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IL-2Rβ Agonist P1–30 Acts in Synergy with IL-2, IL-4, IL-9, and IL-15: Biological and Molecular Effects[1]

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From the sequence of human IL-2 we have recently characterized a peptide (p1–30), which is the first IL-2 mimetic described. P1–30 covers the entire α helix A of IL-2 and spontaneously folds into a α helical homotetramer mimicking the quaternary structure of a hemopoietin. This neocytokine interacts with a previously undescribed dimeric form of the human IL-2 receptor β-chain likely to form the p1–30 receptor (p1–30R). P1–30 acts as a specific IL-2Rβ agonist, selectively inducing activation of CD8 and NK lymphocytes. From human PBMC we have also shown that p1–30 induces the activation of lymphokine-activated killer cells and the production of IFN-γ. Here we demonstrate the ability of p1–30 to act in synergy with IL-2, -4, -9, and -15. These synergistic effects were analyzed at the functional level by using TS1β, a murine T cell line endogenously expressing the common cytokine γ gene and transfected with the human *IL-2Rβ* gene. At the receptor level, we show that expression of human IL-2Rβ is absolutely required to obtain synergistic effects, whereas IL-2Rα specifically impedes the synergistic effects obtained with IL-2. The results suggest that overexpression of IL-2Rα inhibits p1–30R formation in the presence of IL-2. Finally, concerning the molecular effects, although p1–30 alone induces the antiapoptotic molecule bcl-2, we show that it does not influence mRNA expression of c-*myc*, c-*jun*, and c-*fos* oncogenes. In contrast, p1–30 enhances IL-2-driven expression of these oncogenes. Our data suggest that p1–30R (IL-2Rβ)$_2$ and intermediate affinity IL-2R (IL-2Rβγ), when simultaneously expressed at the cell surface, may induce complementary signal transduction pathways and act in synergy. ***The Journal of Immunology*****, 2000, 165: 4312–4318.**

Interleukin-2 is a cytokine critically involved in inflammatory reactions and immune responses (1) (2). It is responsible for cellular expansion of Ag-activated cells and in the negative feedback control of this expansion (2–4). Gene knockout experiments have shown that IL-2 is not essential for some of these activities, indicating that other factors can substitute for IL-2 in vivo (5). In accordance with this, a number of cytokines, namely IL-4, -7, -9, and -15, elicit immunological activities similar to those of IL-2 (6).

The structure of IL-2 (133 aa) is made up of a compact core bundle of four antiparallel α helices (7) (A, BB′, C, and D) connected by three loops (8). Three chains participate in the formation of the IL-2R (9). IL-2Rα is a 55-kDa protein that binds to IL-2 with a K_d value of ~10 nM (10, 11). IL-2Rβ is a 75-kDa protein with a large intracytoplasmic domain (286 aa) that plays a critical role in signal transduction both in vitro and in vivo (12–14). IL-2Rβ also takes part in the formation of the IL-15R (15). The IL-2Rγ-chain is a 64-kDa protein (16), which is shared by the IL-2R, -4R, -7R, -9R, and -15R and is referred to as the common γ-chain (γc).[3] In the human system two IL-2 receptor complexes are able to transduce signals. The association of human IL-2Rβ and IL-2Rγ forms an intermediate affinity receptor with a K_d value of ~1 nM, whereas expression of all three chains leads to the formation of a high affinity IL-2R (K_d ~10 pM). IL-2Rβ recruits protein tyrosine kinase p56^{lck} and the adapter protein Shc, both of which play an essential role in lymphocyte activation (17, 18). Shc invokes the Ras and the phosphatidylinositol 3-kinase (PI3 kinase) signaling pathways that are essential in the control of cellular activation and proliferation. Ras activates the mitogen-activated protein kinase (MAPK) pathway responsible for the up-regulation of the two proto-oncogenes c-*fos* and c-*jun*. Via the Akt protein, PI3 kinase is implicated in the regulation of the proto-oncogene c-*myc* and in the expression of the antiapoptotic molecule bcl-2 (19–22). Another important signaling pathway implicated in the course of IL-2 activation is the Janus kinase (Jak)/STAT pathway (23).

A peptide, named p1–30 (comprising aa 1–30 of human IL-2), which includes the entire α helix A of human IL-2, has been recently characterized in our laboratory (24). At the structural level p1–30 appears to be folded as a cytokine of the hemopoietin family and binds to human IL-2Rβ dimers (p1–30R). Contrary to IL-2, p1–30 is not able to activate Jak1, Jak3, or STAT5 but, strikingly, it induces the phosphorylation of Tyk2. Furthermore, p1–30 induces the activation of p56^{lck} and the phosphorylation of the adaptor protein Shc (24). At the immunological level p1–30 is able to specifically activate NK and CD8 T cells, which constitutively express large amounts of IL-2Rβ. In human PBMC, p1–30 induces lymphokine-activated killer (LAK) cells and leads to the production of IFN-γ. We have also suggested that p1–30 has therapeutic potential (24).

Unexpectedly, we observed that p1–30 not only mimics some of the biological effects of IL-2 but can also act in synergy with this cytokine. This suggests that p1–30 and IL-2 may use distinct signaling pathways for their function. We analyzed the effects of p1–30 on the cytokine-driven response of a mouse cell line transfected with human IL-2Rβ. We demonstrate that peptide p1–30 also acts in synergy with IL-4, -9, and -15. At the receptor level, we demonstrate that this synergy is dependent on human IL-2β

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[3] Abbreviations used in this paper: γc, common γ-chain PI3 kinase, phosphatidylinositol 3-kinase; Jak, Janus kinase; LAK, lymphokine-activated killer; MAPK, mitogen-activated protein kinase; IgG L, IgG light chain; MFI, mean fluorescence intensity.

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expression, whereas IL-2Rα specifically impedes the synergy between p1–30 and IL-2. To address the molecular mechanism that may be involved, we analyzed the expression of the proto-oncogenes c-*myc*, c-*fos*, and c-*jun* as well as that of the antiapoptotic molecule bcl-2 after p1–30, IL-2, and stimulation with p1–30 in the presence of IL-2. Altogether, the results suggest that p1–30 and IL-2 act on different receptors and, under certain experimental conditions, induce complementary signals.

Materials and Methods

Peptide p1–30 and cytokines

Peptide p1–30 was synthesized by the stepwise solid-phase reaction using the boc/trifluoroacetic acid method (25), on a *p*-methylbenzhydrylamine resin with an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Paris, France), as described previously (26). Following purification, the identity of p1–30 was verified by mass spectrometry and amino acid analysis after total hydrolysis.

The cytokines used in this work were human rIL-2 (Chiron Europe, Amsterdam, The Netherlands), purified murine IL-9 (provided by Dr. J. Van Snick, Ludwig Institute, Brussels, Belgium), or human rIL-15 (obtained from Dr. S. Chouaïb, Institut Gustave Roussy, Villejuif, France).

The supernatant of a HeLa cell subline (H28) transfected with the mouse IL-4 expression plasmid pKCR IL-4 Neo, provided by Dr. T. Honjo (Kyoto University, Kyoto, Japan), was used as a source of mouse rIL-4.

Cell lines and proliferation assays

TS1(γm) is a murine T cell line expressing only the murine IL-2Rγ chain, which grows in IL-4 or -9. TS1β(βh, γm) cells are TS1 cells transfected with human IL-2Rβ, which are, in addition, able to grow in IL-2 and -15. TS1$\alpha\beta$(αh, βh, γm) cells are TS1β cells transfected with human IL-2Rα (27). C30.1(αm, βm, γm) is a murine cytotoxic T cell line expressing the three murine IL-2Rα-, β-, and γ-chains, and grows in IL-2 and -4 (28). The murine cell line 8.2(βm, γm) is derived from C30.1 after prolonged culture in IL-4. It expresses mouse IL-2Rβ and mouse IL-2Rγ, but not mouse IL-2Rα, and grows only in IL-4 (29).

Proliferation assays were performed as previously described (29). [^{3}H]TdR incorporation was measured 36 h after stimulation. Human rIL-2, murine rIL-4, murine IL-9, human IL-15, or peptide p1–30 was assayed at the indicated concentrations. For analysis of synergy, various concentrations of cytokines were used at time 0 of the assay in the presence of the indicated concentrations of p1–30. Data shown are from one representative experiment of at least three. Synergy is presented as percent increase in the proliferation obtained above cytokine proliferation alone plus p1–30 proliferation alone and calculated as follows: synergy % = [(pl–30 + cytokine response) − (pl–30 response) − (cytokine response)]/[(pl–30 response) + (cytokine response)] × 100.

FACS analysis

Expression of human IL-2Rβ and murine IL-2Rγ was detected by flow cytometry (29). Mouse anti-human IL-2Rβ mAb CF_1 (Immunotech, Marseille, France) and rat anti-mouse IL-2Rγ mAb TUGm2, provided by Dr. K. Sugamura (University of Sendai, Sendai, Japan), were used for these assays.

Briefly, after 0, 24, or 72 h of activation in the presence of the indicated concentration of IL-2 and/or p1–30, cells (2×10^5 in 200 μl) were labeled with anti-IL-2R mAb followed by anti-mouse or anti-rat FITC-conjugated Ab (Jackson ImmunoResearch, West Grove, PA). Following the staining procedure, cells were washed in RPMI 1640 and fixed in 1% paraformaldehyde. A total of 2×10^4 cells per sample were analyzed with a FACScan flow cytometer using CellQuest 1.2 software (Becton Dickinson, Mountain View, CA).

Bcl-2 immunoprecipitation and Western blot analysis

TS1β cells were washed and then stimulated at 37°C with IL-2 (3 nM) and/or p1–30 (60 μM) for 24 h. Proteins were solubilized from 5×10^6 cells in 125 μl of lysis buffer (50 mM Tris, pH 8, 10% glycerol, 200 mM NaCl, 0.5% Nonidet P-40, and 0.1 mM EDTA) supplemented with each of the following protease inhibitors at 10 μg/ml: leupeptin, aprotinin, and PMSF, and with the phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM). Lysates of 5×10^6 cells were loaded on a 12% SDS-polyacrylamide gel. For immunoprecipitation, lysates from 5×10^6 cells were treated with Ab to mouse bcl-2 (PharMingen, San Diego, CA) for 1 h at 4°C. After electrophoresis, the proteins were transferred to Immobilon membranes (Millipore, Bedford, MA), and immunoblots were incubated with Ab to mouse bcl-2. Subsequent to incubation with an anti-hamster Ig peroxidase-conjugated mAb (Southern Biotechnology Associates, Birmingham, AL), reactive protein bands were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.). Quantification of bcl-2 protein expression levels was accomplished by densitometry. Bands corresponding to the amount of bcl-2 or IgG light chain (IgG L) were measured with NIH Image software (National Institutes of Health, Bethesda, MD). Normalization of the bcl-2 signal to that of IgG L was performed and the bcl-2/IgG L ratio is reported in histogram plots.

Northern blot analysis

Cells were stimulated as described for Western blot analysis and in the legend of the corresponding figure. Total RNA was extracted from TS1β cells using RNA-B solution (Bioprobe System, Montreuil sous Bois, France), following the supplier's recommendations. Northern blot analysis was performed as already described (30). Briefly, 10 μg RNA was electrophoresed on 1% agarose denaturing gel and transferred to a Hybond-N membrane (Amersham). Proto-oncogene mRNA was detected by specific cDNA probes labeled with [α-^{32}P]dCTP. The following probes were used: c-*myc* (30), c-*jun* (31), and c-*fos* (32). A probe for 18S ribosomal RNA was used as control. The hybridization signal was quantified by phosphorus-stimulated luminescence (PhosphorImager, Molecular Dynamics, Evry, France). The oncogene-specific hybridization signal was normalized to the 18-sense signal. The c-*myc*/18-sense, c-*jun*/18-sense, and c-*fos*/18-sense ratios are represented in histogram plots (30).

Results

Peptide p1–30 acts in synergy with IL-2, -4, -9, or -15

As previously demonstrated, p1–30 induces the proliferation of cell lines expressing the human IL-2Rβ-chain (24). Fig. 1*A* shows the proliferation of TS1β cells induced by various concentrations of p1–30 alone (up to 100 μM). Fig. 1*B* shows the titration of p1–30 in the presence of 0.1, 1, or 10 nM of IL-2. A strong synergy

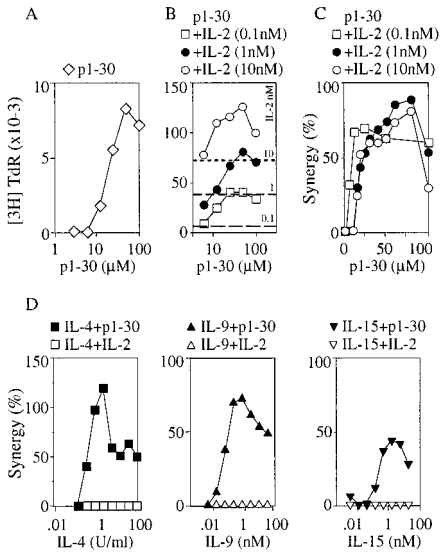

FIGURE 1. p1–30 acts in synergy with IL-2, -4, -9, or -15. *A*, Proliferative responses were assayed on TS1β cells expressing human IL-2Rβ and murine IL-2Rγ (βh^+, γm^+). Cells (10^4/well) were stimulated with various concentrations of p1–30 (up to 100 μM). After 36 h, cells were pulsed for 15 h with 0.5 μCi of [^{3}H]TdR before harvesting and scintillation counting. *B*, TS1β cells were stimulated with p1–30 (60 μM) in the presence of 0.1, 1, or 10 nM of IL-2 and assayed as in (*A*). *C*, Results obtained in (*B*) are presented as percent synergy as described in *Materials and Methods*. *D*, Proliferative responses of TS1β cells, conducted as in (*B*), were induced with various concentrations of IL-4 (from 8×10^{-1} to 100 U/ml), IL-9 (from 8×10^{-2} to 10 nM), or IL-15 (from 8×10^{-2} to 10 nM) in the presence or absence of p1–30 (60 μM) or IL-2 (1 nM). Results are presented as percent synergy.

was observed between p1–30 and IL-2 because the proliferative response obtained with the combination of p1–30 + IL-2 was much greater than the sum of each individual response. Even at 10 nM of IL-2 responsible for a maximal IL-2-induced proliferation, the combination with p1–30 allowed a synergistic response. Fig. 1*C* displays the synergy as calculated in percent increase over the two individual proliferative responses.

To characterize the synergistic capacities of p1–30, we also analyzed its effects in the presence of other cytokines. TS1β cells were stimulated with different concentrations of IL-4, -9, or -15 in the presence or the absence of p1–30 (60 μM). As shown in Fig. 1*D*, a strong synergy was observed with the three lymphokines tested. On the contrary, we were not able to find any synergy with GM-CSF or IL-3 (data not shown). We also tested the possibility of synergistic effects mediated by IL-2 in the presence of IL-4, -9, or -15. Fig. 1*D* clearly shows that, contrary to p1–30, IL-2 is unable to act in synergy with these cytokines. This emphasizes the divergence of p1–30 and IL-2 biological activities.

Effects of p1–30 after prolonged cell starvation of TS1β

When exponentially-growing TS1β cells cultured in IL-2 are immediately assayed for their proliferative capacity they respond to IL-2, -4, -9, -15, or to peptide p1–30 (Fig. 2*A*). After prolonged cell starvation the pattern of cytokine responsiveness is greatly altered (33). Starved TS1β (48 h in 5% FCS) stimulated with either peptide p1–30 (from 2×10^{-1} to 300 μM) or cytokines alone over a wide range of concentrations (IL-2 from 5×10^{-3} to 10 nM, IL-4 from 5×10^{-3} to 10 U/ml, IL-9 from 5×10^{-3} to 10 nM, or IL-15 from 5×10^{-3} to 10 nM) failed to respond (Fig. 2*B*). However, upon introduction of both p1–30 (60 μM) and cytokine, a restoration of the proliferative response was observed and thus strikingly demonstrates the synergistic effect between p1–30 and different cytokines (Fig. 2*C*).

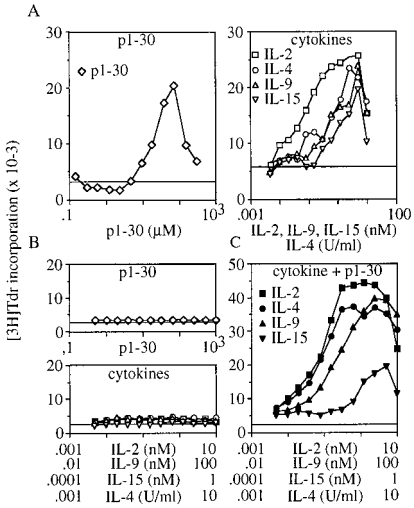

FIGURE 2. Synergistic effect of p1–30 after prolonged cell starvation of TS1β. *A*, Exponentially growing TS1β cells were stimulated with various concentrations of p1–30 (from 2×10^{-1} to 100 μM), IL-2 (from 5×10^{-3} to 10 nM), IL-4 (from 5×10^{-3} to 10 U/ml), IL-9 (from 5×10^{-3} to 10 nM), or IL-15 (from 5×10^{-3} to 10 nM). Proliferative responses are shown. *B*, Impact of cytokine starvation on TS1β cell proliferation. TS1β cells were cultured in cytokine-free medium (48 h) before the proliferation assay. Subsequently proliferative response to p1–30, IL-2, -4, -9, or -15 was measured. *C*, Proliferative response of starved TS1β cells. TS1β cells were cultured in cytokine-free medium for 48 h. Subsequent proliferative response after p1–30 stimulation in the presence of IL-2, -4, -9, or -15 was measured.

Role of human IL-2Rβ-chain in the synergistic effect

The ability of p1–30 to induce proliferation is dependent on human IL-2Rβ-chain expression (24). Here we evaluate the role of human IL-2Rβ-chain on the synergistic effect induced by p1–30. Proliferation assays were performed with various cell lines expressing different combinations of the three IL-2R chains. When stimulated with IL-4 or -9 in the presence of p1–30 no synergy was observed with TS1 cells expressing only murine IL-2Rγ (Fig. 3*A*). After transfection of TS1 cells with the human IL-2Rβ gene (TS1β cells) a synergy is always observed between p1–30 and IL-2, -4, -9, or -15 (Fig. 3*B*).

The same experiments were performed with 8.2 cells (expressing murine IL-2Rβ and γ) and C30.1 cells (expressing murine IL-2Rα, -β, and -γ). These cells were stimulated with p1–30 (60 μM) in the presence of various concentrations of IL-2 or -4, depending on the cell line. With these cell lines, synergy was never observed between the different cytokines and p1–30 (Fig. 1, *C* and *D*). Therefore, unlike human IL-2Rβ, expression of the murine IL-2Rβ-chain does not allow the induction of the p1–30-mediated synergy. This is in agreement with the fact that in the context of the murine IL-2Rβγ receptor, murine IL-2Rβ does not interact with human or murine IL-2 (29). In addition to the essential role of human IL-2Rβ for p1–30-mediated binding and proliferation, our results demonstrate that expression of this chain is also involved in the synergistic effect characterized in this study.

Analysis of the synergistic effect in the presence of IL-2Rα

IL-2Rα is not necessary for the direct p1–30 proliferative effects because TS1β cells do not express IL-2Rα (24). Here, we investigated the influence of IL-2Rα on the p1–30 + IL-2 synergy. This

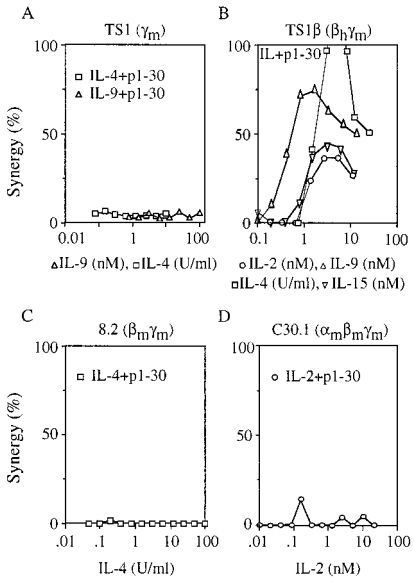

FIGURE 3. The p1–30 synergistic effect is dependent on human IL-2Rβ-chain expression. P1–30 + cytokine proliferative synergy was measured in cell lines expressing different combinations of IL-2R chains. Cell lines were stimulated with p1–30 (60 μM) in the presence or absence of IL-2, -4, -9, and -15 as indicated. Percent synergy was calculated as described in *Materials and Methods*. The origin and the pattern of IL-2R chain expressed by the different cell lines are described in *Materials and Methods*. *A*, TS1 is a murine cell line expressing murine IL-2Rγ (γm). *B*, TS1β is the TS1 cell line transfected with human IL-2Rβ (βh, γm). *C*, The 8.2 murine cell line was derived from C30.1 and expresses murine IL-2Rβ and γ(βm, γm). *D*, C30.1 is an IL-2-dependent murine T cell line expressing murine IL-2Rα, -β, and -γ (αm, βm, γm).

part of the study was performed using TS1$\alpha\beta$ cells (TS1β cells transfected with the *IL-2Rα* gene). At the surface of these cells, IL-2 interacts with the high affinity IL-2R$\alpha\beta\gamma$. Cells were stimulated with various concentrations of IL-2 in the presence or the absence of 60 μM p1–30 (Fig. 4*A*). Under these experimental conditions, IL-2Rα impeded the synergy between p1–30 and IL-2. We have verified that p1–30 induces TS1$\alpha\beta$ cell proliferation, showing that IL-2Rα plays a negative role in the synergistic effect but not in the p1–30-induced proliferative response (Fig. 4*B*). In contrast, Fig. 4*C* shows that under identical experimental conditions, in the presence of IL-2Rα, p1–30 induces a synergistic response when coupled to IL-4, -9, or -15. Therefore, the inhibitory effect of IL-2Rα is specific for the IL-2 system.

IL-2Rγ expression after p1–30 stimulation

On TSI β cells expressing the human IL-2Rβ transgene p1–30 acts in synergy with cytokines IL-2, -4, -9, and -15, all of which bind to receptors containing the common γ-chain. Therefore, modulation of mouse IL-2Rγ expression may be involved in the mechanism explaining synergy. P1–30 stimulation may induce IL-2Rγ overexpression and lead to an increased responsiveness to the cytokine tested. The effect of IL-2 and p1–30 alone and in synergy was tested on IL-2Rγ expression. Cell surface expression levels were measured after 1, 2, or 3 days of stimulation. No significant changes in mean fluorescence intensity (MFI) were detected over time. Fig. 5*A* shows the results at days 1 and 3. The results suggest that under these experimental conditions (in vitro cytokine stimulation and FACS analysis) IL-2Rγ-chain expression is not modulated by p1–30 (Fig. 5*A*). Variations in the expressed IL-2Rβ transgene, although unlikely, were nevertheless verified. TS1β cells were stimulated with p1–30 and IL-2 alone or in combination, and human IL-2Rβ expression was followed by FACS. Cell surface expression was not induced by days 1–3 whatever the stimulation (Fig. 5*B*).

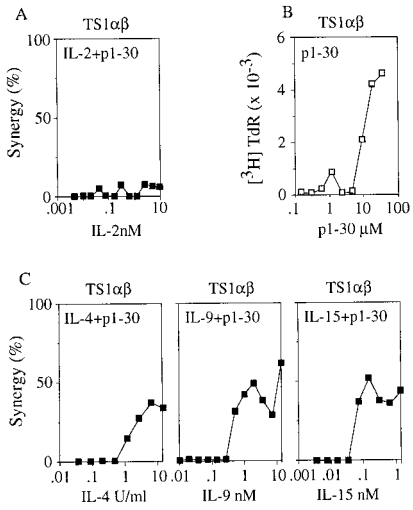

FIGURE 4. IL-2Rα expression specifically impedes the synergy between p1–30 and IL-2. *A*, The proliferative response of TS1$\alpha\beta$ (αh, βh, γm) cells induced by various concentrations of IL-2 (from 5×10^{-3} to 10 nM) in the presence or absence of 60 μM p1–30 was measured. Percent synergy was calculated as in *Materials and Methods*. *B*, P1–30-induced proliferation of TS1$\alpha\beta$ cells with peptide concentrations ranging from 10^{-1} to 75 μM was measured. *C*, The proliferative response of TS1$\alpha\beta$(αh, βh, γm) induced by various concentrations of IL-4 (from 1 to 10 U/ml), IL-9 (from 2×10^{-1} to 10 nM), or IL-15 (from 5×10^{-3} to 1 nM) was also tested in the presence or absence of p1–30 (60 μM). The synergy between p1–30 and IL-4, -9, or -15 was calculated as in *Materials and Methods*.

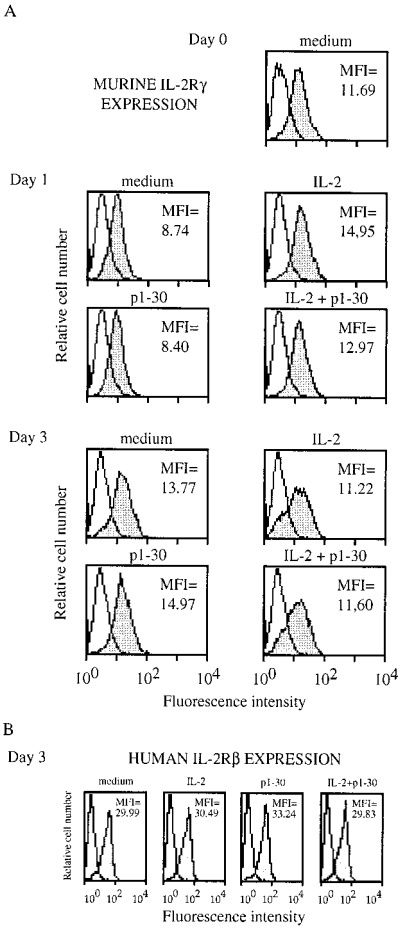

FIGURE 5. Expression of IL-2Rγ after IL-2, p1–30, and IL-2 + p1–30 stimulation. *A*, TS1β cells were stimulated with IL-2 (1 nM), p1–30 (60 μM), or both (1 nM of IL-2 plus 60 μM of p1–30). After 1, 2, or 3 days of stimulation, expression of murine IL-2Rγ was quantified by flow cytometry using mAb TUGm2 (shaded histogram). Background was obtained with isotype-matched control mAb (open histogram). For each day unstimulated cells (medium) serve as control. MFI is shown for days 1 and 3 after stimulation. *B*, FACS analysis of human IL-2Rβ was performed using mAb CF1. TS1β cells were stimulated as described above. MFI results obtained at day 3 are reported.

Synergistic effect at the proto-oncogene level

We previously demonstrated that p1–30, like IL-2, activates $p56^{lck}$ and Shc proteins, which recruit the PI3 kinase and Ras/MAPK pathways. These two pathways lead to the expression of bcl-2 and c-*myc* as well as to the stimulation of c-*jun* and c-*fos* expression, respectively. The proto-oncogene bcl-2 is antiapoptotic, whereas c-*myc*, c-*jun*, and c-*fos* are important stimulators of proliferation. Therefore, we investigated the expression of these oncogenes to explore their possible involvement in the p1–30 cytokine synergy.

We first studied the expression of the PI3 kinase pathway downstream molecules. Bcl-2 mRNA was difficult to measure in TS1β cells; therefore, bcl-2 protein expression was quantified by Western blot analysis. Fig. 6*A* (*left*) shows bcl-2 protein expression in TS1β cells stimulated with p1–30 or IL-2 for 24 h. IL-2 is an efficient inducer of bcl-2 protein expression in this model cell line (34). In contrast, p1–30 only induces a weak expression of bcl-2. Further analysis demonstrated that p1–30 plus IL-2 were unable to

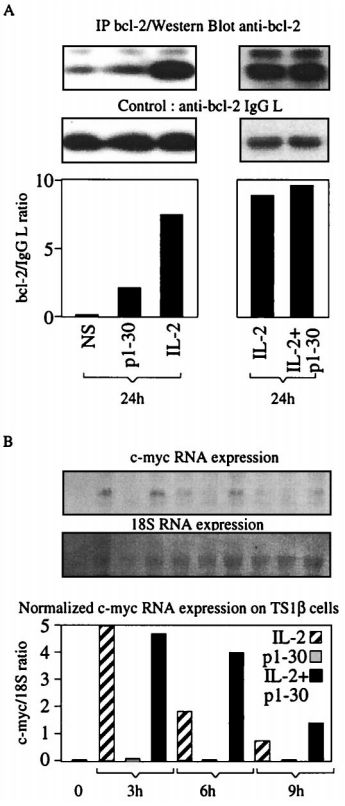

FIGURE 6. bcl-2 protein expression and c-*myc* mRNA induction. *A, Left,* TS1β cells were cultured for 24 h in medium containing 5% FCS in the absence or presence of p1–30 (60 μM) or IL-2 (3 nM). *Right,* TS1β were stimulated with IL-2 or p1–30 + IL-2 for 24 h. *A* and *B,* Cell lysates were immunoprecipitated and analyzed by Western blotting as described in *Materials and Methods*. The bcl-2/IgG L ratio is shown. *B,* TS1β cells were starved in cytokine-free medium for 12 h before stimulation with IL-2 (3 nM), p1–30 (60 μM), or both IL-2 + p1–30. After 3, 6, and 9 h, total RNA was extracted and c-*myc* mRNA and 18S RNA measured by Northern blot as described in *Materials and Methods*. After quantification the c-*myc*/18-sense ratio were calculated.

act in synergy to enhance expression of bcl-2 (Fig. 6*A*, *right*). These results, obtained after immunoprecipitation of the bcl-2 protein, were confirmed by direct Western blotting and analysis of actin as control (data not shown).

c-*myc* RNA expression was also examined on TS1β cells under similar conditions. No c-*myc* RNA was detected after p1–30 stimulation, whereas IL-2 rapidly up-regulated expression of this oncogene (Fig. 6*B*) as previously described (35). Despite the inefficiency of p1–30 alone to up-regulate c-*myc*, the signal detected with p1–30 plus IL-2 was greater than that for IL-2 alone after 6 h of stimulation, suggesting a synergistic effect.

Expression of c-*fos* and c-*jun* was then analyzed by Northern blot 1 and 2 h after TS1β cell stimulation with IL-2 and/or p1–30 (Fig. 7). Compared with unstimulated cells, p1–30 did not induce any expression of these two proto-oncogenes. In contrast, IL-2 rapidly induced c-*fos* and c-*jun* mRNA expression, as previously demonstrated (35). The p1–30-induced RNA expression was followed up to 12 h of stimulation and remained negative (data not shown). However, after 1-h stimulation, c-*fos* and c-*jun* expression was significantly enhanced when cells were stimulated with IL-2 and p1–30 in combination. This suggested that the enhanced c-*fos* and c-*jun* expression obtained with p1–30 + IL-2 may be implicated in the synergistic effect.

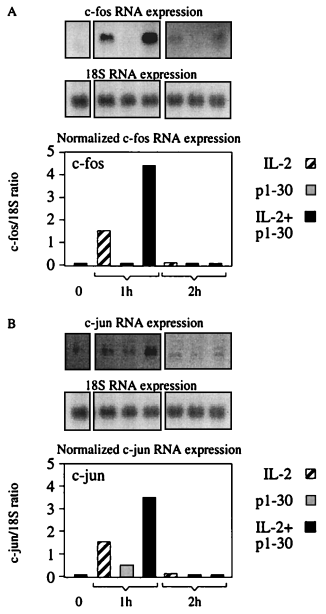

FIGURE 7. c-*fos* and c-*jun* proto-oncogene expression. *A,* TS1β cells were starved in cytokine-free medium for 12 h before stimulation with IL-2 (3 nM), p1–30 (60 μM), or both IL-2 + p1–30. After 1 and 2 h, total RNA was extracted from stimulated TS1β cells, and the c-*fos* mRNA and 18-sense RNA were detected by Northern blot. *B,* Northern blots were analyzed for c-*jun* expression. For (*A*) and (*B*), quantification was performed as described in *Materials and Methods* and the c-*fos*/18-sense or c-*jun*/18-sense ratio are shown.

Discussion

Structure-function studies of the IL-2/IL-2R system led us to the analysis of various IL-2 peptides. Among them, peptide p1–30 was shown to be an IL-2Rβ agonist. It is also the first human IL-2 mimetic characterized (24). Peptides related to erythropoietin (36) and thrombopoietin (37) have been selected by random phage display, whereas p1–30 corresponds to the natural α helix A sequence of IL-2. Experimental data supporting an IL-2/IL-2R model where α helix A of IL-2 is involved in binding to IL-2Rβ have been published (26, 38). This is consistent with the unique properties of p1–30, which behaves as a selective agonist of IL-2Rβ. The association of p1–30 tetramers with dimeric structures of IL-2Rβ, as revealed by ultracentrifugation analysis, suggested that the p1–30 cell surface receptor is made up of IL-2Rβ dimers (24). The major immunological effects of p1–30 consist of triggering human PBMC proliferation and induction of CD8, NK, and LAK responses. While characterizing these effects, we observed that p1–30 possesses the ability to act in synergy with IL-2. Here we confirm and extend this observation, documenting the synergy found between p1–30 and cytokines including IL-4, -9, and -15 (Figs. 1 and 2). In accordance with our previous results, these studies suggest that the receptors for p1–30 and for the cytokines studied are distinct. The mechanism by which synergy is obtained has been investigated at the receptor level, and potential target genes have been identified.

The IL-2Rβ-chain has proven to be critical in the synergistic effect described in this paper (Fig. 3). Simultaneous binding of p1–30 and IL-2 or -15 to IL-2Rβ as a mechanism leading to enhanced multimerization of the chain can be excluded. First of all, it seems unlikely that both p1–30 and IL-2 would bind simultaneously to IL-2Rβ molecules because they recognize the same area of the protein. Indeed, anti-IL-2Rβ mAb A41 neutralizes both p1–30 and IL-2 effects (24). Moreover, this hypothesis could not

explain the observed synergy with IL-4 and -9, whose receptors do not contain IL-2Rβ. At the functional level, these data are in agreement with a model suggesting that p1–30 binds to a receptor composed of preformed IL-2Rβ dimers, which at the surface of TS1β cells can be expressed in the presence of the heterospecific intermediate affinity IL-2R (IL-2R$\beta\gamma$). Binding of p1–30 to $(IL\text{-}2R\beta)_2$ and of IL-2 to IL-2R$\beta\gamma$ would be separate phenomena. Similarly, p1–30R would be expressed independently of IL-4R, -9R, and -15R. These independent interactions would constitute the first necessary steps for the synergistic effect.

In agreement with this model involving p1–30 binding to IL-2Rβ homodimers, we have previously described that the p1–30 peptide interacts with a soluble dimeric form of IL-2Rβ and induces signals (24). In contrast, previously published data suggest that IL-2Rβ dimers are unable to induce signals (39, 40). In these reports, chimeric receptors were constructed with the intracellular region of IL-2Rβ and the extracellular region of either IL-2Rα or GM-CSFR. These receptors did not induce significant proliferation after homodimerization by anti-IL-2Rα mAbs or GM-CSF. Conformational constraints due to the constructs could explain these negative results. Indeed, chimeric receptors composed of the extracellular region of either EPOR (41, 42) or c-*kit* (stem cell factor receptor) (39) coupled to the intracellular region of IL-2Rβ were shown to be capable of inducing marked proliferation after binding to the appropriate ligand.

The fact that IL-2R, -4R, -9R, and -15R share the γc led us to envisage a possible role of γc expression in the synergy effects described in this paper. Indeed, γc could be expressed in limited amounts and control the number of IL-2R, -4R, -9R, and -15R (43). Therefore, P1–30-induced enhancement of γc expression could increase the number of functional receptors at the cell surface and allow a better response to IL-2, -4, -9, or -15. However, analysis of IL-2Rγ expression at the cell surface following stimulation of TS1β cells by p1–30, IL-2, or p1–30 + IL-2 did not show any increase as measured by FACS analysis (Fig. 5). This excludes the possibility that the combination IL-2 + p1–30 could have induced expression of additional IL-2R, -4R, -9R, or -15R, which may have explained an increased response to the corresponding cytokine in the presence of p1–30. These results suggest that modulation of the number of cell surface cytokine receptors does not account for the observed synergistic effect.

The inhibitory role of IL-2Rα in p1–30-IL-2 synergy merits further discussion. When it is expressed on TS1$\alpha\beta$ cells, IL-2Rα specifically impedes the synergistic effect between p1–30 and IL-2 without influencing that observed with IL-4, -9, and -15 (Fig. 4). Previous analysis of clones TS1β and TS1$\alpha\beta$ has shown that IL-2Rβ is expressed in comparable quantities at the surface of these two cell lines, whereas IL-2Rα is expressed in great excess at the surface of TS1$\alpha\beta$ (27). Therefore, it is possible that in the presence of IL-2 and an excess of IL-2Rα, all IL-2Rβ-chains are sequestered to participate in either IL-2/IL-2R$\alpha\beta\gamma$ complex or IL-2/IL-2R$\alpha\beta$ complexes. Indeed, IL-2 binds to the IL-2R$\alpha\beta$ complex ($K_d = 10^{-10}$ M) and to the IL-2R$\alpha\beta\gamma$ complex ($K_d = 10^{-11}$ M) with high affinity. Under these conditions, formation of IL-2Rβ dimers may be inhibited and binding to p1–30 greatly reduced. Alternatively, one may consider that in the absence of IL-2Rα presentation of IL-2 to IL-2Rβ is not optimal (44), which allows the p1–30 effects to be seen.

With the purpose of identifying potential target genes involved in the proliferative synergy between p1–30 and IL-2, the induction of several genes implicated in the cell cycle control of T cell proliferation was analyzed. This includes the antiapoptotic molecule bcl-2 and proto-oncogenes c-*myc*, c-*fos*, and c-*jun*.

Concerning the antiapoptotic protein bcl-2, we observed that it is slightly induced by p1–30 alone and that no enhancement over that of IL-2 alone was found with p1–30 plus IL-2. In the IL-2 system, bcl-2 expression is under the control of the Akt protein kinase, which is regulated by PI3 kinase (19). PI3 kinase may form a complex with Cbl and Grb2. Under IL-2 stimulation, this complex binds to IL-2Rβ through the adaptor protein Shc, and this may explain the recruitment of PI3 kinase to the IL-2Rβ-chain (20). Shc phosphorylation (24) may explain the induction of bcl-2 after p1–30 stimulation. It has been demonstrated that the transfection of BAF/BO3 cells with an active $p56^{lck}$ protein and a constitutively expressed *bcl-2* gene was sufficient to trigger proliferation (35). Therefore, $p56^{lck}$ activation (24) and bcl-2 induction (Fig. 6) may explain the proliferation observed after p1–30 stimulation but not the synergy described in this paper. However, we cannot exclude that other molecules of the bcl-2 family, like BCL-XL, may participate in the synergistic effects by their antiapoptotic activity (21).

Altogether, the potentiation of the c-*myc*, c-*fos*, and c-*jun* mRNA induction may, at least in part, explain the synergistic responses observed when TS1β cells are simultaneously stimulated with p1–30 and IL-2. An influence of p1–30 on the stability of the proto-oncogene mRNA may also participate in the observed effects. C-myc is a key regulator of cell proliferation, activating Cdk2 kinase activity and antagonizing the function of Cdk inhibitors such as p27 (45). c-*fos* and c-*jun* constitute the transcription factor AP-1. An increase in AP-1 DNA binding is generally observed in response to extracellular signals resulting in proliferation (46). Moreover, c-$jun^{-/-}$ mice are defective in primary fibroblast proliferation (47). The proto-oncogenes c-*myc*, c-*fos*, and c-*jun* are not induced by p1–30 alone. However, a potentiation above that of IL-2 alone was observed with IL-2 + p1–30. c-*fos* and c-*jun* are known to be downstream components of the RAS pathway. We have previously showed that p1–30 activates the protein kinase $p56^{lck}$ and the phosphorylation of the adaptor protein Shc that are upstream of RAS (48). In the course of p1–30 stimulation, activation of $p56^{lck}$ and phosphorylation of Shc may not be sufficient to induce c-*fos* and c-*jun* mRNA expression. Additional signals may be required for the full expression of these oncogenes in TS1β cells. In accordance with this hypothesis, previous reports have demonstrated the critical function of Jak3 in IL-2-dependent activation of c-*fos* (49) and the essential role of the C-terminal 68 aa of IL-2Rγ in the IL-2-dependent activation of c-*fos* and c-*jun* (50). Because we have previously determined that p1–30 alone does not act through the IL-2Rγ-chain and does not mediate the activation of Jak3 (24), this may, at least in part, explain its inability to induce c-*jun* and c-*fos*. Concerning the synergistic response, our data suggest that p1–30 may provide a potentiating signal for IL-2 and lead to the proliferative synergy observed. A similar explanation can be applied for the enhancement of c-*myc* expression in the presence of p1–30 + IL-2 because this proto-oncogene is a distal element of the PI3 kinase pathway, which was also reported to be dependant on the C-terminal 30 aa of IL-2Rγ (50) and on the activation of Jak3 (49). More detailed analysis is now required at the molecular level to define the biochemical steps of the Ras/MAPK and Akt/PI3 kinase pathways that may be the targets of p1–30.

The results reported here, using the TS1β cell line as a model, have been confirmed and extended to other systems. When the human T cell line Kit 225 was studied, a similar synergy was observed for the induction of oncogenes such as c-*myc*. Under some experimental conditions, a synergistic effect was also observed for the induction of LAK cells and production of IFN-γ by human PBMC. At the fundamental level, the capacity of the IL-2 mimetic, p1–30, to act in synergy with cytokines like IL-2, -4, -9, and -15 may provide an additional tool to further analyze signal

transduction mechanisms by IL-2Rβ and cross-talk between different molecules involved in the combinative family of cytokine receptors of the hemopoietin class. Furthermore, because p1–30 may have therapeutic potential, as already discussed, its ability to synergize with IL-2 or -15 may have practical implications for the stimulation of lymphocytes, like CD8 and/or NK cells, which constitutively express IL-2Rβ (24, 51).

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