A Single STAT Recruitment Module in a Chimeric Cytokine Receptor Complex Is Sufficient for STAT Activation

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We established a system of receptor chimeras that enabled us to induce heterodimerization of different cytoplasmic tails. Fusion constructs were created that are composed of the extracellular parts of the interleukin-5 receptor α and β chains, respectively, and the transmembrane and intracellular parts of gp130, the signaling factor dimer of the interleukin-6 receptor complex. In COS-7 transfectants we observed a dose-dependent interleukin-5-inducible STAT1 activation for which the presence of both the α and the β chain chimera was needed. No STAT activity was detected if one of the cytoplasmic tails of the receptor complex was deleted, indicating that STAT activity resulted from a receptor dimer rather than from higher receptor aggregates.

We further investigated whether dimerization of STAT1 depends on the juxtaposition of two STAT recruitment modules in a receptor complex. We show that a receptor dimer with only a single STAT1 docking site was still able to lead to STAT1 activation. This indicates that the formation of a paired set of STAT binding sites in a receptor complex is not the prerequisite for STAT factor dimerization. Our findings are discussed in view of alternative STAT dimerization models.

A detailed analysis of interferon (IFN) signaling events first provided insight into a general signaling mechanism, the Jak-STAT pathway, by which many cytokines lead to an altered transcriptional activity (2, 3). This pathway involves the activation of STAT (signal transducers and activators of transcription) factors and other proteins with "matching" SH2 domains upon binding. Subsequently, the STATs dissociate from the receptor and translocate as homo- or heterodimers to the nucleus where they bind to enhancer elements of target genes and influence transcriptional activity (2, 3).

The events leading to STAT factor dimerization are not well understood. Due to receptor dimerization, many cytokines lead to the formation of a paired set of STAT docking sites, e.g. IFNγ signals through a homodimer of STAT1 that binds to γ-interferon-activated sequence elements of IFNγ-regulated genes (4, 5). A single tyrosine residue (Tyr-440) in the α chain of the IFNγ receptor serves as a docking site for STAT1 (6–8). Since IFNγ receptor α chains dimerize upon binding of IFNγ (9), phosphorylation of Tyr-440 forms two juxtaposed docking sites for latent STAT1. Dimerization of STAT1 monomers phosphorylated on a specific tyrosine residue (Tyr-701) might be favored by the presence of another phosphorylated STAT1 monomer in the near proximity (7). In the present study we tested whether the formation of a STAT1 homodimer depends on the presence of two STAT1 docking sites in a dimerized receptor.

For this reason we established a system of receptor chimeras based on the extracellular parts of the human interleukin-5 (IL-5) receptor α and β chains that enabled us to induce heterodimerization of different cytoplasmic tails. We show that a receptor dimer with only a single STAT1 docking site is still able to lead to activated STAT1 dimers, indicating that close juxtaposition of STAT binding sites in a receptor complex is not the prerequisite for STAT factor dimerization.

MATERIALS AND METHODS

Reagents—Human IL-5 was expressed in Sf9 insect cells and purified as described previously (10). 125I-hIL5 was prepared with the IODOGEN iodination agent (Pierce) as described (11). The eukaryotic expression vector pSVLgp130 was generously supplied by Dr. T. Taga (Osaka, Japan). For flow cytometry the monoclonal antibody 16-4 specific for the human IL-5 receptor α chain was obtained from Dianova (Hamburg, Germany).

Construction of the Chimeric Molecules—For amplification of the cDNA encoding the extracellular part of the human IL-5 receptor α chain and human β chain the primers cctgctgacctacATATCATGGCTGCGCATG (α sense), cggaattcATTTCCACATAAATTGTTG (α antisense), getcagagactacATATCATGGCTGCGCATG (β sense), and cggaattcATATCATGGCTGCGCATG (β antisense) were used. Uppercase letters indicate cDNA sequence; underlined are restriction sites for XhoI, EcoRI, and XbaI, which were added to facilitate cloning. The IL-5Re

1 The abbreviations used are: IFN, interferon; SH2, Src homology 2; IL, interleukin; EMSA, electrophoretic mobility shift assay; SIE, sis-inducible element; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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chain sensese primer was designed to provide a “better” Kozak consensus sequence. Therefore a point mutation was introduced, leading to a valine instead of an isoleucine residue at position 2 of the signal peptide of IL-5Rα/gp130. Polymerase chain reactions were performed with 20 ng of plasmid DNA, 20 pmol of each primer, and 1 unit of Vent polymerase (New England Biolabs). The polymerase chain reaction products were digested with XhoI/EcoRI or XbaI/EcoRI, respectively, and inserted into pSVLgp130 cut with the same enzymes, thus replacing the DNA for the extracellular region of gp130 with the one of the IL-5R α or β chain. The resulting plasmids were named pSVL-IL-5Rα/gp130 and pSVL-IL-5Rβ/gp130, respectively. Deletion constructs IL-5Rα/gp130Δcyt and IL-5Rβ/gp130Δcyt lack the gp130 cytoplasmic region apart from the first three residues (NKR). Due to the cloning procedure, they contain additionally three vector-encoded residues (IQT) before termination. The construction of chimeric molecules Eg (EpoR/gp130), Eg/Ab, and Eg/Tyr-γ440 has been described (12). Eg/Ab includes only the box 1 and 2 regions of gp130 and therefore lacks the five carboxyterminal tyrosine residues. Eg/Tyr-γ440 contains additional 7 amino acid residues from the human IFNγ receptor (including Tyr-440), Eg/Ab and Eg/Tyr-γ440 end with a FLAG epitope. From those expression constructs EcoRI/BamHI digested pSVL-IL-5Rα/gp130 and pSVL-IL-5Rβ/gp130, thereby replacing the intracellular part of gp130.

**Cell Transfections**—Simian kidney cells (COS-7, ATCC CRCL 1651) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin. Cells were grown at 37 °C in a water-saturated atmosphere with 5% CO2. 10^7 COS-7 cells were transiently transfected with 20–30 μg of plasmid DNA by electroporation (Gene Pulser, Bio-Rad). Electroporations were performed at 960 microfarads and 230 V. Cells were harvested 48–72 h post transfection.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts were prepared as described (13). Protein concentrations were measured with the Bio-Rad protein assay. A double-stranded mutated SIE oligonucleotide from the c-fos promoter ([m67SIE]: 5’-GAT CGG GGA GGG ATT TAC GGG GAA ATG CTG-3’) was labeled by filling in 5’ protruding ends with the Klenow enzyme, using [α-32P]dATP (10 mCi/ml, 3,000 Ci/mmol). Nucleic extract (2.5 to 5 μg of protein) was incubated with about 10 fmol (5,000 cpm) of probe in gel shift incubation buffer (10 mm HEPES, pH 7.8, 1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 5 μM dithiothreitol, 0.7 μM phenylmethylsulfonyl fluoride, 0.1 mg/ml of poly(dI-dC), and 1 mg/ml bovine serum albumin) for 10 min at room temperature. The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid, and 80% water for 30 min, dried, and autoradiographed.

**Flow Cytometry**—COS-7 cells were released from the dishes by treating them with phosphate-buffered saline, 10 mM EDTA at 37 °C for 10 min. Approximately 10⁶ cells were incubated with monoclonal antibody 16-4 or S-16 for 30 min, followed by treatment with the secondary antibody (goat anti-mouse IgG phycoerythrin-conjugated F(ab’)_2 fragment, Dianova, Hamburg, Germany). Fluorescence was measured on a FACScan (Becton Dickinson).

**Binding Studies**—Upon transfection approximately 10⁵ COS-7 cells were seeded into 24-well plates and incubated for 2 days until they reached 80–90% confluence. Cells were washed twice with cold binding medium (0.2% bovine serum albumin, 20 mM HEPES buffer (pH 7.0) in Dulbecco’s modified Eagle’s medium). 200 μl of binding medium with 125I-IL-5 (10 nM and 2-fold dilutions thereof) were added for 2 h on ice in the presence or absence of 100-fold excess of cold IL-5. Cells were washed three times with cold phosphate-buffered saline containing 1 mM MgCl₂, 0.1 mM CaCl₂, and 0.2% bovine serum albumin. They were lysed in 1 M NaOH overnight before cell-associated radioactivity was measured in a γ counter. Specific binding was calculated as the difference between the binding in the absence (total binding) and presence

**Fig. 1.** IL-5 induces STAT activation in COS-7 cells coexpressing IL-5Rα/gp130 and IL-5Rβ/gp130 chimeras. A, surface expression of the chimeric molecules. COS-7 cells cotransfected with expression plasmids for IL-5Rα/gp130 and IL-5Rβ/gp130 or with the vector pSVL were incubated with monoclonal antibodies specific for the IL-5Rα or β chain or with medium (= control) and consequently with phycoerythrin-conjugated-anti-mouse Ig F(ab’)₂. Surface staining was measured by flow cytometry. In each panel the percentage of positive cells is indicated. B, IL-5 binding to chimeric receptors: affinity conversion upon expression of the IL-5Rβ/gp130. Scatchard plot of 125I-IL-5 binding to COS-7 cells expressing IL-5Rα/gp130 alone (○) or in combination with IL-5Rβ/gp130 (●). The derived affinities (K′) are 1 nM (IL-5Rα/gp130) and 500 pM (IL-5Rβ/gp130). C, STAT chain activation increases dose-dependently with IL-5 concentration. COS-7 cells cotransfected with expression plasmids for IL-5Rα/gp130 and IL-5Rβ/gp130 were incubated with increasing amounts of IL-5 for 30 min. STAT activity in nuclear extracts was measured in an EMSA using the m67SIE probe. The retarded band corresponds to a homodimer of STAT1 (12). Deletion of the IL-5Rα- and the IL-5Rβ-chains is needed for an IL-5-inducible STAT activation in COS-7 transfectants. COS-7 cells expressing the indicated chimeric receptors were stimulated with IL-5 (160 ng/ml), erythropoietin (Epo, 7 units/ml) or left untreated. STAT activity in nuclear extracts was measured in an EMSA using the m67SIE probe.
RESULTS

IL-5 Stimulates STAT Activation in COS-7 Cells Coexpressing IL-5Ra/gp130 and IL-5Rβ/gp130—For the establishment of the system of heterodimeric receptor chimeras, we first made hybrid molecules composed of the extracellular parts of the human interleukin-5 receptor (IL-5R) α and β chains, respectively, and the transmembrane and the intracellular regions of gp130, the signal transducing receptor chain for IL-6-type cytokines. Upon transfection of COS-7 cells with expression plasmids for IL-5Ra/gp130 and IL-5Rβ/gp130, surface expression of the chimeric molecules could be observed in a fraction of the cells. Mock-transfected COS-7 cells were not stained with antibodies recognizing human IL-5R α or β chains (Fig. 1A). Affinity of these chimeric constructs is similar to that seen with wild type IL-5 receptors expressed in COS-1 cells (15, 16); IL-5Ra/gp130 bound IL-5 with a low affinity ($K_d = 1$ nM), and coexpression of IL-5Rβ/gp130 led to a 2-fold increase of binding (Fig. 1B).

Dimerization of gp130 induced by cytokines (17) or agonistic antibodies (18) leads to rapid activation of STAT factors in a variety of cell lines (19, 20). We now tested whether the heterodimeric chimeric receptors have signaling ability. COS-7 transfectedants expressing IL-5Ra/gp130 and IL-5Rβ/gp130 were stimulated (30 min, 37°C) with increasing amounts of recombinant human IL-5; nuclear extracts were prepared and tested in an EMSA. Stimulation with 4 ng of IL-5/ml already led to a signal, and the intensity of the gel shift bands increased dose-dependently until at 80 ng/ml a maximum was reached (Fig. 1C). As shown in Fig. 1D, the shifted band had the same mobility as but a lower intensity than the one observed upon homodimerization of EpoR/gp130 chimeras which have previously been shown to lead to STAT1 activation in COS-7-transfectants (12, 21). Importantly, expression of either the IL-5Ra/gp130 or the IL-5Rβ/gp130 chimera alone did not lead to IL-5-inducible STAT activation (Fig. 1D). Therefore, both the α- and the β-chain chimeras contribute to the signaling ability of the receptor complex.

No Evidence for a Further Ligand-induced Receptor Aggregation—The stoichiometry of the components of the IL-5 receptor is not clear (see “Discussion”). The simplest model of a functional IL-5 receptor system is a dimer comprising one α chain and one β chain, but further receptor chain multimerization cannot be excluded (e.g. Fig. 2A, left panel, see also Ref. 22). Therefore, we investigated whether the observed STAT activation might have resulted from receptor aggregates (see Fig. 2A, right panel).

We generated constructs (IL-5Ra/gp130Δcyt, IL-5Rβ/gp130Δcyt) with a deletion of the whole cytoplasmic region of gp130 and coexpressed them with the “complementary” full-length constructs (IL-5Rβ/gp130 or IL-5Ra/gp130, respectively). However, no IL-5-inducible STAT activation was observed (Fig. 2B), although these cotransfectants bound IL-5 with high affinity (data not shown). We therefore conclude that the STAT activity observed in COS-7 cells expressing IL-5Ra/gp130 and IL-5Rβ/gp130 is unlikely to result from higher aggregates. Thus, the IL-5R-based chimeras are suitable to study signaling of asymmetric receptor dimers.

A Single Tyrosine Module in a Cytokine Receptor Complex Is Sufficient for STAT Activation—We have shown previously that a “tyrosine module” from the interferon γ receptor mediated a specific activation of STAT1 when fused to the membrane proximal box 1/2 region of gp130 (12, 21). In that study chimeric receptors with the extracellular part of the erythropoietin receptor were applied which homodimerized upon stimulation with erythropoietin.

We then replaced the extracellular region of the erythropoietin receptor by the IL-5R α and β chains resulting in constructs IL-5Ra/Tyr-γ440 and IL-5Rβ/Tyr-γ440 carrying a 7-amino acid tyrosine module of the IFNγ receptor distal from the box 1/2 region of gp130. The constructs IL-5Ra/ΔB and IL-5Rβ/ΔB contain the gp130 box 1/2 region but no STAT recruiting tyrosine module. IL-5 stimulation of the two Tyr-γ440 constructs led to a strong STAT1 activation (Fig. 3A), whereas no STAT1 activation was observed upon dimerization of the two ΔB constructs (Fig. 3D). Importantly, dimerization of a single Tyr-γ440 with a ΔB construct resulted in a clear STAT1 activation (Fig. 3, B and C). Therefore, a single tyrosine module in a cytokine receptor complex is sufficient for STAT1 activation. This observation indicates that close contact of STAT factors by juxtaposition of STAT binding sites at dimerized receptors is not necessary for dimerization.
**DISCUSSION**

It was our intention to study whether a single STAT module in a receptor complex is sufficient to achieve STAT activation. For this purpose, an “asymmetric” receptor complex was created with chimeric receptors based on the extracellular parts of the IL-5 receptor. The IL-5 receptor complex is composed of the ligand-binding IL-5Rα subunit and the common β receptor chain (βc) that is shared with the α receptors for IL-3 and GM-CSF and functions as an affinity converter (Ref. 15; reviewed in Ref. 23). Members of this receptor family have already been successfully employed for the construction of chimeric molecules by others (24–26). In this study we generated fusion constructs of the extracellular parts of the IL-5Rα and β chain, respectively, and the transmembrane and intracellular region of gp130, the signal transducing chain of the IL-6 receptor. In COS-7 transfectants expressing the IL-5Rα/gp130 and the IL-5Rβ/gp130 chimeras, we observed an IL-5-inducible STAT activation (Fig. 1). Although “STAT activation” is certainly only one of several possible experimental read-outs to characterize the chimeras functionally, it indicates that they can be used to mimic signaling events involving gp130 dimers just like chimeric receptors that homodimerize upon ligand binding (e.g., via the extracellular region of receptors for erythropoietin, granulocyte colony-stimulating factor, epidermal growth factor, or neurotrophin-3; Refs. 12, 27, and 28).

Active receptor complexes depend on the presence of both IL-5Rα and β chimeric molecules. Expression of the IL-5Rα/gp130 alone did not yield an IL-5-inducible signal. This was expected since cross-linking analyses as well as studies in solution indicated that IL-5, although itself a homodimer, binds only to one IL-5Rα chain (29–32). Similarly, a chimeric molecule consisting of the extracellular domain of murine IL-5Rα and the transmembrane and intracellular regions of βc could mediate proliferation of transfectants only in the presence of a functional βc chain (25).

The simplest model of the active IL-5 receptor complex consists of a heterodimer of one α and one β chain, but there are some reports that members of the IL-3/IL-5/GM-CSF receptor family might undergo further multimerization. 1) The cytoplasmic tail of the βc chain has signaling capacity when dimerized upon triggering of chimeric receptor constructs (25, 33, 34); 2) constitutively active mutants of the βc chain could be isolated (35–37); 3) it was suggested that a ligand-bound active receptor complex might normally contain a βc chain dimer (37); and 4) the occurrence of ligand-independent βc homodimers has recently been reported (38). In addition, unusual affinities were observed when GM-CSF receptor α and β subunits were expressed in COS-1 cells. These data have been discussed in view of possible complex variability (39).

We therefore expressed IL-5Rα/gp130 together with an IL-5Rβ/gp130 construct lacking the whole cytoplasmic region (and vice versa) in COS-7 cells. If multimerization would occur, the proximity of at least two cytoplasmic tails could be expected to result in the activation of Jaks and STATs. However, since such receptor complexes did not signal, we conclude that, at least under our experimental conditions, the STAT activity we observed upon IL-5 stimulation is very likely to result from an αβ receptor dimer rather than from higher receptor aggregates. We cannot exclude, however, the possibility that potential multimers do not signal due to an “incorrect” orientation of their cytoplasmic tails.

The measured affinities of the IL-5Rα/gp130 chimeric receptors are comparable to those of the wild type IL-5 receptor in COS-1 transfectants (15, 16). However, this affinity is lower than the one observed on HL-60 eosinophilic cells (11). Maybe additional, so far unknown receptor components contribute to the high binding affinity in these cells. A potential “γ”-subunit of the GM-CSF receptor has recently been suggested to be present in certain cell types (37). We cannot rule out the possibility that the lack or the presence of unknown receptor components in the COS-transfectants might also have influenced the signals we observed. Additionally, we cannot exclude a potential cross-talk between different receptor systems (such as described for the stem cell factor receptor and the erythropoietin receptor; Ref. 40) that might contribute to the responses we observed.

The mechanisms leading to STAT activation are not well understood. Although STAT activation independent of receptor tyrosine residues has been described (41, 42), the importance of tyrosine residues within the cytoplasmic tail of cytokine receptors has been demonstrated in many reports (e.g., Refs. 6, 12, 28, and 43–45).

The following models for STAT activation at receptors phosphorylated on tyrosine residues have been discussed (Fig. 4).

**Cytokine receptors, usually containing multiple potential binding sites for STAT factors, dimerize upon ligand stimula-**
After STAT binding to their receptor docking sites followed by their tyrosine phosphorylation, the dimerization process might be favored by the presence of other phosphorylated STAT factors in close proximity. The "second" STAT monomer of the dimer could be recruited from a phosphotyrosine residue nearby on the same receptor chain. Alternatively, it might have been bound to the other chain of the receptor dimer before (Fig. 4, upper part). Support for this model comes from the observation that IFNγ receptor chains point-mutated at the tyrosine residue critical for STAT1 binding (Tyr-440 in the human or Tyr-420 in the murine receptor) act as dominant-negative mutants when overexpressed in homologous cells (46). This effect could be explained by a model in which the IFNγ-induced formation of dimerized STAT1 binding sites was the prerequisite for consequent STAT factor dimerization (see also discussion in Ref. 7).

From our studies, however, we conclude that the presence of dimerized STAT recruitment sites in an active receptor complex is not the prerequisite for STAT activation. We rather show that one "tyrosine module" is sufficient to achieve STAT activation.

This finding can be reconciled with other models for STAT activation. One possibility would be that the second STAT factor binds to a phosphorylated STAT monomer already bound to the tyrosine phosphorylated receptor and then becomes phosphorylated (Fig. 4, middle part). The finding that there is no STAT1 phosphorylation in STAT2-deficient fibrosarcoma cells upon stimulation with IFNα could be explained by this model (47). However, the formation of the STAT1/2 heterodimer might be a special case, since it has been demonstrated that STAT1 has a rather high affinity for the phosphorylated peptide of STAT2 (7). According to analogous peptide binding studies, it seems unlikely that STAT1 is also recruited by a receptor-associated tyrosine-phosphorylated STAT1 molecule.

It is also possible that phosphorylated STAT1s dissociate from the receptor, a process that in analogy to the situation of p56

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