Formation of STAT1-STAT2 Heterodimers and Their Role in the Activation of IRF-1 Gene Transcription by Interferon-α*

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Many cytokine and growth factor signaling pathways utilize proteins of the Jak1 and STAT families (1–3). The IFN-α pathway involves activation of TYK2, Jak1, STAT1, and STAT2. The two STAT proteins are phosphorylated on conserved tyrosine residues by the Jak family kinases when IFN-α binds to the receptor complex (3). Activated STAT1 and STAT2 associate with the DNA-binding protein p48 to form the transcription factor ISGF3, which recognizes an interferon-stimulated response element (ISRE, consensus: AGTTTC-NNTTTCTC(C/T)) present in many promoters activated by IFN-α (for examples, see Refs. 4–6). All three proteins of ISGF3 make contact with DNA (7).

IFN-γ triggers the tyrosine phosphorylation of STAT1, but not STAT2 (8). Activated STAT1 forms homodimers, known as GAF (9). In IFN-α signaling, a complex containing STAT1, biochemically similar to GAF, has been reported (10). The GAS DNA sequences (consensus: TTTCNNNAA) recognized by GAF serve as binding sites for various cytokine- or growth factor-activated STAT proteins, including STAT3 homodimers (11, 12), STAT1-STAT2 heterodimers (11, 12), STAT4 (13), STAT5 (14–16), and STAT6 (17).

Transcription of the IRF-1 gene is inducible by both IFN-α and IFN-γ. A 16-kb 5′-flanking region that mediates a response to either IFN-α or -γ does not contain an ISRE, and the induction of IRF-1 by IFN-α is independent of the p48 subunit of ISGF3 (18–20). Transcriptional activation of this IRF-1 promoter segment by IFNs requires a palindromic GAS element, the IR element, which lies about 110 bases upstream of the transcription start site. IFN-inducible transcription factors containing STAT1 bind to the IR element in vitro.

In this report, we demonstrate the formation of a novel IFN-α-inducible DNA-binding factor consisting of STAT1 and STAT2. Although this factor does not include p48, the level of p48 protein does affect the balance between the novel factor and ISGF3. We propose that transcriptional activation of the IRF-1 gene involves interaction of the IR element with either a STAT1-STAT2 heterodimer or a STAT1 homodimer.

MATERIALS AND METHODS

Cells and IFNs—293T cells and mutant cell lines derived from it have been described elsewhere (21). Human recombinant IFN-α (5 × 10⁶ IU/mg) was obtained from Hoffmann LaRoche. IFN-γ (8 × 10⁶ IU/mg) was from Genentech.

RNA Protection Assay—Total RNA was prepared from IFN-treated cells and protection experiments were performed as described by Sambrook et al. (22). The probes used protect 175 bases of IRF-1 or 130 bases of γ-actin mRNAs (23).

EMSA—The oligonucleotides used were: IR element, 5′-GGATTTC-CCCGAAATGACG-3′; EcoGAS, 5′-GATTTCCCCGAAATGACG-3′. Briefly, complementary oligonucleotides, end-labeled with polynucleotide kinase (Boehringer Mannheim) and γ-UPS3P, were annealed by slow cooling. Approximately 20,000 cpm of probe were used per assay. Cytoplasmic extracts were prepared, and assays were carried out as described previously (24, 25). Briefly, the binding reaction was carried out in a total volume of 12.5–20 μl in 20 mM Hepes buffer, pH 7.0, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, and 10% glycerol at room temperature for 20 min. For supershift experiments, the extract was incubated with 1 μl of antibody for 15 min at 4 °C before adding the probe. The antibodies used were against STAT1 (26), STAT2 (27), STAT3 (12), p48 (28), and WAF-1 (Transduction Laboratories). Control rabbit preimmune serum was from Sigma.

Transfections—Wild-type and C-terminal deletion constructs of STAT2 (29) were transfected into U6A cells by the calcium phosphate method (30). STAT2 proteins were analyzed by Western blotting and

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1 Abbreviations used are: Jak, Janus family kinases; including TYK2 and Jak1; EMSA, electrophoretic mobility shift assay; Fc, high affinity immunoglobulin G chain receptor gene; GAF, γ-activated factor; GAS, γ-activated sequence; FN, interferon; IR, inverted repeat; IRF-1, interferon response factor-1; ISGF3, interferon-stimulated gene factor 3; ISRE, interferon-stimulated response element; p48, 48-kDa DNA binding component of ISGF3; SH2, Src-homology domain 2; STAT, signal transducer and activator of transcription; WAF-1, wild-type p53-activated factor 1.
clones expressing similar levels were used. The N2 chimera was constructed by the polymerase chain reaction SOEing technique (31), replacing the N-terminal region of STAT1 (amino acids 1–305) with the corresponding region of STAT2 (amino acids 1–315) (27).

RESULTS

Detection of a GAS-binding Transcription Factor Containing STAT1 and STAT2—Complex formation with various GAS element probes was examined by EMSA using cytoplasmic extracts of 2fTGH cells. At least three IFN-α-inducible DNA-binding factors were detected by using an IR element as probe (Fig. 1A, lane 2). We designate these complexes A, B, and C, from the slowest mobility to the fastest, respectively. The intensity of complex C was at least five times higher than that of complex A, and the intensity of complex B was weakest. Similar results were obtained using an Fcγ GAS probe.2 We used antibodies against STAT1, STAT2, and STAT3 to analyze the components present in these complexes. Complex C, which migrated similarly to the IFN-γ-induced GAF (Fig. 1A, lane 3), was supershifted only by an antibody against STAT1 (Fig. 1B, lane 2). Complex B was supershifted by antibodies against STAT1 or STAT3 (Fig. 1B, lanes 2 and 4). Complex A was supershifted by antibodies against STAT1 or STAT2 (Fig. 1B, lanes 2 and 3). A control antibody against WAF-1 did not supershift any of these complexes (Fig. 1B, lane 5). (The control antibody did generate nonspecific protein-DNA complexes near the top of the gel.) The composition of complexes A, B, and C was analyzed further by comparing 2fTGH and mutant cell extracts. No complex was detected with an extract of U3A cells, which lack STAT12, and complex A was not detected in IFN-α-treated U6A cells (Fig. 1A, lane 5). Complex C was barely detectable in IFN-α-treated U6A cells (Fig. 1A, lane 5), probably due to the weak activation of STAT1 in the absence of STAT2 (23). Thus, it is likely that complex C contains STAT1 homodimers, complex B contains STAT3-STAT1 heterodimers, and complex A contains STAT1-STAT2 heterodimers. To establish that complex A does not contain p48, we performed an EMSA with an extract of IFN-α-treated U2A cells, which lack p48 (Fig. 1C). Complex A was still detectable in these cells, showing that STAT1-STAT2 heterodimers can bind to DNA in the absence of p48. The bands formed with the U2A extract were less intense than those formed with 2fTGH extracts (Fig. 1B) for an unknown reason, possibly clonal variation.

STAT1-STAT2 Heterodimers and STAT1 homodimers Are Both Transcriptional Activators of the IRF-1 Gene—The IR element of the IRF-1 promoter binds IFN-α-inducible factors containing STAT1 and is important for the transcriptional activation of IRF-1 in response to IFNs (18–20). Previously, we showed that IFN-α induction of IRF-1 in U6A cells (lacking STAT2) was greatly reduced (23). We have now manipulated the levels of the heterodimers and homodimers, to evaluate their roles in the activation of IRF-1 transcription. To evaluate the heterodimers, we used a U6A clone, transfected with a STAT2 expression construct, that expresses about 10 times the level of wild-type STAT2. A U6A clone, transfected with the STAT1 expression construct, that expresses wild-type STAT1 at the levels seen in 2fTGH cells. Complex A was detectable in both the U6A and 2fTGH extracts (Fig. 1B, lanes 2 and 3). Complex B was barely detectable in the U6A extract (Fig. 1B, lane 4), but was strong in the 2fTGH extract. Complex C was barely detectable in the 2fTGH extract (Fig. 1B, lane 5), but was strong in the U6A extract. These results suggest that STAT1-STAT2 heterodimers are not sufficient for IRF-1 transcription, but that STAT1 homodimers are.

2 X. Li, S. Leung, and G. R. Stark, unpublished observations.
more STAT2 protein than 2TGH cells.² Parental 2TGH cells have much less heterodimer than homodimer (Fig. 1A, lane 2). The high level of STAT2 in the transfected U6A clone greatly increases the formation of heterodimers and decreases the formation of homodimers in response to IFN-α (Fig. 2, lane 2). The ratio of heterodimers to homodimers in this clone changes to 5:1 compared to a ratio of about 1:5 in parental 2TGH cells (Fig. 1A, lane 2), a 25-fold increase. IFN-α-induced IRF-1 expression was restored in this clone (Fig. 3, U6A/STAT2). The results suggest that the STAT1-STAT2 heterodimer can function as a transcriptional activator of the IRF-1 gene.

It has been proposed that the STAT1 homodimer also functions as a transcriptional activator of this gene in IFN-α-treated cells (20). To evaluate the role of the homodimer in the absence of the heterodimer, we expressed in U6A cells a chimeric protein, designated N2, in which the N-terminal 305 amino acids of STAT1 are replaced by the N-terminal 315 amino acids of STAT2. We observed only STAT1 homodimers in IFN-α-treated U6A/N2 extracts (Fig. 2, lane 5). In response to IFN-α, IRF-1 induction was restored in U6A/N2 cells (Fig. 3), suggesting that STAT1 homodimers are also transcriptional activators of the IRF-1 gene. The N2 protein and endogenous STAT1 were both phosphorylated in response to IFN-α in U6A/N2 cells.² However, we did not detect co-precipitation of N2 with STAT1 in these cells,² suggesting that N2 dimerizes poorly with STAT1. Furthermore, complex C in U6A/N2 extracts is not likely to contain N2/STAT1 heterodimers or N2/STAT1 heterodimers because an antibody against N-terminal STAT2 that could supershift all of complex A in 2TGH cells (Fig. 1B, lane 3) showed only minimal effect on complex C in U6A/N2 cells.²

Although both STAT1-STAT2 heterodimers and STAT1 homodimers can activate IRF-1, it is likely that the heterodimer is more potent. Upon IFN-α treatment, the level of homodimers in U6A/N2 cells was about 10-fold higher than the level of heterodimers in U6A/STAT2 cells (Fig. 2; compare complex C in lane 5 to complex A in lane 2). However, induction of IRF-1 gene expression was stronger in U6A/STAT2 cells than in U6A/N2 cells 4 h after IFN-α treatment (Fig. 3, A and B; compare U6A/STAT2 to U6A/N2). Although there are more STAT1 homodimers than STAT1-STAT2 heterodimers in IFN-α-treated 2TGH cells (Fig. 1A, lane 2), the heterodimers may still contribute to the activation of IRF-1 gene expression, since they are more potent.

The Acidic Domain at the C Terminus of STAT2 Is Required for IFN-α-induced IRF-1 Transcription—The critical role of STAT1-STAT2 heterodimers in IFN-α-induced IRF-1 expression is supported by studies with a series of C-terminal STAT2 deletion mutants. The STAT2 C terminus contains a domain rich in acidic amino acids, believed to be important to transcriptional activation (27). Recently, we expressed a series of C-terminal STAT2 deletion mutants in U6A cells and showed that the acidic domain is important for ISGF3-mediated transcriptional activation in response to IFN-α (29). A U6A clone expressing full-length STAT2 (851 amino acids) was compared to clones expressing similar levels of STAT2 deletions missing 20 (Δ831 construct), 39 (Δ812 construct), or 51 (Δ800 construct) C-terminal amino acids. In all of these clones, the IFN-α-mediated tyrosine phosphorylation of STAT1 was restored to normal levels (29) and heterodimers of STAT1 and the truncated
STAT2 proteins (Fig. 4, complex A) and STAT1 homodimers (Fig. 4, complex C) were formed. Interestingly, IRF-1 gene induction could not be restored by STAT2 mutants with deletion of 51 amino acids (Fig. 5, ∆800) or more. At least three factors contribute to this result. First, the STAT1-∆800-STAT2 heterodimers are likely to be inherently defective in transcriptional activation. Second, the small amounts of STAT1 homodimers formed in these clones will activate IRF-1 transcription only inefficiently. Third, the inactive heterodimers are likely to compete with the homodimers for IR elements. In summary, the segment between amino acids 851 and 800 is important for transcriptional activation. The STAT1 homodimers induced by IFN-α gene transcription mediated by the STAT1-STAT2 heterodimers and also for the transcriptional activity of STAT1-STAT2 heterodimers bound to p48 (ISGF3) (29).

Inhibition of Complex A Formation by p48—Overexpression of p48 should reduce the amount of STAT1-STAT2 heterodimers by promoting ISGF3 formation. In U2A cells (lacking p48), ISGF3 was not formed in response to IFN-α (Fig. 6A, lane 4), but the heterodimer was detected with the IR element probe (Fig. 6B, lane 4). When p48 was overexpressed in U2A cells, the ISGF3 complex was formed (Fig. 6A, lane 2), but the heterodimer (complex A) was not detected, even when twice the amount of extract was used (Fig. 6B, lane 2), suggesting that the level of STAT1-STAT2 heterodimers is affected by the level of p48. Formation of complex C (STAT1 homodimers) was not affected in p48-transfected U2A cells.

**DISCUSSION**

We performed EMSA with various GAS elements (1) and an extract of U6A/STAT2 cells; only the IR and Fcγ elements were found to bind STAT1-STAT2 heterodimers, suggesting that the heterodimers probably prefer GAS elements with the core sequence TCCCC(A/C)GAA. We did not identify a GAS element specific for heterodimers since STAT1 homodimers were detected with the same probes. It will be interesting to determine the optimal GAS sequence for binding heterodimers by use of the polymerase chain reaction (32). However, the optimal sequence for STAT1-STAT2 heterodimers may be no more specific than the optimal sequence for STAT1-STAT3 heterodimers, which also binds to STAT1 homodimers with high affinity (32).

STAT1-STAT2 heterodimers form in U2A cells in the absence of p48. It is likely that p48 binds to preformed heterodimers to form ISGF3, so that the level of p48 can influence the steady-state amount of heterodimer. The expression of p48 is usually low in most cell types but can be induced by IFN-γ.

Thus, the heterodimer may be directed either to form ISGF3 or to bind to a selected set of GAS elements depending on the availability of p48, which may modulate the response to IFN-α in an IFN-γ-dependent manner.

We manipulated the amounts of STAT1-STAT2 heterodimers and STAT1 homodimers in transfected U6A cells to reveal that both can function to stimulate transcription of the IRF-1 gene in response to IFN-α. However, a small amount of heterodimer is sufficient to promote a high level of IRF-1 induction, revealing that this novel factor is a potent transcriptional activator. The STAT1 homodimers induced by IFN-α activate IRF-1 transcription less strongly. Our results suggest that, depending on the level of p48, STAT1-STAT2 heterodimers can play a major role in activating IRF-1 transcription in response to IFN-α.
We showed by deletion analysis that the acidic domain of STAT2 is important for the transcriptional activation of the IRF-1 gene. The same region is also important for transcriptional activation of ISRE-containing genes (29). It is possible that the acidic domain of STAT2 may interact with the same basic transcription factor(s) at the start sites of these genes. We detected truncated heterodimers and STAT1 homodimers in U6A cells transfected with a series of STAT2 proteins carrying C-terminal deletions and found that decreased IRF-1 induction correlated with shortening of the STAT2 acidic domain. The STAT1 homodimers formed in these cells are insufficient to induce IRF-1 transcription, probably because they fail to compete effectively with the defective heterodimers.

Although a STAT1-STAT3 heterodimer (complex B) was detected using the IR element probe, this species is unlikely to be the GAs element of the Fc gene and is a potent transcriptional activator of the IRF-1 gene. Since the IRF-1 protein has been found to be a tumor suppressor (33) and a mediator of apoptosis (34), the STAT1-STAT2 heterodimer may play an important role in the antiproliferative response mediated by type I IFNs.

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