STAT6 Is Required for the Anti-inflammatory Activity of Interleukin-4 in Mouse Peritoneal Macrophages

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Yoshihiro Ohmori‡ and Thomas A. Hamilton

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

Interleukin-4 (IL-4) is an anti-inflammatory cytokine which inhibits many inducible macrophage functions. The present study demonstrates that the ability of IL-4 to inhibit interferon γ (IFNγ)-dependent gene transcription is dependent upon STAT6. IL-4 suppressed IFNγ-induced expression of the MIG (monokine induced by IFNγ) gene, a C-X-C chemokine, in mouse macrophages. IFNγ-induced expression of MIG mRNA was abolished in peritoneal macrophages from Stat1−/− mice, and the suppression of MIG mRNA by IL-4 was abolished in macrophages from Stat6−/− mice. Transient transfection assays using a reporter gene containing the MIG gene promoter or the IFNγ-responsive element (γRE) from the MIG gene revealed that the IFNγ-dependent transcription was suppressed by IL-4, although IL-4 alone had no transactivating function. IFNγ and IL-4 activated STAT1 and STAT6, respectively, and both proteins were able to bind the γRE motif. Furthermore, STAT6 was associated with the co-activator CREB-binding protein in RAW264.7 cells. These observations indicate that STAT6 is necessary for the IL-4-mediated suppression of STAT1-dependent transcription and suggest that STAT6 may directly suppress the Stat1-dependent transcription by competing with STAT1 for occupancy of the γRE motif and/or by competing with limiting quantities of the transcriptional coactivator.

Regulation of inflammatory and immune responses involves intercellular communication through a network of secreted cytokines (1). Cytokines derived from Th helper (Th)1 cells (prototypically IFNγ) promote the development of cellular immunity, while Th2-derived cytokines such as IL-4 promote humoral immunity and antagonize Th1-dependent activities (2–5). At least a portion of the anti-inflammatory function of IL-4 is targeted to mononuclear phagocytes where expression of inducible genes encoding cytokines (e.g. TNFα, IL-1α, IL-1β, various chemokines) (6–16) and cell surface molecules (FcRγ and ICAM-1) (17, 18) is suppressed, while expression of other anti-inflammatory products such as the IL-1 receptor antagonist is amplified (9–11, 19, 20).

The molecular mechanisms mediating cytokine-induced gene transcription have been extensively characterized in recent studies on the Janus kinase (JAK) family of protein tyrosine kinases and signal transducers and activators of transcription (STATs) (21–25). IFNγ has been shown to induce phosphorylation of STAT1, which, in homodimeric form, binds to the IFNγ activation sequence (GAS) found in many IFNγ-inducible genes (26–29). In similar fashion, IL-4 stimulates tyrosine phosphorylation of STAT6 (30–32), which can bind to GAS motifs as well as IL-4-responsive STAT binding elements (SBEs) found in IL-4-inducible genes (20, 33–37). Interestingly, STAT6 exhibits transactivating function only in the context of a subset of SBE sequence motifs (20, 34, 37–40).

IL-4 and IFNγ exhibit antagonistic effects on macrophage gene expression (14–18). We have previously observed that IFNγ-mediated induction and IL-4-mediated suppression utilize the same regulatory sequences (the ISRE in the IP-10 gene and the GAS motif in the IRF-1 gene) (16, 40). Furthermore, IL-4 does not inhibit the activation of STAT1 by IFNγ and IL-4-induced STAT6 is able to bind the IFNγ-responsive site without transactivation (40). Recent studies have shown that STAT1-dependent transcription requires transcriptional coactivators p300 and CREB-binding protein (CBP), which may be present in limiting amounts (41, 42). On the basis of these lines of evidence, we postulated that the inhibitory effect of IL-4 may result, at least in part, from the direct action of IL-4-induced STAT6 on IFNγ-induced STAT1-dependent transcription. The present study was undertaken to determine if IL-4-induced STAT6 is required for the suppressive action of IL-4 on IFNγ-induced gene transcription and to extend our analysis to include the IFNγ-responsive element (γRE), which controls expression of the MIG chemokine gene (43, 44), a third form of IFNγ-sensitive nucleotide regulatory motif. The results demonstrate that IL-4-dependent suppression of IFNγ-induced MIG gene expression is abolished in peritoneal macrophages from mice in which the STAT6 gene has been deleted (45). Both the MIG gene promoter and the γRE from the MIG promoter are sensitive to the stimulatory action of IFNγ and the inhibitory effects of IL-4. Furthermore, we find that STAT6 as well as STAT1 can interact with the coactivator CBP. These results suggest that these two factors may compete for coactivator and/or occupancy of the γRE site with opposite functional consequences.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Recombinant mouse IFNγ and IL-4 were obtained from Life Technologies, Inc. and R & D Systems, Inc. (Minneapolis, MN), respectively. Rabbit polyclonal antibodies to mouse STAT1α (M-23), STAT6 (M-20), CBP (C-20), NFκB1(NLS), and RelA (A) were obtained from Santa Cruz Biotechnology (Hercules, CA). Normal rabbit IgG was obtained from Sigma.

**Mice**—Homozygous STAT1 mutant mice (Stat1−/−) in which the
Stat1 gene has been deleted by homologous recombination were kindly provided by Dr. Robert D. Schreiber (Washington University School of Medicine, St. Louis, MO) (46). Stat6+/— mice were kindly provided by Dr. James N. Ihle (St. Jude Children’s Research Hospital, Memphis, TN) (45). Specific pathogen-free C57Bl/6 mice 9–12 weeks of age were purchased from the Charles River Institute (Stoughton, MA).

**Cell Culture**—Thioglycolate-elicited peritoneal macrophages were prepared as described previously (47) and cultured in RPMI 1640 containing 1-glutamine, penicillin, streptomycin, and 5% fetal bovine serum. The RAW264.7 or ANA-1 (48) mouse macrophage-like cell lines were obtained and cultured in Dulbecco’s modified Eagle’s medium containing 1-glutamine, penicillin, streptomycin, and 10% fetal bovine serum.

**Preparation of RNA and Northern Hybridization Analysis**—Total cellular RNA was extracted by the guanidine isothiocyanate-cesium chloride method (49). Northern hybridization analysis and cDNA probes for mouse MIG and rat GAPDH were described previously (47, 50). Northern blots were also quantified using phosphorescence detection. The relative magnitude of expression was determined for each gene and normalized to values for GAPDH expression in the same experiment.

**Nuclear Run-on Assay**—Cultures of 6 × 10^6 RAW264.7 cells in 150-mm Petri dishes were treated as indicated in the text for 90 min, and nuclei were isolated as described previously (51). Transcription initiated in intact cells was allowed to complete in the presence of [α-32P]UTP (3000 Ci/mmol; Amersham, Arlington Heights, IL). The RNA was isolated and hybridized to slot-blotted plasmid containing specific cDNA insertion (7 ng of DNA/slot) as described elsewhere (52).

**Reporter Plasmids**—A sequence encoding the 5′-flanking promoter/enhancer region of the MIG gene was cloned by PCR using Ultima DNA polymerase (Perkin-Elmer) from a mouse genomic DNA using a set of primers corresponding to the mouse MIG genomic sequence (53) (upstream primer; 5′-CTCGGATGTCGCACTGCCCC-3′, downstream primer; 5′-CTGTTTGGATGGAAGTCCCG-3′). The amplified PCR fragment was subcloned into luciferase reporter construct (pGL2-B, Promega) and the nucleotide sequence confirmed. The sequences corresponded to nucleotide residues −328 through +34 (53). The 5′-half-site of the γRE motif at position −198 to −189 of the MIG promoter was mutated by using two-step PCR amplification (54). The mutant sequence utilized was TCCCggAggATccACT. Lowercase letters represent mutated residues. The mutant sequence, and the underlined sequence is the 5′-half-site of the γRE motif at position −198 to −189. The amplified PCR fragment was subcloned into luciferase reporter construct (pGL2-B, Promega) and the nucleotide sequence confirmed. The sequences corresponded to nucleotide residues −328 through +34 (53). The 5′-half-site of the γRE motif at position −198 to −189 of the MIG promoter was mutated by using two-step PCR amplification (54). The mutant sequence utilized was TCCCggAggATccACT. Lowercase letters represent mutated sequence.

**Transient Transfection—**Luciferase reporter gene plasmids were transiently transfected into RAW264.7 cells by the DEAE-dextran method as described previously (20, 56). Briefly, 15 μg of reporter plasmid DNA and 3 μg of reference plasmid (pRL-TK, Promega) were transfected in the presence of DEAE-dextran (300 μg/ml). To standardize transfection efficiencies, the transfected cells were harvested, pooled, and seeded in four 60-mm culture dishes. After 24 h, the cells were stimulated with IFNγ and/or IL-4 for 8 h prior to analysis of luciferase activity. Firefly and Renilla luciferase activities were assayed using reagents provided by Promega according to the manufacturer’s instructions. Twenty μg of extract protein were utilized in each assay. Immunoprecipitation and Western Blot Analysis—Cultures of 6 × 10^5 RAW264.7 cells (10^7) were treated as indicated in the text. After stimulation, RAW264.7 cells were washed with ice-cold phosphate-buffered saline, harvested, and resuspended in Lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, and 10 mM sodium orthovanadate, 100 mM leupeptin, antipain, aprotinin, and pepstatin) for 10 min on ice. Before pre-clearing the lysate with normal rabbit IgG (Sigma) and Protein G-Sepharose (Amersham Pharmacia Biotech), whole cell lysate (500 μg) of protein were incubated with anti-GBP (1 μg) or normal rabbit IgG and protein G-Sepharose (50% slurry) for 16 h at 4 °C. The immunoprecipitates were washed six times with 1 ml of lysis buffer, eluted with SDS-PAGE sample buffer, resolved on 7.5% SDS-PAGE, and analyzed by densitometry of western blots as described previously (40).

**Electrophoretic Mobility Shift Assay (EMSA)—**Nuclear extracts were prepared as described previously by using a modified method of Dignam et al. (20, 40, 57). The following oligonucleotides were used in EMSA: γRE: 5′-GATCCCCCATATAAACCTCCCCCGTTTATGTGAAATGGATG-3′; mutant: 5′-GATCCCCCATATAAACCTCCCCCGATGTAATGTGAAATGGATG-3′, 5′-half-site: 5′-ATCCCGTACTTAAATCACCTCCC-3′, 3′-half-site: 5′-GATCCCCCATATAAACCTCCC-3′.

For binding reactions, nuclear extracts (5 μg of protein) were incubated in 12.5 μl of total volume containing 20 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 1 mM diithiothreitol, 5% glycerol, 200 μg/ml bovine serum albumin, and 1.25 μg of poly(dI-dC) for 15 min at room temperature. 32P-Labeled oligonucleotide (0.5 ng, 5 × 10^6 cpm) was added to the reaction mixture and incubated for 15 min at room temperature. The reaction products were analyzed by electrophoresis in a 5% polyacrylamide gel with 0.25× TBE buffer (22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA). In some experiments, rabbit antibody to NFκB1 (p50), RelA (p65); STAT1, and STAT6 were added prior to electrophoresis. The dried gels were analyzed by autoradiography and by phosphorimaging detection.

**RESULTS**

**IL-4 Suppresses IFNγ-induced Expression of MIG mRNA—**IL-4 is known to suppress IFNγ-induced gene expression in mononuclear phagocytes (14–18). The gene encodes a C-C Chemokine known to be transcriptionally regulated by IFNγ (43, 44, 53). To determine whether IL-4 can suppress MIG expression, thioglycollate-elicited peritoneal macrophages were pretreated with IL-4 (10 ng/ml) for 15 min, followed by stimulation with different doses of IFNγ for 4 h prior to preparation of total RNA and Northern hybridization analysis. IFNγ induced and IL-4 suppressed levels of MIG mRNA (Fig. 1A). IL-4 did not alter the kinetics of IFNγ-induced MIG mRNA expression but reduced the steady state mRNA levels over the entire time course (Fig. 1B). Quantitatively and qualitatively similar effects of IFNγ and IL-4 on MIG mRNA were seen in the macrophage-like cell lines RAW264.7 (Fig. 1C) and ANA-1 (Fig. 1D). The suppression by IL-4 was mediated predominantly by inhibiting transcription as assessed by nuclear run-on assay in RAW264.7 cells (Fig. 2).

The Effects of IFNγ and IL-4 on MIG Expression Require STAT1 and STAT6, Respectively—STAT1 is believed to be important for most if not all IFNγ-induced gene expression (46, 58), while STAT6 is a primary signaling molecule linked with response to IL-4 (45, 59, 60). To examine the role of IFNγ-induced STAT1 and IL-4-activated STAT6 in control of MIG expression, macrophages from Stat1−/− and Stat6−/− mice were used to measure each response (45, 46) (Fig. 3). As expected, IFNγ-induced MIG mRNA expression was completely abolished in peritoneal macrophages from Stat1−/− mice (Fig. 3A) but remained intact in macrophages from Stat6−/− mice (Fig. 3B). Whereas IFNγ-induced MIG mRNA was inhibited by IL-4 in wild type macrophages, the suppressive effect of IL-4 was entirely lost in macrophages from the Stat6−/− mice (Fig. 3, B and C).

The γRE Motif in the MIG Promoter Is Sensitive to Both IFNγ and IL-4—IFNγ-induced transcription of the MIG gene has been shown to depend upon the γRE motif located at positions −198 to −171 in the MIG promoter (44). To determine whether the suppression of IFNγ-induced MIG gene expression by IL-4 is dependent upon the γRE motif, 328 nt of the 5′-flanking sequence of the MIG gene were linked to the luciferase reporter gene and transiently transfected into RAW264.7 cells. Twenty-four hours after transfection, the cultures were stimulated with IFNγ alone or in the presence of IL-4 for 8 h prior to analysis of luciferase activity. Although IL-4 alone had no effect on luciferase activity, reporter gene expression was markedly elevated in cells stimulated with IFNγ (Fig. 4A). IL-4 added along with IFNγ suppressed the induced luciferase activity by approximately 50%. IL-4 did not modulate the luciferase activity from the cells transfected with cytomegalovirus promoter-driven luciferase construct (data not shown).

To determine if the γRE motif of the MIG gene was the target site for IL-4, one copy of this motif was placed in front of the TK promoter (TK-81) linked to the luciferase reporter gene and transiently transfected in RAW264.7 cells (Fig. 4B). As was observed for the natural MIG promoter, IFNγ stimulated luciferase activity in RAW264.7 cells transfected with TK-81 linked to the luciferase reporter gene.
IL-4 suppresses IFNγ-induced MIG mRNA expression in macrophages. A, thioglycollate broth-elicited peritoneal macrophages were pretreated with IL-4 (10 ng/ml) for 15 min, followed by stimulation with different concentrations of IFNγ for 4 h prior to preparation of total RNA and analysis of specific mRNA levels by Northern hybridization as described under “Experimental Procedures.” Five µg of total RNA were analyzed in each lane. Similar results were obtained in two separate experiments. B, time dependence of MIG mRNA expression in IFNγ- and/or IL-4-stimulated macrophages. Thioglycollate broth-elicited peritoneal macrophages were pretreated with IL-4 (10 ng/ml) for 15 min, followed by stimulation with IFNγ (100 units/ml) for the indicated times prior to analysis of specific mRNA levels as described above. Northern blots were quantified by phosphorimage analysis, and relative mRNA levels are presented as percentage of maximum expression as described under “Experimental Procedures.” C and D, RAW264.7 (C) or ANA-1 (D) macrophages were unstimulated or pretreated with IL-4 (10 ng/ml) for 15 min prior to stimulation with increasing concentrations of IFNγ as indicated for 4 h. Levels of MIG mRNA were determined by Northern hybridization as described above. Similar results were obtained in two separate experiments.

IL-4-induced STAT6 Interacts with Coactivator CBP—The promoter analysis of the MIG gene indicates that the γRE motif is essential for both the IFNγ-induced transcriptional activation of the MIG promoter and the suppressive effect of IL-4. Furthermore, STAT6 is required for the suppressive activity of IL-4. Since STAT1-dependent transcription has been demonstrated to require the presence of the coactivator p300 or CBP (41, 42), it is conceivable that suppression might involve competition between STAT1 and STAT6 for limiting quantities of the coactivator. To explore this possibility, we assessed whether STAT6 can interact with CBP in vivo using a co-immunoprecipitation analysis (Fig. 5). Consistent with previous results (41, 42), STAT1 was present in immunoprecipitates using antibody to CBP in whole cell lysates from RAW264.7 cells, and the association appeared to be independent of stimulation (Fig. 5A). The same CBP immunoprecipitates also contained STAT6 (Fig. 5B). These results indicate that STAT6 as well as STAT1 binds CBP and are consistent with the possibility that STAT6 may compete for coactivator CBP.

IL-4-induced STAT6 Binds to the γRE Motif—An alternative possibility is that IL-4-induced STAT6 might compete for binding of STAT1 to the γRE motif. Since STAT6 can bind to a number of sequence motifs without transactivating function (20, 38, 40), the occupancy of the γRE site by STAT6 might result in suppression and not transactivation. Indeed, although IFNγ could stimulate reporter gene expression from constructs containing the γRE linked to the TK promoter, treatment with IL-4 was entirely inactive. To further test this hypothesis, we determined the ability of STAT1 or STAT6 to bind with the γRE motif (Fig. 6A). Several constitutive γRE binding activities were detected in nuclear extracts from untreated cells (designated as I and II). IFNγ induced two additional γRE binding activities (lane 2, a low and a high mobility complex designated as γRF-1 and γRF-2, respectively). Interestingly, IL-4 induced a single binding complex on the γRE motif (lane 3). When the cells were co-stimulated with IFNγ and IL-4, the pattern of complex formation was essentially the same as seen in nuclear extracts from the cells treated with individual stimuli. As reported previously, IL-4 did not affect the tyrosine phosphorylation or nuclear localization of STAT1 in response to IFNγ or vice versa (40).

To identify the proteins participating in formation of the γRE binding complexes, antibody supershift assays were performed (Fig. 6B). The constitutive binding activities (I and II) in nu-
FIG. 3. STAT6 is indispensable for the IL-4-mediated suppression of the IFNγ-induced, STAT1-dependent expression of MIG mRNA. A, thioglycolate broth-elicited peritoneal macrophages from wild type mice or from Stat1−/− mice were untreated (UT) or stimulated with IFNγ (100 units/ml) for 4 h. Levels of murine MIG mRNA were determined by Northern hybridization. Five μg of total RNA were analyzed in each lane. B, thioglycolate broth-elicited peritoneal macrophages from wild type mice or from Stat6−/− mice were untreated (UT) or pretreated with IL-4 (10 ng/ml) for 15 min, followed by stimulation with IFNγ (100 units/ml) for 4 h prior to analysis of specific mRNA levels by Northern hybridization. Five μg of total RNA were analyzed in each lane. C, Northern blots were quantified by phosphorimaging analysis and relative mRNA levels are presented as percentage of the IFNγ-induced expression. Each column and bar represents the mean ± S.E. from three independent experiments.

FIG. 4. Effects of IFNγ and IL-4 on transcription from the MIG promoter. A, the luciferase reporter construct (shown schematically) was transiently transfected into RAW264.7 cells as described under “Experimental Procedures.” Twenty-four h after transfection, the cells were pretreated with IL-4 (10 ng/ml) for 15 min prior to stimulation with IFNγ (100 units/ml) for 8 h, followed by analysis of cell extracts for luciferase activity. The relative luciferase activity is presented as a percentage of activity obtained in cells stimulated with IFNγ (100 units/ml) alone. Each column and bar represents the mean ± S.E. from three independent experiments. B, RAW264.7 cells were transiently transfected with a luciferase reporter plasmid containing one copy of the MIG γRE motif linked to the TK promoter (TK-81) as shown schematically. After 24 h, the cells were either unstimulated (UT) or treated with IL-4 (10 ng/ml) for 15 min, followed by stimulation with IFNγ (100 units/ml) for 8 h prior to measurement of luciferase activity. The relative luciferase activity is presented as percentage of maximum activity obtained in cells transfected with the reporter plasmid stimulated with IFNγ (100 units/ml). Each column and bar represents the mean ± S.E. from three independent experiments. The -fold induction of stimulated versus unstimulated samples is also indicated over the column.
FIG. 5. Association of STAT1 and STAT6 with CBP in RAW267.4 cells. Whole cell extracts were prepared from RAW264.7 cells either untreated (UT) or pretreated with IL-4 (10 ng/ml) for 15 min, followed by stimulation with or without IFNγ (100 units/ml) for 30 min before lysis the cells. Whole cell extracts (~500 μg of protein) were immunoprecipitated (IP) with anti-CBP antibody or normal rabbit IgG as indicated. CBP-associated proteins were then eluted, and half of the eluted protein was individually separated by 7.5% SDS-PAGE. One μg of whole cell extract was also loaded onto the gel (Input). Proteins were transferred to membrane and each blot was developed with anti-STAT1 (A) and anti-STAT6 (B) antibodies, respectively. The blot in A was subsequently stripped and immunoblotted with anti-CBP antibody (C). Similar results were obtained in two separate experiments.

DISCUSSION

The initiation, magnitude, and resolution of immune-mediated inflammation are regulated at least in part through the action of pro- and anti-inflammatory cytokines (1, 2). For example, the Th1-derived cytokine IFNγ promotes the pro-inflammatory function of macrophages through stimulation of new gene expression, and this is often antagonized by the Th2-derived cytokine IL-4 (2–5). IFNγ and IL-4 are both known to activate distinct members of the STAT family of transcription factors, each of which may exhibit differential transactivating potential depending upon the sequence motif to which they bind (20, 34, 37–40). In consideration of this, we postulated that the antagonistic action of IL-4 on IFNγ-stimulated gene transcription is mediated, at least part, through a direct antagonistic action of IL-4-activated STAT6. The results from the present study demonstrate that STAT6 is indispensable for the negative regulatory function of IL-4 on IFNγ-induced STAT1-dependent transcription of the MIG gene in mouse macrophages, and both factors appear to mediate their action through the γRE motif found in the MIG gene promoter. These conclusions are based on the following observations. 1) IFNγ-induced MIG mRNA expression was suppressed by IL-4 in primary macrophages and in ANA-1 or RAW264.7 macrophage-like cell lines. 2) IFNγ-induced MIG gene expression was abolished in peritoneal macrophages from Stat1−/− mice, while the suppressive effect of IL-4 was abolished in macrophages from Stat6−/− mice. 3) IL-4 suppressed the IFNγ-induced transcription of a luciferase reporter gene linked to either a 328-base pair fragment flanking the transcriptional start site of the MIG gene or to a single copy of the γRE motif from the MIG gene in the context of a heterologous promoter; IL-4 alone showed no independent transactivating function on either construct.

Our findings provide support for two possible mechanisms through which STAT6 may function to directly suppress IFNγ-induced gene expression. One mechanism of STAT6-mediated suppression may involve the participation of transcriptional co-activators such as CBP or p300. It has been reported recently that STAT1-dependent transcription requires either CBP and/or p300 (41, 42). Thus the finding that STAT6 can interact with CBP raises the possibility that STAT6 might sequester limiting quantities of CBP and thereby antagonize the STAT1-dependent transcription (41). The association of both STAT1 and STAT6 with CBP was not altered by stimulation with either IFNγ or IL-4. Thus, these data do not provide any direct evidence of competition between the two STATs for CBP. Nevertheless, further studies of the functional conse-
sequences of STAT6-CBP interaction will be required to determine the physiological significance.

The present results are also consistent with a scenario in which STAT6 and STAT1 compete for occupancy of the γRE regulatory site, as has been suggested previously in studies of the effects of IL-4 on IFNγ-stimulated IRF-1 expression (40). Because STAT6 has poor transactivating function on the MIG promoter, occupancy of the STAT1 binding site by STAT6 would result in reduced transcription. The nature of the regulatory sequence motif that determines both induction and suppression of the MIG gene (γRE) provides some unique features to the potential interactions between SBEs and STAT1 or STAT6. First, the formation of the γRF-1 complex requires both the 3′- and 5′-half-sites of the γRE motif while STAT6 only recognizes the 3′-half-site. Second, the mutant 5′-half-site version of the γRE motif will form γRF-2 but not γRF-1 and is functionally inactive in supporting IFNγ-driven reporter gene transcription. Thus, the γRF-1 complex but not the γRF-2 complex is essential for the IFNγ-induced transcriptional activity of the MIG gene. Finally, IL-4-induced STAT6 is able to bind to the γRE (3′-half-site) without transactivating function. These findings suggest that binding of STAT6 on the 3′-half-site may prevent the formation of the γRF-1 complex, resulting in decreased transcriptional frequency.

STAT6 exhibits the broadest DNA binding specificity of all STAT family members (20, 33–40). However, binding of STAT6 to IFNγ-responsive motifs does not lead to transcriptional activation despite the fact that STAT6 can interact with CBP. In contrast, STAT6 functions as a transcriptional activator when bound to a subset of SBEs including those found in the IgE or IL-1 receptor antagonist genes (20, 34). Thus, the nucleotide sequence of the DNA binding site is a critical determinant of the transcriptional activating function of STAT6. This may be an important distinguishing feature of STAT6, which allows both positive and negative effects on gene transcription.

The ability of STAT6 to compete with STAT1 for binding to the γRE was not detectable by EMSA (Figs. 6 and 7). This result is, however, not surprising since the oligonucleotide concentration in the reaction is in great excess relative to the concentration of STAT1 or STAT6, a condition under which competition between the protein factors will not occur. Reduction of the oligonucleotide concentration to a level at which competition might occur does not allow detection of the complexes (data not shown). Thus EMSA analysis of γRE binding activities using nuclear extracts from macrophages treated with IFNγ and IL-4 would not be expected show reduced formation of the γRF-1 complex.

STAT1 has been shown to exhibit a cooperative binding activity with two or more low affinity SBE motifs, which is mediated by the N-terminal region of the protein (63, 64). Thus, it is likely that the IFNγ-induced γRF-1 complex is a tetrameric form of STAT1. The higher mobility γRF-2 complex appears to be a STAT1 homodimer, which binds to the 3′-half-site of the γRE motif. Interestingly, STAT6 formed only a single complex despite its ability to bind a relatively wide range of SBE motifs (20, 33–40). Whether STAT6 lacks cooperativity in the N-terminal region or the γRE motif is insufficient for the cooperative binding of STAT6 remains to be determined.

Although IL-4-mediated anti-inflammatory function has been found to include both post-transcriptional and translational events (8, 12, 66), the majority of reports have identified transcription as a primary molecular target (8, 14, 16, 40, 67–71). The data in the present study further demonstrate that STAT6 is essential for IL-4-mediated suppression of IFNγ-induced MIG gene expression. Indeed, we have also observed that IL-4-mediated suppression of IFNγ-induced IP-10, IRF-1, ICAM-1, and inducible nitric-oxide synthase gene expression are also abolished in peritoneal macrophages from Stat6−/−
mice (data not shown). IL-13-mediated suppression of inducible nitric-oxide synthase gene expression has also been shown to depend upon STAT6 (72). This collection of studies indicates that IL-4- or IL-13-induced STAT6 can provide negative regulatory function, which is necessary for the anti-inflammatory cytokine action.

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