Recruitment of Stat4 to the Human Interferon-α/β Receptor Requires Activated Stat2

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Stat4 activation is involved in differentiation of type 1 helper (Th1) T cells. Although Stat4 is activated by interleukin (IL)-12 in both human and murine T cells, Stat4 is activated by interferon (IFN)-α only in human, but not murine, CD4+ T cells. This species-specific difference in cytokine activation of Stat4 underlies critical differences in Th1 development in response to cytokines and is important to the interpretation of murine models of immunopathogenesis. Here, we sought to determine the mechanism of Stat4 recruitment and activation by the human IFN-α receptor. Analysis of phosphopeptide binding analysis suggests that Stat4 does not interact directly with tyrosine-phosphorylated amino acid residues within the cytoplasmic domains of either of the subunits of the IFN-α receptor complex. Expression of murine Stat4 in the Stat1-deficient U3A and the Stat2-deficient U6A cell lines shows that IFN-α-induced Stat4 phosphorylation requires the presence of activated Stat2 but not Stat1. Thus, in contrast to the direct recruitment of Stat4 by the IL-12 receptor, Stat4 activation by the human IFN-α receptor occurs through indirect recruitment by intermediates involving Stat2.

IFN-γ production by CD4+ Th1 cells underlies host resistance to many intracellular pathogens (1). The development of Th1 cells was recently shown to involve IL-12 signaling and activation of the transcription factor Stat4 in activated T cells (2–5). In the human system, type I IFNs can also promote Th1 development (6, 7), whereas in the murine system, IFN-α/β do not induce Th1 development either directly or indirectly (8). In murine CD4+ T cells, IL-12 is unique among the known cytokines in activating Stat4 in directing Th1 development. In contrast, in human CD4+ T cells, both IL-12 and IFN-α can activate Stat4 and induce IFN-γ production characteristic of Th1 cells (9, 10). Thus, a key difference between the human and mouse is that IFN-α/β activates Stat4 in human but not mouse T cells (2, 9–11), with important implications for directing Th1 development between these two species.

Given the extensive use of murine models in analyzing the roles of cytokines in pathogen resistance, it is important to understand the basis for any significant difference between murine and human cells that significantly influence cytokine actions.

The IFN-α/β receptor consists of two subunits, IFNAR1 (12, 13) and IFNAR2 (14–16), and uses the Janus kinases, Jak1 and Tyk2, with subsequent phosphorylation of Stat1, Stat2, and Stat3 (reviewed in Ref. 17). In addition, the human IFN-α receptor was recently shown to recruit and activate Stat4 (10). Although the role for Stat4 in human Th1 development has not been formally demonstrated, Stat4 plays a critical role in murine Th1 development (4, 5). It therefore seems likely that the ability of IFN-α to activate Stat4 in human but not mouse cells explains its ability to induce Th1 development in human but not mouse T cells.

In the present study, we wished to define the mechanism of Stat4 recruitment in human IFN-α signaling as a starting point to understand the basis of the species-specific difference in Th1 development. In this report, we demonstrate an important difference between Stat4 activation by the IL-12 and IFN-α signaling pathways. In IL-12 signaling, Stat4 is recruited directly to the receptor complex by the cytoplasmic domain of the IL-12R b2 subunit (18). In contrast, in IFN-α signaling, Stat4 is not recruited directly to the receptor but appears to be indirectly recruited through an intermediate involving activated Stat2.

MATERIALS AND METHODS

Cytokines, Antibodies, and Reagents—Recombinant murine and human IL-12 and human IFN-α/β were kind gifts from Dr. U. Gubler (Hoffmann-LaRoche). Recombinant murine IFN-α A was purchased from BIOSOURCE (Camarillo, CA). Polyclonal antisera specific for both murine and human Stat1, Stat2, Stat3, and Stat4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Stat4 monoclonal antibody, NB34, has been described previously (19). The peroxidase-conjugated anti-phosphotyrosine antibody RC20 was purchased from Transduction Laboratories (Lexington, KY).

Peptide Synthesis—The chicken ovalbumin peptide 323–332 (20), phosphorylated and nonphosphorylated peptides of the human IFN-α/β receptor, consists of two subunits, IFNAR1 (12, 13) and R1 (14) and R2 (21), and the human Stat4-Y694 was synthesized on an Applied Biosystems’ peptide synthesizer, model 430 (Foster City, CA). The peptide sequences for the cytoplasmic domains of the IFN-α/β receptors are as follows: IFNAR1 subunit: Tyr466, RCINYVFPFYPO3SLKPS; Tyr461, SIDEYPO3FSEQPLKNL; Tyr377, DEDHHKYPO3SSQT-SQDSGN; and Tyr321, DSGNYPO3NEDESSEKSEL, IFNAR2 subunit: Tyr326, KWIGYPO3ICLRNSSLKVL; Tyr320, MVEVYPO4INRKKVVWD; Tyr320, KWYDVPO4NYPO4IDEDSDST; Tyr327, SGGGYPO4TSHGDLTVRPL; Tyr311, PEEDYPO4SSTEGSGGRIT; and Tyr322, TSESIDDLDDQGYPO4IMR. The peptide sequences for the control peptides were: hIFN-γY-P-440, TSGGYPO4DPKPHLV; and hStat4-Y-P-696, GDKGYPO4VPSVFIP. The control peptide from the IL-12R b2 cytoplasmic tail was DLPTHDDGYPO4LP5N1ID. All peptides were purified by reverse phase C18-HPLC, and their purity and molecular weights were determined by mass spectrometry. Synthetic
peptides used in this study were determined to be >85% pure and of the correct molecular weight for each species.

Cell Culture—The DO11.10 Th1 clone, 3F6, was maintained by weekly stimulation with irradiated BALB/c spleen cells pulsed with the ovalbumin peptide as described previously (2). The human KIt225 cell line was maintained in complete RPMI 1640 supplemented with 1000 units/ml IL-2 as described (23). Human peripheral blood mononuclear cells were purified by Ficol-Hypaque (Sigma) and stimulated for 3 days in complete RPMI 1640 medium containing 5 μg/ml phytohemagglutinin antigen (PHA, Sigma) and 40 units/ml IL-2. The PHA-blasts were split on day 3 in complete RPMI containing 40 units/ml IL-2 and rested to day 7. The parental 2TGH, the Stat2-deficient U6A, and the Stat1-deficient U6A cells were maintained in complete Dulbecco’s modified Eagle’s medium as described previously (24). The Stat2-complemented U6A (U6R) was maintained in complete Dulbecco’s modified Eagle’s medium containing 400 μg/ml Genetnicin (G418, Life Technologies, Inc.).

Construction of Retroviral Plasmids—The retroviral vector used in this study is a derivative of the murine stem cell virus MSCV2.2 and contains an internal ribosomal entry site and the coding region for green fluorescence protein (GFP) downstream of a unique XhoI cloning site (described in Refs. 25 and 26). The complete coding region of mStat4 and hStat2 was cloned into the XhoI site of the GFPRV vector.

Retroviral Transduction—The Phoenix-Ampho packaging cell line was transfected with the retroviral vectors described above by calcium phosphate precipitation (26). 24 h after transfection, the medium was replaced, and the retroviral supernatant was generated by culturing the cells at 32 °C for 24 h. The 2TGH, U3A, and U6A cell lines were infected by overnight culture in retroviral culture supernatant containing 4 μg/ml polybrene (1,5-dimethyl-1,5-diazaundecamethylene polyethylene polyamphoromide, Sigma). Transduced cells were purified by FACS sorting for GFP expression. Sorted cells were expanded in culture for 1 week and were then determined to be >90% pure and to stably express the retroviral marker protein by post-sort analysis.

Immunoprecipitation and Immunoblotting—Analysis of phosphoryrosine-containing Stat proteins was performed as described previously (2). Briefly, 5 × 10⁶ cells were incubated with the indicated cytokines for 30 min at 37 °C. Whole-cell lysates were prepared, and STAT molecules were precipitated with specific polyclonal antibodies and protein G-Sepharose (Amersham Pharmacia Biotech). Immunoprecipitates were resolved by denaturing SDS-polyacrylamide gel electrophoresis and were transferred to nitrocellulose. Phosphotyrosine-containing proteins was performed as described previously (5). The membranes were then stripped and re-probed with anti-Stat polyclonal antibodies followed by detection with peroxidase-conjugated Gt-anti-Rb Ig (Jackson ImmunoResearch, West Grove, PA).

Electrophoretic Mobility Shift Analysis—Nuclear extracts were prepared from cytokine-treated cells as described previously (2). Binding reactions consisted of 3 μg of nuclear extract, 1 μg of poly(dI:dC) (Amersham Pharmacia Biotech), 10 μM Tris-Cl (pH 7.5), 50 μM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% (v/v) glycerol, and 1 × 10⁵ cpm Klenow-labeled probe in 20-μl reaction volumes. Reactions were incubated at room temperature for 30 min. Supershifting nuclear antibodies were added to some samples (2 μg) and incubated for an additional 30 min at room temperature. DNA-DNA binding complexes were resolved by nondenaturing 4.5% polyacrylamide gel electrophoresis for 2 h at 150 V followed by autoradiography. The DNA probes used in this study were as follows: M67 SIE, GTCGACATTTCCCGTAAATCGTCGA; Fc-Rl, TCGACCATTTTCTCCCTAAATCGTCGA; M67 SIE, GTCGACATTTCCCGTAAATCGTCGA; Fc-Rl, TCGACCATTTTCTCCCTAAATCGTCGA; M67 SIE, GTCGACATTTCCCGTAAATCGTCGA; Fc-Rl, TCGACCATTTTCTCCCTAAATCGTCGA.

For peptide competition studies, nuclear extracts were first denatured with the addition of 200 mM guanidinium HCl for 2 min at room temperature prior to their addition to the DNA binding reaction mixtures as described previously (27). These binding reactions included purified phosphorylated or nonphosphorylated peptides, as indicated in the text, at concentrations ranging from 20 to 100 μM.

RESULTS

Stat4 Does Not Interact with Phosphorylated Tyrosine Sequences from the IFN-α Receptor—First, to confirm the reported differences in Stat4 activation by IFN-α between mouse and human, we compared the murine Th1 clone 3F6 and human Th1 cells derived by PHA activation in vitro as described under “Materials and Methods.” For IL-12 signaling, murine or human Th1 cells were treated with recombinant murine or human IL-12, respectively. For IFN-α signaling, murine and human Th1 cells were treated with hIFN-α/D, which activates both murine and human IFN-α receptors. IL-12 induced the tyrosine phosphorylation of Stat4 in both murine and human T cells (Fig. 1A, lanes 1 and 2). IL-12 also induced tyrosine phosphorylation of Stat3 in both species, although more strongly in human compared with murine cells (Fig. 1A). In contrast, IFN-α induced tyrosine phosphorylation Stat4 and Stat3 only in human T cells but not in murine T cells (Fig. 1A, lanes 3 and 6). In addition, IFN-α induced Stat4 DNA binding.
activity in human (Fig. 1B, lower panel, lanes 6 and 9), but not mouse, Th1 cells (Fig. 1B, upper panel, lanes 6–9). The lack of Stat4 activation by IFN-α in murine T cells was not due to inactivity of the hIFN-α (A/D) at murine hIFN-α receptors, because hIFN-α (A/D) strongly induced Stat1 DNA binding in murine Th1 cells (Fig. 1B, upper panel, lanes 6–9). Furthermore, DNA-binding complexes induced by mIFN-α (A) were similar to hIFN-α (A/D) (not shown). These results confirm the report of Rogge et al. (10) that IFN-α signaling activates Stat4 in human and not murine T cells. However, that recent report did not address the mechanism underlying this difference.

Differential Stat4 activation could be caused by sequence variations in the IFN-α receptor subunits, particularly phosphotyrosine residues within the cytoplasmic domains that may act as binding sites for Stat4. Indeed, amino acid sequences of the IFNAR1 and IFNAR2 subunits are not well conserved between mouse and human (28, 29). Therefore, we examined the ability of specific phosphotyrosine-containing peptides from the IFNAR1 and IFNAR2 subunits to interact with Stat4 complexes by EMSA (Fig. 2). Tyrosine residues within the cytoplasmic domain of the IFNAR1 (Tyr466, Tyr481, Tyr527, and Tyr538) and IFNAR2 (Tyr269, Tyr296, Tyr316, Tyr318, Tyr337, Tyr411, and Tyr512) receptor subunits could serve as recruitment sites for Stat4. Phosphopeptides corresponding to amino acids surrounding each of the potential tyrosines were tested for their abilities to disrupt Stat4 DNA binding activity as a measure of sequence-specific binding (27). As controls, we used phosphotyrosine- and a nonphosphotyrosine-containing peptide consisting of the Stat4 recruitment site from the cytoplasmic domain of the human IL-12R β2 subunit (18) (IL-12Rβ2-T-P-800) as a positive control for Stat4 DNA binding activity and a peptide from the Stat1 recruitment site of the IFN-γ receptor, IFN-γR-Y-440 (positive control for Stat1 DNA binding activity) (30). Nuclear extracts were prepared from hIFN-α (A/D)-treated human Kit225 cells as a source of Stat1 and Stat4 DNA-binding complexes. First, the specificity of these complexes was confirmed by using anti-Stat1 and anti-Stat4 antibodies in supershift assays (Fig. 2, first and second panels). Next, we demonstrated that the hStat4 SH2-dependent phosphopeptide hStat4-Y-P-694 and the IFN-γ receptor phosphopeptide IFN-γR-Y-P-440 potently inhibited Stat4 and Stat1 complexes, respectively, in EMSA (Fig. 2, first and second panels). These data are consistent with the ability of these phosphopeptide sequences to interact with the SH2 domains of Stat4 and Stat1. This inhibition was specific, because the nonphosphorylated versions of these peptides did not block Stat binding in the EMSA.

Next, we asked whether phosphotyrosine peptides from either the IFNAR1 or the IFNAR2 could inhibit Stat1 or Stat4 binding activity by EMSA (Fig. 2, third and fourth panels). All of the phosphopeptides from the IFNAR1 (Tyr466, Tyr481, Tyr527, and Tyr538) and IFNAR2 (Tyr269, Tyr296, Tyr316, Tyr318, Tyr337, Tyr411, and Tyr512) were tested in this EMSA binding assay. Fig. 2 shows two representative experiments from the analysis of phosphopeptides from the IFNAR1 (third panel) and the IFNAR2 (fourth panel). Surprisingly, none of the phosphotyrosine-containing peptides from either the IFNAR1 (Tyr466, Tyr481, Tyr527, and Tyr538) or IFNAR2 (Tyr269, Tyr296, Tyr316, Tyr318, Tyr337, Tyr411, and Tyr512) subunits inhibited Stat4 complex formation. Interestingly, a phosphopeptide containing tyrosine 306 of IFNAR2 (IFN-α-R2-Y-P306) potently inhibited Stat1 binding (Fig. 2, fourth panel). This inhibition was specific, because IFN-α-R2-Y-P306 did not inhibit Stat4 binding. In summary, whereas phosphotyrosine peptide sequences expected to interact with Stat1 did selectively inhibit Stat1 binding in EMSA, none of the phosphotyrosine-containing peptide sequences from either the IFNAR1 or IFNAR2 receptor chain subunits showed significant interaction with Stat4. These results indicate that Stat4 either does not interact with, or interacts only very weakly with, any of the phosphotyrosine-containing regions in the cytoplasmic domain of IFNAR1 and IFNAR2. This finding suggests that, potentially, Stat4 may not be recruited by direct receptor interactions but rather indirectly via an intermediate adapter molecule.

Stat4 Is Recruited to the Human IFN-α Receptor in a Stat2-dependent Manner—Previous studies showed that Stat2 acts as a docking site for the recruitment of Stat1 in IFN-α receptor signaling (31). During IFN-α signaling, Stat2 is first recruited to specific residues from the cytoplasmic domain of the IFNAR1 receptor subunit (27, 32). Stat2 next becomes phosphorylated on tyrosine 690 (33), and the surrounding region (YLKHR) serves as a docking site for the SH2-dependent recruitment of
nor did Stat4 become phosphorylated in response to IFN-α (24, 31, 34). The U6A mutation prevents IFN-α mutation of Stat2 causing a defect in Stat2 protein expression derived from the parental line 2fTGH, has an uncharacterized possible because these cells do not express Stat4 (Fig. 3, mid). Direct use of U6A for analysis of Stat4 activation is not from U6A by stable transfection with a Stat2 expression plasmid. The U6R cell line is derived (Fig. 4). In parental line 2fTGH, IFN-α-induced Stat4 tyrosine phosphorylation (Fig. 4A). In contrast to the absence of Stat2 (Fig. 4A, lane 4) but not in non-Stat4-expressing cells (lane 2). This result demonstrates that murine Stat4 can be recruited and activated by the human IFN-α signaling complex, similar to human Stat4. In the U6A cells, which lack Stat2, IFN-α failed to induce Stat1 phosphorylation (Fig. 4A, lanes 5 and 6). Introduction of murine Stat4 did not affect IFN-α recruitment to Stat2, was required for Stat4 activation by hIFN-α (Fig. 4B). Because Stat1 recruitment to the IFN-α receptor is dependent on Stat2 activation, we wondered whether Stat1, in addition to Stat2, was required for Stat4 activation by hIFN-α. As shown in Fig. 4B, IFN-α-induced tyrosine phosphorylation sites of Stat4 in both the 2fTGH control and in the Stat1-deficient U3A cell line (Fig. 4B, lanes 2 and 4). In contrast, tyrosine phosphorylation of Stat1 was seen only in the parental line as expected (Fig. 4B, lane 2). Thus, these data demonstrate that the acti-

Stat1 (31, 34). Stat1 docking presumably allows for its subsequent phosphorylation by receptor-associated kinases. Based on these observations, we wondered whether Stat4 might be recruited to the receptor complex by a similar STAT-dependent mechanism.

To determine whether Stat4 activation proceeds by a similar Stat1-dependent mechanism, we used cells deficient in specific components of IFN-α signaling to determine which component may be responsible for Stat4 recruitment. The U6A cell line, derived from the parental line 2fTGH, has an uncharacterized mutation of Stat2 causing a defect in Stat2 protein expression (24, 31, 34). The U6A mutation prevents IFN-α-induced phosphorylation of Stat2 and also prevents IFN-α-induced phosphorylation of Stat1 and Stat3 (Fig. 3). The U6R cell line is derived from U6A by stable transfection with a Stat2 expression plasmid. Direct use of U6A for analysis of Stat4 activation is not possible because these cells do not express Stat4 (Fig. 3, bottom panel).

To analyze Stat4 phosphorylation in these cells, we stably expressed Stat4 in 2fTGH, U6A, and U6R cells by retrovirus (Fig. 4). In parental line 2fTGH, IFN-α-induced tyrosine phosphorylation of Stat1 with or without introduction of murine Stat4 (Fig. 4A, lanes 2 and 4). Also, IFN-α-induced tyrosine phosphorylation of Stat4 in Stat4-expressing 2fTGH cells (Fig. 4A, lane 4) but not in non-Stat4-expressing cells (lane 2). This result demonstrates that murine Stat4 can be recruited and activated by the human IFN-α signaling complex, similar to human Stat4. In the U6A cells, which lack Stat2, IFN-α failed to induce Stat1 phosphorylation (Fig. 4A, lanes 5 and 6). Introduction of murine Stat4 did not affect IFN-α activation of Stat1 nor did Stat4 become phosphorylated in response to IFN-α in the absence of Stat2 (Fig. 4A). In contrast, in the Stat2-reconstituted U6R cell line, IFN-α did induce Stat1 and Stat4 tyrosine phosphorylation (Fig. 4A, lane 10). This result suggests that Stat2 participates in the recruitment of both Stat1 and Stat4 to the IFN-α signaling complex. Moreover, Stat2-dependent tyrosine phosphorylation of Stat4 was correlated with activation and phosphorylation of Stat2 in response to IFN-α.
of Stat4 by the hIFN-α receptor requires the activation of Stat2 but not Stat1.

**DISCUSSION**

Several possible mechanisms could account for the differential species-specific activation of Stat4 by type I interferons. First, we examined the possibility that Stat4 was activated by direct receptor recruitment to specific phosphorylated tyrosine residues within the cytoplasmic domains of either the IFNAR1 or IFNAR2 subunits. Stat4 was recently shown to bind to a phosphorylated tyrosine-containing sequence, YLPSNID, at Tyr360 in the cytoplasmic domain of the IL-12R β2 (18). This sequence is conserved between the murine and human IL-12R β2 (35). However, there is no conservation of any similar sequence within the cytoplasmic domains of either the IFNAR1 or IFNAR2. Further, peptide competition analysis presented here showed that Stat4 does not significantly interact with any of the tyrosine-phosphorylated sequences derived from either the IFNAR1 or IFNAR2 subunit (Fig. 2). Thus, although the overall amino acid sequence identity of murine and human Stat2 is only recently cloned and sequenced (38). Interestingly, the sequence divergence between murine and human Stat2 suggests a potential explanation for the functional difference in Stat4 activation.

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