Growth Signal Transduction by the Human Interleukin-2 Receptor Requires Cytoplasmic Tyrosines of the \( \beta \) Chain and Non-tyrosine Residues of the \( \gamma_c \) Chain*

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To evaluate the possible role for receptor-based tyrosine phosphorylation in growth signaling induced by interleukin-2 (IL-2), a series of substitution tyrosine mutants of the IL-2 receptor \( \beta \) and \( \gamma_c \) chains was prepared and analyzed. Concurrent substitution of all six of the cytoplasmic tyrosines present in the \( \beta \) chain markedly inhibited IL-2-induced growth signaling in both pro-B and T cell lines. Growth signaling in a pro-B cell line was substantially reconstituted when either of the two distal tyrosines (Tyr-392, Tyr-510) was selectively restored in the tyrosine-negative \( \beta \) mutant, whereas reconstitution of the proximal tyrosines (Tyr-338, Tyr-355, Tyr-358, Tyr-361) did not restore this signaling function. Furthermore, at least one of the two cytoplasmic tyrosines that is required for \( \beta \) chain function was found to serve as a phosphate acceptor site upon induction with IL-2. Studies employing a chimeric receptor system revealed that tyrosine residues of the \( \beta \) chain likewise were important for growth signaling in T cells. In contrast, although the \( \gamma_c \) subunit is a target for tyrosine phosphorylation in vivo, concurrent substitution of all four cytoplasmic tyrosines of this chain produced no significant effect on growth signaling by chimeric IL-2 receptors. However, deletion of either the Box 1, Box 2, or intervening (V-Box) regions of \( \gamma_c \) abrogated receptor function. Therefore, tyrosine residues of \( \beta \) but not of \( \gamma_c \) appear to play a pivotal role in regulating growth signal transduction through the IL-2 receptor, either by influencing cytoplasmic domain folding or by serving as sites for phosphorylation and subsequent association with signaling intermediates. These findings thus highlight a fundamental difference in the structural requirements for IL-2R\( \beta \) and \( \gamma_c \) in receptor-mediated signal transduction.

Interleukin-2 (IL-2)\(^1\) is a helical cytokine that induces the

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\(^{f}\) The abbreviations used are: IL-2, interleukin-2; IL-2R, interleukin-2 receptor; EPO, erythropoietin; EPOR, erythropoietin receptor; PCR, polymerase chain reaction.

\(^{g}\) Among the earliest biochemical changes induced by ligation of the IL-2 receptor is activation of cytoplasmic tyrosine kinases resulting in the phosphorylation of certain recognized and unrecognized cellular substrates. The biologic relevance of IL-2-induced tyrosine kinase activity is supported by the finding that selective tyrosine kinase inhibitors (herbimycin A and genistein) concomitantly block these intracellular phosphorylation events as well as growth signal transduction (11, 12). Although none of the known IL-2R subunits contain recognizable kinase catalytic domains, tyrosine kinase activity has been immunoprecipitated with the IL-2R (13–18). Recent evidence indicates that the J\( \alpha \)us kinases JAK1 and JAK3 (19–21) as well as various src family kinases (13, 15, 18, 22) are among the signaling molecules that are physically and functionally linked to the IL-2R. However, the specific role of each of these kinases and their substrates in IL-2R signal transduction remains to be defined.

Like many growth factor receptors containing intrinsic tyrosine kinase activity (for review, see Ref. 23), the cytoplasmic domains of the \( \beta \) and \( \gamma \) subunits of the interleukin-2 receptor itself undergo inducible tyrosine phosphorylation upon engagement by IL-2 (24–26). The biological significance of such receptor phosphorylation is poorly defined for cytokine receptors lacking intrinsic tyrosine kinase activity. Since the IL-2 receptor itself is a major substrate of tyrosine phosphorylation following the binding of IL-2, the present investigation was undertaken to determine the potential regulatory role played by the cytoplasmic tyrosine residues of the IL-2R\( \beta \) and \( \gamma_c \) subunits. Our results demonstrate that tyrosines within the cyto-
plasmic tail of IL-2Rβ are critical for full growth signaling in pro-B and T cells. In contrast, the tyrosine residues of the γc chain are dispensable for this function, revealing an important distinction between the IL-2Rβ and γc subunits. These findings, along with a delineation of essential membrane-proximal domains of γc, may have general implications for the functional design of cytokine receptors, particularly those employing the common γc subunit.

MATERIALS AND METHODS

Cell Lines—The cell line BA/F3 (27), an IL-3-dependent murine pro-B cell line, was maintained as described previously (28). Supernatant from WEHI-3 cells (ATCC) was used as a source of IL-3. HT-2, an IL-2-dependent murine helper T cell line (ATCC), was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 55 mm β-mercaptoethanol, 2 mm L-glutamine, and 200 units/ml recombinant human IL-2 (a gift of the Chiron Corp.). Transfection of either BA/F3 or HT-2 cells was performed by electroporation as described previously (28); stable transfectants were obtained by selection in G418 (Genetech, 1 mg/ml, Life Technologies, Inc.) and clones isolated by limiting dilution were screened by radiodigand binding analysis with 125I-IL-2 or 125I-EPO (see below) or by Northern blot analysis to identify clones expressing the transfectant receptor (see text and figure legends). HT-2-EPO was established by transfecting HT-2-EPOβ cells with pEPO-neo and culturing in recombinant human EPO (10 units/ml, Amgen, Inc.) without IL-2. The COS-7 cell line (ATCC) was maintained as described (29).

Proliferation Assays—Conventional 24-h [3H]thymidine incorporation assays and transfection proliferation assays were performed essentially as described previously (28). In transfection studies using the chimeric receptors, HT-2 cells and their derivatives (see text) were transfected with expression plasmids encoding chimeric receptors and were then selected for approximately 10 days in EPO (50 units/ml) in the absence of IL-2; cell growth was assessed by [3H]thymidine incorporation on the indicated days.

Plasmid Constructs—All receptor cDNAs were subcloned into the expression vectors pCMV4 (30), pCMV4Neo (28), or pCMV4Δa (a PCV4 derivative containing a deletion of a vestigial second polylinker downstream of the cytomegalovirus expression cassette). For all constructs requiring synthetic oligonucleotides or PCR reactions, sequences were confirmed by DNA sequencing. The murine EPO cDNA from pXM-nePOR (31) was inserted into the KpnI/XbaI sites of pCMV4Neo to yield pEPO-neo, and the human IL-2Rβ cDNA from pIL2R30 (provided by T. Taniguchi) was inserted into the HindIII/BamHI sites of pCMV4Neo to yield pEPOneo.

The tyrosine substitution mutants of IL-2Rβ and γc (tyrosine (TAC) to phenylalanine (TTC)) were prepared by a combination of oligonucleotide-directed mutagenesis in M13 bacteriophage and PCR-based methods. Primers directing the γc cytoplasmic tail, a full-length cDNA, was obtained by reverse transcription PCR based on the IL-2Rγ sequence reported by Takeshita et al. (32). Deletion and substitution mutants described under “Results” (see figure legends) were prepared by PCR using IL-2Rβ or γc cDNAs as templates.

pEPO-neo, constructed by PCR using an Nhel site at the fusion junction, encodes a chimeric receptor (see Fig. 6A) containing the extracellular domain of the EPOR fused just above the transmembrane segment to the human IL-2Rβ transmembrane and cytoplasmic segments (resulting sequence: ... (EPOR-T-A-S1-G-K-D-IL-2Rβ) ... ). pEPO-neo, also constructed by PCR using the Nhel site, encodes a receptor (see Fig. 6A) containing the extracellular domain of the EPO fused to the human γc transmembrane and cytoplasmic segments (resulting sequence: ... (EPOR-T-A-S1-G-K-D-IL-2Rβ) ... ). Expression plasmids encoding the mutants described in the text were prepared by subcloning appropriate DNA fragments spanning the indicated mutations into the parental pEPO-neo and pEPO-neo plasmids.

Protein Expression and Phosphorylation Studies—COS-7 cells (ATCC) were transfected with the indicated plasmids (see text) using Lipofectamine (Life Technologies, Inc.) per the manufacturer’s instructions. For expression analysis of chimeric receptors, immunoblotting analyses were performed on cell lysates using an anti-EPO N-terminal peptide antisera and 125I-protein A as described previously (31). For phosphorylation analyses, the indicated cell lines were stripped of bound ligands by a 1-min acidic wash (10 mm sodium citrate, 0.14 m NaCl, pH 4) and then were rested in medium without serum or cytokines for 4 h. Cells were then stimulated with either IL-2 (10 mm) or EPO (50 units/ml) for 10 min at 37°C lysed (1% Nonidet P-40, 150 mm NaCl, 20 mm Tris, pH 8.0, 50 mm NaF, 100 mm sodium orthovanadate, 1 mm phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A) and immunoprecipitated with either the anti-IL-2Rβ monoclonal antibody 561 (kindly provided by Dr. R. Robb) or an anti-J AK1 antisera (Upstate Biotechnology, Inc.) and protein A-Sepharose. Immunoblotting studies were performed with anti-phosphotyrosine antibodies (4G10, Upstate Biotechnology, Inc.) per the manufacturer’s instructions followed by ECL (Amersham Corp.) signal development.

Radiodigand Binding and Cross-linking—Equilibrium binding analyses were performed as described (28, 33) with 125I-IL-2 (DuPont NEN) and either IL-2Rβ or γc cDNAs as templates. In transfection assays, performed by electroporation as described above, the transformant cell line was selected for approximately 10 days in EPO (50 units/ml) in the absence of IL-2. The COS-7 cell line (ATCC) was maintained as described (29).

RESULTS

Substitution Mutation of all Six Cytosolic Tyrosine Residues in IL-2Rβ Impairs Growth Signal Transduction in a Transient Assay System—The cytoplasmic tail of the human interleukin-2 receptor (IL-2Rβ) chain contains six tyrosine residues (37) (Fig. 1), including four in the “acidic” region (A) (38) and one in each of two distal segments (B, C) (28). To investigate the possibility that growth signaling through the IL-2R is regulated by tyrosine phosphorylation, a mutant IL-2Rβ chain ([βFJ]) containing concurrent substitutions of phenylalanine at all six cytoplasmic tyrosine positions was prepared and analyzed in a transient assay of lymphocyte growth signal transduction. In this method (28), IL-3-dependent murine pro-B cells

![Fig. 1. Growth signal transduction properties and expression of tyrosine-negative mutant IL-2Rβ chains in transient assays.](image-url)
(BAF3) (27) containing endogenous IL-2Rγ chains are transfected with expression plasmids encoding wild type or mutant IL-2Rβ and selected in medium containing IL-2 in the absence of IL-3. Cells transfected with wild type IL-2Rβ (WT) chains proliferated vigorously as indicated by substantial incorporation of [3H]thymidine within 7 to 9 days, whereas cells receiving the vector control died in culture (Fig. 1A). Using this assay system, lymphocytes transfected with the all tyrosine-negative IL-2Rβ mutant (βYF) demonstrated a dramatically impaired proliferative response to IL-2 (Fig. 1B). Thus, one or more of these cytoplasmic tyrosines of IL-2Rβ appeared to be critically required for full growth signal transduction through the IL-2R.

Two independent types of experiments were performed to ensure that the impaired function of βYF was not simply the result of ineffective surface expression or faulty binding of ligand. First, to monitor surface expression COS cells were transiently transfected with expression vectors encoding the IL-2Rα chain and either native IL-2Rβ or βYF, followed by incubation with [125I]IL-2, chemical cross-linking with disuccinimidyldi carbonate, and immunoprecipitation with the anti-β monoclonal antibody DU-2 (14). Following SDS-polyacrylamide gel electrophoresis, bands of comparable intensity and migration were observed for cells transfected with the wild type β and βYF, indicating the unimpaired surface expression of the mutant βYF receptor (Fig. 1C). To investigate potential changes in receptor affinity, radioligand binding analyses were performed with [125I]IL-2 in COS cells transfected with IL-2Rβ and WT or βYF. These studies revealed the expected single class of intermediate affinity IL-2 binding sites for both WT and βYF (Kd 300–400 pM) (Fig. 1, D and E). Thus, surface expression and ligand binding by βYF appeared indistinguishable from wild type β and therefore do not account for its impaired signaling function in the transfection assay system.

The Tyrosine-negative Mutant of IL-2Rβ Demonstrates Impaired Responsiveness to IL-2 in a Stable Transfectant—To confirm the phenotype of βYF, stable sublines of BAF3 were prepared by transfection with the plasmid pβYFNeo. Radioligand analysis demonstrated that the BafβWT and BafβYF cell lines expressed receptors that bound IL-2 with comparable intermediate affinities (data not shown), although for unknown reasons the BafβYF lines consistently expressed the receptor at somewhat lower levels than did BafβWT (BafβWT, 3000 receptors/cell; BafβYF, 700 receptors/cell). Nevertheless, in analyses of numerous sublines we have seen no correlation between expression levels in this range and proliferative signaling capacity.

Analysis of [3H]thymidine incorporation in response to IL-2 revealed marked unresponsiveness of the stable BafβYF cell line to IL-2 compared with BafβWT (Fig. 2). As expected, the BafβWT cell line demonstrated detectable proliferation even at very low doses of IL-2 (10 pM) well below the Kd of IL-2 binding to IL-2Rβ complexes, whereas the BafβYF line demonstrated no response even at very high doses of IL-2 (100 nM) vastly exceeding the measured Kd. These findings confirmed the impaired proliferation signaling exhibited by the βYF mutant initially detected in the transient system.

Selective Mutation of Individual Tyrosine Residues Does Not Alter IL-2Rβ Growth Signaling in a Pro-B Cell Line—The results in both transient and stable assay systems indicated that at least one tyrosine residue contributes importantly to IL-2R growth signaling competence in pro-B cells. To identify the relevant functional tyrosine residue(s), IL-2Rβ mutants containing selective phenylalanine for tyrosine substitutions were constructed and characterized using the BAF3 transient assay system. Surprisingly, substitution of phenylalanine at Tyr-338 (βY1F), Tyr-355/Tyr-358/Tyr-361 (βY234F), Tyr-392 (βY5F), or Tyr-510 (βY6F) had little or no effect on growth signal transduction in response to IL-2 (Fig. 3). In contrast to βYF, each of these selective tyrosine mutants mediated substantial proliferation; only a subtle compromise in receptor function was intermittently observed with βY5F and βY6F. These results revealed that no single cytoplasmic tyrosine is essential to growth signaling function, implying that a functional redundancy may exist involving two or more of these residues.

Either Tyr-392 or Tyr-510 Alone Is Sufficient to Permit IL-2Rβ Growth Signaling Function in Pro-B Cells—Previous reports with stable transfectants expressing IL-2Rβ mutants had demonstrated that the "A" segment spanning the first four cytoplasmic tyrosine residues is dispensable for growth signaling function (38), an observation confirmed in our previous studies employing the transient assay system in BAF/3 cells (28). This finding implied that the C-terminal tyrosines (Tyr-392 and Tyr-510) may be sufficient for full growth signaling. To evaluate this possibility, a mutant was prepared (βYF:6Y) containing substitutions of phenylalanine for the proximal four tyrosines, leaving the distal tyrosines intact; this mutant mediated a full proliferative response to IL-2 in BAF3 cells (Fig. 3). In contrast, a mutant with phenylalanines replacing exclusively these two distal tyrosines (βY5F6) was substantially impaired in its growth signal transduction capacity in the BAF3 cells, further demonstrating the importance of Tyr-392 and Tyr-510 to growth signaling by IL-2Rβ (Fig. 3).

We further observed that internal deletion of a 119-amino acid cytoplasmic region of IL-2Rβ spanning the A region as well as the contiguous "B" segment exhibited fully preserved growth signaling (Fig. 3, βAAB), suggesting that the first five tyrosines are dispensable. In contrast, extension of this deletion to include the C-terminal region containing the sixth tyrosine (βAAABC) abrogated receptor function (Fig. 3). These results suggested that the sixth tyrosine (Tyr-510) is sufficient to permit growth signal transduction. Indeed, an IL-2Rβ mutant in which only this single tyrosine was restored in the βYF background (βYF:6Y) exhibited substantial IL-2 growth signaling (Fig. 4A).

Although Tyr-510 alone is sufficient for receptor competence, selective substitution of phenylalanine at this position had little effect on the signaling function (Fig. 3). These results strongly implied that at least one other tyrosine site also could support growth signal transduction, a hypothesis that was tested by evaluating additional tyrosine add-back mutants. Interestingly, reconstitution of Tyr-392 (βYF:5Y) substantially
restored the IL-2Rβ signaling function (Fig. 4B). In contrast, restoration of tyrosines in the first four positions in two additional add-back mutants (βYF:234Y and βYF:1Y) failed to reconstitute receptor function (Fig. 4C and D, respectively). Importantly, the βYF:56Y, βYF:56F, βYF:1Y, βYF:234Y, βYF:5Y, and βYF:6Y proteins were all expressed abundantly as detected by immunoblotting analysis (data not shown). Thus, either the fifth tyrosine (Tyr-392) or sixth tyrosine (Tyr-510) is necessary and sufficient for IL-2 growth signaling in BA/F3 cells.

Tyrosine 392 of IL-2Rβ Is Phosphorylated upon Engagement of the IL-2R—The present findings indicating a functional role for certain cytoplasmic tyrosine residues of IL-2Rβ raised the important question of whether or not these tyrosine residues serve as phosphate acceptor sites, a possibility suggested by the recognition that this chain undergoes rapid tyrosine phosphorylation during receptor activation (24, 25). To address this question, stable transfectants of the BA/F3 line were prepared using expression plasmids encoding tyrosine-add-back mutants (βYF:5F, βYF:6Y, and βYF:5Y). Anti-IL-2Rβ immunoprecipitates from cells stimulated with IL-2 for 10 min were subjected to immunoblotting with anti-phosphotyrosine antibody. In contrast, tyrosine phosphorylation analysis of BafβWT (βwt), BafβYF (βYF), and BafβYF:5Y (βYF:5Y) cells yielded no discernible signal (Fig. 5). Anti-IL-2Rβ immunoprecipitates from cells stimulated with IL-2 for 10 min were subjected to immunoblotting with anti-phosphotyrosine antibody, whereas the BafβYF line yielded no discernible signal (Fig. 5).
Like BafβWT, BafβYF:5Y cells also yielded a phosphotyrosine-containing protein band (Fig. 5B). Since this add-back cell line expresses IL-2Rβ chains containing only a single cytoplasmic tyrosine residue (Tyr-392) with all others replaced by phenylalanine, a phosphotyrosine signal generated in the immunoblot experiment is clearly attributable to this tyrosine. These results thus indicated that Tyr-392 of IL-2Rβ serves as a phosphoacceptor site during receptor activation.

Similar experiments were performed with the BafβYF:6Y line to assess the role of Tyr-510 in receptor phosphorylation. Surprisingly, no IL-2Rβ chain tyrosine phosphorylation was detectable in experiments with cells expressing the Ty-510 add-back mutant (data not shown). Such experiments were performed with multiple, independently derived lines, and stimulations were performed for various lengths of time ranging from 3 to 30 min. It remains possible that this functional tyrosine residue of IL-2Rβ does indeed undergo phosphorylation and that this site is perhaps particularly sensitive to phosphatase attack after detergent solubilization of the cells. Nonetheless, phosphorylation of this tyrosine has not yet been detected (see “Discussion”).

Establishment of EPOR/IL-2R Chimeric Receptors to Study the Cytoplasmic Domains of the IL-2Rβ and γc Receptor Subunits in T Cells—To permit study of the functional interactions of the IL-2Rβ and γc cytoplasmic domains in T lymphocytes already expressing endogenous IL-2 receptors, we developed a chimeric receptor system in which the intracellular domains of interest (derived from IL-2Rβ and γc) were fused to an extracellular ligand binding domain not present in the host cell lines (Fig. 6A). Extracellular domains of the homodimeric EPOR extracellular domain were employed for this purpose, since the EPOR, IL-2Rβ, and γc subunits are all members of the cytokine receptor superfamily. Because the EPOR homodimerizes in the presence of EPO, these chimeric receptors were expected to promote dimerization of the IL-2Rβ and/or γc cytoplasmic domains following ligand binding. Plasmids encoding the chimeric EPORβ and EPORγ receptors expressed proteins of the predicted masses as detected by immunoblot analysis of lysates from transfected COS-7 cells (Fig. 6B): the native EPOR and wild type EPOβ and EPOγ constructs yielded bands of approximately 70, 75, and 40 kDa, respectively. Frequently protein doublets were observed with all of these constructs, which result from variable glycosylation.

The IL-2-dependent murine helper T cell line, HT-2, was employed for analysis of EPOβ and EPOγ signaling. Initially, stable HT-2 transfectants expressing the EPOR, EPOβ, or EPOγ subunits were established. In 24-h [3H]thymidine incorporation assays, the EPOR was found to mediate a modest response to EPO, whereas neither of the chimeric receptor subunits alone produced a detectable response in multiple transfected clones (Fig. 6C). The failure of EPOβ and EPOγ to mediate a response was not due to lack of expression, since Northern blotting, Western blotting, and radioligand binding analyses with 125I-EPO confirmed the expression and ligand binding competence of these chimeras in the HT-ZEPOβ and HT-ZEPOγ cell lines (data not shown).

Since neither chimera alone (EPOβ or EPOγ) demonstrated detectable growth signal transduction, combinations of these chimeras in HT-2 cells were tested for growth signaling in response to EPO as a means of promoting heterodimerization of the IL-2Rβ and γc cytoplasmic tails. For these studies the transfection assay originally described for BA/F3 cells (28) was adapted to HT-2 cells. When the EPOγ expression plasmid was introduced by electroporation into multiple HT-2 clones stably expressing EPOβ (HT-ZEPOβ), addition of EPO without IL-2 produced marked proliferation and vigorous incorporation of [3H]thymidine during the 12-day assay (Fig. 7A). Similarly, multiple HT-2 clones stably expressing EPOγ (HT-ZEPOγ) displayed marked proliferative responses to EPO following introduction of the EPOβ expression plasmid (Fig. 7B). Additionally, double transfectants arising from such experiments were easily maintained in long term culture by addition of EPO alone, allowing the isolation of a stable transfected cell line (HT-ZEPOβγ) for further studies of early signal transduction events. Thus, concurrent engagement of both the β and γc subunits...
chimeras is required for effective growth signaling, as has been reported in studies with other chimeric receptors (3, 4).

Tyrosine Residues of IL-2Rβ Are Required for Full Growth Signaling in Mature T Cells—The functional contribution of IL-2Rβ cytoplasmic tyrosines in T cells was assessed using the chimeric receptor system and the HT-2 cell line. HT-2EPOγ cells transfected with expression plasmids encoding either wild type EPOβ or a mutant, tyrosine-negative EPO/IL-2Rβ chimeric (EPOβYF) were selected in EPO and assessed for proliferation. Unlike the parental EPOβ (Fig. 7A), the tyrosine-negative EPOβYF exhibited no detectable growth response to EPO (Fig. 7C). Similarly, stable double transfectants of HT-2 expressing both EPOβYF and EPOγ demonstrated no proliferative response to EPO (data not shown). These findings demonstrated that the cytoplasmic tyrosines of the IL-2Rβ chain strongly influence receptor growth signaling independently of ligand specificity in both pro-B and mature T cells.

To analyze further the disruption in signal transduction by the YF mutant, J anus kinase induction in response to receptor engagement was assessed. Lysates prepared from HT-2 cells stimulated with no cytokine, IL-2, or EPO were subjected to immunoprecipitation with an anti-JAK1 antiserum followed by immunoblot analysis with the anti-phosphotyrosine antibody. Cells expressing chimeric γc chains and either wild type chimeric β chains (HT-2EPOβγ) or tyrosine-negative β chains (HT-2EPOβYFγ) both exhibited strong induction of JAK1 phosphorylation in response to either ligand (Fig. 7D). Likewise, preserved induction of JAK3 phosphorylation by receptor complexes containing EPOβYF was observed in parallel experiments employing an anti-JAK3 antiserum (data not shown).

Since tyrosine phosphorylation of the γc subunit upon ligand binding has been well described (26), we investigated the putative role of the tyrosine residues present in the γc subunit by phenylalanine substitution of all four tyrosine residues (EPOγYF). Surprisingly, growth signal transduction by EPOγYF was nearly indistinguishable from that by EPOγ both in transfection assays (Fig. 8, A and B) and in 24-h [3H]thymidine incorporation assays of stable transfectants arising from transfection of HT-2EPOβ cells with the EPOγYF expression plasmid (Fig. 8, C and D). Thus, the cytoplasmic tyrosine residues of γc appeared to be dispensable for growth signaling, which stands in sharp contrast to their importance in the IL-2Rβ subunit.

Although the tyrosine residues are non-essential, other regions of the γc cytoplasmic tail proved important for growth signaling. EPOγ mutants truncated at the cell membrane (EPOγTM) or at the end of the Box 1 (39) homology region (EPOγ294) mediated no detectable proliferation signaling (Fig. 9). Similarly, internal deletion of Box 1 (EPOγΔBox1), of a segment with distant relationship to the Box 2 motif (EPOγΔBox2), or of the segment connecting Box 1 to Box 2 (EPOγΔV-Box), also abolished proliferation signaling. However, truncation of the γc subunit at the C-terminal end of the Box 2 region (EPOγ336) resulted in levels of growth signaling similar to that obtained with the wild type subunit. Thus, unlike the IL-2Rβ subunit, the distal portion of the γc subunit is dispensable for proliferation signal transduction, and full growth-signaling function resides in the proximal 53 amino acids containing the Box 1, Box 2, and intervening (V-Box) segments.

DISCUSSION

Like many other cytokine receptor systems, the binding of IL-2 to the IL-2R induces the tyrosine phosphorylation of a variety of intracellular substrates, including the IL-2Rβ and γc chains (24–26). Although no tyrosine kinase domain is identifiable within the recognized ligand-binding subunits of the IL-2R, the J anus kinases JAK1 and JAK3 as well as the src family kinase p56Lck and p59Shc are now recognized to associate noncovalently with the cytoplasmic tails of IL-2R subunits (10, 15, 19, 40). The activation of such receptor-associated kinases may represent a mechanism for signal transduction that is fundamentally the same as that for receptors containing intrinsic kinase activity. Indeed, as in such kinase-containing receptors, some evidence has accumulated from mutagenesis and in vitro analyses that certain tyrosine residues of the IL-4 and interferon receptors are crucial for signal transduction competence (41–44).

The present studies were undertaken to evaluate the poten-
The potential regulatory role of cytoplasmic tyrosines of the IL-2Rβ and γc chains. In these studies employing both native and chimeric receptors, substitution of phenylalanine for all six cytoplasmic tyrosine residues of IL-2Rβ substantially impaired growth signaling in both a pro-B and a mature T cell line (Figs. 1 and 7). A panel of add-back mutants revealed that both Tyr-392 and Tyr-510 individually exhibit signaling potential in the BA/F3 pro-B cell line while the four more proximal tyrosines demonstrate no functional capacity in this specific cellular environment (Fig. 4). We conclude from these experiments that, in BA/F3 cells, the two C-terminal cytoplasmic tyrosines serve important but redundant functions in determining the signal transduction competence of the IL-2Rβ chain.

The finding that C-terminal tyrosines of IL-2Rβ influence growth signaling in this system appears to contrast with an earlier report that the IL-2Rβ segment encompassing these tyrosines is dispensable for proliferative signaling (38). However, point substitutions and deletions of identical regions may have different phenotypic consequences, particularly if the protein region in question exerts regulatory effects via conformational changes. For example, the C terminus of IL-2Rβ may negatively regulate proximal domains through steric hindrance, which might be relieved by receptor activation. Such a model would also explain the negative regulatory domain identified within the EPOR C terminus (45). A deletion mutant thus may obscure a role of tyrosine residues within this region. Therefore, we conclude that tyrosines within the IL-2Rβ cytoplasmic tail are indeed important for the growth signaling competence of IL-2Rβ.

The mechanism(s) underlying the importance of Tyr-392 and Tyr-510 to IL-2R function remain uncertain. In the platelet-derived growth factor receptor system, several distinct signaling pathways are activated selectively by individual phosphotyrosine residues through interactions with proteins via SH2 domains (46, 47). Recent reports have described the inducible binding of p52shc to the IL-2Rβ chain upon the binding of IL-2 (48, 49), although the molecular basis of this interaction is unknown. Similarly, phosphatidylinositol 3-kinase has also been found to associate with the IL-2Rβ chain in the presence of IL-2 (50, 51), an event which may be facilitated by phosphorylation of IL-2Rβ Tyr-392 as revealed in studies with phosphopeptides (51). Finally, following completion of the present
work, we (52) and others (53) have demonstrated that phosphopeptides encompassing either Tyr-392 or Tyr-510 are potent and specific inhibitors of the in vitro DNA binding activity of STAT-5, a STAT factor that is regulated by the IL-2R (52–54). Interestingly, tyrosine residues of IL-2Rβ are dispensable for Janus kinase activation by the IL-2R (Fig. 7B) but are essential for the effective induction of STAT-5 (52). Together, these findings are consistent with the popular model of cytokine receptor function (55) in which ligand-induced phosphorylation of certain tyrosine residues of the receptor is a critical step in the generation of downstream intracellular signals.

Convincing demonstration of the model for IL-2R function requires identification of the sites of IL-2-induced tyrosine phosphorylation of IL-2Rβ in vivo. The present studies demonstrate that Tyr-392 serves as a phosphate acceptor site upon exposure of BA/F3 transfectants to IL-2 (Fig. 5). Unexpectedly we failed to detect phosphorylation of Tyr-510 in parallel experiments. It is possible that this lack of detection results from technical problems, such as insensitivity of the assay method or contaminating phosphatase activity released during cell lysis. Alternatively, this observation may indicate that Tyr-510 function is entirely independent of its phosphorylation status. Indeed, the published evidence supporting a critical role for receptor phosphotyrosines in the JAK–STAT pathway is largely circumstantial. For example, experimental demonstration of direct interactions between STAT factors and phosphotyrosine-containing receptor segments has proven difficult in most circumstances, and heavy emphasis has been placed instead on in vitro peptide approaches (44). Therefore, the lack of detectable phosphorylation of Tyr-510 in the present studies raises the possibility that this and perhaps other tyrosine residues of IL-2Rβ exert crucial influences on the tertiary conformation of IL-2Rβ independently of their phosphorylation status. Although we tend to favor the tyrosine phosphorylation model, rigorous consideration of the published data demands further studies to distinguish effectively between these interpretations.

Other cytokine receptor superfamily members (1) may similarly be influenced by tyrosines. Functionally important tyrosine residues within the cytoplasmic domains of the IL-4 and interferon-γ receptors have been described recently (41–43), although the significance of IL-4R phosphorylation has been disputed (56). The functional redundancy described here for the distal IL-2Rβ tyrosines may also be a feature of the human IL-4 receptor that could explain the incomplete impairment of function reported upon substitution of phenylalanine for Tyr-497 in the IL-4 receptor (41). Further investigation is needed to clarify these events within the IL-2R.

The EPOR/IL-2R chimeric system also permitted an assessment of the role of tyrosine and other residues within the γc cytoplasmic tail for growth signaling in T cells. In contrast to the IL-2Rβ chain, the γc subunit functioned fully in the absence of all four of its cytoplasmic tyrosine residues (Fig. 8). This finding indicates that growth signaling intermediates interacting with the γc tail do so independently of phosphotyrosine docking sites, even though one or more of these tyrosine sites is phosphorylated after IL-2 stimulation in vivo. In view of the fact that both the IL-4 and IL-2 receptors employ the γc subunit, these observations raise the intriguing possibility that the longer, unique chain in each receptor provides the docking sites for the specific signaling intermediates engaged by each receptor complex. In this arrangement, the shared γc subunit would participate in general initiation of the signaling process, whereas the specialized subunits would contain unique sites for the inducible binding of specific components, such as STAT factors. Other cytokine receptors might employ a similar functional configuration. Of course, it remains possible that components involved in other pathways not measured here (such as differentiation) do indeed depend upon these γc tyrosine sites. Although the tyrosines of γc proved to be dispensable for growth signaling by the IL-2R, a panel of truncation and internal deletion mutants revealed other elements within γc that are critical for growth signaling in the T cell line. Remarkably, the C-terminal 33 amino acids of γc are fully dispensable for growth signaling (Fig. 9), indicating that the proximal 53 amino acids are sufficient for full growth signal transduction. Mutations within this membrane-proximal region abrogated signaling function. For example, extension of the truncation N-terminal to a gestrical “Box 2” motif (39) abolished the signaling function, as did internal deletion of the 14 amino acids constituting a “Box 1” motif, the 14 amino acids constituting this gestrical Box 2 motif, or the 26 amino acids connecting Box 1 to Box 2 (V-Box) (Fig. 5). These observations in T cells extend the studies by others which employed certain truncated γc subunits expressed in heterologous cell types (57–59) and demonstrate clearly that the γc tail is needed for growth signal transduction by IL-2R heterodimers in T cells. Importantly, the impairment of these γc domains undoubtedly contributes to the pathologic effects manifested in the X-linked severe combined immunodeficiency syndrome (60).

The recognition that the growth signaling function of γc resides in a relatively small portion of the cytoplasmic tail and that this segment functions independently of tyrosine residues is consistent with the receptor model described above. The essential, membrane-proximal region of γc has been shown to be crucial for the assembly of the Janus kinase JAK3 with γc (10, 40). Perhaps the primary function of γc in the IL-2, IL-4, and other receptors is to convey JAK3 into the receptor complex upon engagement of the appropriate ligand, which would thus allow trans-activation of JAK1 and JAK3 bound to their respective receptor subunits. Subsequent signaling activities may focus primarily upon the extended cytoplasmic tail of the unique IL-2Rβ chain, including the inducible binding and activation of specific factors. Further studies are needed to determine whether or not the γc chain has additional functions in addition to its conveyance role. One or both of the Janus kinases may be involved in phosphorylation substrates within the receptor complex. The present findings provide a rationale for further investigation of these intracellular events.

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REFERENCES
1. Bazan, J.-F. (1990a) Proc. Natl. Acad. Sci. U.S.A. 87, 6934–6938
2. Goldsmith, M. A., and Greene, W. C. (1994) in The Cytokine Handbook (Thompson, A., ed) pp. 57–80, Academic Press, Ltd., London
3. Nakamura, Y., Russell, S. M., Mess, S. A., Friedman, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S., and Leonard, W. J. (1994) Nature 369, 401–404
4. Nelson, B. H., Lord, J. D., and Greenberg, P. D. (1994) Nature 369, 333–336
5. Grinstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., Beers, C., and Leonard, W. J. (1994) Nature 369, 401–404
6. Giri, J. G., Adelstein, S., Eisenman, J., Shanebeck, K., and Leonard, W. J. (1994) Science 264, 965–968
7. Nakamura, Y., Russell, S. M., Mess, S. A., Friedman, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S., and Leonard, W. J. (1994) Nature 369, 401–404
8. Nakamura, Y., Russell, S. M., Mess, S. A., Friedman, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S., and Leonard, W. J. (1994) Nature 369, 401–404
9. Giri, J. G., Adelstein, S., Eisenman, J., Shanebeck, K., and Leonard, W. J. (1994) Science 264, 965–968
10. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K., and Sugamura, K. (1993) Science 262, 874–877
11. Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S., and Sugamura, K. (1994) Science 263, 1453–1454
12. Kondo, M., Nakamura, M., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X., and Leonard, W. J. (1993) Science 262, 1877–1880
13. Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Bacon, C. M., Friedmann, M., Berg, M., McVicar, D. W., Withuhn, B. A., Silvennoinen, O., Goldman, A. S., Schmalstieg, F. C., Ilie, J. N., O’shea, J. J., and Leonard, W. J. (1994) Science 266, 1042–1045
