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Research Article

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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. 99:169–177.)

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 the insulin receptor substrate-1 (IRS-1)

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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.

(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 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. $40–60 \times 10^6$ 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

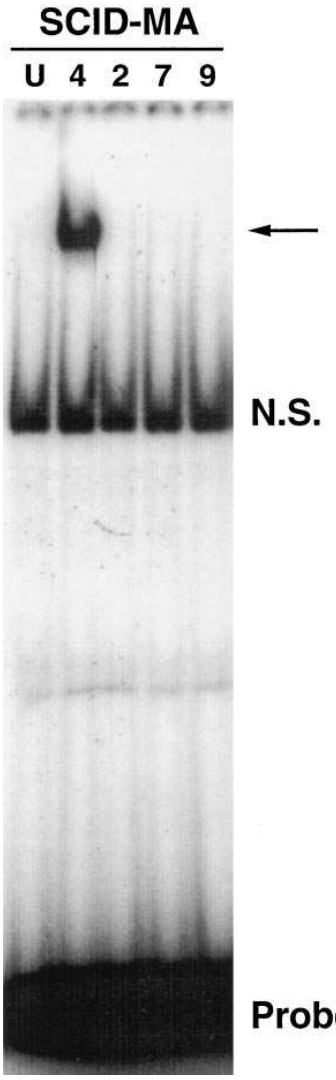

Figure 1. The γ_c mutation in an X-SCID patient results in multiple cytokine receptor signaling defects. 40×10^6 cells/sample were unstimulated (*U*) or stimulated for 15 minutes with various cytokines: IL-4 (*4*, 100 ng/ml), IL-2 (*2*, 10 nM), IL-7 (*7*, 100 U/ml), and IL-9 (*9*, 100 U/ml). EMSA of 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.

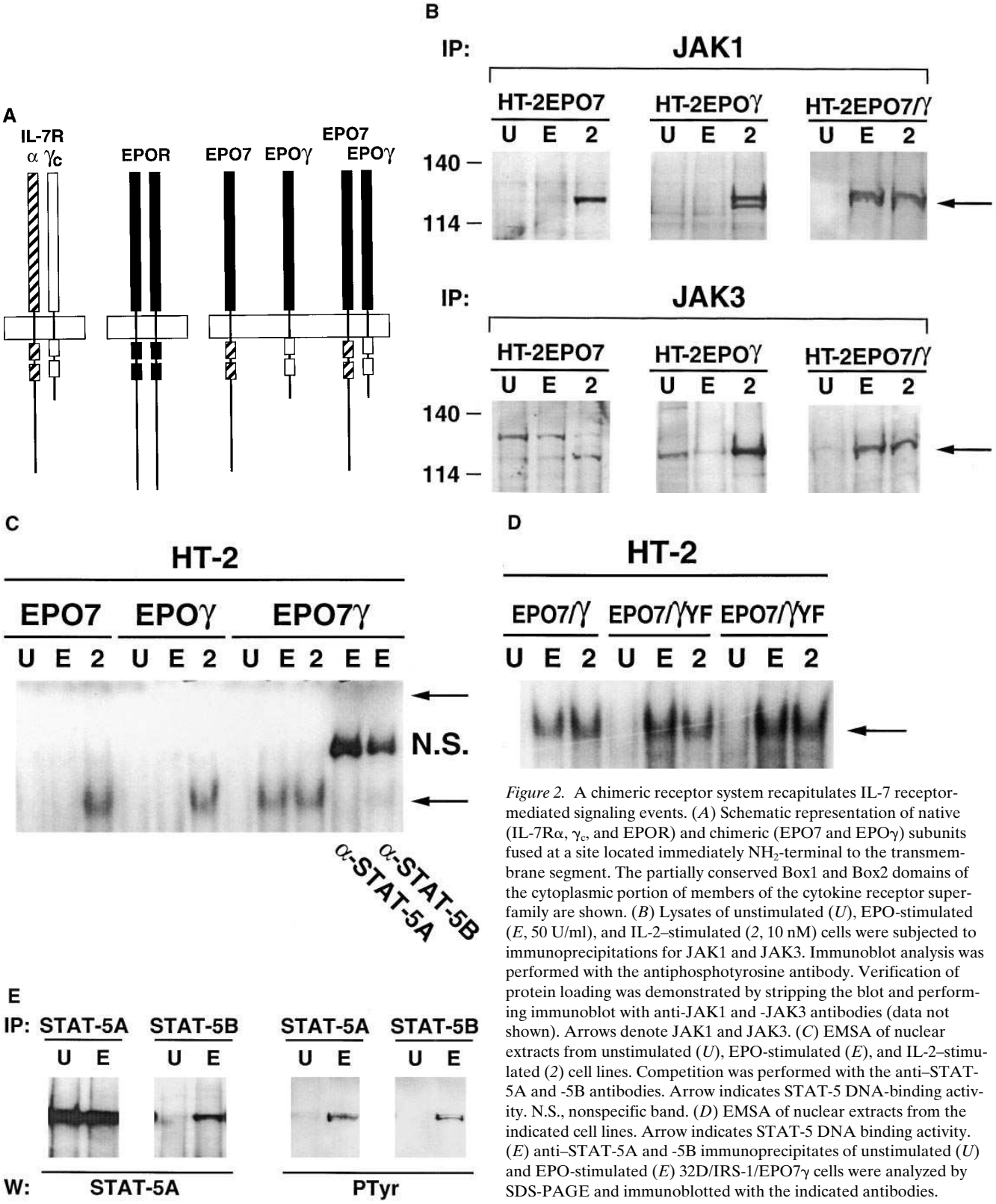

Figure 2. A chimeric receptor system recapitulates IL-7 receptor-mediated signaling events. (*A*) Schematic representation of native (IL-7Rα, γ_c, and EPOR) and chimeric (EPO7 and EPOγ) subunits fused at a site located immediately NH_2-terminal to the transmembrane segment. The partially conserved Box1 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-

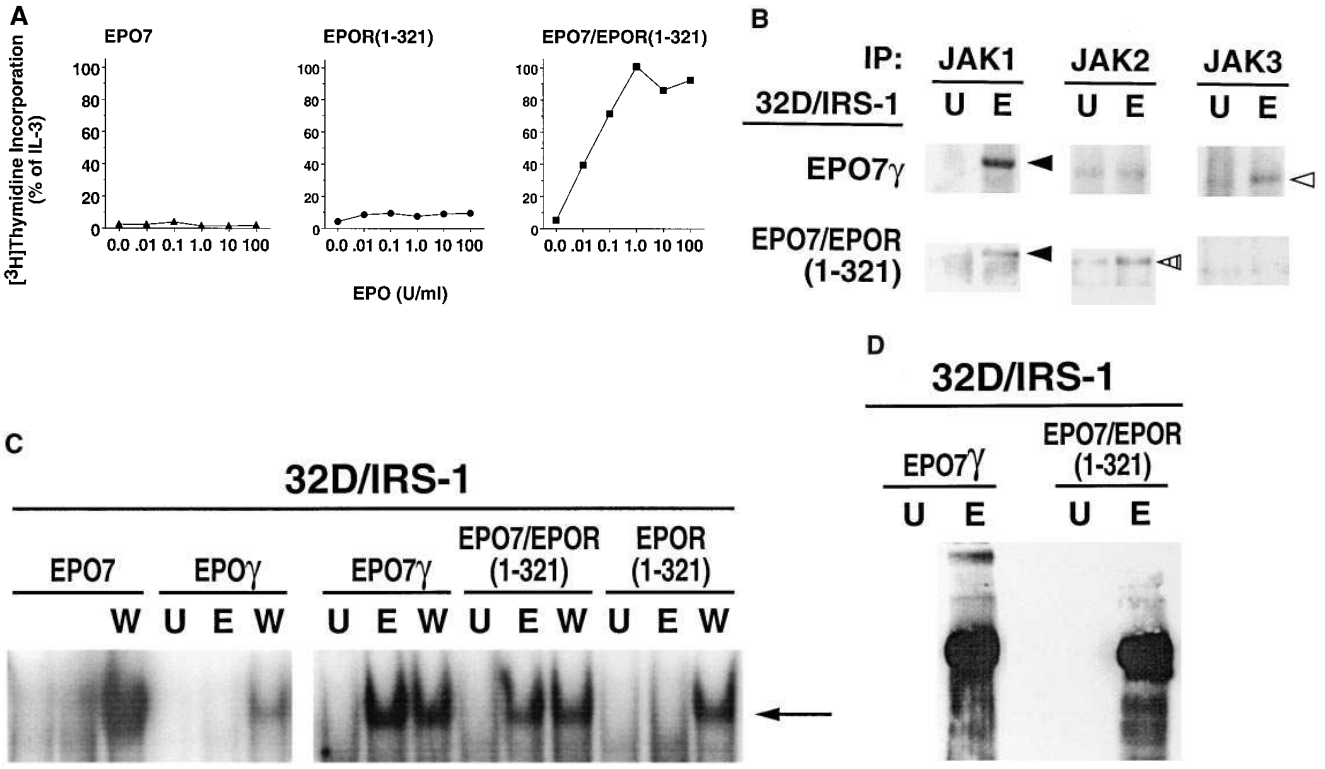

Figure 3. The functional replacement of γ_c by a truncated EPOR does not alter specific signaling events. (*A*) Stable 32D/IRS-1 transfectants were stimulated with EPO in [^{3}H]thymidine incorporation experiments. (*B*) Serial immunoprecipitates of JAK1, JAK2, and JAK3 from lysates of the indicated 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 the 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 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

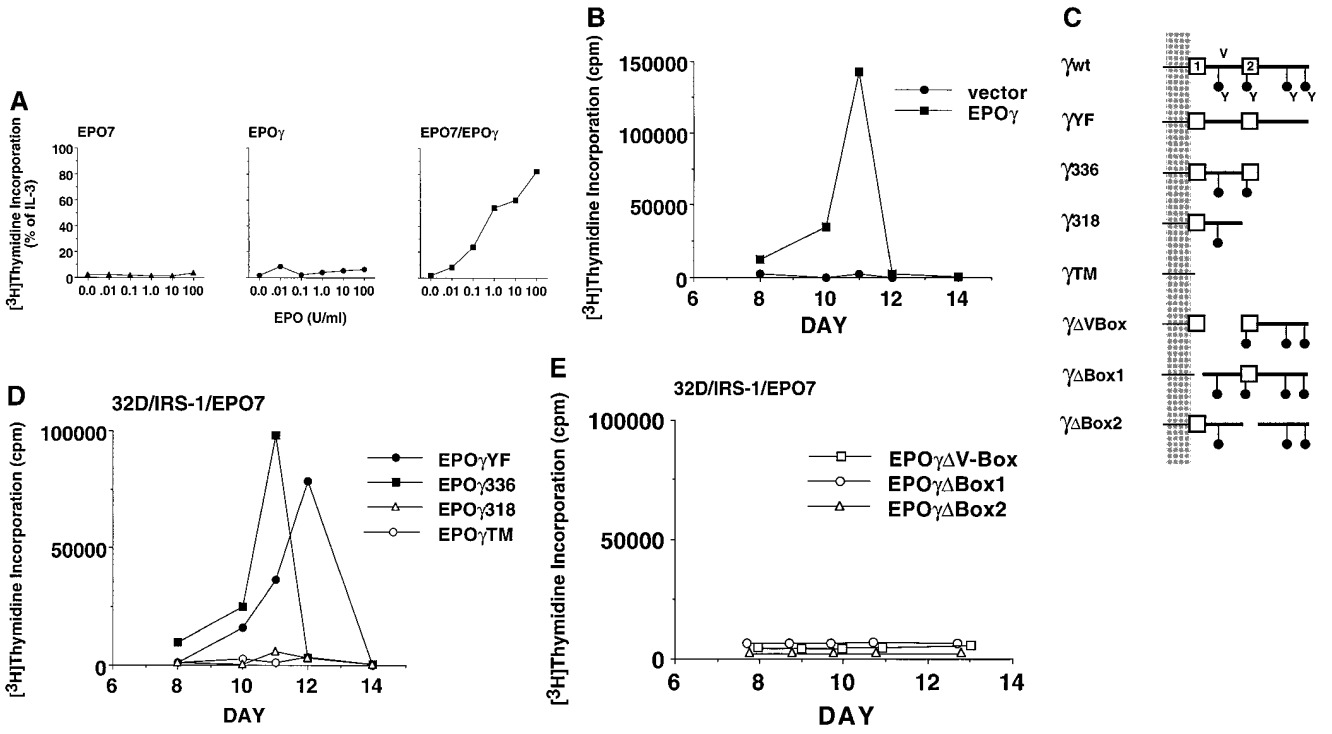

Figure 4. Heterodimerization of the IL-7Rα and γ_c cytoplasmic domains results in the activation of IL-7–specific signaling events. (*A*) Stable 32D/IRS-1 transfectants were stimulated with EPO in [^{3}H]thymidine incorporation experiments. (*B*) Transient transfection assay of 32D/IRS-1/EPO7 cells demonstrates 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.

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,

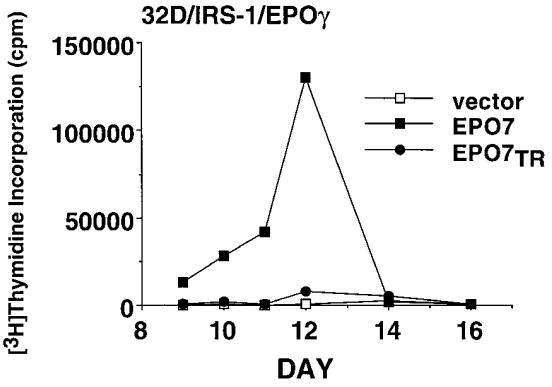

Figure 5. The cytoplasmic domain of the IL-7Rα subunit is required for proliferation signaling. Transient transfection assay was performed in 32D/IRS-1/EPOγ cells.

downstream signaling events such as proliferation were unaffected by the substitution of the γ_c cytoplasmic domain by the truncated EPOR subunit.

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 anti-phosphotyrosine 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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