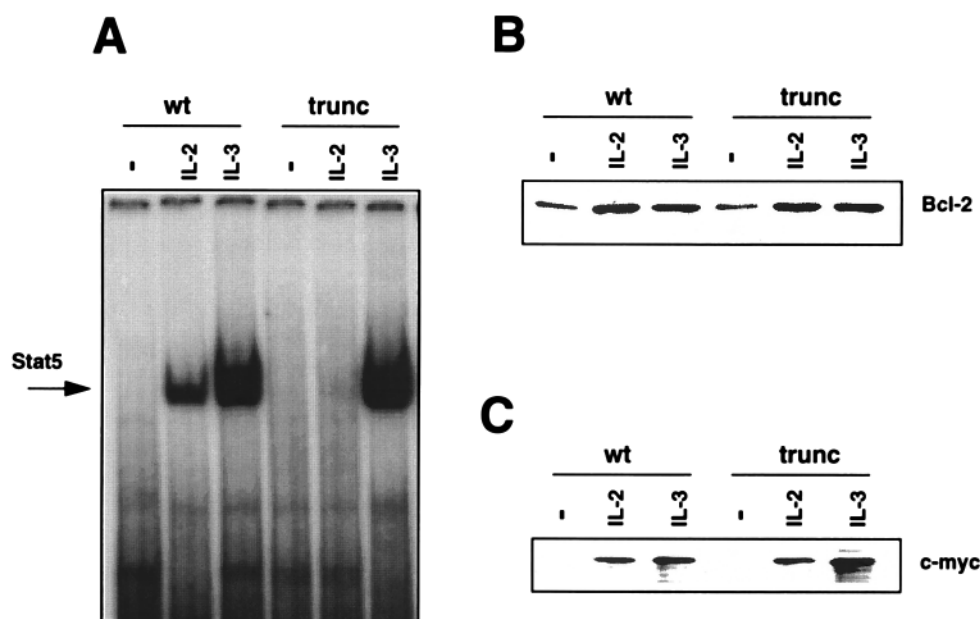
Cells were starved for 3 h and then stimulated with the appropriate cytokine for 30 min at 37°C. After washing with PBS, they were resuspended for 30 min in lysis buffer (0.5% Nonidet P-40, 50 mM Tris (pH 8.0), 10% glycerol, 0.1 mM EDTA, 200 mM NaCl, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 0.5 mM PMSF, and protease inhibitor mixture). Lysates were clarified and incubated with 1 ng of <sup>32</sup>P-labeled oligonucleotide in reaction buffer (40 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.5 mM DTT, 20 mM HEPES (pH 7.9), 6% glycerol, and 0.1 mg/ml poly(dIdC)) for 20 min at room temperature. To determine IL-2-induced DNA-binding activity, we used the GAS sequence found in the FC $\gamma$ RI promoter (5'-atgcGTATTTCCCA GAAAAGGAAC). For the IL-4-mediated DNA-binding activity, we used the GAS sequence in the C $\epsilon$  promoter (5'-actgCACTTCCCAAGAA CAGA). Polyacrylamide gels (4.5%) containing 0.22 $\times$  Tris-borate-EDTA were prerun for 1 h at 200 V. After loading the samples, the gels were run at 290 V for approximately 2 h. Afterward, gels were dried and exposed to film. For gel supershift experiments the reaction mixture was supplemented with 1  $\mu$ g of the appropriate anti-STAT3, anti-STAT5, or anti-STAT6 Abs (Santa Cruz Biotechnology, Santa Cruz, CA).

## Results

### The truncated huIL-2R $\beta$ does not signal STAT5 activation

To address the role of STAT5 in regulating cell growth in response to IL-2, we have made a huIL-2R $\beta$  truncation terminating at aa 378 similar to the h-truncated huIL-2R $\beta$  described previously (15). This deletion mutant lacks Y392 and Y510 that are required for the IL-2-mediated STAT5 activation (21, 23, 24). We then transfected the myeloid cell line 32D with the wt or truncated huIL-2R $\beta$ . 32D is a murine IL-3-dependent cell line that expresses the  $\alpha$ - and  $\gamma$ -chains, but not the  $\beta$ -chain of the IL-2R complex; therefore, it does not respond to murine IL-2 (39). Transfection of 32D cells with the wt huIL-2R $\beta$  enables them to respond to human IL-2 (21, 36, 39). As previously described (23), IL-2 induced STAT5 DNA-binding activity in 32D cells transfected with the wt but not with the truncated huIL-2R $\beta$  (Fig. 1A). All these cell lines showed STAT DNA-binding activity in response to IL-3 (Fig. 1A) and IL-4 (see Fig. 7). By contrast, the induction of Bcl-2 (19) and *c-myc* (16–18) is dependent on the most membrane proximal domain of the huIL-2R $\beta$ . Therefore, the truncated huIL-2R $\beta$  was able to up-regulate the expression of Bcl-2 (Fig. 1B) and *c-myc* (Fig. 1C) upon stimulation with IL-2. Although some variation can be observed between different cell lines, the levels of Bcl-2 were quite similar in cells expressing the wt or truncated huIL-2R $\beta$  stimulated with IL-2 or IL-3 (Fig. 1B). In addition, we observed no significant differences in *c-myc* levels in cells expressing the wt huIL-2R $\beta$  stimulated with IL-2 or IL-3. However, we found that the level of *c-myc* in cells bearing the truncated huIL-2R $\beta$  stimulated with IL-2 was consistently less than in cells treated with IL-3. We believe this to be due to the instability of *c-myc* protein (40) since about 50% of these cells stimulated with IL-2 were dead (see below and Fig. 5).

**FIGURE 1.** Regulation of cellular messengers by the truncated form of the huIL-2R $\beta$ . **A**, 32D cells expressing the wt or truncated huIL-2R $\beta$  (trunc) were cultured in the absence or presence of IL-2 (200 U/ml) or IL-3 (5% WEHI-3-conditioned media) for 30 min, and STAT DNA-binding activity was analyzed using the GAS sequence contained in the FC $\gamma$ RI promoter. **B**, Cells were starved for 18 h, and then they were stimulated or not with IL-2 or IL-3 for 20 h. Afterward, total cell lysate was separated by SDS-PAGE, and transferred protein was immunoblotted with an anti-Bcl-2 Ab. **C**, Same as **B**, but *c-myc* was detected in nuclear extracts with an anti-*c-myc* Ab. Similar results were observed in at least three different clones.



#### *The truncated huIL-2R $\beta$ signals short- but not long-term cell proliferation*

Several studies have analyzed the role that STAT5 plays in the IL-2-mediated cell proliferation using different huIL-2R $\beta$  constructs impaired in their ability to signal STAT5 activation (21, 23, 35, 36). These studies demonstrated that the proliferation mediated by IL-2 was totally dependent (35, 36), partially dependent (21), or completely independent (23) on the expression of STAT5-docking sites. Since those studies were performed in either short-term (21, 23) or long-term (35, 36) cell cultures, we analyzed the ability of IL-2 to signal proliferation in both short- and long-term cultures. We found that IL-2 was able to induce cell proliferation in a short-term culture in those cells expressing the truncated form of the huIL-2R $\beta$  (Fig. 2A). After 24 h of stimulation with IL-2, cells expressing the truncated huIL-2R $\beta$  incorporated 40% to 90% [ $^3$ H]thymidine with respect to cells expressing the wt huIL-2R $\beta$ . At this time, cells expressing the wt huIL-2R $\beta$  responded to IL-2 with [ $^3$ H]thymidine incorporation that was  $\sim$ 50% of the levels of [ $^3$ H]thymidine uptake elicited by IL-3. Cells expressing the truncated huIL-2R $\beta$  responded to IL-2 with  $\sim$ 20 to 45% [ $^3$ H]thymidine uptake with respect to that elicited by IL-3. However, the results were quite different in long-term assays. IL-2 was able to maintain long-term proliferation in cells expressing the wt huIL-2R $\beta$ ; these cells showed a high incorporation of [ $^3$ H]thymidine after 48, 72, and 96 h of stimulation with IL-2. In contrast, IL-2 did not promote [ $^3$ H]thymidine incorporation in 32D cells expressing the truncated huIL-2R $\beta$  in long-term experiments; in fact, the incorporation of [ $^3$ H]thymidine declined with time until it almost disappeared at 72 to 96 h. Similar results were obtained using a wide range of IL-2 concentrations (36) (see Fig. 8A).

#### *The truncated huIL-2R $\beta$ does not signal protection from apoptosis*

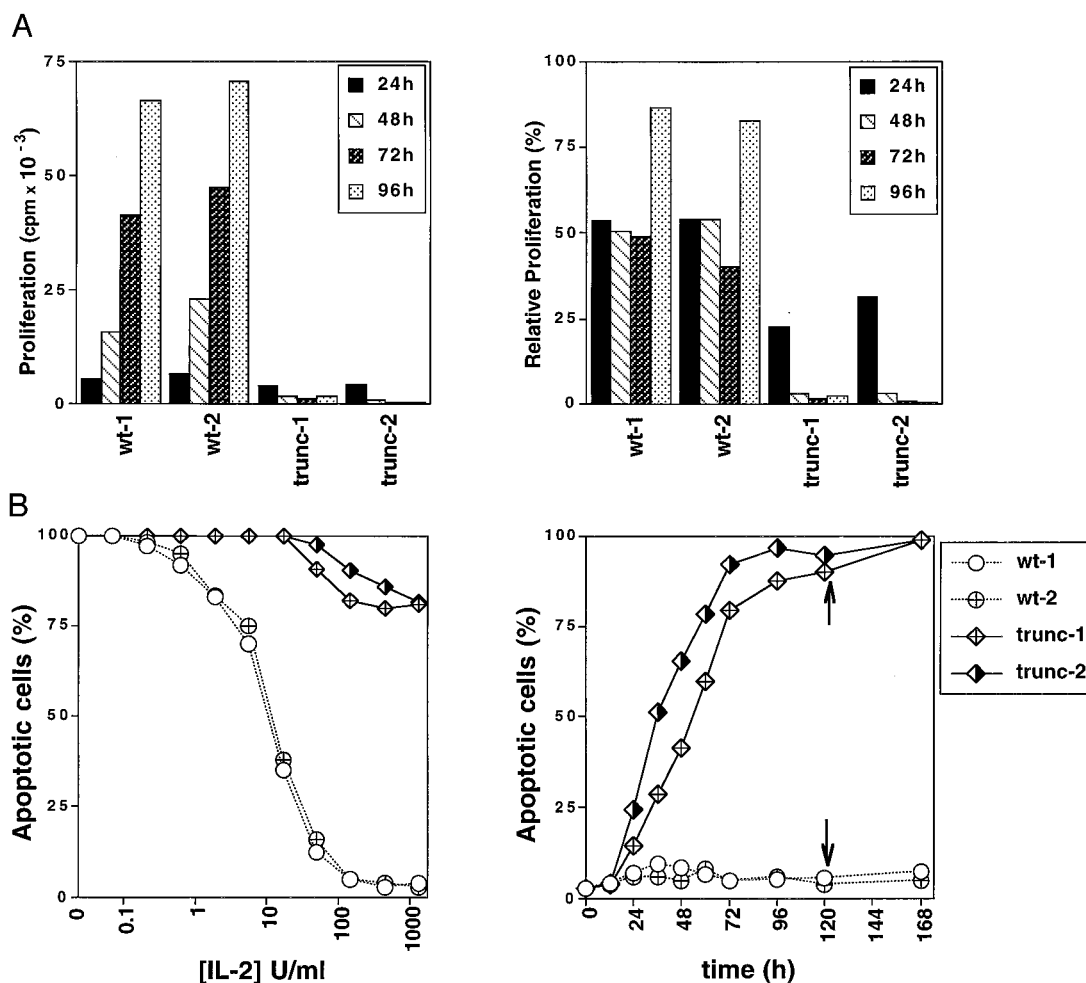
The previous results may be due to the inability of the truncated receptor either to transmit a proliferative signal or to protect cells from apoptosis. Therefore, we first examined the ability of this truncated receptor to protect cells from apoptosis. We analyzed, by propidium iodide staining of nuclear DNA, the ability of IL-2 to protect 32D cells expressing the wt or the truncated huIL-2R $\beta$  from apoptosis induced by IL-3 withdrawal. We found that IL-2

protected 32D cells expressing the wt but not the truncated huIL-2R $\beta$  from apoptosis after IL-3 withdrawal (Fig. 2B). As we expected, IL-2 was a potent survival factor for those cells expressing the wt huIL-2R $\beta$ ; in fact, IL-2 can be used as a long-term growth factor. Conversely, those cells expressing the truncated huIL-2R $\beta$  died over time in the presence of IL-2 although there was a slight and transient protection at high concentrations of IL-2. Even though after 24 h of stimulation with IL-2 most of these cells were still alive (only 15–25% apoptotic cells), after 4 days of culture most of them were dead ( $>$ 90% apoptotic cells). Furthermore, IL-2 could not replace IL-3 as a long-term growth factor in cells expressing the truncated form the huIL-2R $\beta$ .

Although the truncation of the cytoplasmic domain of the huIL-2R $\beta$  does not affect the extracellular IL-2-binding properties (15), it is possible that after long-term cultures the number of receptors and/or the IL-2 available could affect the ability of the truncated huIL-2R $\beta$  to signal protection from apoptosis. However, this is not the case since the levels of huIL-2R $\beta$  expression remained basically unchanged in all cell types during short- and long-term cultures (Fig. 3). In addition, even with continual addition of 300 U/ml of IL-2 to the cultures every 24 h in a 5-day assay, the truncated huIL-2R $\beta$  was still unable to prevent cell death (data not shown). Therefore, a difference in the level of IL-2R expression and/or the availability of IL-2 do not explain the differential ability of the receptors to signal protection from apoptosis.

#### *The truncated huIL-2R $\beta$ can signal cell cycle progression*

It has been shown that 32D cells undergo G<sub>1</sub> arrest and subsequent cell death by apoptosis after IL-3 withdrawal when they are cultured in the absence of cytokines (41, 42). However, we observed that while IL-2 did not protect 32D cells expressing the truncated huIL-2R $\beta$  from apoptosis, it promoted cell cycle progression. When we analyzed the cell cycle distribution only in living cells, by gating for the cell population with  $\geq$  2N DNA, we observed that the cell cycle profiles of 32D cells expressing the wt or the truncated huIL-2R $\beta$  stimulated with IL-2 were quite similar (Fig. 4). After 36 h in the absence of IL-2, cells were G<sub>1</sub> arrested; about 94% of the living cells expressing the wt or the truncated huIL-2R $\beta$  were in the G<sub>1</sub> phase of the cell cycle. In the presence of IL-2, however, 41% and 47% of the living cells expressing the wt and



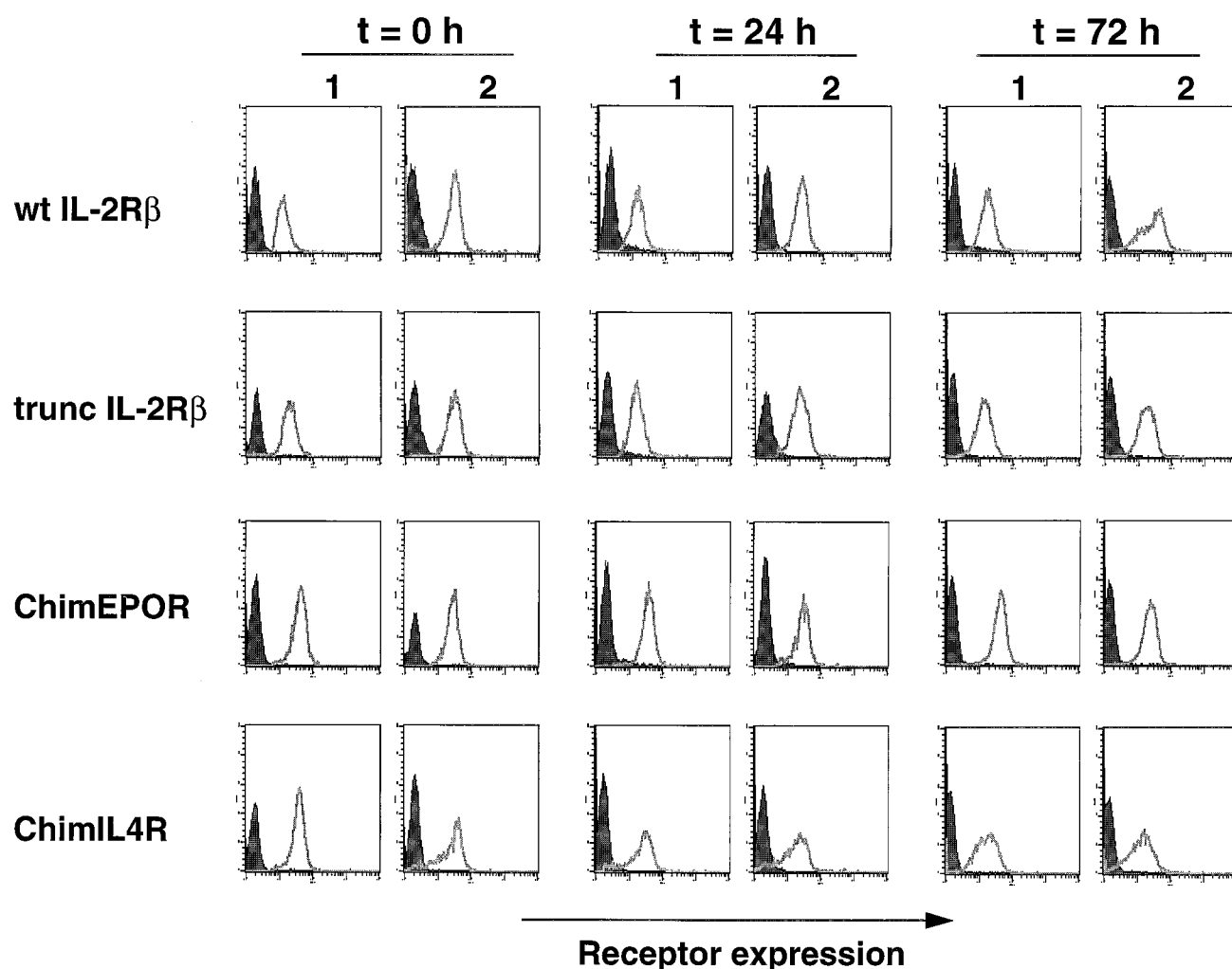
**FIGURE 2.** Proliferation and apoptosis in 32D cells expressing the wt or truncated huIL-2R $\beta$  in response to IL-2. *A*, Cells were cultured in the presence of IL-2 (200 U/ml) for 24, 48, 72, and 96 h. [ $^3$ H]thymidine was added for the last 4 h of culture before harvesting (*left*). In the *right panel*, relative proliferation indicates the incorporation of [ $^3$ H]thymidine in cells stimulated with IL-2 with respect to the incorporation of [ $^3$ H]thymidine in the same cell line stimulated with IL-3 (5% WEHI-3-conditioned medium). Cells expressing the wt and the truncated huIL-2R $\beta$  showed similar [ $^3$ H]thymidine uptake at all time points tested in response to IL-3. *B*, 32D cells expressing wt (circles) or truncated (diamonds) huIL-2R $\beta$  were cultured for 72 h in the presence of increasing amounts of IL-2 (*left*), or they were cultured in the presence of IL-2 (200 U/ml) for the indicated period of time (*right*). Afterward, the cellular DNA content was analyzed, and the apoptotic cells were defined as those with less than 2N DNA content. All cell lines stimulated with IL-3 showed a similar percentage of apoptotic cells with only 3 to 5% in all time points tested. The arrow indicates where the cells were refed. Two representative clones expressing the wt or the truncated huIL-2R $\beta$  are shown.

the truncated huIL-2R $\beta$ , respectively, were in the G<sub>1</sub> phase of the cell cycle while more than 50% of cells were in the S and G<sub>2</sub>/M phases in both cell populations (Fig. 4). At that time, 52% of total cells expressing the truncated huIL-2R $\beta$  were apoptotic while only 9% of wt huIL-2R $\beta$  expressing cells were dead. Furthermore, after 72 h of stimulation with IL-2 when most cells expressing the truncated huIL-2R $\beta$  were dead (89%), the living cells demonstrated an active cell cycle progression profile with 44% cells in G<sub>1</sub> and 56% of them were cycling (S + G<sub>2</sub>/M). This distribution is similar to the cell cycle distribution observed in 32D cells expressing the wt huIL-2R $\beta$  with 40% cells in G<sub>1</sub> and 60% in the cell cycle.

These experiments were performed using an asynchronous cell population. To determine whether the truncated huIL-2R $\beta$  was able to signal entry into cell cycle from G<sub>1</sub>, we cultured 32D cells in the absence of exogenous cytokines for 18 h to induce G<sub>1</sub> arrest. As we and others have previously shown (39, 40), after such culture about 80 to 85% of cells were in G<sub>1</sub>. G<sub>1</sub>-arrested 32D cells were incubated in the presence or absence of IL-2 for an additional 24 to 48 h, and then cell cycle was analyzed (Fig. 5). Since the cells had been preincubated without stimulus, they died faster;

therefore, the times of incubation were shorter than in those cells that were not arrested (compare Figs. 4 and 5). IL-2 induced an active cell cycle progression both in 32D cells expressing the wt or the truncated huIL-2R $\beta$ . Approximately 50% of living cells expressing the wt or the truncated huIL-2R $\beta$  were in the S + G<sub>2</sub>/M phases after 24 h and 48 h of stimulation with IL-2 even though after 48 h of stimulation with IL-2 most cells expressing the truncated huIL-2R $\beta$  were dead (89% apoptotic cells). Conversely, those cells that had been cultured in the absence of cytokines for an additional 24 h remained G<sub>1</sub> arrested with greater than 90% of cells in the G<sub>1</sub> phase. These results demonstrate that the truncated form of the huIL-2R $\beta$  is able to transmit signals that promote cell cycle progression but not protection from apoptosis.

As mentioned above, 32D cells undergo G<sub>1</sub> arrest after factor withdrawal and subsequently die by apoptosis. However, cells expressing the truncated huIL-2R $\beta$  did not suffer G<sub>1</sub> arrest when stimulated with IL-2 even though they were not protected from apoptosis. To determine at what stage of the cell cycle these cells died, we used the TUNEL assay. The TUNEL assay consists of an enzymatic incorporation of digoxigenin-nucleotides into the DNA



**FIGURE 3.** Analysis of receptor expression. 32D cells expressing the different receptor constructs used in this study, wt huIL-2R $\beta$ , truncated huIL-2R $\beta$ , chimEPOR, and chimIL4R, were cultured in the presence of IL-2 (200 U/ml) for the indicated period of time (0, 24, and 72 h). Then, cell surface expression of the receptors was analyzed by FACScan using an anti-huIL-2R $\beta$  Ab (open area). Closed areas show the profile of cells stained in the absence of anti-huIL-2R $\beta$  Ab as indicated in *Materials and Methods*. Two representative clones of each (1 and 2) are shown.

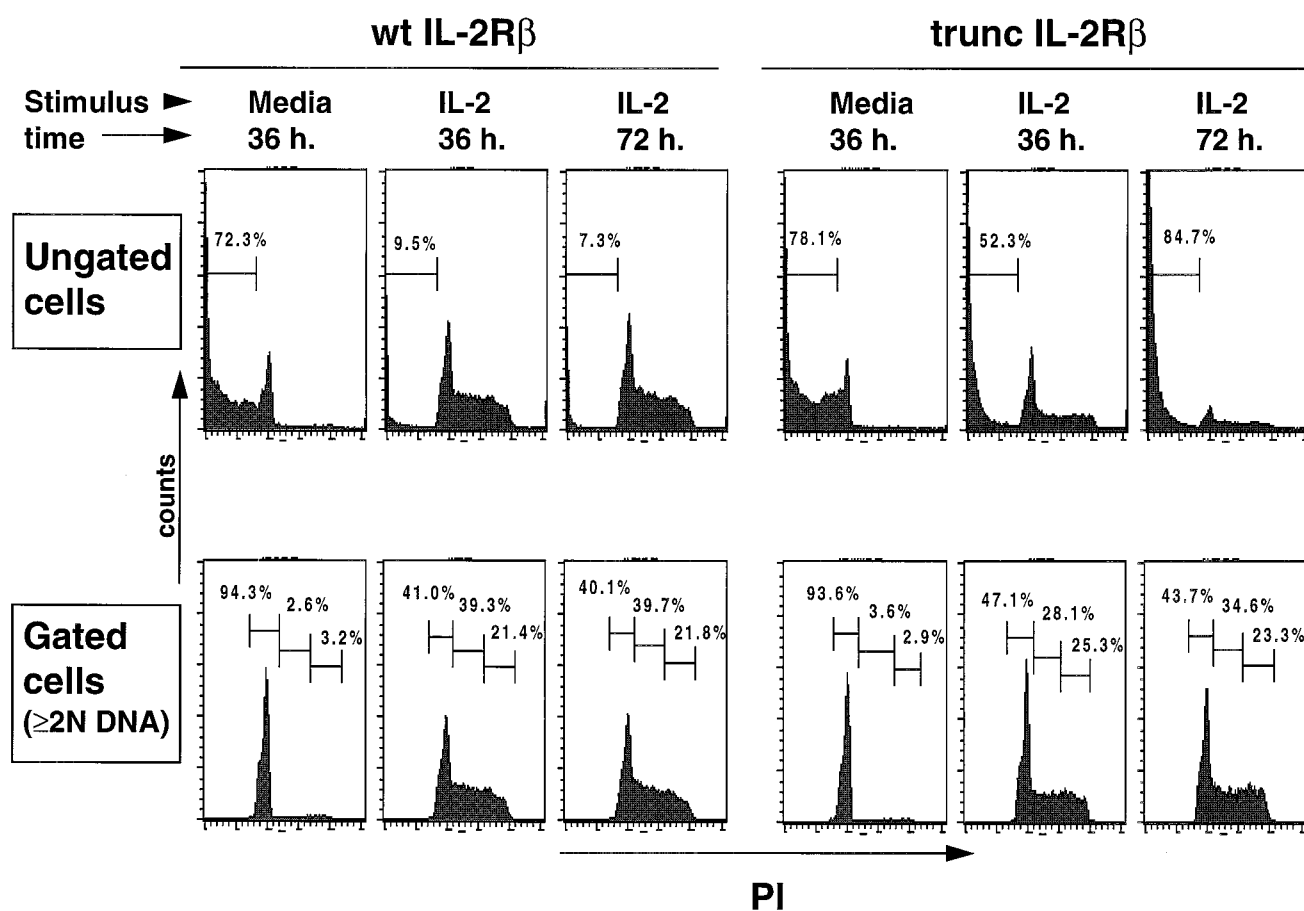
fragments characteristic of apoptosis, which combined with a propidium iodide staining of genomic DNA allows the determination of the phase in which the cells die. When 32D cells expressing the wt or the truncated huIL-2R $\beta$  were cultured for 48 h in the absence of IL-2, all of them incorporated digoxigenin-nucleotides in the G<sub>0</sub>/G<sub>1</sub> phase, which indicates they died in this phase of the cell cycle as expected (Fig. 6). In contrast, cells expressing the wt huIL-2R $\beta$  stimulated with IL-2 were not TUNEL positive. Interestingly, approximately 50% of the cells expressing the truncated huIL-2R $\beta$  stimulated with IL-2 incorporated the digoxigenin-nucleotides into the G<sub>0</sub>/G<sub>1</sub> phase. These data suggest that, like unstimulated cells, 32D cells expressing the truncated huIL-2R $\beta$  stimulated with IL-2 died by apoptosis in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle.

*STAT5 activation is related to protection from apoptosis and long-term growth*

So far, the only characterized signal transduction factor that is not activated by the truncated huIL-2R $\beta$  is STAT5 (19, 21, 23, 24) (Fig. 1). It has been shown that the truncated huIL-2R $\beta$  is either unable to or vastly impaired at signaling STAT5 activation since it lacks Y392 and Y590, which are the docking sites for STAT5 proteins (21, 23, 24). To delineate the contribution of STAT5 to

the IL-2-induced protection from apoptosis, we reconstituted the ability of the truncated receptor to activate STAT5 by a gain of function approach. The erythropoietin (EPO) receptor (EPOR) has been shown to induce STAT5 activation in response to EPO, and the cytoplasmic Y343 plays an important role in the recruitment of STAT5 in response to EPO (43, 44). Therefore, we transplanted a short fragment of the EPOR (aa 317–367) containing Y343 to the truncated huIL-2R $\beta$  (chimEPOR). As shown in Figure 7A, transplantation of this fragment from the EPOR reconstituted the ability of IL-2 to induce STAT5 activation. IL-2 promoted STAT5 DNA-binding activity in those cells expressing the chimEPOR, and furthermore, an anti-STAT5 Ab recognized this complex. In addition, we transplanted the STAT6 domain of the huIL-4R $\alpha$ , comprising aa 558–657, to the truncated huIL-2R $\beta$  (chimIL4R; previously termed chim2) (36). In Figure 7B, we show that IL-2 was able to induce STAT6 activation in 32D cells expressing the chimIL4R, but not in those cells expressing the wt or the truncated huIL-2R $\beta$ . All cell lines tested expressed a similar number of receptors before and after culture in the presence of IL-2 (Fig. 3).

Afterward, we analyzed the ability of these chimeric receptors to promote cell proliferation and protection from apoptosis. The chimEPOR, which was able to activate STAT5, completely reconstituted the ability of IL-2 to transmit a proliferative response (Fig.



**FIGURE 4.** Cell cycle analysis of 32D cells in response to IL-2. 32D cells expressing the wt or the truncated huIL-2R $\beta$  were stimulated with nothing or IL-2 for 36 and 72 h. Then, the percentage of apoptotic cells in the whole cell population was analyzed (*upper graphics*). The cell cycle distribution was analyzed only in living cells by gating cells having  $\geq 2N$  DNA content (*lower graphics*). Cells cultured in the absence of IL-2 for 72 h are not shown since all of them were dead. This figure shows a representative of at least three different experiments.

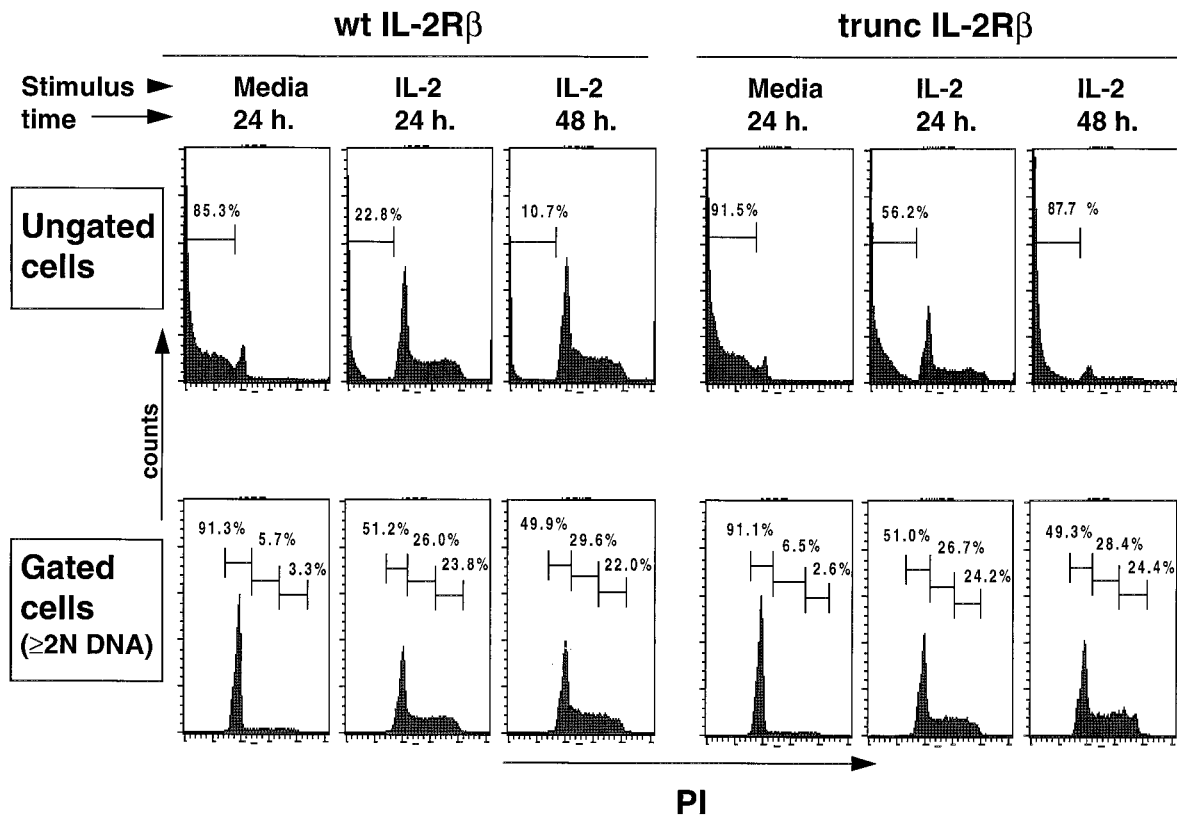
8A and B). 32D cells expressing the chimEPOR proliferated in response to IL-2, both in short- and long-term cultures, as well as those cells expressing the wt huIL-2R $\beta$ . In contrast, cells expressing the chimIL4R, which activates STAT6 but not STAT5, did not proliferate well in response to IL-2 (36) (Fig. 8B); in fact, the response of these cells to IL-2 was similar to those cells expressing the truncated huIL-2R $\beta$ . Finally, we obtained similar results when we analyzed the ability of these IL-2R constructs to regulate protection from apoptosis (Fig. 8C). The chimEPOR, but not the chimIL4R, was able to protect 32D cells from apoptosis in the presence of IL-2 after IL-3 withdrawal to the same extent as the wt huIL-2R $\beta$ . In addition, IL-2 could act as a long-term growth factor in those cells expressing the chimEPOR, but not in cells expressing the chimIL4R or the truncated huIL-2R $\beta$ . Therefore, the reconstitution of the ability of the IL-2R to signal STAT5 activation was directly related to its ability to promote protection from apoptosis and long-term cell growth.

## Discussion

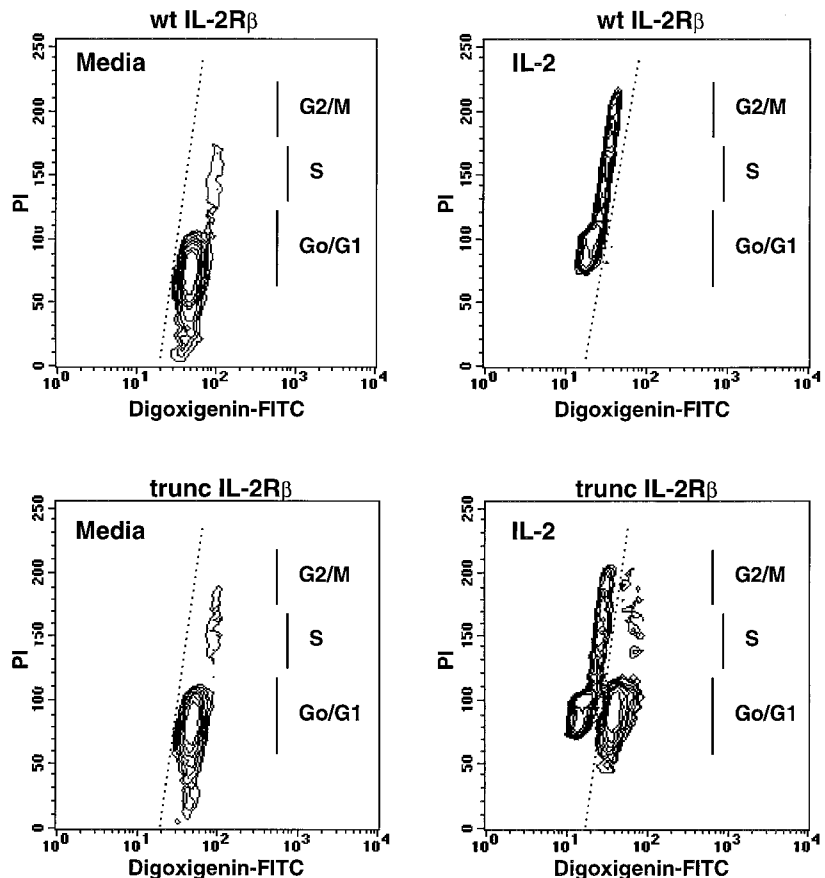
IL-2 can promote proliferation, differentiation, and protection from apoptosis in a wide variety of cell types. This is achieved through activation of several cellular proteins that subsequently mediate these responses. Among these, STAT5 plays a role in the regulation of the transcription of IL-2-inducible genes. However, the function that STAT5 plays in the IL-2-mediated cell growth has not been clearly defined. Since cell growth can be viewed as a

regulated process that requires both mitogenic and anti-apoptotic activities, we sought to discern the role that STAT5 plays in the IL-2-mediated regulation of mitogenesis and apoptosis. To this end, we have used the IL-3-dependent cell line 32D, which had been transfected with either the wt huIL-2R $\beta$  or truncated form of this receptor. The truncated huIL-2R $\beta$  lacks Y392 and Y510, which have been shown to recruit STAT5 after IL-2 engagement; therefore cells expressing this receptor were unable to activate STAT5 function whereas other proteins were still activated. In this study, we have shown that 32D cells expressing the truncated huIL-2R $\beta$  were capable of entering the cell cycle in response to IL-2, but IL-2 did not prevent them from apoptosis in long-term cultures. This defect could be completely reversed by restoring the ability of the truncated huIL-2R $\beta$  to activate STAT5. These results suggest that IL-2-induced STAT5 activation is not required for cells to enter the cell cycle but rather functions to mediate long-term protection from apoptosis.

These results increase our understanding of the role that STAT5 plays in IL-2-mediated cell growth. Previous studies have focused on the ability of IL-2R $\beta$  mutants lacking the ability to activate STAT5 to promote cell proliferation. However, the specific role that apoptosis plays in this process has not been addressed. Several laboratories have found that STAT5 activation is dispensable for IL-2-induced cell growth, while others have shown that the inability of IL-2 to activate STAT5 partially or completely blocked cell growth in response to IL-2. Goldsmith et al. observed a complete

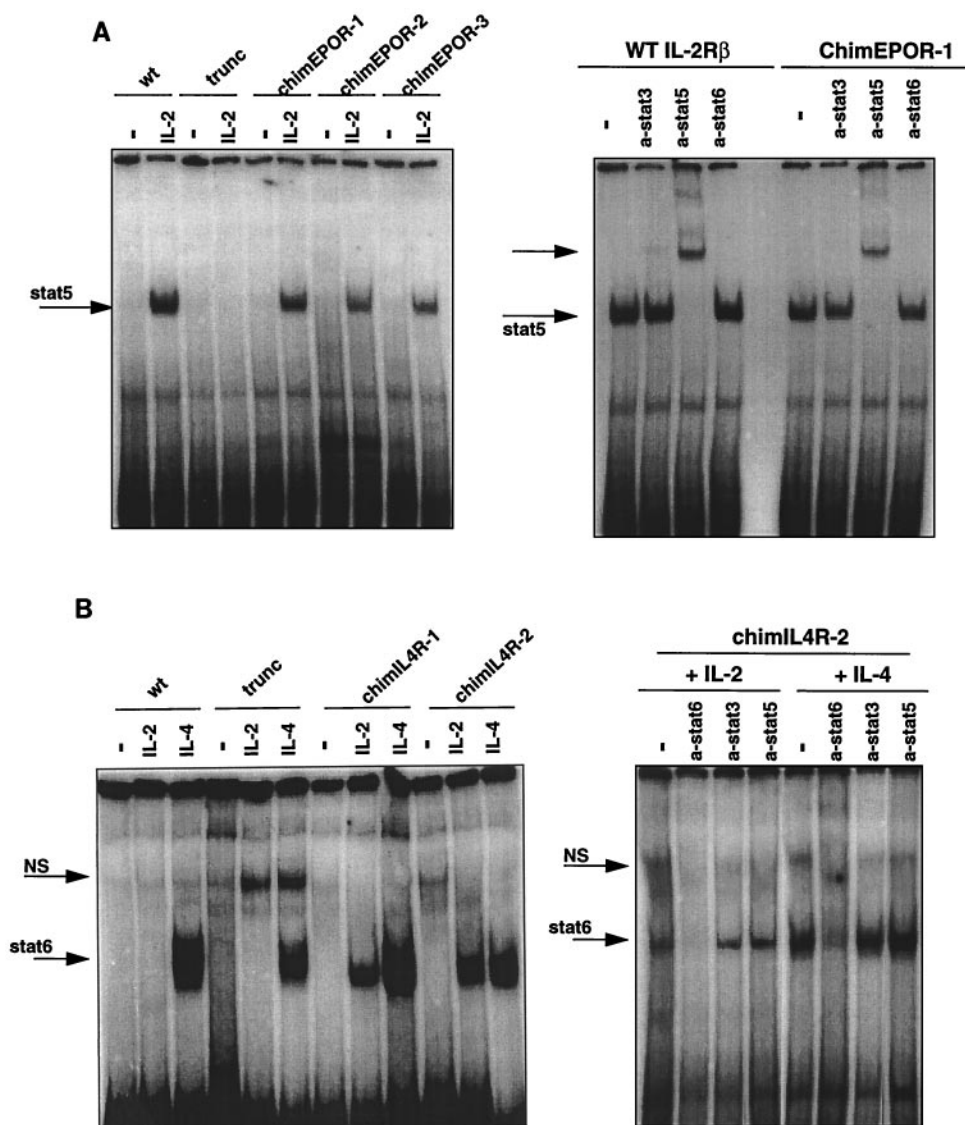


**FIGURE 5.** Cell cycle analysis in synchronized cells in response to IL-2. 32D cells expressing the wt or truncated huIL-2R $\beta$  were cultured in the absence of any cytokine for 18 h to induce G<sub>1</sub> arrest. Afterward, G<sub>1</sub>-arrested cells were incubated in the presence or absence of IL-2 for an additional 24 and 48 h. Both the percentage of apoptotic cells (*upper graphics*) and cell cycle distribution in living cells (*lower graphics*) were analyzed as in Figure 4. All cells were dead after 48 h of culture without stimulation. Graphics are representative of at least three different cell lines.



**FIGURE 6.** TUNEL assay of apoptotic cells. 32D cells expressing the wt or the truncated huIL-2R $\beta$  were cultured in the presence or absence of IL-2 for 48 h. Harvested cells were stained using the TUNEL assay in combination with propidium iodide staining of DNA. Apoptotic cells incorporated digoxigenin-labeled dNTPs, which can be detected with an appropriate FITC-conjugated Ab. Dashed lines separated nonapoptotic (*left*) from apoptotic cells (*right*). This figure is representative of three similar experiments.



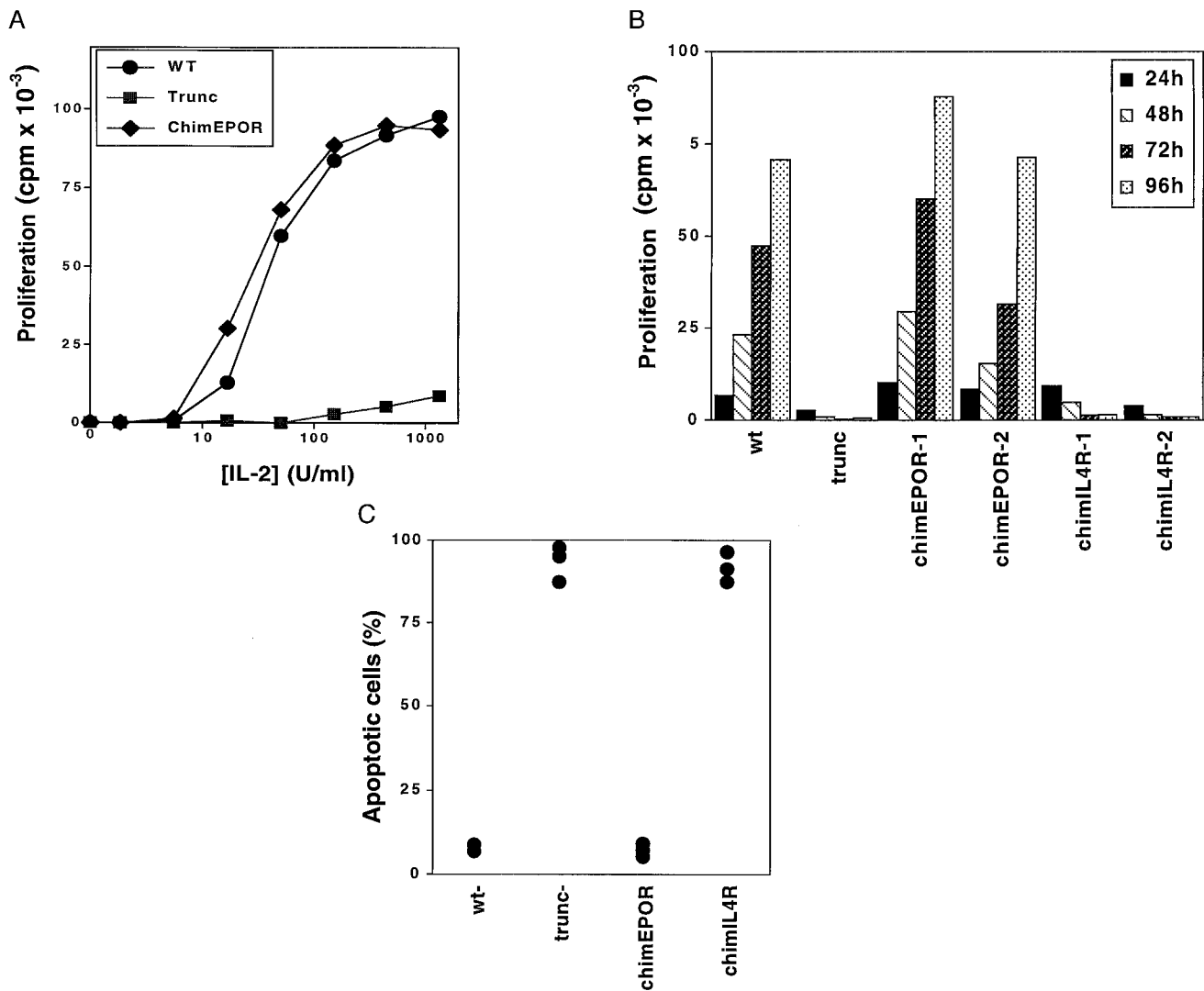


**FIGURE 7.** Analysis of STAT DNA-binding activity in 32D cells expressing different chimeric receptors in response to IL-2. *A*, 32D cells expressing wt huIL-2R $\beta$ , truncated huIL-2R $\beta$ , or chimEPOR receptors were treated with IL-2 for 30 min. STAT5 DNA-binding activity was analyzed using the GAS sequence contained in the FC $\gamma$ RI promoter. The *right panel* shows the supershift activity of an anti-STAT5 Ab (*upper arrow*). Anti-STAT3 and anti-STAT6 Abs were included as control. *B*, 32D cells expressing the different huIL-2R $\beta$  constructs were stimulated with IL-2 or IL-4 (10 ng/ml) for 30 min. Then, STAT6 DNA-binding activity was determined in cell extracts using the GAS sequence in the promoter of C $\epsilon$  gene. The *right panel* shows the ability of an anti-STAT6 Ab to block the cytokine-induced DNA-binding activity. Anti-STAT3 and anti-STAT5 Abs were included as control. NS indicates nonspecific binding.

inhibition of proliferation in response to IL-2 in Ba/F3 cells expressing a form of huIL-2R $\beta$  which had mutated both Y392 and Y590 to phenylalanine (35). In contrast, Fujii et al. detected no significant role of these tyrosines in cell proliferation since the truncated huIL-2R $\beta$  was able to signal cell proliferation almost as well as the wt huIL-2R $\beta$  (23). In addition, Friedmann et al. observed a greatly diminished response in 32D cells when both Y392 and Y510 were changed to phenylalanine (21). These differences may be due to the fact that STAT5 activation is required to protect cells from apoptosis, which is essential for long-term survival, but not to induce cell cycle progression. We have observed that 32D cells expressing the truncated huIL-2R $\beta$  incorporated about 40 to 90% of thymidine in response to IL-2 when compared with cells bearing the wt huIL-2R $\beta$  in short-term experiments (24 h), which agrees with data reported by Fujii et al. (23) and Friedmann et al. (21). Cells cultured for extended periods of time (longer than 48 h)

did not incorporate a significant amount of thymidine in response to IL-2, which is consistent with the results obtained by Goldsmith et al. (35). These results suggest that proper activation of STAT5 is required for IL-2-mediated cell growth by regulating long-term survival.

To promote cell growth successfully, growth factors should be able to signal both progression through the cell cycle and long-term survival. Both *c-myc* (45, 46) and *shc* (47, 48) have been associated with the induction of cell proliferation. Induction of *c-myc*, whose activation has been linked to the serine-rich domain of the huIL-2R $\beta$ , has been suggested to play a critical role in cell cycle progression in response to IL-2 (16–18). Strong evidence indicates that Y338, in the acidic region of the huIL-2R $\beta$ , is the docking site for *shc* (20, 21). Although some reports indicated that Y338 does not play a principal role in the IL-2-mediated cell proliferation (20, 35), Friedmann et al. have shown that the mutation



**FIGURE 8.** Proliferation and apoptosis in 32D cells expressing the chimeric receptors in response to IL-2. *A*, 32D cells expressing wt huIL-2R $\beta$  (circle), truncated huIL-2R $\beta$  (square), or chimEPOR (diamond) receptors were cultured for 72 h in the presence of various amounts of IL-2, and proliferation was analyzed by the incorporation of [<sup>3</sup>H]thymidine during the last 4 h of culture. *B*, 32D cells expressing the indicated constructs of the huIL-2R $\beta$  were incubated in the presence of 200 U/ml of IL-2 for 24, 48, 72, and 96 h, and proliferation was then measured as in *A*. All cell lines had similar proliferation levels in response to IL-3. Two representative clones expressing each chimeric receptor are shown. *C*, 32D cells expressing the indicated constructs were stimulated with 200 U/ml of IL-2 for 80 h; afterward, the percentage of apoptotic cells was determined as indicated in Figure 3. At this time, all unstimulated cells were dead. Three different clones of each huIL-2R $\beta$  construct are shown.

Y338F greatly diminished 32D cell proliferation in response to IL-2 (21). In addition, a huIL-2R $\beta$  that lacks the acidic region was partially impaired at signaling cell cycle progression (16). We have observed that the truncated form of the huIL-2R $\beta$  is able to induce the expression or activation of several important cellular messengers such as Bcl-2, *c-myc* (Fig. 1), and *shc* (data not shown), which is consistent with previous observations (20, 21, 34). Therefore, the activation of *c-myc* and *shc* could explain the ability of the truncated receptor to promote transition from G<sub>1</sub> to S into the cell cycle. Taken together, the membrane-proximal region of the cytoplasmic domain of the huIL-2R $\beta$  can be responsible for the induction of cells to cycle while the carboxyl-terminal region can regulate long-term protection from apoptosis through the activation of STAT5.

Although the truncated receptor was not able to signal extended protection from apoptosis, it could support Bcl-2 protein levels as well as the wt huIL-2R $\beta$ . It has been published that Bcl-2 induction is dependent on the serine-rich domain of the huIL-2R $\beta$  (19).

In addition, it has been shown that Bcl-2 overexpression, which did not influence cell cycle progression, extended the short-term survival of 32D cells after IL-3 deprivation, yet cells still died (49, 50). Therefore, the induction of Bcl-2 expression may be responsible for the short delay of apoptosis observed in the presence of IL-2 by those cells expressing the truncated huIL-2R $\beta$ . These results suggest that Bcl-2 can play a critical role in the short-term survival, but additional proteins regulated by STAT5 may play a fundamental function in the long-term survival mediated by IL-2. In preliminary studies designed to identify STAT5-regulated proteins, we analyzed the contribution of caspases in this process; however, we have not been able to complement the defect of the truncated huIL-2R $\beta$  by using caspase inhibitors such as Z-YVAD, Z-DEVD, and Z-VAD, or the overexpression of the viral IL-1 $\beta$ -converting enzyme inhibitor CrmA.

We have shown that transplantation of a STAT5 domain from the EPOR, but not the STAT-6 domain from the IL-4R $\alpha$ , to the truncated form of the huIL-2R $\beta$  completely restores the capacity

of IL-2 to act as a potent growth factor able to signal long-term protection from apoptosis. Although Y392, Y510, and surrounding amino acids may be docking sites for several unknown proteins (51), so far, the only signal transduction factor known to depend on the carboxyl-terminal domain of the huIL-2R $\beta$  is STAT5 (19, 21, 23, 24). Moreover, transplantation of a region from the EPOR containing Y343 to the huIL-2R $\beta$  restored STAT5 activation and protection from apoptosis, strongly suggesting that STAT5 is involved in anti-apoptotic processes.

The concept that STAT5 regulates long-term survival is supported by studies presented by Mui et al. (33). They observed that a dominant-negative STAT5 inhibited the IL-3-induced Ba/F3 cell proliferation. Furthermore, a detailed analysis of their data indicates that the dominant-negative STAT5 construct could inhibit the ability of IL-3 to signal protection from apoptosis while, similar to our results, the cells were still progressing within the cell cycle. They also found that both *c-myc* and cyclin expression were induced by IL-3 in the presence of the dominant-negative STAT5. Several studies suggest that STAT5 may play a similar role in T cells. The constitutive activation of STAT5 corresponded with the development of IL-2-independent growth of human T cells infected with human T cell lymphotropic virus 1 (52). In addition, T cell hyporesponsiveness in animals bearing tumors was correlated with a decrease in the expression of STAT5 (53). It will be important to directly test the role of STAT5 in regulating the survival of T cells in response to IL-2.

Interestingly, Fukada et al. have been able to distinguish mitogenic and anti-apoptotic signals mediated by the gp130 receptor (54). Their data also indicate that STAT3 is involved in the regulation of apoptosis. Although they did not exclude a possible role of STAT3 in mitogenesis, the function of STAT5 and STAT3 in cell growth could be similar, regulating apoptosis instead of cell cycle. Moreover, the ability of STAT5 and STAT3, but not STAT6, to mediate protection from apoptosis is consistent with the concept that the function of STAT proteins may be revealed by their evolutionary origin (55). STAT3, STAT5a, and STAT5b are linked on the same region of mouse chromosome 11. STAT6, together with STAT2, is mapped to chromosome 10. It is interesting to speculate that the STAT proteins linked on the same chromosome may have arisen by duplication of a common ancestral gene, thereby imparting common function (56). Our results, together with those performed by Fukada et al., indicate that some STAT proteins can be involved in cell growth processes by regulating long-term survival. However, additional experiments will be necessary to characterize the proteins that mediate the anti-apoptotic effect.

## Acknowledgments

We acknowledge Dr. Warren Leonard for the wt huIL-2R $\beta$ -pME18S plasmid and critical reading of the manuscript and Dr. Jacalyn Pierce for the parental 32D cells.

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