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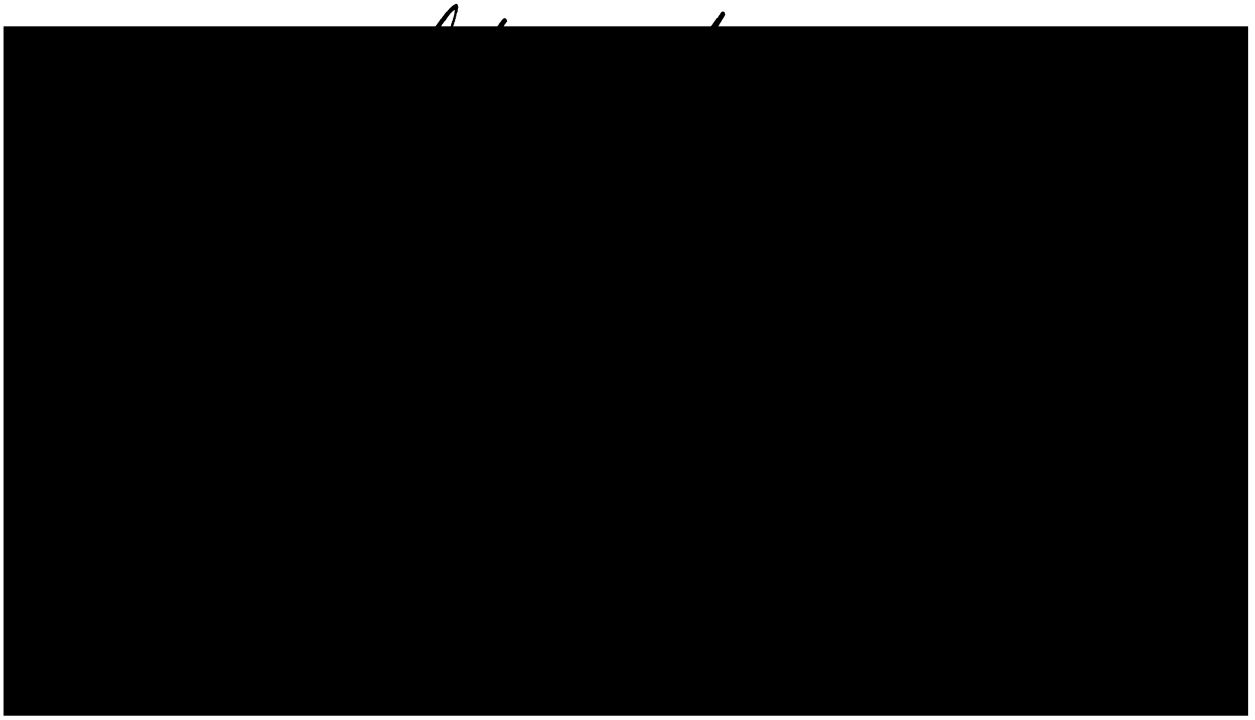

Dedication

This dissertation is dedicated to my parents Susan Kao
and Jeng Lai who have made all things possible.

Preface

With the completion of my dissertation, I realize that many wonderful colleagues and friends have made this work and my education possible. I can only begin to express my admiration and gratitude to those individuals with whom I have had the opportunity to work. First, I am indebted to Mark Goldsmith and Warner Greene for their joint supervision of my doctoral work and for the opportunity to be a part of their unique and productive collaboration. I thank Mark for his valuable guidance and scientific enthusiasm. I thank Warner for his commitment to excellence and steadfast support. Their dedication and passion for their work as both scientist and physician will always serve as an inspiration to me. I appreciate the dedication and candor that Sarah Gaffen shared with me during our various collaborations. I thank Kathleen Liu for her scientific precision and forthright perspective. I appreciate the refreshing eagerness that Jaime Molden brought to our work. I thank Weiduan Xu for her good humor and her careful attention to our experiments. Working with them and the other members of the laboratory group has taught me many valuable lessons. I can only hope that the magnanimous spirit and camaraderie of the laboratory group continue to flourish. I look forward to working with these talented people in the future. Finally, I thank my family and Teresa Lin for their love and understanding during this unfamiliar process.

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Much of the work in this dissertation has been submitted and/or published in various scientific journals. Chapter 2 of this thesis is a reprint of the material as it appears in **The Journal of Biological Chemistry**. The material from Chapter 3 is a reprint of the article published in **The Proceedings of the National Academy of Sciences.** In Chapter 4, the two papers are reprinted as they appear in **The Proceedings of the National Academy of Sciences** (Chapter 4A) and **The Journal of Biological Chemistry** (Chapter 4B), respectively. Chapter 5 is in press and is reproduced from **The Journal of Clinical Investigation** by copyright permission of The American Society for Clinical Investigation. Chapter 6 is a reprint of the work as it appears in **The EMBO Journal** by permission of Oxford University Press. Chapter 7 is a manuscript that has been submitted to **Science**.

The Functional Architecture of Hematopoietic Cytokine Receptor Complexes

Stephen Y. Lai

Cytokines direct a multitude of biological functions within a delicate regulatory network that controls the growth and differentiation of many cell populations. Within the last two decades, the identification of many of these factors, their cognate receptors and interacting cytoplasmic signaling intermediates has provided important insights into how cells communicate and respond to changes in their external environment. While each cytokine activates a unique profile of signaling events, many cytokine receptor complexes share receptor subunits and utilize similar signaling intermediates. The ability of these receptors to employ common signaling components, yet orchestrate specific cellular responses, remains one of the most intriguing puzzles in cytokine biology. The research described in this dissertation focused on the molecular factors that regulate cytokine receptor assembly and the specific roles of the individual receptor chains in subsequent signaling activity. To accomplish these studies, a chimeric receptor system was employed, involving the fusion of the extracellular region of the erythropoietin receptor to the transmembrane and cytoplasmic domains of various receptor subunits. This general strategy permitted the selective activation of different combinations of receptor subunit cytoplasmic domains in physiologically relevant cells that displayed the native cytokine receptor under investigation. The structure/function analyses of the interleukin-2 (IL-2), IL-4 and IL-7 receptor complexes that share the γ_c subunit have led to a conceptual framework now advanced in the "trigger-driver" model that describes the modular organization of heteromeric receptor complexes. Furthermore, these studies have revealed a novel functional interplay between the intracellular domains of the IL-2Rβ and γ_c chains, indicating a previously unappreciated mechanism for regulation of cytokine receptor signaling. These findings provide the basis for future studies of related cytokine receptor systems.

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Chapter 1

Introduction

I. Overview

Cytokines direct vital biological processes including cellular proliferation, differentiation, apoptosis and regulation of immune responses (1). These ligands act by engaging one or more receptor subunits expressed on the cell surface. Cytokine binding drives assembly of the receptor subunits and associated cellular signaling molecules into a competent receptor signal transduction complex that promotes multiple biochemical events. These modifications lead to the altered regulation of downstream gene transcription and subsequent biological effects. Control of these cytokine signaling programs occurs at multiple levels, ranging from the specific ligand/receptor complex interactions to the activation of distinct combinations of transcription factors that regulate gene expression. With the cloning and characterization of many cytokines and their receptors, these factors have been widely used in the treatment of malignancies, infections and immune disorders (2). The clinical application of these cytokines has been guided by the detailed analysis of their signal transduction activities. Several general features of cytokines emerged from these biological studies. First, each factor mediates a wide range of biological effects. Second, each cytokine activates a specific profile of signaling events and functions. Finally, while each factor has specific signaling functions, some signaling effectors and functions are shared by one or more related cytokines. Since the molecular determinants that define these cytokine receptor signal transduction characteristics have not been well characterized, a primary goal of this project was to delineate those factors that contribute to the assembly of functional cytokine receptor complexes and activate specific intracellular signaling programs.

II. The Cytokine Receptor Complex

Cytokines are small proteins or polypeptides that are produced and secreted by discrete cell populations to mediate a wide array of functions that collectively form a complex, but flexible, regulatory network. While these cytokines display little primary sequence similarity, the available structural data for these factors define several cytokine families (3,4). For example, the hematopoietic cytokines display a common tertiary structure in the antiparallel arrangement of four α helices (3). Moreover, while most of these cytokines are biologically active as monomers or dimers, interleukin-12 (IL-12) is an example of a heterodimeric factor (5).

The biological actions of cytokines are mediated through their association with distinct receptor complexes composed of one or more receptor subunits. These receptor subunits are quite diverse, ranging from those that contain intrinsic tyrosine or serine/

threonine kinase domains to those that are related to the G-protein-coupled seven transmembrane spanning receptors. Most of these receptor subunits are type I integral membrane proteins that are heterogeneous in primary sequence and size. However, some structural characteristics of cytokine receptor subunits indicate an evolutionary relationship prompting their classification within a cytokine receptor superfamily (Figure 1a) (6). Particular features of these receptor subunits divide members of this superfamily into related Class I (e.g. IL-2Rβ, gp130) and Class II [e.g., interferon-γ (IFN-γ) receptor] receptor subunits. The Class I receptor subunits, which are the primary focus of the present work, share three extracellular motifs: fibronectin-like domains, a conserved tetrad of cysteines with canonical spacing and a Trp-Ser-X-Trp-Ser motif adjacent to the transmembrane domain (6). Moreover, these subunits contain two small, partially conserved cytoplasmic regions referred to as Box1 and Box2 (7,8). These membrane-proximal Box regions and other cytoplasmic receptor elements mediate interactions with specific cellular signaling intermediates (9,10).

The cytokine receptor complex is typically composed of one or more receptor subunits. The structural data for the growth hormone receptor (GHR) and erythropoietin receptor (EPOR) complexes indicate that these receptor complexes contain two identical receptor subunits. In contrast, many hematopoietic cytokine receptor complexes are composed of two or more different receptor subunits. For example, the high-affinity IL-2R complex contains the IL-2Rα, IL-2Rβ and γ_c subunits (11), although IL-2Rβ and γ_c are sufficient for receptor signaling (12,13). While each of the receptor subunits contributes to the high-affinity binding of ligand, the individual role of the distinct receptor subunits within the complex for signal transduction has not been well characterized. Thus, the functional differences in signaling stemming from the physical asymmetry of heterodimeric receptors remains poorly defined.

An additional level of structural complexity within the receptor complex involves the stoichiometry of receptor subunits. Unlike the GHR and EPOR complexes, recent crystallographic analysis of the tyrosine kinase domains of the fibroblast growth factor receptor (FGFR) strongly suggest that the FGFR complex contains more than two FGFR subunits (14). Moreover, the IL-6R complex, which was originally believed to be heterodimeric (IL-6Rα/gp130), is actually a tetrameric receptor complex composed of two gp130 and two IL-6Rα subunits (15). Similar multimeric arrangements may exist in other homomeric and heteromeric cytokine receptor complexes.

Finally, analysis of many cytokine receptor complexes has led to the identification of another characteristic structural feature. Within the cytokine receptor superfamily, several subfamilies of receptors are recognized to share common receptor subunits (Figure 1B). For example, the interleukin-3 (IL-3), interleukin-5 (IL-5) and granulocyte/macrophage

colony stimulating factor (GM-CSF) receptors share a common β_c subunit (16). Each of these receptor complexes contains a unique receptor subunit (IL-3Rα, IL-5Rα and GM-CSFRα) that determines ligand-binding specificity. The β_c subunit is characterized by a long cytoplasmic tail and appears to be necessary for both high-affinity ligand binding and intracellular signal transduction. Moreover, these three receptor complexes have very similar biological functions, perhaps due to their use of a shared β_c chain. In a second receptor subfamily, the IL-6, IL-11, ciliary neurotrophic factor (CNTF), oncostatin M (OSM) and leukemia inhibitory factor (LIF) receptor complexes include different combinations of gp130 and LIFRβ, which may account for their functional redundancy (17,18). In another receptor subfamily, the common γ_c subunit is employed within receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 (19-25). The critical role of γ_c in receptor signaling has been revealed through γ_c mutations which lead to X-linked severe combined immunodeficiency (X-SCID) (26,27), although the precise molecular basis for these pathogenic effects remains unclear. In contrast to the other described receptor subfamilies, the shared subunit in this subfamily (γ_c) has a relative short cytoplasmic domain, while the partner subunit has a longer intracellular tail. Interestingly, these receptor complexes often regulate different signaling programs and distinct biological outcomes. For example, signaling through the IL-2R complex leads to the activation of the signal transducer and activator of transcription (STAT) factor (28), STAT-5 (29-34), while signaling through the IL-4R complex activates STAT-6 (35). Finally, an additional level of subunit sharing is also evident. The IL-4Rα subunit not only combines with γ_c to form an IL-4R complex, but also pairs with IL-13Rα to form the IL-13R complex (36). Moreover, the IL-2R and IL-15R complexes both employ the γ_c and IL-2Rβ subunits. Thus, formation of cytokine receptor complexes can involve the extensive sharing of receptor subunits, although the functional contributions of these individual receptor chains to signaling remains unresolved.

III. Signal Transduction Through the Cytokine Receptor Complex

Activation of signaling through many classes of receptors requires the ligand-mediated oligomerization of the receptor subunits (37,38). Following this event, cytokine receptor complexes employ a wide array of cellular intermediates to produce effective signaling programs (10,39). This section focuses on cytokine receptor subunit oligomerization and those signaling events that are relevant to cytokines that bind several members of the γ_c-containing receptor subfamily. In particular, the activation of proximal signaling intermediates and distal gene transcription events through the IL-2R complex has

been well examined. Additionally, signaling through the IL-4R and IL-7R complexes will be discussed.

A. Ligand-mediated Receptor Subunit Oligomerization: Activation of Cytokine Receptor Signal Transduction

Activation of receptor complex signaling through these signaling elements depends upon ligand-mediated oligomerization of the receptor subunits. In the erythropoietin receptor (EPOR) system, a point mutation that introduces a new cysteine residue into the EPOR extracellular domain results in constitutive, erythropoietin-independent signal activation, through the disulfide-linking of two EPOR subunits (40). Biophysical studies of the receptors for growth hormone (GH), prolactin (PRL) and erythropoietin (EPO) demonstrate sequential binding of ligand by two receptor subunits (41-44), suggesting that the ligand serves to stabilize the receptor subunit alignment within the receptor complex for signaling activation. Furthermore, studies of ligand-receptor interactions have defined particular residues of the receptor ligand-binding domain that interact with distinct ligand moieties (45-49). These results demonstrate that the ligand-receptor interfaces impose a specific spatial conformation on the bound ligand and receptor subunits. Moreover, the crystal structures of the GHR (50) and PRL receptor (51) with bound cytokine and the EPOR (52) with a mimetic peptide agonist further demonstrate the critical orientation of ligand with the receptor subunits. Presumably, these carefully orchestrated extracellular ligand-receptor interactions juxtapose the cytoplasmic subunit domains within the receptor complex in an appropriate manner for signaling activation.

B. Proximal Signaling Events: Activation of Cellular Signaling Intermediates

The ligand-mediated engagement of select receptor subunits leads to the assembly of those receptor subunits and a characteristic set of cytoplasmic proteins that initiate a cascade of post-translational modifications such as the tyrosine phosphorylation and oligomerization of downstream signaling molecules. The importance of the receptor-associated tyrosine kinase activity has been demonstrated by the abrogation of intracellular phosphorylation as well as proliferation by selective tyrosine kinase inhibitors (53,54). Although the cytokine receptor subunits do not contain the consensus sequence (Gly-X-Gly-X-X-Gly) of the ATP-binding motif characteristic of intrinsic tyrosine kinase domains (55), multiple cellular kinases appear to physically associate with specific portions of the various receptor subunits (9,10).

The phosphorylation and catalytic induction of the Janus kinases (JAKs) are among the earliest signaling events activated by the cytokine receptor complex (28). Activation of these kinases (JAK1, JAK2, JAK3 or Tyk2) by the receptor complex leads to the phosphorylation and multimerization of STAT factors which then translocate to the nucleus and modulate gene expression. This JAK-STAT pathway was first identified through the elegant isolation and reconstitution of somatic mutants defective in interferon-α (IFN-α) and IFN-γ signaling (56,57). Subsequent studies have demonstrated that this pathway is involved in signaling not only through all of the cytokine receptors (58), but also through other receptor types including the epidermal growth factor receptor (EGFR) (59,60) which contains an intrinsic tyrosine kinase domain and the seven-transmembrane angiotensin II receptor which associates with G-proteins (61). In the IL-2, IL-4 and IL-7 receptor complexes, JAK1 and JAK3 are activated upon ligand-binding (62-66). However, activation of these proximal kinases leads to the activation of different STAT factors by these receptors. While IL-2 and IL-7 both promote the activation of STAT-5 (29-34), IL-2 also directs the induction of STAT-3 in activated peripheral blood lymphocytes (32). Furthermore, IL-4 leads to the activation of STAT-6 (35), but not STAT-5. Although recent observations suggest that the STAT factors (39,67) and certain receptor tyrosine residues (10) may be targets for JAK kinase activity, the substrates for the JAKs and the transcriptional events regulated by the various STAT factors continue to be the focus of current JAK-STAT research.

Other protein tyrosine kinases such as the members of the src-like kinase family, including p56lck and p59fyn, have also been observed to associate with the IL-2Rβ subunit (68-70). These kinases and p53/56lyn are activated in various cellular contexts following IL-2 stimulation (70). Similarly, engagement of the IL-7R complex results in the activation of these same src-like kinases (71-73). However, the specific contribution of these src-like kinases to the various biological effects of IL-2 and IL-7 has not been well-established (10,74).

Several lines of evidence also suggest the involvement of the highly conserved Shc/ Grb2/mSOS1 pathway by many cytokine receptors (75,76). IL-2 promotes the tyrosine phosphorylation of the adaptor protein p52Shc (77,78) and its association with a specific subregion of IL-2Rβ (79). Additionally, IL-2 induces the expression and catalytic activity of the serine/threonine kinase Raf-1 (80,81). These activities lead to the formation of the GTP-bound form of p21ras (82). Moreover, a potential downstream target of Raf-1, the rapamycin-sensitive ribosomal S6 kinase (p70S6k), is activated by IL-2 and is likely involved in distal signaling events that lead to cellular proliferation (83-85). In contrast, the IL-4R may induce proliferation through a p21ras-independent pathway(s) as numerous studies have documented the lack of p21ras activation by IL-4 (86-88). Furthermore, no IL-4-

mediated activation of Shc or mSOS1 has been detected (89). However, recent reports of mitogen-activated protein kinase (MAPK) activation in certain cellular contexts suggest that this issue is not completely resolved (90).

Additional signaling pathways have also been associated with cytokine receptor function. The tyrosine kinase p72Syk associates with the IL-2R complex (91), although its precise role in signaling remains undefined. IL-2, IL-4 and IL-7 lead to the activation of phosphatidylinositol-3-kinase (PI_3K) (92-96). These receptor subunits do not appear to engage PI_3K directly, rather an intermediate adaptor protein may be involved (97). The insulin receptor substrate-1 (IRS-1) and the highly-related 4PS molecules are likely candidates. Each protein contains multiple binding sites for PI_3K and the phosphorylation of these adaptor molecules has been detected upon IL-4 stimulation (98). A homologous protein IRS-2 has been recently characterized and shown to associate with the IL-2R and IL-7R (99). Finally, tyrosine phosphatases may perform as important a role in cytokine receptor signaling as the tyrosine kinases (100). The tyrosine phosphatase, SH-PTP1, is involved in the regulation of EPOR (101) and prolactin receptor (PRLR)(102) signaling. However, the functional role of phosphatases in cytokine receptor complexes will require further examination.

C. Distal Signaling Events: Regulation of Gene Transcription

Cytokines activate many distal signaling events that are placed within the broad categories of proliferation and differentiation. While these effects result from the transcriptional regulation of numerous genes by these cytokines, the direct link between many of the proximal signaling events and these distal nuclear effects has yet to be established. IL-2 induces transcription of one of its own receptor subunits, IL-2Rα (103-107). Moreover, IL-2R-signaling leads to the induction of c-fos, c-myc and pim-1 (108-110). Interestingly, IL-2 directs transcription of members of the cyclin family and the cdc2 family which are closely linked to progression through the cell cycle (110). Additionally, IL-2R signaling regulates a delicate balance between the p21 and p27kip1 cell-cycle arrest proteins (111). The induction of bcl-2 by IL-2 may mediate related anti-apoptosis events (74,112,113). Recent studies have also demonstrated that IL-2 upregulates expression of various C-C chemokine receptors (114 and W. Xu, manuscript submitted), including CCR5, a co-receptor for the human immunodeficiency virus-1 (HIV-1). This may be one of the molecular causes of the transient increase in viral burden seen in HIV-infected patients receiving IL-2 therapy (115,116). Finally, distal signaling events mediated by the IL-4R and IL-7R are less well-defined, although CD23 (117) and c-myc induction by IL-4 have been reported (118).

IV. Structure/Function Relationships Within Cytokine Receptor Subunits

Mutational analyses of the cytokine receptor subunits have defined particular amino-acid motifs and structural elements necessary for specific signaling events. These domains may serve as docking sites for signaling molecules or may be critical for the structural integrity of the cytoplasmic domain. A number of critical regions have been defined within the cytoplasmic tail of IL-2Rβ (Figure 1A). A large C-terminal deletion of IL-2Rβ (140 aa) in IL-2RβΔBC resulted in a significant loss of growth signaling (119), similar to an "H" mutant reported by others (8). However, deletion of the C domain (94 aa) in IL-2RβΔC had little effect on proliferation. Together, these mutants indicate that the distal portion of IL-2Rβ may contain redundant signaling elements that are revealed only when they are all absent. Internal deletion of a more proximal domain (IL-2RβΔA) and a larger deletion of the A and B regions (119 aa distal to Box2) in IL-2RβΔAB did not affect growth signaling (119). This large internal deletion encompasses a previously described "acidic" region that is responsible for p56lck activation (68) and transcriptional induction of c-fos (109), indicating that these events are not critical for proliferation. While initial evaluation of these large domains focused primarily on proliferation, other signaling events related to cellular differentiation may require these domains (W. Xu, manuscript submitted).

Interestingly, these large distal domains of IL-2Rβ contain six individual tyrosine residues, and one or more of these residues is phosphorylated upon ligand binding (120-122). Specific amino-acid motifs surrounding the phosphotyrosine residues are recognized and bound by cellular signaling intermediates through distinct elements including the SH2 (src-homology domain 2) and PTB (phosphotyrosine-binding) domains (123,124). Within the platelet-derived growth factor receptor (PDGFR), these phosphotyrosine-containing motifs have been shown to serve as docking sites for specific signaling molecules that mediate defined portions of the signaling program (125,126). Studies of IL-2Rβ should lead to the assignment of some signaling molecules and events that have been associated with the large distal domains to these phosphotyrosine-containing elements.

Within the membrane-proximal portion of IL-2Rβ, focused mutational analysis has also revealed crucial signaling elements. Deletion of a serine-rich segment (IL-2RβSD), which contains the conserved Box2 domain, abolished growth signaling, activation of tyrosine kinase activity and the transcriptional induction of c-fos and c-myc (8,109,127). Moreover, mutational analysis of the Box1 and Box2 domains revealed a single non-conserved residue within each segment (Asp^{258} and Leu^{299}; Figure 1) that is critical for growth signaling (119). Interestingly, alanine scan mutations in the intervening V-Box also defined several critical elements, suggesting that this non-conserved segment also has an important function

in IL-2R signaling (128). As in other receptor subunits (129,130), one or more of these elements may be crucial for the association of a Janus kinase, specifically JAK1 in the case of IL-2Rβ. Alternatively, some of these elements may have other functional roles. Thus, critical domains for various signaling events are distributed along the entire length of the IL-2Rβ cytoplasmic tail.

Although less complete, mutational analysis of the IL-4Rα subunit also reveals a similar domain structure. Several regions distal to the conserved Box1 and Box2 domains are critical for proliferation (131,132) and the induction of c-myc (118). Additionally, an insulin receptor motif present in one of these domains is necessary for the association and phosphorylation of IRS-1 (133), which has been previously linked to PI_3K and cell growth (94). A single tyrosine residue within this insulin receptor motif is critical for this IRS-1-mediated signaling activity. Two distal tyrosine residues within IL-4Rα may serve as redundant docking sites for STAT-6 (35). More recent studies have distinguished the domains necessary for growth signaling from those that direct differentiation events, including expression of CD23, a class II MHC antigen ($I\text{-}A^d$) and the germline ε constant region (Iε) (134). Interestingly, transposing certain IL-4Rα domains to the IL-2Rβ subunit confers distinct IL-4R signaling events to this hybrid receptor chain (135). The modular nature of such receptor motifs was similarly demonstrated with the transposition of the STAT-3-binding motif from the gp130 subunit to the EPOR chain (136). Thus, these mutational studies demonstrate modularity within the receptor subunit through the association of specific signaling functions with distinct subregions of the receptor chain. Given the combinatorial use of defined amino acid motifs in receptor subunits and signaling molecules, we hypothesized that such modular design principles may also govern the assembly of the receptor complex. To investigate this possibility, we established a general method for the analysis of cytokine receptor subunit function.

V. A General Strategy for Analysis of the Molecular Basis of Signal Transduction Specificity Through Cytokine Receptors

With the increased understanding of the individual cytokines, the various receptor complexes and their roles within complex regulatory networks, several general principles that govern the overall regulation of cellular function and communication by cytokines have emerged. First, cytokines are **pleiotropic** given their ability to direct many diverse biological functions. Second, individual cytokine/receptor systems produce distinct and **specific** biological effects. Third, despite the specific nature of their overall signaling

program, various cytokines are **redundant** because they produce certain similar biological effects.

Characterization of receptor complex design should identify critical factors that contribute to these features of receptor signal transduction. The assembly of the receptor complex involves the juxtaposition of the cytoplasmic domains of the receptor subunits and the recruitment of a distinct set of signaling molecules to the receptor chains. These essential proximal steps result in the engagement of multiple signaling pathways leading to various downstream effects. Analysis of the structural features of the individual receptor subunits might reveal general principles that predict the functional contribution of those subunits to signaling by the receptor complex. These findings might also provide a rationale for the shared use of a common receptor subunit in multiple receptor complexes and lead to insights regarding the regulation of signal transduction activation by the receptor complex. Finally, understanding the relationship between receptor design and signaling function may lead to a more effective use of cytokines in clinical therapy.

Thus, the present studies were designed to achieve the following goals: 1) the development of a system to study the relationship between various elements within the receptor subunit and signaling function; 2) structure/function analysis of the elements within the receptor subunits that contribute to signaling specificity; and 3) elaboration of models relating the functional architecture of receptor complexes to the specific nature of the receptor-mediated signaling program.

The work in this dissertation focused upon the related IL-2, IL-4 and IL-7 cytokine receptor complexes. This receptor subfamily was ideal for these studies because each employs the γ_c subunit in addition to a unique receptor subunit (IL-2Rβ, IL-4Rα and IL-7Rα, respectively). This receptor arrangement facilitated the analysis of individual receptor subunit function and the general architecture of heteromeric receptor complexes.

References

1. T. Kishimoto, T. Taga, S. Akira, *Cell* **76**, 253-62 (1994).
2. C. Haworth, R. N. Maini, M. Feldmann, in *The Cytokine Handbook* A. Thomson, Eds. (Academic Press, Ltd, London, 1994) pp. 461-88.
3. J. F. Bazan, *Immunol Today* **11**, 350-4 (1990).
4. J. L. Boulay, W. E. Paul, *Current Biology* **3**, 573-81 (1993).
5. M. J. Brunda, *J. Leukocyte Bio.* **55**, 280-8 (1994).
6. J.-F. Bazan, *PNAS USA* **87**, 6934-8 (1990).
7. M. Murakami, et al., *PNAS USA* **88**, 11349-53 (1991).
8. M. Hatakeyama, H. Mori, T. Doi, T. Taniguchi, *Cell* **59**, 837-45 (1989).
9. A. F. Wilks, A. G. Harpur, *BioEssays* **16**, 313-20 (1994).
10. T. Taniguchi, *Science* **268**, 251-5 (1995).
11. M. A. Goldsmith, W. C. Greene, in *The Cytokine Handbook* A. Thomson, Eds. (Academic Press, Ltd, London, 1994) pp. 57-80.
12. Y. Nakamura, et al., *Nature* **369**, 330-3 (1994).
13. B. H. Nelson, J. D. Lord, P. D. Greenberg, *Nature* **369**, 333-6 (1994).
14. M. Mohammadi, J. Schlessinger, S. R. Hubbard, *Cell* **86**, 577-87 (1996).
15. G. Paonessa, et al., *EMBO J* **14**, 1942-51 (1995).
16. A. Miyajima, T. Hara, T. Kitamura, *TIBS* **17**, 378-82 (1992).
17. N. Y. Ip, et al., *Cell* **69**, 1121-32 (1992).
18. N. Stahl, G. D. Yancopoulos, *J. Neruobio.* **25**, 1454-66 (1994).
19. T. Takeshita, et al., *Science* **257**, 379-82 (1992).
20. M. Kondo, et al., *Science* **262**, 1874-7 (1993).
21. M. Noguchi, et al., *Science* **262**, 1877-80 (1993).
22. S. M. Russell, et al., *Science* **262**, 1880-3 (1993).
23. J. G. Giri, et al., *EMBO J* **13**, 2822-30 (1994).
24. M. Kondo, et al., *Science* **263**, 1453-4 (1994).
25. Y. Kimura, et al., *Intl Immunol* **7**, 115-20 (1995).
26. M. Noguchi, et al., *Cell* **73**, 147-57 (1993).
27. J. M. Puck, et al., *Human Mol Gen* **2**, 1099-104 (1993).
28. J. E. Darnell, I. M. Kerr, G. R. Stark, *Science* **264**, 1415-21 (1994).
29. S. L. Gaffen, et al., *PNAS USA* **92**, 7192-6 (1995).
30. H. Fujii, et al., *PNAS USA* **92**, 5482-6 (1995).
31. J. Hou, U. Schindler, W. J. Henzel, S. C. Wong, S. L. McKnight, *Immunity* **2**, 321-9 (1995).

32. J.-X. Lin, et al., *Immunity* **2**, 331-9 (1995).
33. K. C. Gilmour, R. Pine, N. C. Reich, *PNAS USA* **92**, 10772-6 (1995).
34. D. A. Frank, M. J. Robertson, A. Bonni, J. Ritz, M. E. Greenberg, *PNAS USA* **92**, 7779-83 (1995).
35. J. Hou, et al., *Science* **265**, 1701-6 (1994).
36. D. J. Hilton, et al., *PNAS USA* **93**, 497-501 (1996).
37. A. Ullrich, J. Schlessinger, *Cell* **61**, 203-12 (1990).
38. C.-H. Heldin, *Cell* **80**, 213-23 (1995).
39. J. N. Ihle, *Nature* **377**, 591-4 (1995).
40. S. S. Watowich, D. J. Hilton, H. F. Lodish, *MCB* **14**, 3535-49 (1994).
41. M. Ultsch, A. M. de Vos, A. A. Kossiakoff, *JMB* **222**, 865-8 (1991).
42. B. C. Cunningham, et al., *Science* **254**, 821-825 (1991).
43. G. Fuh, et al., *Science* **256**, 1677-80 (1992).
44. D. J. Matthews, R. S. Topping, R. T. Cass, L. B. Giebel, *PNAS USA* **93**, 9471-6 (1996).
45. B. C. Cunningham, J. A. Wells, *Science* **244**, 1081-5 (1989).
46. B. C. Cunningham, P. Jhurani, P. Ng, J. A. Wells, *Science* **243**, 1330-6 (1989).
47. S. H. Bass, M. G. Mulkerrin, J. A. Wells, *PNAS USA* **88**, 4498-502 (1991).
48. B. C. Cunningham, J. A. Wells, *PNAS USA* **88**, 3407-11 (1991).
49. J. A. Wells, et al., *Rec. Prog. in Hormone Res.* **48**, 253-275 (1993).
50. A. de Vos, M. Ultsch, A. A. Kossiakoff, *Science* **255**, 306-12 (1992).
51. W. Somers, M. Ultsch, A. M. de Vos, A. A. Kossiakoff, *Nature* **372**, 478-81 (1994).
52. O. Livnah, et al., *Science* **273**, 464-71 (1996).
53. I. Merida, G. N. Gaulton, *JBC* **265**, 5690-4 (1990).
54. H. Otani, et al., *PNAS USA* **89**, 2789-93 (1992).
55. S. K. Hanks, A. M. Quinn, T. Hunter, *Science* **241**, 42-52 (1988).
56. S. Pellegrini, J. John, M. Shearer, I. M. Kerr, G. R. Stark, *MCB* **9**, 4605-12 (1989).
57. L. Velazquez, M. Fellous, G. R. Stark, S. Pellegrini, *Cell* **70**, 313-22 (1992).
58. C. Schindler, J. E. Darnell, in *Ann Rev Biochem* **64**, 621-651 (1995).
59. K. Shual, et al., *Nature* **366**, 580-3 (1993).
60. D. W. Leaman, et al., *MCB* **16**, 369-75 (1996).
61. M. B. Marrero, et al., *Nature* **375**, 247-50 (1995).
62. V. A. Boussiotis, et al., *Science* **266**, 1039-42 (1994).
63. J. A. Johnston, et al., *Nature* **370**, 151-3 (1994).

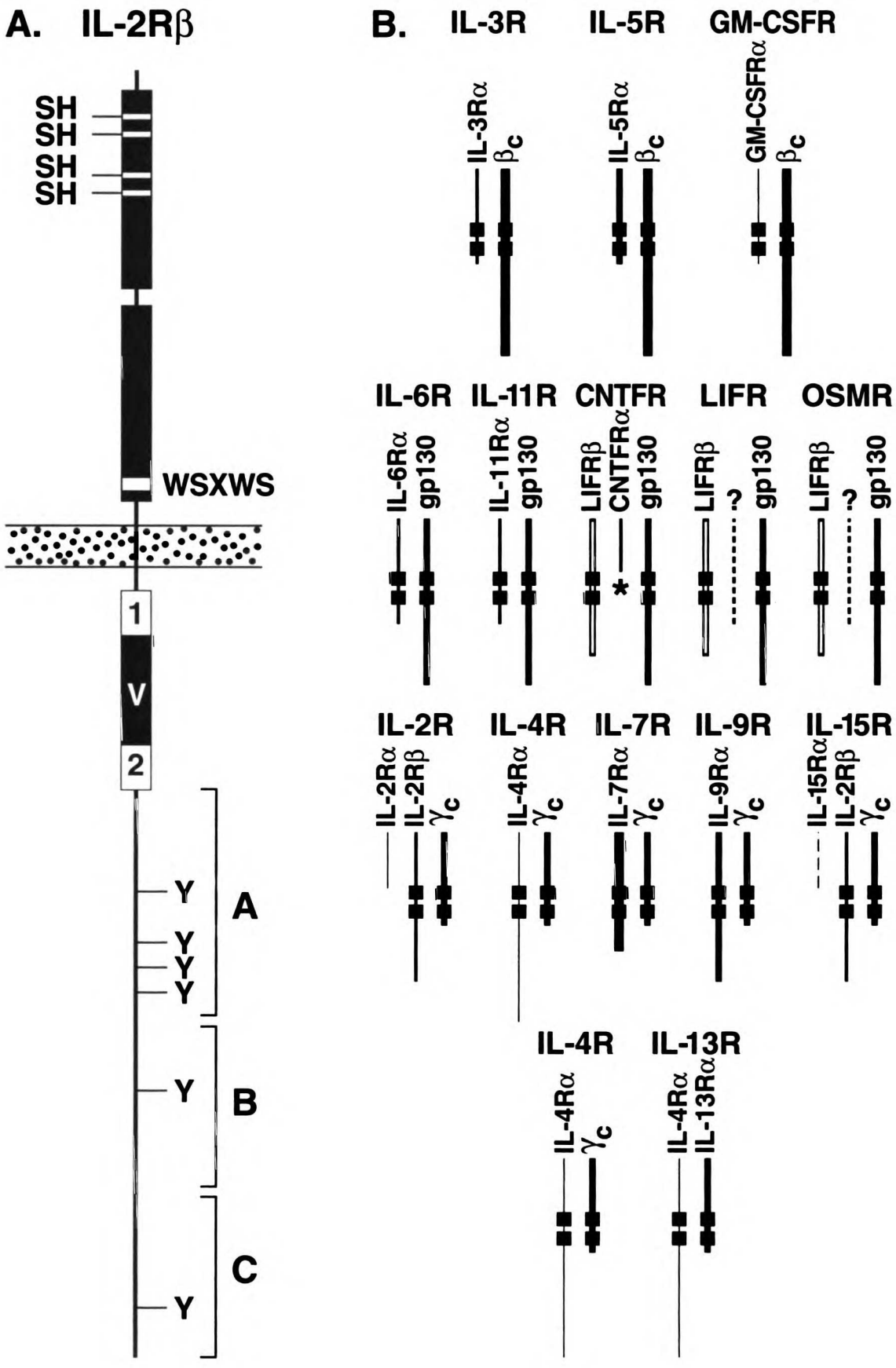
64. T. Miyazaki, et al., *Science* **266**, 1045-7 (1994).
65. S. M. Russell, et al., *Science* **266**, 1042-5 (1994).
66. B. A. Witthuhn, et al., *Nature* **370**, 153-7 (1994).
67. T. S. Migone, et al., *Science* **269**, 79-81 (1995).
68. M. Hatakeyama, et al., *Science* **252**, 1523-8 (1991).
69. Y. Minami, et al., *EMBO J* **12**, 759-68 (1993).
70. N. Kobayashi, et al., *PNAS USA* **90**, 4201-5 (1993).
71. A. R. Venkitaraman, R. J. Cowling, *PNAS USA* **89**, 12083-7 (1992).
72. P. Seckinger, M. Fougereau, *J Immunol* **153**, 97-109 (1994).
73. T. H. Page, F. V. Lali, B. M. Foxwell, *Euro J Immunol* **25**, 2956-60 (1995).
74. T. Miyazaki, et al., *Cell* **81**, 223-31 (1995).
75. J. Downward, J. Graves, D. Cantrell, *Imm Today* **13**, 89-92 (1992).
76. L. M. Karnitz, R. T. Abraham, *Curr. Op. Imm.* **7**, 320-6 (1995).
77. G. A. Evans, O. M. Z. Howard, R. Erwin, W. L. Farrar, *Biochem J* **294**, 339-42 (1993).
78. L. A. Burns, L. M. Karnitz, S. L. Sutor, R. T. Abraham, *JBC* **268**, 17659-61 (1993).
79. G. A. Evans, et al., *JBC* **270**, 28858-63 (1996).
80. A. Zmuidzinas, H. J. Mamon, T. M. Roberts, K. A. Smith, *MCB* **11**, 2794-803 (1991).
81. B. Turner, et al., *PNAS USA* **88**, 1227-31 (1991).
82. J. D. Graves, et al., *J Immunol* **148**, 2417-22 (1992).
83. C. J. Kuo, et al., *Nature* **358**, 70-3 (1992).
84. V. Calvo, C. M. Crews, T. A. Vik, B. E. Bierer, *PNAS USA* **89**, 7571-5 (1992).
85. N. Terada, et al., *Biochem Biophys Res Commun* **186**, 1315-21 (1992).
86. V. Duronio, M. J. Welham, S. Abraham, P. Dryden, J. W. Schrader, *PNAS USA* **89**, 1587-91 (1992).
87. T. Satoh, M. Nakafuku, A. Miyajima, Y. Kaziro, *PNAS USA* **88**, 3314-8 (1991).
88. M. J. Welham, V. Duronio, J. W. Schrader, *JBC* **269**, 5865-73 (1994).
89. M. J. Welham, V. Duronio, K. B. Leslie, D. Bowtell, J. W. Schrader, *JBC* **269**, 21165-76 (1994).
90. S. Wery, M. Letourneur, J. Bertoglio, J. Pierre, *JBC* **271**, 8529-32 (1996).
91. Y. Minami, et al., *Immunity* **2**, 89-100 (1995).
92. J. A. Augustine, S. L. Sutor, R. T. Abraham, *MCB* **11**, 4431-40 (1991).
93. I. Merida, E. Diez, G. N. Gaulton, *J Immunol* **147**, 2202-7 (1991).
94. L.-M. Wang, et al., *EMBO J* **11**, 4899-908 (1992).

95. H. K. Dadi, C. M. Roifman, *J Clin Invest* **92**, 1559-63 (1993).
96. K. Izuhara, N. Harada, *JBC* **268**, 13097-102 (1993).
97. M. G. J. Myers, et al., *JBC* **269**, 28783-9 (1994).
98. L.-M. Wang, et al., *Science* **261**, 1591-4 (1993).
99. J. A. Johnston, et al., *JBC* **270**, 28527-30 (1995).
100. N. K. Tonks, B. G. Neel, *Cell* **87**, 365-8 (1996).
101. U. Klingmuller, U. Lorenz, L. C. Cantley, B. G. Neel, H. F. Lodish, *Cell* **80**, 729-38 (1995).
102. S. Ali, et al., *EMBO J.* **15**, 135-42 (1996).
103. J. M. Depper, et al., *PNAS USA* **82**, 4230-4 (1985).
104. K. A. Smith, D. A. Cantrell, *PNAS USA* **82**, 864-8 (1985).
105. G. Le Gros, P. S. Shackell, G. J. Le, J. D. Watson, *J Immunol* **138**, 478-83 (1987).
106. J. H. Kehrl, et al., *JCI* **81**, 200-5 (1988).
107. J. J. Ruegemer, et al., *J Immunol* **144**, 1767-76 (1990).
108. F. Dautry, D. Weil, J. Yu, A. Dautry-Varsat, *JBC* **263**, 17615-20 (1988).
109. M. Hatakeyama, A. Kawahara, H. Mori, H. Shibuya, T. Taniguchi, *PNAS USA* **89**, 2022-6 (1992).
110. H. Shibuya, M. Yoneyama, T. J. Ninomiya, K. Matsumoto, T. Taniguchi, *Cell* **70**, 57-67 (1992).
111. J. Nourse, et al., *Nature* **372**, 570-3 (1995).
112. G. Deng, E. R. Podack, *PNAS USA* **90**, 2189-93 (1993).
113. H. Otani, M. Erdos, W. J. Leonard, *JBC* **268**, 22733-6 (1993).
114. P. Loetscher, M. Seitz, M. Baggiolini, B. Moser, *JEM* **184**, 569-77 (1996).
115. A. Kinter, A. S. Fauci, *Imm. Res.* **15**, 1-15 (1996).
116. M. C. Sneller, *Antiviral Res.* **29**, 105-9 (1996).
117. D. H. Conrad, et al., *J Immunol.* **139**, 2290-6 (1987).
118. A. Pernis, et al., *PNAS USA* **92**, 7971-5 (1995).
119. M. A. Goldsmith, W. Xu, M. C. Amaral, E. S. Kuczek, W. C. Greene, *JBC* **269**, 14698-704 (1994).
120. H. Asao, T. Takeshita, M. Nakamura, K. Nagata, K. Sugamura, *JEM* **171**, 637-44 (1990).
121. G. B. Mills, et al., *JBC* **265**, 3561-7 (1990).
122. M. A. Goldsmith, et al., *JBC* **270**, 21729-37 (1995).
123. Z. Songyang, et al., *Cell* **72**, 767-78 (1993).
124. W. M. Kavanaugh, C. W. Turck, L. T. Williams, *Science* **268**, 1177-9 (1995).

125. A. Kazlauskas, J. A. Cooper, *Cell* **58**, 1121-33 (1989).
126. M. Valius, A. Kazlauskas, *Cell* **73**, 321-34 (1993).
127. I. Merida, P. Williamson, W. A. Kuziel, W. C. Greene, G. N. Gaulton, *JBC* **268**, 6765-70 (1993).
128. K. D. Liu, S. Y. Lai, M. A. Goldsmith, W. C. Greene, *JBC* **270**, 22176-81 (1995).
129. B. A. Witthuhn, et al., *Cell* **74**, 227-36 (1993).
130. N. Stahl, et al., *Science* **263**, 92-5 (1994).
131. N. Harada, G. Yang, A. Miyajima, M. Howard, *JBC* **267**, 22753-8 (1992).
132. D. C. Seldin, P. Leder, *PNAS USA* **91**, 2140-4 (1994).
133. A. D. Keegan, et al., *Cell* **76**, 811-20 (1994).
134. J. J. Ryan, et al., *Immunity* **4**, 123-32 (1996).
135. H. Y. Wang, W. E. Paul, A. D. Keegan, *Immunity* **4**, 113-21 (1996).
136. N. Stahl, et al., *Science* **267**, 1349-53 (1995).

Figure Legends

Figure 1. Structural features of the cytokine receptor subunits and their classification within subfamilies of the cytokine receptor superfamily. (A) The characteristics of receptor subunits within the cytokine receptor superfamily are highlighted in the IL-2Rβ chain. These features include the conserved tetrad of extracellular cysteine residues (SH), the membrane-proximal WSXWS motif and the short, partially conserved proximal Box1 (1) and Box2 (2) elements. The cytoplasmic domain of the IL-2Rβ subunit is also composed of a variable region (V-Box; V) between Box1 and Box2 and six distal tyrosine residues present within 3 large domains referred to as A, B and C. (B) The shared use of certain receptor subunits defines several receptor subfamilies within the cytokine receptor superfamily. The β_c chain is shared by members of the IL-3/IL-5/GM-CSF receptor subfamily. Combinations of gp130 and LIFRβ are present in receptor complexes within the IL-6/IL-11/CNTF/LIF/OSM receptor subfamily. The '*' represents the glycosylphosphatidylinositol (GPI)-anchor for CNTFRα to the cell surface. Members of the IL-2/IL-4/IL-7/IL-9/IL-15 receptor subfamily share the γ_c chain. In some cases, receptor subunits are present within receptor complexes that are members of more than one receptor subfamily. For example, IL-4Rα is present in both the IL-4R complex (within the γ_c-containing subfamily) and the IL-13R complex (a γ_c-independent receptor). See text for further discussion.


A. IL-2Rβ
SH
SH
SH
SH
WSXWS
1
V
2
Y
Y
Y
Y
A
Y
B
Y
C
B. IL-3R
IL-3Rα
βc
IL-5R
IL-5Rα
βc
GM-CSFR
GM-CSFRα
βc
IL-6R
IL-6Rα
gp130
IL-11R
IL-11Rα
gp130
CNTFR
LIFRβ
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gp130
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LIFRβ
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gp130
OSMR
LIFRβ
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gp130
IL-2R
IL-2Rα
IL-2Rβ
γc
IL-4R
IL-4Rα
γc
IL-7R
IL-7Rα
γc
IL-9R
IL-9Rα
γc
IL-15R
IL-15Rα
IL-2Rβ
γc
IL-4R
IL-4Rα
γc
IL-13R
IL-4Rα
IL-13Rα

Chapter 2

Growth Signal Transduction by the Human Interleukin-2 Receptor Requires Cytoplasmic Tyrosines of the β Chain and Non-tyrosine Residues of the γ_c Chain

Prologue

The work described in this chapter focused on establishing an experimental system for structure/function analysis of cytokine receptor subunits. Efforts to create such a system began with the heteromeric IL-2 receptor complex, which requires IL-2Rβ and γ_c cytoplasmic domains for receptor signaling to occur. Early structural work on the IL-2R complex was directed at IL-2Rβ because of the availability of IL-2Rβ-negative cell lines (1,2). IL-2Rβ tyrosine reconstitution studies described in the present chapter employed this strategy. However, this approach was not possible for γ_c due to the lack of γ_c-negative lymphoid cell lines. Additionally, these mutational studies might be more informative if they were performed in the context of IL-2-responsive cells. Therefore, we established a chimeric receptor system in which the extracellular domain of a heterologous receptor subunit was fused to the cytoplasmic portions of the receptor chains of interest. These chimeric receptor complexes could be engaged selectively by a ligand that did not normally have any effect on the cell line being studied. Given the ability to engage particular receptor subunits, we have been able to dissect the architecture of multiple receptor complexes with this chimeric receptor system.

The following paper, reproduced from the journal in which it was published, describes studies of the functional role of the IL-2Rβ cytoplasmic tyrosine residues, the establishment of a chimeric receptor system for the examination of the IL-2R complex and the functional analysis of the γ_c subunit. My participation in these studies included intellectual contributions, preparation of some mutants and other necessary reagents and experimental data which are depicted in Figures 2, 5, 7, 8 and 9. W. Xu had an extensive role in the establishment of important cell lines and the generation of experimental data. L. J. Parent was responsible for the initial set of IL-2Rβ mutants, M. C. Amaral established and characterized several IL-2Rβ stable cell lines and E. S. Kuczek and G. B. Mills created a number of γ_c mutants. K. L. Tarr and G. D. Longmore provided reagents and technical analysis of the chimeric receptor system. M. A. Goldsmith made certain IL-2Rβ and γ_c mutants and worked jointly with W. C. Greene in the overall supervision of the project.

References

1 M.A. Goldsmith, et al. *JBC* **269**, 14698-14704 (1994).

2 M. Hatakeyama, et al. *Cell* **59**, 837-845 (1989).

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Growth Signal Transduction by the Human Interleukin-2 Receptor Requires Cytoplasmic Tyrosines of the β Chain and Non-tyrosine Residues of the γ_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 β and γ_c chains was prepared and analyzed. Concurrent mutation of all six of the cytoplasmic tyrosines present in the β 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 β 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 β 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 β chain likewise were important for growth signaling in T cells. In contrast, although the γ_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 γ_c abrogated receptor function. Therefore, tyrosine residues of β but not of γ_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-2Rβ and γ_c in receptor-mediated signal transduction.

Interleukin-2 (IL-2)[1] is a helical cytokine that induces the proliferation of T and B lymphocytes as well as the expression of a number of immune effector functions by binding to the heterotrimeric IL-2 receptor complex (IL-2R). The 70–75-kDa β (IL-2Rβ) and 64-kDa γ (γ_c) subunits of the IL-2R share structural homology with other members of a cytokine receptor superfamily (1) and together form a receptor complex that is competent to bind IL-2 with intermediate affinity and to transduce growth and differentiation signals (reviewed in Ref. 2). As in other receptor systems, evidence has accumulated indicating that signal transduction is initiated upon ligand-induced heterodimerization of the β and γ_c cytoplasmic tails (3, 4). Interestingly, IL-2Rβ is also employed in the receptor for IL-15 (5, 6), whereas γ_c participates in the formation of the receptors for IL-4 (7), IL-7 (8, 9), IL-9 (10), and IL-15 (6).

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 coimmunoprecipitated with the IL-2R (13–18). Recent evidence indicates that the Janus 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 β and γ 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-2Rβ and γ_c subunits. Our results demonstrate that tyrosines within the cyto-

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[g] Supported by the National Institutes of Health.

[1] The abbreviations used are: IL-2, interleukin-2; IL-2R, interleukin-2 receptor; EPO, erythropoietin; EPOR, erythropoietin receptor; PCR, polymerase chain reaction.

plasmic tail of IL-2Rβ are critical for full growth signaling in pro-B and T cells. In contrast, the tyrosine residues of the γ_c chain are dispensable for this function, revealing an important distinction between the IL-2Rβ and γ_c subunits. These findings, along with a delineation of essential membrane-proximal domains of γ_c, may have general implications for the functional design of cytokine receptors, particularly those employing the common γ_c 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 μM 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 (Geneticin, 1 mg/ml, Life Technologies, Inc.) and clones isolated by limiting dilution were screened by radioligand binding analysis with ^{125}I-IL-2 or ^{125}I-EPO (see below) or by Northern blot analysis to identify clones expressing the transfected receptor (see text and figure legends). HT-2EPOβγ was established by transfecting HT-2EPOβ cells with pEPOγneo 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 [^{3}H]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 [^{3}H]thymidine incorporation on the indicated days.

Plasmid Constructs—All receptor cDNAs were subcloned into the expression vectors pCMV4 (30), pCMV4Neo (28), or pCMV4Δ (a pCMV4 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 EPOR cDNA from pXMnEPOR (31) was inserted into the *KpnI/XbaI* sites of pCMV4Neo to yield pEPORneo, and the human IL-2Rβ cDNA from pIL2R30 (provided by T. Taniguchi) was inserted into the *Hin*dIII/*Bam*HI sites of pCMV4Neo to yield pβneo.

The tyrosine substitution mutants of IL-2Rβ and γ_c (tyrosine (TAC) to phenylalanine (TTC)) were prepared by a combination of oligonucleotide-directed mutagenesis in M13 bacteriophage and PCR-based methods. For constructs involving the γ_c 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 γ_c cDNAs as templates.

pEPOβneo, constructed by PCR using an *Nhe*I site at the fusion junction, encodes a chimeric receptor (see Fig. 6*A*) 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-S)-(G-K-D-IL-2Rβ) . . .). pEPOγneo, also constructed by PCR using the *Nhe*I site, encodes a receptor (see Fig. 6*A*) containing the extracellular domain of the EPOR fused to the human γ_c transmembrane and cytoplasmic segments (resulting sequence: . . . (EPOR-T-A-S)-(S-K-E-γ_c) . . .). Expression plasmids encoding the mutants described in the text were prepared by subcloning appropriate DNA fragments spanning the indicated mutations into the parental pEPOβneo and pEPOγneo plasmids.

Protein Expression and Phosphorylation Studies—COS-7 cells (ATCC) were transfected with the indicated plasmids (see text) using Lipofectamine (Life Technologies, Inc.) as per the manufacturer's instructions. For expression analysis of chimeric receptors, immunoblotting analyses were performed on cell lysates using an anti-EPOR N-terminal peptide antiserum and ^{125}I-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 nM) 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 μM 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-JAK1 antiserum (Upstate Biotechnology, Inc.) and protein A-Sepharose. Immunoblotting studies were performed with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Inc.) per the manufacturer's instructions followed by ECL (Amersham Corp.) signal development.

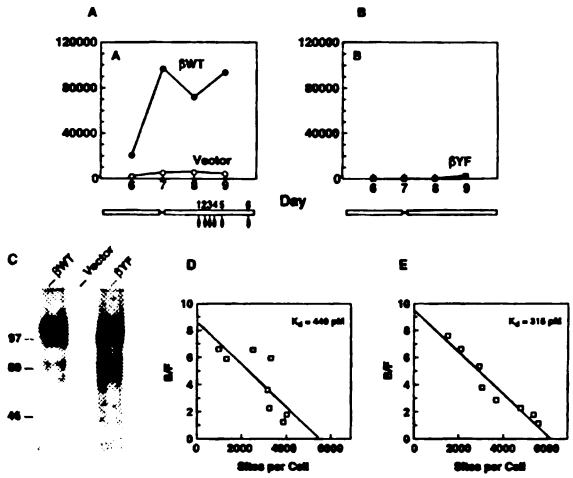

FIG. 1. **Growth signal transduction properties and expression of tyrosine-negative mutant IL-2β chains in transient assays.** *A*, proliferation of BA/F3 cells transfected with expression vector encoding wild type IL-2Rβ (*βWT, closed circles*) or empty vector (*Vector, open circles*), as measured by incorporation of [^{3}H]thymidine on the indicated days following initiation of IL-2 selection (10 nM) in the absence of IL-3. Each data point is the mean of triplicates, and each experiment shown is representative of several independent experiments. The relative positions of the six cytoplasmic tyrosine residues of IL-2Rβ are indicated by *closed symbols* in the schematic: *1*, Tyr-338; *2*, Tyr-355; *3*, Tyr-358; *4*, Tyr-361; *5*, Tyr-392; *6*, Tyr-510. *B*, proliferation of cells transfected with vector encoding tyrosine-negative mutant of IL-2Rβ (*βYF, closed squares*). *C*, surface expression of IL-2Rβ and βYF mutant receptors. Autoradiograph of immunoprecipitates of COS cells cotransfected with vectors encoding wild type human IL-2Rα and either IL-2Rβ (*βWT*) or the tyrosine-negative mutant (*βYF*) prepared following disuccinimidyl suberate-mediated cross-linking with ^{125}I-IL-2. Molecular mass markers are shown on *left* (kDa). D and E, equilibrium ^{125}I-IL-2 binding analysis of COS cells cotransfected with vectors encoding wild type human IL-2Rγ and either IL-2Rβ (*D*) or βYF (*E*).

Radioligand Binding and Cross-linking—Equilibrium binding analyses were performed as described (28, 33) with ^{125}I-IL-2 (DuPont NEN) and either BA/F3 cell lines or COS-7 cells transfected by the DEAE-dextran method (34) with human IL-2Rγ and the indicated IL-2Rβ expression plasmids. Cross-linking analyses were performed by cotransfecting COS-7 cells with IL-2Rα and IL-2Rβ expression plasmids followed by incubation with ^{125}I-IL-2, cross-linking with disuccinimidyl suberate (Pierce), immunoprecipitation with the anti-β monoclonal antibody DU-2 (14), and analysis by SDS-polyacrylamide gel electrophoresis and autoradiography, as described previously (35, 36).

RESULTS

Substitution Mutation of all Six Cytoplasmic Tyrosine Residues in IL-2Rβ Impairs Growth Signal Transduction in a Transient Assay System—The cytoplasmic tail of the human interleukin-2 receptor (IL-2R) β 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 (βYF) 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

(BA/F3) (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 (βWT) chains proliferated vigorously as indicated by substantial incorporation of [^{3}H]thymidine within 7 to 9 days, whereas cells receiving the vector control died in culture (Fig. 1*A*). 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. 1*B*). 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 ^{125}I-IL-2, chemical cross-linking with disuccinimidyl suberate, 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. 1*C*). To investigate potential changes in receptor affinity, radioligand binding analyses were performed with ^{125}I-IL-2 in COS cells transfected with IL-2Rγ and βWT or βYF. These studies revealed the expected single class of intermediate affinity IL-2 binding sites for both βWT and βYF (K_d 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 BA/F3 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 [^{3}H]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 K_d of IL-2 binding to IL-2Rβγ complexes, whereas the BafβYF line demonstrated no response even at very high doses of IL-2 (100 nM) vastly exceeding the measured K_d. 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 BA/F3 transient assay system. Surprisingly, substitution of phenylalanine at Tyr-338 (βY1F), Tyr-355/Tyr-358/Tyr-361 (βY234F), Tyr-392

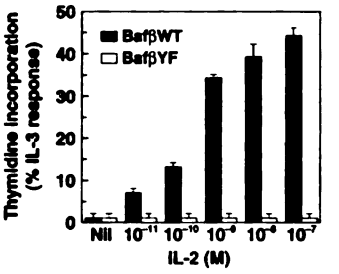

FIG. 2. **Growth signaling and expression characteristics of stable transfectants expressing tyrosine-negative IL-2Rβ chains.** BA/F3 cells expressing wild type IL-2Rβ (*BafβWT*) or tyrosine-negative mutant IL-2Rβ chains (*BafβYF*) were analyzed. Cells stimulated for 24 h with IL-2 at the indicated concentration (in the absence of IL-3) were pulsed with [^{3}H]thymidine for the final 4 h of the culture and harvested. Results are expressed relative to the level of incorporation occurring with IL-3 stimulation (100%); *error bars* represent standard errors of the mean (n = 3).

(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 BA/F3 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:56Y) 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 BA/F3 cells (Fig. 3). In contrast, a mutant with phenylalanines replacing exclusively these two distal tyrosines (βY56F) was substantially impaired in its growth signal transduction capacity in the BA/F3 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, β*ΔAB*), suggesting that the first five tyrosines are dispensable. In contrast, extension of this deletion to include the C-terminal region containing the sixth tyrosine (βΔABC) 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. 4*A*).

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

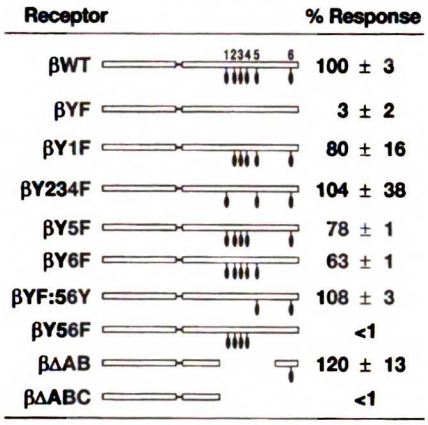

FIG. 3. **Peak proliferative responses of various tyrosine and deletion mutants of IL-2Rβ.** Transient proliferation assays were performed as in Fig. 1. Peak [^{3}H]thymidine incorporation is shown as a percentage of the peak response by βWT transfectants in each assay. Each value is the mean of triplicate determinations with standard errors of the mean, and results shown are representative of multiple independent experiments. *βWT*, wild type IL-2Rβ; *βYF*, tyrosine-negative IL-2Rβ; *βY1F*, β with Tyr-338 (1) mutated to Phe; *βY234F*, β with Tyr-355 (2), Tyr-358 (3), and Tyr-361 (4) mutated to Phe; *βY5F*, β with Tyr-392 (5) mutated to Phe; *βY6F*, β with Tyr-510 (6) mutated to Phe; *βYF:56Y*, β with Tyr-338 (1), Tyr-355 (2), Tyr-358 (3), and Tyr-361 (4) mutated to Phe; *βY56F*, β with Tyr-392 (5) and Tyr-510 (6) mutated to Phe; *βΔAB*, IL-2Rβ deleted from amino acid 313–431; *βΔABC*, IL-2Rβ truncated after amino acid 312.

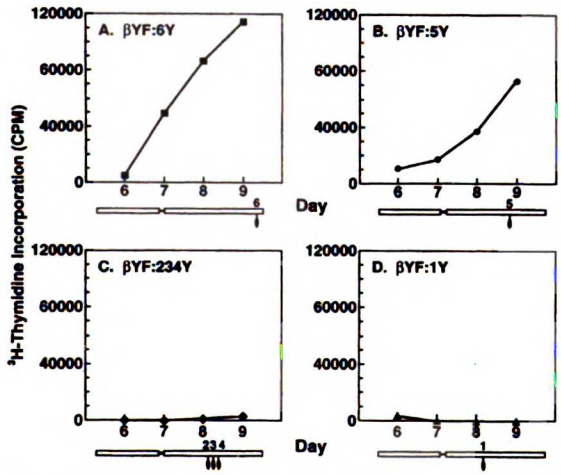

FIG. 4. **Proliferative responses of IL-2Rβ mutants with selective reconstitution of cytoplasmic tyrosines.** Transient proliferation assays were performed as in Fig. 1. *A*, *βYF:6Y*, βYF with Phe-510 (6) back-mutated to Tyr; *B*, *βYF:5Y*, βYF with Phe-392 (5) back-mutated to Tyr; *C*, *βYF:234Y*, βYF with Phe-355 (2), Tyr-358 (3), and Tyr-361 (4) back-mutated to Tyr; *D*, *βYF:1Y*, βYF with Phe-338 (1) back-mutated to Tyr. Constructs were prepared by recombination of cytoplasmic restriction fragments derived from mutants shown in Fig. 3 and were verified by DNA sequence analysis.

restored the IL-2Rβ signaling function (Fig. 4*B*). 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. 4, *C* and *D*, respectively). Importantly, the βYF:56Y, βY56F, βYF:1Y, βYF234Y, βYF:5Y, and βYF:6Y proteins were all expressed abundantly as detected by immunoblotting analysis (data not shown). Thus,

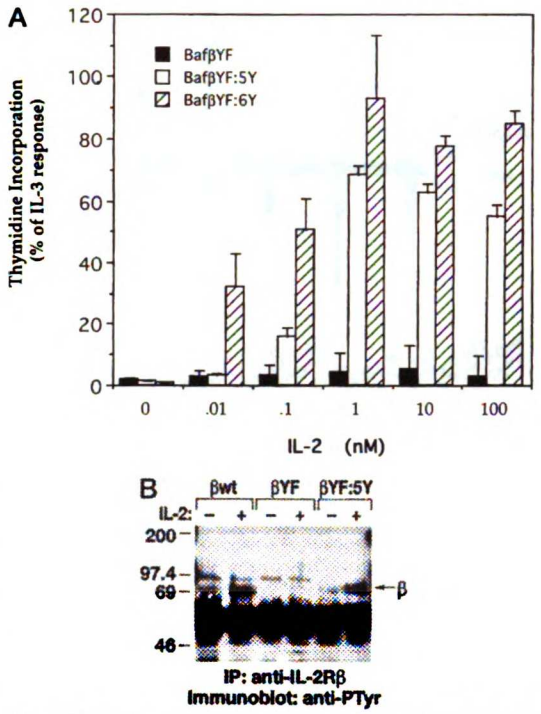

FIG. 5. **Signaling function and receptor phosphorylation in stable transfectants expressing IL-2Rβ tyrosine add-back mutants.** *A*, BA/F3 cells expressing tyrosine-negative IL-2Rβ (*BafβYF*) or IL-2Rβ tyrosine add-back mutants with restoration of Tyr-392 (*BafβYF:5Y*) or Tyr-510 (*BafβYF:6Y*) IL-2Rβ chains (*BafβYF*) were analyzed. *A*, [^{3}H]Thymidine incorporation assay with the indicated doses of IL-2, as described in the legend to Fig. 2. *B*, tyrosine phosphorylation analysis of BafβWT (*βwt*), BafβYF (*βYF*), and BafβYF:5Y (*βYF:5Y*). Anti-IL-2Rβ immunoprecipitates from cells stimulated with IL-2 for 10 min were subjected to immunoblotting with anti-phosphotyrosine antibody.

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 (pβYF:5YNeo and pβYF:6YNeo). Both of the resulting cell lines (BafβYF:5Y and BafβYF:6Y) proliferated vigorously in IL-2 despite the unresponsiveness of the BafβYF line (Fig. 5). These results confirmed in permanent BA/F3 cell lines the reconstitution of growth signaling function upon restoration of either Tyr-392 or Tyr-510.

Phosphorylation studies were next performed using these stable transfectants. In these experiments, cell lines were rested without growth factors and then exposed to IL-2. Stimulated cells were lysed, immunoprecipitated with anti-IL-2Rβ monoclonal antibody, and then subjected to immunoblot analysis with anti-phosphotyrosine antibody. Upon induction with IL-2 the BafβWT line yielded a strong phosphotyrosine signal at the appropriate molecular weight for IL-2Rβ chains, whereas the BafβYF line yielded no discernible signal (Fig. 5).

Like BafβWT, BafβYF:5Y cells also yielded a phosphotyrosine-containing protein band (Fig. 5*B*). 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 phosphate acceptor 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 Tyr-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 γ_c Receptor Subunits in T Cells—To permit study of the functional interactions of the IL-2Rβ and γ_c 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 γ_c) were fused to an extracellular ligand binding domain not present in the host cell lines (Fig. 6*A*). Extracellular domains of the homodimeric EPOR extracellular domain were employed for this purpose, since the EPOR, IL-2Rβ, and γ_c 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 γ_c cytoplasmic domains following ligand binding. Plasmids encoding the chimeric EPOβ and EPOγ receptors expressed proteins of the predicted masses as detected by immunoblot analysis of lysates from transfected COS-7 cells (Fig. 6*B*): 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 [^{3}H]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. 6*C*). 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 ^{125}I-EPO confirmed the expression and ligand binding competence of these chimeras in the HT-2EPOβ and HT-2EPOγ 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 γ_c 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-2EPOβ), addition of EPO without IL-2 produced marked proliferation and vigorous incorporation of

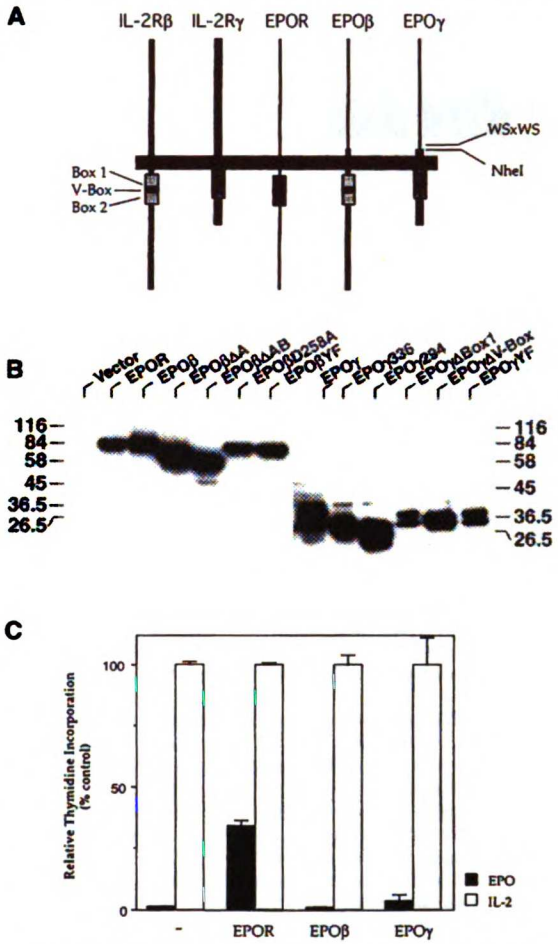

FIG. 6. **Chimeric EPOR/IL-2R receptor subunits.** *A*, schematic representation of native (*IL-2Rβ*, γ_c, and *EPOR*) and chimeric (EPOβ and EPOγ) subunits fused at a unique *Nhe*I site located immediately N-terminal to the transmembrane segment. Some conserved features of the cytokine receptor superfamily shown are indicated (the extracellular WSXWS sequence and the intracellular signaling domain, including Box 1, Box 2, and the intervening V-(variable) Box). *B*, immunoblot analysis of native and chimeric receptor subunits. The EPOR and EPOβ variants are shown on the *left*, and the EPOγ variants are shown on the *right*. The βΔA, βΔAB, and βD258A mutations have been described (28); in the βYF and γYF cytoplasmic tails all tyrosines (TAC) have been replaced with phenylalanines (TTC). See Fig. 9 legend for description of additional mutant γ_c cytoplasmic tails. *C*, parental HT-2 cells (−) or stable transfectants expressing EPOR, EPOβ, or EPOγ were stimulated with either EPO (*closed bars*, 50 units/ml) or IL-2 (*open bars*, 10 nM) for 24 h, and [^{3}H]thymidine incorporation was assessed as in Fig. 2. Results are expressed relative to the level of incorporation occurring with IL-2 stimulation (100%); *error bars* represent standard errors of the mean (n = 3).

[^{3}H]thymidine during the 12-day assay (Fig. 7*A*). Similarly, multiple HT-2 clones stably expressing EPOγ (HT-2EPOγ) displayed marked proliferative responses to EPO following introduction of the EPOβ expression plasmid (Fig. 7*B*). 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-2EPOβγ) for further studies of early signal transduction events. Thus, concurrent engagement of both the β and γ_c

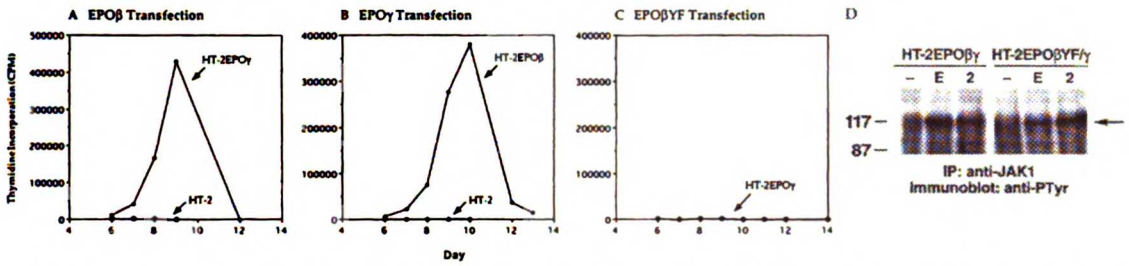

FIG. 7. **Functional analyses of EPOR/IL-2R chimeras.** *A*, parental HT-2 cells or HT-2EPOγ cells described in the legend to Fig. 6 were transfected with the EPOβ expression plasmid, selected in EPO (50 units/ml) without other cytokines, and assayed for growth by measuring [^{3}H]thymidine incorporation on the indicated days. *B*, parental HT-2 cells or HT-2EPOβ cells described in Fig. 6 were transfected with the EPOγ expression plasmid, selected in EPO, and assayed for growth. *C*, HT-2EPOγ cells were transfected with the EPOβYF expression plasmid, selected in EPO, and assayed for growth. Each experiment shown was performed multiple times with similar results. *D*, to assess phosphorylation of JAK1 during receptor activation, the stable transfectants HT-2EPOβγ and HT-2EPOβYF/γ were stimulated with no cytokine (−), EPO (*E*), or IL-2 (*2*) followed by immunoprecipitation with the anti-JAK1 antiserum and immunoblotting with the anti-phosphotyrosine antibody.

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 EPOR/IL-2Rβ chimera (EPOβYF) were selected in EPO and assessed for proliferation. Unlike the parental EPOβ (Fig. 7*A*), the tyrosine-negative EPOβYF exhibited no detectable growth response to EPO (Fig. 7*C*). Similarly, stable double transfectants of HT-2 expressing both EPOβYF and EPOγ demonstrated no proliferation 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 βYF mutant, Janus 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 an anti-phosphotyrosine antibody. Cells expressing chimeric γ_c chains and either wild type chimeric β chains (HT-2EPOβγ) or tyrosine-negative β chains (HT-2EPOβYF/γ) both exhibited strong induction of JAK1 phosphorylation in response to either ligand (Fig. 7*D*). Likewise, preserved induction of JAK3 phosphorylation by receptor complexes containing EPOβYF was observed in parallel experiments employing an anti-JAK3 antiserum (data not shown). Therefore, at least one early phase of receptor-mediated signaling by the βYF mutant is intact despite the failure to achieve full growth signaling.

Characterization of Cytoplasmic γ_c Mutant Function in T Lymphocytes—Development of the chimeric receptor system also permitted an examination in T cells of the functional contributions of tyrosine residues and other elements within the γ_c cytoplasmic tail. We therefore introduced EPOγ mutants into the HT-2EPOβ stable cell line for functional analysis in the transfection assay. Protein expression from the various mutant EPOγ chimeric constructs was first verified by immunoblot analysis of lysates from transiently transfected COS-7 cells (Fig. 6*B*). As predicted, the substitution mutant construct (EPOγYF, see below) produced protein comparable with that of the wild type EPOγ construct, and the deletion mutants (EPOγ336, EPOγ294, EPOγΔBox1, and EPOγΔV-Box) produced slightly faster migrating species.

Since tyrosine phosphorylation of the γ_c subunit upon ligand binding has been well described (26), we investigated the putative role of the tyrosine residues present in the γ_c 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 [^{3}H]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 γ_c 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 γ_c 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 γ_c 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 γ_c 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 γ_c chains (24–26). Although no tyrosine kinase domain is identifiable within the recognized ligand-binding subunits of the IL-2R, the Janus kinases JAK1 and JAK3 as well as the *src* family kinase $p56^{lck}$ and $p59^{fyn}$ 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-

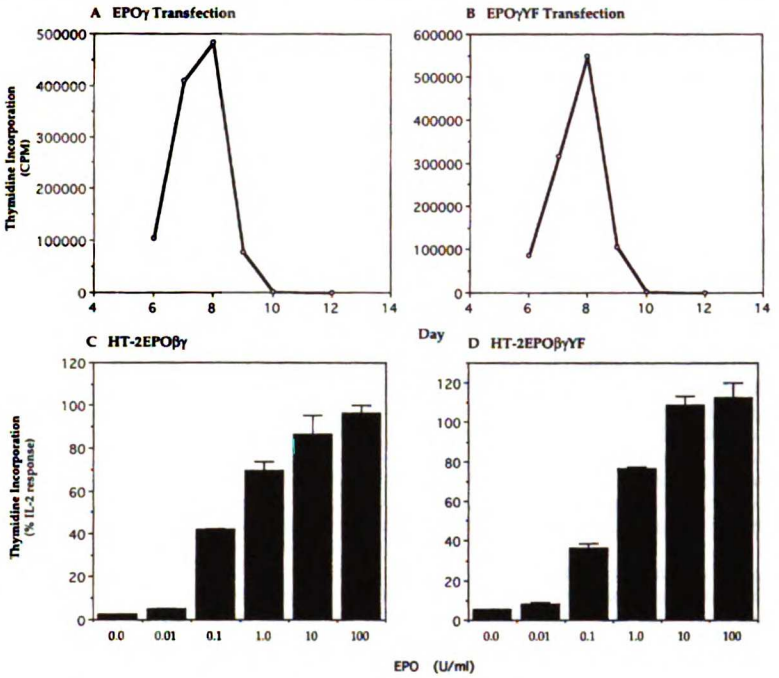

FIG. 8. **Preserved growth signal transduction function of tyrosine-negative γ_c chains.** *A* and *B*, transfection growth assays were performed by transfecting HT-2EPOβ cells with expression vectors encoding either EPOγ (*A*) or EPOγYF (*B*) and selecting in EPO, as in Fig. 7. *C* and *D*, stable HT-2 transfectants expressing EPOβ and either EPOγ (*C*) or EPOγYF (*D*) were analyzed by 24-h [^{3}H]thymidine incorporation assays, as in Fig. 6*C*. Shown is the dose response to EPO for each cell line relative to the response to IL-2, with *closed bars* representing the means and error bars indicating the standard errors of the mean (n = 3).

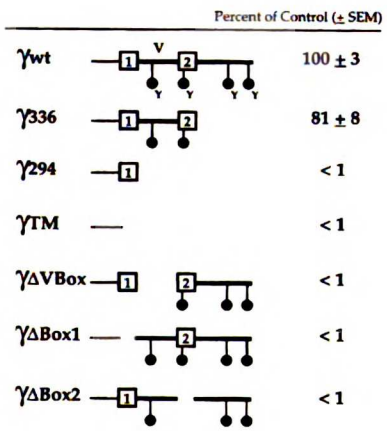

FIG. 9. **Functional analyses of EPOR/γ_c chimeras in transfection assays of proliferation.** Transfection growth assays using the HT-2EPOβ line as a host to assess the responses of the the indicated EPOγ mutants. The γ336, γ294, and γTM mutants are truncated immediately after amino acids 336, 294, and 286, respectively, in the mature γ_c protein. γΔBox 1 is deleted of residues 281–294, γΔV-Box is deleted of residues 295–320, and γΔBox 2 is deleted of residues 321–334. Results are expressed as the incorporation of [^{3}H]thymidine for each line relative to that of the wild type (*γwt*) cytoplasmic tail, with standard errors of the mean ($n \geq 3$).

tial regulatory role of cytoplasmic tyrosines of the IL-2Rβ and γ_c 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 p52^{shc} 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. 7*D*) 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 significance of this model for IL-2R function requires identification of the sites of IL-2-induced tyrosine phosphorylation of IL-2Rβ *in vivo*. The present studies demonstrated 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 γ_c cytoplasmic tail for growth signaling in T cells. In contrast to the IL-2Rβ chain, the γ_c 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 γ_c 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 γ_c 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 γ_c 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 γ_c tyrosine sites.

Although the tyrosines of γ_c proved to be dispensable for growth signaling by the IL-2R, a panel of truncation and internal deletion mutants revealed other elements within γ_c that are critical for growth signaling in the T cell line. Remarkably, the C-terminal 33 amino acids of γ_c 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 vestigial "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 vestigial 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 γ_c subunits expressed in heterologous cell types (57–59) and demonstrate clearly that the γ_c tail is needed for growth signal transduction by IL-2R heterodimers in T cells. Importantly, the impairment of these γ_c 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 γ_c 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 γ_c has been shown to be crucial for the assembly of the Janus kinase JAK3 with γ_c (10, 40). Perhaps the primary function of γ_c 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 γ_c 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* (Thomson, A., ed) pp. 57–80, Academic Press, Ltd., London
3. Nakamura, Y., Russell, S. M., Mess, S. A., Friedmann, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S., and Leonard, W. J. (1994) *Nature* **369**, 330–333
4. Nelson, B. H., Lord, J. D., and Greenberg, P. D. (1994) *Nature* **369**, 333–336
5. Grabstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., Beers, C., Richardson, J., Schoenborn, M. A., Ahdieh, M., Johnson, L., Alderson, M. R., Watson, J. D., Anderson, D. M., and Giri, J. G. (1994) *Science* **264**, 965–968
6. Giri, J. G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. (1994) *EMBO J.* **13**, 2822–2830
7. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K., and Sugamura, K. (1993) *Science* **262**, 1874–1877
8. Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S., and Sugamura, K. (1994) *Science* **263**, 1453–1454
9. Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X., and Leonard, W. J. (1993) *Science* **262**, 1877–1880
10. Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Bacon, C. M., Friedmann, M., Berg, M., McVicar, D. W., Witthuhn, B. A., Silvennoinen, O., Goldman, A. S., Schmalstieg, F. C., Ihle, J. N., O'Shea, J. J., and Leonard, W. J. (1994) *Science* **266**, 1042–1045

11. Merida, I., Diez, E., and Gaulton, G. N. (1991) *J. Immunol.* **147,** 2202–2207
12. Otani, H., Siegel, J. P., Erdos, M., Gnarra, J. R., Toledano, M. B., Sharon, M., Mostowski, H., Feinberg, M. B., Pierce, J. H., and Leonard, W. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2789–2793
13. Kobayashi, N., Kono, T., Hatakeyama, M., Minami, Y., Miyazaki, T., Perlmutter, R. M., and Taniguchi, T. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4201–4205
14. Fung, M. R., Scearce, R. M., Hoffman, J. A., Peffer, N. J., Hammes, S. R., Hosking, J. B., Schmandt, R., Kuziel, W. A., Haynes, B. F., Mills, G. B., and Greene, W. C. (1991) *J. Immunol.* **147,** 1253–1260
15. Hatakeyama, M., Kono, T., Kobayashi, N., Kawahara, A., Levin, S. D., Perlmutter, R. M., and Taniguchi, T. (1991) *Science* **252,** 1523–1528
16. Michiel, D. F., Garcia, G. G., Evans, G. A., and Farrar, W. L. (1991) *Cytokine* **3,** 428–438
17. Garcia, G. G., Evans, G. A., Michiel, D. F., and Farrar, W. L. (1992) *Biochem. J.* **285,** 851–856
18. Minami, Y., Kono, T., Yamada, K., Kobayashi, N., Kawahara, A., Perlmutter, R. M., and Taniguchi, T. (1993) *EMBO J.* **12,** 759–768
19. Tanaka, N., Asao, H., Ohbo, K., Ishii, N., Takeshita, T., Nakamura, M., Sasaki, H., and Sugamura, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7271–7275
20. Kawamura, M., McVicar, D. W., Johnston, J. A., Blake, T. B., Chen, Y., Lal, B. K., Lloyd, A. R., Kelvin, D. J., Staples, J. E., Ortaldo, J. R., and O'Shea, J. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6374–6378
21. Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T., and Ihle, J. N. (1994) *Nature* **370,** 153–157
22. Horak, I. D., Gress, R. E., Lucas, P. J., Horak, E. M., Waldmann, T. A., and Bolen, J. B. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1996–2000
23. Ullrich, A., and Schlessinger, J. (1990) *Cell* **61,** 203–212
24. Asao, H., Takeshita, T., Nakamura, M., Nagata, K., and Sugamura, K. (1990) *J. Exp. Med.* **171,** 637–644; Correction (1990) *J. Exp. Med.* **171,** 2183
25. Mills, G. B., May, C., McGill, M., Fung, M., Baker, M., Sutherland, R., and Greene, W. C. (1990) *J. Biol. Chem.* **265,** 3561–3567
26. Asao, H. (1992) *FEBS Lett.* **304,** 141–145
27. Palacios, R., and Steinmetz, M. (1985) *Cell* **41,** 727–734
28. Goldsmith, M. A., Xu, W., Amaral, M. C., Kuczek, E. S., and Greene, W. C. (1994) *J. Biol. Chem.* **269,** 14698–14704
29. Ganchi, P. A., Sun, S.-C., Greene, W. C., and Ballard, D. W. (1992) *Mol. Biol. Cell.* **3,** 1339–1352
30. Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* **264,** 8222–8229
31. Yoshimura, A., Longmore, G., and Lodish, H. F. (1990) *Nature* **348,** 647–649
32. Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M., and Sugamura, K. (1992a) *Science* **257,** 379–382
33. Robb, R. J., Greene, W. C., and Rusk, C. M. (1984) *J. Exp. Med.* **160,** 1126–1146
34. Aruffo, A. (1991) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 16.13.1–16.13.7, John Wiley & Sons, New York
35. Dukovich, M., Wano, Y., Bich-Thuy, L. T., Katz, P., Cullen, B. R., Kehrl, J. H., and Greene, W. C. (1987) *Nature* **327,** 518–522
36. Siegel, J. P., Sharon, M., Smith, P. L., and Leonard, W. J. (1987) *Science* **238,** 75–78
37. Hatakeyama, M., Tsudo, M., Minamoto, S., Kono, T., Doi, T., Miyata, T., Miyasaka, M., and Taniguchi, T. (1989a) *Science* **244,** 551–556
38. Hatakeyama, M., Mori, H., Doi, T., and Taniguchi, T. (1989b) *Cell* **59,** 837–845
39. Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T., and Kishimoto, T. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11349–11353
40. Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z.-J., Oishi, I., Silvennoinen, O., Witthuhn, B. A., Ihle, J. N., and Taniguchi, T. (1994) *Science* **266,** 1045–1047
41. Keegan, A. D., Nelms, K., White, M., Wang, L.-M., Pierce, J. H., and Paul, W. E. (1994) *Cell* **76,** 811–820
42. Cook, J. R., Jung, V., Schwartz, B., Wang, P., and Pestka, S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11317–11321
43. Farrar, M. A., Campbell, J. D., and Schreiber, R. D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11706–11710
44. Hou, J., Schindler, U., Henzel, W. J., Ho, T. C., Brasseur, M., and McKnight, S. L. (1994) *Science* **265,** 1701–1706
45. D'Andrea, A. D., Yoshimura, A., Youssoufian, H., Zon, L. I., Koo, J. W., and Lodish, H. F. (1991) *Mol. Cell. Biol.* **11,** 1980–1987
46. Kazlauskas, A., and Cooper, J. A. (1989) *Cell* **58,** 1121–1133
47. Valius, M., and Kazlauskas, A. (1993) *Cell* **73,** 321–334
48. Burns, L. A., Karnitz, L. M., Sutor, S. L., and Abraham, R. T. (1993) *J. Biol. Chem.* **268,** 17659–17661
49. Ravichandran, K. S., and Burakoff, S. J. (1994) *J. Biol. Chem.* **269,** 1599–1602
50. Remillard, B., Petrillo, R., Maslinski, W., Tsudo, M., Strom, T. B., Cantley, L., and Varticovski, L. (1991) *J. Biol. Chem.* **266,** 14167–14170
51. Truitt, K. E., Mills, G. B., Turck, C. W., and Imboden, J. B. (1994) *J. Biol. Chem.* **269,** 5937–5943
52. Gaffen, S. L., Lai, S. Y., Xu, W., Gouillex, F., Groner, B., Goldsmith, M. A., and Greene, W. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7192–7196
53. Lin, J.-X., Migone, T.-S., Tsang, M., Friedmann, M., Weatherbee, J. A., Zhou, L., Yamauchi, A., Bloom, E. T., Mietz, J., John, S., and Leonard, W. J. (1995) *Immunity* **2,** 331–339
54. Hou, J., Schindler, U., Henzel, W. J., Wong, S. C., and McKnight, S. L. (1995) *Immunity* **2,** 321–329
55. Darnell, J. E., Kerr, I. M., and Stark, G. R. (1994) *Science* **264,** 1415–1421
56. Seldin, D. C., and Leder, P. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2140–2144
57. Asao, H., Takeshita, T., Ishii, N., Kumaki, S., Nakamura, M., and Sugamura, K. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4127–4131
58. Ishii, N., Asao, H., Kimura, Y., Takeshita, T., Nakamura, M., Tsuchiya, S., Kono, T., Maeda, M., Uchiyama, T., and Sugamura, K. (1994) *J. Immunol.* **153,** 1310–1317
59. Kawahara, A., Minami, Y., and Taniguchi, T. (1994) *Mol. Cell. Biol.* **14,** 5433–5440
60. Noguchi, M., Yi, H., Rosenblatt, H. M., Filipovich, A. H., Adelstein, S., Modi, W. S., McBride, O. W., and Leonard, W. J. (1993) *Cell* **73,** 147–157

Chapter 3

The Molecular Role of the Common γ_c Subunit in Signal Transduction Reveals Functional Asymmetry Within Multimeric Cytokine Receptor Complexes

Prologue

To understand the structural arrangement of the IL-2 receptor, we examined the functional role of each receptor subunit within the receptor complex. These studies were facilitated by the ability to control subunit pairings in the chimeric receptor system. Furthermore, these experiments were performed concurrently with studies that identified a novel IL-2-directed signaling event and assigned specific signaling events to particular elements of the IL-2Rβ chain (Chapter 4). These studies demonstrated the separation of distinct functions between IL-2Rβ and γ_c within the IL-2R complex. Thus, these studies led to the conception of the "trigger-driver" model which explains the functional basis for the employment of different receptor subunits within heteromeric receptor complexes. This general model of cytokine receptor architecture presented here provides testable hypotheses that directed the studies found in two subsequent chapters (Chapters 5 and 6). The following paper, reproduced from the journal in which it was published, details the studies that provide the basis for the "trigger-driver" model.

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The molecular role of the common γ_c subunit in signal transduction reveals functional asymmetry within multimeric cytokine receptor complexes

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ABSTRACT **The specific signal transduction function of the γ_c subunit in the interleukin (IL) 2, IL-4, IL-7, IL-9, and IL-15 receptor complexes remains undefined. The present structure–function analyses demonstrated that the entire cytoplasmic tail of γ_c could be functionally replaced in the IL-2 receptor (IL-2R) signaling complex by a severely truncated erythropoietin receptor cytoplasmic domain lacking tyrosine residues. Heterodimerization of IL-2Rβ with either γ_c or the truncated erythropoietin receptor chain led to an array of specific signals normally derived from the native IL-2R despite the substitution of Janus kinase JAK2 for JAK3 in the receptor complex. These findings thus suggest a model in which the γ_c subunit serves as a common and generic "trigger" chain by providing a nonspecific Janus kinase for signaling program initiation, while signal specificity is determined by the unique "driver" subunit in each of the γ_c-containing receptor complexes. Furthermore, these results may have important functional implications for the asymmetric design of many cytokine receptor complexes and the evolutionary design of receptor subfamilies that share common trigger or driver subunits.**

An emerging paradigm in signal transduction by many classes of transmembrane receptors is the requirement for dimerization of receptor subunits for the generation of effective signaling (1, 2). Structural studies of the growth hormone receptor (3) and mutational studies of the erythropoietin receptor (EPOR) extracellular domain (4, 5) have provided strong evidence that these receptors assemble as homodimers to bind their ligands. Other receptor types exemplified by the interleukin 2 receptor (IL-2R) are activated by multimerization of two or more distinct receptor subunits. Indeed, heterodimerization of the IL-2Rβ and γ_c subunits, which are both members of the cytokine receptor superfamily (6, 7), is sufficient to activate the IL-2R signal transduction program leading to cellular proliferation (8, 9).

Recent studies have demonstrated the physical association of various cytoplasmic signaling intermediates with the IL-2Rβ and γ_c subunits, including the Janus kinases JAK1 and JAK3, respectively (10, 11). Ligand binding to a cytokine receptor complex typically results in the activation of such Janus kinases leading to phosphorylation and concomitant induction of STAT factors (signal transducers and activators of transcription) (12). Further studies have characterized several STAT-like DNA binding activities that are involved in the interleukin (IL) 2-signaling pathway (13, 14). STAT-5 induction in particular has been functionally linked to the IL-2Rβ subunit through a redundant tyrosine-containing motif found in several cytokine receptors (15–17).

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The specific function(s) of each receptor subunit within a dimeric receptor complex and the interplay between these subunits are not well understood. Furthermore, individual receptor subunits are frequently shared by multiple receptor complexes (18). For example, the γ_c subunit participates in distinct heterodimers of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptor complexes (11, 19–23), which appears to explain why many mutations of the γ_c gene (*IL2RG*) result clinically in the X chromosome-linked severe combined immunodeficiency (24, 25).

Initial structure–function studies have revealed distinct requirements for the cytoplasmic portions of the IL-2Rβ and γ_c subunits for growth signaling (15, 26), which implies that these chains may play different roles in the IL-2R complex. The γ_c-containing subfamily of receptor complexes thus serves as a useful model for investigating the hypothesis that the principle of modularity applies generally to the overall receptor design, with each subunit in a cytokine receptor heterodimer subsuming distinct specialized functions. The current studies were, therefore, undertaken to define the specific role of the γ_c subunit in the IL-2R complex.

MATERIALS AND METHODS

Cell Lines and Reagents. HT-2 cells (American Type Culture Collection) and stable transfectants were prepared and maintained as described (26). All receptor cDNAs were subcloned into the expression vectors pCMV4neo (27) or pXM (5). The anti-phosphotyrosine monoclonal antibody (4G10) and polyclonal anti-JAK1, anti-JAK2, and anti-JAK3, and anti-SHC [poly(IgG) control] rabbit antisera were obtained from Upstate Biotechnology (Lake Placid, NY). MOPC195 (IgG2b control) and chicken poly(IgG) were from Cappel Laboratories. Anti-CD25 (3C7) was from PharMingen. Fluorescein-conjugated goat anti-mouse antibody was obtained from Becton Dickinson. STAT-5 chicken antiserum was provided by B. Groner (28).

Biological Assays. Twenty-four-hour [^{3}H]thymidine incorporation assays were performed as described (26). Transfection growth assays were performed essentially as described (27) with erythropoeitin (EPO) (50 units/ml) selection in the absence of IL-2. For phosphorylation analysis 3×10^7 HT-2 cells were washed in calcium-and-magnesium-free phosphate-buffered saline (CMF-PBS), stripped of cell-bound ligand for 1 min in 10 mM sodium citrate, pH 4.0/140 mM NaCl, and

Abbreviations: IL, interleukin; IL-2R, IL-2 receptor; EPO, erythropoietin; EPOR, EPO receptor; STAT, signal transducer and activator of transcription; FcγR1, IgG Fc receptor; EMSA, electrophoretic mobility shift assay.

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rested for 4 h in RPMI 1640 medium containing 1% bovine serum albumin. After stimulation with IL-2 (10 nM) or EPO (50 units/ml), cells were washed in CMF-PBS and lysed [1% Nonidet P-40/20 mM Tris·HCl, pH 8.0/150 mM NaCl/50 mM NaF/100 mM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/leupeptin (10 mg/ml)/aprotinin (10 mg/ml)/pepstatin A (1 mg/ml)]. Immunoprecipitations were performed with the appropriate antibodies. Negative control immunoprecipitations were performed with nonimmune rabbit serum (data not shown). Immunoblot analysis was performed with the anti-phosphotyrosine antibody (4G10) by the manufacturer's instructions with detection by ECL (Amersham) signal development. For DNA binding studies, 2.5×10^7 HT-2 cells were rested and stimulated as described above. After cytokine stimulation, mini nuclear extracts were prepared and electrophoretic mobility shift assays (EMSAs) were performed as described by using the IgG Fc receptor ($F_c\gamma R1$) STAT response element (15). For surface phenotyping, HT-2 stable transfectants were maintained in HT-2 medium with EPO (5 units/ml) without IL-2 for at least 48 h. About 1×10^6 cells per sample were washed twice in PBS and incubated with anti-CD25 (3C7) for 1 h on ice. After washing twice with PBS/0.1% fetal calf serum, cells were incubated with the fluorescein-conjugated goat anti-mouse antibody and analyzed on a FACScan (Becton Dickinson).

RESULTS

Heterodimerization of IL-2Rβ and γ_c Is Required for Activation of the JAK–STAT Pathway. The IL-2R signaling complex was reconstituted in a chimeric receptor system containing the extracellular portion of the EPOR fused to the transmembrane and cytoplasmic domains of the IL-2Rβ and γ_c subunits (26). Stable transfectants expressing either the EPOβ or EPOγ subunits individually or together were established in HT-2 cells, an IL-2-dependent helper T-cell line. In 24-h [^{3}H]thymidine incorporation assays, neither of these chimeric receptor subunits alone mediated a detectable proliferative response (Fig. 1*A*). In contrast, HT-2 cells expressing both EPOβ and EPOγ subunits concurrently demonstrated a strong proliferation signal in response to EPO and were sustainable in long-term culture with EPO (Fig. 1*A*). Thus, as reported by others (8, 9), heterodimerization of the IL-2Rβ and γ_c subunits is necessary and sufficient for IL-2R-mediated growth signaling in T cells.

To determine the molecular mechanisms of signal transduction upon receptor subunit heterodimerization, we examined one of the earliest events in IL-2R activation, the phosphorylation of JAK1 and JAK3, Janus kinases that are physically associated with the IL-2Rβ and γ_c subunits, respectively (10, 11, 29–31). Anti-phosphotyrosine immunoblot analysis of JAK1 and JAK3 immunoprecipitates from HT-2 stable transfectant cell lines confirmed the phosphorylation of these kinases upon IL-2 stimulation through activation of endogenous IL-2R (Fig. 1*B*). In contrast, EPO stimulation of stable transfectants expressing either EPOβ or EPOγ alone induced no detectable response. However, EPO-induced tyrosine phosphorylation of both JAK1 and JAK3 was restored when EPOβ and EPOγ were expressed together in the same cell (Fig. 1*B*). Therefore, Janus kinase induction occurs concomitantly with ligand-induced heterodimerization of the IL-2Rβ and γ_c subunits.

We further evaluated the role of heterodimerization in downstream signaling events by examining the induction of STAT DNA-binding activity to a probe corresponding to the $F_c\gamma R1$ STAT response element in an EMSA. Stimulation of HT-2 cells with IL-2 induced a DNA-binding complex with the $F_c\gamma R1$ probe as observed (15), but no activity was detectable in EPO-stimulated cells expressing either EPOβ or EPOγ alone (Fig. 1*C*). A similar specific DNA-binding activity was

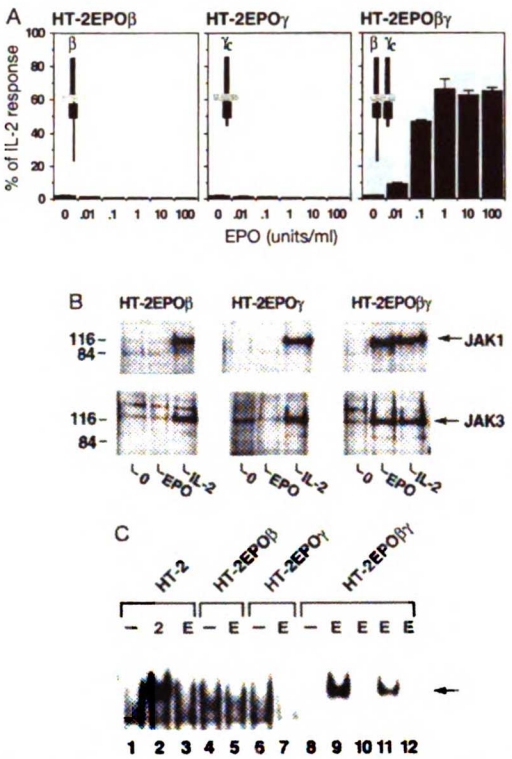

FIG. 1. Heterodimerization of IL-2Rβ and γ_c results in proliferation and activation of the JAK–STAT pathway. (*A*) Stable HT-2 transfectants were stimulated with EPO in [^{3}H]thymidine incorporation experiments. Results are expressed as a percentage of IL-2-induced incorporation for each respective line; error bars represent the SEM (n = 3). (*B*) Lysates from HT-2 stable cell lines rested (lanes 0) or stimulated with EPO (50 units/ml) or IL-2 (10 nM) were subjected to immunoprecipitation with anti-JAK1 (*Upper*) or anti-JAK3 (*Lower*) antisera. Immunoblot analysis was performed with an anti-phosphotyrosine antibody (4G10). The arrows highlight the bands representing JAK1 and JAK3, respectively. (*C*) EMSA of nuclear extracts from cell lines as described above that were unstimulated (lanes –) or treated with IL-2 (lane 2) or EPO (lanes E). The arrow indicates the band representing the IL-2-specific DNA-binding factor. Competition was performed with unlabeled $F_c\gamma R1$ probe (lane 10), 4G10 (lane 12), and the isotope-matched control antibody MOPC195 (lane 11).

induced by EPO in cells expressing both EPOβ and EPOγ (HT-2EPOβγ) cells with rapid induction kinetics similar to those of known STAT factors (Fig. 1*C* and ref. 15); this activity was abolished specifically by incubation with an anti-phosphotyrosine antibody (Fig. 1*C*) and was partially supershifted by an antibody to STAT-5 (Fig. 2*D*), but not reproducibly by control antibodies or antibodies to other known STAT factors (ref. 15 and data not shown). This chimeric receptor system thus appears to recapitulate the early and late IL-2R signal transduction program.

The γ_c Subunit Is Replaceable in the IL-2R Complex for Growth Signaling. Previous studies revealed that the γ_c function is largely independent of its C-terminal 35 amino acids and of tyrosine residues throughout its intracellular tail (15, 26). Given the minimal structural requirements for γ_c signaling competence, we hypothesized that the γ_c chain performs a general role in signaling activation, rather than a specific role in defining the signaling program of the IL-2R and other receptor complexes that utilize γ_c. To evaluate this hypothesis,

we explored the consequences of substituting another receptor subunit for γ_c in the IL-2R complex. Specifically, a severely truncated EPOR mutant, EPOR(1–321) was introduced as a replacement for the γ_c subunit. This EPOR(1–321) mutant contains only the 73-amino acid membrane-proximal cytoplasmic region that mediates association with JAK2 (32) but lacks the C-terminal 162 amino acids including all of the tyrosine residues that could bind STAT factors or other SH2-containing signaling intermediates. Expression of either EPOβ or the truncated EPOR(1–321) mutant alone in HT-2 cells was not sufficient for proliferation signaling in response to EPO as measured in 24-h [^{3}H]thymidine incorporation assays (Figs. 1*A* and 2*A*). In contrast, the hybrid EPOβ/EPOR(1–321) complex effectively supported full growth signaling and sustained long-term proliferation of these cells in response to EPO (Fig. 2*A*). Finally, expression of the EPOR(1–321) in an HT-2 cell line expressing EPOγ did not restore growth signaling (data not shown). Thus, like truncated γ_c subunits (ref. 26 and data not shown), the proximal portion of the EPOR cytoplasmic tail can functionally replace the γ_c chain for proliferation signaling in the EPOβ/EPOR(1–321) heterodimer.

JAK3 Is Functionally Replaceable by JAK2 in the IL-2R Complex. The intracellular signals derived from the EPOβ/EPOR(1–321) heterodimer were next analyzed by examining the pattern of Janus kinase activation. IL-2 stimulation of the native IL-2R complexes in the HT-2EPOβ/EPOR(1–321) cells resulted in the induction of JAK1 and JAK3 (Fig. 2*B*), as expected. However, EPO stimulation resulted in phosphorylation of JAK1 and JAK2, but not of JAK3; although the phosphorylation of JAK1 by this hybrid receptor appears to be somewhat less than that by the native IL-2R, quantitative comparisons are difficult in this system. These results are consistent with the fact that JAK1 physically associates with IL-2Rβ and JAK2 associates with EPOR. The absence of JAK3 phosphorylation is expected due to the exclusion of γ_c from the EPOβ/EPOR(1–321) heterodimers.

The significance of JAK2 activation in the receptor complex for growth signaling was emphasized further by comparing the signaling capabilities of various EPOR mutants paired with the EPOβ subunit. As with the stable EPOβ/EPOR(1–321) transfectant, expression of the truncated EPOR(1–321) mutant in the HT-2EPOβ cell line restored full growth signaling as measured in a transient transfection assay (Fig. 2*C*). However, expression of a more severely truncated EPOR chain [EPOR(1–256)] lacking the conserved domains responsible for JAK2 binding (4) was incapable of growth signaling when paired with EPOβ (Fig. 2*C*), supporting the requirement for concurrent activation of JAK2. In addition, expression of EPOR-W282R, an EPOR mutant that cannot activate JAK2 kinase function (32, 33), failed to reconstitute the proliferative signal in HT-2EPOβ cells (Fig. 2*C*). Therefore, growth signaling by EPOβ requires dimerization with a second receptor chain that is competent to convey a functional Janus kinase into the receptor complex.

As demonstrated, the stimulation of the EPOβ subunit alone did not activate any detectable STAT DNA-binding activity (Fig. 1*C*). Furthermore, no STAT factors were induced upon EPO stimulation of the EPOR(1–321) chain alone (Fig. 2*D*). In contrast, STAT-5 was strongly induced upon stimulation of both the EPOβ/EPOγ and the hybrid EPOβ/EPOR(1–321) receptor heterodimers. No other recognized STAT factors have been detected in these complexes by supershift analyses with available STAT-specific antibodies (data not shown). Thus, these experiments confirm that replacement of γ_c and JAK3 by the EPOR(1–321) and JAK2 results in a signaling program that faithfully recapitulates the events derived from the native IL-2Rβ–γ_c complex.

Induction of the IL-2Rα (CD25) Subunit Expression Requires the IL-2Rβ Subunit but Not γ_c. To determine whether

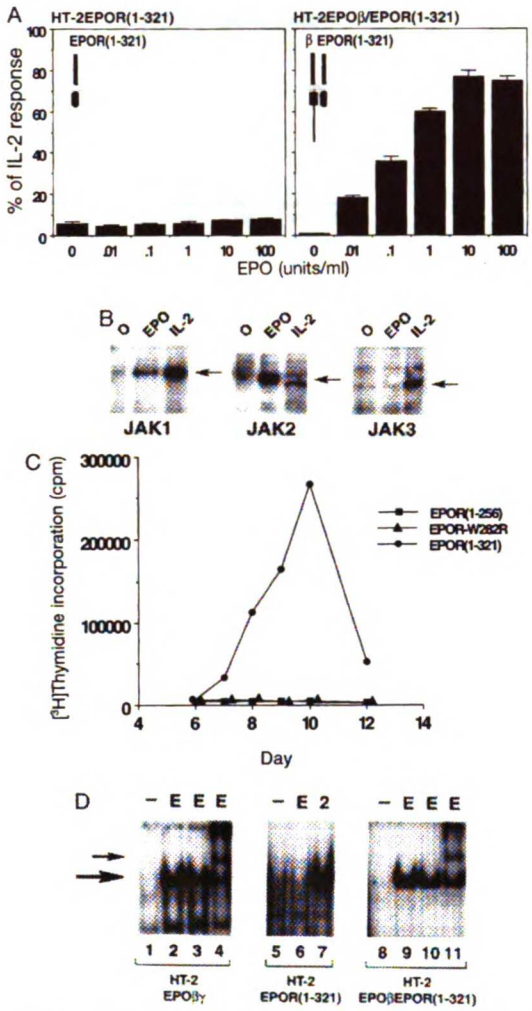

FIG. 2. Specificity of IL-2R-derived signals is driven by the IL-2Rβ subunit. (*A*) [^{3}H]Thymidine incorporation assays were performed as described in Fig. 1. (*B*) Lysates of unstimulated (lanes 0), IL-2-stimulated (IL-2), or EPO-stimulated (EPO) cells were subjected to serial immunoprecipitation for JAK1, JAK3, and JAK2. Immunoblot analysis was performed with the anti-phosphotyrosine antibody. Arrows highlight the bands representing JAK1, JAK2, and JAK3, respectively. (*C*) Transfection assay of HT-2EPOβ cells with the indicated expression plasmids. (*D*) EMSA of nuclear extracts from unstimulated (lanes −), EPO-stimulated (lanes E), or IL-2-stimulated (lane 2) cell lines. Large arrow indicates the IL-2-induced STAT-5 factor; small arrow indicates complex supershifted by STAT-5 antibody. Competition was performed with the STAT-5 antibody (lanes 4 and 11) and a poly(IgG) control (lanes 3 and 10).

other aspects of the signaling program are similarly linked to the IL-2Rβ subunit, an IL-2-inducible transcriptional event was examined in the context of the hybrid EPOβ/EPOR(1–321) receptor. Like other T cells (34), HT-2 cells up-regulate the transcription and subsequent cell surface expression of IL-2Rα (CD25) in response to ligand-mediated activation of the IL-2R (35). Accordingly, the HT-2EPO$\beta\gamma$ cell line demonstrated increased surface expression of IL-2Rα in response to EPO (Fig. 3) or IL-2 (data not shown). In contrast,

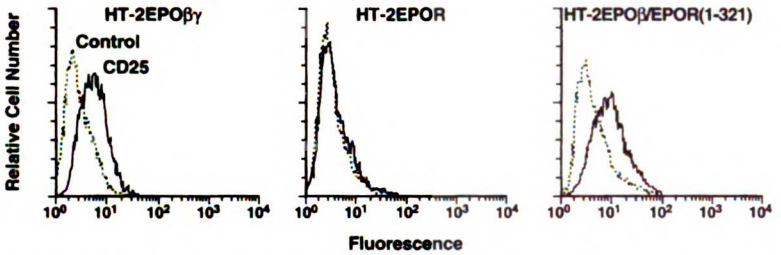

FIG. 3. Induction of IL-2Rα (CD25) depends upon IL-2Rβ. Cells were stained with the anti-CD25 monoclonal antibody 3C7 (solid line) or isotype-matched control antibody (broken line) and assayed by flow cytometry.

HT-2EPOR cells showed negligible expression of IL-2Rα in the presence of EPO (Fig. 3). Notably, the EPO-stimulated HT-2EPOβ/EPOR(1–321) cell line expressed the IL-2Rα subunit at levels similar to the HT-2EPOβγ cell line. These results further demonstrate that the signals from the hybrid EPOβ/EPOR(1–321) receptor are indistinguishable from the IL-2R-derived signals and specifically depend upon the IL-2Rβ subunit and its associated signaling molecules, rather than upon γ_c.

DISCUSSION

By using a chimeric receptor system, we have analyzed further the molecular mechanisms of heterodimeric receptor signaling. Surprisingly, a truncated EPOR mutant and its associated JAK2 can act in concert with the IL-2Rβ cytoplasmic subunit without altering the specificity of the resultant signaling events. Collectively, these results with the IL-2R as a model system provide insights into the specific functional architecture of γ_c-containing receptors and suggest certain design principles that may apply to other dimeric receptor systems.

(*i*) By all examined criteria, the cytoplasmic domain of the γ_c subunit appears to be replaceable by a severely truncated EPOR cytoplasmic tail. Although it remains possible that the γ_c chain itself regulates functions not measured in these studies (36), the γ_c subunit appears to be largely dispensable for a variety of IL-2-specific signals, including proliferation of a T-cell line, induction of a STAT factor, and transcriptional regulation of the IL-2Rα gene. The only important functional element of the γ_c subunit appears to be the JAK-binding domain, rather than the tyrosine residues or other C-terminal motifs. Therefore, it is possible that the γ_c chain serves primarily to transport a Janus kinase into the receptor complex for initiation of the signaling cascade.

(*ii*) The pairing of Janus kinases in a dimeric receptor appears to be governed by certain rules of compatibility. Specifically, while both the JAK1–JAK3 (IL-2Rβ–γ_c) and the JAK1–JAK2 pairs [EPOβ/EPOR(1–321)] effectively mediate signal transduction, homodimerization of either JAK1 (EPOβ) or JAK3 (EPOγ) in this setting is ineffective (Fig. 1*B*). Thus, Janus kinase pairings in a functional receptor complex are characterized by substantial, though incomplete, flexibility. Furthermore, Janus kinases within a receptor complex exhibit overlapping substrate specificities. For example, an IL-2R complex retains signal transduction competence and specificity despite activation of JAK2 rather than JAK3, although the small decrease in JAK1 phosphorylation by the hybrid receptor may reflect somewhat imperfect substitution of JAK3 by JAK2. The present direct demonstration is nonetheless consistent with prior circumstantial evidence of such kinase flexibility (15–17, 28). Thus, signal transduction specificity is determined largely by factors other than the specific Janus kinases.

(*iii*) The present findings clearly implicate the IL-2Rβ chain as the subunit responsible for specificity in many aspects of IL-2R signal transduction. Unlike the γ_c chain, the C-terminal region of the IL-2Rβ is critical for specification of the subsequent signaling program, presumably through the tyrosine-containing motifs. In the case of the JAK–STAT pathway, the different pairs of Janus kinases activated by either the EPOβ/EPOγ or the EPOβ/EPOR(1–321) receptor complexes result in the induction of the same STAT factor. This interaction provides a biochemical mechanism by which specificity is regulated through an individual receptor subunit, as suggested by recent studies identifying tyrosine-based motifs that determine STAT factor binding to a receptor (15, 17, 37).

Thus, receptor heterodimers appear to manifest a modular architecture. In the case of the IL-2R, the IL-2Rβ subunit functions as the "driver" subunit in the receptor complex by defining the exact nature of the signaling program through specific intracellular motifs, while the γ_c subunit may function predominantly as a relatively nonspecific "trigger" element in the initiation of signal transmission (Fig. 4). Although it remains to be determined whether γ_c contributes any signaling specificity whatsoever, it is tempting to speculate that other multimeric cytokine receptors employ a similar degree of subunit specialization. The structure–function features of the interferon γ receptor appear to follow such principles (38–40). Other members of the cytokine receptor superfamily with short cytoplasmic tails such as MPL (41), the interferon α receptor (42), or the low-affinity IL-12 receptor (43) may also serve as trigger subunits in their respective receptor complexes. In the case of homodimeric receptors, each subunit may function as a trigger chain for its partner subunit.

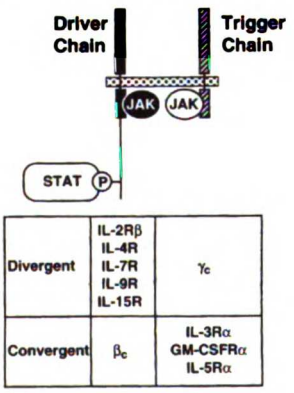

FIG. 4. Heterodimeric receptors regulate signal transduction specificity through two distinct evolutionary strategies. Members of "divergent" receptor subfamilies share a common "trigger" chain involved in signaling initiation, while members of "convergent" subfamilies share a common "driver" subunit that binds specific signaling intermediates. The figure depicts receptor heterodimerization leading to Janus kinase (JAK) dimerization and activation, receptor tyrosine phosphorylation, and STAT factor (STAT) association with a phosphotyrosine motif in the cytoplasmic tail of the "driver" subunit.

(*iv*) Finally, two basic variations are now recognizable in the evolutionary design of receptor subfamilies employing a shared subunit (Fig. 4). In a "convergent" type of architecture, multiple cytokines exploit the same signal transducing or driver receptor subunit and its associated signaling program. For example, members of the IL-3/IL-5/granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor subfamily utilize a common signal transduction subunit (i.e., β_c) and the members of this subfamily all regulate the induction of the same STAT activity (44, 45). A similar shared driver function may apply to the IL-4 and IL-13 receptors (data not shown). In a "divergent" arrangement now exemplified by the IL-2/IL-4/IL-7/IL-9/IL-15 receptor subfamily, the common chain (i.e., γ_c) provides a general trigger function, while the specialized driver receptor subunits activate distinct signaling events. Specifically, engagement of the receptors for IL-2, IL-4, and IL-9 results in activation of distinct profiles of STAT factors (refs. 15, 16, 46, and 47 and data not shown). Therefore, despite shared use of the γ_c chain, these receptor complexes exhibit distinct downstream signaling events, reflecting the presence of different driver subunits containing specific tyrosine-based motifs. Certain other receptors (e.g., the gp130/LIFRβ subfamily) may exhibit features of both the divergent and convergent strategies. Further studies may delineate additional receptor subfamilies that utilize a common trigger or driver subunit, and the use of structure–function polarity within such receptors to execute specific signaling programs.

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1. Heldin, C.-H. (1995) *Cell* **80,** 213–223.
2. Ullrich, A. & Schlessinger, J. (1990) *Cell* **61,** 203–212.
3. de Vos, A., Ultsch, M. & Kossiakoff, A. A. (1992) *Science* **255,** 306–312.
4. Watowich, S. S., Hilton, D. J. & Lodish, H. F. (1994) *Mol. Cell. Biol.* **14,** 3535–3549.
5. Yoshimura, A., Longmore, G. & Lodish, H. F. (1990) *Nature (London)* **348,** 647–649.
6. Bazan, J.-F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6934–6938.
7. Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T. & Kishimoto, T. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11349–11353.
8. Nakamura, Y., Russell, S. M., Mess, S. A., Friedmann, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S. & Leonard, W. J. (1994) *Nature (London)* **369,** 330–333.
9. Nelson, B. H., Lord, J. D. & Greenberg, P. D. (1994) *Nature (London)* **369,** 333–336.
10. Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z.-J., Oishi, I., Silvennoinen, O., Witthuhn, B. A., Ihle, J. N. & Taniguchi, T. (1994) *Science* **266,** 1045–1047.
11. Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Bacon, C. M., Friedmann, M., Berg, M., McVicar, D. W., Witthuhn, B. A., Silvennoinen, O., Goldman, A. S., Schmalstieg, F. C., Ihle, J. N., O'Shea, J. J. & Leonard, W. J. (1994) *Science* **266,** 1042–1045.
12. Darnell, J. E., Kerr, I. M. & Stark, G. R. (1994) *Science* **264,** 1415–1421.
13. Beadling, C., Guschin, D., Witthuhn, B. A., Ziemiecki, A., Ihle, J. N., Kerr, I. M. & Cantrell, D. A. (1994) *EMBO J.* **13,** 5605–5615.
14. Gilmour, K. C. & Reich, N. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6850–6854.
15. Gaffen, S. L., Lai, S. Y., Xu, W., Gouillex, F., Groner, B., Goldsmith, M. A. & Greene, W. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7192–7196.
16. Hou, J., Schindler, U., Henzel, W. J., Wong, S. C. & McKnight, S. L. (1995) *Immunity* **2,** 321–329.
17. Lin, J.-X., Migone, T.-S., Tsang, M., Friedmann, M., Weatherbee, J. A., Zhou, L., Yamauchi, A., Bloom, E. T., Mietz, J., John, S. & Leonard, W. J. (1995) *Immunity* **2,** 331–339.
18. Miyajima, A., Kitamura, T., Harada, N., Yokota, T. & Arai, K. (1992) *Annu. Rev. Immunol.* **10,** 295–331.
19. Giri, J. G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D. & Anderson, D. (1994) *EMBO J.* **13,** 2822–2830.
20. Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S. & Sugamura, K. (1994) *Science* **263,** 1453–1454.
21. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-I. & Sugamura, K. (1993) *Science* **262,** 1874–1877.
22. Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X. & Leonard, W. J. (1993) *Science* **262,** 1877–1880.
23. Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E. & Leonard, W. J. (1993) *Science* **262,** 1880–1883.
24. Noguchi, M., Yi, H., Rosenblatt, H. M., Filipovich, A. H., Adelstein, S., Modi, W. S., McBride, O. W. & Leonard, W. J. (1993) *Cell* **73,** 147–157.
25. Puck, J. M., Deschenes, S. M., Porter, J. C., Dutra, A. S., Brown, C. J., Willard, H. F. & Henthorn, P. S. (1993) *Hum. Mol. Genet.* **2,** 1099–1104.
26. Goldsmith, M. A., Lai, S. Y., Xu, W., Amaral, M. C., Kuczek, E. S., Parent, L. J., Mills, G. B., Tarr, K. L., Longmore, G. D. & Greene, W. C. (1995) *J. Biol. Chem.* **270,** 21729–21737.
27. Goldsmith, M. A., Xu, W., Amaral, M. C., Kuczek, E. S. & Greene, W. C. (1994) *J. Biol. Chem.* **269,** 14698–14704.
28. Wakao, H., Gouilleux, F. & Groner, B. (1994) *EMBO J.* **13,** 2182–2191.
29. Johnston, J. A., Kawamura, M., Kirken, R. A., Chen, Y., Blake, T. B., Shibuya, K., Ortaido, J. R., McVicar, D. W. & O'Shea, J. J. (1994) *Nature (London)* **370,** 151–153.
30. Tanaka, N., Asao, H., Ohbo, K., Ishii, N., Takeshita, T., Nakamura, M., Sasaki, H. & Sugamura, K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7271–7275.
31. Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T. & Ihle, J. N. (1994) *Nature (London)* **370,** 153–157.
32. Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O. & Ihle, J. N. (1993) *Cell* **74,** 227–236.
33. Miura, O., Cleveland, J. L. & Ihle, J. N. (1993) *Mol. Cell. Biol.* **13,** 1788–1795.
34. Goldsmith, M. A. & Greene, W. C., in *The Interleukin-2 Receptor*, eds. LeRoith, D. & Bondy, C. (JAI, Greenwich, CT), in press.
35. Ruegemer, J. J., Ho, S. N., Augustine, J. A., Schlager, J. W., Bell, M. P., McKean, D. J. & Abraham, R. T. (1990) *J. Immunol.* **144,** 1767–1776.
36. Asao, H., Takeshita, T., Ishii, N., Kumaki, S., Nakamura, M. & Sugamura, K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4127–4131.
37. Stahl, N., Farruggella, T. J., Boulton, T. G., Zhong, Z., Darnell, J. E., Jr., & Yancopoulos, G. D. (1995) *Science* **267,** 1349–1353.
38. Bach, E. A., Aguet, M. & Schreiber, R. D. (1995) *FASEB J.* **9,** A1021 (abstr.).
39. Farrar, M. A., Campbell, J. D. & Schreiber, R. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11706–11710.
40. Greenlund, A. C., Farrar, M. A., Viviano, B. L. & Schreiber, R. D. (1994) *EMBO J.* **13,** 1591–1600.
41. Vigon, I., Mornon, J.-P., Cocault, L., Mitjavila, M.-T., Tambourin, P., Gisselbrecht, S. & Souyri, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5640–5644.
42. Uze, G., Lutfalla, G. & Gresser, I. (1990) *Cell* **60,** 225–234.
43. Chua, A. O., Chizzonite, R., Desai, B. B., Truitt, T. P., Nunes, P., Minetti, L. J., Warrier, R. R., Presky, D. H., Levine, J. F., Gately, M. K. & Gubler, U. (1994) *J. Immunol.* **153,** 128–136.
44. Gouilleux, F., Pallard, C., Dusanter-Fourt, I., Wakao, H., Haldosen, L.-A., Norstedt, G., Levy, D. & Groner, B. (1995) *EMBO J.* **14,** 2005–2013.
45. Mui, A. L.-F., Wakao, H., O'Farrell, A.-M., Harada, N. & Miyajima, A. (1995) *EMBO J.* **14,** 1166–1175.
46. Hou, J., Schindler, U., Henzel, W. J., Ho, T. C., Brasseur, M. & McKnight, S. L. (1994) *Science* **265,** 1701–1706.
47. Yin, T., Tsang, M. L.-S. & Yang, Y.-C. (1994) *J. Biol. Chem.* **269,** 26614–26617.

Chapter 4

Signaling Through the Interleukin 2 Receptor β Chain Activates a STAT-5-like DNA-Binding Activity

Distinct Tyrosine Residues Within the Interleukin-2 Receptor β Chain Drive Signal Transduction Specificity, Redundancy, and Diversity

Prologue

With the establishment of the chimeric receptor system, our evaluation of the IL-2 receptor complex focused upon two related goals. First, evaluation of functional roles of the receptor subunits within the IL-2R complex led to the trigger-driver model (Chapter 3). This molecular dissection relied upon previous structure/function analysis of the receptor subunits themselves (Chapter 2) (1,2). Another goal of these studies was to identify other signaling molecules involved in IL-2R signaling processes and to delineate the subunit elements associated with these cytoplasmic intermediates. Moreover, mapping of specific signaling events to the IL-2Rβ subunit provides further confirmation of the "trigger-driver" model.

The following chapter includes two papers, reproduced from the journals in which they were published. During a very productive period in our laboratory, I was fortunate to collaborate with a postdoctoral fellow, Sarah Gaffen, on the studies described in chapters 3 and 4. While I led the studies described in chapter 3, Sarah was responsible for directing the work in this chapter. Many of the experiments performed for the studies in these chapters were performed by both of us for independent confirmation. The first paper in this chapter describes the identification of the STAT factor activated by the IL-2R complex. The second paper describes the assignment of specific proximal signaling events to particular tyrosine residues of the IL-2Rβ subunit.

References

1 M.A. Goldsmith, et al. *JBC* **269**, 14698-704 (1994) .

2 M. Hatakeyama, et al. *Cell* **59**, 837-845 (1989).

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Immunology

Signaling through the interleukin 2 receptor β chain activates a STAT-5-like DNA-binding activity

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ABSTRACT **To explore the possible involvement of STAT factors ("signal transducers and activators of transcription") in the interleukin 2 receptor (IL-2R) signaling cascade, murine HT-2 cells expressing chimeric receptors composed of the extracellular domain of the erythropoietin receptor fused to the cytoplasmic domains of the IL-2Rβ or -γ_c chains were prepared. Erythropoietin or IL-2 activation of these cells resulted in rapid nuclear expression of a DNA-binding activity that reacted with select STAT response elements. Based on reactivity with specific anti-STAT antibodies, this DNA-binding activity was identified as a murine homologue of STAT-5. Induction of nuclear expression of this STAT-5-like factor was blocked by the addition of herbimycin A, a tyrosine kinase inhibitor, but not by rapamycin, an immunophilin-binding antagonist of IL-2-induced proliferation. The IL-2Rβ chain appeared critical for IL-2-induced activation of STAT-5, since a mutant β chain lacking all cytoplasmic tyrosine residues was incapable of inducing this DNA binding. In contrast, a γ_c mutant lacking all of its cytoplasmic tyrosine residues proved fully competent for the induction of STAT-5. Physical binding of STAT-5 to functionally important tyrosine residues within IL-2Rβ was supported by the finding that phosphorylated, but not nonphosphorylated, peptides corresponding to sequences spanning Y392 and Y510 of the IL-2Rβ tail specifically inhibited STAT-5 DNA binding.**

The cytokine interleukin 2 (IL-2) plays a central role in the immune response, serving as a primary regulator of T-cell proliferation and differentiation as well as a mediator of NK- and B-cell development and differentiation (1, 2). The IL-2 receptor (IL-2R) is a multisubunit complex composed of three recognized proteins, α, β, and a common chain, γ_c, shared with the IL-4, IL-7, IL-9, and IL-15 receptors (3–6). The IL-2Rβ and -γ_c chains contain cytoplasmic domains of 385 and 86 amino acids, respectively, and are sufficient to transduce signals after IL-2-mediated dimerization (7–9). In particular, the cytoplasmic domain of the IL-2Rβ chain is associated with the Janus-family kinase JAK1, while γ_c is found with JAK3 (10, 11). Thus, a major early signaling event produced by IL-2 binding is tyrosine phosphorylation of several substrates, including the IL-2Rβ and -γ_c chains themselves as well as JAK1 and JAK3 (12–15).

Cytokine receptors and their associated JAKs have been shown to mediate tyrosine phosphorylation and concomitant activation of STAT factors ("signal transducers and activators of transcription") (reviewed in ref. 16), which are found in a latent, unphosphorylated state in the cytoplasm (17, 18). Upon receptor stimulation, they likely physically associate with the activated receptor tail through their SH2 domains (19, 20), become phosphorylated, dissociate from the receptor, and then dimerize via these SH2 domains (21). Dimerization is thought to allow nuclear import, where the STAT factors subsequently regulate transcription of target genes. At least four STAT response elements are the targets of a rapidly induced DNA-binding activity after IL-2R stimulation (22–26). Thus, to study the role of STAT factors in IL-2 signaling, we made use of an IL-2R signaling system in the IL-2-dependent, CD4+ murine T-cell line HT-2. In this system, chimeric receptors containing the extracellular domain of the erythropoietin (EPO) receptor fused to the transmembrane and cytoplasmic domains of the IL-2Rβ and -γ_c chains (EPOβ and EPOγ) are stably expressed in HT-2 cells. Heterodimerization of EPOβ/EPOγ results in proliferation and induced phosphorylation of the IL-2R and JAK1 and JAK3 (M.A.G., S.Y.L., W.X., and W.C.G., unpublished data). With this system, mutations in the β and γ_c receptors can be readily introduced into the cellular context of the IL-2-dependent HT-2 cells and their functional consequences assessed.

The present studies demonstrate that IL-2R ligation in HT-2 cells induces binding of a STAT-5-like factor to specific STAT response elements. STAT-5 induction is strictly dependent on the IL-2Rβ chain; however, its induction appears to be insufficient to trigger proliferation signaling.

MATERIALS AND METHODS

Cell Culture, Preparation of Cell Lines, and Cytokine Stimulations. HT-2 transfectant cell lines were prepared as will be reported elsewhere. HT-2 cells were cultured in RPMI 1640 medium, 10% fetal bovine serum (FBS) (GIBCO/BRL), 2 mM glutamine, 0.05 mM 2-mercaptoethanol, and 1 nM recombinant IL-2 (rIL-2) (Chiron). HT-2EPOβγ, HT-2EPOβ/γYF, HT-2EPOβΔA/γ, and HT-2EPOβΔAB/γ cells were grown in this medium with 5 units of EPO per ml (Amgen) in place of IL-2. HT-2EPOβYF/γ stable transfectants were obtained by selection in hygromycin B (Boehringer Mannheim) and clones were selected by limiting dilution. HeLa cells were grown in Dulbecco's modified Eagle medium with 10% FBS. For cytokine stimulations, cells were washed twice in phosphate-buffered saline (PBS), stripped by a 30-sec incubation in 10 mM sodium citrate and 140 mM NaCl, and incubated for 4 hr in serum- and growth factor-free RPMI 1640 medium containing 1% bovine serum albumin (fraction V, Sigma). Stimulations were for various periods of time (typically 15 min) with 10 ng of interferon γ per ml (IFN-γ, PharMingen), 10 nM IL-2, or 50 units of EPO per ml in serum- and growth factor-free media. Herbimycin A (HA; BRL) and rapamycin (Calbiochem) were added 4 hr prior to and maintained during cytokine stimulations.

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Abbreviations: IL, interleukin; R, receptor; EMSA, electrophoretic mobility shift assay; HA, herbimycin A; PRL, prolactin; STAT, signal transducer and activator of transcription; EPO, erythropoietin; IFN, interferon; JAK, Janus kinase; FBS, fetal bovine serum; r, recombinant.

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Nuclear Extracts. Ten to 50 million cells were stimulated as described above and washed in PBS. Nuclear extracts were prepared as described (27) in the presence of 1 mM sodium orthovanadate and the following protease inhibitors: antipain, 0.5 mg/ml; aprotinin, 0.5 mg/ml; bestatin, 0.75 mg/ml; leupeptin, 0.5 mg/ml; pepstatin A, 0.05 mg/ml; phosphoramidon, 1.4 mg/ml; and soybean trypsin inhibitor, 0.5 mg/ml (Sigma).

Electrophoretic Mobility Shift Assay (EMSA). Oligonucleotide probes were end-labeled with [γ-^{32}P]dATP (Amersham) and polynucleotide kinase (New England Biolabs), and binding assays were performed with 10^5 cpm of probe, 2 μg of poly[d(I-C)], and 10 μg of nuclear extract as described (28). Preincubations of nuclear extracts with different antibodies were performed in the absence of poly[d(I-C)] and binding buffer for 45 min on ice prior to initiation of the binding assay by addition of radiolabeled probe. Preincubations with peptides were performed for 45 min at 25°C with all binding reagents except probe. STAT-1 and STAT-5 C-terminal antibodies were obtained from Transduction Laboratories (Lexington, KY); STAT-2, -3, and -6 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); 4G10 and anti-Shc (poly-IgG control) were from Upstate Biotechnology (Lake Placid, NY); MOPC 195 (IgG2b control) and UPC10 (ascites control) were from Cappel; and phosphoserine and phosphothreonine antibodies were from Sigma. Phosphopeptides were synthesized by C. Turck (University of California, San Francisco) and solubilized in PBS at a final pH of 7–8.

Preparation of STAT-5 N-Terminal Antisera. Anti-STAT-5 was produced in chickens against a glutathione *S*-transferase (GST)–STAT-5 fusion protein containing an N-terminal fragment spanning residues 6–160 (29). Antibodies from yolk egg extracts were precipitated and purified with polyethylene glycol and resuspended in 10 mM phosphate buffer, pH 7.5/100 mM NaCl/0.01% sodium azide as described (30).

RESULTS

Identification of IL-2-Inducible DNA-Binding Complexes in HT-2EPO$\beta\gamma$ Cells. To identify an IL-2-inducible DNA-binding activity in wild-type HT-2 and HT-2EPO$\beta\gamma$ cells, cells maintained in IL-2 or EPO were cultured in the absence of these factors for 4 hr and then stimulated for 15 min with saturating quantities of IL-2 (10 nM) or EPO (50 units/ml). Nuclear extracts were prepared and EMSAs were performed with ^{32}P-radiolabeled oligonucleotides (19–30 bp in length) corresponding to STAT-responsive elements found in the promoters of FcγR1, IRF-1, Ly6E, and SIE (16, 22, 24, 25, 31, 32). These probes revealed a specific pattern of IL-2-inducible DNA-binding activity. Specific binding to the FcγR1 element was observed in wild-type HT-2 cells induced with IL-2 as well as in HT-2EPO$\beta\gamma$ cells induced with EPO (Fig. 1). This DNA binding activity was not detected in HT-2 cells expressing EPOβ or EPOγ alone (data not shown), and allowed to react with both the FcγR1 and the IRF-1 probes, since the addition of a 100-fold molar excess of either unlabeled oligonucleotide as a competitor blocked binding to either probe (lanes 5 and 6). In contrast, oligonucleotides containing two other STAT-responsive elements, Ly6E and SIE, previously shown to bind IL-2-induced nuclear factors in other cell types (22, 25), did not significantly compete for binding (lanes 7 and 8). An additional, faster migrating DNA-binding complex was variably observed that was not reproducibly competitively inhibited by any of the unlabeled competitor oligonucleotides (Fig. 1 and data not shown) and thus appeared to reflect an extract-dependent, nonspecific protein–DNA interaction.

Characterization of IL-2-Binding Activity. To assess the role of tyrosine phosphorylation in the IL-2 induction of the observed DNA-binding activity, HT-2EPO$\beta\gamma$ cells were stimulated with EPO in the presence of a protein tyrosine kinase inhibitor, HA, which was added 4 hr prior to EPO stimulation

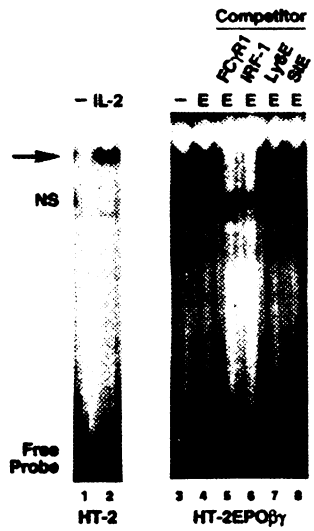

FIG. 1. IL-2-induced DNA-binding activity is selective for specific STAT response elements. HT-2EPO$\beta\gamma$ cells were incubated in medium alone and stimulated for 15 min with 10 nM IL-2 (lane 2) or 50 units of EPO (E) per ml (lane 4), and nuclear extracts were prepared. The specific probe employed was ^{32}P-end-labeled FcγRI. Binding assays and EMSAs were performed in the presence or absence of 50 ng of unlabeled competitor oligonucleotides (lanes 5–8). Sequences of oligonucleotides: FcγRI, GTATTTCCCAGAAAAGGAC; IRF-1, GCCTGATTTCCCCGAAATGACGG; Ly6E, GATCATATTCCTGTAAGTG; SIE, GTGCATGGTTCAGCCATCTTGTCTACAATTC. Arrow, inducible band; NS, nonspecific band.

(Fig. 2*A*). The presence of HA completely blocked the induction of this DNA-binding activity (lane 2), whereas equivalent concentrations of dimethyl sulfoxide used to dissolve the HA had no effect on its induction (lane 2). In contrast to HA, treatment of HT-2EPO$\beta\gamma$ cells with the IL-2 signaling antagonist rapamycin, at a concentration known to inhibit T-cell proliferation (refs. 33 and 34 and data not shown), did not impair the activation of this DNA-binding activity (lane 4).

To examine further the requirements for tyrosine phosphorylation, nuclear extracts were incubated with monoclonal antibodies specific for phosphotyrosine for 45 min prior to EMSA. As shown in Fig. 2*B* (lane 4), the addition of 4G10 anti-phosphotyrosine antibody almost completely abrogated DNA-binding activity. In contrast, addition of anti-phosphoserine or anti-phosphothreonine antibodies had no effect on DNA binding (lanes 6 and 7). The critical involvement of phosphotyrosine residues in DNA-binding competence is another characteristic feature of STAT factors.

The kinetics of induction of this DNA-binding activity were next examined. Maximal activity was observed within 15 min of EPO stimulation of the HT-2EPO$\beta\gamma$ cells, while a 70% decline in total binding occurred over the ensuing 4 hr (Fig. 2*C*, lanes 2–5). These activation kinetics are similar to those observed with many other STAT factors, in which binding to the target promoter sequences occurs quickly and decays over time (31, 35).

Identification of the IL-2-Induced DNA-Binding Activity as a Murine STAT-5 Homologue. Studies were next performed to assess whether the detected IL-2-induced DNA-binding activity could be attributed to any of the six, currently identified members of the STAT family. It has been previously demonstrated that HT-2 cells do not express STAT-4 (36). To screen the remainder of the STAT proteins, nuclear extracts from EPO-stimulated HT-2EPO$\beta\gamma$ cells were incubated with

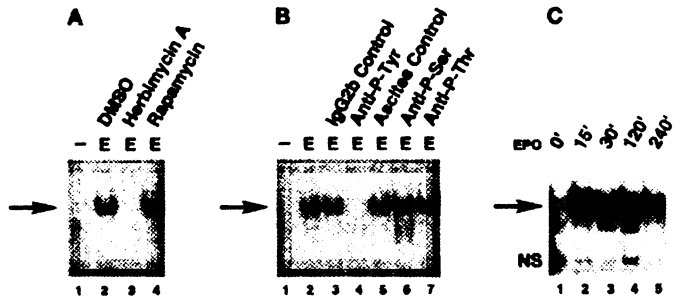

FIG. 2. IL-2 receptor-induced DNA-binding activity shares characteristics of a STAT factor. (*A*) HT-2EPOβγ cells were stimulated with EPO (E) and nuclear extracts were prepared as detailed in the legend to Fig. 1 after a prior 4-hr incubation in 0.5% dimethyl sulfoxide (DMSO), HA (0.5 μg/ml), or rapamycin (2 ng/ml). HA and rapamycin were dissolved in DMSO to a final concentration of 0.5%. (*B*) HT-2EPOβγ cells were stimulated with EPO and nuclear extracts were prepared (see legend to Fig. 1). Nuclear extracts were incubated with 3 μg of MOPC 195 IgG2b control, 3 μg of 4G10 anti-phosphotyrosine, 5 μg of UPC10 ascites control, 5 μg of anti-phosphoserine, or 5 μg anti-phosphothreonine antibodies for 45 min at 4°C prior to EMSA. (*C*) Time course of IL-2R-induced STAT expression. HT-2EPOβγ cells were stimulated with EPO for the indicated times and nuclear extracts were prepared (see legend to Fig. 1). Arrows and NS are as in Fig. 1.

STAT-specific antibodies prior to EMSA. Fig. 3 shows that the EPO-induced complex was neither competitively inhibited nor supershifted by preincubation with antibodies to STAT-1, -2, -3, and -6 (lanes 3–8) or ISGF3γ (p48) (data not shown). However, antibodies specific for an N-terminal fragment of sheep STAT-5 resulted in a new DNA protein complex with a slower mobility in IL-2-induced HT-2EPOβγ cells (lanes 12 and 13) but had no effect on IFNγ-induced STAT-1 in HeLa cells (data not shown). Notably, the original DNA-binding complex was not completely shifted by addition of anti-STAT-5 antibodies, suggesting that there may be a second protein involved in the complex that is antigenically distinct from the known STAT factors. Interestingly, antibodies raised against a C-terminal protein fragment of sheep STAT-5 had no effect on the DNA-binding complex (lane 14). This finding raises the possibility that this factor may be a distinct protein that shares STAT-5 homology at its N terminus. Thus, the DNA-binding activity induced in HT-2EPOβγ cells appears to correspond to a murine STAT-5-like protein.

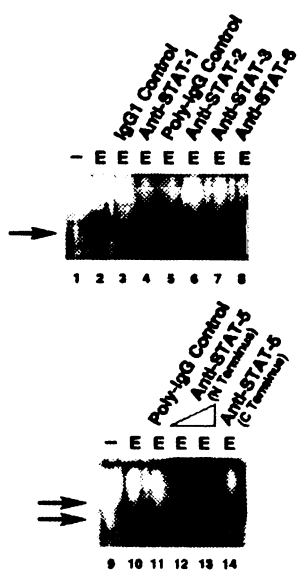

FIG. 3. The IL-2-induced DNA-binding complex is a STAT-5-like protein. HT-2EPOβγ cells were stimulated with EPO (E) for 15 min and nuclear extracts prepared (see legend to Fig. 1). Nuclear extracts were incubated with 2 μg of LFA-1β IgG1 control, 2 μg of anti-human STAT-1, 3 μg of anti-human Shc poly-IgG control, 3 μg of anti-human STAT-2, 3 μg of anti-mouse STAT-3, 3 μg of anti-human STAT-6, 1 μl (lane 12) or 2 μl (lane 13) of anti-sheep STAT-5 (N terminus), or 2 μg of anti-sheep STAT-5 (C terminus) as detailed in the legend to Fig. 2*B*. Arrows, specific, cytokine-inducible band (*Lower*) and supershifted band (*Upper*).

STAT-5 Induction Is Mediated Through the IL-2Rβ Chain. The HT-2 EPOβγ chimera system allows testing of β and γ_c mutants in the context of endogenous, wild-type β and γ_c chains. To determine whether the tyrosine residues within the γ_c subunit play a role in the induction of the STAT-5-like activity, nuclear extracts were prepared from HT-2EPOβ/γYF cells, a cell line in which all four of the cytoplasmic tyrosines within EPOγ had been substituted with phenylalanine residues (M.A.G., *et al.*, unpublished data). As shown in Fig. 4*A*, these cells remained fully capable of inducing STAT-5 in response to EPO (lane 4).

To determine whether the tyrosine residues of the IL-2Rβ chain were necessary for STAT-5 induction, a chimeric EPOβ fusion gene in which all cytoplasmic tyrosine residues of β were replaced with phenylalanine (M.A.G., *et al.*, unpublished data) was introduced into HT-2 cells stably expressing the EPOγ construct (HT-2EPOβYF/γ). Transgene expression was confirmed by RNA Northern analysis (data not shown). In marked contrast to cells expressing the γYF mutation, six independently derived HT-2EPOβYF/γ cell lines were incapable of activating STAT-5 DNA-binding activity in response to EPO (two representative transfectants are shown, Fig. 4*B*, *Upper*). As a control for the integrity of the nuclear extracts, DNA-binding activity to the ubiquitous transcription factor SP-1 was assessed and found to be equivalent in all extracts (Fig. 4*B*, *Lower*). This result provided strong evidence that the IL-2Rβ chain was critical for induction of the STAT-5-like factor.

To localize further the region within the IL-2Rβ chain involved in STAT-5 activation, nuclear extracts were prepared from cells expressing EPOγ and either of two deletion mutants in the β chain, EPOβΔA and EPOβΔAB (38). Both of these mutant β chains were competent to induce STAT-5 fully (Fig. 4*A*), indicating that sequences in the A and B regions of the IL-2Rβ subunit are unnecessary for STAT-5 induction, thus implicating the most distal tyrosine (Y510) in inducible STAT-5 DNA binding.

STAT-5 DNA Binding Is Inhibited by Phosphotyrosine-Containing Peptides from the IL-2Rβ Chain. In previously described cytokine-induced STAT activation systems, direct association of the cytokine receptor with its cognate STAT factor through SH2-phosphotyrosyl interactions has been pro-

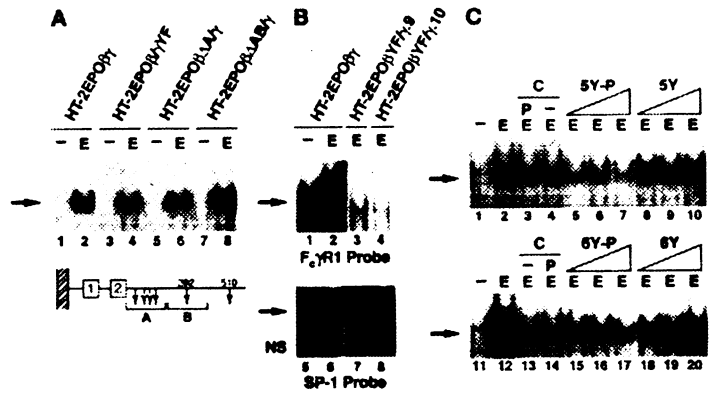

FIG. 4. STAT-5 induction is mediated through the IL-2Rβ chain. (*A*) STAT-5 induction is independent of γ_c tyrosine residues and the IL-2Rβ AB region. Indicated cell lines were stimulated with EPO (E), nuclear extracts were prepared, and EMSAs were performed as described in the legend to Fig. 1. The IL-2Rβ chain transmembrane and cytoplasmic regions are represented. Conserved box 1 and box 2 regions (37), tyrosine residues (Y), and A and B regions are indicated. (*B*) Tyrosine residues of the IL-2Rβ chain are required for STAT-5 induction. Indicated cell lines were stimulated with EPO and nuclear extracts were prepared. EMSA was performed with the FcγR1 or SP-1 probe (TCGAGAGGTGGGTG-GAGTTTCGCG), as indicated. (*C*) Certain tyrosine-phosphopeptides derived from the IL-2Rβ cytoplasmic tail inhibit STAT-5 DNA binding. HT-2EPOβγ cells were stimulated with EPO and nuclear extracts were prepared. Nuclear extracts were incubated with phosphorylated (P) or unphosphorylated (−) peptides at concentrations of 680 μM (lanes 3, 4, 7, 10, 13, 14, 17, and 20), 425 μM (lanes 6, 9, 16, and 19), or 250 μM (lanes 5, 8, 15, and 18). Peptide sequences: C, TPSLLEYDQNAL; 5Y, SGEDDAYCTFPS; 6Y, PLNTDAYLSLQE. Arrows, specific, cytokine-induced bands. NS, nonspecific band.

posed (32, 39). Together, the HT-2EPOβΔAB/γ and HT-2EPOβYF/γ mutants suggested that Y510 was critical for STAT-5 induction. However, previous studies of receptor mutants have implicated a redundant function for Y392 and Y510 in proliferation signaling in the Ba/F3 cell line (M.A.G., *et al.*, unpublished data). Further, Y392 has been clearly shown to be phosphorylated *in vivo* (M.A.G., *et al.*, unpublished data). Therefore, to delineate the potential role of phosphorylated tyrosine residues within the IL-2Rβ tail in STAT-5 activation, synthetic phosphopeptides corresponding to these receptor sites and their nonphosphorylated counterparts were added to EMSA reactions. A peptide corresponding to a random rearrangement of the amino acids surrounding IL-2Rβ Y510 was employed as a control (C). Peptides derived from the IL-2Rβ Y392 and Y510 were designated 5Y and 6Y, respectively. Fig. 4*C* shows that neither the control peptides nor nonphosphorylated 5Y and 6Y peptides affected STAT-5 DNA binding. However, addition of the phosphorylated 5Y and 6Y peptides markedly inhibited DNA binding in a dose-dependent fashion (lanes 5–7 and 15–17). Since the amino acid sequences surrounding these tyrosines are similar (*Discussion*), it is not unexpected that both sequences when phosphorylated can form binding motifs recognized by STAT-5. These data suggest that STAT-5 activation likely involves physical assembly at Y392 and/or Y510 of the IL-2Rβ chain, further supporting a central role for IL-2Rβ in the activation of STAT-5.

DISCUSSION

The present studies identify an IL-2-inducible DNA-binding activity in murine T cells that exhibits activation kinetics and tyrosine phosphorylation dependence characteristic of a STAT factor. These observations are fully consistent with its identification as a murine homologue of STAT-5, based on reactivity with anti-sheep STAT-5 antibodies.

STAT-5 was originally identified as a prolactin (PRL)-induced DNA-binding activity present in sheep and murine mammary epithelial cells (29, 40, 41). PRL and IL-2 induce a STAT-like DNA-binding activity in rat Nb2 cells (24), and it has been suggested that nuclear PRL may be required for IL-2-induced proliferation in this cell line (42). The fact that the IL-2 and PRL receptors utilize a common or closely related STAT factor raises the intriguing notion of convergent signaling through these disparate receptors in specific cell types.

The pattern of STAT activation by IL-2 varies considerably, depending upon the specific cellular environment and STAT response element tested. For example, in HT-2 cells, IL-2 induces STAT-5 binding to some (FcγR1 and IRF-1), but not all (Ly6E and SIE), STAT response sequences (Fig. 1). Similarly, in human T cells and NK cells, IL-2 induces STAT-5 binding to the FcγR1 element (32). In human lymphocytes, IL-2 also induces STAT-1 binding with the FcγR1 site (22, 32), while constitutive binding of STAT-3 is observed with this element (32). Using the Ly6E and SIE elements as probes, STAT-1-binding activity has been detected in human T cells and promonocytes (22, 25) but is lacking in HT-2 cells. We conclude that multiple STAT factors may be involved in IL-2 signaling and that different patterns of STAT factor induction may occur in different cellular environments.

The emerging picture of STAT factor activation suggests a direct physical association of the factor with its inducing receptor mediated through the same SH2-phosphotyrosyl interactions that subsequently allow for its dimerization and nuclear import (21, 32, 39). This model was strengthened by the finding that tyrosine-phosphorylated peptides derived from the IL-4R block STAT-6 binding to DNA, presumably by preventing its dimerization (32). We now describe studies in the IL-2R system that are consistent with this paradigm. Notably, our studies indicate that the IL-2Rβ chain, and not γ_c, is principally responsible for STAT-5 induction. Indeed, a γ_c mutant chain lacking all four cytoplasmic tyrosine residues continued to promote normal STAT-5 induction when paired with IL-2Rβ (Fig. 4A). In contrast, an IL-2Rβ mutant receptor lacking all cytoplasmic tyrosines failed to induce STAT-5 activity (Fig. 4). Further evidence indicating that STAT-5 activation is mediated exclusively through the β chain was obtained in studies of a hybrid receptor containing wild-type EPOR and EPOβ chains without γ_c; this hybrid complex, but not the EPOR homodimer, induced STAT-5 identically to the HT-2EPOβγ cells. Data supporting the direct physical assembly of STAT-5 with the IL-2Rβ chain were obtained in the present peptide competition experiments in which phosphor-

ylated, but not nonphosphorylated, peptides spanning either Y392 or Y510 of IL-2Rβ specifically blocked STAT-5 DNA binding (Fig. 4*C*). The ability of the EPOβΔAB mutant receptor (which lacks Y392 and four proximal cytoplasmic tyrosines) to induce STAT-5 compared to the inability of the EPOβYF (lacking all six tyrosines) to induce STAT-5 suggests that there may be functional redundancy of these two tyrosine residues. Such a functional redundancy in Y392 and Y510 has also been found for growth signaling in pro-B cells. In addition, Y392 has been documented as a site for IL-2-induced phosphorylation *in vivo* (M.A.G., *et al.*, unpublished data). Together, these observations strongly suggest that the specificity of IL-2-induced activation of STAT-5 lies in the IL-2Rβ cytoplasmic domain and that specific phosphotyrosines within this chain mediate binding of STAT-5 to the receptor.

Recent studies have led to the identification of a tyrosine-containing motif within the gp130-containing family of cytokine receptors that regulates the specificity of STAT factor binding (20). In a parallel fashion, we note that the sequences surrounding Y392 and Y510 of the IL-2Rβ share certain similarities with each other as well as with sequences present in other select cytokine receptors such as the EPO, IL-3, IL-4, and growth hormone receptors. This general sequence, DAYXS/TLX/P, may represent a second motif for cytokine receptor–STAT association. Consistent with this model, the EPO and growth hormone receptors have been shown to induce STAT-5 (unpublished data; ref. 43). However, the tyrosine-containing regions within the PRLR fail to conform to the proposed IL-2Rβ motif, yet STAT-5 is induced through this receptor. Thus, more than one STAT-5 motif may exist, or different STAT-5 isoforms may display different receptor specificities. With sufficient knowledge of the range of such motifs, it may be possible to predict on the basis of a receptor's primary sequence and state of phosphorylation *in vivo* which STAT factors will be induced following receptor ligation.

In conclusion, the role of STAT-5 in signal transduction through the IL-2R as described in this work adds to the rapidly expanding picture of STAT factor diversity in cytokine receptor signaling and reinforces the importance of the JAK–STAT pathway in IL-2R signaling. At present, the precise targets of STAT-5 action remain unknown. Further understanding of the range of STAT factors induced and the identity of the cellular genes that they regulate will help dissect at a molecular level the underlying basis for IL-2 signaling.

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1. Goldsmith, M. A. & Greene, W. C. (1994) in *The Cytokine Handbook*, ed. Thomson, A. (Paston, Norfolk, England), pp. 57–80.
2. Smith, K. A. (1992) *Curr. Opin. Immunol.* **4**, 271–276.
3. Noguchi, M., Nakamura, Y., Russel, S. M., Ziegler, S. F., Tsang, M., Cao, X. & Leonard, W. J. (1993) *Science* **262**, 1877–1880.
4. Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedman, M. C., Miyajima, A., Puri, R. K., Paul, W. E. & Leonard, W. J. (1993) *Science* **262**, 1880–1883.
5. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-i. & Sugamura, K. (1993) *Science* **262**, 1874–1877.
6. Grabstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., Beers, C., Richardson, J., Schoenborn, M. A., Ahdieh, M., Johnson, L., Alderson, M. R., Watson, J. D., Anderson, D. M. & Giri, J. G. (1994) *Science* **264**, 965–968.
7. Nelson, B. H., Lord, J. D. & Greenberg, P. D. (1994) *Nature (London)* **369**, 333–336.
8. Nakamura, Y., Russell, S. M., Mess, S. A., Friedmann, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S. & Leonard, W. J. (1994) *Nature (London)* **369**, 330–333.
9. Taniguchi, T. & Minami, Y. (1993) *Cell* **73**, 5–8.
10. Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Bacon, C. M., Friedmann, M., Berg, M., McVicar, D. W., Whitthuhn, B. A., Silvennoinen, O., Goldman, A. S., Schmalsteig, F. C., Ihle, J. N., O'Shea, J. J. & Leonard, W. J. (1994) *Science* **266**, 1042–1045.
11. Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z.-J., Oishi, I., Silvennoinen, O., Witthuhn, B. A., Ihle, J. N. & Taniguchi, T. (1994) *Science* **266**, 1045–1047.
12. Johnston, J. A., Kawamura, M., Kirken, R. A., Chen, Y.-Q., Blake, T. B., Shibuya, K., Ortaldo, J. R., McVicar, D. W. & O'Shea, J. J. (1994) *Nature (London)* **370**, 151–153.
13. Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T. & Ihle, J. N. (1994) *Nature (London)* **370**, 153–157.
14. Asao, H., Takeshita, T., Nakamura, M., Nagata, K. & Sugamura, K. (1990) *J. Exp. Med.* **171**, 637–644.
15. Sugamura, K., Takeshita, T., Asao, H., Kumaki, S., Ohbo, K., Ohtani, K. & Nakamura, M. (1990) *Lymphokine Res.* **9**, 539–543.
16. Darnell, J. E., Kerr, I. M. & Stark, G. R. (1994) *Science* **264**, 1415–1421.
17. Schindler, C., Shuai, K., Prezioso, V. R. & Darnell, J. E. (1992) *Science* **257**, 809–815.
18. Shuai, K., Stark, G. R., Kerr, I. M. & Darnell, J. E. (1993) *Science* **261**, 1744–1746.
19. Heim, M. H., Kerr, I. M., Stark, G. R. & Darnell, J. E. (1995) *Science* **267**, 1347–1349.
20. Stahl, N., Farruggella, T. J., Boulton, T. G., Zhong, Z., Darnell, J. E. & Yancopoulos, G. D. (1995) *Science* **267**, 1349–1353.
21. Shuai, K., Horvath, C. M., Huang, L. H. T., Qureshi, S. A., Cowburn, D. & Darnell, J. E. (1994) *Cell* **76**, 821–828.
22. Beadling, C., Gischin, D., Witthuhn, B. A., Ziemiecki, A., Ihle, J. N., Kerr, I. M. & Cantrell, D. A. (1994) *EMBO J.* **13**, 5605–5615.
23. Finbloom, D. S., Petricoin, E. F., Hackett, R. H., David, M., Feldman, G. M., Igarashi, K.-i., Fibach, E., Weber, M. J., Thorner, M. O., Silva, C. M. & Larner, A. C. (1994) *Mol. Cell. Biol.* **14**, 2113–2118.
24. Gilmour, K. C. & Reich, N. C. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 6850–6854.
25. Lamb, P., Kessler, L. V., Suto, C., Levy, D. E., Seidel, H. M., Stein, R. B. & Rosen, J. (1994) *Blood* **83**, 2063–2071.
26. Hou, J., Schindler, U., Henzel, W. J., Wong, S. C. & McKnight, S. L. (1995) *Immunity* **2**, 321–329.
27. Schreiber, E., Matthias, P., Muller, M. M. & Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419–6420.
28. Latchman, D. S. (1993) *Transcription Factors: A Practical Approach* (IRL, New York).
29. Wakao, H., Gouilleux, F. & Groner, B. (1994) *EMBO J.* **13**, 2182–2191.
30. Carroll, S. B. & Stollar, B. D. (1983) *J. Biol. Chem.* **258**, 24–26.
31. Kotanides, H. & Reich, N. C. (1993) *Science* **262**, 1265–1267.
32. Hou, J., Schindler, U., Henzel, W. J., Ho, T. C., Brasseur, M. & McKnight, S. L. (1994) *Science* **265**, 1701–1706.
33. Dumont, F. J., Staruch, M. J., Koprak, S. L., Melino, M. R. & Sigal, N. H. (1990) *J. Immunol.* **144**, 251–258.
34. Juo, C. J., Chung, J., Florentino, D. F., Flanagan, W. M., Blenis, J. & Crabtree, G. R. (1992) *Nature (London)* **358**, 70–73.
35. Schindler, C., Kashleva, H., Pernis, A., Pine, R. & Rothman, P. (1994) *EMBO J.* **13**, 1350–1356.
36. Zhong, Z., Wen, Z. & Darnell, J. E. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 4806–4810.
37. Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T. & Kishimoto, T. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11349–11353.
38. Goldsmith, M. A., Xu, W., Amaral, M. C., Kuczek, E. S. & Greene, W. C. (1994) *J. Biol. Chem.* **269**, 14698–14704.
39. Greenlund, A. C., Farrar, M. A., Viviano, B. L. & Schreiber, R. D. (1994) *EMBO J.* **13**, 1591–1600.
40. Gouilleux, F., Wakao, H., Mundt, M. & Groner, B. (1994) *EMBO J.* **13**, 4361–4369.
41. Welte, T., Garimorth, K., Philipp, S. & Doppler, W. (1994) *Mol. Endocrinol.* **8**, 1091–1102.
42. Clevenger, C. V., Altmann, S. W. & Prystowsky, M. (1991) *Science* **253**, 77–79.
43. Gouilleux, F., Pallard, C., Dusanter-Fourt, I., Wakao, H., Haldosen, L.-A., Norstedt, G., Levy, D. & Groner, B. (1995) *EMBO J.* **14**, 2005–2013.

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Distinct Tyrosine Residues within the Interleukin-2 Receptor β Chain Drive Signal Transduction Specificity, Redundancy, and Diversity*

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To explore the basis for interleukin (IL)-2 receptor (IL-2R) signaling specificity, the roles of tyrosine-based sequences located within the cytoplasmic tails of the β and γ_c chains were examined in the murine helper T cell line HT-2. Activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, cellular proliferation, and the induction of various genes were monitored. All four of the cytoplasmic tyrosine residues as well as the distal portion of the γ_c proved dispensable for the entire spectrum of IL-2R signaling responses studied. Conversely, select tyrosine residues within the β chain were essential and differentially required for various signaling events. Specifically, activation of c-*fos* gene expression was found to occur exclusively through the most membrane proximal tyrosine, Tyr-338, whereas proliferation and the activation of STAT-5 were induced either through Tyr-338 or through the two C-terminal tyrosine residues, Tyr-392 and Tyr-510. These tyrosine residues mediated the induction of two different STAT-5 isoforms, which were found to form heterodimers upon receptor activation. In contrast to the tyrosine dependence of c-*fos* and STAT-5 induction, *bcl-2* gene induction proceeded independently of all IL-2Rβ tyrosine residues. Thus, the tyrosine-based modules present within the IL-2Rβ cytoplasmic tail play a critical role in IL-2R signaling, mediating specificity, redundancy, and multifunctionality.

A select group of cytokines is responsible for coordinating a diverse array of biologic responses including hematopoiesis, neurological development, and control of the immune system. Understanding the molecular mechanisms by which such a limited number of cytokines regulate a variety of cellular responses has remained a central goal in the field of signal transduction (1). Cytokines act by binding to specific cell surface receptors, many of which are members of a single receptor superfamily (2). One striking feature of this cytokine receptor superfamily is the shared use of common receptor subunits to generate combinatorial diversity. For example, the interleukin (IL)[1]-2 receptor is composed of three subunits, α, β, and γ (reviewed in Ref. 3). The β and γ chains are shared by the IL-15 receptor, while the γ subunit also participates in the formation of the IL-4, -7, and -9 receptors (thus termed γ_c for "γ common") (4–7). Since most immune cells express receptors for multiple cytokines, signal integrity must somehow be preserved despite the use of overlapping signal transduction systems.

Considerable evidence has emerged supporting the view that cytokine receptors are composed of a series of functional signaling "modules." At the level of the entire multimeric receptor, each individual subunit may be considered a distinct module, potentially serving a specialized function within the overall receptor complex. Moreover, individual receptor subunits contain within their cytoplasmic domains combinations of functional peptide sequences that link the receptor to a distinct array of intracellular signaling pathways. For example, the particular sequence surrounding receptor tyrosine residues may impart specificity by permitting the docking of particular SH2- or PTB-containing proteins (8, 9) such as "signal transducer and activator of transcription" (STAT) proteins (10). Ultimately, the combinatorial effects of these modular domains help to generate an integrated signal from the receptor that is unique to each cytokine.

The IL-2/IL-2 receptor (IL-2R) system exhibits the hallmark pleiotropy of the cytokine family. Studies dissecting the functional role of the IL-2R have yielded valuable information pertaining to the general principles underlying cytokine signaling. First, chimeric receptors have been employed to demonstrate that heterodimerization of receptor subunits is critical for signal transduction (11–13). For example, when the extracellular domains of the β and γ_c chains are replaced with the erythropoietin (EPO) receptor extracellular domain and expressed in IL-2-dependent T cells, co-expression of these chimeric receptors is necessary to recapitulate IL-2 signaling in response to EPO (11, 14). Similar results have been obtained with other chimeric signaling systems (12, 13). Moreover, since the IL-15 receptor also contains the IL-2Rβ and γ_c chains (7), these chimeras presumably also reflect signaling mechanisms induced by this cytokine.

A second principle that applies generally to cytokine recep-

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[1] The abbreviations used are: IL, interleukin; IL-2R, interleukin-2 receptor; EPO, erythropoietin; JAK, Janus kinase; STAT, signal transducer and activator of transcription; EMSA, electrophoretic mobility shift assay; ECL, enhanced chemiluminescence; GAPD, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; kb, kilobase pair(s).

tors is the physical association of the receptor subunits with members of the Janus family of tyrosine kinases (JAKs, reviewed in Ref. 15). The IL-2Rβ and γ_c subunits bind to JAK1 and JAK3, respectively (16–19); upon heterodimerization of the receptor by ligand, both the JAKs and the receptor subunits themselves become tyrosine-phosphorylated (14, 20, 21). JAK1 and JAK3 phosphorylation is associated with an increase in their enzymatic activities (15), while phosphorylation of the IL-2R subunits creates binding sites for cytoplasmic effector molecules. For example, the signaling adapter Shc has been shown to associate with the IL-2Rβ chain through a single tyrosine residue, Tyr-338 (22). In addition, phosphorylation and nuclear transport of the STAT factor STAT-5 is dependent upon tyrosine residues located in the IL-2Rβ chain (23–28).

Although heterodimerization of β and γ_c is necessary for IL-2R signaling, studies examining the relative contributions of the individual subunits have suggested that the γ_c subunit may play a more limited role in this signaling cascade. For example, all four of the tyrosine residues of γ_c are dispensable for both proliferation and STAT-5 activation (11, 23). Further recent studies have revealed that the entire γ_c chain and its associated JAK3 molecule can be replaced by a heterologous receptor that binds JAK2 without apparent disruption of IL-2-specific signaling (14). These observations suggest that β and γ_c contribute quite differently to the IL-2 signaling program, with γ_c acting mainly to trigger the signaling cascade, while β serves to drive signaling specificity. However, it is possible that some as yet unstudied downstream signaling events might be more dependent on the γ_c chain.

In the present report, the participation of the γ_c and β chains in determining specificity in both early and downstream IL-2 signaling pathways has been defined in detail. In particular, these studies assigned the molecular basis of signal transduction specificity largely to individual tyrosine-containing peptides within the cytoplasmic portion of the IL-2 receptor β chain. The findings strongly support a separation of function between IL-2Rβ and γ_c within the receptor complex, and reveal that the tyrosine residues of IL-2Rβ exhibit a high degree of specificity as well as multifunctionality in coupling to various signaling pathways.

MATERIALS AND METHODS

Cell Culture—The cell line HT-2, an IL-2-dependent murine helper T cell line (ATCC) was cultured in RPMI, 10% fetal bovine serum, antibiotics, and either 1 nM human IL-2 (Chiron) or 5 units/ml erythropoietin (EPO, Amgen) as described previously (11). HT-2EPOβ-containing cell lines were generated by transfecting HT-2EPOγ cells by electroporation and selecting stable transfectants by limiting dilution either in hygromycin B (Boehringer Mannheim) and G418 (Life Technologies, Inc.) as described previously (29) (HT-2EPOβYF:1Y, HT-2EPOβYF:234Y, HT-2EPOβYF:5Y, HT-2EPOβYF:6Y cell lines) or in 5 units/ml EPO (HT-2EPOβYF:1Y, EPOβYF:5Y, EPOβYF:6Y, HT-2EPOβYF:56Y, HT-2EPOβYF:1234Y cell lines). No significant phenotypic differences were observed between cell lines generated by selection in G418/hygromycin B *versus* EPO.[2]

Cytokine Stimulations—For immunoprecipitations and nuclear extract preparation, cells were incubated for 2–4 h in RPMI with antibiotic and 1% bovine serum albumin (fraction V, Sigma) without growth factor as described previously (23). For RNA analyses, cells were incubated for 15 h in RPMI with antibiotic and 10% fetal bovine serum (Life Technologies, Inc.) in the absence of growth factor. Cells were stimulated for the indicated time intervals in 5–20 ml of the appropriate medium plus growth factor: IL-2 (10 nM), murine IL-4, (100 units/ml, Genzyme), human EPO (50–100 units/ml).

Plasmid Constructs—All receptor cDNAs were subcloned into the expression vectors pCMV4 or pCMV4neo (29). Tyrosine substitution mutants in the IL-2Rβ chain were created as described previously (29). These mutations were transferred into the pEPOβneo backbone by replacing the *AflII/Bam*HI fragment within the cytoplasmic tail of EPOβ with an equivalent fragment containing the βYF:1Y, βYF:234Y, βYF:5Y or βYF:6Y, βYF:56Y, and βYF:1234Y (formerly βYF:56F) (11) mutations. Mutations were confirmed by sequencing. The pME18 s-STAT-5A and pME18s-STAT-5B constructs were generously provided by Dr. A. Mui (30).

RNA Preparation and Northern Blot Analysis—Cytoplasmic RNA was prepared from 1–2 × 10^7 cells using an RNeasy mRNA kit (Qiagen) and quantified by UV spectrophotometry. Denaturing gels were run with 10 μg of RNA/lane and blotted to Zeta Probe membranes (Bio-Rad) as described elsewhere (31). Hybridization was at 42 °C for 12–16 h. Probes were random prime-labeled using a Megaprime labeling kit (Amersham Corp.) and [α-^{32}P]dATP and [α-^{32}P]dCTP (Amersham Corp.). The glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA was from ATCC (32), the c-*fos* cDNA was provided by Dr. I. Verma, and the *bcl-2* cDNA was provided by Dr. S. Korsmeyer. DNA fragments used to label probes were as follows: a 1.2-kb *HindIII/KpnI* fragment of the extracellular murine EPO receptor, a 2.1-kb *Eco*RI murine c-*fos* cDNA fragment from pMc-fos (33), a 0.9-kb *PstI* murine *bcl-2* fragment from pBS-bcl-2 (34), and a 1.0-kb *Eco*RI fragment from GAPD.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extractions and EMSA were performed using the FcγRI oligonucleotide as described previously (23, 35). Antibody supershifts were performed by preincubating nuclear extracts with 1 μl of STAT-5A or STAT-5B antisera (see below), 3 μg of 4G10 (anti-phosphotyrosine), or 3 μg of MOPC195 (IgG2b control) antibodies for 30 min on ice prior to the binding reaction.

Protein Expression in COS-7 Cells—COS-7 cells (ATCC) were transfected with the indicated plasmids using Lipofectamine (Life Technologies, Inc.) per the manufacturer's instructions. Nuclear extracts were prepared from 1–2 million transfected cells as described previously (35).

Immunoprecipitations—Cells were lysed (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 8.0, 50 mM NaF, 100 uM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A) and immunoprecipitated with 1 μl of anti-STAT-5A, anti-STAT-5B, or anti-JAK1 antiserum (Upstate Biotechnology, Inc.) per 20–30 million cells using protein A-agarose (Boehringer Mannheim). Immunoblotting studies were performed with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) or anti-STAT-5A or anti-STAT-5B followed by enhanced chemiluminescence (ECL) (Amersham Corp.) signal development.

Proliferation Assays—Conventional 48-h [^{3}H]thymidine incorporation assays and transient proliferation assays were performed as described previously (29). Data are expressed as a percentage of [^{3}H]thymidine incorporation of cells treated with 10 nM IL-2.

Antibodies—Anti-STAT-5A and anti-STAT-5B antibodies were generated in rabbits against the C-terminal peptides specific for mouse STAT-5A (LDARLSPPAGLFTSARSSLS) or mouse STAT-5B (MDSQWIPHAQS), and were used 1:50,000 in Western blotting and 1 μl/500 μg in immunoprecipitations.

RESULTS

Role of the IL-2Rβ and γ_c Tyrosine Residues in Downstream Signaling Events

Proliferation Signaling—All facets of IL-2R signaling examined thus far appear to require heterodimerization of the IL-2Rβ and γ_c chains; however, the relative contributions of these individual subunits to specific signaling processes may be distinct. In order to explore the functions of these chains in an IL-2-responsive cellular environment, this laboratory has developed a chimeric receptor system in which the extracellular domain of the EPO receptor (EPOR) is fused to the cytoplasmic domains of the IL-2Rβ and γ_c chains, thus forming EPOβ and EPOγ (Fig. 1*A*). When expressed in IL-2-dependent helper HT-2 cells, EPO stimulation recapitulates all IL-2-mediated signaling events so far examined (11, 14, 23). In the present studies, a series of mutant tyrosine constructs were created to delineate the role of individual IL-2Rβ tyrosine residues in IL-2R signaling (Fig. 1*A*). These constructs were introduced into HT-2EPOγ cells (11), and receptor gene expression in the stable transfectants was assessed by RNA blotting analysis to identify positive clones for further study. Relative levels of EPOβ and EPOγ mRNA were approximately equivalent in all clones, indicative of similar expression levels of receptors (data

[2] S. L. Gaffen, unpublished observations.

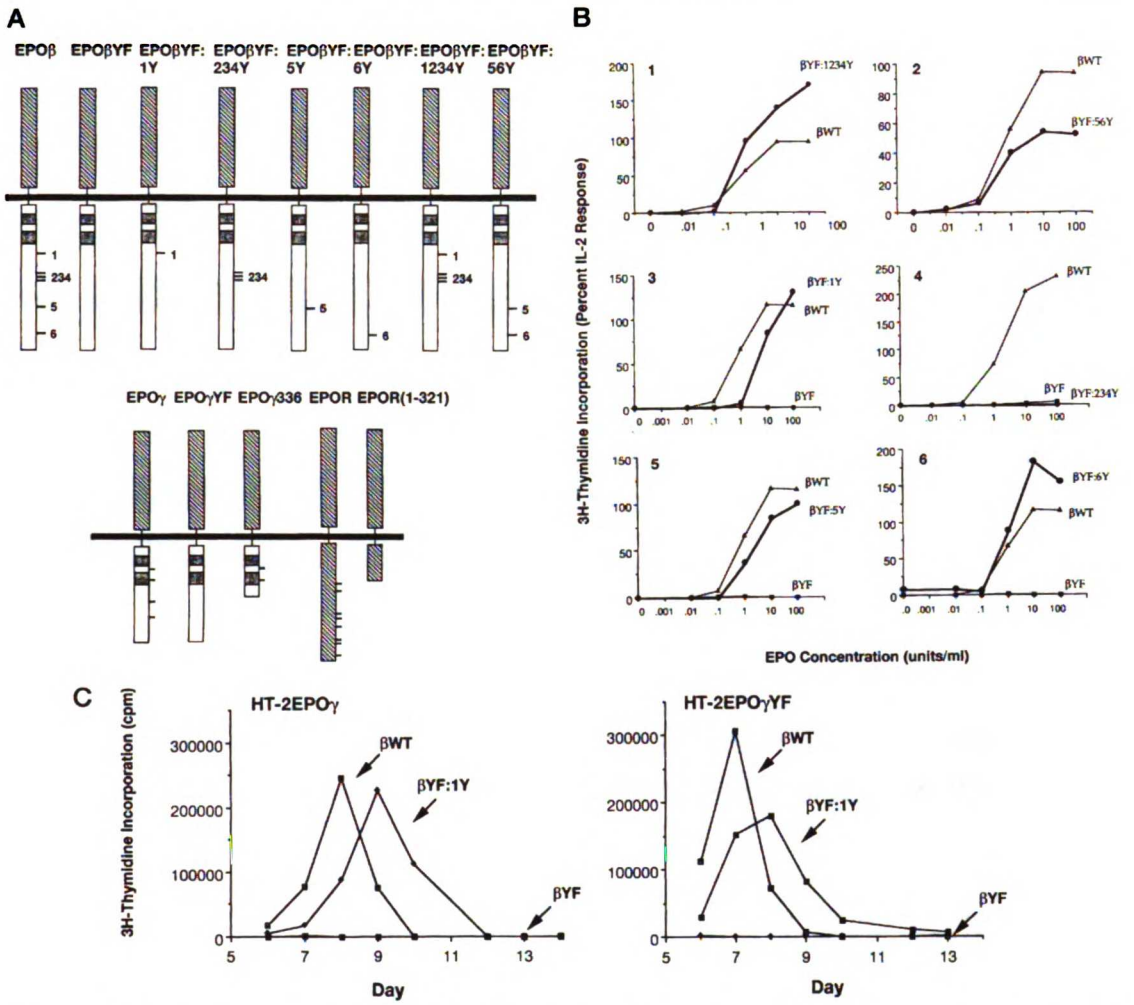

FIG. 1. *A*, diagrams of chimeric receptor constructs. *Hatched boxes* represent EPO receptor sequence, and *open boxes* represent IL-2R sequences. ***Shaded boxes*** within IL-2R sequences correspond to conserved Box1 and Box2 regions (53). Tyrosine residues are indicated by ***horizontal bars*** and are numbered as follows: *Y1* = Tyr-338, *Y2* = Tyr-355, *Y3* = Tyr-358, *Y4* = Tyr-361, *T5* = Tyr-392, and *Y6* = Tyr-510. *B*, multiple tyrosine residues of the IL-2Rβ chain can support proliferation signaling. HT-2EPOβγ, HT-2EPOβYF/γ, HT-2EPOβYF:1Y/γ, HT-2EPOβYF:234Y/γ, HT-2EPOβYF:5Y/γ, HT-2EPOβYF:6Y/γ, HT-2EPOβYF:1234Y/γ, and HT-2EPOβYF:56Y/γ cells were stimulated for 48 h with EPO at the indicated concentrations. Cells were pulsed with [^{3}H]thymidine for the final 4 h of the culture and harvested. Results are expressed relative to the level of incorporation occurring with stimulation in 10 nM IL-2 (100%). Each data point is the mean of triplicates, and is representative of three experiments. *C*, a single tyrosine residue within IL-2Rβ is sufficient for growth signaling in the absence of γ_c tyrosine residues. HT-2EPOγ (*left panel*) or HT-2EPOγYF (*right panel*) cells were transiently transfected with the EPOβYF, EPOβYF:1Y, or EPOβ (*βWT*) constructs as described previously (29), and [^{3}H]thymidine incorporation was measured at the indicated time points after transfection. Each data point is the mean of triplicates, and is representative of four experiments.

not shown). As observed previously (11), the wild type HT-2EPOβγ cells (*βWT*) exhibited a vigorous proliferative dose response to EPO (Fig. 1*B*, *panels 1–6*), whereas the HT-2EPOβYF/γ cells failed to proliferate (Fig. 1*B*, *panels 3–6*). HT-2 cell lines expressing either the two most distal tyrosines of β (*EPOβYF:56Y/γ*, *panel 2*) or the first four membrane-proximal tyrosines of β (*EPOβYF:1234Y/γ*, *panel 1*) both showed a strong proliferative response to EPO and could support long term growth in EPO. Moreover, certain cell lines expressing a single β tyrosine residue, EPOβYF:1Y/γ, EPOβYF:5Y/γ, or EPOβYF:6Y/γ, supported proliferation and long term growth comparably to those expressing wild type EPOβγ (*panels 3*, *5*, and *6*). Finally, cells expressing EPOβYF:234Y/γ did not proliferate significantly or support long term growth in EPO (Fig. 1*B*, *panel 4*). It is not clear whether or not the minor variations in proliferation among the EPOβγ, EPOβYF:1Y, EPOβYF:5Y, and EPOβYF:6Y cell lines represent important differences, and we do not attempt to draw quantitative conclusions from these data. In conclusion, these experiments indicate that HT-2 cellular proliferation depends on select tyrosines within the IL-2Rβ chain, namely 1Y, 5Y, or 6Y, which are functionally independent of one another. Moreover, the specific tyrosines capable of supporting proliferation only partially overlap with those found to be important in a pro-B

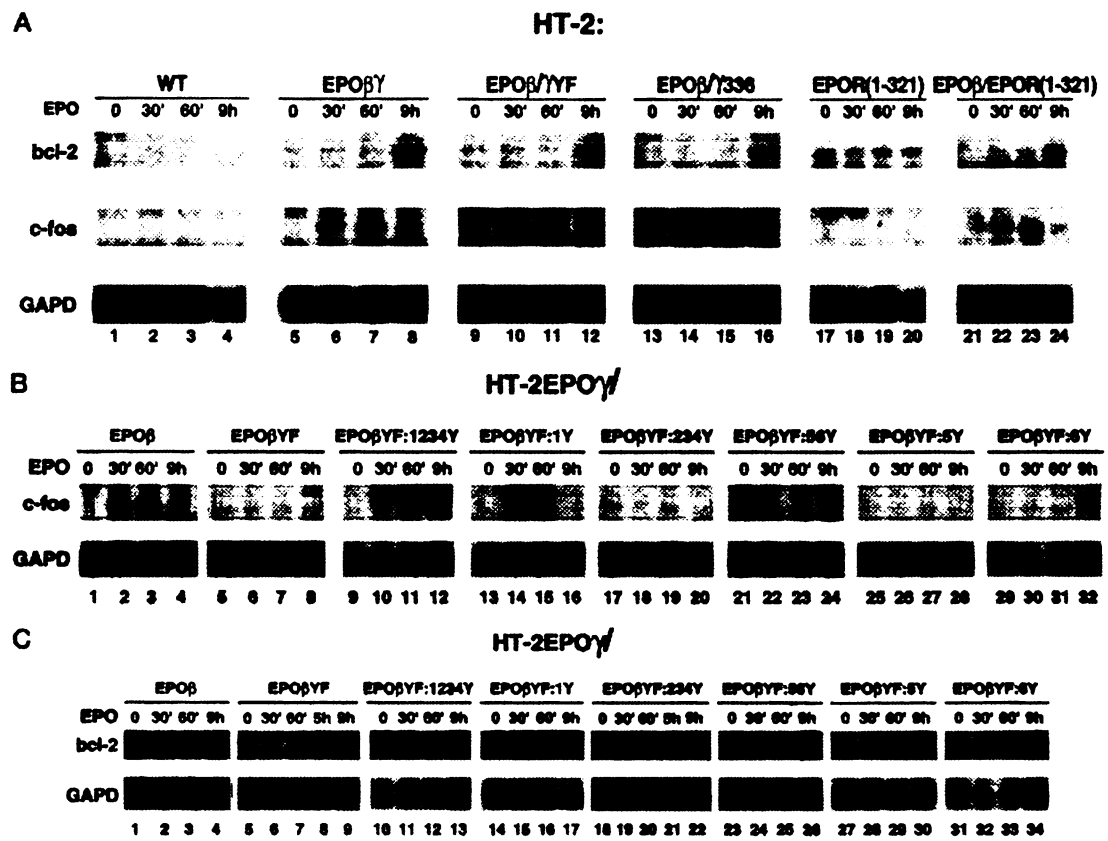

FIG. 2. *A*, the γ_c chain is not required to specify activation of the *bcl-2* and c-*fos* proto-oncogenes. HT-2, HT-2EPOβγ, HT-2EPOβ/γYF, HT-2EPOβ/γ336, HT-2EPOR(1–321), and HT-2EPOβ/EPOR(1–321) cells were incubated in media without growth factor for 15 h and stimulated with 50 units/ml EPO for 0, 30, 60 min or 9 h, as indicated. Total cellular RNA was prepared, separated by Northern blotting, and probed with *bcl-2*, c-*fos* or GAPD cDNA probes. *B*, a single tyrosine residue of the IL-2Rβ chain is necessary and sufficient for induction of c-*fos* gene expression. HT-2EPOβγ, HT-2EPOβYF/γ, HT-2EPOβYF:1234Y/γ, HT-2EPOβYF:1Y/γ, HT-2EPOβYF:234Y/γ, HT-2EPOβYF:56Y/γ, HT-2EPOβYF:5Y/γ, and HT-2EPOβYF:6Y/γ cells were stimulated with EPO as described in *A*. Total cellular RNA was separated by Northern blotting and probed with c-*fos* and GAPD cDNA probes. *C*, induction of *bcl-2* gene expression occurs via a tyrosine-independent pathway. HT-2EPOβγ, HT-2EPOβYF/γ, HT-2EPOβYF:1234Y/γ, HT-2EPOβYF:1Y/γ, HT-2EPOβYF:234Y/γ, HT-2EPOβYF:56Y/γ, HT-2EPOβYF:5Y/γ, and HT-2EPOβYF:6Y/γ cells were stimulated with EPO as described in *A*. Total cellular RNA was prepared, separated by Northern blotting, and probed with *bcl-2* and GAPD cDNA probes.

cell line, Ba/F3 (11), indicating that IL-2 signaling in T cells may differ in certain ways from that in pro-B cells (see "Discussion").

Prior studies have shown that the EPOβ/γYF-expressing cell lines proliferate in response to EPO equivalently to cells expressing wild type EPOβγ receptors (11). Combined with the data presented above (Fig. 1*B*), it appeared that a single tyrosine residue of the IL-2Rβ chain was sufficient to support growth signaling. However, it remained formally possible that tyrosines of the γ_c chain could play a compensatory role in the context of a mutant β chain containing only a single tyrosine residue. Therefore, the ability of the IL-2Rβ tyrosine mutants to proliferate when paired with a γ_c chain lacking all of its cytoplasmic tyrosine residues (EPOγYF) was assessed. As shown in Fig. 1*C*, both EPOβ and EPOβYF:1Y induced proliferation equally well when transiently transfected into HT-2EPOγ cells and HT-2EPOγYF cells, whereas EPOβYF failed to mediate the proliferative response in either case. Similar results were obtained for the EPOβYF:5Y and the EPOβYF:6Y (data not shown). Therefore, a single tyrosine residue in the β chain of the IL-2 receptor complex is both necessary and sufficient to support proliferation signaling in HT-2 cells.

Induction of Gene Expression—A second important outcome of IL-2R signaling is the induced expression of a number of specific genes (36, 37). To assess the relative roles of the β and γ_c subunits in gene induction, cells expressing various combinations of mutant receptors were stimulated for varying lengths of time. Cytoplasmic RNA was analyzed by Northern blotting with probes to the c-*fos* and *bcl-2* proto-oncogenes (33, 34). As shown in Fig. 2*A*, EPO stimulation of HT-2EPOβγ cells induced expression of these genes with reproducible and characteristic time courses (*lanes 5–8*), whereas EPO stimulation of HT-2 cells failed to induce these genes (*lanes 1–4*). To assess the role of the γ_c tyrosine residues and distal tail in induction of these genes, HT-2EPOβ/γYF or HT-2EPOβ/γ336 cells were examined. These cell lines were found to induce transcription of both c-*fos* and *bcl-2* identically to the wild type controls (Fig. 2*A*, *lanes 9–16*). Thus, neither the tyrosine residues nor the distal 35 amino acids of the γ_c chain appear necessary for the induction of c-*fos* or *bcl-2* in this cellular context.

To determine whether any other portion of the γ_c chain was required for the induction of these genes, EPOγ was replaced

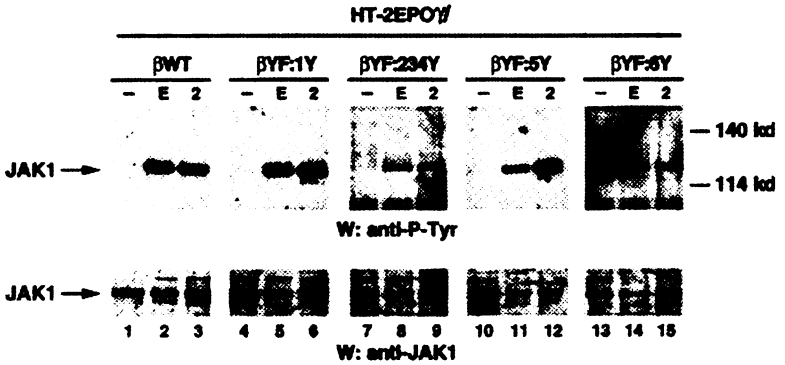

FIG. 3. **IL-2-induced JAK1 phosphorylation occurs independently of IL-2Rβ tyrosine residues.** HT-2EPOβγ, HT-2EPOβYF:1Y/γ, HT-2EPOβYF:234Y/γ, HT-2EPOβYF:5Y/γ, and HT-2EPOβYF:6Y/γ cells were incubated in the absence of growth factor for 4 h and stimulated for 15 min with 50 units/ml EPO (*E*) or 10 nM IL-2 (*2*). JAK1 immunoprecipitates were prepared, immunoblotted with anti-phosphotyrosine (4G10) antibodies (*top*) or stripped and reprobed with anti-JAK1 antibodies (*bottom*), and visualized by ECL. *Arrows* indicate JAK1. Migration of molecular weight markers is indicated in *top panel*.

with a truncated version of the erythropoietin receptor, EPOR(1–321), that contains no tyrosine residues (14). Stimulation of cells expressing EPOR(1–321) alone failed to induce transcription of the c-*fos* or *bcl-2* genes (Fig. 2*A*, *lanes 17–20*). In contrast, co-expression of EPOR(1–321) with a wild type EPOβ chain induced expression of these genes that was indistinguishable from cells expressing wild type EPOγ (*lanes 21–24*). Thus, γ_c can be replaced entirely with a heterologous receptor subunit with no apparent change in downstream activation of a variety of IL-2-induced genes. These data further indicate that JAK3, which binds to γ_c (16, 17), can be replaced by JAK2, which binds to the EPOR (38), without altering signaling specificity from the IL-2 receptor.

Since the tyrosine residues within the γ_c chain appeared to provide no detectable specificity to the IL-2 signaling program, the role of the six tyrosine residues within the cytoplasmic tail of the IL-2Rβ chain was examined. HT-2EPOγ cells expressing the EPOβ tyrosine addback constructs (Fig. 1*A*) were stimulated, and Northern blots were prepared. As illustrated in Fig. 2*B*, c-*fos* induction was dependent on tyrosine-containing sequences in the β chain, since HT-2EPOβYF/γ cells failed to induce these genes in response to receptor activation (Fig. 2*B*, *lanes 5–8*). Indeed, c-*fos* transcription was linked to a single tyrosine residue, as only EPOβ constructs that retain the most membrane proximal tyrosine, Y1 (Tyr-338), supported its transcriptional induction (Fig. 2*B*, *lanes 9–16*). Thus, a single tyrosine residue within IL-2Rβ is critical for the induction of c-*fos*. These data further indicate that the mechanism of c-*fos* induction is likely to occur via pathways linked to Shc, an adapter molecule we have previously shown to be exclusively engaged by the first tyrosine (Tyr-338) of the IL-2Rβ chain (22).

In contrast to the regulation of c-*fos*, the induced expression of the *bcl-2* proto-oncogene was found to be independent of tyrosine residues. All β mutants examined, including a mutant lacking all six cytoplasmic tyrosine residues (HT-2EPOβYF), induced *bcl-2* transcription in response to ligand (Fig. 2*C*). Thus, at least one downstream signaling event is independent of tyrosine residues within the IL-2 receptor, while others are governed by individual tyrosines.

Role of the IL-2Rβ Tyrosine Residues in Proximal Signaling Events

JAK1 Activation—Proliferation and gene induction are both relatively late events in signaling through the IL-2R. One of the best characterized signaling pathways initiated rapidly upon receptor ligation is the phosphorylation of the Janus kinases JAK1 and JAK3 (18, 19), followed by the activation of STAT factors (23–28, 39). To assess the role of tyrosine residues within the IL-2Rβ chain in Janus kinase activation, cells expressing tyrosine mutant EPOβ chains were stimulated with EPO or IL-2 as a positive control, and JAK1 immunoprecipitates were prepared for immunoblotting with 4G10 anti-phosphotyrosine antibodies. As shown in Fig. 3, stimulation of all of the EPOβ tyrosine mutant cell lines led to phosphorylation of JAK1 (*top panel*). The blots were stripped and reprobed with anti-JAK1 antibodies to ensure equivalent loading of the gels (*bottom panel*). These results were consistent with the previous demonstration that JAK1 and JAK3 activation is independent of IL-2Rβ tyrosine residues (data not shown) (11). The slight differences in relative JAK1 induction among cell lines may reflect variations in either endogenous JAK1 levels and/or receptor expression. However, it should be noted that these results serve as independent confirmation that each of the mutant tyrosine receptors is expressed at the cell surface and is capable of initiating signaling.

STAT-5 Activation—One consequence of Janus kinase activation in the IL-2 receptor system is the phosphorylation, nuclear import, and DNA binding activity of several STAT factors, notably factors related to STAT-5 (23–28, 40). In particular, stimulation of either native or chimeric IL-2 receptors expressed on HT-2 cells induces a nuclear DNA binding complex that is recognized by antibodies raised against sheep STAT-5/MGF (23). We examined the nature of the DNA binding complex in more detail than has been previously described. In particular, two murine isoforms of STAT-5 (STAT-5A and STAT-5B) have been identified recently that differ at their C termini from the originally identified sheep STAT-5/MGF (30, 41). Antibodies were raised against the unique, C-terminal portions of STAT-5A and STAT-5B. These antisera exhibited no detectable cross-reactivity by immunoblotting of cell lysates from COS-7 cells expressing either STAT-5A or STAT-5B alone (Fig. 4*A*). To evaluate the specificity of these antibodies in EMSA, either STAT-5A or STAT-5B was expressed with JAK1 in COS7 cells to permit constitutive, receptor-independent phosphorylation of the STATs in an overexpression system. Nuclear extracts were prepared from transfected COS7 cells, and an EMSA was performed using an oligonucleotide corresponding to the FcγRI STAT response element (39) as described previously (23). JAK1 overexpression in COS7 cells resulted in a DNA binding complex that represents endogenous STAT-1 (Fig. 4*B*, *lane 4*, and data not shown). However, when JAK1 was co-expressed with STAT-5A or STAT-5B, DNA binding complexes were observed that co-migrated with STAT-5. Importantly, addition of anti-STAT-5A and -5B antisera to the EMSA reaction caused selective supershifting of their respective DNA binding complexes, as demonstrated by the appearance of a slower migrating complex present at the top of the gel (Fig. 4*B*, *lanes 9, 10, 15, 16, 19,* and *20*), and these supershifted bands were accompanied by a corresponding diminution of the original DNA binding complexes. The anti-STAT-5A antiserum

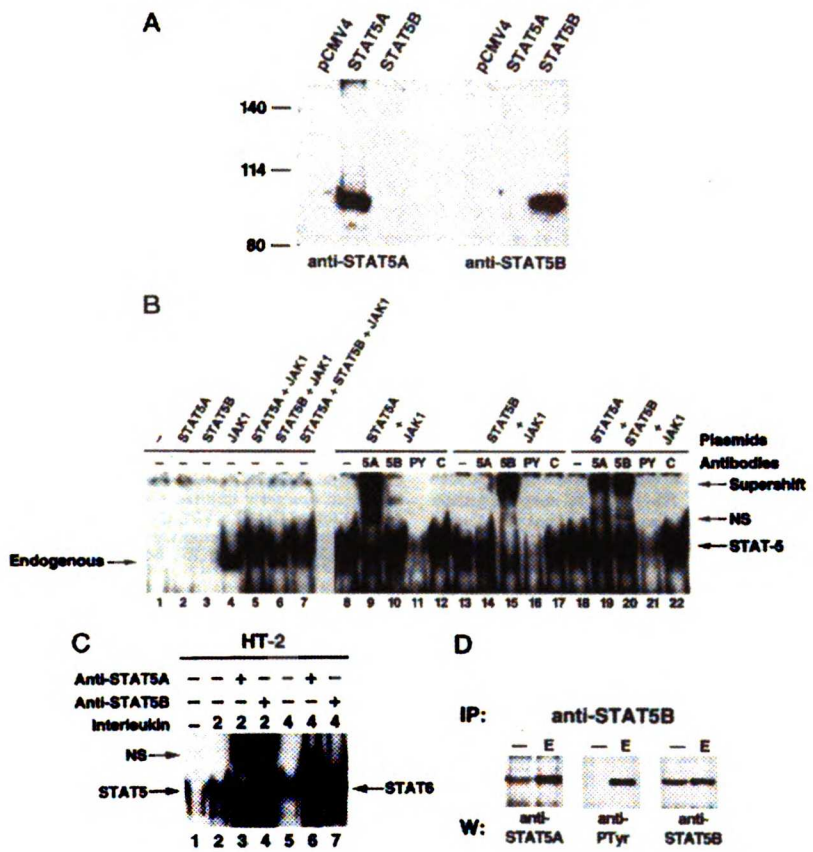

FIG. 4. *A*, anti-STAT-5A and anti-STAT-5B antibodies are specific in immunoblot analysis. Vectors containing STAT-5A, STAT-5B and a control vector (*pCMV4*) were transfected alone in COS7 cells, and cellular lysates prepared 48 h post-transfection. Lysates were separated by 8.75% SDS-polyacrylamide gel electrophoresis and immunoblotted with either STAT-5A or STAT-5B antisera, as indicated. Blots were visualized by ECL. Migration of molecular weight markers is shown. *B*, anti-STAT-5A and anti-STAT-5B antibodies exhibit specific supershifting in EMSA. COS-7 cells were transfected with the pCMV4 (*lane 1*), pCMV4-JAK1 (*lanes 4–22*), pME18s-STAT-5A (*lanes 2, 5, 7, 8–12,* and *18–22*) or pME18s-STAT-5B (*lanes 3, 6, 7,* and *13–22*) vectors. Nuclear extracts were prepared 48 h post transfection, and EMSA was performed as described under "Materials and Methods." The specific probe employed was ^{32}P-end-labeled FcγRI STAT-response element (GTATTTCCCAGAAAAAGGAC) (39). Nuclear extracts were incubated on ice with 1 μl of anti-STAT-5A (*5A, lanes 9, 14,* and *19*), 1 μl of anti-STAT-5B (*5B, lanes 10, 15,* and *20*), 3 μg of 4G10 (*PY, lanes 11, 16,* and *21*), or 3 μg of MOPC195 IgG2b control antibodies (*C, lanes 12, 17,* and *22*) for 30 min prior to EMSA. The *top arrow* indicates supershifted bands, and the *bottom arrow* indicates STAT-5 DNA binding complexes. *Endogenous* represents a STAT-1 DNA binding activity that appears constitutively in the presence of JAK1 overexpression (data not shown). *C*, IL-2R signaling induces both STAT-5A and STAT-5B in HT-2 cells. HT-2 cells were incubated in the absence of IL-2 for 2 h and stimulated for 15 min with 10 nM IL-2 (*2*) (*lanes 2–4*) or 100 u/ml murine IL-4 (*4*) (*lanes 5–7*), and nuclear extracts were prepared as described in *B*. Nuclear extracts were incubated with 1 μl of anti-STAT-5A (*lanes 3* and *6*) or anti-STAT-5B (*lanes 4* and *7*) for 45 min on ice prior to EMSA. *Arrows* indicate STAT-5 and STAT-6; *NS*, nonspecific band. *D*, IL-2 induces heterodimerization of STAT-5A and STAT-5B in HT-2 cells. HT-2EPOβγ cells were stimulated with EPO as described in *C*, and cellular lysates were immunoprecipitated with 1μl anti-STAT-5B. Immunoprecipitates were separated by 8.75% polyacrylamide gel electrophoresis, immunoblotted with anti-STAT-5A, anti-phosphotyrosine (*PTyr*, 4G10), or anti-STAT-5B antibodies as indicated.

caused such a supershift only in extracts from STAT-5A transfectants, and the anti-STAT-5B antiserum caused a supershift only in extracts from STAT-5B transfectants. In addition, a nonspecific (*NS*) reactivity was present in the anti-STAT-5A and -5B antisera that was also found in preimmune sera (Fig. 5, *lanes 5* and *6*), but the bands representing specific supershifts are nonetheless diagnostic for the presence of STAT-5A or -5B.

In order to determine precisely which STAT-5 homologue(s) were induced by IL-2 in T cells, HT-2 cells were stimulated with IL-2 or IL-4 as a negative control and EMSA was performed as described in Fig. 4*B*. As shown in Fig. 4*C*, the IL-2-induced DNA binding complex reacted with both antisera specific for either STAT-5A or STAT-5B by causing a diminution in the DNA binding complex with a corresponding appearance of a specific supershift at the top of the gel (Fig. 4*C*, *lanes 3* and *4*). These findings imply that the IL-2R mediates the induction of both STAT-5A and STAT-5B, consistent with previous observations in the IL-3R system (30). This reactivity was not observed with preimmune sera from the same animals (Fig. 5, *lanes 5* and *6*), nor were IL-4-induced DNA binding complexes containing STAT-6 affected in this way (Fig. 4*C*, *lanes 6* and *7*). The finding that virtually the entire DNA binding complex induced by IL-2 in HT-2 cells was competed with anti-STAT-5A antibodies suggested that all of the DNA binding complexes contained STAT-5A (Fig. 4*C*, *lane 3*). However, the fact that only a partial supershift of the complex was achieved with anti-STAT-5B (Fig. 4*C*, *lane 4*) argued that the nucleoprotein complexes represented a combination of STAT-5A homodimers and STAT-5A/STAT-5B heterodimers.

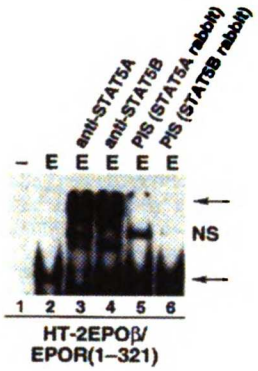

FIG. 5. **The γ_c chain can be replaced with a heterologous receptor subunit in STAT-5A/B induction.** HT-2EPOβ/EPOR(1–321) cells were stimulated with EPO (*E*) and nuclear extracts subjected to EMSA as described in Fig. 4*B*. Nuclear extracts were also incubated with 1 μl of specific preimmune serum (*PIS*) for 30 min on ice prior to EMSA (*lanes 5* and *6*). The *top arrow* indicates the supershifted band, and the *bottom arrow* indicates STAT-5. *NS*, nonspecific DNA binding complex.

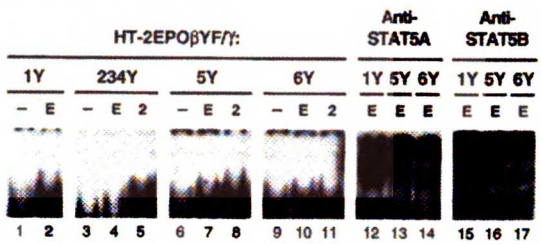

FIG. 6. **Redundant tyrosine residues of the IL-2Rβ chain induce STAT-5A/5B in HT-2 cells.** HT-2EPOβYF:1Y/γ, HT-2EPOβYF:234Y/γ, HT-2EPOβYF:5Y/γ, and HT-2EPOβYF:6Y/γ cells were stimulated with 50 units/ml EPO (*E*) (*lanes 2, 4, 7, 10,* and *12–17*) or 10 nM IL-2 (*2*) (*lanes 5, 8,* and *11*) and nuclear extracts subjected to EMSA and antibody competitions performed as described in Fig. 4*B*.

To assess directly whether signaling through the IL-2R induces heterodimerization of STAT-5A and -5B, lysates from uninduced or EPO-induced HT-2EPOβγ cells were immunoprecipitated with anti-STAT-5B antibodies and immunoblotted with anti-STAT-5A antisera. As shown in Fig. 4*D*, the presence of STAT-5A was dramatically increased in anti-STAT-5B immunoprecipitates following induction of the IL-2R, indicating that STAT-5B and STAT-5A form heterodimers primarily after stimulation (*panel 1*). The low level of background bands seen in the uninduced cells may suggest that some STAT-5A and STAT-5B molecules are preassociated prior to receptor stimulation, but this association is significantly and reproducibly enhanced upon ligation of the receptor. Since dimerization of STAT factors is dependent on reciprocal SH2/phosphotyrosine interactions (39), the phosphorylation status of the STAT-5B immunoprecipitates was assessed by stripping the blot and re-probing with anti-phosphotyrosine antibodies. Indeed, STAT-5B was found to be phosphorylated only after EPO stimulation (Fig. 4*D*, *panel 2*). Finally, efficient immunoprecipitation of STAT-5B was confirmed by stripping and reprobing the blot with anti-STAT-5B (Fig. 4*D*, *panel 3*). Of course, these data do not rule out the possibility that STAT-5A and STAT-5B homodimers are also present in the DNA binding complexes in addition to the STAT-5A/B heterodimers. Nevertheless, IL-2R stimulation induces the DNA binding activities of both STAT-5A and STAT-5B, involving the formation of heterodimers (and possibly homodimers) *in vivo*.

Neither the Four Cytoplasmic Tyrosine Residues of γ_c nor JAK3 Are Required for STAT-5A/B Induction

Previous studies have demonstrated that a truncated EPO receptor (EPOR(1–321)) is capable of functionally substituting for EPOγ in the IL-2 receptor complex, even though it physically associates with and activates JAK2 rather than JAK3 (Fig. 2*A*) (14, 38). To determine whether the γ_c chain exerts any influence on the specific composition of the DNA binding complex induced by the IL-2R, supershift analyses were performed with nuclear extracts prepared from HT-2EPOβ/EPOR(1–321) cells. Consistent with previous observations, STAT-5A and -5B were both significantly induced in HT-2EPOβ/EPOR(1–321) cells (Fig. 5), indicating heterodimer formation. In addition, STAT-5A and STAT-5B heterodimers were also induced in HT-2EPOβ/γYF and HT-2EPOβ/γ336 cells (data not shown). These results confirmed that the γ_c chain plays no role in determining the specificity of the STAT-5 isoform induced following ligation of the IL-2R, and that JAK3 can be replaced with JAK2 in the receptor complex without adverse functional effects in the activation of select STAT factors.

Specific Tyrosine Residues of the IL-2Rβ Chain Regulate STAT-5A/B Induction

Since tyrosine residues within the γ_c subunit failed to specify STAT-5A/B induction (Fig. 5) (23), studies were performed to delineate the role of the IL-2Rβ chain in this process. Previous studies have established that one or more tyrosine residues of the IL-2Rβ are required for STAT-5 activation, because a mutant IL-2Rβ chain lacking all cytoplasmic tyrosine residues failed to induce STAT-5 DNA binding activity in HT-2 cells (23). The abilities of the EPOβ tyrosine reconstitution mutants to activate STAT-5A and -5B were therefore tested in EMSA. Consistent with prior *in vitro* peptide competition data (23, 26), HT-2 cells expressing either of the two C-terminal tyrosine residues (EPOβYF:5Y/γ or EPOβYF:6Y/γ) each induced STAT-5A and STAT-5B in response to EPO (Fig. 6, *lanes 7* and *10*). Furthermore, supershift analyses suggested that typical STAT-5A/B heterodimers were assembled via these receptors (Fig. 6, *lanes 12–17*). In contrast, HT-2EPOβYF:234Y/γ cells failed to induce significant STAT-5A/B DNA binding activity, despite the activation of Janus kinases in these cells (Fig. 3). Unexpectedly, HT-2 cells expressing EPOβYF:1Y/γ also proved competent to activate STAT-5A/B (Fig. 6, *lane 2*). The relatively higher levels of STAT-5 induction by the EPOβYF:1Y cell line as compared to the EPOβYF:5Y and EPOβYF:6Y cell lines shown in this gel were not consistently observed; thus all three mutants appear to activate STAT-5A/B roughly equivalently. In addition, EPOβYF:1Y induced both STAT-5A/B in cells expressing EPOγYF, indicating that γ_c tyrosine residues do not exhibit a compensatory function for absent IL-2Rβ residues (data not shown). These results underscore both redundancy and specificity within the IL-2R system, since STAT-5A/B can be activated through three different tyrosine residues but not by three other cytoplasmic tyrosines.

DISCUSSION

The IL-2 Receptor Is Composed of Distinct, Functional Modules—One of the paradoxes in cytokine receptor signal transduction is that a high degree of signaling specificity is achieved even though receptor subunits and intracellular signaling intermediates are shared among multiple receptor types. In this regard, the γ_c subunit corresponds to a relatively short chain that participates in the IL-2, -4, -7, -9, and -15 receptors (4–7). However, γ_c appears to confer no detectable specificity to the IL-2 signaling program. Indeed, none of the four cytoplasmic

tyrosine residues within γ_c nor distal sequences within the γ_c tail is required for effective signaling, including proliferation, induction of the c-*fos* and *bcl-2* proto-oncogenes, or STAT-5A and -5B activation (Figs. 1*C*, 2*A*, and 5) (14). Furthermore, while JAK3 is bound to proximal sequence elements in γ_c, this cytoplasmic domain and Janus kinase can be replaced by a truncated form of the erythropoietin receptor bound to JAK2 with no loss of signaling specificity (Figs. 2*A* and 5) (14). Nevertheless, γ_c-associated JAK activity appears to be necessary for native IL-2R signaling, since a dominant negative JAK3 mutant lacking intrinsic kinase activity effectively disrupts signal transduction (42). In addition, natural mutations of γ_c that abrogate JAK3 binding lead to clinically significant immunodeficiencies (43). Taken together, our present findings support the notion that γ_c plays an important role in the initiation of transmembrane signaling but is likely dispensable for subsequent execution and completion of specific signals. Accordingly, we have suggested that γ_c and its associated JAK3 function as a "trigger" chain in the IL-2R complex (14).

In contrast, the subunits that pair with γ_c in the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors have relatively long cytoplasmic domains that typically associate with multiple signaling intermediates. For example, while the IL-2Rβ chain binds specifically to such molecules as Shc and STAT-5 (22–24, 26–28), the IL-4R subunit binds to STAT-6 (44, 45), thus specifying a very different signaling program. The present findings provide further molecular evidence that these longer subunits play a major role in driving the specificity of the signaling program; thus, we have referred to this class of receptor subunits as "driver" chains (14).

Signaling Modules Are Specific, Redundant, and Multifunctional—The findings reported here illustrate several properties of the IL-2 receptor "driver" subunit, the IL-2Rβ chain, that characterize its mechanism of signaling. First, within the IL-2Rβ chain, there are multiple functional, tyrosine-based peptide modules that exhibit a high degree of specificity in coupling to signaling pathways (summarized in Fig. 7*B*). For example, the ability of an individual tyrosine residue to drive a specific signal is manifested in the activation of c-*fos* gene expression. Data presented here illustrate that Y1 (Tyr-338) is uniquely required for inducing c-*fos* mRNA levels (Fig. 2*B*), while another recent study has shown that Y1 (Tyr-338) is the only cytoplasmic tyrosine residue within IL-2Rβ that binds to the Shc adapter molecule after IL-2Rβ phosphorylation (22). Taken together, these observations suggest that c-*fos* is likely induced via the Ras-Raf pathway that is activated by Shc (reviewed in Ref. 46). These experiments have also shown that the c-*fos* and STAT-5 pathways appear to be distinct. STAT-5 can be activated through other tyrosine residues (Y5 (Tyr-392) and Y6 (Tyr-510)) that fail to induce c-*fos* or bind Shc (Figs. 2*B* and 6) (22). However, since both pathways are induced through an IL-2Rβ mutant that contains only Y1 (Tyr-338), these studies do not preclude the possibility that STAT-5 activation is necessary but not sufficient for AP-1 induction by this receptor (47).

Second, in contrast to the selective activation of c-*fos* by Y1 (Tyr-338), there is marked redundancy in the abilities of certain tyrosine residues within IL-2Rβ to mediate other signaling events. For example, Y1 (Tyr-338), Y5 (Tyr-392), and Y6 (Tyr-510) all promote activation of STAT-5 nuclear import and DNA binding activity in HT-2 cells (Fig. 6), despite clear sequence differences in the amino acid sequences flanking Tyr-338 and Tyr-392/Tyr-510 (Fig. 7*A* and see below). Importantly, efficient STAT5A/5B heterodimerization is induced via an IL-2Rβ mutant retaining a single STAT-binding motif, indicating that the receptor does not necessarily form a multivalent "platform" to

A

Receptor	Tyrosine	Sequence							
IL-2Rβ	338	H	Q	G	Y	F	F	F	H
IL-2Rβ	392	D	D	A	Y	C	T	F	P
IL-2Rβ	510	T	D	A	Y	L	S	L	Q
EPO	367	Q	D	T	Y	L	V	L	D
IL-3	881	Q	D	-	Y	L	S	L	P
IL-3	765	F	E	D	Y	V	E	L	P
IL-7	409	Q	E	A	Y	V	T	M	S
GH	548	E	D	I	Y	I	T	T	E
GH	577	P	D	-	Y	T	S	I	H
PRL	382	L	D	-	Y	L	D	P	T
		X	D	A	Y	L	S/T	L	X

B

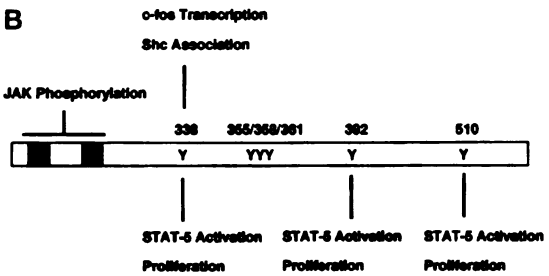

FIG. 7. *A*, amino acid motifs flanking tyrosine residues of STAT-5-inducing receptors. *B*, specific signaling events mediated by IL-2Rβ tyrosine residues located in the cytoplasmic tail. Conserved Box1 (*1*) and Box2 (*2*) motifs are *shaded* (53), and relative positions of the six tyrosine (*Y*) residues are indicated.

which multiple STATs are recruited simultaneously in order to direct heterodimer or homodimer formation. Similar redundancy among Y1 (Tyr-338), Y5 (Tyr-392), and Y6 (Tyr-510) is observed in the activation of cellular proliferation (Fig. 1) (11), although STAT-5 activation and proliferation are probably independent events (23, 24). The biological importance of such functional redundancy is unknown, but it may allow for amplification of signaling, as has been demonstrated for repetitive tyrosine-based motifs present in the T cell receptor complex (48).

Third, the studies performed here indicate that individual tyrosine-based motifs in the IL-2Rβ chain can serve multiple, apparently independent functions. For example, as described above, Y1 (Tyr-338) couples both to a Shc-dependent pathway as well as to the JAK-STAT pathway (Fig. 7*B*). Moreover, two isoforms of STAT-5 are induced through single tyrosine residues, indicating at least some potential for multifunctionality through these sequences. Since both STAT-1 and STAT-3 have been implicated in IL-2 signaling in other systems (26, 40), it is likely that more STAT factors may couple to some or all of the receptor tyrosine residues.

Interestingly, no obvious function for the Tyr-234 (Tyr-355/Tyr-358/Tyr-361) tyrosine cluster has yet been detected in HT-2 cells. Although it is unknown whether the restriction of function of Tyr-234 occurs at the level of phosphorylation or at the level of binding to particular signaling intermediates, it is nonetheless clear that there is strict selectivity in which tyrosines are capable of mediating such events.

Finally, at least some signaling pathways are coupled to the IL-2R through a tyrosine-independent mechanism, as represented by induction of the proto-oncogene *bcl-2*. It has been previously shown that *bcl-2* induction is independent of JAK3 activation (42), but the data presented here represent the first direct demonstration that *bcl-2* induction is also independent of tyrosine residues within the IL-2 receptor. Further studies are needed to determine how tyrosine-independent pathways are

initiated by cytokine receptors after receptor activation. Other studies with the growth hormone (GH) and erythropoietin receptor have suggested that receptor tyrosine residues are apparently not absolutely essential for activation of proliferation and the JAK-STAT pathway in these receptor systems (49, 50). It remains to be determined whether these observations represent a fundamental difference in the mechanism of signaling between the EPOR/GHR and the IL-2 receptors, or whether low levels of endogenous EPO and GH receptors might contribute to the observed functionality of the transfected receptor mutants.

Cell Context Specificity of STAT-binding Motifs—Among cytokine receptors it is often possible to identify putative functional "driver" modules based on sequence alone, such as IRS-1- or STAT-binding motifs (10, 44). The present studies reveal additional complexities underlying STAT-5-activation motifs and the role of additional cellular factors in permitting STAT activation. First, in HT-2 cells, we have shown that two isoforms of STAT-5 (STAT-5A and -5B) are induced by IL-2 stimulation through three different tyrosine residues within IL-2Rβ (Figs. 4 and 6). Surprisingly, however, although the sequences surrounding Y5 (Tyr-392) and Y6 (Tyr-510) are quite similar to tyrosine motifs found in other STAT-5-inducing receptors (D A Y L S/T L), the functionally redundant Y1 (Tyr-338) is notably divergent from these in primary sequence (N Q G Y F F F) (Fig. 7*A*). Moreover, the context of the prolactin receptor tyrosine residue that induces transcription through a STAT-response element differs somewhat from these motifs (LDYLDPT) (51). Therefore, STAT-5 appears to be activated through as many as three different tyrosine-based sequences.

In addition, there appears to be an influence of cellular environment on STAT-5 activation. First, in the pro-B/myeloid cell line Ba/F3, a truncated IL-2Rβ chain retaining Y1 (Tyr-338) fails to activate STAT-5 DNA binding, although it still maintains the ability to induce cellular proliferation (24). These observations contrast with the present observation that Y1 (Tyr-338) induces STAT-5A/B in IL-2-dependent T cells (Fig. 6). Second, the effects of point substitutions at Y1 (Tyr-338) on proliferation signaling appear to vary depending on cellular context (11). Moreover, the EPOR can induce STAT-5 only in certain cellular contexts[3] (52). These data suggest that the type of cell chosen for receptor studies may be important in dissecting specific consequences of cytokine signaling. In summary, a full understanding of STAT-activation motifs and their functions in different cellular environments will require further study.

Modularity within the Cytokine Receptor Superfamily—In summary, the present studies reveal that tyrosine-based sequences located in the IL-2Rβ chain exhibit specificity, redundancy and multifunctionality in regulating signaling events initiated by IL-2. Since the signaling portion of IL-15 receptor contains both the IL-2Rβ and γ_c chains (7), these findings probably apply to this cytokine as well. It is likely that analogous modules in "driver" subunits within other receptor complexes also exhibit these characteristics, and that the combinatorial association of various modules within a receptor complex permits a diverse range of biologic consequences. For example, some domains may have synergistic or inhibitory influences on one another. A more complete understanding of such interactions is essential to developing strategies to manipulate these intracellular processes for clinical benefit.

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[3] S. Y. Lai, unpublished observations.

REFERENCES

1. Paul, W. E., and Seder, R. A. (1994) *Cell* **76**, 241–251
2. Bazan, J. F. (1990) *Proc. Nat. Acad. Sci. U. S. A.* **87**, 6934–6938
3. Goldsmith, M. A., and Greene, W. C. (1994) *Interleukin-2 and the Interleukin-2 Receptor Complex*, Paston Press, Ltd., Norfolk, England
4. Noguchi, M., Nakamura, Y., Russel, S. M., Ziegler, S. F., Tsang, M., Cao, X., and Leonard, W. J. (1993) *Science* **262**, 1877–1880
5. Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedman, M. C., Miyajima, A., Puri, R. K., Paul, W. E., and Leonard, W. J. (1993) *Science* **262**, 1880–1883
6. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-i., and Sugamura, K. (1993) *Science* **262**, 1874–1877
7. Giri, J. G., Ahdieh, M., Eisenmann, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. (1994) *EMBO J.* **13**, 2822–2830
8. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* **72**, 767–778
9. Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1995) *Science* **268**, 1177–1180
10. Stahl, N., Farruggella, T. J., Boulton, T. G., Zhong, Z., Darnell, J. E., and Yancopoulos, G. D. (1995) *Science* **267**, 1349–1353
11. Goldsmith, M. A., Lai, S. Y., Xu, W., Amaral, M. C., Kuczek, E. S., Parent, L. J., Mills, G. B., Tarr, K. L., Longmore, G. D., and Greene, W. C. (1996) *J. Biol. Chem.* **270**, 21729–21737
12. Nelson, B. H., Lord, J. D., and Greenberg, P. D. (1994) *Nature* **369**, 333–336
13. Nakamura, Y., Russell, S. M., Mess, S. A., Friedmann, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S., and Leonard, W. J. (1994) *Nature* **369**, 330–333
14. Lai, S. Y., Xu, W., Gaffen, S., Liu, K. D., Greene, W. C., and Goldsmith, M. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 231–235
15. Ihle, J. N. (1995) *Nature* **377**, 591–594
16. Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Bacon, C. M., Friedmann, M., Berg, M., McVicar, D. W., Whitthuhn, B. A., Silvennoinen, O., Goldman, A. S., Schmalsteig, F. C., Ihle, J. N., O'Shea, J. J., and Leonard, W. J. (1994) *Science* **266**, 1042–1045
17. Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z.-J., Oishi, I., Silvennoinen, O., Witthuhn, B. A., Ihle, J. N., and Taniguchi, T. (1994) *Science* **266**, 1045–1047
18. Johnston, J. A., Kawamura, M., Kirken, R. A., Chen, Y.-Q., Blake, T. B., Shibuya, K., Ortaldo, J. R., McVicar, D. W., and O'Shea, J. J. (1994) *Nature* **370**, 151–153
19. Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T., and Ihle, J. N. (1994) *Nature* **370**, 153–157
20. Sugamura, K., Takeshita, T., Asao, H., Kumaki, S., Ohbo, K., Ohtani, K., and Nakamura, M. (1990) *Lymphokine Res.* **9**, 539–543
21. Asao, H., Takeshita, T., Nakamura, M., Nagata, K., and Sugamura, K. (1990) *J. Exp. Med.* **171**, 637–644
22. Evans, G. A., Goldsmith, M. A., Johnston, J. A., Xu, W., Weiler, S. R., Erwin, R., Howard, O. M. Z., Abraham, R. T., O'Shea, J. J., Greene, W. C., and Farrar, W. L. (1995) *J. Biol. Chem.* **270**, 28858–28863
23. Gaffen, S. L., Lai, S. Y., Xu, W., Gouilleux, F., Groner, B., Goldsmith, M. A., and Greene, W. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7192–7196
24. Fujii, H., Nakagawa, Y., Schindler, U., Kawahara, A., Mori, H., Gouilleux, F., Groner, B., Ihle, J. N., Minami, Y., Miyazaki, T., and Taniguchi, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5482–5486
25. Hou, J., Schindler, U., Henzel, W. J., Wong, S. C., and McKnight, S. L. (1995) *Immunity* **2**, 325–330
26. Lin, J.-X., Migone, T.-S., Tsang, M., Friedmann, M., Weatherbee, J. A., Zhou, L., Yamauchi, A., Bloom, E. T., Mietz, J., John, S., and Leonard, W. J. (1995) *Immunity* **2**, 331–339
27. Gilmour, K. C., Pine, R., and Reich, N. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10772–10776
28. Frank, D. A., Robertson, M. J., Bonni, A., Ritz, J., and Greenberg, M. E. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7779–7783
29. Goldsmith, M. A., Xu, W., Amaral, M. C., Kuczek, E. S., and Greene, W. C. (1994) *J. Biol. Chem.* **269**, 14698–14704
30. Mui, A. L.-F., Wakao, H., O'Farrell, A.-M., Harada, N., and Miyajima, A. (1995) *EMBO J.* **14**, 1166–1175
31. Lamson, G., and Koshland, M. E. (1984) *J. Exp. Med.* **160**, 877–892
32. Adams, M. D., Dubnick, M., Kerlavage, A. R., Moreno, R., Kelley, J. M., Utterback, T. R., Nagle, J. W., Fields, C., and Venter, J. C. (1992) *Nature* **355**, 632–634
33. van Beveren, C., Van Straaten, F., Curran, T., Muller, R., and Verma, I. M. (1983) *Cell* **32**, 1241–1255
34. Nunez, G., London, L., Hockenberry, D., Alexander, M., McKearn, J. P., and Korsmeyer, S. J. (1990) *J. Immunol.* **144**, 3602–3610
35. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419–6420
36. Hatakeyama, M., Kawahara, A., Mori, H., Shibuya, H., and Taniguchi, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2022–2026
37. Miyazaki, T., Liu, Z.-J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E. L., Perlmutter, R. M., and Taniguchi, T. (1995) *Cell* **81**, 223–231
38. Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ihle, J. N. (1993) *Cell* **74**, 227–236
39. Darnell, J. E., Kerr, I. M., and Stark, G. R. (1994) *Science* **264**, 1415–1421
40. Beadling, C., Gischin, D., Witthuhn, B. A., Ziemiecki, A., Ihle, J. N., Kerr, I. M., and Cantrell, D. A. (1994) *EMBO J.* **13**, 5605–5615
41. Azam, M., Erdjument-Bromage, H., Kiedler, B. L., Xia, M., Quelle, F., Basu, R., Saris, C., Tempst, P., Ihle, J. N., and Schindler, C. (1995) *EMBO J.* **14**,

1402–1411
42. Kawahara, A., Minami, Y., Miyazaki, T., Ihle, J. N., and Taniguchi, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8724–8728
43. Russell, S. M., Tayebi, N., Nakajima, H., Reidy, M. C., Roberts, J. L., Aman, M. J., Migone, T.-S., Noguchi, M., Markert, M. L., Buckley, R. H., O'Shea, J. J., and Leonard, W. J. (1995) *Science* **270,** 797–799
44. Keegan, A. D., Nelms, K., White, M., Wang, L.-M., Pierce, J., and Paul, W. E. (1994) *Cell* **76,** 811–820
45. Hou, J., Schindler, U., Henzel, W. J., Ho, T. C., Brasseur, M., and Mcknight, S. L. (1994) *Science* **265,** 1701–1706
46. Pronk, G. J., and Bos, J. L. (1994) *Biochim. Biophys. Acta* **1198,** 131–134
47. Coffer, P., Lutticken, C., Van Puijenbroek, A., Klop-de Jonge, M., Horn, F., and Kruijer, W. (1995) *Oncogene* **10,** 985–994
48. Weiss, A., and Littman, D. R. (1994) *Cell* **76,** 263–274
49. Wang, Y.-D., Wong, K., and Wood, W. I. (1995) *J. Biol. Chem.* **270,** 7021–7024
50. Damen, J. E., Wakao, H., Miyajima, A., Krosi, J., Humphries, R. K., Cutler, R. L., and Krystal, G. (1995) *EMBO J.* **14,** 5557–5568
51. Lebrun, J.-J., Ali, S., Goffin, V., Ullrich, A., and Kelly, P. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4031–4035
52. Wakao, H., Harada, N., Kitamura, T., Mui, A. L.-F., and Miyajima, A. (1995) *EMBO J.* **14,** 2527–2535
53. Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T., and Kishimoto, T. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11349–11353

Chapter 5

The Shared γ_c Subunit Within the Human Interleukin-7 Receptor Complex: A Molecular Basis for the Pathogenesis of X-Linked Severe Combined Immunodeficiency

Prologue

Following the characterization of the IL-2 receptor system and the description of receptor complex architecture in the "trigger-driver" model, we examined other receptor complexes that employ the γ_c chain. The IL-7R complex was of particular interest because of recent gene-ablation studies. Specifically, deletion of the gene for IL-7 (1) or the IL-7R α subunit (2) resulted in severe developmental defects in the murine immune system that paralleled those defects found in human X-linked severe combined immunodeficiency (X-SCID) (3). Thus, the studies presented here examined the role of receptor subunit heterodimerization for signaling activation by IL-7R and the functional role of each subunit within the IL-7R complex. The findings were very analagous to those for the IL-2R complex and provide additional support for the "trigger-driver" model. The following paper, as presented here, is in press.

References

1 U. von Freeden-Jeffry, et al. *JEM* **181**, 1519-26 (1995).

2 J.J. Peschon, et al. *JEM* **180**, 1955-60 (1994).

3 M.E. Conley *Ann Rev Immunol* **10**, 215-38 (1992).

Shared γ_c Subunit Within the Human Interleukin-7 Receptor Complex

A Molecular Basis for the Pathogenesis of X-Linked Severe Combined Immunodeficiency

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Abstract

Genetic evidence suggests that mutations in the γ_c receptor subunit cause X-linked severe combined immunodeficiency (X-SCID). The γ_c subunit can be employed in receptor complexes for IL-2, -4, -7, -9, and -15, and the multiple signaling defects that would result from a defective γ_c chain in these receptors are proposed to cause the severe phenotype of X-SCID patients. Interestingly, gene disruption of either IL-7 or the IL-7 receptor (IL-7R) α subunit in mice leads to immunological defects that are similar to human X-SCID. These observations suggest the functional importance of γ_c in the IL-7R complex. In the present study, structure/function analyses of the IL-7R complex using a chimeric receptor system demonstrated that γ_c is indeed critical for IL-7R function. Nonetheless, only a limited portion of the cytoplasmic domain of γ_c is necessary for IL-7R signal transduction. Furthermore, replacement of the γ_c cytoplasmic domain by a severely truncated erythropoeitin receptor does not affect measured IL-7R signaling events. These findings support a model in which γ_c serves primarily to activate signal transduction by the IL-7R complex, while IL-7Rα determines specific signaling events through its association with cytoplasmic signaling molecules. Finally, these studies are consistent with the hypothesis that the molecular pathogenesis of X-SCID is due primarily to γ_c-mediated defects in the IL-7/IL-7R system. (*J. Clin. Invest.* 1997. 99:■■■■–■■■■.) Key words: interleukin-7 • receptor • X-linked severe combined immunodeficiency • signal transduction • specificity

Introduction

Various cytokines contribute to the development and regulation of lymphocytes. A number of cytokines including IL-2, -4, -7, -9, and -15 engage receptor complexes composed of a specific subunit in conjunction with the shared γ_c chain (1–9). Human mutations in γ_c have been linked to X-linked severe combined immunodeficiency (X-SCID),[1] a disease characterized by severe lymphopenia and recurring persistent infections in the first months of life (10–12). Additionally, gene deletion studies in mice of γ_c have revealed a similar immunodeficiency (13, 14). The wide array of receptor signaling defects that result from γ_c mutations are hypothesized to lead to the severe immunological defects found in both X-SCID patients and γ_c-deletion murine models.

Recent studies, however, suggest that more selective signaling defects due to γ_c mutations may cause X-SCID and the murine immunodeficiency syndrome. Gene ablation of IL-2 (15) and IL-2 receptor (IL-2R)β/IL-15Rβ (16) do not cause early developmental defects of the immune system. Furthermore, IL-4 gene-ablation studies do not detect early lymphopoietic and functional disturbances, suggesting that γ_c-independent forms of the IL-4R are biologically active (17–21). Additionally, the role of γ_c in the IL-9R complex remains undefined. The γ_c subunit does not appear to modulate IL-9R binding affinity for IL-9 (8) and IL-9 may act primarily on mast cells (22). In contrast, gene disruption of either IL-7 (23) or the IL-7Rα subunit (24) leads to severe developmental perturbations. Thus, signaling defects in the IL-7/IL-7R system resulting from changes in γ_c may be sufficient to account for the developmental anomalies that lead to X-SCID.

IL-7 was identified as a factor secreted by stromal cells of the bone marrow and thymus that stimulates proliferation of immature B and T lymphocytes (25–31). The biological effects of IL-7 are mediated through a receptor complex containing the IL-7Rα subunit (32) and the γ_c chain (3, 6, 9). Both of these receptor subunits are members of the cytokine receptor superfamily (33), sharing a number of structural features including two partially conserved, intracellular regions termed Box1 and Box2 that are involved in Janus kinase (JAK) association (7, 34–36). Engagement of these subunits by IL-7 leads to the activation of the Janus kinases, JAK1 and JAK3, and the subsequent induction of a signal transducer and activator of transcription (STAT) factor, STAT-5 (7, 19, 37). Additionally, activation of the src-like kinase family members p56lck, p59fyn, and p53/p56lyn has been reported in various cellular contexts (38–40). Downstream of the activation of tyrosine kinases is the activation of

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1. *Abbreviations used in this paper:* CMF-PBS, calcium- and magnesium-free PBS; EMSA, electrophoretic mobility shift assay; EPOR, erythropoietin receptor; IRS-1, insulin receptor substrate-1; JAK, Janus kinase; STAT, signal transducer and activator of transcription; X-SCID, X-linked severe combined immunodeficiency.

the insulin receptor substrate-1 (IRS-1) (41) and phosphatidylinositol-3-kinase (42, 43). Collectively, these and related specific molecular events culminate in differentiation and proliferation by IL-7.

While many of the specific signaling events activated by the IL-7R complex have been delineated, the functional roles of the receptor subunits themselves in generating these signals are relatively undefined. However, the heterodimeric structure of the IL-7R complex suggests distinct functions for the individual receptor subunits. Thus, the present studies were undertaken to identify the functional roles of IL-7Rα and γ_c within the IL-7R complex. These observations define the structural determinants of signaling specificity within the IL-7R complex and further clarify mechanisms associated with the molecular pathogenesis of X-SCID.

Methods

Cell lines and reagents. HT-2, an IL-2–dependent murine helper T cell line (American Type Culture Collection, Rockville, MD) was cultured in RPMI 1640 supplemented with 10% FBS, 55 μM β-mercaptoethanol (β-ME), 2 mM L-glutamine, and 200 U/ml recombinant human IL-2 (a generous gift of Chiron Corp, Emeryville, CA). 32D/IRS-1, a pro-myeloid cell line stably expressing the IRS-1, was maintained in 32D medium (RPMI 1640 containing 10% FBS and 5% WEHI 3B-conditioned medium). The SCID-MA cell line was maintained in RPMI 1640 with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, and 5 μM β-ME.

Electroporation of cells was performed as described previously (44), and stable transfectants were obtained by selection in G418 (1 mg/ml Geneticin; GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD). Clones isolated by limiting dilution were screened by Northern blot analysis to identify clones expressing the transfected receptor subunit(s). HT-2 stable cell lines expressing two receptor subunits were derived from cells already expressing either the ropoietin (EPOγ) or EPOγYF chain. After electroporation, stable transfectants were isolated by selection in G418 (1 mg/ml) and hygromycin B (500 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) and screened by Northern blot analysis. The antiphosphotyrosine monoclonal antibody (4G10), anti–JAK1, –JAK2, and –JAK3 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Human IL-4 and IL-9 were obtained from R & D Systems, Inc. (Minneapolis, MN). Recombinant human IL-7 was from Genzyme, Corp. (Cambridge, MA) and recombinant human EPO was the generous gift of Ortho Diagnostic Systems Inc. (Raritan, NJ).

Plasmid constructs. All receptor cDNAs were subcloned into the expression vectors pCMV4Neo (44) or pCMV4Hygro, a derivative of pCMV4 (45) containing a hygromycin B–resistance gene as a selectable marker. The cytoplasmic portion of the IL-7Rα subunit was isolated by PCR from PBMC cDNA. pEPO7neo was constructed by PCR using a NheI site at the fusion junction. The chimeric receptor subunit contains the extracellular domain of the erythopoietin receptor (EPOR) fused just above the transmembrane segment to the human IL-7Rα transmembrane and cytoplasmic domains (resulting sequence: . . . (EPOR-T-A-S)-(I-N-N-IL-7α) . . .). The EPOγ and the chimeric γ_c receptor mutants were constructed as previously described (46). A stop codon was introduced at the HindIII site present in the coding sequence of EPO7 to create the $EPO7_{TR}$ subunit that lacks the distal 100 amino acid residues. For all constructs requiring synthetic oligonucleotides or PCR reactions, sequences were confirmed by DNA sequencing.

JAK and IRS-1 phosphorylation studies. 40 × 10^6 cells were washed twice in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), stripped of cell-bound ligand for 1 min in 10 mM sodium citrate, pH 4.0, and 140 mM NaCl and rested for 4 h or overnight in RPMI 1640 medium containing 1% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO). After stimulation with the appropriate factor, cells were washed in CMF-PBS and lysed (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl, 50 mM NaF, 100 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A. Immunoprecipitations were performed with the indicated antibodies and protein A-Sepharose. Immunoblot analysis was performed with the appropriate antibodies with detection by ECL (Amersham Corp., Arlington Heights, IL) signal development. For JAK analysis, blots were stripped (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl, pH 6.7) for 30 min at 55°C and reprobed with anti–JAK1 and –JAK3 antisera to verify equivalent protein loading (data not shown).

Electrophoretic mobility shift assay. 40–60 × 10^6 cells were rested and stimulated as described above and washed in CMF-PBS. Nuclear extracts were prepared as described (47) in the presence of 1 mM sodium orthovanadate and the following protease inhibitors (in μg/ml): antipain 0.5, aprotinin 0.5, bestatin 0.75, leupeptin 0.5, pepstatin A 0.05, phosphoramidon 1.4, and soybean trypsin inhibitor 0.5 (Sigma Chemical Co.). The IgG Fc receptor STAT response element probe was end-labeled with [γ-^{32}P]dATP (Amersham Corp.) and polynucleotide kinase (New England Biolabs Inc., Beverly, MA). DNA binding studies were performed with 10^5 cpm probe, 3 μg poly[d(I-C)] and 10 μg nuclear extract as described (47*a*). Preincubation of nuclear extracts with different antibodies were performed in the absence of

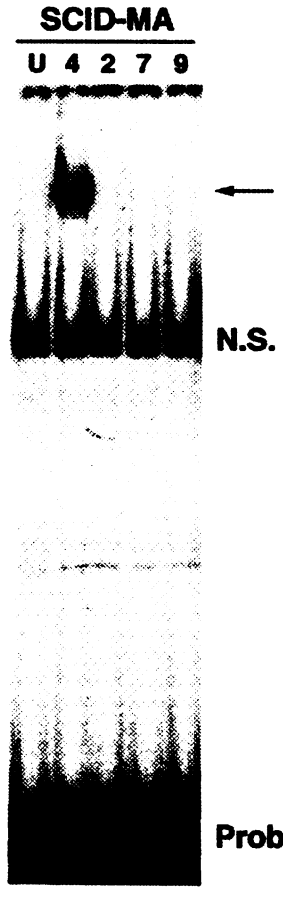

Figure 1. The γ_c mutation in an X-SCID patient results in multiple cytokine receptor signaling defects. 40 × 10^6 cells/sample were unstimulated (*U*) or stimulated for 15 minutes with various cytokines: IL-4 (4,100 ng/ml), IL-2 (2,10 nM), IL-7 (7,100 U/ml), and IL-9 (9,100 U/ml). EMSA of prepared nuclear exracts are shown. Cytokine bioactivity was verified by stimulation of various responsive cell lines and the integrity of the nuclear extracts was verified by EMSA with the SP-1 probe (data not shown). Arrow denotes IL-4–induced STAT-6 DNA-binding activity.

poly[d(I-C)] and binding buffer for 45 min on ice before initiation of the binding assay by addition of radiolabeled probe.

Proliferation assays. Conventional 24-h [^{3}H]thymidine (DuPont-NEN, Boston, MA) incorporation assays were performed as previously described (44). Briefly, 32D cells were counted, washed twice in CMF-PBS and resuspended at 10^6 cells/ml of 32D medium without the WEHI 3B–conditioned medium supplement. 10^5 cells per well were grown in the indicated concentrations of EPO for 24 h with [^{3}H]thymidine incorporation measured in the last 4 h. Transient transfection assays of EPO-induced proliferation were performed as described previously (44). [^{3}H]Thymidine incorporation was measured on days 8–16 after transfection.

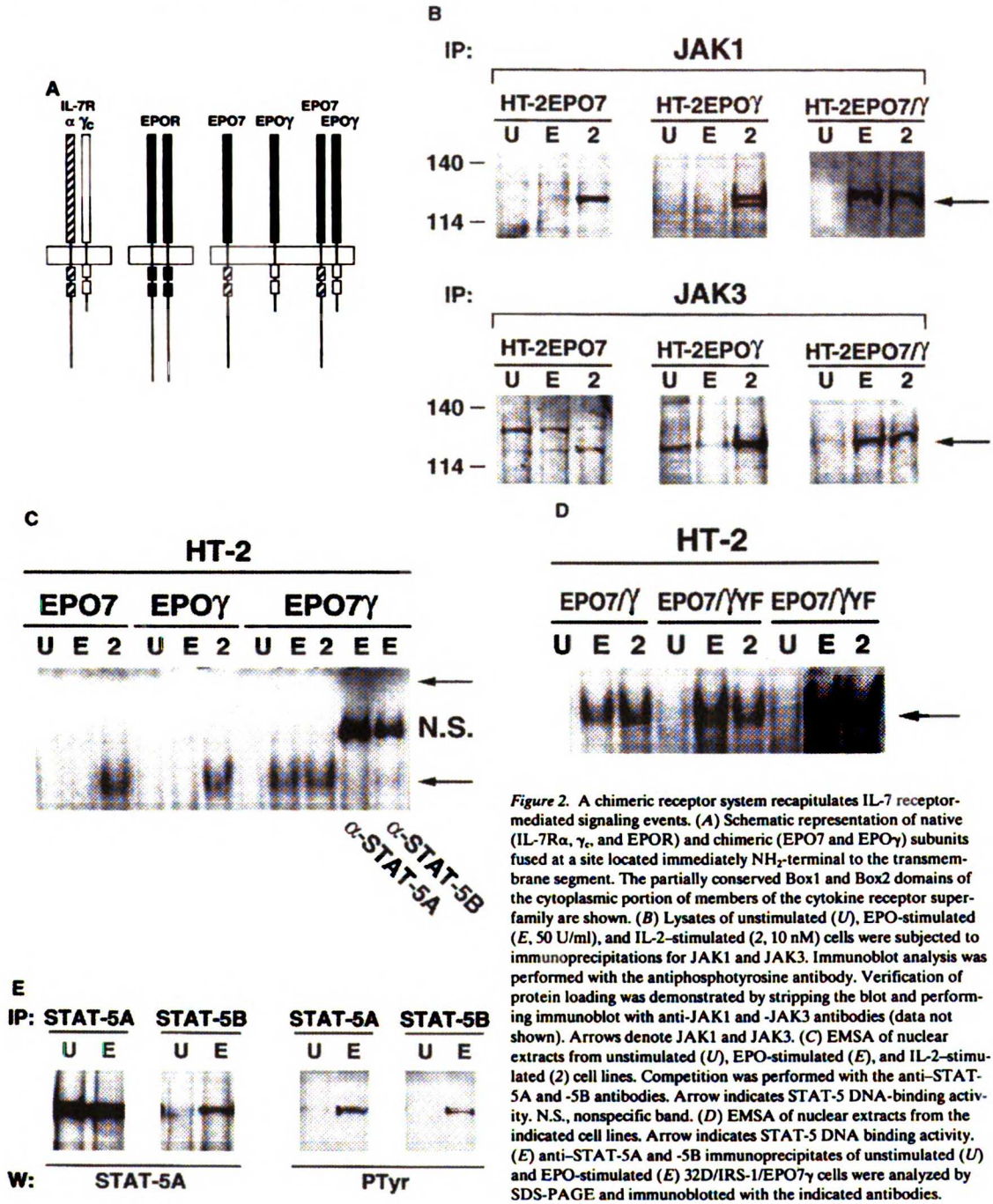

Figure 2. A chimeric receptor system recapitulates IL-7 receptor-mediated signaling events. (*A*) Schematic representation of native (IL-7Rα, γ_c, and EPOR) and chimeric (EPO7 and EPOγ) subunits fused at a site located immediately NH_2-terminal to the transmembrane segment. The partially conserved Box1 and Box2 domains of the cytoplasmic portion of members of the cytokine receptor superfamily are shown. (*B*) Lysates of unstimulated (*U*), EPO-stimulated (*E*, 50 U/ml), and IL-2–stimulated (*2*, 10 nM) cells were subjected to immunoprecipitations for JAK1 and JAK3. Immunoblot analysis was performed with the antiphosphotyrosine antibody. Verification of protein loading was demonstrated by stripping the blot and performing immunoblot with anti-JAK1 and -JAK3 antibodies (data not shown). Arrows denote JAK1 and JAK3. (*C*) EMSA of nuclear extracts from unstimulated (*U*), EPO-stimulated (*E*), and IL-2–stimulated (*2*) cell lines. Competition was performed with the anti-STAT-5A and -5B antibodies. Arrow indicates STAT-5 DNA-binding activity. N.S., nonspecific band. (*D*) EMSA of nuclear extracts from the indicated cell lines. Arrow indicates STAT-5 DNA binding activity. (*E*) anti–STAT-5A and -5B immunoprecipitates of unstimulated (*U*) and EPO-stimulated (*E*) 32D/IRS-1/EPO7γ cells were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

Results

B cells from an X-SCID patient are unresponsive to multiple cytokines that bind to receptor complexes containing the γ_c subunit. Epstein-Barr virus–transformed B cell lines derived from X-SCID patients have various mutations in the γ_c gene. For example, the SCID-MA cell line contains a mutation that results in the lack of detectable mRNA transcripts for the γ_c subunit (reference 12 and data not shown). To assess the functional consequences of this genetic defect, SCID-MA cells were stimulated with cytokines that employ γ_c-containing receptor complexes (Fig. 1). The activation of STAT factors was evaluated in an electrophoretic mobility gel shift assay (EMSA) using an oligonucleotide probe that contains the IgG Fc receptor promoter element. SCID-MA cells retained responsiveness to IL-4, as demonstrated by the retarded mobility of the radiolabeled probe and anti–STAT-6 antibody competition (Fig. 1 and data not shown). Previous studies are consistent with the hypothesis that IL-4 may engage another receptor complex(es) that does not require the γ_c subunit (17–21). However, in the absence of a functional γ_c chain, SCID-MA cells were unresponsive to IL-2, -7, and -9, raising the possibility that γ_c is critical for signaling by these receptor systems. Since IL-2 and IL-9 do not appear to affect early lymphocyte development, further analysis of the IL-7R complex and its link to X-SCID was undertaken.

A chimeric receptor system recapitulates JAK-STAT signaling of the IL-7 receptor complex. To investigate the functional roles of the individual receptor subunits in the IL-7 receptor complex, chimeric receptor subunits were formed by fusing the extracellular domain of the EPOR to the intracellular portions of IL-7Rα and γ_c to form EPO7 and EPOγ, respectively (Fig. 2 *A*). These chimeric receptor subunits were evaluated for their abilities to recapitulate IL-7 receptor signaling, specifically through the JAK-STAT pathway. Stable transfectants expressing EPO7 or EPOγ were established in HT-2 cells, a murine helper T cell line. Antiphosphotyrosine immunoblot analysis of JAK kinase immunoprecipitates from HT-2 stable transfectants demonstrated the phosphorylation of JAK1 and JAK3, but not JAK2, in response to a positive control ligand, IL-2 (Fig. 2 *B* and data not shown). Upon EPO stimulation, neither the HT-2EPO7 nor the HT-2EPOγ cell lines demonstrated an increase in the phosphorylation of JAK1 and JAK3 when compared with unstimulated cells. In contrast, a stable cell line expressing both EPO7 and EPOγ subunits, HT-2EPO7γ, displayed a marked increase in tyrosine phosphorylation of JAK1 and JAK3 in response to EPO (Fig. 2 *B*). Therefore, activation of the chimeric receptor system leads to the induction of the same JAK kinases as those reportedly linked to the native IL-7 receptor complex (7, 37, 48).

Activation of JAK kinases in cytokine receptor complexes typically leads to the recruitment and activation of STAT fac-

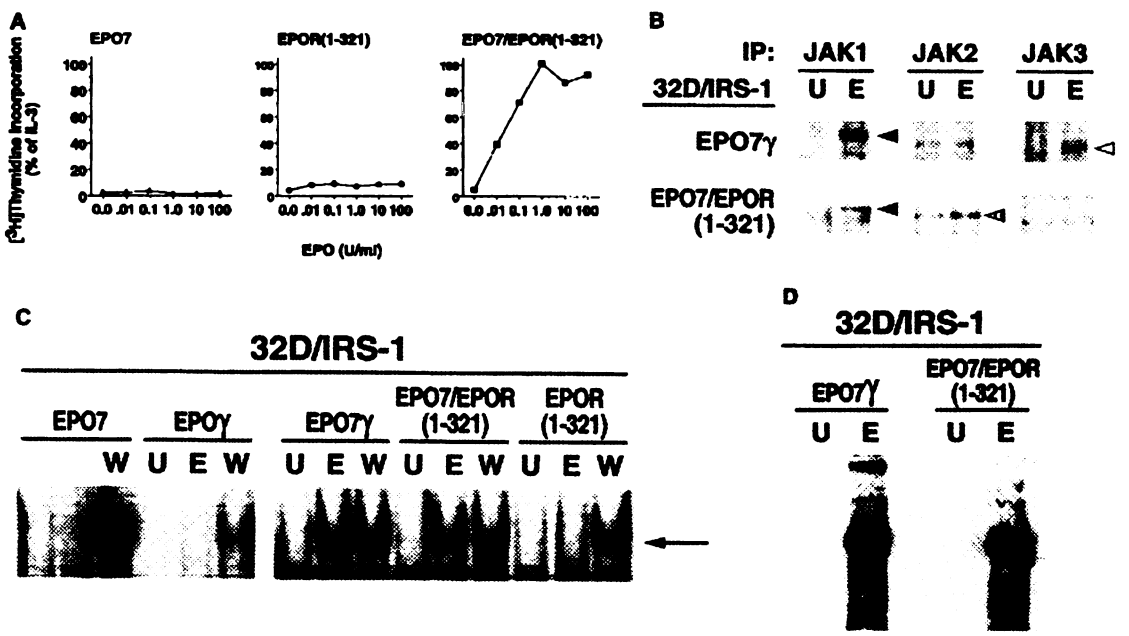

Figure 3. The functional replacement of γ_c by a truncated EPOR does not alter specific signaling events. (*A*) Stable 32D/IRS-1 transfectants were stimulated with EPO in [^{3}H]thymidine incorporation experiments. (*B*) Serial immunoprecipitates of JAK1, JAK2, and JAK3 from lysates of the indicated cell lines. Immunoblot analysis was performed with the antiphosphotyrosine antibody. Verification of protein loading was demonstrated by stripping the blot and performing immunoblot analysis with the anti–JAK1, –JAK2 and –JAK3 antibodies. Arrowheads denote JAK1 (*solid*), JAK2 (*hatched*), and JAK3 (*open*). (*C*) EMSA of nuclear extracts from the indicated 32D/IRS-1 stable transfectants that were unstimulated (*U*) or treated with EPO (*E*) or Wehi 3B-conditioned media (*W*, 10%). Arrow depicts STAT-5 DNA-binding activity. (*D*) anti–IRS-1 immunoprecipitates of lysates from the indicated cell lines that were unstimulated (*U*) or EPO stimulated (*E*). Immunoblotting was performed with the antiphosphotyrosine antibody.

tors (49). As demonstrated by EMSA using a probe corresponding to the IgG Fc receptor STAT responsive element, EPO stimulation of HT-2EPO7 and HT-2EPOγ cell lines did not result in activation of a specific DNA-binding activity (Fig. 2 *C*). Like the native IL-7 receptor complex (19), engagement of both chimeric receptor subunits by EPO stimulation of the HT-2EPO7γ cell line resulted in the activation of STAT-5 (Fig. 2 *C*). This DNA-binding complex contained both STAT-5A and -5B, as demonstrated by specific antibody competition that diminished the presence of the retarded nucleoprotein complex.

To examine the IL-7R complex in another cell context, the chimeric receptor system was also established in 32D/IRS-1, a promyeloid cell line that expresses IRS-1 (50). As in the HT-2 cell line, EPO stimulation of 32D/IRS-1 stable transfectants expressing EPO7 or EPOγ alone did not affect the phosphorylation of JAK1 or JAK3 (data not shown). EPO stimulation of a 32D/IRS-1 stable cell line expressing both EPO7 and EPOγ (32D/IRS-1/EPO7γ) resulted in a marked increase in the phosphorylation of JAK1 and JAK3 (Fig. 3 *B*). Additionally, EPO stimulation of 32D/IRS-1/EPO7 and 32D/IRS-1/EPOγ cell lines did not result in the activation of DNA-binding activity (Fig. 3 *C*). However, EPO stimulation of a 32D cell line expressing both EPO7 and EPOγ, 32D/IRS-1/EPO7γ, led to the activation of a DNA-binding complex (Fig. 3 *C*). To determine the composition of the DNA-binding complex, 32D/IRS-1/EPO7γ cellular lysates were subjected to immunoprecipitation with the STAT-5A and -5B antibodies after activation of the EPO7/EPOγ heterodimer. Anti-STAT-5A immunoblot analysis of the immunoprecipitates demonstrated the coimmunoprecipitation of STAT-5B with STAT-5A after EPO stimulation (Fig. 2 *E*). Similarly, immunoprecipitates of the STAT-5A antibody demonstrated the EPO-induced association of STAT-5B (data not shown). Therefore, as in the IL-2R system (51), signaling through an IL-7Rα/γ_c heterodimer results in the activation and heterodimerization of STAT-5A and -5B. Thus, these findings demonstrate that signaling by the IL-7 receptor complex through the JAK-STAT pathway is reproduced by the chimeric receptor system in two cytokine-dependent hematopoietic cell lines. Furthermore, heterodimerization of the cytoplasmic tails of IL-7Rα and γ_c are required to activate signal transduction by the IL-7R complex.

The cytoplasmic tyrosines of the γ_c subunit are not required for JAK-STAT signaling by the IL-7R complex. Previous studies in the IL-2 and IL-4 receptor complexes demonstrated that the four cytoplasmic tyrosine residues of the γ_c subunit were

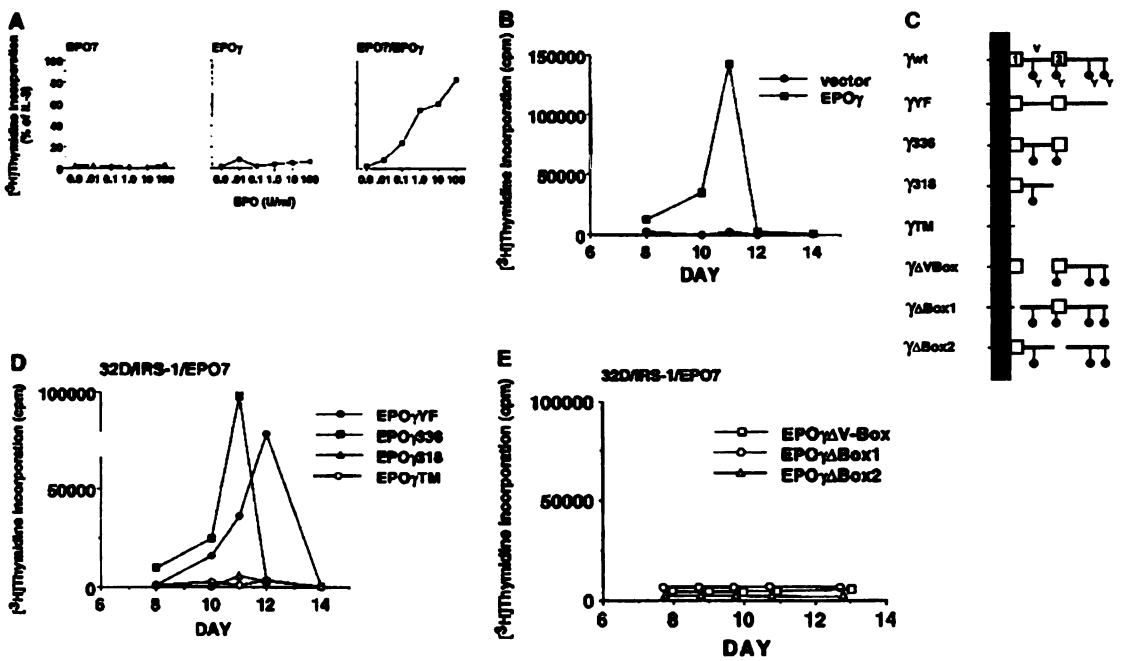

Figure 4. Heterodimerization of the IL-7Rα and γ_c cytoplasmic domains results in the activation of IL-7-specific signaling events. (*A*) Stable 32D/IRS-1 transfectants were stimulated with EPO in [^{3}H]thymidine incorporation experiments. (*B*) Transient transfection assay of 32D/IRS-1/EPO7 cells demonstrates that EPO-induced proliferation requires the presence of both EPO7 and EPOγ. (*C*) Schematic diagram of the γ_c mutants. γ336, γ318 and γTM mutants are truncated immediately after amino acids 336, 318, and 286, respectively. γΔBox1 lacks residues 281–294, γΔV-BOX lacks residues 295–320, and γΔBox2 lacks residues 321–334. (*D*) Transfection assay of 32D/IRS-1/EPO7 cells with various EPOγ mutants demonstrates the critical role of the cytoplasmic membrane proximal region of γ_c for growth signaling. (*E*) Evaluation of internal deletion mutants of γ_c by the transient transfection assay. Each transfection assay done in 32D/IRS-1/EPO7 cells included EPOγ as a positive control.

dispensable for signal transduction in response to those cytokines (20, 46, 51). To investigate the functional role of the tyrosine residues of the γ_c subunit in the IL-7R complex, HT-2 stable cell lines were established that expressed EPO7 and EPOγYF, a chimeric γ_c mutant in which all four cytoplasmic tyrosine residues are replaced by phenylalanines. Like wild-type HT-2EPO7γ cells, EPO-stimulation of two distinct HT-2EPO7/γYF cell lines resulted in the activation of a DNA-binding activity (Fig. 2 *D*). Additionally, antibody competition experiments confirmed the presence of STAT-5A and -5B in this DNA-binding complex (data not shown). Thus, as in other γ_c-containing receptor complexes, the specific JAK-STAT signaling events directed by the IL-7 receptor complex are independent of the tyrosine residues of the γ_c subunit.

Only the membrane-proximal portion of the γ_c subunit is necessary for growth signaling by the IL-7R. Growth signaling mediated by the chimeric EPO7/EPOγ heterodimer was evaluated in 32D/IRS-1 stable cell lines. In [^{3}H]thymidine incorporation assays, EPO stimulation of the 32D/IRS-1/EPO7 and 32D/IRS-1/EPOγ cell lines did not induce detectable proliferative responses (Fig. 4 *A*). In contrast, 32D/IRS-1/EPO7γ cells demonstrated a strong, dose-dependent proliferation in response to EPO. Additionally, a transient transfection assay originally established to study the IL-2R (44) was employed to evaluate IL-7R complex subunit requirements. In such experiments, expression of the EPOγ expression plasmid in the 32D/IRS-1/EPO7 cell line restored EPO-induced proliferation signaling, as measured by [^{3}H]thymidine incorporation (Fig. 4 *B*). Similarly, introduction of the EPO7 subunit into the 32D/IRS-1/EPOγ cell line also restored growth signaling in response to EPO (Fig. 5). Thus, heterodimerization of the IL-7Rα and γ_c subunits is necessary and sufficient for IL-7–mediated growth signaling.

Establishment of the transfection assay in these cells permitted the rapid assessment of various γ_c mutants in the IL-7R complex. The γ_c mutants included the previously described EPOγYF lacking all four cytoplasmic tyrosine residues, various truncation mutants, and internal deletion mutants lacking specific membrane-proximal regions of the γ_c subunit (Fig. 4 *C*). Expression of these chimeric γ_c mutants had been previously verified (44). As with JAK-STAT signaling, growth signaling was intact with the EPOγYF mutant (Fig. 4 *D*). Additionally, truncation of the γ_c subunit to the Box2 region (EPOγ336) did not adversely affect EPO-mediated proliferation. However, more severe truncations of the chimeric γ_c mutant (EPOγTM and EPOγ318) resulted in the abrogation of growth signaling, demonstrating the importance of the membrane-proximal regions of γ_c in IL-7R function (Fig. 4 *D*). This finding was further verified by internal deletion mutants of the Box1, V-Box, and Box2 regions of the γ_c chimeric receptor, each of which also abolished proliferation signaling (Fig. 4 *E*). Therefore, only the membrane-proximal regions of the γ_c subunit are necessary and sufficient for growth signaling mediated by the IL-7Rα/γ_c heterodimer.

The γ_c subunit is functionally replaceable in the IL-7R complex for signal transduction. Previous studies of the IL-2R complex demonstrated that the γ_c subunit serves primarily to activate receptor signaling, rather than to determine specific signaling events (52). Since comparable minimal structural elements of the γ_c subunit are required for IL-7R signaling, it is reasonable to predict that γ_c may perform a similar function in the IL-7Rα/γ_c heterodimer. To test this hypothesis, the γ_c subunit was replaced by a heterologous receptor subunit in the chimeric receptor system. Specifically, the EPOγ subunit was substituted by a truncated EPOR mutant, EPOR(1-321), which retains only the membrane-proximal cytoplasmic region that mediates JAK2 association (53). In [^{3}H]thymidine-incorporation assays, 32D/IRS-1 stable cell lines expressing EPO7 or EPOR(1-321) alone did not demonstrate a detectable proliferation signal in response to EPO (Fig. 3 *A*). However, EPO induced a vigorous growth response in cells that expressed both receptor subunits [32D/IRS-1/EPO7/EPOR(1-321)]. Thus, downstream signaling events such as proliferation were unaffected by the substitution of the γ_c cytoplasmic domain by the truncated EPOR subunit.

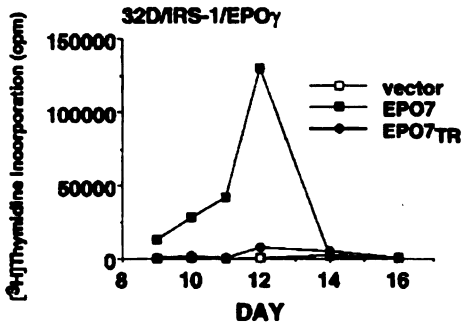

Figure 5. The cytoplasmic domain of the IL-7Rα subunit is required for proliferation signaling. Transient transfection assay was performed in 32D/IRS-1/EPOγ cells.

Distinct molecular signaling events were evaluated to examine other potential consequences of this receptor subunit substitution. As described above, the tyrosine phosphorylation of JAK1 and JAK3 was increased upon EPO-stimulation of the 32DEPO7γ cell line (Fig. 3 *B*). In the 32DEPO7/EPOR(1-321) cell line, EPO stimulation resulted in the increased tyrosine phosphorylation of JAK1 and JAK2, but not of JAK3. Furthermore, this replacement of JAK3 by JAK2 in the IL-7R complex did not alter the specificity of the JAK-STAT pathway. EMSA of nuclear extracts from 32D/IRS-1/EPO7 and 32D/IRS-1/EPOR(1-321) cells demonstrated no detectable DNA-binding activity in response to EPO (Fig. 3 *C*). In contrast, EPO stimulated the induction of STAT-5 in 32D/IRS-1/EPO7/EPOR(1-321) cells (Fig. 3 *C* and data not shown). Additionally, signaling through the IL-7R complex results in the activation of IRS-1 (41). Immunoblot analysis with the antiphosphotyrosine antibody of anti–IRS-1 immunoprecipitates demonstrated no detectable increase in phosphorylation of IRS-1 in EPO-stimulated 32D/IRS-1/EPO7 or 32D/IRS-1/EPOγ cells (data not shown). In contrast, IRS-1 demonstrated a marked EPO-dependent increase in tyrosine phosphorylation in 32D/IRS-1/EPO7γ cells (Fig. 3 *D*). Similarly, IRS-1 was phosphorylated upon EPO-induced heterodimerization of the IL-7Rα and truncated EPOR(1-321) cytoplasmic tails. Therefore, a variety of specific signaling events mediated by the IL-7R complex occur independently of the presence of the γ_c subunit. The γ_c subunit therefore appears to serve primarily to activate signaling by the IL-7R, rather than to specify signaling events directed by the receptor complex.

The distal portion of the IL-7Rα subunit is required for growth signaling. Because the major role of the γ_c subunit within the IL-7 receptor complex appears to be during initiation of receptor signaling, it was hypothesized that the IL-7Rα subunit itself serves to direct distinct signaling events. Specifically, the IL-7Rα subunit contains three tyrosine residues in the distal portion of the cytoplasmic tail, which may serve as docking sites for various signaling molecules. To evaluate the functional contribution of IL-7Rα in the receptor complex, a chimeric receptor containing the extracellular portion of EPOR and a truncated cytoplasmic portion of the IL-7Rα subunit lacking the three distal tyrosines ($EPO7_{TR}$) was constructed. Expression of the $EPO7_{TR}$ subunit was verified by immunoblotting of lysates from transfected COS cells (data not shown). In a transient transfection assay, expression of the wild-type EPO7 subunit in 32D/IRS-1/EPOγ cells led to the proliferation of these cells in response to EPO (Fig. 5). Strikingly, introduction of the $EPO7_{TR}$ subunit into this same cell line did not restore growth signaling. Thus, the IL-7Rα subunit and specifically, its distal cytoplasmic domain, are critical for proliferation signaling in the IL-7 receptor complex.

Discussion

Genetic analysis indicates that human X-SCID results from mutations in the γ_c subunit (11, 12), a receptor chain shared by several cytokine receptors (1–9). While recent observations suggest that the most critical functional defects in X-SCID are manifested in the IL-7R system (23, 24, 54, 55), the specific role of γ_c in that receptor complex remains poorly defined. The present studies analyzing the functional architecture of the IL-7R complex demonstrate the essential requirement of γ_c for proper IL-7R function. Furthermore, these studies define the role of γ_c in the IL-7R complex, leading to a potential molecular mechanism for the pathogenesis of X-SCID.

Structure/function analysis using a chimeric receptor system demonstrated the absolute requirement for heterodimerization of the IL-7Rα and γ_c cytoplasmic domains to activate IL-7–specific signaling events. The EPO7/EPOγ heterodimer activated STAT-5A and -5B in both HT-2 and 32D/IRS-1 stable transfectants. However, in the HT-2 cellular context, STAT-5 activation alone was not sufficient to mediate proliferation (data not shown), a finding that is presently being investigated. In the 32D/IRS-1 stable cell line, these molecular events were not affected by mutations in the γ_c subunit that COOH terminally truncated the cytoplasmic domain to the membrane-proximal Box2 region or converted the four cytoplasmic tyrosine residues to phenylalanines. However, more severe truncation mutants or internal deletions of the membrane-proximal region of γ_c that binds JAK3 (7) abolished proliferation signaling. These results complement recent studies in which a severely truncated γ_c subunit acted in a dominant-negative fashion to inhibit IL-7–mediated growth signaling (56) and demonstrate the critical contribution of the membrane-proximal segment of γ_c to IL-7R function. In contrast, a relatively distal truncation of the IL-7Rα subunit completely abrogated growth signaling by the IL-7R complex in the present system. Thus, while both receptor subunits were observed to be essential for IL-7R signaling competence, the structural requirements for these subunits were quite distinct.

Collectively, these findings demonstrate an asymmetric structure/function organization in the IL-7R complex that is strikingly similar to the "trigger-driver" arrangement of the IL-2R complex (20). In this configuration, specific receptor signaling events are determined by a distinct profile of signaling intermediates that physically associate with a single "driver" subunit, represented by the IL-7Rα chain. For example, these interactions may be mediated by conserved SH2 or PTB domains within signaling molecules that interact with phosphorylated tyrosine residues embedded within specific peptide motifs of such receptor subunits (57, 58). Indeed, in the IL-7Rα chain, removal of the three distal tyrosine residues was sufficient to abolish growth signaling by IL-7R. This finding is consistent with the recent studies of the murine IL-7Rα subunit, which demonstrated the importance of a distal tyrosine residue for proliferation in B cell lymphopoiesis (59). Thus, the IL-7Rα subunit functions as a "driver" subunit in determining the specific signaling events mediated by the IL-7R complex.

As in another γ_c-containing receptor, the IL-2R system, the γ_c subunit transports JAK3 into the IL-7R complex to activate receptor signaling. The initimate association of γ_c and JAK3 is confirmed by patients with JAK3 mutations who have immunodeficiencies that are very similar to X-SCID (60). Indeed, γ_c- and JAK3-deletion mice share similar immunodeficiency phenotypes (13, 14, 61). Furthermore, the nonfunctional γ_c mutants employed in the present studies affected similar portions of the γ_c subunit as mutations found in X-SCID patients (11, 12), and disrupted the membrane-proximal region of γ_c reported to mediate association with JAK3 (7). However, the requirement for JAK3 itself within this signaling complex is not absolute. Functional replacement of the γ_c cytoplasmic domain by EPOR(1-321) did not alter downstream signaling events measured in the chimeric IL-7R system. Furthermore, signaling via the EPO7/EPOR(1-321) heterodimer depended on the engagement of JAK1 and JAK2, but not JAK3 (data not shown). Importantly, this substitution of JAK2 for JAK3 in this complex did not affect events such as cellular proliferation, phosphorylation of IRS-1, or induction of STAT-5A or -5B. Therefore, various signaling events previously thought to be linked to JAK3 itself may instead be coupled to the IL-7Rα "driver" chain (62). Thus, the present studies support the model that γ_c and JAK3 are not required for specific signaling events, but act instead as a relatively generic "trigger" for activation of receptor-mediated signal transduction.

Several experimental approaches have demonstrated the important role of the IL-7/IL-7R system to early lymphocyte development (23, 24, 54, 55). The delineation of the functional role of the γ_c subunit within the IL-7R complex provides a possible molecular mechanism for the pathogenesis of X-SCID. γ_c defects that prevent the transport of JAK3 into the receptor complex would prevent IL-7–mediated signaling and the subsequent development of B and T cells. Based on the present findings, the critical step affected by mutations in the γ_c subunits appears to be the initiation of IL-7R signal transduction. Interestingly, unlike γ_c-deficient mice and X-SCID patients, IL-7– and IL-7Rα–deletion mice retain functional natural killer cells (63), raising the possibility that an unrecognized cytokine(s) may also employ γ_c-containing receptor complex(es). Nevertheless, the developmental defects for T and B cells are at least as severe as those for X-SCID individuals and γ_c-deficient mice. Therefore, these findings suggest that the earliest signaling events that would affect lymphocyte development in X-SCID appear to be mediated by the IL-7R complex.

While the γ_c subunit is present in other receptor complexes, such as the IL-2R, -4R, -9R, and -15R, these receptors appear to function in the later stages of lymphoid development and regulation. Thus, the early block of lymphocyte development due to defects in IL-7R function would mask potential functional aberrations due to the lack of signaling by these receptors.

The present studies define the role of γ_c in the IL-7R complex and provide a possible molecular mechanism for the pathogenesis of X-SCID. These studies also define further at least two distinct classes of receptors that employ the γ_c subunit. First, the IL-7R and -2R complexes exhibit a strict requirement for γ_c in the receptor complex. In these receptor systems, γ_c initiates signaling by transporting the associated JAK3 into the receptor complex, while IL-7Rα and -2Rβ function in their respective receptor complexes to mediate specific signaling events. Interestingly, a similar functional arrangement of the individual receptor subunits has recently been demonstrated in the interferon-γ receptor complex (64). In the second class of receptors, represented by the IL-4R, γ_c is not absolutely essential for receptor function. In SCID-MA cells, the lack of detectable γ_c expression (reference 12 and data not shown) does not affect IL-4 responsiveness (Fig. 1 and reference 20). This observation supports the recent findings that the IL-4R complex may exist in multiple forms (17–21). In certain cell lines derived from X-SCID patients, mutant forms of γ_c may act in a dominant negative fashion to inhibit the IL-4–mediated activation of JAK3 and STAT-6 (65). In the absence of γ_c, another receptor subunit, perhaps the IL-13Rα and/or other unidentified partner chains, may perform a similar role as that of the γ_c subunit during initiation of signaling through the receptor complex (17–21, 66). This may account for the responsiveness of some gggccc-negative cells to IL-4. Additionally, circumstantial evidence also exists for a homomeric form of IL-4R that consists of only the IL-4Rα subunit (reference 20 and data not shown). Further studies are in progress to determine the functional role of γ_c in the other receptors, such as the IL-9R complex, and to understand the structure/function arrangements of heterodimeric receptor systems.

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References

1. Takeshita, T., H. Asao, K. Ohtani, N. Ishii, S. Kumaki, N. Tanaka, H. Munakata, M. Nakamura, and K. Sugamura. 1992. Cloning of the gamma chain of the human IL-2 receptor. *Science (Wash. DC).* 257:379–382.

2. Kondo, M., T. Takeshita, N. Ishii, M. Nakamura, S. Watanabe, K.-I. Arai, and K. Sugamura. 1993. Sharing of the interleukin-2 (IL-2) receptor γ chain between receptors for IL-2 and IL-4. *Science (Wash. DC).* 262:1874–1877.

3. Noguchi, M., Y. Nakamura, S.M. Russell, S.F. Ziegler, M. Tsang, X. Cao, and W.J. Leonard. 1993. Interleukin-2 receptor γ chain: a functional component of the interleukin-7 receptor. *Science (Wash. DC).* 262:1877–1880.

4. Russell, S.M., A.D. Keegan, N. Harada, Y. Nakamura, M. Noguchi, P. Leland, M.C. Friedmann, A. Miyajima, R.K. Puri, W.E. Paul, and W.J. Leonard. 1993. Interleukin-2 receptor γ chain: a functional component of the interleukin-4 receptor. *Science (Wash. DC).* 262:1880–1883.

5. Giri, J.G., M. Ahdieh, J. Eisenman, K. Shanebeck, K. Grabstein, S. Kumaki, A. Namen, L.S. Park, D. Cosman, and D. Anderson. 1994. Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2822–2830.

6. Kondo, M., T. Takeshita, M. Higuchi, M. Nakamura, T. Sudo, S. Nishikawa, and K. Sugamura. 1994. Functional participation of the IL-2 receptor γ chain in IL-7 receptor complexes. *Science (Wash. DC).* 263:1453–1454.

7. Russell, S.M., J.A. Johnston, M. Noguchi, M. Kawamura, C.M. Bacon, M. Friedmann, M. Berg, D.W. McVicar, B.A. Witthuhn, O. Silvennoinen, et al. 1994. Interaction of IL-2Rβ and γc chains with Jak1 and Jak3: implications for XSCID and XCID. *Science (Wash. DC).* 266:1042–1045.

8. Kimura, Y., T. Takeshita, M. Kondo, N. Ishii, M. Nakamura, J. Van Snick, and K. Sugamura. 1995. Sharing of the IL-2 receptor gamma chain with the functional IL-9 receptor complex. *Intl. Immunol.* 7:115–120.

9. Ziegler, S.E., K.K. Morella, D. Anderson, N. Kumaki, W.J. Leonard, D. Cosman, and H. Baumann. 1995. Reconstitution of a functional IL-7 receptor demonstrates that the IL-2 receptor gamma chain is required for IL-7 signal transduction. *Eur. J. Immunol.* 25:399–404.

10. Conley, M.E. 1992. Molecular approaches to analysis of X-linked immunodeficiencies. *Ann. Rev. Immunol.* 10:215–238.

11. Noguchi, M., H. Yi, H.M. Rosenblatt, A.H. Filipovich, S. Adelstein, W.S. Modi, O.W. McBride, and W.J. Leonard. 1993. Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell.* 73:147–157.

12. Puck, J.M., S.M. Deschenes, J.C. Porter, A.S. Dutra, C.J. Brown, H.F. Willard, and P.S. Henthorn. 1993. The interleukin-2 receptor γ chain maps to Xq13.1 and is mutated in X-linked severe combined immunodeficiency, SCIDX1. *Hum. Mol. Genet.* 2:1099–1104.

13. Cao, X., E.W. Shores, J. Hu-Li, M.R. Anver, B.L. Kelsall, S.M. Russell, J. Drago, M. Noguchi, A. Grinberg, E.T. Bloom, et al. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. *Immunity.* 2:223–238.

14. DiSanto, J.P., W. Muller, D. Guy-Grand, A. Fischer, and K. Rajewsky. 1995. Lymphoid development in mice with a targeted deletion of the interleukin-2 receptor γ chain. *Proc. Natl. Acad. Sci. USA.* 92:377–381.

15. Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature (Lond.).* 352:621–624.

16. Suzuki, H., T.M. Kundig, C. Furlonger, A. Wakeham, E. Timms, T. Matsuyama, R. Schmits, J.J. Simard, P.S. Ohashi, and H. Griesser. 1995. Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. *Science (Wash. DC).* 268:1472–1476.

17. He, Y.-W., and T.R. Malek. 1995. The IL-2 receptor γ_c chain does not function as a subunit shared by the IL-4 and IL-13 receptors: implication for the structure of the IL-4 receptor. *J. Immunol.* 155:9–12.

18. Keegan, A.D., J.A. Johnston, P.J. Tortolani, L.J. McReynolds, C. Kinzer, J.J. O'Shea, and W.E. Paul. 1995. Similarities and differences in signal transduction by IL-4 and IL-13: analysis of Janus kinase activation. *Proc. Natl. Acad. Sci. USA.* 92:7681–7685.

19. Lin, J.-X., T.-S. Migone, M. Tsang, M. Friedmann, J.A. Weatherbee, L. Zhou, A. Yamauchi, E. T. Bloom, J. Mietz, S. John, and W. J. Leonard. 1995. The role of shared receptor motifs and common stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity.* 2:331–339.

20. Lai, S.Y., J. Molden, K.D. Liu, J.M. Puck, M.D. White, and M.A. Goldsmith. 1996. Interleukin 4–epecific signal transduction events are driven by homotypic interactions on the interleukin-4 receptor α subunit. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:4506–4514.

21. Palmer-Crocker, R.L., C.C.W. Hughes, and J.S. Pober. 1996. IL-4 and IL-13 activate the JAK2 tyrosine kinase and Stat6 in cultured human vascular endothelial cells through a common pathway that does not involve the γ_c chain. *J. Clin. Invest.* 98:604–609.

22. Renauld, J.C., A. Kermouni, A. Vink, J. Louahed, and J. Van Snick. 1995. Interleukin-9 and its receptor: involvement in mast cell differentiation and T cell oncogenesis. *J. Leukocyte Biol.* 57:353–360.

23. von Freeden-Jeffry, U., P. Vieira, L.A. Lucian, T. McNeil, S.E. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL-7)-7 gene-deleted mice identifies IL-7 as a nonredunant cytokine. *J. Exp. Med.* 181:1519–1526.

24. Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Maraskovsky, B.C. Gliniak, L.S. Park, S.F. Ziegler, D.E. Williams, C.B. Ware, et al. 1994. Early lymphocyte expansion is severely impaired in interleukin-7 receptor-deficient mice. *J. Exp. Med.* 180:1955–1960.

25. Namen, A.E., A.E. Schmierer, C.J. March, R.W. Overell, L.S. Park,

D.L. Urdal, and D.Y. Mochizuki. 1988. B cell precursor growth-promoting activity: purification and characterization of a growth factor active on lymphocyte precursors. *J. Exp. Med.* 167:988–1002.

26. Namen, A.E., S. Lupton, K. Hjerrild, J. Wignall, D.Y. Mochizuki, A. Schmierer, B. Mosley, C.J. March, D. Urdal, and S. Gillis. 1988. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature (Lond.).* 333:571–573.

27. Conlon, P.J., P.J. Morrissey, R.P. Nordan, K.H. Grabstein, K.S. Prickett, S.G. Reed, R. Goodwin, D. Cosman, and A.E. Namen. 1989. Murine thymocytes proliferate in direct response to interleukin-7. *Blood.* 74:1368–1373.

28. Goodwin, R.G., S. Lupton, A. Schmierer, K.J. Hjerrild, R. Jerzy, W. Clevenger, S. Gillis, D. Cosman, and A.E. Namen. 1989. Human interleukin 7: Molecular cloning and growth factor activity on human and murine B-lineage cells. *Proc. Natl. Acad. Sci. USA.* 86:302–306.

29. Takeda, S., S. Gillis, and R. Palacios. 1989. In vitro effects of recombinant interleukin-7 on growth and differentiation of bone marrow pro-B- and pro-T-lymphocyte clones and fetal thymocyte clones. *Proc. Natl. Acad. Sci. USA.* 86:1634–1638.

30. Okazaki, H., M. Ito, T. Sudo, M. Hattori, S. Kano, Y. Katsura, and N. Minato. 1989. IL-7 promotes thymocyte proliferation and maintains immunocompetent thymocytes bearing alpha beta or gamma delta T-cell receptors in vitro: synergism with IL-2. *J. Immunol.* 143:2917–2922.

31. Edington, H., and M.T. Lotze. 1994. Interleukin-7. *In* The Cytokine Handbook. Academic Press Inc., Orlando, FL. 169–184.

32. Goodwin, R.G., D. Friend, S.F. Ziegler, R. Jerzy, B.A. Falk, S. Gimpel, D. Cosman, S.K. Dower, C.J. March, A.E. Namen, and L.S. Park. 1990. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell.* 60:941–951.

33. Bazan, J.-F. 1990. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA.* 87:6934–6938.

34. Murakami, M., M. Narazaki, M. Hibi, H. Yawata, K. Yasukawa, M. Hamaguchi, T. Taga, and T. Kishimoto. 1991. Critical cytoplasmic region of the interleukin-6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc. Natl. Acad. Sci. USA.* 88:11349–11353.

35. Miyazaki, T., A. Kawahara, H. Fujii, Y. Nakagawa, Y. Minami, Z.-J. Liu, I. Oishi, O. Silvennoinen, B.A. Witthuhn, J.N. Ihle, and T. Taniguchi. 1994. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science (Wash. DC).* 266:1045–1047.

36. Wilks, A.F., and A.G. Harpur. 1994. Cytokine signal transduction and the JAK family of protein tyrosine kinases. *Bioessays.* 16:313–320.

37. Boussiotis, V.A., D.L. Barber, T. Nakarai, G.J. Freeman, J.G. Gribben, G.M. Bernstein, A.D. D'Andrea, J. Ritz, and L.M. Nadler. 1994. Prevention of T cell anergy by signaling through the γ_c chain of the IL-2 receptor. *Science (Wash. DC).* 266:1039–1042.

38. Venkitaraman, A.R., and R.J. Cowling. 1992. Interleukin-7 receptor functions by recruiting the tyrosine kinase p59fyn through a segment of its cytoplasmic tail. *Proc. Natl. Acad. Sci. USA.* 89:12083–12087.

39. Seckinger, P., and M. Fougereau. 1994. Activation of src family kinases in human pre-B cells by IL-7. *J Immunol.* 153:97–109.

40. Page, T.H., F. V. Lali, and B.M. Foxwell. 1995. Interleukin-7 activates p56lck and p59fyn, two tyrosine kinases associated with the p90 interleukin-7 receptor in primary human T cells. *Eur. J. Immunol.* 25:2956–2960.

41. Johnston, J.A., L.M. Wang, E.P. Hanson, X.J. Sun, M.F. White, S.A. Oakes, J.H. Pierce, and J.J. O'Shea. 1995. Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells: potential role of JAK kinases. *J. Biol. Chem.* 270:28527–28530.

42. Dadi, H.K., and C.M. Roifman. 1993. Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor on human thymocytes. *J. Clin. Invest.* 92:1559–1563.

43. Dadi, H.K., S. Ke, and C.M. Roifman. 1993. Interleukin-7 receptor mediates the activation of phosphatidylinositol-3 kinase in human B-cell precursors. *Biochem. Biophys. Res. Commun.* 192:459–464.

44. Goldsmith, M.A., W. Xu, M.C. Amaral, E.S. Kuczek, and W.C. Greene. 1994. The cytoplasmic domain of the IL-2 receptor β chain contains both unique and functionally redundant signal transduction elements. *J. Biol. Chem.* 269:14698–14704.

45. Andersson, S., D.L. Davis, H. Dahlback, H. Jornvall, and D.W. Russell. 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* 264:8222–8229.

46. Goldsmith, M.A., S.Y. Lai, W. Xu, M.C. Amaral, E.S. Kuczek, L.J. Parent, G.B. Mills, K.L. Tarr, G.D. Longmore, and W.C. Greene. 1995. Growth signal transduction by the human IL-2 receptor requires cytoplasmic tyrosines of the β chain and non-tyrosine residues of the γ_c chain. *J. Biol. Chem.* 270: 21729–21737.

47. Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffer. 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.

47a. Latchman, D.S. 1993. Transcription Factors: A Practical Approach. IRL Press at Oxford University Press, Oxford, UK. 1–26.

48. Musso, T., J.A. Johnston, D. Linnekin, L. Varesio, T.K. Rowe, J.J. O'Shea, and D.W. McVicar. 1995. Regulation of JAK3 expression in human monocytes: phosphorylation on response to interleukins 2, 4, and 7. *J. Exp. Med.* 181:1425–1431.

49. Darnell, J.E., I.M. Kerr, and G.R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science (Wash. DC).* 264:1415–1421.

50. Wang, L.-M., M.G. Myers, X.-J. Sun, S.A. Aaronson, M. White, and J.H. Pierce. 1993. IRS-1: essential for insulin- and IL-4–stimulated mitogenesis in hematopoietic cells. *Science (Wash. DC).* 261:1591–1594.

51. Gaffen, S.L., S.Y. Lai, M. Ha, X. Liu, L. Hennighausen, W.C. Greene, and M.A. Goldsmith. 1996. Distinct tyrosine residues within the IL-2 receptor β chain drive signal transduction specificity, redundancy and diversity. *J. Biol. Chem.* 271:21381–21390.

52. Lai, S.Y., W. Xu, S.L. Gaffen, G.D. Longmore, W.C. Greene, and M.A. Goldsmith. 1996. The molecular role of the common γ_c subunit in signal transduction reveals functional asymmetry within multimeric cytokine receptor complexes. *Proc. Natl. Acad. Sci. USA.* 93:231–235.

53. Witthuhn, B.A., F.W. Quelle, O. Silvennoinen, T. Yi, B. Tang, O. Miura, and J.N. Ihle. 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorated and activated following stimulation with erythropoietin. *Cell.* 74:227–236.

54. Grabstein, K.H., T.J. Waldschmidt, F.D. Finkelman, B.W. Hess, A.R. Alpert, N.E. Boiani, A.E. Namen, and P.J. Morrissey. 1993. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. *J. Exp. Med.* 178:257–264.

55. Sudo, T., S. Nishikawa, N. Ohno, N. Akiyama, M. Tamakoshi, H. Yoshida, and S.-I. Nishikawa. 1993. Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proc. Natl. Acad. Sci. USA.* 90:9125–9129.

56. Kawahara, A., Y. Minami, and T. Taniguchi. 1994. Evidence for a critical role for the cytoplasmic region of the interleukin 2 (IL-2) receptor gamma chain in IL-2, IL-4, and IL-7 signaling. *Mol. Cell. Biol.* 14:5433–5440.

57. Songyang, Z., S.E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Haser, F. King, T. Roberts, S. Ratnofsky, and R.J. Lechleider. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell.* 72:767–778.

58. Kavanaugh, W.M., and L.T. Williams. 1994. An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science (Wash. DC).* 266: 1862–1865.

59. Corcoran, A.E., F.M. Smart, R.J. Cowling, T. Crompton, M.J. Owen, and A.R. Venkitaraman. 1996. The interleukin 7 receptor α chain transmits distinct signals for proliferation and differentiation during B lymphopoiesis. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:1924–1932.

60. Russell, S.M., N. Tayebi, H. Nakajima, M.C. Riedy, J.L. Roberts, M.J. Aman, T.S. Migone, M. Noguchi, M.L. Markert, and R.H. Buckley. 1995. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science (Wash. DC).* 270:797–800.

61. Nosaka, T., J.M. van Deursen, R.A. Tripp, W.E. Theirfelder, B.A. Witthuhn, A.P. McMickle, P.C. Doherty, G.C. Grosveld, and J.N. Ihle. 1995. Defective lymphoid development in mice lacking Jak3. *Science (Wash. DC).* 270:800–802.

62. Sharfe, N., H. Dadi, and C.M. Roifman. 1995. JAK3 protein tyrosine kinase mediates interleukin 7–induced activation of phosphatidylinositol-3' kinase. *Blood.* 86:2077–2085.

63. Maki, K., S. Sunaga, Y. Komagata, Y. Kodaira, A. Mabuchi, H. Karasuyama, K. Yokomuro, J.-I. Miyazaki, and K. Ikuta. 1996. Interleukin 7 receptor–deficient mice lack γδ T cells. *Proc. Natl. Acad. Sci. USA.* 93:7172–7177.

64. Bach, E.A., J.W. Tanner, S. Marsters, A. Ashkenazi, M. Aguet, A.S. Shaw, and R.D. Schreiber. 1996. Ligand-induced assembly and activation of the gamma interferon receptor in intact cells. *Mol. Cell. Biol.* 16:3214–3221.

65. Izuhara, K., T. Heike, T. Otsuka, K. Yamaoka, M. Mayumi, T. Imamura, Y. Niho, and N. Harada. 1996. Signal transduction pathway of interleukin-4 and interleukin-13 in human B cells derived from X-linked severe combined immunodeficiency patients. *J. Biol. Chem.* 271:619–622.

66. Hilton, D.J., J.-G. Zhang, D. Metcalf, W.S. Alexander, N.A. Nicola, and T.A. Willson. 1996. Cloning and characterization of a binding subunit of the IL-13 receptor that is also a component of the IL-4 receptor. *Proc. Natl. Acad. Sci. USA.* 93:497–501.

Chapter 6

Interleukin-4-Specific Signal Transduction Events are Driven by Homotypic Interactions of the Interleukin-4 Receptor α Subunit

Prologue

With the characterization of the functional role of γ_c within the IL-2R and IL-7R complexes, we turned our attention to the IL-4R complex. Although previous work had demonstrated the presence of γ_c within the IL-4R complex (1,2), the existence of other IL-4R complexes with different subunit configurations has been subsequently reported (3-5). In many of those studies, an alternative subunit, sometimes referred to as γ', is hypothesized to replace γ_c in the IL-4R heterodimeric complex. However, those studies did not address the possibility that IL-4Rα alone could be competent for IL-4-mediated signaling activity in the absence of γ_c. Therefore, we reconstituted the IL-4R complex in a chimeric receptor system to analyze the functional roles of the individual receptor subunits. Interestingly, we found that the IL-4Rα subunit alone was competent to mediate IL-4-specific signaling events. The following paper, reproduced from the journal in which it was published, examines the role of γ_c in the IL-4R complex and provides evidence for alternative forms of the IL-4R complex that do not require γ_c.

References

1 M. Kondo, et al., *Science* **262**, 1877-80 (1993).

2 S.M. Russell, et al., *Science* **262**, 1880-3 (1993).

3 Y.-W. He, T.R. Malek *J Immunol* **155**, 9-12 (1995).

4 A.D. Keegan, et al. *PNAS USA* **92**, 7681-5 (1995).

5 J.-X. Lin et al. *Immunity* **2**, 331-9 (1995).

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Interleukin-4-specific signal transduction events are driven by homotypic interactions of the interleukin-4 receptor α subunit

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Interleukin-4 (IL-4) exerts its effects through a heterodimeric receptor complex (IL-4R), which contains the IL-4Rα and γ_c subunits. IL-4Rα also functions with other partner subunits in several receptor types, including the IL-13 receptor. To examine the roles of the individual subunits within IL-4R complexes, we employed a chimeric system that recapitulates native IL-4R function as verified by the activation of the kinases, JAK1 and JAK3, and induction of STAT-6. When a mutant γ_c subunit in which the four cytoplasmic tyrosines were converted to phenylalanine was paired with the cytoplasmic domain of the IL-4Rα chain, specificity within the JAK–STAT pathway was not altered. Signaling events were examined further in cells expressing the IL-4Rα chimera alone without the γ_c chimera. Ligand-induced homodimerization of these receptors activated the IL-4 signaling program despite the absence of γ_c, including induction of JAK1 and STAT-6, phosphorylation of the insulin-related substrate 1 and cellular proliferation. Thus, homotypic interactions of the IL-4Rα subunit are sufficient for the initiation and determination of IL-4-specific signaling events, and such interactions may be integral to signaling through IL-4R complexes.

Keywords: interleukin-4/JAK–STAT/receptor/signal transduction/specificity

Introduction

Interleukin-4 (IL-4) exerts pleiotropic effects on multiple cell lineages (reviewed in Beckmann *et al.*, 1992; Banchereau and Rybak, 1994; Keegan *et al.*, 1994). These widely different functions include proliferation and differentiation of B and T lymphocytes. Stimulation of B cells with IL-4 leads to heavy chain class switching to IgE and the induction of major histocompatibility complex class II molecules. Furthermore, IL-4 up-regulates the expression of CD23, the low affinity Fc receptor for IgE. IL-4 also acts as a potent inducer of cytotoxic T cells (Widmer *et al.*, 1987) and can antagonize responses to IL-2 by lymphocytes under specific conditions (Tigges *et al.*, 1989; Tanaka *et al.*, 1993). *In vitro* studies have demonstrated the ability of IL-4 to act in concert with known colony-stimulating factors either to stimulate or to suppress colony formation of hematopoietic progenitor cells (Broxmeyer *et al.*, 1988); IL-4 also exerts an inhibitory effect on growth of certain human carcinoma cells (Murata *et al.*, 1996).

The activation of cellular signaling events by IL-4 depends upon ligand binding to a receptor complex that employs the IL-4Rα subunit. This 140 kDa subunit is sufficient to permit high affinity binding of the IL-4 ligand (Mosley *et al.*, 1989; Harada *et al.*, 1990). Additionally, the γ_c subunit associates with IL-4Rα in the presence of IL-4 to promote a modest increase in receptor binding affinity (Kondo *et al.*, 1993; Russell *et al.*, 1993). Engagement of the heterodimeric IL-4Rα–γ_c complex by IL-4 results in the association and activation of signaling intermediates, such as the insulin receptor substrate-1 (IRS-1), that lead to proliferation and various differentiation events (Wang *et al.*, 1993; Seldin and Leder, 1994; Pernis *et al.*, 1995).

Both IL-4Rα and γ_c are members of the cytokine receptor superfamily; they contain the canonically spaced extracellular cysteine residues, the juxtamembrane WSXWS motif and the partially conserved membrane-proximal Box 1 and Box 2 regions in their cytoplasmic tails (Bazan, 1990; Murakami *et al.*, 1991). Interestingly, IL-4R is one member of a receptor subfamily in which the γ_c chain is paired with different partner subunits to bind distinct cytokines. Other receptor complexes recognized to employ the γ_c subunit include receptors for IL-2, IL-7, IL-9 and IL-15 (Takeshita *et al.*, 1992; Noguchi *et al.*, 1993a; Giri *et al.*, 1994; Kondo *et al.*, 1994). Mutations in the γ_c subunit have been proposed to cause the global signaling defects that lead to X-linked severe combined immunodeficiency (X-SCID) because of the general use of γ_c in multiple receptor complexes (Noguchi *et al.*, 1993b; Puck *et al.*, 1993). This hypothesis is partially supported by experimental deletion of the γ_c gene in mice, which results in severe early lymphoid developmental defects (Cao *et al.*, 1995; DiSanto *et al.*, 1995).

Like the γ_c chain, the IL-4Rα subunit functions as a modular receptor component that can be employed in multiple receptor complexes. For example, IL-4Rα apparently is employed in the IL-13 receptor complex (Lefort *et al.*, 1995; Lin *et al.*, 1995). In addition to forming a heterodimer with γ_c to bind IL-4, IL-4Rα may also partner with a second, uncharacterized subunit(s) to mediate IL-4-dependent signals in cells that do not express γ_c (Hou *et al.*, 1994; He and Malek, 1995). The presence of IL-4Rα in multiple receptor complexes would explain the relatively wider tissue distribution of the IL-4Rα subunit expression compared with that of the γ_c chain (Beckmann *et al.*, 1992; Takeshita *et al.*, 1992), and implies that IL-4Rα has γ_c-independent function(s).

The use of individual receptor subunits in multiple

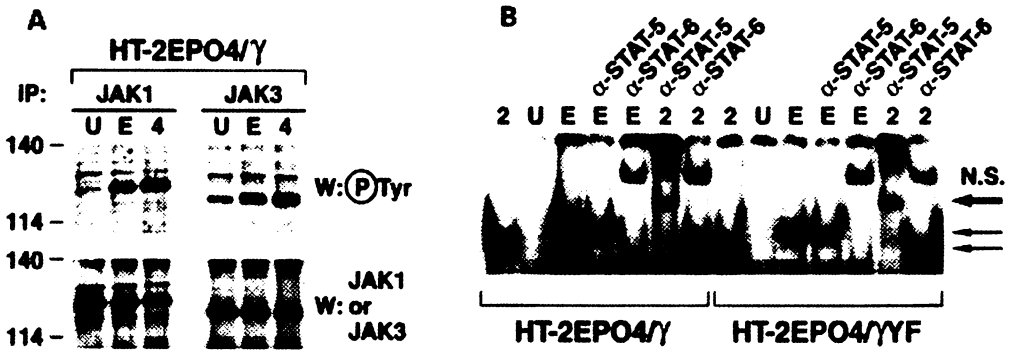

Fig. 1. The IL-4Rα–γ_c heterodimer mediates activation of the JAK–STAT pathway. (A) Lysates from HT-2EPO4/γ cells unstimulated (U) or treated with EPO (E, 50 U/ml) or IL-4 (4, 50 U/ml) were subjected to serial immunoprecipitation with anti-JAK1 and anti-JAK3 antisera. Immunoblotting was performed with an anti-phosphotyrosine antibody (4G10). Blots were then stripped and re-probed with anti-JAK1 or anti-JAK3 antisera to verify even sample loading. (B) EMSA of nuclear extracts from HT-2EPO4/γ and HT-2EPO4/γYF cells that were rested (U) or stimulated with IL-2 (2, 10 nM) or EPO (E, 50 U/ml). Competition was performed with the anti-STAT-5 and anti-STAT-6 antibodies. The lower two arrows highlight EPO-induced (top) and IL-2-induced (bottom) bands. The bold arrow indicates the complex supershifted by anti-STAT5 antibody. N.S., non-specific band.

receptor complexes appears to be an important means of defining and regulating different signaling events in several biological systems (Schlessinger and Ullrich, 1992). Members of the epidermal growth factor (EGF) receptor family, for example, are employed in an array of different receptor multimers to regulate ligand binding specificity and to direct distinct signaling programs in response to various related growth factors (Earp *et al.*, 1995). Analogously, both the IL-4Rα and γ_c subunits may modulate these activities in different cytokine receptor complexes. The current studies, therefore, were undertaken to define the functional roles of the IL-4Rα and γ_c chains and to explore the combinatorial use of these chains in different receptor complexes. Collectively, the present findings suggest that the IL-4Rα subunit serves as the primary determinant of signaling specificity in various receptor complexes, and that homotypic interactions of IL-4Rα may be integral to the signal transduction process.

Results

A chimeric receptor system recapitulates native IL-4R function

To examine the function of individual receptor subunits within the heterodimeric IL-4R signaling complex, a chimeric receptor system was employed in which the extracellular domain of the erythropoietin receptor (EPOR) was fused to the transmembrane and cytoplasmic regions of IL-4Rα (EPO4) and γ_c (EPOγ). Stable transfectants expressing both the EPO4 and EPOγ subunits (HT-2EPO4/γ) were established in HT-2 cells, an IL-2-dependent helper T-cell line. To verify the integrity of this chimeric receptor system, activation of the JAK–STAT pathway was examined as a representative early event in IL-4R signaling. First, anti-phosphotyrosine immunoblot analysis of JAK1 and JAK3 immunoprecipitates from HT-2EPO4/γ cells demonstrated the induction of these kinases, but not of JAK2 and Tyk2, following stimulation with IL-4 (Figure 1A and data not shown). Similarly, EPO stimulation of these cells led to the selective phosphorylation of JAK1 and JAK3, demonstrating the concurrent engagement of both the IL-4Rα and γ_c cytoplasmic tails in this chimeric model (Figure 1A). Second, electrophoretic gel mobility shift assay (EMSA) demonstrated the retardation of an oligonucleotide probe containing the FcγR1 STAT response element upon stimulation with IL-4 (Figure 2A). This finding is consistent with earlier reports that STAT-6 DNA binding activity is induced by the IL-4R complex following JAK1 and JAK3 activation (Hou *et al.*, 1994). Likewise, EPO stimulation of the HT-2EPO4/γ cell line led to the activation of a DNA binding complex (Figure 1B). The composition of the EPO-induced binding complex was revealed by pre-incubation of the nuclear extracts with various anti-STAT antibodies. The anti-STAT-6 antibody abrogated the EPO-stimulated DNA binding complex, but had no effect on the STAT-5 DNA binding activity that resulted from IL-2 stimulation (Figure 1B). Furthermore, an anti-STAT-5 antibody further retarded the mobility of the IL-2-induced DNA binding activity, but not of the EPO-stimulated complex (Figure 1B). Thus, EPO stimulation of a cell line expressing both the EPO4 and EPOγ receptor subunits reconstituted specific JAK–STAT signaling events of the native IL-4R complex.

A γ_c subunit lacking tyrosine residues is permissive for IL-4R signal transduction

In the IL-2R complex, the tyrosine residues of the γ_c subunit become phosphorylated upon ligand binding (Takeshita *et al.*, 1992), but do not appear to affect signaling events (Goldsmith *et al.*, 1994). To evaluate the functional contribution of the γ_c cytoplasmic tyrosines within the IL-4R complex, a stable cell line was established that expresses the wild-type EPO4 subunit and a chimeric γ_c mutant subunit in which all four cytoplasmic tyrosine residues were replaced by phenylalanines (EPOγYF). EMSA demonstrated that EPO-induced engagement of the IL-4Rα and γYF cytoplasmic tails resulted in a DNA binding complex indistinguishable from that induced by the wild-type heterodimeric receptor (Figure 1B). As with the wild-type IL-4Rα–γ_c complex, this DNA binding complex was abolished by incubation of the HT-2EPO4/γYF nuclear extract with the anti-STAT-6 antibody. In contrast, pre-incubation of the HT-2EPO4/γYF nuclear extract with the anti-STAT-5 antibody did not affect the

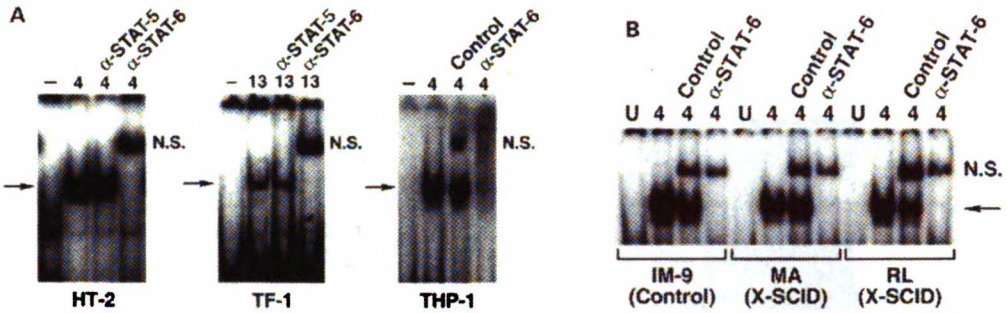

Fig. 2. Various receptors employing the IL-4Rα subunit activate STAT-6 DNA binding activity. (A) EMSA of nuclear extracts from HT-2 (left), TF-1 (middle) and THP-1 (right) cells that were unstimulated (U) or treated with IL-4 (4, 50 U/ml) or IL-13 (13, 20 U/ml). Competition was performed with the anti-STAT-5, anti-STAT-6 and pre-immune antisera (Control). Arrows highlight the IL-4- and IL-13-induced bands. N.S., non-specific band. (B) EMSA of nuclear extracts from the IM-9 cell line and the X-SCID cell lines, MA and RL, rested (U) or treated with IL-4 (4, 10 ng/ml). Anti-STAT-6 and pre-immune antisera (Control) were used in competition studies. The arrow highlights the IL-4-induced band. N.S., non-specific band.

DNA binding complex (Figure 1B), although the anti-STAT-5 antibody did cause the expected supershift of the IL-2-induced DNA binding activity. Thus, specific signaling events directed by the IL-4R heterodimeric complex do not require the tyrosine residues of the γ_c subunit. The γ_c subunit, therefore, seems to be involved primarily in the initiation of signaling by the receptor complex, rather than in the determination of specific signaling events within the JAK–STAT pathway through tyrosine-based sequences of γ_c.

The IL-4Rα subunit determines specific signaling events in multiple receptor complexes

Based on the results above, the IL-4Rα and its associated signaling molecules appear to be responsible for determining the specific signaling events induced by the heterodimeric IL-4Rα–γ_c receptor complex. Since the IL-4Rα subunit is involved in multiple receptor complexes, each of these receptor types might be predicted to activate similar specific signaling events. In the IL-13R complex, the IL-4Rα subunit is thought to serve an integral role by pairing with the recently characterized IL-13Rα subunit in the absence of γ_c (Zurawski *et al.*, 1995; Hilton *et al.*, 1996). Consistent with the participation of IL-4Rα in this complex, stimulation of an erythroleukemia cell line, TF-1, with IL-13 also resulted in the activation of STAT-6 (Figure 2A, middle). This finding implies that signaling specificity within this receptor complex is likewise derived from the common IL-4Rα chain.

Evidence for another configuration of IL-4Rα-containing receptors is found in a human colon carcinoma cell line, in which IL-4 receptor complexes are competent to bind the IL-4 ligand despite the lack of detectable γ_c subunit expression (Murata *et al.*, 1996). These receptors apparently contain IL-4Rα chains without a known partner subunit. Similarly, such receptors are also expressed by a human monocyte cell line, THP-1, which does not express mRNA encoding the γ_c subunit (Takeshita *et al.*, 1992). As confirmed here (Figure 2A, right), stimulation of THP-1 cells by IL-4 nonetheless results in the selective induction of STAT-6 (Hou *et al.*, 1994). Furthermore, B cell lines derived from patients with X-SCID, which do not express detectable mRNA encoding γ_c (MA) or which contain an extracellular mutation in γ_c that prevents ligand binding (RL) (Puck *et al.*, 1993), retain responsiveness to IL-4. As with IM-9, a human lymphoblast cell line, stimulation of the X-SCID MA and RL cell lines by IL-4 led to the activation of STAT-6 (Figure 2B). Therefore, this class of receptor complexes is capable of activating the same signaling event as other classes of IL-4Rα-containing receptor complexes, despite the absence of γ_c. Thus, in all of these receptor complexes, the IL-4Rα chain is responsible for signal transduction specificity as measured by activation of STAT-6, apparently regardless of the nature of the partner subunit.

The IL-4Rα subunit alone mediates specific signaling events

Because of the range of receptor configurations that retain IL-4-specific signaling specificity, the IL-4Rα subunit alone may be competent to form a functional receptor complex without other receptor subunit partners. To test this hypothesis, the EPO4 receptor subunit was stably expressed alone in HT-2 cells to generate the HT-2EPO4 cell line. As expected, stimulation of HT-2EPO4 cells with IL-2 or IL-4 resulted in distinct DNA binding complexes corresponding to STAT-5 and STAT-6, respectively (Figure 3B). Strikingly, stimulation of the HT-2EPO4 cell line by EPO resulted in the strong activation of a DNA binding complex that had a similar migration to that of the IL-4-induced STAT-6 complex. Moreover, the specific nature of this complex was demonstrated by the abolition of this EPO4-mediated activity upon pre-treatment with the anti-STAT-6, but not with the anti-STAT-5, antibody (Figure 3B). Thus, engagement of the IL-4Rα cytoplasmic tails without the γ_c subunit leads to the activation of STAT-6 DNA binding activity as detected by EMSA.

As shown earlier, engagement of the IL-4Rα and γ_c subunits in the heterodimeric IL-4R complex results in the activation of JAK1 and JAK3 as well as the subsequent induction of the STAT-6 DNA binding complex (Figure 1). Similarly, binding of the EPO4 subunit in the absence of chimeric EPOγ chains resulted in the activation of STAT-6, implying that activation of the JAK–STAT pathway by the IL-4R can occur independently of γ_c chains. As demonstrated in a previous report, engagement of the EPOγ subunit alone did not result in the detectable

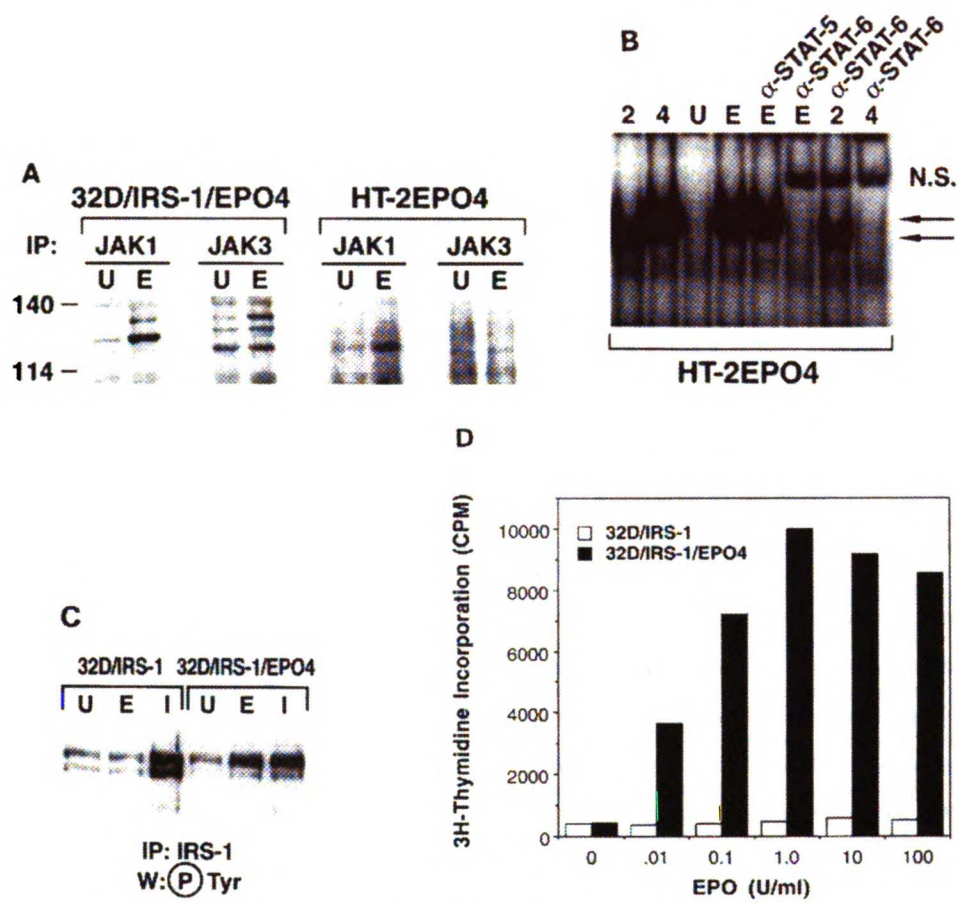

Fig. 3. Homodimerization of IL-4Rα cytoplasmic domains leads to the activation of IL-4-specific signaling events. (**A**) Lysates of the indicated cell lines rested (U) or treated with EPO (E, 50 U/ml) were subjected to immunoprecipitation with anti-JAK1 antiserum. Immunoblot analysis was performed with the anti-phosphotyrosine antibody. (**B**) EMSA of nuclear extracts from HT-2EPO4 cells that were rested (U) or stimulated with IL-2 (2, 10 nM), IL-4 (4, 50 U/ml) or EPO (E, 50 U/ml). Competition was performed with the anti-STAT-5 and anti-STAT-6 antibodies. Arrows indicate the IL-2-induced (upper) and IL-4-induced (bottom) bands. (**C**) Lysates of the indicated cell lines that were rested (U) or treated with EPO (E, 50 U/ml) or insulin (I, 1 μg/ml) were subjected to immunoprecipitation by the anti-IRS-1 antiserum. Immunoblotting was performed with the anti-phosphotyrosine antibody. Even sample loading was verified by stripping the blot and re-probing with the anti-IRS-1 antiserum (data not shown). (**D**) Stable 32D transfectants were stimulated with EPO in [^{3}H]thymidine incorporation assay experiments to evaluate the proliferative response of cells expressing the EPO4 chimeric subunit. Maximum [^{3}H]thymidine incorporation due to stimulation by EPO was 50% of incorporation measured when cells were grown in 5% WEHI 3B-conditioned medium (data not shown).

activation of either the JAK kinases or any STAT factor (Lai *et al.*, 1996). To determine the specific nature of JAK kinase activation by EPO4 homodimers, anti-JAK immunoprecipitates prepared from HT-2EPO4 cells were immunoblotted with an anti-phosphotyrosine antibody. EPO stimulation of these cells resulted in the weak, but reproducibly detectable, activation of JAK1, but not of JAK3 (Figure 3A), in contrast to the strong activation of both kinases by the heterodimeric IL-4Rα–γ_c complexes (Figure 1A). Similar results were observed in an IL-4-responsive pro-myeloid cell line, 32D/IRS-1, previously used for studies of IL-4R-mediated signaling through IRS-1 (Wang *et al.*, 1993). These cells were transfected with the EPO4 expression vector, and the resulting 32D/IRS-1/EPO4 cell line was used to examine IL-4Rα-specific signaling events. As with the HT-2EPO4 cell line, EPO stimulation of 32D/IRS-1/EPO4 cells activated JAK1, but not JAK3 (Figure 3A); no activation of either JAK2 or Tyk2 was observed in either cell line (data not shown). These results with the stably transfected HT-2 and 32D cell lines may be expected, since JAK3 depends upon its association with the γ_c cytoplasmic tail for transport into a receptor complex, while JAK1 presumably associates with the IL-4Rα cytoplasmic tail. Thus, low level activation of JAK1 alone, but not JAK3, appears to be sufficient for the robust activation of STAT-6 DNA binding activity by the IL-4Rα subunit.

The IL-4Rα cytoplasmic tail without γ_c mediates IRS-1 phosphorylation and cellular proliferation

The observation that stimulation of 32D/IRS-1/EPO4 cells by EPO resulted in JAK1 phosphorylation and STAT-6

activation suggested that homodimers of the IL-4Rα cytoplasmic domain mediate signaling events comparable with those generated by the heterodimeric IL-4R complex. Previous work in 32D/IRS-1/IL-4R cells demonstrated the linkage of the IL-4R to the phosphorylation of IRS-1 and related molecules, and correlated this event with cellular proliferation (Wang *et al.*, 1993). Therefore, to determine the role of the IL-4Rα chain in such processes, these events were investigated in the 32D/IRS-1/EPO4 cell line. Anti-phosphotyrosine immunoblotting of IRS-1 immunoprecipitates from 32D/IRS-1 and 32D/IRS-1/EPO4 cells demonstrated strong IRS-1 phosphorylation following the stimulation of endogenous insulin receptors (Figure 3C). While EPO stimulation of parental 32D/IRS-1 cells did not enhance IRS-1 phosphorylation, 32D/IRS-1/EPO4 cells demonstrated a marked increase in IRS-1 phosphorylation upon treatment with EPO (Figure 3C).

Furthermore, to determine the role of IL-4Rα cytoplasmic tails in cellular proliferation, these transfectants were employed in conventional [^{3}H]thymidine incorporation experiments. While 32D/IRS-1 cells were unresponsive to EPO at all concentrations, 32D/IRS-1/EPO4 cells demonstrated a clear dose-dependent proliferation response to EPO stimulation (Figure 3D). Both the activation of IRS-1 and the induction of cellular proliferation through EPO4 chains in the absence of γ_c demonstrate the critical and unique role of the IL-4Rα subunit in defining IL-4R complex signaling events. These findings suggest that IL-4Rα homomers represent a novel class of functional IL-4 receptors with preserved signal transduction competence and specificity.

Discussion

In this study, a chimeric receptor system was employed to examine the functional architecture of the IL-4R complex. In the heterodimeric pairing of the IL-4Rα chain with the shared γ_c chain, various cellular signaling events were activated. These processes were not altered detectably when all four cytoplasmic tyrosine residues of the γ_c chain were converted to phenylalanines. Consistent with recent mutational studies of the IL-4Rα subunit (Ryan *et al.*, 1996; Wang *et al.*, 1996), this finding suggests that specific signaling events derived from the IL-4R heterodimeric complex depend primarily upon particular peptide motifs contained within the IL-4Rα subunit, rather than upon the tyrosine-containing sequences of the γ_c chain.

Since the combination of IL-4Rα and γ_c chains has been demonstrated previously to increase the affinity of the IL-4R complex for the ligand (Kondo *et al.*, 1993; Russell *et al.*, 1993), it is likely that in cells expressing the γ_c subunit, IL-4 would be bound predominantly by IL-4Rα–γ_c heterodimeric complexes. Consistent with this model is the finding that expression of cytoplasmic truncation mutants of γ_c inhibits responses to IL-4 in some cellular contexts by forming IL-4Rα–γ_c heterodimers that bind IL-4 but are incompetent to transduce signals (Kawahara *et al.*, 1994). Therefore, in the heterodimeric IL-4Rα–γ_c configuration, a functionally intact γ_c chain appears to be required for optimal receptor function.

Evidence exists, however, suggesting that γ_c is not an obligate component of the IL-4R signaling apparatus in all receptor configurations. For example, certain cells naturally containing IL-4Rα subunits but lacking γ_c chains have also been reported to display IL-4R-specific signaling responses to IL-4 (Hou *et al.*, 1994; He and Malek, 1995). Additionally, specific γ_c mutations in several cell lines from patients with X-SCID lead to the lack of γ_c expression on the cell surface without concomitant loss of responsiveness to IL-4 and IL-13 (Matthews *et al.*, 1995). The present studies, likewise, provide functional examples of cell lines derived from human patients with X-SCID that display a preserved STAT-6 response to IL-4 despite the absence of functional γ_c chains (Figure 2B). There are two possible explanations for such observations. First, it is possible that the IL-4Rα chain associates with another unidentified receptor subunit to form heteromeric complexes that are competent to execute signal transduction. For example, although IL-4Rα forms a heterodimeric complex with IL-13Rα to bind IL-13, it remains to be determined whether this heterodimer also forms functional IL-4 receptors lacking γ_c chains. The second alternative, which is supported by the present findings, is that homotypic interactions of IL-4Rα are capable of transducing specific signaling events, including induction of JAK1, activation of STAT-6 DNA binding activity, phosphorylation of IRS-1 and cellular proliferation (Figure 3; Wang *et al.*, 1996). Together, these findings demonstrate that the IL-4Rα chain alone is capable of coupling directly to specific signaling pathways, and suggest that the γ_c subunit represents only one of the various partner chains that may form functional receptor complexes with IL-4Rα to activate cellular signaling pathways. For example, activation of the IL-13R complex, presumably due to the heterodimerization of IL-4Rα with IL-13Rα, leads to the induction of STAT-6 (Figure 2A, middle), a DNA binding factor previously proposed to associate with specific phosphotyrosines of the IL-4Rα cytoplasmic domain (Hou *et al.*, 1994). This observation confirms earlier reports suggesting that the IL-4- and IL-13-induced STAT activities are related (Kohler *et al.*, 1994; Lin *et al.*, 1995), and further assigns signaling specificity to the IL-4Rα subunit. Furthermore, this ability of the IL-4Rα subunit to direct signaling events independently of the γ_c subunit is consistent with the wider pattern of IL-4Rα expression compared with the distribution of γ_c chains (Beckmann *et al.*, 1992; Takeshita *et al.*, 1992).

These results parallel certain structure–function relationships previously described for the IL-2 receptor complex (Lai *et al.*, 1996). In that receptor system, the IL-2Rβ subunit directs the specific nature of the signaling events derived from IL-2 binding and has therefore been designated a 'driver' subunit. The γ_c subunit primarily functions to provide JAK3 to permit receptor signaling initiation, and was thus termed the 'trigger' subunit. As in the IL-2 receptor complex, the γ_c subunit in the heterodimeric IL-4Rα–γ_c complex appears to function mainly to transport JAK3 into the receptor complex for initiation of the signaling cascade. Our studies do not formally exclude the possibility that γ_c itself directs particular signaling events within some receptor contexts. A prior report suggested that the prevention of T cell anergy is mediated through γ_c-associated signaling pathways (Boussiotis *et al.*, 1994). Although those studies do not address the possibility that such signals are due to subunits present in other γ_c-containing receptor complexes (e.g. IL-15R), the γ_c subunit

itself may modulate or differentially regulate signaling events directed by its partner subunit. Nevertheless, the ability of the IL-4Rα subunit to direct IL-4R-specific signaling events in the absence of γ_c indicates that the IL-4Rα subunit is the 'driver' subunit in the IL-4R complexes.

A variety of observations have led to the hypothesis that JAK3 is predominantly responsible for signal transduction specificity in γ_c-containing receptors. Studies with dominant-negative strategies suggested that the γ_c-associated JAK3 kinase plays an important role in signal transduction by IL-2R and related receptors (Kawahara *et al.*, 1995). Likewise, biochemical analysis implied the possible role of JAK3 activation in signaling through IL-4R (Fenghao *et al.*, 1995; Malabarba *et al.*, 1995). JAK3 itself has also been implicated in the activation of specific signaling pathways, such as the induction of STAT-5, by recent studies of HTLV-I-transformed T-cells (Migone *et al.*, 1995). Furthermore, both JAK3 gene deletion mice and human subjects with JAK3 mutations display phenotypic abnormalities that suggest a crucial role for JAK3 in lymphoid cell development (Park *et al.*, 1995; Russell *et al.*, 1995). However, in the present study, engagement of JAK3 was dispensable in the activation of specific signaling events normally associated with the IL-4Rα–γ_c heterodimeric complex, such as the induction of STAT-6, the activation of IRS-1 and cellular proliferation. Rather, these signaling events could also be activated through a homodimeric IL-4Rα receptor complex lacking γ_c and its associated JAK3. These findings are consistent with prior functional analyses of the role of Janus kinases within the IL-2R complex (Gaffen *et al.*, 1996; Lai *et al.*, 1996). In these studies, replacement of the cytoplasmic region of γ_c and its associated JAK3 in the IL-2R complex by a heterologous receptor domain and an alternate JAK did not alter specific signaling outcomes. Thus, while JAK3 may be crucial for signaling by certain cytokine receptors during lymphoid development, this kinase is not essential for the activation of certain specific signaling events by homomeric IL-4Rα receptor complexes.

In the heterodimeric IL-4R complex, ligand binding leads to the activation of JAK1 and JAK3. Although others have reported the additional induction of Tyk2 by IL-4 in some cell types (Murata *et al.*, 1996), we did not detect activation of this kinase in the present system by either heterodimeric or homomeric IL-4 receptors (data not shown). In the present chimeric receptor system, homodimerization of the IL-4Rα cytoplasmic tails was sufficient to activate JAK1 at least modestly, without detectable induction of JAK2, Tyk2 or JAK3. Although the possibility certainly exists that JAK3 may augment phosphorylation of JAK1 in the heterodimeric IL-4Rα–γ_c complex, moderate activation of JAK1 alone, in the absence of JAK3, appeared to be sufficient to induce IL-4R complex signaling events. Interestingly, although JAK2 is activated independently of other JAK kinases in a variety of receptor types (e.g. the homodimeric erythropoietin and growth hormone receptors), activation of JAK1 alone by a cytokine receptor complex has not been reported previously. This finding raises the possibility that other native receptor complexes may also employ JAK1 exclusively. Surprisingly, we found previously that homodimerization of other receptor chains that normally heterodimerize with γ_c was insufficient to activate JAK1 and further

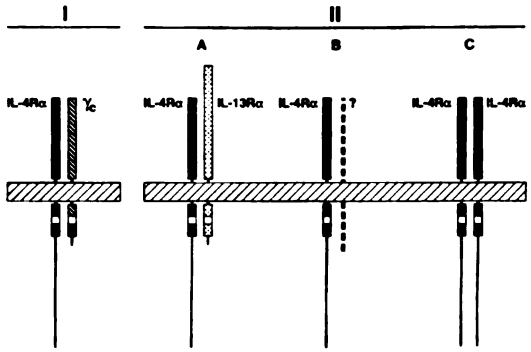

Fig. 4. Multiple forms of the IL-4 receptor complex. IL-4 activates cellular signaling events through several potential receptor complexes. Type I receptor complexes are composed of the IL-4Rα and γ_c subunits. Current evidence also supports the possible existence of several Type II receptor complexes for IL-4. See text for further discussion.

signaling responses (Lai *et al.*, 1996 and data not shown). The contrast between IL-4Rα and these other receptor subunits suggests the possibility that a previously unrecognized factor influencing receptor competence is the compatibility of the cytoplasmic domains of two subunits within a receptor complex. Further study is needed to determine whether or not such a constraint upon receptor activation is imposed by direct subunit–subunit interactions.

Recent reports and the present study collectively demonstrate the existence of at least two types of receptor complexes that bind to IL-4 (Figure 4). Type I receptors employ the IL-4Rα and γ_c subunits in a heterodimeric complex (He and Malek, 1995). The nature of the other type(s) of IL-4 receptor complex is less clearly delineated. IL-4Rα and the recently cloned IL-13Rα subunit form a complex that binds IL-13, but the capacity of such a heterodimer to serve as an IL-4 receptor (here termed Type IIA, Figure 4) has not been well established (Hilton *et al.*, 1996). IL-4Rα may also form a receptor complex (Type IIB) with another as yet unidentified subunit, termed γ′ (Keegan *et al.*, 1995; Lin *et al.*, 1995), which may be either the IL-13Rα chain itself or a novel protein. Since the IL-13Rα gene is available, this issue now can be addressed directly. The present studies support the simplest model, in which γ_c-independent responsiveness to IL-4 is mediated through a homomeric IL-4Rα receptor complex (Type IIC), in which no additional receptor subunits are necessary for the initiation and specification of IL-4-directed signaling events. Although direct evidence for the existence of these homomeric receptors in nature is not available, such a functional receptor complex conceivably could represent a novel class of receptors for IL-4, IL-13 or other as yet unidentified cytokines.

While cytokine receptor complexes typically have been depicted as dimeric or trimeric combinations of individual subunits, conventional descriptions may not delineate the actual stoichiometry of the functional signaling complex. Signaling by cytokine receptor complexes may result from multimeric arrangements of the heterodimeric units, as reported for the IL-6 receptor (Paonessa *et al.*, 1995). Specifically, the heterodimeric IL-4Rα–γ_c pairing may

serve as a functional unit within a larger complex to permit signal transduction. Thus, the functional pairing of IL-4Rα chains in the present chimeric system may represent essential homotypic subunit interactions within these multimeric receptor complexes. Nevertheless, the presence of IL-4Rα in multiple receptor complexes for IL-4 and IL-13 demonstrates a general principle of receptor subunit modularity: in all of the receptor complexes that employ the IL-4Rα subunit, IL-4Rα and its associated intracellular molecules are principally responsible for directing specific signaling events. These conclusions may have implications regarding structure–function principles that govern other receptor families with shared subunits.

Materials and methods

Cell lines and reagents

HT-2, an IL-2-dependent murine helper T cell line [American Type Culture Collection (ATCC)] was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 55 μM β-mercaptoethanol (β-ME), 2 mM L-glutamine and 200 U/ml recombinant human IL-2 [a gift of Chiron Corp. (Emeryville, CA)]. 32D/IRS-1, a pro-myeloid cell line stably expressing the IRS-1, was cultured in 32D medium (RPMI 1640 containing 10% FBS and 5% WEHI 3B-conditioned medium). THP-1, a human monocyte cell line (ATCC), was grown in RPMI 1640 supplemented with 10% FBS and 55 μM β-ME. TF-1, a human erythroleukemia cell line (ATCC), was maintained in RPMI 1640 with 10% FBS and 1 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF, Genzyme). IM-9, a human lymphoblast cell line (ATCC), was cultured in RPMI 1640 with 10% FBS. The X-SCID cell lines were maintained in RPMI 1640 with 10% FBS, 10 mM HEPES, 2 mM L-glutamine and 55 μM β-ME.

Transfection of either HT-2 or 32D/IRS-1 cells was performed by electroporation as described previously (Goldsmith *et al.*, 1994). Stable transfectants were obtained by selection in G418 (Geneticin, 1 mg/ml, GIBCO Life Technologies), and clones isolated by limiting dilution were screened by Northern blot analysis to identify clones expressing the transfected receptor subunit. Stable HT-2 transfectants expressing two receptor subunits were derived from cells already expressing either the EPOγ or EPOγYF chain. Following electroporation, stable transfectants were isolated by selection in G418 (1 mg/ml) and hygromycin B (500 μg/ml, Boehringer Mannheim) and screened by Northern blot analysis. The anti-phosphotyrosine monoclonal antibody (4G10), anti-JAK1, anti-JAK2 and anti-JAK3 were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-STAT-5 antiserum was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-STAT-6 antiserum and the control pre-immune serum were kindly provided by S.McKnight. Anti-IRS-1 antibody was prepared as previously described (Wang *et al.*, 1993). Human and murine recombinant IL-4 was obtained from Genzyme (Cambridge, MA), and recombinant human EPO was the generous gift of Ortho Biotech Inc. (Raritan, NJ).

Plasmid constructs

All receptor cDNAs were subcloned into the expression vectors pCMV4Neo (Goldsmith *et al.*, 1994) or pCMV4Hygro, a derivative of pCMV4 (Andersson *et al.*, 1989) containing a hygromycin B resistance gene as a selectable marker. pEPO4neo was constructed by PCR using an *Nhe*I site at the fusion junction. The chimeric receptor subunit contains the extracellular domain of the EPOR fused just above the transmembrane segment to the human IL-4Rα transmembrane and cytoplasmic domains [resulting sequence: . . . (EPOR-T-A-S)-(R-E-P-IL-4Rα). . .]. The EPOγ and EPOγYF receptor subunits were constructed as previously described (Goldsmith *et al.*, 1994). For all constructs requiring synthetic oligonucleotides or PCR reaction, sequences were confirmed by DNA sequencing.

Electrophoretic mobility shift assay (EMSA)

For this assay, $20–40\times10^6$ cells were rested and stimulated as described above and washed in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). Nuclear extracts were prepared as described (Schreiber *et al.*, 1989) in the presence of 1 mM sodium orthovanadate and the following protease inhibitors: antipain, 0.5 mg/ml; aprotinin, 0.5 mg/ml; bestatin, 0.75 mg/ml; leupeptin, 0.5 mg/ml; pepstatin A, 0.05 mg/ml; phosphoramidon 1.4 mg/ml; and soybean trypsin inhibitor, 0.5 mg/ml (Sigma). The IgG Fc receptor (FcγR1) STAT response element probe was end-labeled with [γ-^{32}P]dATP (Amersham) and polynucleotide kinase (New England Biolabs). DNA binding studies were performed with 10^5 c.p.m. of probe, 3 μg of poly[d(I–C)] and 10 μg of nuclear extract as described (Latchman, 1993). Pre-incubations of nuclear extracts with different antibodies were performed in the absence of poly[d(I–C)] and binding buffer for 45 min on ice prior to initiation of the binding assay by addition of radiolabeled probe.

JAK and IRS-1 phosphorylation studies

For cytokine stimulation, $20–40\times10^6$ cells were washed twice in CMF-PBS, stripped of cell-bound ligand for 1 min in 10 mM sodium citrate, pH 4.0/140 mM NaCl and rested for 4 h in RPMI 1640 medium containing 1% bovine serum albumin (fraction V, Sigma). After stimulation with the appropriate factor, cells were washed in CMF-PBS and lysed [1% NP-40/20 mM Tris–HCl, pH 8.0/150 mM NaCl/50 mM NaF/100 mM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/leupeptin (10 μg/ml)/aprotinin (10 μg/ml)/pepstatin A (1 μg/ml)]. Immunoprecipitations were performed with the indicated antibodies and protein A–Sepharose. Immunoblot analysis was performed with the anti-phosphotyrosine antibody (4G10) according to the manufacturer's instructions, with detection by ECL (Amersham) signal development. For JAK analysis, blots were stripped (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris–HCl pH 6.7) for 30 min at 55°C and re-probed with anti-JAK1 and anti-JAK3 antisera to verify even protein loading.

Proliferation assays

Conventional 24 h [^{3}H]thymidine incorporation assays were performed as previously described (Goldsmith *et al.*, 1994). Briefly, 32D cells were counted, washed twice in CMF-PBS and resuspended at 10^6 cells/ml of 32D medium without the WEHI 3B-conditioned medium supplement. A total of 10^5 cells per well were grown in the indicated concentrations of EPO for 24 h, with [^{3}H]thymidine incorporation measured in the last 4 h.

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References

Andersson,S., Davis,D.L., Dahlback,H., Jornvall,H. and Russell,D.W. (1989) Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.*, **264**, 8222–8229.

Banchereau,J. and Rybak,M.E. (1994) Interleukin-4. In Thompson,A.W. (ed.), *The Cytokine Handbook*. Academic Press Ltd, San Diego, CA, pp. 99–126.

Bazan,J.-F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl Acad. Sci. USA*, **87**, 6934–6938.

Beckmann,M.P., Cosman,D., Fanslow,W., Maliszewski,C.R. and Lyman,S.D. (1992) The IL-4 receptor: structure, function, and signal transduction. In Kishimoto,T. (ed.), *Interleukins: Molecular Biology and Immunology*. Karger, Basel, Vol. 51, pp. 107–134.

Boussiotis,V.A., Barber,D.L., Nakarai,T., Freeman,G.J., Gribben,J.G., Bernstein,G.M., D'Andrea,A.D., Ritz,J. and Nadler,L.M. (1994) Prevention of T cell anergy by signaling through the γ_c chain of the IL-2 receptor. *Science*, **266**, 1039–1042.

Broxmeyer,H.E., Lu,L., Cooper,S., Tushinski,R., Mochizuki,D., Rubin, B.Y., Gillis,S. and Williams,D.E. (1988) Synergistic effects of purified recombinant human and murine B cell growth factor-1/IL-4 on colony

formation *in vitro* by hematopoietic progenitor cells. *J. Immunol.*, **141**, 3852–3862.

Cao,X. *et al.* (1995) Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. *Immunity*, **2**, 223–238.

DiSanto,J.P., Muller,W., Guy-Grand,D., Fischer,A. and Rajewsky,K. (1995) Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. *Proc. Natl Acad. Sci. USA*, **92**, 377–381.

Earp,H.S., Dawson,T.L., Li,X. and Yu,H. (1995) Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Res. Treatment*, **35**, 115–132.

Fenghao,X., Saxon,A., Nguyen,A., Ke,Z., Diaz-Sanchez,D. and Nel,A. (1995) IL4 activates a signal transducer and activator of transcription (Stat) protein which interacts with an interferon-γ activation site-like sequence upstream of the Ie exon in a human B cell line. *J. Clin. Invest.*, **96**, 907–914.

Gaffen,S.L., Lai,S.Y., Ha,M., Liu,X., Hennighausen,L., Greene,W.C. and Goldsmith,M.A. (1996) Distinct tyrosine residues within the IL-2 receptor β chain drive signal transduction specificity, redundancy and diversity. *J. Biol. Chem.*, in press.

Giri,J.G. *et al.* (1994) Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.*, **13**, 2822–2830.

Goldsmith,M.A., Xu,W., Amaral,M.C., Kuczek,E.S. and Greene,W.C. (1994) The cytoplasmic domain of the IL-2 receptor β chain contains both unique and functionally redundant signal transduction elements *J. Biol. Chem.*, **269**, 14698–14704.

Harada,N., Castle,B.E., Gorman,D.M., Itoh,N., Schreurs,J., Barrett,R.L., Howard,M. and Miyajima,A. (1990) Expression cloning of a cDNA encoding the murine interleukin 4 receptor based on ligand binding. *Proc. Natl Acad. Sci. USA*, **87**, 857–861.

He,Y.-W. and Malek,T.R. (1995) The IL-2 receptor γ_c chain does not function as a subunit shared by the IL-4 and IL-13 receptors: implication for the structure of the IL-4 receptor *J. Immunol.*, **155**, 9–12.

Hilton,D.J., Zhang,J.-G., Metcalf,D., Alexander,W.S., Nicola,N.A. and Willson,T.A. (1996) Cloning and characterization of a binding subunit of the IL-13 receptor that is also a component of the IL-4 receptor. *Proc. Natl Acad. Sci. USA*, **93**, 497–501.

Hou,J., Schindler,U., Henzel,W.J., Ho,T.C., Brasseur,M. and McKnight,S.L. (1994) An interleukin-4-induced transcription factor: IL-4 Stat. *Science*, **265**, 1701–1706.

Kawahara,A., Minami,Y. and Taniguchi,T. (1994) Evidence for a critical role for the cytoplasmic region of the interleukin 2 (IL-2) receptor γ chain in IL-2, IL-4, and IL-7 signaling. *Mol. Cell. Biol.*, **14**, 5433–5440.

Kawahara,A., Minami,Y., Miyazaki,T., Ihle,J.N. and Taniguchi,T. (1995) Critical role of the IL-2 receptor γ-chain-associated Jak3 in the IL-2-induced *c-fos* and *c-myc*, but not *bcl-2*, gene induction. *Proc. Natl Acad. Sci. USA*, **92**, 8724–8728.

Keegan,A.D., Nelms,K., Wang,L.M., Pierce,J.H. and Paul,W.E. (1994) IL-4 receptor: signaling mechanisms. *Immunol. Today*, **15**, 423–432.

Keegan,A.D., Johnston,J.A., Tortolani,P.J., McReynolds,L.J., Kinzer,C., O'Shea,J.J. and Paul,W.E. (1995) Similarities and differences in signal transduction by IL-4 and IL-13: analysis of Janus kinase activation. *Proc. Natl Acad. Sci. USA*, **92**, 7681–7685.

Kohler,I., Alliger,P., Minty,A., Caput,D., Ferrara,P., Holl-Neugebauer,B., Rank,G. and Rieber,E.P. (1994) Human IL-13 activates the IL-4-dependent transcription factor NF-IL4 sharing a DNA binding motif with an interferon-γ-induced nuclear binding factor. *FEBS Lett.*, **345**, 187–192.

Kondo,M., Takeshita,T., Ishii,N., Nakamura,M., Watanabe,S., Arai,K.-I. and Sugamura,K. (1993) Sharing of the interleukin-2 (IL-2) receptor γ chain between receptors for IL-2 and IL-4. *Science*, **262**, 1874–1877.

Kondo,M., Takeshita,T., Higuchi,M., Nakamura,M., Sudo,T., Nishikawa,S. and Sugamura,K. (1994) Functional participation of the IL-2 receptor γ chain in IL-7 receptor complexes. *Science*, **263**, 1453–1454.

Lai,S.Y., Xu,W., Gaffen,S.L., Longmore,G.D., Greene,W.C. and Goldsmith,M.A. (1996) The molecular role of the common γ_c subunit in signal transduction reveals functional asymmetry within multimeric cytokine receptor complexes. *Proc. Natl Acad. Sci. USA*, **93**, 231–235.

Latchman,D.S. (1993) *Transcription Factors: A Practical Approach*. IRL Press at Oxford University Press, Oxford.

Lefort,S., Vita,N., Reeb,R., Caput,D. and Ferrara,P. (1995) IL-13 and IL-4 share signal transduction elements as well as receptor components in TF-1 cells. *FEBS Lett.*, **366**, 122–126.

Lin,J.-X. *et al.* (1995) The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity*, **2**, 331–339.

Malabarba,M.G., Kirken,R.A., Rui,H., Koettnitz,K., Kawamura,M., O'Shea,J.J., Kalthoff,F.S. and Farrar,W.L. (1995) Activation of JAK3, but not JAK1, is critical to IL4 stimulated proliferation and requires a membrane-proximal region of IL4 receptor α. *J. Biol. Chem.*, **270**, 9630–9637.

Matthews,D.J. *et al.* (1995) Function of the IL-2 receptor γ-chain in biologic responses of X-linked severe combined immunodeficient B cells to IL-2, IL-4, IL-13, and IL-15. *Blood*, **85**, 38–42.

Migone,T.S., Lin,J.X., Cereseto,A., Mulloy,J.C., O'Shea,J.J., Franchini,G. and Leonard,W.J. (1995) Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-1. *Science*, **269**, 79–81.

Mosley,B. *et al.* (1989) The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. *Cell*, **59**, 335–348.

Murakami,M., Narazaki,M., Hibi,M., Yawata,H., Yasukawa,K., Hamaguchi,M., Taga,T. and Kishimoto,T. (1991) Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc. Natl Acad. Sci. USA*, **88**, 11349–11353.

Murata,T., Noguchi,P.D. and Puri,R.K. (1996) Receptors for IL-4 do not associate with the common γ chain, and IL-4 induces the phosphorylation of JAK2 tyrosine kinase in human colon carcinoma cells. *J. Biol. Chem.*, **270**, 30829–30836.

Noguchi,M., Nakamura,Y., Russell,S.M., Ziegler,S.F., Tsang,M., Cao,X. and Leonard,W.J. (1993a) Interleukin-2 receptor γ chain: a functional component of the interleukin-7 receptor. *Science*, **262**, 1877–1880.

Noguchi,M., Yi,H., Rosenblatt,H.M., Filipovich,A.H., Adelstein,S., Modi,W.S., McBride,O.W. and Leonard,W.J. (1993b) Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell*, **73**, 147–157.

Paonessa,G., Graziani,R., De Serio,A., Savino,R., Ciapponi,L., Lahm,A., Salvati,A.L., Toniatti,C. and Ciliberto,G. (1995) Two distinct and independent sites on IL-6 trigger gp 130 dimer formation and signalling. *EMBO J.*, **14**, 1942–1951.

Park,S.Y. *et al.* (1995) Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity*, **3**, 771–782.

Puck,J.M., Deschenes,S.M., Porter,J.C., Dutra,A.S., Brown,C.J., Willard,H.F. and Henthorn,P.S. (1993) The interleukin-2 receptor γ chain maps to Xq13.1 and is mutated in X-linked severe combined immunodeficiency, SCIDX1. *Hum. Mol. Genet.*, **2**, 1099–1104.

Russell,S.M. *et al.* (1993) Interleukin-2 receptor γ chain: a functional component of the interleukin-4 receptor. *Science*, **262**, 1880–1883.

Russell,S.M. *et al.* (1995) Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science*, **270**, 797–800.

Ryan,J.J., McReynolds,L.J., Keegan,A., Wang,L.-H., Garfein,E., Rothman,P., Nelms,K. and Paul,W.E. (1996) Growth and gene expression are predominantly controlled by distinct regions of the human IL-4 receptor. *Immunity*, **4**, 123–132.

Schlessinger,J. and Ullrich,A. (1992) Growth factor signaling by receptor tyrosine kinases. *Neuron*, **9**, 383–391.

Schreiber,E., Matthias,P., Muller,M.M. and Schaffer,W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.*, **17**, 6419.

Seldin,D.C. and Leder,P. (1994) Mutational analysis of a critical signaling domain of the human interleukin 4 receptor. *Proc. Natl Acad. Sci. USA*, **91**, 2140–2144.

Takeshita,T., Asao,H., Ohtani,K., Ishii,N., Kumaki,S., Tanaka,N., Munakata,H., Nakamura,M. and Sugamura,K. (1992) Cloning of the γ chain of the human IL-2 receptor. *Science*, **257**, 379–382.

Tanaka,T., Hu-Li,J., Seder,R.A., Fazekas de St Groth,B. and Paul,W.E. (1993) IL-4 suppresses IL-2 and interferon-γ production by naive T cells stimulated by accessory cell-dependent receptor engagement. *Proc. Natl Acad. Sci. USA*, **90**, 5914–5918.

Tigges,M.A., Casey,L.S. and Koshland,M.E. (1989) Mechanism of interleukin-2 signaling: mediation of different outcomes by a single receptor and transduction pathway. *Science*, **243**, 781–786.

Wang,H.Y., Paul,W.E. and Keegan,A.D. (1996) IL-4 function can be transferred to the IL-2 receptor by tyrosine containing sequences found in the IL-4 receptor α chain. *Immunity*, **4**, 113–121.

Wang,L.-M., Myers,M.G., Sun,X.-J., Aaronson,S.A., White,M. and Pierce,J.H. (1993) IRS-1: essential for insulin- and IL-4-stimulated mitogenesis in hematopoietic cells. *Science*, **261**, 1591–1594.

Wang,L.-M. *et al.* (1996) The insulin receptor substrate-1-related 4PS substrate but not the interleukin-2Rγ chain is involved in interleukin-13-mediated signal transduction. *Blood*, **86**, 4218–4227.

Widmer,M.B., Acres,R.B., Sassenfeld,H.M. and Grabstein,K.H. (1987) Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. *J. Exp. Med.*, **166**, 1447–1455.

Zurawski,S.M., Chomarat,P., Djossou,O., Bidaud,C., McKenzie,A.N.J., Miossec,P., Banchereau,J. and Zurawski,G. (1995) The primary binding subunit of the human IL-4 receptor is also a component of the IL-13 receptor. *J. Biol. Chem.*, **270**, 13869–13878.

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Chapter 7

An Intracellular Regulatory Domain in γ_c Inhibits Signaling in the Interleukin-2 Receptor Complex

Prologue

The modular design of the cytokine receptor complex provides a molecular basis for the specificity of signal transduction by various receptors. The current paradigm suggests that the oligomerization of receptor subunits leads to the approximation of associated signaling molecules, which is sufficient to promote signal transduction activity. In this model, the receptor subunit serves principally as a passive docking platform for cellular signaling intermediates. Recent analysis of a mutant IL-2Rβ subunit has revealed an active functional role for the cytoplasmic domains of the receptor subunits. The present studies describe the analysis of this IL-2Rβ mutant and the identification of an inhibitory domain within γ_c. Additionally, we describe a model of regulated inhibition that involves the cooperative interaction of IL-2Rβ and γ_c. The following manuscript, as presented here, has been submitted to a journal for possible publication.

Abstract

Cytokine receptor signaling is activated by ligand-induced assembly of an intracellular complex composed of one or more receptor subunits and distinct cytoplasmic signaling molecules (1-3). The oligomerization of the receptor subunits reconfigures the associated signaling molecules in a spatial orientation that promotes their activation and function. Our studies of the human interleukin-2 receptor (IL-2R) now reveal a previously unrecognized mechanism for the inhibition of signaling by this cytokine receptor. This regulated inhibition involves a functional interplay between a distal inhibitory domain located within the cytoplasmic tail of the γ_c subunit and the conserved Box1 element present in the proximal intracellular region of IL-2Rβ. Such an inhibitory interplay between the cytoplasmic tails of two different cytokine receptor subunits represents a novel form of regulation for the cytokine receptors. Since γ_c also participates in the formation of the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 (4,5), the inhibitory domain in the distal tail of γ_c may modulate signal transduction by multiple cytokine receptors.

The high-affinity IL-2R is composed of the IL-2Rα, IL-2Rβ and γ_c chains (6). Heterodimerization of the IL-2Rβ and γ_c subunits is sufficient for IL-2R-mediated growth signaling in T cells (7,8), while the IL-2Rα chain plays a major role in determining the affinity of the receptor for IL-2 (9). To facilitate the mutational analysis of the IL-2R complex in T cells expressing native IL-2R, we have established a chimeric receptor system involving fusion of the extracellular portion of the erythropoietin receptor (EPOR) to the transmembrane and cytoplasmic domains of the IL-2Rβ and γ_c subunits, thus forming EPOβ and EPOγ, respectively (Fig. 1) (8). In combination, these chimeras faithfully execute all of the known steps in the IL-2R signaling pathway when expressed in HT-2 cells, a murine, IL-2-dependent helper T cell line (Fig. 2) (8,10,11).

Our previous mutational analysis of the intracellular domain of IL-2Rβ had identified a single amino acid residue (Asp^{258}) in a conserved membrane-proximal cytoplasmic region termed Box1 that was required for growth signaling (12). Box1, as well as a second 15 amino acid proximal cytoplasmic element termed Box2, are conserved elements found in most members of the cytokine receptor superfamily (Fig. 1) (13). When tested in the chimera system, conversion of Asp^{258} to Ala ($D^{258}A$) predictably abolished EPO-mediated proliferative signaling when paired with EPOγ in multiple HT-2 stable transfectants (HT-2EPOβ-D^{258}A/γ) (Fig. 2a, lower left panel). Further analysis of this abortive signaling revealed that neither the IL-2Rβ-associated Janus kinase JAK1 nor the γ_c-associated Janus kinase JAK3 (5,14) were activated following EPO addition, as assessed by tyrosine auto-phosphorylation (Fig. 2b). However, IL-2 stimulation of the native IL-2 receptors also present on these cells readily activated these JAK kinases. Predictably, the JAK-dependent induction of the transcription factor, STAT-5, was also absent following EPO stimulation as assessed in electrophoretic mobility shift assays (EMSA) (Fig. 2c). Finally, EPO ligation of the EPOβ-D^{258}A/EPOγ heterodimer also failed to induce *c-fos* mRNA expression. Of note, this otherwise inactive EPOβ-D^{258}A/EPOγ heterodimer pair was capable of inducing *bcl-2* mRNA expression which appears to proceed through a JAK-independent (15) and IL-2Rβ tyrosine-independent mechanism (16) (Fig. 2d). This latter finding confirms that these receptor subunits are effectively expressed at the cell surface and are capable of binding ligand. Together, these results indicate that alteration of a single amino acid residue in the Box1 region of the IL-2Rβ cytoplasmic domain markedly compromises JAK-dependent signaling through the IL-2R complex.

IL-2Rβ normally recruits distinct signaling molecules into the receptor complex to drive specific signaling events (16,17). In contrast, the γ_c subunit functions primarily to transport the pre-associated JAK3 kinase into the IL-2R complex to initiate the signaling

process (11,18). Confirmation of this "trigger-driver" model for the IL-2R derives in part from the finding that the γ_c cytoplasmic domain can be fully replaced with a severely truncated tail from the EPOR (EPOR(1-321)) (Fig. 2a, upper right panel) (11) retaining only the membrane-proximal portion of the receptor tail that associates with JAK2 (19). The consequent substitution of the EPOR-associated JAK2 kinase for JAK3 does not appear to affect adversely any measured IL-2R signaling events (11,16).

Although EPOβ-D^{258}A could not effectively partner with EPOγ to activate the JAK-STAT pathway or induce cell growth, we tested the ability of the truncated EPOR subunit to functionally pair with the EPOβ-D^{258}A mutant. Surprisingly, EPO-induced engagement of EPOβ-D^{258}A and EPOR(1-321) pair resulted in significant restoration of growth signaling (Fig. 2a, right lower panel). In addition, the EPOβ-D^{258}A/EPOR(1-321) heterodimer mediated effective activation of JAK1 and JAK2, but not JAK3 (Fig. 2b). STAT-5 activation was also readily detected (Fig. 2c). Finally, the replacement of γ_c by EPOR(1-321) in the heterodimer with IL-2Rβ-D^{258}A led to full restoration of *c-fos* mRNA induction by EPO (Fig. 2d). Thus, the EPOβ-D^{258}A mutant is not fundamentally altered in its ability to associate with cellular signaling intermediates. Instead, the single amino acid alteration in the Box1 domain of IL-2Rβ somehow alters the ability of this chain to cooperate with γ_c subunits to mediate effective signaling.

In view of the effective signaling of the EPOβ-D^{258}A and heterologous EPOR(1-321), we re-focused our attention on the γ_c subunit asking whether suppressor mutations could be identified in this chain that would relieve the signaling block obtained with EPOβ-D^{258}A. We reasoned that the identification of such γ_c mutants could provide important insights into the molecular basis for the abortive signaling. These studies revealed that truncation of the γ_c tail at the end of the Box2 domain (EPOγ336, Fig. 3a) restored growth signaling with EPOβ-D^{258}A (Fig. 3b). These experiments involved transient transfection of the indicated subunits into HT-2 cells stably expressing EPOβ-D^{258}A, followed by serial measurement of EPO-induced [^{3}H]thymidine incorporation . These findings suggest that the C-terminal 33 amino-acids of γ_c are somehow involved in the abortive signaling obtained when EPOγ is paired with EPOβ-D^{258}A.

Based on these results, we hypothesized that this terminal domain of γ_c might normally inhibit interactions between γ_c and IL-2Rβ that are critical for effective signaling. To test this hypothesis, the distal 33-amino-acid γ_c cytoplasmic region was directly fused to the carboxy-terminus of the truncated EPOR(1-321) subunit, thus forming EPOR(1-321)-γ_c and tested for proliferative signaling with EPOβ-D^{258}A in the transient transfection assay. As expected, introduction of EPOR(1-321) into HT-2EPOβ or HT-2EPOβ-D^{258}A cells conferred EPO-mediated proliferation signaling as measured by [^{3}H]thymidine incorporation

(Fig. 3c). Strikingly, pairing of EPOR(1-321)-γ_c with EPOβ-D^{258}A failed to support EPO-mediated proliferation (Fig. 3c), although this construct did signal when paired with the wildtype IL-2Rβ tail (EPOβ). These findings indicate that the distal portion of the γ_c cytoplasmic tail contains a transposable inhibitory domain that may interact with the IL-2Rβ subunit to inhibit receptor signaling.

According to the current paradigm, receptor subunits principally serve as passive docking platforms for cellular signaling intermediates (2,20). For example, γ_c associates with JAK3 (5,14) and functions primarily to transport this kinase into the receptor complex to initiate signaling (11,18). While various experimental approaches have failed to identify additional γ_c-associated signaling molecules (data not shown), our present work suggests a new role for γ_c in the regulation of signal transduction through the IL-2R complex. Unlike potential cytoplasmic interactions of the integrin receptor subunits (21) or phosphorylation-dependent interactions of the TGFβ receptor subunits (22) which act to stabilize these receptor complexes and enhance their function, the interplay of the distal γ_c domain with IL-2Rβ serves to negatively regulate receptor function.

The functional complementation observed in the pairing of IL-2Rβ-D^{258}A with the truncated EPOR(1-321) chain, but not with γ_c, suggests that the dynamic interplay within the receptor heterodimer of IL-2Rβ and γ_c leads to an equilibrium between at least two different receptor configurations that are termed 'active' and 'inactive' in our current model (Fig. 4). In the wildtype receptor complex, the functional inhibitory interaction between the Asp^{258} residue of IL-2Rβ and the inhibitory domain of γ_c ('inactive' state) is overcome by IL-2 binding, favoring an 'active' receptor conformation that allows receptor signaling to proceed. However, in the case of the mutant IL-2Rβ-D^{258}A chain, the functional interplay of Box1 with the inhibitory domain of γ_c is so strong that it can not be overcome by IL-2 binding, leading to capture of the receptor in an 'inactive' state (Fig. 4). Unlike γ_c, the heterologous EPOR(1-321) and truncated γ_c (γ336) successfully pair with the IL-2Rβ-D^{258}A mutant because they lack the inhibitory domain in the distal tail of γ_c. It is possible that this inhibitory function of the γ_c tail may safeguard against inadvertent receptor signaling in the absence of ligand due to subunit collisions in the membrane. Alternatively, the inhibitory domain within γ_c may engage IL-2Rβ and terminate signaling by the 'active' receptor complex. Such an interaction could produce a form of receptor desensitization resulting in down-regulation of signaling activity by the activated receptor complex.

Collectively, the present observations reveal for the first time a role for the cytokine receptor subunits themselves in the direct regulation of signal transduction by the IL-2R complex. This novel regulatory mechanism may involve a direct physical interaction between the cytoplasmic domains of IL-2Rβ and γ_c, or a more indirect interaction with an intermediate

signaling molecule such as JAK3 (5). Given the lack of similarity between the inhibitory domain of γ_c and other known proteins (data not shown), examination of the functional role and molecular mechanism of this regulation is currently ongoing. Furthermore, since γ_c participates in the IL-2, IL-4, IL-7, IL-9 and IL-15 receptor complexes (4,5), studies are in progress to determine whether the distal domain of γ_c exerts a similar inhibitory role in these cytokine receptor systems.

References

1. J. A. Wells, A. M. de Vos, *Ann Rev of Biochem* **65**, 609-634 (1996).
2. J. N. Ihle, *Nature* **377**, 591-594 (1995).
3. C.-H. Heldin, *Cell* **80**, 213-223 (1995).
4. T. Takeshita, et al., *Science* **257**, 379-82 (1992); M. Kondo, et al., *Science* **262**, 1874-1877 (1993); M. Noguchi, et al., *Science* **262**, 1877-1880 (1993); S. M. Russell, et al., *Science* **262**, 1880-83 (1993); J. G. Giri, et al., *EMBO J* **13**, 2822-2830 (1994); M. Kondo, et al., *Science* **263**, 1453-1454 (1994); Y. Kimura, et al., *Intl Immunol* **7**, 115-120 (1995); S. E. Ziegler, et al., *Eur J Immunol* **25**, 399-404 (1995).
5. S. M. Russell, et al., *Science* **266**, 1042-1045 (1994).
6. Reviewed in T. Taniguchi, Y. Minami, *Cell* **73**, 5-8 (1993); M. A. Goldsmith, W. C. Greene, in *The Cytokine Handbook* A. Thomson, Eds. (Academic Press, Ltd, London, 1994) pp. 57-80; W. J. Leonard, *Ann Rev of Med* **47**, 229-39 (1996).
7. B. H. Nelson, J. D. Lord, P. D. Greenberg, *Nature* **369**, 333-336 (1994); Y. Nakamura, et al., *Nature* **369**, 330-333 (1994).
8. M. A. Goldsmith, et al., *JBC* **270**, 21729-21737 (1995).
9. R. J. Robb, W. C. Greene, C. M. Rusk, *JEM* **160**, 1126-1146 (1984).
10. S. L. Gaffen, et al., *PNAS USA* **92**, 7192-7196 (1995).
11. S. Y. Lai, et al., *PNAS USA* **93**, 231-235 (1996).
12. M. A. Goldsmith, W. Xu, M. C. Amaral, E. S. Kuczek, W. C. Greene, *JBC* **269**, 14698-14704 (1994).
13. M. Hatakeyama, H. Mori, T. Doi, T. Taniguchi, *Cell* **59**, 837-845 (1989); M. Murakami, et al., *PNAS USA* **88**, 11349-11353 (1991).
14. T. Miyazaki, et al., *Science* **266**, 1045-1047 (1994).
15. A. Kawahara, Y. Minami, T. Miyazaki, J. N. Ihle, T. Taniguchi, *PNAS USA* **92**, 8724-8728 (1995).
16. S. L. Gaffen, et al., *JBC* **271**, 21381-21390 (1996).
17. M. C. Friedmann, T. S. Migone, S. M. Russell, W. J. Leonard, *PNAS USA* **93**, 2077-82 (1996).
18. B. H. Nelson, J. D. Lord, P. D. Greenberg, *MCB* **16**, 309-317 (1996).
19. B. A. Witthuhn, et al., *Cell* **74**, 227-236 (1993).
20. T. Taniguchi, *Science* **268**, 251-255 (1995).
21. R. Briesewitz, A. Kern, E. E. Marcantonio, *Molec Bio Cell* **6**, 997-1010 (1995).

22. R.-H. Chen, H. L. Moses, E. M. Maruoka, R. Derynk, M. Kawabata, *JBC* **270**, 12235-41 (1995).
23. E. Schreiber, P. Matthias, M. M. Muller, W. Schaffer, *Nucleic Acids Res* **17**, 6419 (1989).
24. D. S. Latchman, *Transcription Factors: A practical approach*. D. R. a. B. D. Hames, Eds., The Practical Approach Series (Oxford University Press, Oxford, 1993).
25. G. Lamson, M. E. Koshland, *JEM* **160**, 877-92 (1984).
26. S. S. Watowich, D. J. Hilton, H. F. Lodish, *MCB* **14**, 3535-3549 (1994).
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Figure Legends

Figure 1. Different 'partner' receptor subunits interact with the IL-2Rβ subunit to form functional receptor signal transduction complexes. (Top) The cytoplasmic domain of the IL-2Rβ subunit can be divided into distinct functional domains: Box1 and Box2 are short, membrane-proximal regions shared by members of the cytokine receptor superfamily. The distal portion of the IL-2Rβ cytoplasmic tail contains six tyrosine-containing motifs which engage various signaling molecules. (Bottom) The chimeric receptor subunits were constructed by overlap PCR through the fusion of the extracellular domain of EPOR to the transmembrane and cytoplasmic segments of IL-2Rβ and γ_c to form EPOβ (resulting sequence: ... (EPOR-**T-A-S**)-(**G-K-D**-IL-2Rβ) ...) and EPOγ (resulting sequence: ... (EPOR-**T-A-S**)-(**S-K-E**-γ_c) ...), respectively (8). The chimeric receptor subunit EPOγ as well as the truncated EPOR(1-321) can be paired with EPOβ to reconstitute receptor signaling. Stable expression of any of the individual receptor subunits alone failed to mediate detectable signaling activity in HT-2 cells (data not shown).

Figure 2. Substitution of aspatic acid 258 with alanine in the proximal Box1 region of EPOβ abrogates JAK/STAT signaling when paired with EPOγ but not when paired with EPOR(1-321). (a) Stable HT-2 transfectants ($5X10^4$ cells/well; HT-2EPOβγ, HT-2EPOβ/EPOR(1-321), HT-2EPOβ-D^{258}A/EPOγ and HT-2EPOβ-D^{258}A/EPOR(1-321) were washed, grown without IL-2 in the indicated doses of EPO (Epogen, Amgen, Inc.) for 24 hours, and assayed for [^{3}H]-thymidine incorporation as previously described (12). (b) Detergent lysates (1% NP-40, 20 mM Tris pH 8.0, 150 mM NaCl, 50 mM NaF, 100 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A) from stable HT-2 transfectants (HT-2EPOβγ, HT-2EPOβ-D^{258}A/γ and HT-2EPOβ-D^{258}A/EPOR(1-321)) rested (U) or stimulated with EPO (E, 50 U/ml) or IL-2 (2, 10 nM) for 15 minutes were subjected to serial immunoprecipitation with anti-JAK1, anti-JAK2 and anti-JAK3 antisera (Upstate Biotechnology Incorporated). Immunoblotting was performed with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology Incorporated) per the manufacturer's instructions with detection by ECL (Amersham Corp.) signal development. Blots were stripped (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris-HCL pH 6.7; 30 min at 55°C) and re-probed with the respective anti-JAK antisera to verify equivalent protein loading (data not shown). Arrowheads denote JAK1 (solid), JAK2 (hatched) and JAK3 (open). (c) Electrophoretic mobility shift assay (EMSA) of nuclear extracts prepared as described (10,23) from cells unstimulated (U) or treated with EPO (E, 50 U/ml) or IL-2 (2, 10 nM) for 15 minutes. Extracts (10 μg) were incubated for 15 minutes

in DNA binding reactions (20 μl) containing ^{32}P-labeled FcγR1 (GTATTTCCCAGAAAAAGGAC) probe (24). Anti-STAT-5 antibody (3 ug) was incubated with extracts for 45 minutes on ice prior to the DNA-binding reaction. Resultant nucleoprotein complexes were resolved on native 5% polyacrylamide gels and visualized by autoradiography. Cell lines are as described above. The bold arrow indicates the band corresponding to STAT-5 and the thin arrow depicts the antibody-induced supershifted complex. (d) Northern analysis of total cellular RNA prepared from 10^7 cells of the indicated cell lines using the RNeasy system (Qiagen). Denaturing gels were run with 10 μg of RNA/ lane and blotted to Zeta Probe membranes (Bio-Rad) (25). Probes were random prime-labeled using a Megaprime kit (Amersham Corp.) with [α-^{32}P]dATP and [α-^{32}P]dCTP (Amersham Corp.).

Figure 3. A transposable inhibitory domain in the distal tail of the γ_c receptor subunit regulates IL-2R complex signal transduction. (a) Schematic diagram of the EPOR(1-321) which retains the membrane proximal Box1 and Box2 domains of EPOR (26), EPOγ336 which lacks the distal 33-amino acid region) and EPOR(1-321)/γ_c which was constructed using overlap PCR (resulting sequence: . . . (EPOR-**A-G-D**)-(**P-K-G**-γ_c). . .). (b) HT-2EPOβ-D^{258}A cells were transfected with the EPOγ, EPOγ336 and EPOR(1-321) expression plasmids or empty vector (data not shown) and selected in EPO(50 U/ml) as previously described (12). Proliferation was measured by incorporation of [^{3}H]thymidine on the indicated days following EPO addition. (c) HT-2EPOβ and HT-2EPOβ-D^{258}A cells were transfected with EPOR(1-321) and EPOR(1-321)/γ_c or empty vector and assayed for proliferative responses to EPO as described above.

Figure 4. A model for the regulated inhibition of signal transduction by the IL-2 receptor complex. (Top) Upon ligand-binding, IL-2Rβ and γ_c are in an equilibrium between the 'active' and 'inactive' receptor complex configurations. In the wildtype receptor complex, the functional interaction between the receptor subunits favors the 'active' conformation that promotes signal transduction. (Bottom) Heterodimerization of IL-2Rβ-D^{258}A and γ_c arrests the receptor complex in the 'inactive' configuration. See text for further discussion.

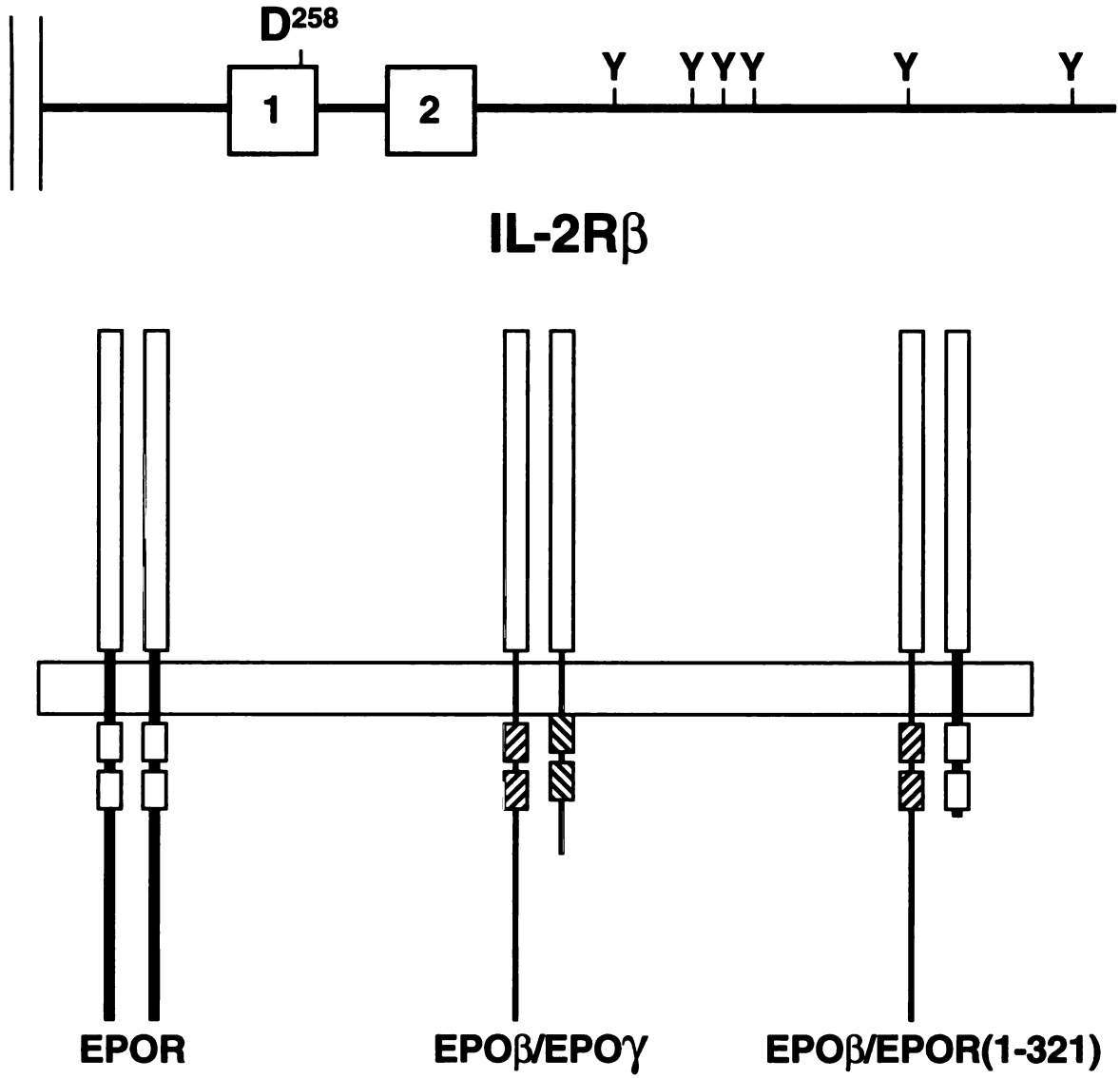

Figure 1

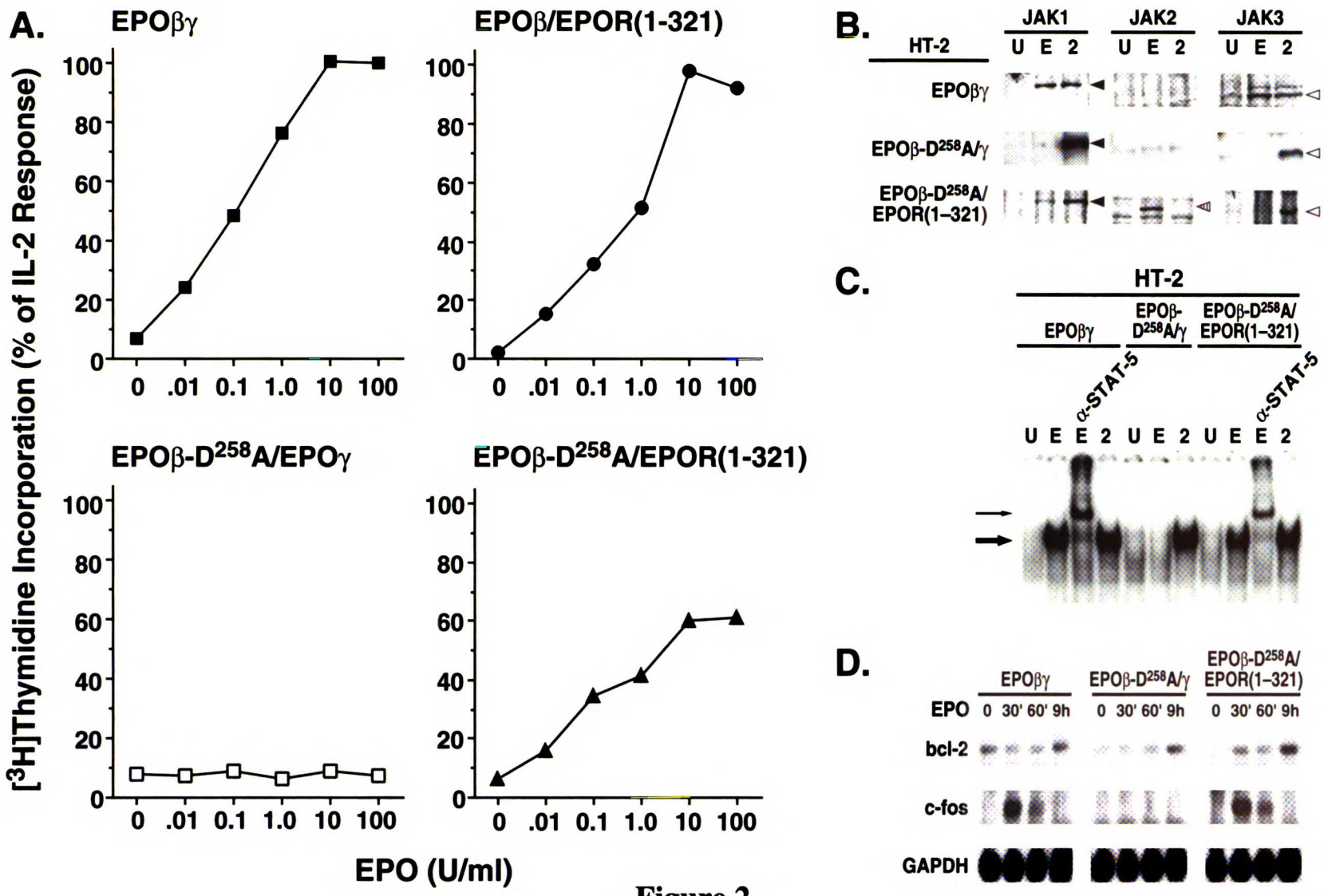
A.
EPOβγ
EPOβ/EPOR(1-321)
EPOβ-D258A/EPOγ
EPOβ-D258A/EPOR(1-321)
[3H]Thymidine Incorporation (% of IL-2 Response)
0
20
40
60
80
100
0 .01 0.1 1.0 10 100
EPO (U/ml)
B.
HT-2
JAK1
JAK2
JAK3
U E 2
EPOβγ
EPOβ-D258A/γ
EPOβ-D258A/
EPOR(1–321)
C.
HT-2
EPOβγ
EPOβ-
D258A/γ
EPOβ-D258A/
EPOR(1–321)
α-STAT-5
α-STAT-5
U E E 2 U E 2 U E E 2
D.
EPOβγ
EPOβ-D258A/γ
EPOβ-D258A/
EPOR(1–321)
EPO 0 30' 60' 9h 0 30' 60' 9h 0 30' 60' 9h
bcl-2
c-fos
GAPDH

Figure 2

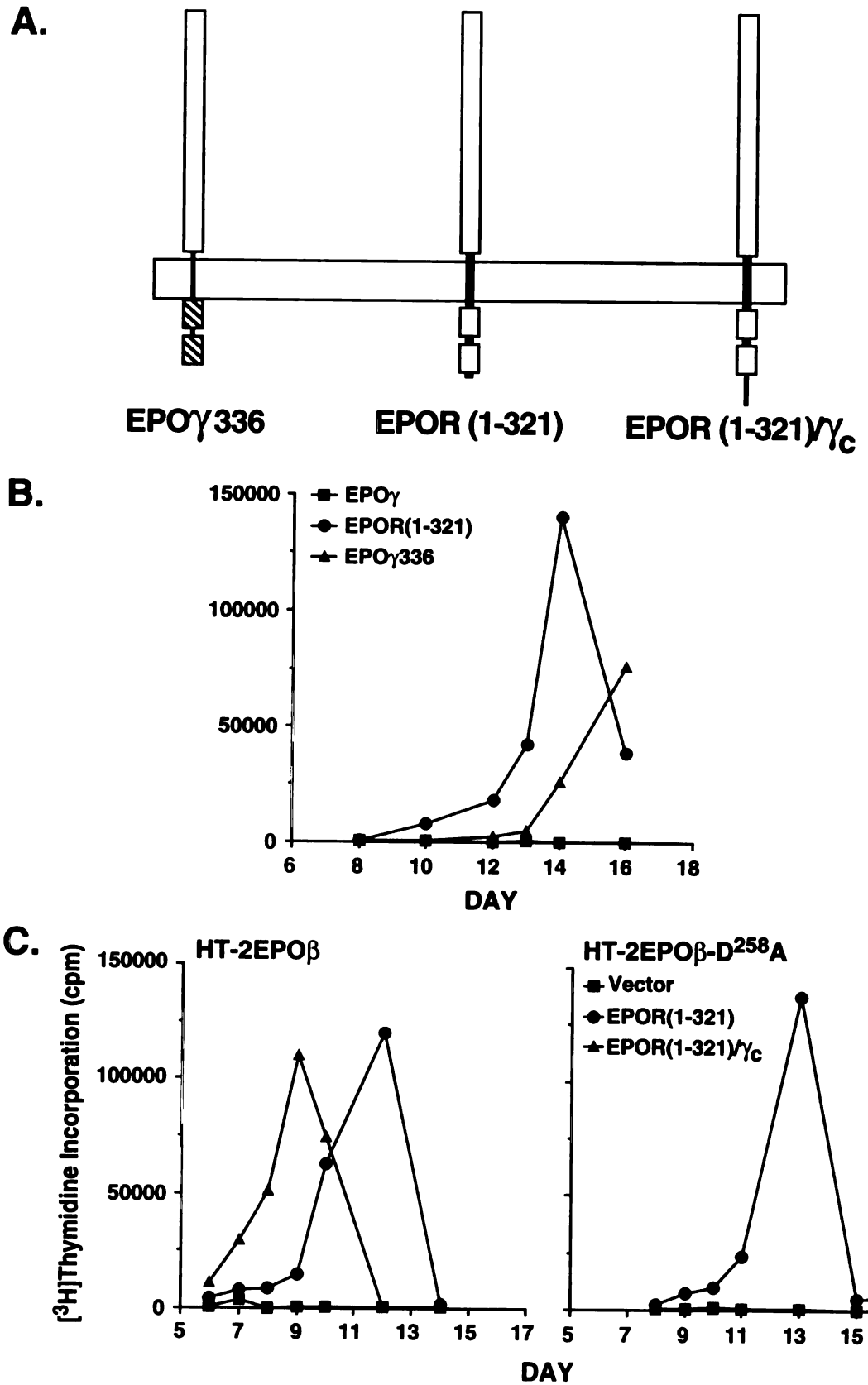
A.
EPOγ336
EPOR (1-321)
EPOR (1-321)/γc
B.
150000
100000
50000
0
EPOγ
EPOR(1-321)
EPOγ336
6
8
10
12
14
16
18
DAY
C.
[3H]Thymidine Incorporation (cpm)
HT-2EPOβ
HT-2EPOβ-D258A
Vector
EPOR(1-321)
EPOR(1-321)/γc
5
7
9
11
13
15
17
DAY

Figure 3

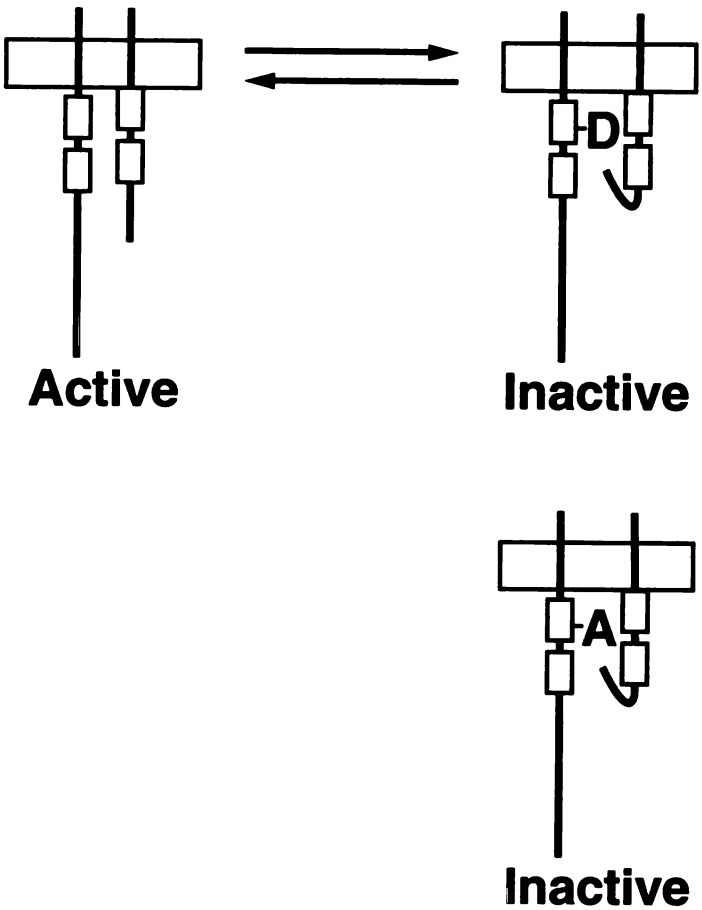

Figure 4

Chapter 8

Conclusions

Molecular analyses of cytokine receptor complexes that share the γ_c chain reveal distinct functional roles for the individual receptor subunits in signal transduction. In the present chapter, functional data regarding the IL-2, IL-4 and IL-7 receptor complexes are reviewed within the context of general principles that determine cytokine receptor signaling specificity. These general principles are used as the basis for a conceptual framework within which the functional architecture of cytokine receptor complexes and the role of individual receptor subunits in the regulation and execution of signaling activity can be understood. Finally, possible future directions and studies that derive from this model are discussed.

I. A Chimeric Receptor System

Fusion or chimeric proteins have been widely employed in the functional analyses of receptor systems (1,2). This approach facilitated the analysis of receptor complexes in cells that express the native receptor, by permitting activation of these receptor systems with a heterologous ligand. In the case of the IL-2R, a wide array of extracellular domains, including EPOR (3,4), IL-2Rα (5), c-kit (6) and GM-CSF (6), have been fused to the cytoplasmic portions of IL-2Rβ and γ_c, leading to the functional reconstitution of IL-2-specific signaling through alternate ligands in these systems. The relative ease with which the extracellular domains could be substituted suggests that many receptors have similar requirements for the alignment of their respective cytoplasmic domains, or that some conformational flexibility exists in the multimerization process. While the spatial relationships of the receptor subunits are not well understood, the requirement for subunit heterodimerization in the IL-2R and IL-7R for effective signal transduction was clearly demonstrated (Chapters 2,3 and 5). Moreover, establishment of this chimeric receptor system led to detailed structure/function analysis of the various receptor subunits through mutagenesis. While the lack of a γ_c-negative lymphoid cell line had previously precluded specific analysis of this receptor subunit, the structural requirements of γ_c were easily characterized in the chimeric receptor system. Finally, this system has proven useful in the dissection of the modular design of receptor complexes because of the ability to pair heterologous receptor subunits in hybrid signaling complexes.

II. Determinants of Signal Transduction Specificity

In the elucidation of factors that contribute to signaling specificity through cytokine receptors, the arrangement of the receptor complex may be viewed as the combinatorial use of modular components to fashion unique signaling systems. At the level of the receptor

complex, these modular components are the receptor subunits themselves which are engaged by the ligand. These interactions typically involve ligand association with a single receptor subunit (7,8), leading to the recruitment of one or more partner subunits that may modulate the affinity of the receptor complex for the ligand (e.g., the IL-4R and IL-7R). Additionally, as in the IL-6R (9), the ligand-receptor interactions may require precise stoichiometry of receptor subunits to promote signal transduction. This ligand-mediated recruitment of one or more receptor subunits serves as the first level of signaling specificity. Moreover, the receptor subunits themselves are composed of distinct elements or modules that recruit select cellular proteins into the receptor complex. These elements are unique amino acid sequences (10,11) that may contain serine/threonine or tyrosine residues that must be phosphorylated to serve as docking sites for cellular signaling molecules (10). Finally, these signaling molecules also contain transposable modules such as the SH2 and PTB domains that serve to engage the specific receptor motifs (12,13). Collectively, the combinatorial arrangement of these various elements define the unique signal transduction program of a receptor complex.

A. Signal Transduction Specificity Derives from a Single Receptor Subunit Within the Receptor Complex

The establishment of the chimeric receptor system permitted a detailed analysis of the functional architecture of the cytokine receptor complex. The determinants of specificity at the receptor complex level were a primary focus of this project. The resulting studies of the IL-2R, IL-4R and IL-7R identified critical signaling elements contained within the individual receptor subunits and defined the functional roles of the subunits within the receptor complex.

Previous analysis of the IL-2Rβ subunit had identified large regions of the cytoplasmic domain that were necessary for proliferation (14). More detailed analysis of the IL-2Rβ subunit defined critical residues within the partially conserved Box1 and Box2 regions that were crucial for growth signaling (15). In the present studies of the IL-2Rβ subunit (Chapter 2), particular attention was given to the functional roles of the six cytoplasmic tyrosine residues. Conversion of these tyrosine residues to phenylalanines in the IL-2RβYF mutant rendered the receptor complex deficient in proliferation signaling (3). Reconstitution studies demonstrated that the distal tyrosines, Y-392 and Y-510, individually were sufficient to reconstitute growth signaling when paired with γ_c in the pro-B cell line, Ba/F3. Additionally, the functional role of Y-392 could be correlated with the phosphorylation of that residue. Evaluation of the IL-2Rβ tyrosine mutants through the chimeric receptor system in HT-2

cells, a murine IL-2-dependent helper T cell line, confirmed the importance of the distal tyrosines for growth signaling, as well as for the activation of STAT-5A and STAT-5B (Chapter 4). Interestingly, the first tyrosine (Y-338) was also sufficient for STAT-5A/B activation and growth signaling in a T cell environment. Moreover, both p52Shc and elements that mediated the induction of c-fos were recruited to Y-338, but not to other IL-2Rβ tyrosines. Thus, most of the cellular events examined could be assigned to particular elements of the IL-2Rβ chain.

Complementary observations were made in the studies of the IL-7R and IL-4R. A truncation mutant of IL-7Rα that removed the three intracellular tyrosine residues abolished growth signaling by the IL-7R complex (Chapter 5). Similar studies in an IL-7Rα-deficient cell line demonstrated the role of a specific tyrosine residue of IL-7Rα for proliferation (16). The concept that signaling events could be assigned to a single subunit in the receptor complex was exemplified more clearly in the IL-4R complex. In previous studies, essential cytoplasmic regions of IL-4Rα for growth signaling had been identified (17-20). Moreover, IL-4Rα, without γ_c, was sufficient to mediate IL-4-specific signaling events, including proliferation, the activation of STAT-6 and IRS-1 phosphorylation (Chapter 6). These findings and recent observations (21-27) also support the model that multiple forms of the IL-4R complex exist. Furthermore, recent observations suggest that receptor complexes may employ multiple IL-4Rα subunits alone, possibly in a homodimeric form (data not shown).

Collectively, the structure/function analysis of the IL-2R, IL-4R and IL-7R revealed an important characteristic shared by these complexes with regard to signal transduction specificity. The IL-2Rβ, IL-4Rα and IL-7Rα subunits have relatively long cytoplasmic tails that contain multiple tyrosine residues. Upon ligand binding, one or more of these tyrosine residues is phosphorylated (3,16,28-33). Furthermore, the association of signaling molecules such as Shc and IRS-1 to specific tyrosine residues in these receptor complexes has been observed (18,34). Strikingly, the recruitment of a distinct profile of signaling molecules to the receptor complex depends primarily upon elements within the IL-2Rβ, IL-4Rα and IL-7Rα subunits, rather than upon γ_c. A single receptor subunit within these cytokine receptor complexes is primarily responsible for determining the specific signaling programs that are activated.

B. The Functional Role of the γ_c Subunit: Activation of Receptor Complex Signal Transduction

The structure/function analysis of the γ_c subunit was greatly facilitated by the chimeric receptor system. While some previous mutational analysis had been performed in 3T3

fibroblasts (35), the relevance of these studies to lymphoid cells was unclear. In contrast, our studies were performed in HT-2 cells, a murine helper T cell line. Conversion of the four cytoplasmic tyrosine residues to phenylalanines in the γYF mutant did not affect measured signaling events of the IL-2, IL-4 or IL-7 receptors (Chapters 2, 5 and 6). This finding is particularly surprising because the γ_c subunit becomes tyrosine phosphorylated when the IL-2R is activated (36), suggesting other roles for these phosphotyrosines.

The actual physical requirements of γ_c for signaling through these receptor complexes were quite minimal. Mutational analysis of γ_c demonstrated similar structural requirements in the IL-2 and IL-7 receptors (Chapter 2, 4B and 5). Truncation mutants of γ_c to the Box2 region (EPOγ336) could pair with IL-2Rβ and IL-7Rα, respectively, to promote growth signaling. However, more severe truncations and internal deletion mutants within the Box1 and Box2 domains abolished proliferation signaling (Chapters 2 and 5; data not shown). Presumably, the membrane-proximal portion of γ_c containing the Box1 and Box2 regions mediates the association of JAK3 to γ_c (37). Interestingly, IL-4Rα could mediate IL-4-specific signaling events independently of γ_c (Chapter 6). This mutational analysis and similar observations (60) strongly suggested that the primary function of γ_c was the recruitment of JAK3 to activate receptor signaling.

The minimal structural requirements for γ_c signaling function suggested that this subunit served a critical, but more general role within the receptor complex. Interestingly, a heterologous receptor subunit could functionally replace γ_c in the IL-2R and IL-7R complexes (Chapter 3, 4B and 5). Pairing of either IL-2Rβ or IL-7Rα with a severely truncated EPOR chain was sufficient to activate signaling by these hybrid receptor complexes. With the use of a truncated EPOR subunit, a different JAK kinase, JAK2, was introduced into these receptor systems. However, this difference did not alter any of the other measured signaling events mediated by the hybrid receptor complexes as compared to the native receptor systems. Importantly, γ_c could be replaced effectively only by forms of EPOR that were capable of associating with and mediating the activation of JAK2. Moreover, the requirement of a second subunit in the IL-2R and IL-7R complexes was demonstrated by the failure of homodimers of the IL-2Rβ or IL-7Rα cytoplasmic domains to function. Thus, these findings confirmed our hypothesis that γ_c serves primarily to activate the signaling complex by transporting JAK3 into the receptor complex, although γ_c may still mediate other activation events or affect as yet unmeasured signaling events.

III. The Functional Interaction of Receptor Subunits Within the IL-2R Complex

Previous mutational analysis of the membrane-proximal region of IL-2Rβ identified a single critical amino acid residue (Asp258) in the Box1 domain necessary for proliferation (15). Although mutagenesis of various receptor subunits has revealed critical domains necessary for the association of particular cytoplasmic signaling intermediates, the present work indicates that Asp258 may be involved in a novel functional interaction (Chapter 7). This potential interaction was revealed by the context-specific nature of the IL-2Rβ-D258A mutation. The IL-2Rβ-D258A mutation resulted in an IL-2Rβ-D258A/γ_c heterodimer that was almost completely non-functional. In contrast, the pairing of IL-2Rβ-D258A with a truncated EPOR(1-321) subunit restored proliferation and all measured IL-2-specific signaling events, including activation of STAT-5 and induction of c-fos gene expression. Similarly, the IL-2Rβ-D258A mutant could be functionally complemented by a truncation mutant of γ_c, lacking the carboxy-terminal 33 amino acids of this chain.

Interestingly, induction of bcl-2 gene expression was not altered by the IL-2Rβ mutation in any of the heteromeric receptor complexes, although expression of c-fos was differentially affected. The distinct nature of bcl-2 induction is consistent with previous studies which have demonstrated that IL-2Rβ tyrosine residues (38) and the JAK kinases (39) are not necessary. Signaling events that regulate apoptosis, including the upregulation of bcl-2, may not be subject to this form of regulation. Alternatively, the rapid activation of the c-fos pathway may demand more stringent control to avoid inadvertent activation. Thus, this functional interaction of IL-2Rβ and γ_c may serve to regulate many, but not all aspects of signaling activity by the receptor complex.

The restoration of much of the IL-2R-mediated signaling activity through the pairing of IL-2Rβ-D258A and the truncated γ_c chain suggested that the C-terminal portion of γ_c may contain an inhibitory domain. Specifically, the membrane-proximal Asp258 residue might functionally interact with this distal 33-amino acid domain of γ_c to prohibit signaling. Moreover, the alteration within IL-2Rβ-D258A might prevent the normal regulation of this interaction, leading to a non-functional receptor complex. To test this model, the C-terminal γ_c domain was transposed to the distal portion of EPOR(1-321) to create EPOR(1-321)-γ_c. As with EPOR(1-321), the fusion subunit EPOR(1-321)-γ_c could pair with wildtype IL-2Rβ for receptor signaling. However, the functional pairing of EPOβ-D258A and EPOR(1-321) was not recapitulated by the heterodimer of EPOβ-D258A and EPOR(1-321)-γ_c. These observations suggest that the distal portion of γ_c contains a transposable inhibitory domain that directly or indirectly interacts with IL-2Rβ. While this functional interaction between

receptor subunits may serve to negatively regulate receptor function, the precise molecular nature of this regulatory mechanism remains unclear. Interestingly, comparison of the distal segment of γ_c with other known proteins reveals no sequence similarities (data not shown). Further studies may reveal the direct physical engagement of these receptor subunits or the presence of an intermediate molecule.

IV. The Modular Design of the Cytokine Receptor Complex

A. Distinct Modules Within the Cytokine Receptor Subunit Determine Specific Signaling Events

A detailed analysis of the tyrosine-containing elements within IL-2Rβ demonstrated several important characteristics of cytokine receptor subunit design. As described in the Introduction, various signaling molecules associate with phosphorylated tyrosine residues through SH2 or PTB domains that recognize phosphotyrosines within the context of particular amino acid sequences or motifs (12,13). Studies of IL-2Rβ determined the particular tyrosine residues necessary for the activation of select signaling events (Chapter 2 and 4). The specificity of these modules was clearly demonstrated by the assignment of c-fos gene induction only to the proximal tyrosine-containing module (Tyr-338). Moreover, any one of three tyrosine-containing modules was sufficient for proliferation and STAT-5 activation (Chapter 4), demonstrating the marked redundancy present in cytokine receptor subunit architecture, similar to the findings within the PDGFR (40,41). Examination of the tyrosine-containing motifs within IL-2Rβ revealed that both Y-392 and Y-510 are contained within very similar amino acid motifs that were also found in other receptor subunits. Interestingly, the proximal tyrosine (Y-338) is embedded in an unrelated amino acid sequence, raising the possibility that this module may engage STAT-5 and growth signaling elements in a manner distinct from those employed by the two more distal tyrosine-containing elements. Indeed, STAT-5 DNA binding could be effectively inhibited by phosphorylated peptides corresponding to the two distal tyrosine-containing motifs, but not by the peptide corresponding the sequences surrounding the proximal tyrosine (Chapter 4A and data not shown). Finally, these phosphotyrosine elements are not dedicated to the engagement of a single signaling pathway. As described above, Tyr-338 engages STAT-5 and elements leading to c-fos induction. Moreover, activation of STAT-5 results in the induction of both two highly-related STAT elements, STAT-5A and STAT-5B. Indeed, all three tyrosine-containing elements that activate STAT-5 recruit both STAT-5A and STAT-5B to the IL-2Rβ subunit. Given their ability to engage more than one signaling molecule, the phosphotyrosine-

containing modules of IL-2Rβ are multifunctional. Thus, many signaling pathways are engaged by the receptor complex through the recruitment of distinct signaling molecules to select motifs contained within the cytoplasmic domain of the receptor subunit. The lack of similarity among the other IL-2Rβ tyrosine-containing elements and any of the presently defined motifs (12) does not suggest what other signaling functions are mediated through the IL-2R complex. Nonetheless, the combinatorial use of these multifunctional elements within different receptor subunits results in the diversity of signaling events activated by different receptor complexes.

B. The Functional Roles of the Individual Cytokine Receptor Subunits

Since receptor subunits contain amino acid motifs that associate with distinct signaling molecules, the engagement of one or more receptor subunits by a particular cytokine may determine the precise array of signaling pathways that are activated by a receptor complex. A receptor subunit present in several receptor complexes should promote similar signaling events through the recruitment of the same signaling molecules to these different receptor complexes. Conversely, the different receptor subunits employed in distinct receptor complexes should direct different signaling events through the engagement of discrete signaling molecules. In examining the functional contribution of the individual receptor subunits to signal transduction, the γ_c-containing receptor complexes were an ideal receptor subfamily to analyze since each receptor in this family contains the shared γ_c chain, as well as a distinct second subunit.

Studies of these receptor complexes clearly demonstrated that the measured signaling events depend upon the presence of the unique second subunit to drive the specific signaling program of each of these receptor complexes. In these heterodimeric receptor complexes, IL-2Rβ, IL-4Rα and IL-7Rα have relatively long cytoplasmic domains composed of tyrosine-containing elements and other amino acid motifs that serve as binding sites for intracellular signaling molecules. Studies of the IL-2R and IL-7R demonstrate the requirement of tyrosine-containing amino acid motifs for particular signaling events. These subunits are termed "driver" chains because they determine the specific signaling events of the receptor complex. Additional support for this "driver" subunit function is found in some forms of the IL-4R complex which elicit IL-4-specific signaling events from a receptor complex containing IL-4Rα, without γ_c.

While "driver" subunits determine signal transduction specificity, the γ_c subunit performs a more general function. As demonstrated in human X-SCID (42,43) and murine gene-ablation studies (44,45), the γ_c subunit and its associated kinase JAK3 are obviously

critical for the development of the immune system and signal transduction through γ_c-containing receptors. Moreover, the nearly identical immunodeficiency syndromes due to mutations in either γ_c or JAK3 (46) suggest an intimate relationship between these two molecules. The presence of γ_c was necessary in the IL-2R and IL-7R complexes, as neither IL-2Rβ nor IL-7Rα alone were capable of productive signaling in lymphoid cells. However, γ_c and JAK3 could be functionally replaced by a truncated EPOR subunit and its associated JAK2 in the IL-2R and IL-7R complexes, without altering subsequent signaling events. Thus, γ_c is termed the "trigger" subunit because it initiates signal transduction by transporting JAK3 into the receptor complex.

Rather than mediating specific signaling events, γ_c performs a critical role within the receptor complex that is not linked to specificity. These results do not necessarily conflict with previous studies that employed dominant negative forms of γ_c (47). In those studies, truncated γ_c subunits abolished IL-2, IL-4 and IL-7-specific signaling events. Although certain signaling events were assigned to γ_c based upon those studies, loss of signaling activity by the γ_c-containing receptor complexes is likely to be a result of the overall signaling deficit caused by the inability of γ_c to introduce a JAK3 molecule into the receptor complex to activate signal transduction. Furthermore, other work has suggested that prevention of T cell anergy is directed through γ_c (48). However, those studies do not exclude the likely role of other γ_c-containing receptor complexes in generating these signals.

The functional dissection of the receptor complex revealed a direct parallel between the physical asymmetry of receptor subunit structures and the distinct functional roles of these subunits. This difference is embodied in the "trigger-driver" model of heteromeric cytokine receptor complexes. Within this paradigm, receptor subunits have distinct and separable functions. The "driver" subunit contains the structural elements to associate with the distinct array of cellular intermediates that determine the specific signaling program. The "trigger" subunit is pre-associated with a JAK kinase and functions primarily to transport this kinase into the receptor complex to activate the signaling process. The intriguing observation of γ_c-independent forms of the IL-4R complex suggests that IL-4Rα may act as a "trigger" subunit for its partner IL-4Rα chain, or that other "trigger" subunits may be present in these alternate IL-4R complexes. Moreover, the existence of other "trigger-driver" arrangements has been supported by recent studies of the IL-12R (49) and Class II members of the cytokine receptor superfamily, including IFN-α/β (O.R. Colamonici, personal communication) and IFN-γ receptor complexes (50). In the case of the IFN-γ receptor, replacement of the entire cytoplasmic portion of the putative IFN-γ "trigger" chain by JAK2 resulted in no alteration of signaling.

The dichotomy of receptor subunit functions in the "trigger-driver" model provides

a useful conceptual framework for understanding signal transduction by heteromeric cytokine receptor complexes and the shared use of subunits in certain receptor subfamilies. However, the complete separation of function suggested by this model may be idealized. Signaling by other heteromeric cytokine receptor complexes may require the recruitment of signaling intermediates to more than one receptor subunit. Furthermore, the current studies and supporting evidence are focused on heteromeric receptor complexes. Some of the general principles embodied in the "trigger-driver" model may be applicable to other cytokine receptor complexes that employ a homomeric receptor subunit arrangement. Finally, unlike many signaling events which can be attributed to an individual receptor subunit, bcl-2 induction is independent of tyrosine-containing motifs within IL-2Rβ, functional elements of γ_c and JAKs (38,39), and appears to depend only upon receptor subunit heterodimerization.

The recognition of individual receptor subunit functions within the heteromeric cytokine receptor complex also provides a rationale for receptor subfamilies that share a common subunit. Based on these studies, two basic arrangements may be proposed regarding the evolutionary design of these receptor complexes. Within the "convergent" arrangement exemplified by the IL-3/IL-5/GM-CSF receptor subfamily, members employ a common signal transduction subunit (i.e., β_c). Predictably, the shared use of this "driver" subunit leads to the activation of similar signaling events. In the "divergent" type of architecture, the use of a shared "trigger" chain simply provides a general activation function. For example, the IL-2/IL-4/IL-7/IL-9/IL-15 receptor subfamily members all have receptor complexes that employ γ_c for signaling initiation, but the use of different "driver" subunits leads to distinct signaling profiles. Thus, the functional polarity of the "trigger" and "driver" subunits are the structural basis for specificity and redundancy in these receptor subfamily arrangements.

C. Regulated Inhibition of Signal Transduction Within the IL-2R Complex

The activation of signal transduction by cytokine receptors requires the multimerization of the individual receptor subunits through ligand-binding (51). The physical approximation of the receptor subunits and their associated signaling intermediates promotes the activation of multiple signaling pathways. In the current paradigm, receptor subunits are thought to serve primarily as passive docking platforms for signaling intermediates. Analysis of the IL-2Rβ-D258A mutant (Chapter 7), though, suggests that the receptor subunits may perform an active role in the dynamic regulation of signaling by the receptor complex.

As noted above, the γ_c subunit functions primarily to transport JAK3 into the receptor complex to activate signaling function and is employed by many different receptor complexes

(e.g. IL-2, IL-4, IL-7, IL-9 and IL-15) (36,52-57). The activation of one or more of these receptor signaling programs has profound effects upon cellular fate(s). Thus, the precise regulation of signal transduction by γ_c seems necessary. As described above, the IL-2Rβ-D258A mutant revealed a functional interaction between IL-2Rβ and γ_c that may be involved in the regulation of receptor signaling activity. Furthermore, detailed analysis of the receptor complex led to the identification of an inhibitory domain within the C-terminal portion of γ_c.

The regulatory function served by the cooperative engagement of IL-2Rβ and γ_c is not well-understood. In a speculative model, ligand-binding leads to the approximation of the IL-2Rβ and γ_c subunits. IL-2Rβ and γ_c functionally interact to form a receptor complex that is in an equilibrium between 'active' and 'inactive' states. The existence of these alternative states is suggested by the ability of the mutant IL-2Rβ-D258A subunit to signal when paired with a truncated EPOR subunit, but not with γ_c. In the 'inactive' form, the inhibitory domain of γ_c functionally engages the Asp258 residue of IL-2Rβ. Within the wildtype receptor complex, this inhibitory interaction can be overcome, leading to an equilibrium that favors an 'active' conformation that allows receptor signaling to proceed. In the case of the IL-2Rβ-D258A mutant, the equilibrium between the two receptor states favors the 'inactive' state, as the interaction of IL-2Rβ with the inhibitory domain of γ_c can not be released. Future studies that are beyond the scope of the present work will be needed to define the precise molecular basis of the functional interaction between IL-2Rβ and γ_c and to understand its regulatory effects on signaling by the receptor complex.

V. Future Directions

The general model developed in these studies provide the basis for further analysis of signaling through heteromeric cytokine receptor complexes. While recent reports have demonstrated the requirement of IL-9Rα and γ_c heterodimerization for IL-9R signaling (57), current studies in the chimeric receptor system are examining the functional role of γ_c in the IL-9R complex. Other studies may be directed at understanding the compatibility of "trigger" and "driver" subunits. Specifically, while a truncated EPOR subunit can functionally replace γ_c in some receptor complexes, we have found that the cytoplasmic domains of the granulocyte/macrophage colony-stimulating factor (GM-CSF) α and IL-12Rα chains do not pair with IL-2Rβ to restore growth signaling (data not shown). These potential "trigger" subunits may not be interchangeable with γ_c due to structural constraints within the receptor complex that may be critical for receptor function. Additional studies may define those factors that affect receptor subunit compatibility.

While the "trigger-driver" model provides a conceptual framework for understanding the signaling function of heteromeric receptors, perhaps those general principles may be applied to other receptor arrangements. A considerable number of cytokine receptors employ multimers of a single receptor chain (e.g. EPOR, GHR, PRLR). In these receptor complexes, it is possible that each receptor subunit functions both as a "driver" subunit in the specification of signaling events and as a "trigger" subunit for its partner subunit(s). To test this hypothesis, a chimeric receptor system has been established in which the cytoplasmic domain of EPOR is joined to the extracellular portion of IL-2Rβ and γ_c to form βEPOR and γEPOR, respectively. Activation of this chimeric receptor would require IL-2-binding through the heterodimeric extracellular domains. Once preliminary studies verify reconstitution of EPOR signaling by this chimeric receptor system, the use of a heterodimeric ligand-binding element will permit the pairing of different EPOR mutants. Specifically, a limited portion of EPOR will be evaluated for its ability to activate signaling through its full-length partner. The molecular analysis of the EPOR complex may provide further insights regarding the signaling principles governing homomeric receptor complexes and may lead to further support for the trigger-driver model.

Analysis of signaling through the cytokine receptor complex has relied upon a few well-characterized signaling events such as proliferation, activation of IRS-1 and the JAK-STAT pathway. Further studies of the γ_c-containing receptor complexes will focus on other signaling events, such as the activation of MAPK and PI3K (K.D. Liu, data not shown). Additionally, assays to evaluate other biological effects such as anti-apoptosis are being established (J. Bauer, data not shown). However, the in vitro analysis of receptor complexes is limited by the relatively low number of known signaling pathways. Clearly, systematic approaches, such as the isolation of somatic signaling mutants, to identify other signaling pathways would be invaluable. These other signaling events could be evaluated within the context of the trigger-driver model. These novel signaling pathways could also be linked to specific elements within the various receptor subunits.

Differences in receptor signaling due to the use of different cell lines demonstrated another limitation of the *in vitro* analysis of cytokine receptor signaling. Specifically, the IL-4R does not mediate proliferation in all cell lines (Lai, data not shown). Similarly, EPOR signaling does not function completely in many T cell lines (58 and Lai, data not shown). While these cell context effects are being investigated, in vivo studies might provide additional insights regarding receptor subunit structure. With the availability of the γ_c-deficient mouse, it may be possible to evaluate γ_c-mutants in an animal model. As a direct test of the trigger-driver model, γ_c-deficient mice could be reconstituted with a fusion protein composed of the extracellular γ_c domain and the cytoplasmic portion of the EPOR subunit.

These studies would examine the ability of the intracellular domain of EPOR to substitute for γ_c in a whole-organism context.

Finally, additional studies may continue to characterize the molecular features of regulated inhibition in the IL-2R complex. While the functional evidence for this inhibitory mechanism exists in the IL-2R, other γ_c-containing receptor complexes such as IL-7R and IL-9R may be examined for similar context-dependent mutations in the membrane-proximal Box1 region. These studies might provide evidence for a similar mode of signaling regulation in these receptor complexes. Other studies may also examine if other signaling events (e.g., MAPK activation) are similarly regulated by this mechanism. Lastly, the functional subunit interactions that govern signal transduction from the receptor complex may be transient and difficult to detect. This novel regulatory mechanism may involve physical interaction between IL-2Rβ and γ_c, or may require a bridging molecule, like JAK3 (37). Several biochemical and biophysical approaches are available and may be further pursued. An attractive mechanism for this regulatory function would involve the direct physical interaction between the membrane-proximal Asp258 residue and the C-terminal portion of the γ_c subunit. Biochemical studies might demonstrate this interaction. While the IL-2Rβ and γ_c subunits do not associate detectably in the absence of ligand (data not shown), perhaps the mutation within IL-2Rβ-D258A stabilizes the interaction with the γ_c chain. Such an association may be detectable in a yeast two-hybrid system or in co-immunoprecipiation studies. However, the likely transient nature of this potential subunit interaction may require that a very sensitive detection systems such as the BioCore be employed.

VI. The Modular Architecture of the Cytokine Receptor Complex Defines the General Features of Signal Transduction

The primary goal of this dissertation project was the delineation of elements within the receptor complex that define and regulate the signal transduction program. The structure/ function analysis of heteromeric receptor complexes that employ the γ_c subunit provided a general model of receptor architecture. Functional interactions between the receptor subunits may be important in the regulation of signaling by the receptor complex. Furthermore, the individual receptor subunits perform distinct and separable functions within the receptor complex. These subunits and their activities are modular entities that can be employed in a combinatorial fashion to create receptor complexes with distinct signaling properties. Moreover, this modular design provides a molecular rationale for the specificity, redundancy and pleiotropy of signaling events generated by hematopoeitic cytokine receptors. Specificity is the product of distinct sequence motifs or elements within the receptor subunit which

direct signaling events through the recruitment of select signaling molecules. The combination of multiple sequence elements in the receptor tail results in the activation of many signaling events, leading to the pleiotropic effects of cytokines. Finally, redundancy derives from the employment of similar amino acid motifs by two different receptor subunits which results in the activation of the same signaling events. At the level of the receptor complex, redundancy may result from the shared use of a 'driver' subunit by multiple receptor complexes (e.g. IL-3R/IL-5R/GM-CSFR subfamily). The "trigger-driver" model and the supporting evidence provide a rationale for these signaling characteristics and highlight the natural economy of receptor subunit usage in cytokine receptor complexes.

Clearly, this paradigm may represent an idealized separation of subunit function. In an intermediate arrangement, each subunit may be responsible for a subset of signaling events. In a receptor subfamily including IL-6, IL-11, CNTF, LIF and OSM receptor complexes, an α subunit mediates ligand binding, while the gp130 and LIFRβ receptor chains are employed in homodimeric or heterodimeric combinations for signal transduction (59). Perhaps each of these receptor subunits contributes to the signaling activities directed by members of this receptor subfamily. However, differences in signaling events activated by these receptors have yet to be reported (59). Additionally, each subunit in a homodimeric receptor (e.g., EPOR) is identical and presumably recruits the same signaling molecules into the receptor complex. The possibility that signaling by these receptors may be predicted by the "trigger-driver" model awaits further study. The establishment of the chimeric receptor system and of a general model for the architecture of cytokine receptor complexes provides the basic foundation for the examination of this and other related issues.

References

1. A. C. Chan, D. M. Desai, A. Weiss, *Ann Rev Immunol* **12**, 555-92 (1994).
2. A. L. DeFranco, et al., *Ann NY Acad Sci* **766**, 195-201 (1995).
3. M. A. Goldsmith, et al., *JBC* **270**, 21729-37 (1995).
4. S. Minamoto, J. Treisman, W. D. Hankins, K. Sugamura, S. A. Rosenberg, B*lood* **6**, 2281-7 (1995).
5. Y. Nakamura, et al., N*ature* **369**, 330-3 (1994).
6. B. H. Nelson, J. D. Lord, P. D. Greenberg, N*ature* **369**, 333-6 (1994).
7. T. Kishimoto, S. Akira, T. Taga, S*cience* **258**, 593-7 (1992).
8. N. Stahl, G. D. Yancopoulos, C*ell* **74**, 587-90 (1993).
9. G. Paonessa, et al., E*MBO J* **14**, 1942-51 (1995).
10. A. F. Wilks, A. G. Harpur, B*ioEssays* **16**, 313-20 (1994).
11. J. N. Ihle, B. A. Witthuhn, F. W. Quelle, K. Yamamoto, O. Silvennoinen, *Ann Rev Immunol* **13**, 369-98 (1995).
12. Z. Songyang, et al., C*ell* **72**, 767-78 (1993).
13. W. M. Kavanaugh, C. W. Turck, L. T. Williams, S*cience* **268**, 1177-9 (1995).
14. M. Hatakeyama, H. Mori, T. Doi, T. Taniguchi, C*ell* **59**, 837-45 (1989).
15. M. A. Goldsmith, W. Xu, M. C. Amaral, E. S. Kuczek, W. C. Greene, *JBC* **269**, 14698-704 (1994).
16. A. E. Corcoran, et al., E*MBO J* **15**, 1924-32 (1996).
17. N. Harada, G. Yang, A. Miyajima, M. Howard, *JBC* **267**, 22753-8 (1992).
18. A. D. Keegan, et al., C*ell* **76**, 811-20 (1994).
19. D. C. Seldin, P. Leder, *PNAS USA* **91**, 2140-4 (1994).
20. A. Pernis, et al., *PNAS USA* **92**, 7971-5 (1995).
21. Y.-W. He, T. R. Malek, J *Immunol* **155**, 9-12 (1995).
22. A. D. Keegan, et al., *PNAS USA* **92**, 7681-5 (1995).
23. J.-X. Lin, et al., I*mmunity* **2**, 331-9 (1995).
24. W. Kammer, et al., J*BC* **271**, 23634-7 (1996).
25. H. Kotanides, N. C. Reich, J*BC* **271**, 25555-61 (1996).
26. S. Y. Lai, et al., E*MBO J* **15**, 4506-14 (1996).
27. R. L. Palmer-Crocker, C. C. W. Hughes, J. S. Pober, J*CI* **98**, 604-9 (1996).
28. H. Asao, T. Takeshita, M. Nakamura, K. Nagata, K. Sugamura, *JEM* **171**, 637-44 (1990).
29. G. B. Mills, et al., *JBC* **265**, 3561-7 (1990).

30. A. R. Venkitaraman, R. J. Cowling, *PNAS USA* **89**, 12083-7 (1992).
31. L.-M. Wang, et al., E*MBO J* **11**, 4899-908 (1992).
32. K. Izuhara, N. Harada, *JBC* **268**, 13097-102 (1993).
33. C. Smerz-Bertling, A. Duschl, *JBC* **270**, 966-70 (1995).
34. G. A. Evans, et al., *JBC* **270**, 28858-63 (1996).
35. H. Asao, et al., *PNAS USA* **90**, 4127-31 (1993).
36. T. Takeshita, et al., S*cience* **257**, 379-82 (1992).
37. S. M. Russell, et al., S*cience* **266**, 1042-5 (1994).
38. S. L. Gaffen, et al., *JBC* **271**, 21381-90 (1996).
39. A. Kawahara, et al., *PNAS* **92**, 8724-8 (1995).
40. A. Kazlauskas, J. A. Cooper, C*ell* **58**, 1121-33 (1989).
41. M. Valius, A. Kazlauskas, C*ell* **73**, 321-34 (1993).
42. M. Noguchi, et al., C*ell* **73**, 147-57 (1993).
43. J. M. Puck, et al., H*uman Mol Gen* **2**, 1099-104 (1993).
44. X. Cao, et al., I*mmunity* **2**, 223-38 (1995).
45. J. P. DiSanto, W. Muller, D. Guy-Grand, A. Fischer, K. Rajewsky, *PNAS USA* **92**, 377-81 (1995).
46. S. M. Russell, et al., S*cience* **270**, 797-800 (1995).
47. A. Kawahara, Y. Minami, T. Taniguchi, *MCB* **14**, 5433-40 (1994).
48. V. A. Boussiotis, et al., *Science* **266**, 1039-42 (1994).
49. D. H. Presky, et al., P*NAS* **93**, 14002-7 (1996).
50. E. A. Bach, et al., *MCB* **16**, 3214-21 (1996).
51. C.-H. Heldin, *Cell* **80**, 213-23 (1995).
52. M. Kondo, et al., S*cience* **262**, 1874-7 (1993).
53. M. Noguchi, et al., S*cience* **262**, 1877-80 (1993).
54. S. M. Russell, et al., S*cience* **262**, 1880-3 (1993).
55. J. G. Giri, et al., *EMBO J* **13**, 2822-30 (1994).
56. M. Kondo, et al., S*cience* **263**, 1453-4 (1994).
57. Y. Kimura, et al., I*ntl Immunol* **7**, 115-20 (1995).
58. H. Wakao, N. Harada, T. Kitamura, A. L. Mui, A. Miyajima, E*MBO J* **14**, 2527-35 (1995).
59. N. Stahl, G. D. Yancopoulos, J. *Neruobio.* **25**, 1454-66 (1994).
60. B.H. Nelson, J.D. Lord, P.D. Greenberg, *MCB* **16**, 309-17 (1996).