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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

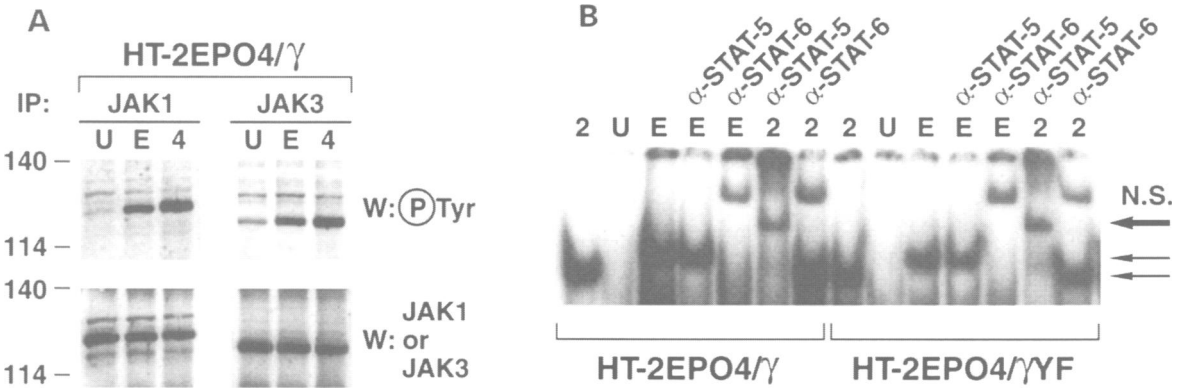

Fig. 1. The IL-4Rα–γ_c heterodimer mediates activation of the JAK–STAT pathway. (**A**) Lysates from HT-2EPO4/γ cells unstimulated (U) or treated with EPO (E, 50 U/ml) or IL-4 (4, 50 U/ml) were subjected to serial immunoprecipitation with anti-JAK1 and anti-JAK3 antisera. Immunoblotting was performed 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

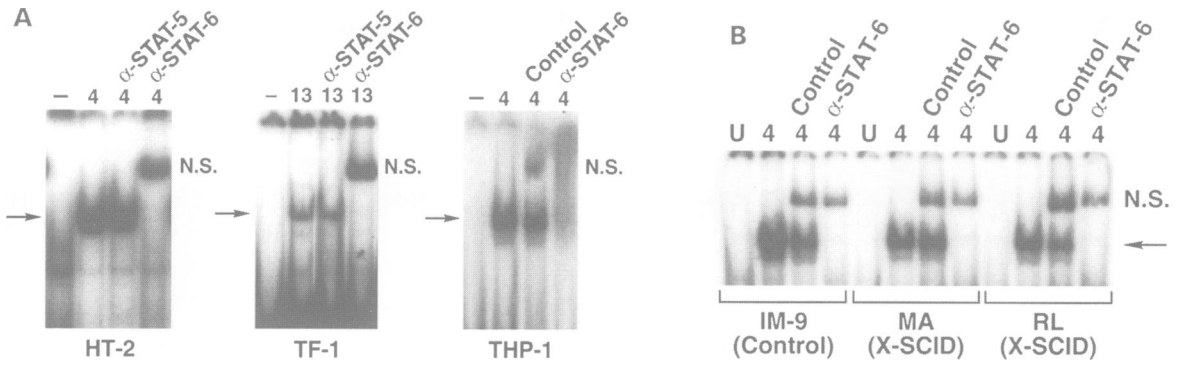

Fig. 2. Various receptors employing the IL-4Rα subunit activate STAT-6 DNA binding activity. (**A**) EMSA of nuclear extracts from HT-2 (left), TF-1 (middle) and THP-1 (right) cells that were unstimulated (U) or treated with IL-4 (4, 50 U/ml) or IL-13 (13, 20 U/ml). Competition was performed with the 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

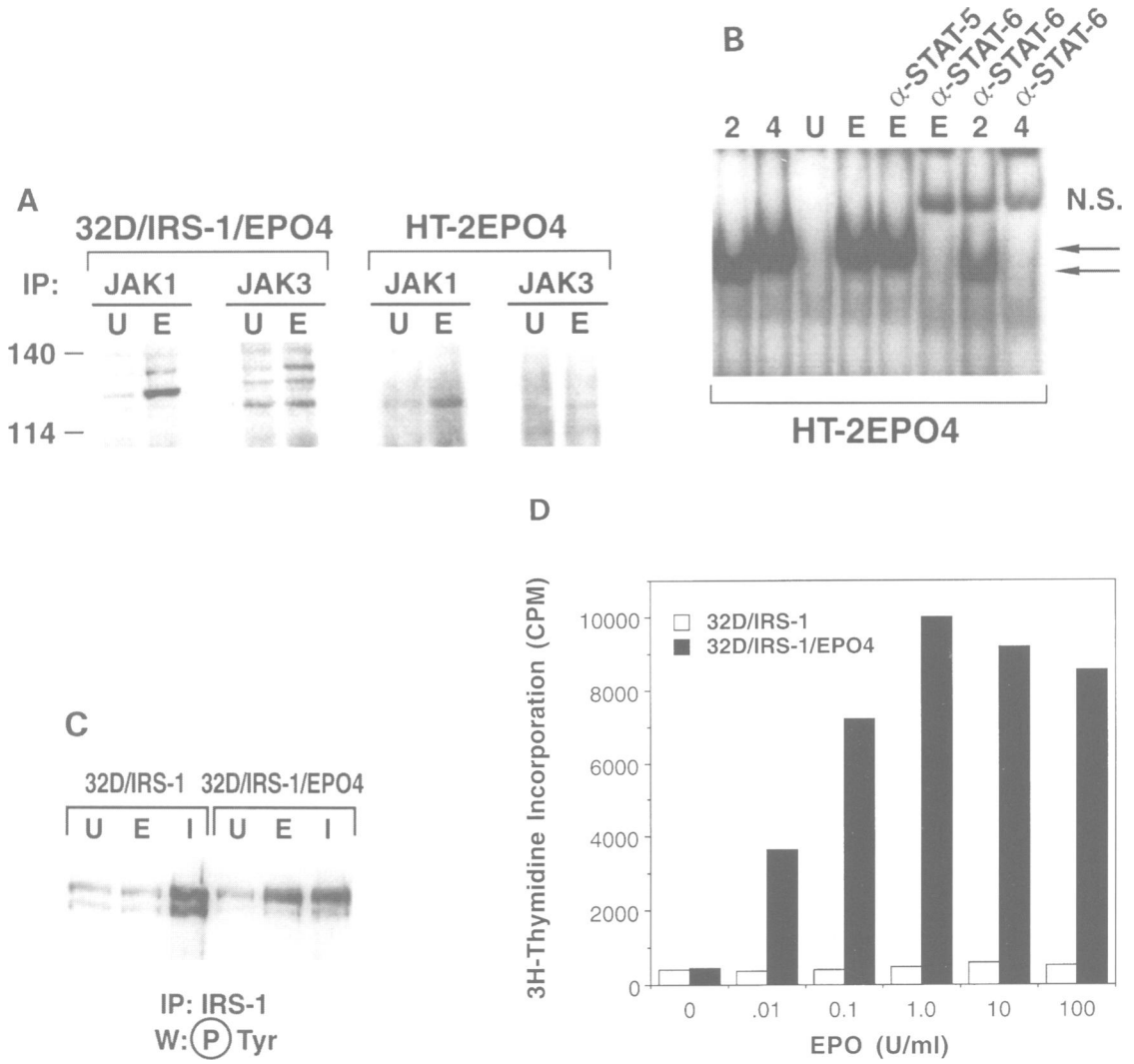

Fig. 3. Homodimerization of IL-4Rα cytoplasmic domains leads to the activation of IL-4-specific signaling events. (**A**) Lysates of the indicated cell lines rested (U) or treated with EPO (E, 50 U/ml) were subjected to immunoprecipitation with anti-JAK1 antiserum. Immunoblot analysis was performed with 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

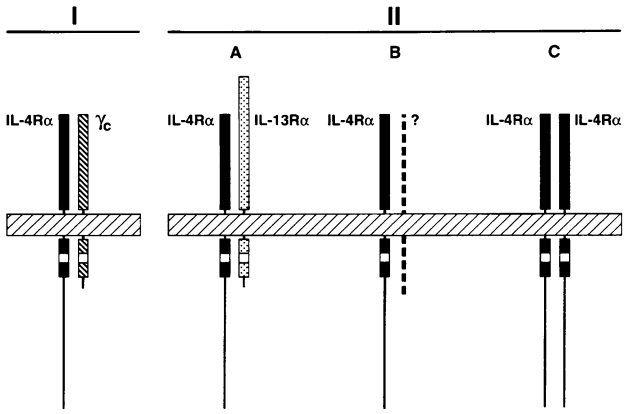

Fig. 4. Multiple forms of the IL-4 receptor complex. IL-4 activates cellular signaling events through several potential receptor complexes. Type I receptor complexes are composed of the IL-4Rα and γ_c subunits. Current evidence also supports the possible existence of several Type II receptor complexes 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, 20–40×10^6 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, 20–40×10^6 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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