Identification of a STAT4 Binding Site in the Interleukin-12 Receptor Required for Signaling*

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The specificity of the various STAT SH2 domains for different tyrosine-containing peptides enables cytokines to activate different signaling pathways and to induce distinct patterns of gene expression. We show that STAT4 has a unique peptide specificity and binds to the peptide sequence pYLPNSID (where pY represents phosphotyrosine). This motif is found at tyrosine residue 800 in the β2 subunit of the interleukin-12 receptor and is required for DNA binding and transcriptional activity of STAT4. Our data demonstrate that transfection of interleukin-12 receptor β1 and β2 subunits is sufficient for STAT4 activation but not for STAT1 or STAT3 activation.

IL-12 is a heterodimeric cytokine secreted from antigen presenting cells in response to bacteria and intracellular parasites and thus plays an important role in host defense against bacterial pathogens (1). IL-12 promotes the proliferation of T cells and NK cells and is required for the differentiation of T cells into the Th1 subset of T helper cells. Th1 cells are critical for cell-mediated immune responses, because they secrete IFN-γ, which enhances the activity of cytotoxic T cells and NK cells (1–3). In addition to these essential functions, Th1-dominated responses are associated with pathologic autoimmune and inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (4). Given this potential for immunopathology, it is important to understand the mechanisms that control the Th1 response to develop possible therapeutic interventions.

IL-12 signals through the IL-12 receptor, which is composed of at least two subunits designated β1 and β2 (5, 6). Both IL-12 receptor subunits are members of the hematopoietin receptor superfamily and have strong homology to the gp130 receptor (5). The β1 receptor, although a low affinity binder of IL-12, is not capable of transducing an IL-12-mediated signal (6). A second subunit of the IL-12 receptor was subsequently identified that when coexpressed with the β1 subunit forms a high affinity receptor for IL-12 and confers IL-12 signaling (6). IL-12R β2 expression is differentially regulated in Th1 versus Th2 cells (7). Th1 cells but not Th2 cells express the β2 subunit of the IL-12 receptor (8). Following T cell activation, IL-12 and IFN-γ treatment induces β2 expression, whereas IL-4, a cytokine produced by Th2 cells, inhibits β2 expression resulting in loss of IL-12 signaling (7, 8). Therefore, expression of the β2 subunit of the IL-12 receptor is a crucial determinant of Th1 versus Th2 development.

Cytokine binding to its receptor leads to activation of JAK kinases that phosphorylate the receptor on tyrosines located in the intracellular domain. The phosphorylated regions are binding sites for signal transduction molecules called STATs that are rapidly recruited to the receptor and tyrosine phosphorylated by JAK kinases. Tyrosine phosphorylation of STAT proteins induces their dimerization and translocation to the nucleus where they bind to specific DNA sequences and regulate transcription. IL-12 stimulation results in TYK2 and JAK2 phosphorylation and interaction of TYK2 with the β1 subunit of the IL-12 receptor and interaction of JAK2 with the β2 subunit (9, 10). Unlike STAT4, whose expression is limited to lymphoid and spermatogonia cells, JAK2 and TYK2 are ubiquitously expressed (11, 12).

In response to IL-12, STAT4 has been shown to be tyrosine phosphorylated and activated in Th1 lymphocytes and NK cells (9, 13). In addition, it has been reported that STAT1, STAT3, and STAT5 can be activated in response to IL-12 (13–15).

The specificity of the various STAT SH2 domains for different tyrosine-containing peptides enables cytokines to activate different signaling pathways and to induce distinct patterns of gene expression. We show that STAT4 has a unique peptide specificity and binds to the peptide sequence pYLPNSID (where pY represents phosphotyrosine). This motif is found at tyrosine residue 800 in the β2 subunit of the interleukin-12 receptor and is required for DNA binding and transcriptional activity of STAT4. Our data demonstrate that transfection of interleukin-12 receptor β1 and β2 subunits is sufficient for STAT4 activation but not for STAT1 or STAT3 activation.

STAT proteins, by way of their SH2 domains, interact with specific phosphotyrosine residues on cytokine receptors (16). Although the phosphotyrosine binding sites of all the other known STAT proteins have been identified, the tyrosine docking site of STAT4 has not previously been reported. The β2 subunit of the IL-12 receptor contains three tyrosines in its cytoplasmic domain, whereas the β1 subunit contains no tyrosine residues in its cytoplasmic domain (5, 6). Therefore, given the importance of tyrosine phosphorylation in cytokine signaling pathways, we focused on the three tyrosines of the β2 subunit as possible docking sites for the STAT4 signaling protein in response to IL-12.

We determined that STAT4 is activated through interaction with the tyrosine at amino acid residue 800 in the IL-12 receptor β2 subunit. STAT4 activation depends on interaction with the peptide sequence pYLPNSID (where pY represents phosphotyrosine), through providing evidence for the specificity of the STAT4 SH2 domain for a unique binding site in the IL-12 receptor. In addition, we show that neither STAT1 nor STAT3 is activated directly through the IL-12 receptor.
STAT4 Binding Site in the IL-12 Receptor

and 3′-GGGGTTGGGACTGAGAATGCGAGG-primer by β2 Y767F, 5′-G

TTAAGCGGCAGGACG-3′ and 3′-GACCATGCTCCTCTGTGC-5′; and for β2 Y767F, 5′-G

CTAAGATTGTTGCATTCCGACG-3′ and 3′-GGGGTCTTAAAAGTTAGGTT-5′. Mutations changing the tyrosine to phenylalan

ine were blocked with 5% milk, Tris-buffered saline, 0.06% Tween 20 and beads. Proteins were resolved on 12% SDS-polyacrylamide gels (Fisher) with anti-M2 Flag antibody (Kodak, Sigma) and 25 μl of binding buffer (100 mM KCl, 25 mM HEPES (pH 7.9), 0.1 mM EDTA, 5 mM MgCl2, 200 μg/ml bovine serum albumin, 10% glycerol), and 1 μg of poly(dI-dC) in a final volume of 15 μl. Reactions were incubated at room temperature for 5 min before 1 μl of probe was added for 15 min at room temperature. oligonucleotides containing two high affinity STAT consensus binding sites, (5′-AACAATCTCAGCGAGTGCTCCGGAAAAATT-3′ and its complement) were annealed and kinnased to generate labeled DNA for the EMSA.

**Immunoblot Analysis—** Transfected cells were lyzed in TNT buffer (200 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 1 mM vanadate, 1 mM β-glycerophosphate, 5 mM NaF, and a complete protease inhibitor tablet (Boehringer Mannheim)). Extracts were immunoprecipitated with anti-M2 Flag antibody (Kodak, Sigma) and 25 μl of protein G beads. Proteins were resolved on 12% SDS-polyacrylamide gels (Fisher) and transferred to Immobilon-P membrane (Millipore). Membranes were blocked with 5% milk, Tris-buffered saline, 0.1% Tween 20 and incubated with anti-M2 Flag antibody (10 μg/ml) and horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000, Amersham Pharmacia Biotech). ECL (Amersham Pharmacia Biotech) was used for detection.

**Fluorescence Polarization—** Tyrosine phosphorylated forms of IL-12Rβ2 peptides Tyr100 (SHEGpYLPSID), Tyr276 (LVDLpYKVLESR), and Tyr278 (CACKpYPIAEEK) were synthesized as described previously (19). Peptides were purified as described in Schindler et. al. (20). Fluorescence polarization was measured in an FPM-1 analyzer (Jolly Consulting). The sequence of the fluorescent peptide was SFDPyDMPHVVL. The binding affinity of STAT4 for the fluorescent peptide is similar to its affinity for Tyr800 peptide. A fluorescent peptide is SFDpYDMPHVL. The binding affinity of STAT4 for the fluorescent peptide was measured in an FPM-1 analyzer (Jolly Consulting). The IC50 was 2.9 μM, whereas the other peptides SFDPyDMPHVVL had IC50 of greater than 100 μM is equivalent to nonspecific binding. STAT4 binds to the mutant peptide approximately 8-fold better than it does to the wild-type sequence, SFDPyDMPHVVL. This is a mutated version of the STAT1 binding site derived from the IFN-γ receptor. STAT4 binds to the mutant peptide approximately 95% homogeneity by nickel affinity chromatography (Qiagen).

**Transactivation Reporter Assay—** COS cells were plated at 2 × 105 well (35 mm) on 6-well plates. Cells were transfected the next day using Prefection Mammalian Transfection System calcium phosphate method (Promega) with 1 μg/well of STAT4 expression plasmid, 1.5 μg/well of each IL-12R subunit expression plasmid, and 1 μg/well of IRF-1 luciferase reporter plasmid. The IRF-1 reporter contains two copies of the p82 subunit at the C-terminal histidine tag in a baculovirus expression system. The protein was purified to approximately 95% homogeneity by nickel affinity chromatography (Qiagen).

**RESULTS**

**STAT4 Protein Has the Highest Affinity for pYLPSND1 Sequence in IL-12 Receptor—** It seemed likely that STAT4 phosphorylation in IL-12 is dependent on interaction with one of the three tyrosines in the cytoplasmic domain of the IL-12R β2 subunit, because the β1 subunit cytoplasmic domain contains no tyrosines. We tested the specificity of the STAT4 SH2 domain for phosphopeptides corresponding to tyrosine-containing regions of the IL-12R β2 subunit by fluorescence polarization competition binding assays. The sequence of the fluorescent peptide was SFDPyDMPHVVL. This is a mutated version of the STAT1 binding site derived from the IFN-γ receptor. STAT4 binds to the mutant peptide approximately 8-fold better than it does to the wild-type sequence, SFDPyDMPHVVL. The peptide containing tyrosine 800 with the sequence pYLPSND1 had an IC50 of 2.9 μM, whereas the other peptides SFDPyLpPVEKL, SFDPyYpKL and pYFIAEEK (Tyr278) had IC50 values of greater than 100 μM, equivalent to nonspecific background binding (Fig. 1A). These results indicate that STAT4 has the highest affinity for the peptide sequence that contains tyrosine 800 in the IL-12R β2 subunit, suggesting that this is the in vivo docking site for STAT4.

**The Tyrosine at Amino Acid 800 in the IL-12 Receptor Is Required for STAT4 Activation—** We reconstituted IL-12 receptor signaling in 293 cells to determine whether the β1 and β2 subunits of the IL-12 receptor are sufficient for STAT4 activation in response to IL-12. Both subunits of the IL-12 receptor and STAT4 were transfected into 293 cells, and STAT4 activation was tested by EMSA using nuclear extracts isolated from the transfected cells after addition of IL-12. IFN-γ stimulation has also been shown to activate STAT4 (21) and was used as a control for activation of STAT4 in these experiments. STAT4 was activated to bind DNA following IL-12 addition (Fig. 1B, lane 3) and IFN-γ addition (Fig. 1B, lane 10) but was not activated in the absence of IL-12 addition (Fig. 1B, lane 2). The β1 and β2 subunits of the IL-12 receptor were sufficient for activation of STAT4 by IL-12 in 293 cells. Both of the IL-12 receptor subunits were required for activation of STAT4 be-
cause neither one alone resulted in STAT4 DNA binding (data not shown).

IL-12R β2 subunit mutants that each contain one of the tyrosines in the cytoplasmic domain changed to phenylalanine were then analyzed for their ability to recruit STAT4. IL-12 β2 receptor mutants at both tyrosine 678 and tyrosine 767 were capable of activating STAT4, whereas the mutant at tyrosine 800 was not able to activate STAT4 (Fig. 1B, lanes 4–6). When anti-STAT4 antiserum was added to the binding reactions, the STAT4 DNA binding complex was removed, indicating that STAT4 protein was responsible for binding to the probe (Fig. 1B, lanes 7–9 and I). This evidence, along with the in vitro peptide binding data, indicates that the STAT4 SH2 domain binds to a specific phosphorylated tyrosine in the IL-12R β2 subunit that contains the sequence PYLPSNID.

IL-12-dependent transcriptional activation through STAT4 was also tested by co-transfecting the IL-12 receptor subunits, STAT4, and an IRF-1 luciferase reporter gene into cells and measuring luciferase assay following treatment with IL-12. STAT1 can induce the IRF-1 gene (22), and STAT4 has the same DNA binding specificity as STAT1 (23). Therefore, given that IL-12 stimulation has also been shown to induce IRF-1 gene expression in human peripheral blood lymphocytes, we used the IRF-1 STAT binding site for our reporter assays. We determined that in the transient transfection assay, STAT4 transcriptional activation of the IRF-1 gene was dependent on IL-12 and was induced 4-fold in IL-12 treated transfected cells (Fig. 2A). The IL-12 receptor mutants β2-Y678F and β2-Y767F were also able to induce transcriptional activation of the IRF-1 gene to levels equivalent to wild-type IL-12 β2 receptor (Fig. 2A). However, STAT4-mediated transcriptional activation with the β2-Y800F mutant of IL-12R β2 was not detected. Expression of each of the mutant β2 subunits was confirmed by Western blot (Fig. 2B). Protein levels of the IL-12R β1 subunit and STAT4 were also shown to be equivalent in each transfection (data not shown). We conclude that the tyrosine at amino acid 800 in IL-12R β2 is necessary for efficient activation of STAT4 for both DNA binding and transcriptional activation.

Fig. 2. A, transcriptional activation of STAT4 via the IL-12R and the IL-12R β2 mutants was measured by transfecting 293 cells in duplicate with indicated IL-12R constructs, STAT4, IRF-1 luciferase, and CMV β-galactosidase. After 24 h, indicated transfected cells were treated with IL-12 at 10 ng/ml for 4 h and assayed for luciferase assay. Relative luciferase activity was determined by normalizing for β-galactosidase activity and taking the average of three experiments. B, immunoblot analysis was used to analyze expression of IL-12R β2 in 293 cells following transfection with wild-type IL-12R β2 (wt), β2-Y678F, β2-Y767F, or β2-Y800F. The sample in lane 1 was prepared from cells transfected with the empty expression vector.

**IL-12RB1 and β2 Are Not Sufficient for STAT1 or STAT3 Activation**—It has previously been reported that IL-12 is able to induce tyrosine phosphorylation and DNA binding of STAT3 and STAT1 in addition to STAT4 (13, 14). We tested the ability of IL-12 to activate STAT1 and STAT3 through the IL-12 receptor in our 293 transfection system. Following transfection of 293 cells with the IL-12 receptor subunits and STAT4, STAT1, or STAT3, nuclear extracts were prepared and analyzed for DNA binding activity by EMSA using a high affinity double STAT consensus site as probe. Although we detected STAT4 DNA binding following activation with IL-12, we were unable to detect DNA binding by STAT1 or STAT3 (Fig. 3), even though their expression levels were equivalent to STAT4 expression (data not shown). STAT1 was capable of being activated for DNA binding by IFN-γ as seen in lane 7 of Fig. 3. Our results indicate that in this system, STAT1 and STAT3, unlike STAT4, cannot be activated by IL-12 through the IL-12 receptor. In addition, we also tested the ability of STAT1 or STAT3 to be activated when STAT4 is co-transfected into 293 cells. When extracts from IL-12 stimulated 293 cells co-transfected with the IL-12R subunits and STAT4 plus STAT1 or STAT4 plus STAT3 were analyzed by EMSA, supershift, and co-immunoprecipitation experiments, only STAT4 homodimer complexes were detected (data not shown). These data suggest that neither STAT1 nor STAT3 is activated by IL-12 through an interaction with STAT4.

**DISCUSSION**

IL-12 stimulation results in activation of STAT4 through interaction with the IL-12 receptor (13, 24). In response to IL-12 stimulation, STAT4 is tyrosine phosphorylated and is competent for DNA binding and transcriptional activation of target genes. STAT4 function is necessary for mediating the IL-12 response and is crucial for Th1 development and efficient IFN-γ production as shown by STAT4 knock-out studies (25, 26).

Receptor binding by STAT proteins has been shown to be specific and dictated by the SH2 domain of the STAT proteins (reviewed in Refs. 16, 27, and 28). STAT interaction with cytokine receptors depends on the phosphotyrosine residue in the receptor and the amino acid residues C-terminal to the phosphotyrosine that mediate specificity. In this paper, we demonstrate that STAT4 is activated by interacting with the IL-12
receptor and that the interaction of the STAT4 SH2 domain is specific for the peptide sequence pYLPNID, the peptide at tyrosine 800 in the IL-12R β2 subunit. This tyrosine at amino acid 800 in the IL-12R β2 subunit is required for STAT4 DNA binding activity and transcriptional activation of the IRF-1 gene. STAT4 is not capable of binding the other peptide sequences at tyrosine residues 678 or 679 of the IL-12R β2 subunit, and these peptide sequences are not required for the activation of STAT4. The IL-12R peptide sequence pYLPNID appears to be a specific protein binding site for STAT4. Other STATs, including STAT1, which has a closely related SH2 domain, cannot recognize the IL-12R pYLPNID peptide.3

STAT1 and STAT3 are activated by a number of cytokines (28, 29). Additionally, it has been reported that STAT1 and STAT3 are activated in response to IL-12. Jacobson et al. (13) reported that IL-12 induced weak tyrosine phosphorylation of STAT3 and DNA binding activity of STAT3 in addition to STAT4. They showed that STAT3 was part of a IL-12-induced DNA complex that included STAT4, suggesting that STAT3 and STAT4 form heterodimers. Yu et al. (14) demonstrated DNA-protein complexes containing STAT4, STAT1α, and STAT3 following treatment of NK cells with IL-12. These reports, however, did not address the issue of whether STAT1, STAT3, or STAT4 directly interacts with the IL-12 receptor. Interestingly, in our experiments, neither STAT1 nor STAT3 was activated by IL-12 through the transfected IL-12 receptor subunits under conditions in which STAT4 was phosphorylated. Perhaps STAT1 and STAT3 could be recruited to the IL-12 receptor through another factor that functions as an adaptor protein and is not present in 293 cells.

For certain SH2-containing proteins, the +1 and -3 positions in phosphotyrosine peptides have been shown to confer binding specificity (30). All the known docking sites for STAT3, for instance, have a glutamine residue at the +3 position relative to tyrosine. None of the three tyrosine peptides in the IL-12 β2 receptor contain a glutamine in the +3 position, nor do they share amino acid sequence similarities to the STAT1 docking site in the IFN-γ receptor, pYDKPH (31, 32). This is consistent with our results that STAT1 and STAT3 are not activated directly by contact with the IL-12 receptor.

The specificity of the various STAT protein SH2 domains for different tyrosine-containing peptides results in activation of specific cytokine signaling pathways and the induction of distinct gene expression patterns. We conclude that STAT4 has a unique peptide binding specificity among the STAT family and binds to the peptide sequence pYLPNID. The unique specificity of STAT4 for its tyrosine-containing peptide sequence helps explain the distinct effects of IL-12 signaling.

STAT binding peptides derived from cytokine receptors have the ability to disrupt STAT dimers and inhibit STAT tyrosine phosphorylation in vitro (19, 31, 33). It may be possible to develop therapeutically useful compounds that, analogous to the receptor-derived peptides, will block the STAT-receptor interaction, thus preventing STAT activation and subsequent signaling.

The ability to reconstitute IL-12 signaling by transfecting the β1 and β2 subunits of the IL-12 receptor will facilitate the analysis of IL-12 receptor structure and function. In addition to activation of the JAKs resulting in STAT tyrosine phosphorylation, IL-12 also stimulates STAT4 serine phosphorylation possibly by mitogen-activated protein kinase or Lck (21, 34, 35). It will be interesting to determine which regions of the receptor are required for triggering these signaling pathways.

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