Chimeric Erythropoietin-Interferon γ Receptors Reveal Differences in Functional Architecture of Intracellular Domains for Signal Transduction

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Binding of interferon gamma (IFN-γ) causes oligomerization of the two interferon γ receptor (IFN-γR) subunits, receptor chain 1 (IFN-γR1, the ligand-binding chain) and the second chain of the receptor (IFN-γR2), and causes activation of two Jak kinases (Jak1 and Jak2). In contrast, the erythropoietin receptor (EpoR) requires only one receptor chain and one Jak kinase (Jak2). Chimeras between the EpoR and the IFN-γR1 and IFN-γR2 chains demonstrate that the architecture of the EpoR and the IFN-γR complexes differ significantly. Although IFN-γR1 alone cannot initiate signal transduction, the chimera EpoR/γR1 (extracellular/intracellular) generates slight responses characteristic of IFN-γ in response to Epo and the EpoR/γR1-EpoR/γR2 heterodimer is a fully functional receptor complex. The results demonstrate that the configuration of the extracellular domains influences the architecture of the intracellular domains.

The interferon γ (IFN-γ) receptor complex consists of at least two receptor components, a ligand binding chain and a signal transducing chain, each of which is a member of the class II cytokine receptor family (1, 2). Isolation of the two chains of the interferon γ receptor (IFN-γR) has permitted an analysis of the contributions of each to the signal transduction mechanism. The first chain of the receptor (IFN-γR1) binds ligand (3–9). The second chain of the receptor (IFN-γR2) does not bind ligand by itself but is required for signal transduction (3, 10–16). A large body of experiments has elucidated the involvement of the Jak-Stat pathway in signaling by various cytokines (for reviews, see Refs. 17–22). The Janus kinases (or Jaks) are a family of receptor-associated soluble tyrosine kinases with four known members, Tyk2, Jak1, Jak2 and Jak3. Two of the kinases, Jak1 and Jak2, are required for signal transduction by IFN-γ. Further analyses of the interactions have shown that the IFN-γR1 chain binds Jak1 (16, 23, 24) and the intracellular domain of the IFN-γR2 chain brings Jak2 into the signal transduction complex (16). Upon binding of the ligand, IFN-γ, to the IFN-γR1 chain, activation of Jak1 and/or Jak2 by reciprocal transphosphorylation causes the phosphorylation of IFN-γR1 (16, 25). Stat1γ, a latent cytoplasmic transcription factor (26), binds to the phosphorylated IFN-γR1, undergoes tyrosine phosphorylation (27), and forms homodimers that translocate to the nucleus and initiate transcription of IFN-γ inducible genes (for reviews see Refs. 17 and 21).

As with other cytokine receptors, oligomerization upon ligand binding is the first step in the signaling cascade of IFN-γ. IFN-γ is a non-covalent symmetrical homodimer (28) that binds to IFN-γR1 with a stoichiometry of 1:2 (29, 30). It is known that a species-specific interaction between the extracellular domains of the IFN-γR1 and IFN-γR2 subunits is essential for signaling (10–12, 31–33). The IFN-γR2 subunit does not by itself bind the ligand, but can be cross-linked to IFN-γ when both IFN-γR1 and IFN-γR2 chains are present (16). Several lines of evidence (16, 34) suggest that the IFN-γ signaling complex contains two IFN-γR1 chains, two IFN-γR2 chains and one IFN-γ homodimer.

The erythropoietin (Epo) receptor, EpoR, is a member of the class I cytokine receptor subfamily. A single chain encodes both ligand-binding and signal-transducing functions. Epo induces homodimerization of the receptor to initiate signal transduction (for reviews, see Refs. 18, 19, and 35). Jak2 is associated with the cytoplasmic domain of the EpoR and is activated upon ligand-induced dimerization of the receptor (36). Strikingly an Arg → Cys mutation in the extracellular domain of EpoR results in ligand independent dimerization/oligomerization and constitutive, ligand-independent activation of Jak2 and mitogenesis (37, 38).

In this study we used chimeric EpoR, IFN-γR1, and IFN-γR2 constructs to investigate the differences between the architecture of Epo and IFN-γ receptor complexes and shed light on the requirement for one or two receptor-associated tyrosine kinases and the necessity for one or two distinct transmembrane chains for effective signal transduction.

MATERIALS AND METHODS

Reagents, Restriction Endonucleases, and Other Enzymes—Recombinant human erythropoietin was a gift from Dr. Lawrence Blatt of Amgem. Restriction endonucleases were from Boehringer Mannheim and New England Biolabs; T4 DNA ligase was from U. S. Biochemical Corp.; [α-32P]dCTP was from DuPont NEN. All other reagents were of analytical grade and were purchased from Sigma.

Cells and Media—CHO-B7 cells represent the Chinese hamster ovary cell line (CHO-K1) containing a transfected human HLA-B7 gene (12). The 16-9 hamster × human somatic hybrid cell line is a CHO-K1 derivative containing a translocation of the long arm of human chromosome 6 and the human HLA-B7 gene (13). These cells were main-
tained in Ham's F-12 medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal bovine serum (Sigma). Transfections were carried out with the DOTAP transfection reagent (Boehringer Mannheim) according to the manufacturer's protocol and the transfected cells were maintained in F-12 medium containing 450 μg/ml Genetec (antibiotic G418). Uptake of Hu-IFN-R1 and Hu-IFN-R2 cDNAs from cDNA plasmids p18R, pEpoR, pEpoR2IC/R2, and pEpoR1IC/R1 and cloned into the intracellular domain of the various receptors were treated with T4 DNA ligase, and the constructs were centrifuged for 5 min at full speed in a microcentrifuge and the supernatant was recovered for use in the assay and stored at −80 °C.

EMSA reactions contained 2.5 μl of the whole cell extracts, 1 ng P-labeled probe (specific activity approximately 107 cpm/μg), 24 μg/ml bovine serum albumin, 160 μg/ml poly(dI-dC), 20 mM HEPES, pH 7.9, 1 mM MgCl2, 40 μg/ml FicolII (Pharmacia Biotech Inc.), 40 mM KCl, 0.1 mM EDTA, and 0.5 mM dithiothreitol in a total volume of 12.5 μl. For the supershift assay, 1 μl of a 1:10 dilution of anti-Stat1a antibody was included in the reaction. Competition experiments contained a 100-fold excess of the unlabeled oligonucleotide. Reactions were incubated at 24 °C for 20 min. Then 8 μl of the reaction mixture was electrophoresed at 400 V for 3–4 h at 4 °C on a 5% polyacrylamide (19:1, acrylamide: bisacrylamide) gel. The dried gel was exposed to Kodak XAR-5 film with an intensifying screen for 12 h at −80 °C.

Antibodies—Rabbit anti-Jak1 antibody was developed against a synthetic peptide (KTLIEKRYFESCRPVTSPC) corresponding to the end of the second kinase-like domain of murine Jak1. Rabbit anti-Stat1a antibody, raised against the carboxyl-terminal region of Stat1a, was a gift from James Darssler (Rabbit anti-Jak2 (catalogue no. SC-294) and rabbit anti-Stat5 antibody (catalogue no. SC-835) were from Santa Cruz Biotechnology. Monoclonal anti-phosphotyrosine antibody was purchased from Sigma (catalogue no. P3300).

RESULTS

Construction of Chimeric Receptors—The schematic illustration of the various chimeric receptor molecules that were produced is shown in Fig. 1. In one set of chimeric constructs, the extracellular domain of the EpoR was spliced to the transmembrane domain and the cytoplasmic domain of each of the two IFN-γR subunits. In the other set of chimeras, the transmembrane and intracellular domain of EpoR was fused to the extracellular domain of IFN-γR1 and IFN-γR2.

Class I MHC Antigen Induction—To investigate the role of the intracellular domain of IFN-γR2 in the signal transduction complex of IFN-γ, we constructed a chimeric receptor chain consisting of the extracellular domain of IFN-γR2 and the intracellular domain of EpoR. This chimeric construct, γR2/EpoR, and the native IFN-γR2 subunit were separately transfected into CHO-B7 as well as CHO-16-9 cells. The ability of the transfected cDNA to transduce a signal upon induction with IFN-γ was assessed by measurement of enhanced MHC class I antigen expression in the transfected cells and by activation of Stat1a. CHO-B7 cells transfected with IFN-γR2 or γR2/EpoR cDNA showed no response to Hu-IFN-γ as they lack the ligand-binding receptor subunit, Hu-IFN-γR1 (data not shown). Parental CHO-16-9 cells, which contain human chromosome 9q and express the Hu-IFN-γR1 subunit, showed no induction of MHC class I antigens in response to Hu-IFN-γ (Fig. 2, panel A) but were highly stably transfected with expression vectors encoding Hu-IFN-γR2 cDNA or γR2/EpoR chimeras, exhibited enhanced cell surface expression of class I MHC antigens in response to Hu-IFN-γ (Fig. 2, panels B and C). To assess whether the expression of the intracellular domain of EpoR could induce the intracellular domain of IFN-γR2 subunit, we measured the induction of MHC class I antigens as a function of IFN-γ concentration. As depicted in Fig. 3, there was a slight lower induction of MHC class I antigens in the cells.
containing the chimeric γR2/EpoR than in the cells containing the native Hu-IFN-γR2 chain at each concentration of Hu-IFN-γ used. Nevertheless, the fact that the EpoR intracellular domain can be substituted for the Hu-IFN-γR2 intracellular domain shows that another sequence that can recruit Jak2 into the signal transduction complex can substitute for the intracellular domain of Hu-IFN-γR2.

Various chimeric receptors between the EpoR and Hu-IFN-γR1 and Hu-IFN-γR2 subunits were constructed in order to gain an understanding of the events leading to signal transduction. CHO-16-9 cells were stably transfected with expression vectors coding for EpoR, EpoR/γR1, EpoR/γR2, the combination of EpoR/γR1 and EpoR/γR2, and γR1/EpoR(p91). In response to Epo, the EpoR transfectants showed no response (Fig. 2, panel D). The EpoR/γR1 transfectants showed a slight enhancement of expression of MHC class I antigens (Fig. 2, panel E), which shows that the intracellular domain of the Hu-IFN-γR1 chain, by itself, can recruit all the requisite components for signal transduction. At lower concentrations of Epo (less than 100 units/ml), there was little or no increased MHC class I antigen expression in these cells (Fig. 4). The transfec-
tants containing both EpoR/γR1 and EpoR/γR2 chains exhibited substantial expression of MHC class I antigens (Fig. 2, panel F; Fig. 4). Cells transfected with the expression vector coding for EpoR(p91) chimeric cDNA (EpoR with the p91 recruitment site from IFN-γR1) respond to Epo with enhanced

FIG. 1. Schematic representation of native and chimeric receptors. EpoR, γR1, and γR2 represent the wild-type human erythropoietin receptor, Hu-IFN-γR1, and Hu-IFN-γR2, respectively. The various chimeric constructs were made by joining the extracellular domain (top) with the transmembrane and cytoplasmic domains (bottom) of the receptors indicated. The EpoR(p91) and EpoR/γR2(p91) chimeras contain the 5-amino acid Stat1α recruitment site of Hu-IFN-γR1 (25, 45, 50) at the 3’ end of the intracellular domain of EpoR and IFN-γR2, respectively.

FIG. 2. Induction of class I MHC surface antigens. Induction of class I MHC surface antigens by Hu-IFN-γ or Epo, as indicated, of the parental 16-9 cells expressing only Hu-IFN-γR1 (panel A) and of 16-9 cells expressing Hu-IFN-γR1 along with various transfected receptor chains: IFN-γR2 (panel B), γR2/EpoR (panel C), EpoR (panel D), EpoR/γR1 (panel E), both EpoR/γR1 and EpoR/γR2 (panel F), γR1/EpoR(p91) (panel G), EpoR(p91) (panel H), and EpoR/γR2(p91) (panel I). Cells were treated with Hu-IFN-γ at 1,000 units/ml, or Epo at 100 units/ml for 72 h as described (32). Class I MHC antigens were detected by flow cytometry after treatment of cells with mouse anti-human-HLA-B7 monoclonal antibody (W6/32), followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The unshaded regions represent untreated cells, and shaded regions represent cells treated with Hu-IFN-γ (panels A–C and G) or Epo (panels D–F, H, and I).
expression of class I MHC antigens, while the γR1/EpoR(γR1) transfectants were unresponsive (Fig. 2, panels H and G, respectively). Furthermore, the γR1/γR2(γp91) receptor chain is unable to transduce a signal upon binding ligand, whereas the cells expressing the EpoR/γR2(γp91) chimeric receptor exhibited enhanced class I MHC antigen expression in response to activation by Epo (Fig. 2, panel I).

Activation of Stat Proteins—We analyzed Stat activation in cells expressing wild-type and chimeric receptors in response to IFN-γ and Epo. As shown in Fig. 5, IFN-γ stimulation resulted in Stat1α activation in transfected 16-9 cells expressing native IFN-γR2 or γR2/EpoR chains. Similarly, Epo caused activation of Stat1α in transfected cell lines expressing EpoR/γR1 and both EpoR/γR1 and EpoR/γR2 receptor chains (Fig. 6). Consistent with the small enhancement in surface expression of class I MHC antigens in cells expressing EpoR/γR1 in response to Epo, Stat1α activation was also lower in these cells compared to cells expressing both EpoR/γR1 and EpoR/γR2 chains. Activation of p91 was also observed in cells expressing the EpoR(γp91) and EpoR/γR2(γp91) chains (Fig. 6). Furthermore, cells expressing those chimeric receptors containing the EpoR intracellular domain, except γR1/EpoR(γp91) and γR1/EpoR, exhibited activation of Stat5 in addition to Stat1α (Fig. 5; data with γR1/EpoR were negative similar to results with γR1/EpoR(γp91)). Stat5 is phosphorylated on tyrosine in response to Epo (19, 20). Both Stat1α and Stat5 are supershifted by the addition of anti-Stat1α and anti-Stat5 antibodies, respectively. Addition of 100-fold molar excess of unlabeled GAS oligonucleotide eliminates both Stat1α and Stat5 activated complexes.

Activation of Jak Kinases—IFN-γ activates Jak1 and Jak2 kinases (46), whereas Epo activates Jak2 (36) during signal transduction. Thus, we tested the ability of the various chimeric receptors to activate Jak1 and Jak2 kinases in response to binding of ligand. Phosphorylation of Jak1 and Jak2 (Fig. 7) was examined by immunoprecipitation of cellular lysates with anti-phosphotyrosine antibodies, followed by a Western blot visualized with specific anti-Jak1 and anti-Jak2 antibodies. Both Jak1 and Jak2 were phosphorylated in response to Hu-IFN-γ treatment in 16-9 cells expressing parental IFN-γR2 or chimeric γR2/EpoR receptors. Induction with Epo phosphorylated both Jak1 and Jak2 kinase in the cell line expressing both EpoR/γR1 and EpoR/γR2 chains. In the cell line expressing only the chimeric EpoR/γR1 receptor, only Jak1 kinase was phosphorylated in response to Epo. The cell line transfected with the γR1/EpoR chimeric receptor did not exhibit phosphorylation of either Jak1 or Jak2 kinase upon IFN-γ treatment.

**DISCUSSION**

For hormones, growth factors and cytokines, the conversion of the extracellular ligand-binding event to the intracellular signal involves a change in the oligomeric structure of the

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2 S. Kotenko, unpublished observation.

3 G. Muthukumaran, S. Kotenko, R. Donnelly, J. N. Ihle, and S. Pestka, unpublished results.
receptor. Depending on the ligand, this can take the form of receptor homodimers (Epo, growth hormone), heterodimers (ciliary neurotrophic factor, leukemia inhibitory factor), homotrimers (tumor necrosis factor), and more complex assemblies (reviewed in Ref. 47). In the case of IFN-γ, the oligomerization involving IFN-γR1 and IFN-γR2 initiates the signal transduction events: activation of Jak1 and Jak2, phosphorylation of IFN-γR1 on Tyr-457 (16, 25), followed by phosphorylation and activation of Stat1α (27). A major function of receptor dimerization is to bring two receptor-associated kinases together for transactivation and phosphorylation of the receptor chains. The cytoplasmic domain of the IFN-γR2 subunit serves to bring Jak2 kinase into the signal transduction complex (16). This is a crucial event since deletion of the membrane-proximal region of the intracellular domain of the IFN-γR2 chain, which encompasses the Jak2 association site, completely abrogates its ability to transduce signals in response to IFN-γ (16), and cells lacking Jak2 do not respond to IFN-γ (46). This is further supported by the observation that the IFN-γR2/EpoR chimeric receptor, which recruits Jak2, is almost as effective as the native IFN-γR2 chain in supporting signal transduction in response to IFN-γ (Figs. 2, 5, and 7). The IFN-γR2 subunit is a helper receptor subunit with a Jak2 association site, but no Stat recruitment site; its intracellular domain can be substituted with the cytoplasmic domain of any receptor subunit that can bring a Jak kinase to the IFN-γ receptor complex to support signal transduction (40).

The requirement for two distinct Jak kinases in the IFN-γ signaling pathway was demonstrated with the use of kinase-deficient cell lines (46, 48). Based on our results with the chimeric erythropoietin-interferon γ receptors, we propose that this reflects two features characteristic of the IFN-γ receptor complex: the unique properties of the receptor relative to the positioning of the Jak, and the idea that Jak1 is relatively ineffective in one or more of the following phosphorylation steps (trans-phosphorylation of itself, phosphorylation of IFN-γR1, and activation of Stat1α). The presence of Jak2 facilitates effective phosphorylation of the above steps. In contrast to the growth hormone receptor (49) and the EpoR (37) complexes, when one IFN-γ homodimer binds two IFN-γR1 molecules, the two receptor subunits do not interact with one another and are separated by 27 Å (50) at their closest point. Therefore, although the IFN-γR1 chain possesses both a Jak1 association site and a Stat1α recruitment site, alone it is unable to transduce a signal on homodimerization as the two Jak1 kinases are not in physical proximity to permit transphosphorylation (Fig.

**Fig. 6.** Electrophoretic mobility shift assays of cells expressing chimeric receptors. Clones of transfected 16-9 cells stably expressing EpoR/γR1, EpoR(p91), or EpoR/γR2(p91) chimeric receptor subunits, or both EpoR/γR1 and EpoR/γR2 chimeric receptors were treated with erythropoietin, at 100 units/ml for 15 min at 37 °C. Whole cell extracts were made and the electrophoretic mobility shift assay performed. As shown in the figure, induction with Epo causes activation of Stat1α in cells expressing EpoR/γR1, EpoR(p91), and EpoR/γR2(p91), as well as in those cells expressing both EpoR/γR1 and EpoR/γR2. Addition of anti-Stat1α antibody to the reaction mixture caused the Stat1α complex to be shifted.

**Fig. 7.** Phosphorylation of Jak1 and Jak2 kinases. Untreated cells or cells treated with Hu-IFN-γ or Epo were lysed and immunoprecipitated (I.P.) with monoclonal anti-phosphotyrosine antibodies (anti-P-Tyr panels), polyclonal anti-Jak1 antibodies (anti-Jak1 panels) or anti-Jak2 antibodies (anti-Jak2 panels) as described under "Materials and Methods." The cell lines are indicated on the figure and are defined in the legend to Fig. 1. Immunoprecipitates were resolved on SDS-PAGE, transferred to PVDF membranes and the blots probed with anti-Jak1 or anti-Jak2 antibodies as noted under the respective horizontal panels.
The structure of the heterodimer of EpoR/EpoR complex therefore must be the EpoR/EpoR homodimer, permits transactivation of the EpoR/EpoR complex. The EpoR/EpoR homodimer binds to two IFN-γR1 chains, followed by its interaction with two IFN-γR2 chains. The associated Jak2 andJak1 kinases activate one another by transphosphorylation, with subsequent phosphorylation and dimerization of Stat1α. This is consistent with the results of Briscoe et al. (53). Crystallographic analysis of the IFN-γR1 and IFN-γR2 subunits per complex. The IFN-γR1 homodimer is barely active (Figs. 2, 4, and 7) indicate that Jak2 is more effective at phosphorylating role for Jak1 in the IFN-γ receptor complexes.

We propose that the multichain cytokine class II receptors have two major chains exemplified by the IFN-γ receptor complex (Fig. 2B). The ligand binding chain (IFN-γR1) and the accessory chain (IFN-γR2; helper receptor) serve as a foundation for the functional IFN-γ receptor complex (16, 40). The geometry of the IFN-γR1 chain is such that its homodimerization yields a non-functional intracellular receptor complex. The accessory chain complements this function (Fig. 8A). The question arises: why should two separate chains have evolved when one in the correct configuration would suffice? We postulate that the presence of two distinct chains provides for more effective control and fine tuning of responses to ligand. For example, the differences in response of Th1 and Th2 cells to IFN-γ result from the lack of expression of the IFN-γR2 chain in the Th2 subset (55–57) and allows exquisite fine tuning of sensitivity to IFN-γ. It is also possible that receptors with multiple chains could recruit additional factors into the complex to generate a wider variety of intracellular signals. This could explain how receptors with multiple subunits could activate a greater number of specific pathways and signals than those with fewer elements in the receptor complex. Our experiments begin to provide an insight into these possibilities.

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