Interleukin-4/STAT6 Represses STAT1 and NF-κB-dependent Transcription through Distinct Mechanisms*

Yoshihiro Ohmori† and Thomas A. Hamilton

From the Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195

STAT6 mediates interleukin-4 (IL-4)-dependent positive and negative regulation of inflammatory gene expression. In the present report we examined the molecular mechanisms involved in IL-4-induced repression of reporter gene transcription driven by STAT1 and/or NF-κB. Transient expression of STAT6 in a STAT6-deficient cell line (HEK 293) conferred sensitivity to IL-4 for STAT6-dependent transcription and for repression of interferon-γ (IFNγ)/STAT1- and/or tumor necrosis factor-α (TNFα)/NF-κB-driven reporter gene expression. In cells transfected with a deletion mutant of STAT6 lacking its transactivating domain, IL-4 could not mediate either positive or negative control of reporter gene expression. Overexpression of CREB-binding protein dramatically enhanced IL-4/STAT6-stimulated transcription and overcame IL-4-mediated repression of TNFα/NF-κB-dependent but not IFNγ/STAT1-dependent transcription. A single amino acid change in the DNA-binding domain of STAT6 (H415A) selectively reduced the affinity of STAT6 for IL-4-responsive STAT sequence motifs (N4) without affecting the affinity for IFNγ-responsive (GAS) sequences (N3) and, accordingly, eliminated transcription from an IL-4-responsive promoter. Interestingly, this mutation eliminated IL-4-mediated suppression of reporter gene transcription stimulated by TNFα/NF-κB but retained nearly full capacity to suppress IFNγ/STAT1-stimulated transcription. Taken together these results demonstrate that STAT6 mediates suppression of STAT1 and NF-κB-dependent transcription by distinct mechanisms. Both processes are dependent upon the STAT6 transactivation domain and may involve sequestration of necessary but different transcriptional coactivator proteins. These two suppressive mechanisms are controlled differentially by the nature of the STAT6 DNA-binding site (i.e. N3 versus N4).

Cytokines mediate much of the cell to cell communication that is necessary for control of immune-mediated inflammation. In many circumstances, this is achieved through interplay between distinct classes of cytokines that promote either cooperative or antagonistic effects (1–3). The T helper cell type I (Th1)1-derived cytokine IFNγ promotes the development of cell-mediated immunity (3–6) and often functions cooperatively with TNF to regulate pro-inflammatory gene expression in a variety of cell types (7, 8). In contrast, the Th2-derived cytokine IL-4 promotes humoral immunity and differentiation of Th2 cells (1, 3, 5, 6, 9) in part via antagonistic effects on Th1-cytokine-driven gene expression (3, 5, 10–12).

Intracellular signaling events linked with response to cytokine-receptor interaction have been extensively studied. Signal transducers and activators of transcription (STATs) are latent cytoplasmic transcription factors that are phosphorylated at a single tyrosine residue via members of the Jak kinase family following ligand-receptor interaction, assemble in dimeric form, translocate to the nucleus, and bind to specific DNA sequence motifs (13–15). Prototypically, IFNγ activates STAT homodimers that bind to IFNγ activation sequences (GAS) composed of two inverted repeats separated by three intervening nucleotides (N3 site, TTCnnnGAA) and promote transcription of IFNγ-responsive genes (14, 16). Likewise, IL-4 leads to activation of STAT6 (17–19), which can stimulate gene transcription by binding to a sequence that resembles the GAS motif but instead contains four intervening nucleotides between the inverted repeat (N4 site, TTCCnnnGAA) (20–24). Interestingly, STAT6 can also bind N3 GAS elements but with little or no transactivation potential (23, 25, 26).

Although the negative regulatory role of IL-4 in immune inflammatory reactions has been well delineated (3, 5, 10–12, 27–34), the molecular mechanisms involved remain incompletely understood. We have previously demonstrated that IL-4 does not inhibit tyrosine phosphorylation, nuclear translocation, nor DNA binding activity of STAT1 and that STAT6 is required for inhibition of IFNγ-inducible gene transcription in mouse macrophages (26, 35). Because the cis-elements required for IFNγ-induced transcription (ISRE, GAS, and γRE) are also sufficient for IL-4-mediated inhibition and because STAT6 homodimers are capable of binding to these sites without transactivating function (26, 35, 36), it has been suggested that inhibition might involve competition between transactivating STAT1 and non-transactivating STAT6 for the N3 sites found in the promoters of sensitive genes (26). An analogous mechanism has been reported for inhibition of E-selectin gene transcription by IL-4, where STAT6 competes with NF-κB for an overlapping binding site (37). A second hypothesis is that IL-4-activated STAT6 competes with STAT1 for limiting quantities of transcriptional coactivators such as the CREB-binding protein (CBP) (35). Previous studies have shown that repression of AP-1-dependent transcription by nuclear hormone receptor or STAT1 results from competition for limiting amounts of CBP.

* This work was supported by United States Public Health Service Grant CA62220. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Center for Molecular Biology, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado, Saitam 350-0283, Japan. Fax: 0492-87-6857; E-mail: ohmori@dent.meikai.ac.jp.

The abbreviations used are: Th, T helper cells; IFN, interferon; TNF, tumor necrosis factor; STAT, signal transducers and activators of transcription; SBE, STAT-binding element; GAS, IFNγ-activated sequence; ISRE, interferon regulatory factor-1; NF-κB, nuclear factor κB; CBP, CREB-binding protein; TK, thymidine kinase; TAD, transactivation domain; EMSA, electrophoretic mobility shift assay; IL-4, interleukin-4; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; IL-1ra, IL-1 receptor antagonist.
of the coactivator CBP/p300 (38, 39). Consistent with this are several reports demonstrating that CBP can interact with both STAT1 and STAT6 and can function as coactivator for both STAT1- and STAT6-driven transcription (35, 40, 41). In contrast to such direct effects, STAT6 may also function indirectly in negative regulation of transcription. A recent report demonstrated that the transactivating domain of STAT6 is required to suppress IFNγ-stimulated transcription (42). Overexpression of CBP did not, however, overcome this suppression suggesting the following: 1) that competition for CBP was not the mechanism of suppression and 2) that STAT6 might mediate suppression indirectly through induction of a gene product that can inhibit IFNγ-induced transcription (42).

The present study was undertaken to elucidate the molecular mechanisms by which IL-4-activated STAT6 inhibits IFNγ- and TNFα-induced interferon regulatory factor-1 (IRF-1) (43) gene transcription. The results demonstrate that reconstitution of STAT6 expression in a STAT6-deficient cell line confers sensitivity to IL-4-mediated repression of IFNγ- and TNFα-induced gene transcription. Consistent with and extending prior work, deletion of the STAT6 transactivation domain (TAD) abrogates its potential to suppress both IFNγ/STAT1- and TNFα/NF-kB-mediated transcription. Overexpression of CBP can overcome suppression of NF-kB- but not STAT1-driven transcription. A STAT6 mutant protein that contains a single amino acid change in the DNA-binding domain is unable to drive transcription from an IL-4-responsive promoter (N4) and to repress NF-kB-dependent transcription but retains the capacity for inhibition of IFNγ/STAT1-driven transcription. These findings demonstrate that IL-4, via activation of STAT6, can suppress inflammatory gene transcription through distinct mechanisms that may involve sequestration of transcriptional coactivators. This activity is dependent upon the specificity and avidity of DNA binding.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—**Recombinant human IL-4, IFNγ, and TNFα were obtained from R & D Systems (Minneapolis, MN). Antibodies to STAT1α (M-20), STAT1β (M-23), STAT6 (p84/p91) (E3), STAT6 (A-2), CBP (C-20), NF-kB p50(NLS), and NF-kB p65(ΔA) were obtained from Santa Cruz Biotechnology. Anti-phospho-STAT1 (S277) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Normal rabbit IgG and purumycin were obtained from Sigma.

**Cloning of the Mouse STAT6 and Expression Constructs—**The cDNA encoding mouse full-length STAT6 (44) is isolated from mouse spleen cDNA (BALB/c male, CLONTECH, Palo Alto, CA) by polymerase chain reaction (PCR) using Pfu DNA polymerase (Stratagene, La Jolla, CA). The 5′-end of the cDNA was generated by 5′-rapid amplification of cDNA ends reaction (CLONTECH) according to the manufacturer's protocol. Each amplified PCR fragment was assembled into pcmDNA3 (Invitrogen, Carlsbad, CA). Mutations in the DNA-binding domain of STAT6 were generated by substituting an EcoR47III/BstEI-digested fragment (138 base pairs) in the DNA-binding domain of STAT6 with mutant fragments generated by PCR. For the DNA-binding domain mutant EAA, Val-411, Val-412, and Ile-413 were substituted to Glu-Ala-Ala. For DNA-binding domain mutant H415A, His-415 was substituted to Ala-Ala. For DNA-binding domain mutant H415A, Val-411, Val-412, and Ile-413 were substituted to Glu-Ala-Ala. Normal rabbit IgG and purumycin were obtained from Sigma.

**Electrophoretic Mobility Shift Assay (EMSA)—**The following oligonucleotides were used in EMSA: IL-1ra SBE1 (21), 5′-gatcGCTTCTTCTCCAGAGATCAATG-3′; IFN-γ GAS (7, 45), 5′-cagCGTCGATATCCCGAGATGCGC-3′; and IRF-1 NLS (7, 45), 5′-tgatcGCGGGAATCCCGCTG-3′. The underlined sequences represent the consensus sequences for STAT binding. EMSA was performed as described previously (7, 26). For oligonucleotide competition assay, the indicated molar excess of cold oligonucleotide was added in the binding reaction, and relative binding activity was assessed by phosphorescence detection.

**Preparation of DNA and Northern Hybridization Analysis for Luciferase Expression—**Total cellular RNA was extracted by the guanidine isothiocyanate-cesium chloride method (48). Northern hybridization analysis was described previously (49). DNA probe for luciferase was prepared from pGL2 luciferase plasmid (Promega).

**RESULTS**

**Ectopic Expression of STAT6 in STAT6-deficient Cells Confers Sensitivity for IL-4-mediated Inhibition of IFNγ- and TNFα-induced IRF-1 Gene Transcription—**We have shown previously that STAT6 is necessary for the inhibitory activity of IL-4 on IFNγ-stimulated, STAT1-dependent transcription in mouse macrophages (35). To evaluate the structural features of STAT6 that are necessary for IL-4-induced transcriptional repression, we used a model system in which the inhibitory action of IL-4 is reconstituted in a STAT6-deficient cell line by transfecting wild type STAT6. HEK 293 cells have been shown to lack detectable STAT6 but retain other IL-4 signaling components necessary for STAT6 activation (25). As previously reported, transfected wild type STAT6 translocated to the nucleus in response to IL-4 and exhibited binding activity specific for an oligonucleotide containing the SBE1 from the IL-1ra gene, a high affinity STAT6-binding motif (N4 motif, TCC-nnnnGAA) (21). No detectable DNA binding activity was observed in nuclear extracts from cells transfected with vector (pcmDNA3) alone. HEK 293 cells were cotransfected with STAT6 and one of several reporter gene constructs to assess the positive and negative function of the overexpressed protein. Strong IL-4-dependent luciferase expression was obtained from a plasmid containing the consensus site of the SBE1 site (pTK-SBE1) (21) (Fig. 1B). IL-4-activated STAT6 also has the capacity to inhibit IFNγ and/or TNFα-induced transcription of luciferase under control of a 1.3-kilobase pair fragment of the IRF-1 gene promoter (7, 45) (Fig. 1C) and of TNFα-stimulated transcription from a plasmid where the reporter gene was linked to five copies of a consensus NF-kB-binding site (pTKxB2 luc) (Fig. 1D). Luciferase activity in HEK 293 cells
cotransfected with empty vector was not altered by treatment with IL-4. IFNγ and TNFα have previously been shown to cooperate for activation of the IRF-1 promoter by inducing STAT1 and NF-κB, respectively (7, 50). Although TNFα alone produced only a small increase in promoter activity (Fig. 1C; ~2-fold induction), this was also inhibited in IL-4-treated, STAT6-transfected cells (~50% inhibition), and this effect of IL-4/STAT6 was confirmed using the κB-dependent promoter (Fig. 1D). The inhibition of transcriptional activity was not due to reduced levels of endogenous STAT1 and/or RelA (p65) protein as determined by Western blotting analysis (data not shown). Thus, inhibitory activity of IL-4 on IFNγ- and/or TNFα-induced transcriptional activity was reconstituted by transfecting with STAT6.

IL-4-induced STAT6 Does Not Inhibit Activation of STAT1 or NF-κB—Although we have previously reported that IL-4 does not inhibit DNA binding activity of IFNγ-induced STAT1 in mouse macrophages (26, 35), it has been reported to inhibit NF-κB binding activity in certain cell types (33, 51). To determine whether IL-4 acts by inhibiting the activation or activity of IFNγ-induced STAT1 and/or TNFα-induced NF-κB, we assessed the sequence-specific DNA binding activities in IFNγ- and/or TNFα-stimulated HEK 293 cell nuclear extracts. As seen in Fig. 2A, IL-4 had no effect on IFNγ- and TNFα-induced

![EMSA](#)

**FIG. 1.** STAT6 confers IL-4-mediated transcriptional repression of STAT1- and NF-κB-dependent transcriptions. **A,** IL-4-induced DNA binding activity of STAT6 in 293 cells transfected with STAT6 expression plasmid. 293 cells were transiently transfected with vector alone or an expression plasmid encoding mouse STAT6. Twenty four hours after stimulation, the transfected cells were either untreated or treated with IL-4 (10 ng/ml) for 30 min before preparation of nuclear extracts. DNA binding activity was analyzed by EMSA using radiolabeled oligonucleotides corresponding to the SBE1 motif from the IL-1ra gene (21). **B,** STAT6 can mediate IL-4-dependent transcription. 293 cells were transiently cotransfected with varying concentrations of the STAT6 expression plasmid and a luciferase reporter construct containing three copies of the IL-1ra SBE1 linked to the TK minimum promoter (pTK-SBE1luc). Twenty four hours after transfection, the cells were either unstimulated (UT) or treated with IL-4 (10 ng/ml) for 8 h and assayed for luciferase activity. The relative luciferase activity is presented as fold induction in IL-4-stimulated samples as compared with untreated samples. Each column and bar represents the mean ± S.E. from three independent experiments. **C,** IL-4-activated STAT6 represses IFNγ- and TNFα-simulated IRF-1 promoter activity. 293 cells were transiently cotransfected with either empty vector or the STAT6 expression plasmid and the IRF-1 luciferase reporter constructs (pGL-IRF-1luc). Twenty four hours after transfection, the cells were either untreated (open column) or pretreated with IL-4 (10 ng/ml; filled column) for 15 min followed by stimulation with IFNγ and/or TNFα (10 ng/ml each) for 8 h as indicated prior to measurement of luciferase activity. The relative luciferase activity is shown as fold induction compared with unstimulated samples. Each column and bar represents the mean ± S.E. from four independent experiments. Asterisks denote a statistically significant difference from cultures without IL-4 treatment (*, p < 0.01; **, p < 0.05). **D,** IL-4-induced STAT6 represses TNFα-simulated NF-κB-dependent transcription. 293 cells were transiently cotransfected with either empty vector or the STAT6 expression plasmid and a TK luciferase reporter construct containing five copies of the IP-10 κB2 site (46) linked to the TK minimum promoter (pTKκB2luc). Twenty four hours after transfection, the cells were either untreated (open column) or pretreated with IL-4 (10 ng/ml; filled column) for 15 min followed by stimulation with TNFα (10 ng/ml each) for 8 h prior to measurement of luciferase activity. The relative luciferase activity is shown as a percentage of activity obtained in cells transfected with empty vector stimulated with TNFα. Each column and bar represent the mean ± S.E. from three independent experiments.
Transcriptional Repression by STAT6

GAS binding activity. Antibody supershift assay showed that the GAS-binding complex in IFNγ- and TNFα-treated cells contained STAT1, NF-κB1(p50), and RelA(p65) (lanes 10 and 13). NF-κB has been reported previously to bind to the IRF-1 GAS motif (7, 50). IL-4 treatment induced a slowly migrating complex in the STAT6-transfected cells (lanes 6 and 8), and this complex was fully reactive with anti-STAT6 antibody (lanes 11 and 12). In similar experiments using a probe for NF-κB, IL-4 treatment of STAT6-transfected cells did not alter NF-κB binding activity (Fig. 2B). Thus, these results indicate that the STAT6-mediated inhibition of IRF-1 promoter activity is not due to interference with STAT1 and/or NF-κB DNA binding activities. The phosphorylation of STAT1 on Ser-727, which has been shown to be necessary for optimal transactivating function of STAT1(52), was also not altered in IL-4-treated cells (data not shown).

Rationale for Mechanistic Analysis of IL-4/STAT6-dependent Suppression of IFNγ/TNFα-induced Gene Expression—The available experimental evidence suggests several potential mechanisms for the STAT6-mediated repression of inflammatory gene transcription. First, STAT6 may compete with STAT1 for occupancy of the GAS motifs that are necessary for response to IFNγ. Second, STAT6 may compete with STAT1 and/or NF-κB for limiting quantities of a transcriptional coactivator such as CBP/p300. Finally, STAT6 might induce an inhibitory gene product. Each of these hypothetical scenarios would depend upon distinct features of STAT6 activity. For example, if inhibition depends on competition for occupancy of the IRF-1 GAS, DNA binding activity would be required whereas transactivating function would be unnecessary. If the inhibition is mediated by competition for limiting transcriptional coactivators, the TAD of STAT6 would be required, and repression should be rescued by overexpression of the requisite coactivator. Finally, if inhibition depends upon one or more STAT6-directed inhibitory gene products, transcriptionally active STAT6 would be required as would protein synthesis. To discriminate among these possibilities, we carried out a series of experiments using different structural forms of STAT6 in the HEK 293 reconstitution system.

Role of the STAT6 TAD in IL-4-mediated, STAT6-dependent Repression of IRF-1 Promoter Activity—First, to examine whether the STAT6 TAD is required for the transcriptional repression, a deletion mutant was generated (dTAD, Fig. 3A). The mutant dTAD translocated to the nucleus upon IL-4 stimulation (Fig. 3B, lanes 8 and 9), and both wild type and dTAD STAT6 bound comparably to the SBE1 and GAS motifs (Fig. 3C). The dTAD mutant was unable to support IL-4-dependent transcription from the SBE1-containing promoter as expected (Fig. 4A). In cells transfected with dTAD STAT6, IL-4-mediated suppression of IFNγ and/or TNFα-stimulated IRF-1 promoter activity was abolished (Fig. 4B) as was suppression of TNFα-stimulated, NF-κB-dependent luciferase expression from pTkB2luc (Fig. 4C). These results confirm a prior report (42) that the TAD of STAT6 is required for the inhibition of IFNγ/STAT1-dependent transcription and extend this observation to include response to TNFα via NF-κB. Because both wild type and dTAD forms of STAT6 exhibit comparable binding affinity for the N3 GAS site (Fig. 3C, lanes 13–20) but the dTAD form is inactive as a suppressor, simple competition between STAT1 and STAT6 for GAS motifs appears to be an unlikely mechanism for IL-4-mediated suppression.

Suppression of NF-κB but Not STAT1-dependent Transcription Is Rescued by Overexpression of CBP—The requirement for the STAT6 TAD in suppressive function suggests that STAT6 might compete with STAT1 and/or NF-κB for limiting quantities of a transcriptional coactivator. In this regard, CBP, a known coactivator for STAT1 and NF-κB, has been shown also to interact with STAT6 (35, 40, 41). To test this hypothesis, we analyzed whether overexpression of CBP could relieve STAT6-mediated repression of IFNγ- and/or TNFα-induced transcriptional activities. Overexpression of CBP potentiated the ability of IL-4 to drive transcription from the IL-4-responsive promoter (Fig. 5A). Basal as well as TNFα- and IFNγ-induced IRF promoter-driven reporter activities were all enhanced in cells simultaneously transfected with the CBP expression vector (Fig. 5B). STAT6-mediated inhibition of the IFNγ- and IFNγ/TNFα-induced promoter activity was not relieved. Increased concentrations of CBP expression plasmid (up to 4 μg) showed no further effect on the repression of the IRF-1 promoter activity (data not shown). However, as shown previously TNFα-induced IRF-1 promoter activity was inhibited by IL-4 in STAT6-transfected cells (Fig. 5B, lane 7). The inhibition of TNFα-induced transcription from the IRF-1 promoter was restored by overexpression of CBP (lanes 7 versus lanes 15). This finding was confirmed using the NF-κB-dependent reporter system (Fig. 5C). IL-4/STAT6-mediated inhibition of TNFα-stimulated, NF-κB-dependent transcription was restored by CBP overexpression, suggesting that STAT6 and NF-κB may compete for limiting quantity of CBP. The altered promoter activities were not due to changes in the expression of endogenous RelA and STAT1 proteins (Fig. 5D). These results suggest that STAT6 inhibits NF-κB-dependent transcription by sequestering CBP, whereas STAT1-dependent transcription is not rate-limited by CBP in this setting.
pGL-IRF-1luc and empty vector and then stimulated with IFN-γ. The relative luciferase activity is shown as a percentage of activity obtained in cells transfected with pGL-IRF-1luc and empty vector.

Numbers denote amino acid position. A, IL-4-dependent nuclear localization of mutant STAT6 gene products. 293 cells were transfected with empty vector or expression plasmids encoding the wild type or mutant STAT6 proteins as indicated. Twenty four hours after transfection, the cells were either untreated or treated with IL-4 (10 ng/ml) for 30 min before the preparation of cytoplasmic and nuclear extracts. Ten μg of each extract were separated by SDS-PAGE on 7.5% gels and subjected to Western blot analysis for STAT6. Similar results were obtained from three separate experiments.

DNA binding affinity was analyzed by EMSA using radiolabeled oligonucleotides corresponding to the IL-1ra SBE1 (N4) motif in the presence of increasing amount (5-, 25-, and 125-fold excess) of unlabeled oligonucleotide competitors. The amount of IL-4-induced STAT6 binding was quantified by PhosphorImager analysis following EMSA. Complex formation in the absence of competitor was taken as 100%.

Twenty four hours after transfection, the cells were either untreated or treated with IL-4 (10 ng/ml) for 8 h prior to measurement of luciferase activity. The relative luciferase activity is shown as a percentage of the activity in cells stimulated with IL-4.

Each column and bar represents the mean ± S.E. from the three independent experiments.

STAT6 N4-specific DNA Binding Activity Is Differentially Required for IL-4-mediated Suppressive Function—To evaluate the third hypothesis, we examined the requirement for STAT6 DNA binding activity in IL-4-mediated suppression. To accomplish this we took advantage of the properties of single amino acid mutation in the DNA-binding domain of STAT6 (H415A) (Fig. 3A). This STAT6 mutant was fully competent for IL-4-induced tyrosine phosphorylation (data not shown) and nuclear translocation (Fig. 3B, lanes 12, 17, and 18). The DNA binding affinity of H415A for the SBE1 (N4) motif was significantly reduced (Fig. 3C, lanes 9–12), although the DNA-binding affinity for the GAS (N3) motif from the IRF-1 promoter was almost comparable to that of dTAD and wild type STAT6 (lanes 21–24). The ability of H415A to mediate IL-4-stimulated transcription from pTK-SBE1luc was, as expected, severely compromised (Fig. 6A). This mutant, however, still exhibited the capacity to mediate IL-4-dependent inhibition of IFN-γ and IFN-γ/TNFα-induced IRF-1 promoter activity (Fig. 6B). Inter-
FIG. 5. Overexpression of CBP rescues IL-4-mediated repression of NF-κB but not STAT1-dependent transcription. A, overexpression of CBP potentiates IL-4-induced STAT6-dependent transcription. 293 cells were transiently cotransfected with either empty vector or the STAT6 expression plasmid along with the pTK-SBE1 luciferase reporter construct in the presence or absence of CBP expression plasmid (2 μg). Twenty four hours after transfection, the cells were stimulated as described in the legend to Fig. 4. Each column and bar represents the mean ± S.E. from five independent experiments. Asterisks denote a statistically significant difference from cultures without IL-4 treatment (*, p < 0.01; **, p < 0.05). B, overexpression of CBP rescues STAT6-mediated repression of NF-κB-dependent transcription. 293 cells were transiently cotransfected with either empty vector or the STAT6 expression plasmid and pTK-SBE1 in the presence or absence of CBP (2 μg) expression plasmid. Twenty four hours after transfection, the cells were stimulated as described in the legend to Fig. 4. Each column and bar represents the mean ± S.E. from five independent experiments. C, overexpression of CBP rescues STAT6-mediated repression of NF-κB-dependent transcription. 293 cells were transiently cotransfected with either empty vector or the STAT6 expression plasmid and pTK-SBE1luc. Twenty four hours after transfection, the cells were stimulated as described in the legend to Fig. 4. Each column and bar represents the mean ± S.E. from three independent experiments. D, overexpression of CBP does not increase endogenous STAT1 or RelA protein levels. 293 cells were transfected with expression plasmids as described above. Twenty four hours after transfection, total cellular lysates were prepared. Ten μg of total cellular lysates were separated by SDS-PAGE on 7.5% gels and subjected to Western blot analysis for CBP, RelA, and STAT1. Similar results were obtained from two separate experiments.

FIG. 6. DNA-binding domain mutant STAT6 H415A mediates repression of IFN-γ-stimulated IRF-1 transcription but not NF-κB-dependent transcription. A, transactivating function of STAT6 H415A on pTK-SBE1luc. 293 cells were transiently cotransfected with STAT6 expression plasmid and pTK-SBE1luc. Twenty four hours after transfection, the cells were stimulated as described in the legend to Fig. 4. Each column and bar represents the mean ± S.E. from three independent experiments. B, effect of overexpression of CBP on STAT6-mediated repression of IRF-1 promoter activity. 293 cells were transiently cotransfected with either empty vector or the STAT6 expression plasmid and pGL-IRF-1. Twenty four hours after transfection, the cells were stimulated as described in the legend to Fig. 4. Each column and bar represents the mean ± S.E. from three independent experiments. Asterisks denote a statistically significant difference from cultures without IL-4 treatment (*, p < 0.01; **, p < 0.05). C, effect of STAT6 H415A on transcriptional repression of TNFα-NF-κB-dependent transcription. 293 cells were transiently cotransfected with STAT6 expression plasmid and pTKbB2luc reporter construct. Twenty four hours after transfection, the cells were stimulated as described in the legend to Fig. 4. Each column and bar represents the mean ± S.E. from three independent experiments.

DISCUSSION

IL-4 antagonizes IFN-γ-induced gene expression, at least in part, through modulation of transcription (26, 30, 32, 35, 36). We have previously reported the requisite role of IL-4-activated STAT6 in the negative regulation of IFN-γ-stimulated, STAT1-dependent transcription in macrophages (35). These studies also demonstrated that the cis-acting sequences necessary for both IFN-γ-stimulated transcription and IL-4-mediated suppression are identical (26, 35, 36). Surprisingly, IL-4 did not inhibit any aspect of IFN-γ-driven STAT1 activation in these experiments (26, 35). These findings suggested three non-mutually exclusive hypotheses. First, STAT6, which can bind to IFN-γ-responsive GAS motifs without transactivation, could compete with

estingly, the inhibition of TNFα-stimulated transcription from the IRF-1 promoter and from pTKbB2luc was abolished in H415A-transfected cells (Fig. 6B, lane 7 versus 11 and Fig. 6C, lane 4 versus 6).

STAT6-mediated Repression of IRF-1 Transcription Is Independent of Protein Synthesis—Since H415A still exhibits substantial DNA binding activity for the IRF-1 GAS (N3), and the transactivating domain of STAT6 is required for transcriptional repression, it is possible that a STAT6-inducible inhibitory gene product is generated. Thus we tested whether protein synthesis is required for the STAT6-mediated inhibition by evaluating levels of luciferase reporter gene mRNA in the presence or absence of the protein synthesis inhibitor puromycin. As shown in Fig. 7, stimulation with IFN-γ and/or TNFα induced expression of luciferase mRNA in cells transfected with IRF-1 promoter-reporter (Fig. 7, A and B) or pTKbB2luc (Fig. 7, C and D). The expression of luciferase mRNA in either promoter context was inhibited by IL-4 when the cells were transfected with STAT6, and this inhibition was maintained in the presence of puromycin.

DISCUSSION

IL-4 antagonizes IFN-γ-induced gene expression, at least in part, through modulation of transcription (26, 30, 32, 35, 36). We have previously reported the requisite role of IL-4-activated STAT6 in the negative regulation of IFN-γ-stimulated, STAT1-dependent transcription in macrophages (35). These studies also demonstrated that the cis-acting sequences necessary for both IFN-γ-stimulated transcription and IL-4-mediated suppression are identical (26, 35, 36). Surprisingly, IL-4 did not inhibit any aspect of IFN-γ-driven STAT1 activation in these experiments (26, 35). These findings suggested three non-mutually exclusive hypotheses. First, STAT6, which can bind to IFN-γ-responsive GAS motifs without transactivation, could compete with
STAT1 for site occupancy thereby preventing transcription. Second, STAT6 could compete with STAT1 for limiting quantities of a transcriptional coactivator such as CBP or p300. Finally, STAT6, through binding to IL-4-responsive sequence elements, might induce an inhibitory gene product. In the present report we have evaluated these three scenarios by examining the ability of wild type and mutated forms of STAT6 to reconstitute IL-4-induced transcription and IL-4-dependent suppression of IFN-γ/STAT1- and/or TNFα/NF-κB-reporter gene expression in a STAT6-deficient cell line. The results of these studies support the following conclusions. 1) STAT6 mediates suppression of IFN-γ/STAT1- and TNFα/NF-κB-dependent transcription via distinct molecular mechanisms. Both mechanisms of suppression depend upon the transactivation domain of STAT6 suggesting the importance of interaction with transcriptional coactivators or corepressors. 2) Suppression of NF-κB-dependent transcription is restored by overexpression of the coactivator CBP supporting the possibility that this mechanism involves sequestration of this important protein. In contrast IL-4-mediated suppression of IFN-γ/STAT1-stimulated transcription was insensitive to overexpression of CBP. 3) A mutation in the DNA-binding domain of STAT6 selectively influenced STAT6 DNA binding preference leading to a blockade in IL-4-driven transcription. The ability of STAT6 to suppress NF-κB-mediated transcription was also lost, but IFN-γ/STAT1-stimulated transcription remained STAT6-sensitive. Thus different suppressive mechanisms appear to be influenced by the nature of the DNA-binding site (i.e., N3 versus N4). Furthermore, these findings along with the failure of puromycin to block STAT6-dependent suppressive activities suggest strongly that suppression is not a result of IL-4-stimulated corepressor gene expression.

Our initial studies of the inhibitory effect of IL-4 on IFN-γ-inducible gene expression led to the hypothesis that STAT6 suppresses STAT1-dependent transcription by competing with STAT1 for occupancy of a common DNA-binding site (26). A recent report demonstrated a requirement for the transactivation domain of STAT6 in suppressing IFN-γ-driven transcription from the IRF-1 promoter even though the wild type and TAD deletion forms of STAT6 exhibited equivalent affinity for the N3 GAS motif (42). This indicates that the inhibition is not due to blocking of STAT1 occupancy of the IRF-1 GAS by transcriptionally inactive STAT6. The behavior of the TAD deletion is confirmed here and extended to include suppression of TNFα/NF-κB-driven transcription as well.

The anti-inflammatory activity of IL-4 has also been shown to target NF-κB-dependent gene expression (33, 37, 51, 53). In several cases this has been reported to involve either the inhibition of NF-κB-specific DNA binding activity or competition between STAT6 and NF-κB for a shared or overlapping binding site. In addition, IL-4 has been shown to inhibit TNF-dependent NF-κB activation by down-regulation of TNF receptors (54). We did not, however, observe inhibition of NF-κB activation or DNA binding activity by IL-4. Furthermore, since our NF-κB-responsive promoter contains no sites for STAT6 binding, the idea of competition for site occupancy can be essentially ruled out. The present results suggest a novel alternative route for the antagonistic effect of IL-4 on NF-κB-dependent transcription: IL-4-activated STAT6 and NF-κB may compete for a limited supply of CBP thus providing a mechanistic explanation for the ability of IL-4 to suppress NF-κB-dependent transcription without affecting its DNA binding activity. The concept of competition between activated transcription factors for interaction with limited availability transcriptional coactivators as a mechanism of selective gene regulation has received appreciable attention in the last several years (38, 39, 55). Previous studies have shown that STAT1-dependent transcription requires both CBP and p/CIP, a member of the p160 coactivator family (56). Additionally, CBP and p/CIP have been shown to associate with other cofactors (57, 58). Thus, it is conceivable that STAT6 may sequester CBP and/or other members of this coactivator family. In this regard, STAT6 TAD has been shown to interact with CBP, and CBP functions as a coactivator for STAT6, STAT1, and NF-κB (39–41, 59, 60). The ability to overcome STAT6-mediated suppression of TNFα/NF-κB by overexpressing CBP provides compelling support for this hypothesis. A recent report has shown that prolactin-activated STAT5b inhibits NF-κB-dependent transcription by competing for CBP (61). It is intriguing, however, that, in agreement with the recent study of Goenka et al. (42), overexpression of CBP...
does not prevent the suppression of IFNγ/STAT1-driven gene expression. This suggests, as mentioned above, that STAT6 TAD might interact with other coactivator or corepressor proteins providing the potential for distinct functional outcomes.

Evaluation of the importance of DNA binding activity in IL-4/STAT6-dependent inhibitory function can also provide insight into mechanism. The study by Goenka et al. (42) examined several different DNA-binding domain mutations and reported that suppressive activity was lost in each. Because both the TAD and DNA binding activity appeared to be required for suppression, these authors proposed the hypothesis that IL-4 suppresses gene expression via the STAT6-mediated induction of a new gene product with repressor activity. In this prior study, however, the functional characteristics of the DNA-binding mutants were not fully evaluated. Indeed, one of the mutations used was in the SH2 domain of STAT6 that had been shown previously to compromise completely the ability of the resulting protein to respond to IL-4 stimulation (25). Our own experience with a 3-amino acid mutation in the DNA-binding domain at residues 411–413 showed the resulting protein product to be inactive as it was neither phosphorylated on tyrosine nor translocated to the nucleus following treatment of the cells with IL-4 (data not shown). Thus the loss of suppressive activity in this mutation was relatively uninformative regarding the DNA-binding activity of STAT6 as a contributing feature of suppressive function.

The mutation in residue 415 from His to Ala was, however, particularly informative. While the protein was responsive to IL-4 and translocated to the nucleus following stimulation, its DNA-binding activity was selectively altered such that affinity for the N4 IL-4-responsive motif was markedly reduced without loss of apparent binding affinity for the IFNγ-responsive, N3 sites (Fig. 3). This mutant was predictably unable to support IL-4-stimulated gene transcription but retained nearly full suppressive activity on IFNγ/STAT1-dependent reporters, suggesting strongly that induction of a repressor gene is not involved. Whereas the possibility of a STAT6-inducible inhibitory gene product cannot be completely ruled out, several additional lines of evidence further support that STAT6-mediated inhibition of the STAT1-driven transcription is not dependent on a newly expressed gene product. First, the inhibition occurs in the presence of the protein synthesis inhibitor puromycin (Fig. 7). We did not use cycloheximide since this inhibitor has been shown to induce multiple pharmacological effects unrelated to inhibition of protein synthesis (62, 63). Second, time course studies have shown that IL-4-induced, STAT6-mediated inhibition occurs within 2 h after stimulation in HEK 293 cells as well as macrophages (data not shown) (35). Finally, the nature and mechanism for the inhibitory gene product has not been clarified. As we have demonstrated, IL-4 does not affect tyrosine phosphorylation, nuclear translocation, or DNA-binding activity of IFNγ-induced STAT1 (26). Furthermore, IL-4 did not affect Ser-727 phosphorylation of STAT1, which is required for maximal trans-acting function of STAT1 (52) (data not shown).

Although STAT6 H415A supported IL-4-mediated suppression of IFNγ/STAT1-induced reporter gene expression, the ability to suppress TNFα/NF-κB-dependent transcription was abolished. This could occur through one of several different mechanisms. For example it is possible that mutations within the DNA-binding domain might induce a conformational change in STAT6 structure that selectively disrupts coactivator interaction necessary for suppression of NF-κB-dependent transcription. Alternatively, N4-specific but not N3-specific DNA binding activity of STAT6 may be required for repression of NF-κB-dependent transcription. This is suggested by correlation between the selective suppressive and DNA binding activities of the H415A mutant STAT6 protein. This may be particularly important if the sequestration of cofactor occurs only when STAT6 is bound to DNA in the nucleus; thus binding of STAT6 to N4 but not N3 sites may allow recruitment of a coactivator such as CBP. An analogous circumstance has been reported in which a cofactor is sequestered by a DNA-bound acidic activator (64, 65). In this scenario, the antagonistic or cooperative effect of STAT6 would depend upon the promoter context (21, 66, 67). Because the H415A mutant retains suppressive activity toward IFNγ/STAT1- but not TNFα/NF-κB-mediated responses, N3 binding specificity may be required in the former and N4 in the latter. Since we do not have a STAT6 mutant form which exhibits selective loss of N3-specific binding, the physiological significance of the N3 binding activity of STAT6 remains unknown. Whereas the H415A mutant has lost IL-4-mediated transcriptional activity, this does not appear to be required for suppression of TNFα/NF-κB reporter gene expression since this is independent of protein synthesis.

In summary, the requirement for the STAT6 TAD in suppression of both STAT1- and NF-κB-driven transcription suggests one of two broad hypotheses. First, STAT6 may be required to drive transcription of new genes encoding repressor proteins. Alternatively, STAT6 may be required to interact with other proteins via the TAD. Since new protein synthesis is not required for suppression of either STAT1- or NF-κB-dependent transcription and IL-4-driven, N4-dependent transcription is not required for suppression of IFNγ/STAT1-mediated reporter gene expression, it is highly unlikely that IL-4 induces the expression of one or more repressor proteins. The ability of CBP to rescue IL-4-mediated suppression of NF-κB-dependent transcription argues in favor of the hypothesis that STAT6-mediated suppression involves sequestration of this important protein. We have not identified the nature of interacting proteins that might be involved in suppression of STAT1-dependent transcription. Although it remains reasonable to suggest that this process will also involve sequestration of a coactivator, it is also possible that it will involve interaction of STAT6 with a corepressor protein that may depend upon interaction with the N3 motifs found in IL-4 suppressible, IFNγ-inducible genes.

REFERENCES
Interleukin-4/STAT6 Represses STAT1 and NF-κB-dependent Transcription through Distinct Mechanisms

Yoshihiro Ohmori and Thomas A. Hamilton

J. Biol. Chem. 2000, 275:38095-38103. doi: 10.1074/jbc.M006227200 originally published online September 11, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006227200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 66 references, 39 of which can be accessed free at http://www.jbc.org/content/275/48/38095.full.html#ref-list-1