In Vitro Activation of the Transcription Factor Gamma Interferon Activation Factor by Gamma Interferon: Evidence for a Tyrosine Phosphatase/Kinase Signaling Cascade

KENICHI IGARASHI, MICHAEL DAVID, DAVID S. FINBLOOM, AND ANDREW C. LARNER*

Division of Cytokine Biology, Center for Biologies Evaluation and Research, 8800 Rockville Pike, Bethesda, Maryland 20892

Received 29 September 1992/Returned for modification 2 November 1992/Accepted 17 December 1992

Although it has been well documented that the biological activities of gamma interferon (IFN-γ) are initiated through interaction with its cell surface receptor, the signal transduction mechanisms which mediate the effects of this cytokine have remained unclear. In order to facilitate a better understanding of IFN-γ signaling, we have designed an assay using human fibroblast cell homogenates in which IFN-γ activates the formation of the IFN-γ activation factor (GAF) transcription complex. GAF mediates the rapid transcriptional activation of the guanylate-binding protein gene by IFN-γ. Activation of GAF in homogenates required ATP, but not Ca++ or GTP. Fractionation of homogenates indicated that both the pellet (18,000 × g) and the remaining cytoplasmic fraction were required for GAF activation by IFN-γ. In intact cells and cell homogenates, the activation of GAF was prevented by the specific tyrosine kinase inhibitor genistein. Treatment of GAF-containing nuclear extracts with either monoclonal antiphosphotyrosine antibody or protein tyrosine phosphatase prevented the assembly of the transcription complex, indicating that its formation required phosphorylation of tyrosine residues. Furthermore, the tyrosine phosphatase inhibitors phenylarsine oxide and zinc chloride also inhibited GAF formation in vitro, but only if these agents were added to cell homogenates before IFN-γ was added. The addition of either agent 5 min after IFN-γ had no effect. These results provide the first evidence for an IFN-γ-regulated tyrosine phosphatase/kinase signaling cascade that permits this cytokine to activate the transcription of an early-response gene.

Gamma interferon (IFN-γ), like IFN-α and IFN-β, controls a diverse set of biological responses including antiviral protection and antiproliferative activity. IFN-γ also plays a prominent role in inflammation, tissue repair, and host defense. Both IFN-γ and IFN-α are responsible for the induction of a set of early-response genes, and some of these genes are induced by both cytokines, such as major histocompatibility complex class 1 genes (26). Another gene, one that encodes the guanylate-binding protein (GBP), is also induced by both IFNs (6). Cellular genes that are rapidly induced by IFN-α contain within their promoters an IFN-stimulated response element (ISRE), which is necessary and sufficient for the activation of these genes by IFN-α. A multicomponent transcription factor, IFN-stimulated gene factor 3 (ISGF3), forms in the cytoplasm of IFN-α-treated cells and then translocates to the nucleus, where it binds to the ISRE (13, 19). Much less is known about the regulatory elements in cellular genes that are stimulated by IFN-γ. Although IFN-γ-activated cellular genes contain an ISRE (21, 23, 25), this element does not appear to be sufficient for IFN-γ to mediate its effects. No other common response element has been identified within the promoters of IFN-γ-activated genes. Within the GBP gene promoter is an IFN-γ activation sequence (GAS), which overlaps an ISRE. The GAS element itself, in combination with the ISRE, is necessary for IFN-γ to induce the expression of GBP (21). Similar to IFN-α activation of ISGF3, IFN-γ activates a protein(s) termed the IFN-γ-activating factor (GAF) in the cytoplasm, which then translocates to the nucleus, binds to the GAS, and activates expression of the GBP gene (8).

Since GAF is rapidly induced by IFN-γ, GAF is an ideal candidate to use as a marker of IFN-γ-regulated signal transduction. We have taken advantage of an in vitro signaling system that has recently been described for the activation of ISGF3 by IFN-α (4). By a similar approach, the activation of GAF by IFN-γ has been explored in both intact cells and homogenates and found to be regulated in a manner similar to that of ISGF3.

MATERIALS AND METHODS

Cells and culture medium. Primary human fibroblasts (BUD-8) from the American Type Culture Collection (ATCC 1554-CRL), were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

IFNs and reagents. Recombinant human IFN-γ was a generous gift from Genentech Corp. Recombinant IFN-α2a was provided by Hoffmann LaRoche.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed essentially as previously described for exonuclease protection assay detection of GAF (8). A 32P-end-labeled synthetic oligonucleotide (1.0 ng) with the sequence (double stranded) of the GBP gene (−133 to −103 bp) S'AACTTTCCAGTTCCATATTACTCAAATC' was used for detection of GAF1, -2, and -3. The entire sequence of the oligonucleotide which contained both the ISRE and GAS elements was needed to detect GAF1, -2, and -3. The synthetic oligonucleotide probe used for competition experiments that contained the ISRE region of GAS that corresponded to sequences −138 to −109 bp (8). This region in the absence of the complete GAS element which extends from approximately −116 to −105 bp has been demonstrated not to inhibit GAF formation by exonuclease protection (8).

* Corresponding author.
Preparation and fractionation of fibroblast nuclear extracts and homogenates. Nuclear extracts were prepared as previously described (20) from cells which were incubated with or without IFN-γ (2 ng/ml) for 30 min at 37°C. Homogenates were made from fibroblasts (15 to 20 confluent 150-cm² plates) which were trypsinized, collected by centrifugation, washed with phosphate-buffered saline, and resuspended in 1 to 1.5 ml of reaction buffer [20 mM MgCl₂, 100 mM NaF, 100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.9), 200 µM sodium ascorbate, 12 mM phosphoenolpyruvate, 4 mM ATP, pyruvate kinase (30 µg/ml), 2 mM ethylene-glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 600 µM α-toluenesulfonyl fluoride (PMSF)]. Originally, 50 µM guanosine-5'-O-(3-thiotriphosphate) (GTPγS) was also included in the reaction buffer, but this reagent was subsequently found not to be necessary. Sodium fluoride was also not required in the reaction buffer but was routinely included to maintain isotonicity (data not shown). Cells were subjected to Dounce homogenization in stainless steel apparatus, and portions (50 µl) of the lysate were incubated without or with 10 ng of IFN-γ for the indicated times at 30°C. The incubation was terminated by the addition of an eightfold volume of ice-cold stop solution (1 mM MgCl₂, 10 mM KCl, 20 mM HEPES [pH 7.9], 20% glycerol, 500 µM dithiothreitol, 250 µM PMSF, 0.1% Nonidet P-40). The mixture was vortexed and centrifuged at 18,000 × g for 5 min, and the supernatant was assayed for GAF by EMSA with the 32P-labeled oligonucleotide probe as described above.

Homogenates used for fractionation were centrifuged for 5 min at 18,000 × g. The supernatant (SUP) was placed on ice, and the sedimented material (MB) was washed in 1 ml of a solution containing 20 mM MgCl₂, 100 mM HEPES (pH 7.0), 200 µM sodium ascorbate, and 600 µM PMSF, centrifuged again at 18,000 × g and resuspended in reaction buffer. Aliquots (25 µl) of MB or SUP were incubated alone or with 10 ng of IFN-γ at 30°C, placed on ice, and the reaction was terminated by the addition of ice-cold stop solution. The mixture was then assayed by EMSA in a 6% native acrylamide gel.

Antiphosphotyrosine immunoprecipitation. Nuclear extracts prepared from fibroblasts treated with IFN-γ were incubated with 5-µg amounts of a monoclonal antiphosphotyrosine antibody (Upstate Biotechnology) at 4°C for 3 to 5 h prior to the addition of 32P-labeled probe. GAF levels were then assayed by EMSA.

Treatment of fibroblast extracts with tyrosine phosphatase. Nuclear extracts were prepared from cells treated with IFN-γ for 30 min as described above. Recombinant tyrosine phosphatase isolated from Yersinia enterocolitica was expressed and purified as described (17, 27, 29). Native or mutated forms of the enzyme (1 µg) were incubated for 15 min at 30°C with nuclear extracts (50 µl) prior to the addition of the GAF probe and subsequent EMSA.

RESULTS
As described by Decker et al. (6), BUD-8 cells, like other primary human fibroblasts, showed a rapid, protein synthesis-independent induction of the GBP gene by IFN-γ. Initial investigations which characterized IFN-γ-induced formation of GAF and its interaction with GAS used exonuclease protection assays (8). These assays defined the boundaries of GAS, with regards to IFN-γ activation of GBP. Binding of GAF to this site has been correlated with the rapid transcriptional activation of GBP by IFN-γ (7, 8, 21). Because EMSAs are in general more sensitive and convenient to use, we designed EMSA to detect IFN-γ-induced GAF formation in human fibroblasts (Fig. 1). EMSAs were performed with an oligonucleotide probe which contained both GAS and the adjacent ISRE in the GBP promoter. Nuclear extracts, prepared from fibroblasts incubated with IFN-γ for 30 min contained three complexes labeled GAF1, GAF2, and GAF3 that were absent in untreated cells (lanes 1 versus 2). Similar to the results reported by Decker et al. (6), kinetic analysis revealed GAF formation 15 min after treatment of cells with IFN-γ. Formation of the complex was maintained in nuclear extracts for 1 h and then began to decline (data not shown). A constitutive complex labeled C was also noted. The addition of a 100-fold molar excess of unlabeled oligonucleotide corresponding to the labeled probe displaced binding of all the IFN-γ-inducible complexes as well as the constitutive complex (lane 3), while the addition of an oligonucleotide composed of only the ISRE portion of the GAS element inhibited only the binding of the constitutive complex (lane...
were ATP in the reaction buffer
probe (lane 9). slower
unlabeled nuclear
performed buffer and
trypsinized homogenates.
The sedimented fraction
with homogenates (B)
and 2 for the
1636 IGARASHI ET AL.
FIG. 2 A) GAF1 formation is induced by IFN-γ in fibroblast homogenates. BUD-8 cells (20 confluent 150-cm² plates) were trypsinized and homogenized in reaction buffer. Aliquots (50 µl) were incubated at 30°C for the times (in minutes) indicated over the lanes either alone or with 10 ng of IFN-γ, and EMSAs were performed with the GAF probe. A complex with same characteristics as those of GAF1 was formed with IFN-γ treatment (see lanes 1 and 2 for the migration patterns of GAF1, -2, and -3 prepared from nuclear extracts). GAF1 formed in homogenates was displaced by unlabeled probe (lane 9), was NEM resistant (lane 11), and required ATP in the reaction buffer (lane 10). The band which migrates slightly slower than GAF1 may be GAF2, since it was displaced by unlabeled probe (lane 9). However, this complex is not inducible in homogenates with IFN-γ. The other complexes present in the homogenates are nonspecific, in that they were not displaced by unlabeled probe (lane 9). Incubation of homogenates with IFN-α (2,000 U) did not result in the formation of GAF1 (lane 12). Abbreviations: COMP, unlabeled probe; CTL, control; NE, nuclear extract. (B) Characterization of BUD-8 cell homogenate fractions required for IFN-γ-induced GAF1 formation. Homogenates prepared from BUD-8 cells were centrifuged at 18,000 × g for 5 min at 4°C. The sedimented fraction (MB) was washed in 1 ml of wash buffer and centrifuged again (18,000 × g for 5 min). Activation of GAF1 occurred in neither the MB fraction (lane 1) nor the SUP fraction from the initial centrifugation (lane 2) after incubation with IFN-γ for 30 min at 30°C. However, incubation of both the MB and SUP with IFN-γ permitted GAF1 activation (lanes 3 versus 4). Treatment of the SUP with NEM and then with dithiothreitol (DTT) prior to being mixed with membranes permitted GAF1 formation by IFN-γ (lane 5), while treatment of the MB with NEM prior to incubation with IFN-γ in the presence of the SUP prevented activation of GAF1 (lane 6). CTL, control.
kinase (Fig. 3). The tyrosine kinase inhibitor genistein prevented IFN-γ induction of all three GAF complexes when added to intact cells (Fig. 3A). The same reagent was also added to homogenates 5 min before or 5 min after the addition of IFN-γ, and under both conditions GAF1 formation was blocked (Fig. 3B). This result suggested that an IFN-γ activated tyrosine kinase was required for the formation of the GAF1 DNA-binding complex.

Since the formation of the GAF complex(es) was inhibited by genistein, experiments to determine the role of phosphor-ylated tyrosine residues in the formation of the transcription complex were performed. Nuclear extracts from IFN-γ-treated fibroblasts were incubated with an monoclonal antitrophosphotyrosine antibody to investigate whether GAF1, -2, and -3 contained phosphotyrosine (Fig. 4A). The antiphosphotyrosine antibody prevented formation of GAF1, -2, and -3 (lanes 3 to 5), while nonspecific IgG had no effect (lane 2), suggesting that these transcription complexes contained tyrosine phosphate. To determine whether phosphor-ylated tyrosine residues were required for assembly of GAF1, -2, or -3, nuclear extracts prepared from IFN-γ-treated cells were incubated with purified recombinant protein tyrosine phosphatase from Y. enterocolitica (17) (Fig. 4B). The addition of tyrosine phosphatase to extracts prevented GAF1, -2, and -3 formation (lane 1 versus 2), while the addition of a recombinant mutant form of the enzyme, which has a cysteine-to-serine substitution in its active site and has no enzymatic activity, had no effect (lane 4).

Incubation of tyrosine phosphatase in the presence of the tyrosine phosphatase inhibitor vanadate abrogated the ability of tyrosine phosphatase to disrupt GAF (lane 3).

Activation of ISGF3 by IFN-α in vitro required both a tyrosine kinase and phosphatase (5). Experiments using cell homogenates were performed to determine whether GAF1 formation also required IFN-γ activation of a tyrosine phosphatase (Fig. 5). The addition of either the tyrosine phosphatase inhibitor phenylarsine oxide (PAO) or zinc chloride (15, 16) blocked formation of GAF1. These agents also prevented GAF formation in vivo (data not shown). However, in contrast to genistein, both PAO and zinc chloride inhibited IFN-γ signaling only when added before the cytokine (lanes 3 and 4), whereas genistein blocked formation of GAF1 when it was added to the incubation mixture either before or after the cytokine (Fig. 3B). This observation suggested that a tyrosine phosphatase is not necessary in order to maintain the activation process.

**DISCUSSION**

One of the main obstacles in the study of the signal transduction mechanisms by which IFN-γ exerts its biological effects has been the absence of reproducible effects of IFN-γ in a cell-free system. Recently, an assay has been designed where IFN-α activates the formation of the transcription factor ISGF3 in HeLa cell homogenates (4). Results from this system suggested that a membrane-associated tyrosine phosphatase and tyrosine kinase were necessary for activation of ISGF3 by IFN-α (5). We have now applied the same methodology to investigate the mechanisms by which IFN-γ activates the transcription factor GAF, which is responsible for stimulating the expression of the cellular GBP gene (7, 8, 21). Activation of GAF1 in fibroblast homogenates required both ATP and the combination of a membrane-enriched and cytoplasmic fraction. Although the binding of GAF1 to DNA is NEM resistant, formation of the complex following IFN-γ activation in vitro required a
NEM-sensitive membrane-associated component (Fig. 2B). Since the activation of GAF1 by IFN-γ is not as sensitive as the in vitro activation of ISGF3 by IFN-α, it has not been possible to determine definitively whether activation of GAF1 requires only a plasma membrane-enriched fraction of fibroblasts. However, the fact that the membranes contained a NEM-sensitive component needed for signaling is consistent with a kinase and/or phosphatase activity being membrane-associated (14). Studies to address this important question are in progress.

Since activation of ISGF3 by IFN-α involved a tyrosine phosphatase/kinase signaling cascade (5, 12, 28), experiments were performed to explore the role of an IFN-γ-mediated tyrosine kinase and phosphatase activity in GAF formation (Fig. 3, 4, and 5). Since the tyrosine kinase inhibitor genistein blocked IFN-γ-stimulated formation of GAF both in vivo and when added to homogenates, these results indicated that a tyrosine kinase was required for signaling. These results were supported by the observations that treatment of GAF with either a monoclonal antiphosphotyrosine antibody or tyrosine phosphatase prevented GAF assembly. Therefore, tyrosine phosphorylation of the protein(s) which constitute these factors was necessary for their ability to either self-associate or to bind to GAS.

Two well-characterized inhibitors of tyrosine phosphatases (15, 16) PAO and zinc chloride, also prevented formation of GAF1 by IFN-γ. The fact that these agents inhibited signaling only when added to homogenates prior to the addition of IFN-γ not only reinforced the specificity of their effects but also implied that continuous activation of a tyrosine phosphatase was not necessary to maintain GAF activation but was required only during the initial phase of the signaling cascade. The functioning of this putative tyrosine phosphatase in IFN-γ activation of GAF needs clarification. De-phosphorylation of tyrosine residues is a well-described mechanism of activation of the Src family of protein tyrosine kinases (2). It is notable that a tyrosine

with native enzyme and 1 mM vanadate (lane 3), or with the mutant phosphatase (lane 4). After incubation, samples were subjected to EMSA using the GAF probe. Abbreviations: CTRL, control; PTP, protein tyrosine phosphatase; VAN, vanadate; mut., mutant; NUCL., nuclear.
kinase highly related to or the same as Tyk2 has been cloned which restored IFN-α-induced gene expression in an unresponsive cell line (28). The carboxy-terminal region of this kinase is highly homologous to the Src domain which contains Tyr-527 (2, 3). Tyr-527 in Src needs to be dephosphorylated for kinase activity. We speculate that the function of both the IFN-γ-activated tyrosine phosphatase activity that we describe here and the IFN-α-induced tyrosine phosphatase activity required for ISGF3 formation (5) may be to dephosphorylate Tyk2 or a related member of this family of enzymes.

The spatial relationship between the tyrosine kinase and phosphatase that are integral to activation of GAF and the IFN-γ receptor is also uncertain. It is becoming increasingly clear that the IFN-γ receptor is composed of two or more subunits: the cloned 90-kDa ligand-binding protein and a transducing component encoded on chromosome 21. It has been reported that the 90-kDa IFN-γ receptor becomes phosphorylated on serine and threonine when exposed to the ligand (18, 24). Other evidence has suggested that a tyrosine at position 440, an asparagine at 441, and a histidine at 444, all in the cytoplasmic domain of the IFN-γ receptor are needed for signal transduction (10). Although the 90-kDa receptor has no kinase domains, other receptor-associated proteins may contain one or more enzymatic activities. Indeed, antibodies against the 90-kDa protein have been shown to communoprecipitate two proteins (11). Experiments to determine whether this communoprecipitate contains a tyrosine kinase or phosphatase are in progress.

Although it is known that many of the biological activities of IFN-γ and IFN-α are identical and that these cytokines induce the transcription of a set of overlapping early-response genes, it is also clear that they interact with distinct cell surface receptors (26). At present, it appears that IFN-γ may activate genes through several distinct signaling pathways. For instance, the cellular gene γ1 and HLA-DR appear to be activated in response to IFN-γ through a protein kinase C-dependent mechanism (1, 9), while the results reported here indicate that activation of GAF requires a tyrosine phosphatase/kinase signaling cascade. The existence of several IFN-γ-regulated signaling pathways is also suggested by the isolation of cell lines which are selectively defective in IFN-γ-inducible expression of some cellular genes, while induction of other genes remained unaffected (22). The recent observations that IFN-α activation of the transcription factor ISGF3 also involves a tyrosine phosphatase/kinase signaling cascade, which has properties that are very similar or identical to those of IFN-γ activation of GAF suggests that a family of ligand-receptor activated tyrosine phosphatases/kinases may function to regulate the transcription of IFN-activated genes (5, 12, 28). Alternatively, one phosphatase and kinase may regulate expression of both IFN-α- and IFN-γ-activated transcription factors. Such a mechanism would imply that the specificity of this enzyme(s) for a given IFN-α- or IFN-γ-activated transcription factor would be determined by the cell surface receptor inducing the formation of a macromolecular complex which perhaps includes target peptides, the specific receptors, and the signal transducing enzymes. Understanding the specificity of these novel signaling cascades will require purification of the enzymes and the transcription factors which they phosphorylate.

ACKNOWLEDGMENTS

We thank G. Romero for critical reading of the manuscript. J. E. Dixon and Z.-Y. Zhang generously provided purified protein tyrosine phosphatase and the inactive mutant.

M.D. was supported by a Schrödinger fellowship from the Fonds zur Förderung der wissenschaftlichen Forschung from Austria.

REFERENCES


