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

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**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 $Ca^{2+}$ 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-$\alpha_{2a}$ 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 $^{32}$P-end-labeled synthetic oligonucleotide (1.0 ng) with the sequence (double stranded) of the GBP gene (−133 to −103 bp) 5′AAGTACTTTCAGTTTCATATTACTCTAAATC3′ 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 −103 bp has been demonstrated not to inhibit GAF formation by exonuclease protection (8).

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**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-$cm^2$ 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 $MgCl_2$, 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) (GTP-γ-S) 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 $MgCl_2$, 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 $^{32}P$-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 $MgCl_2$, 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 $^{32}P$-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

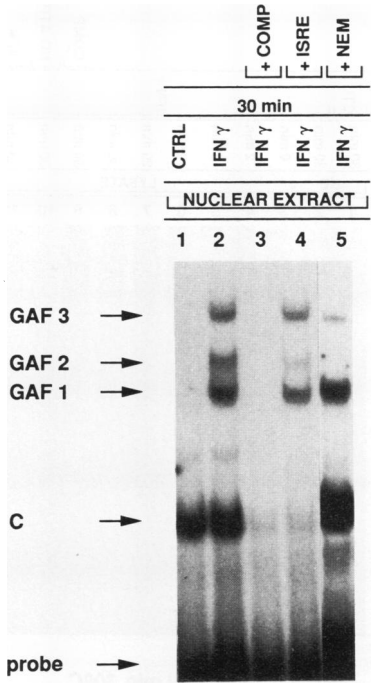


FIG. 1. Activation by IFN-γ of formation of GAF in nuclear extracts of BUD-8 cells. Confluent plates (150 $cm^2$) were incubated without (lane 1) or with IFN-γ (lanes 2 to 5) for 30 min prior to preparation of nuclear extracts. GAF formation was assayed by EMSA using a $^{32}P$-labeled oligonucleotide and 10 μg of protein. The IFN-γ-inducible complexes are labeled GAF1, GAF2, and GAF3. A constitutive complex (C) was also present. The relative amount of C varied from extract to extract. All complexes could be displaced by a 100-fold molar excess of the unlabeled probe (lane 3), but only GAF2 and C were displaced by the addition of a 100-fold molar excess of an oligonucleotide that contained only the ISRE region of GAS (lane 4). GAF1 and C were resistant to treatment with NEM, while GAF2 was sensitive to NEM and GAF3 showed variable sensitivity to NEM (lane 5). Treatment of extracts with NEM was performed as previously described (20). Preparation of nuclear extracts from cells incubated with IFN-α and assayed by EMSA with this same probe showed formation of a complex with a mobility similar to that of GAF2, but no GAF1 or GAF3 was observed in these samples (data not shown). Abbreviations: CTRL, control; COMP, unlabeled probe.

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

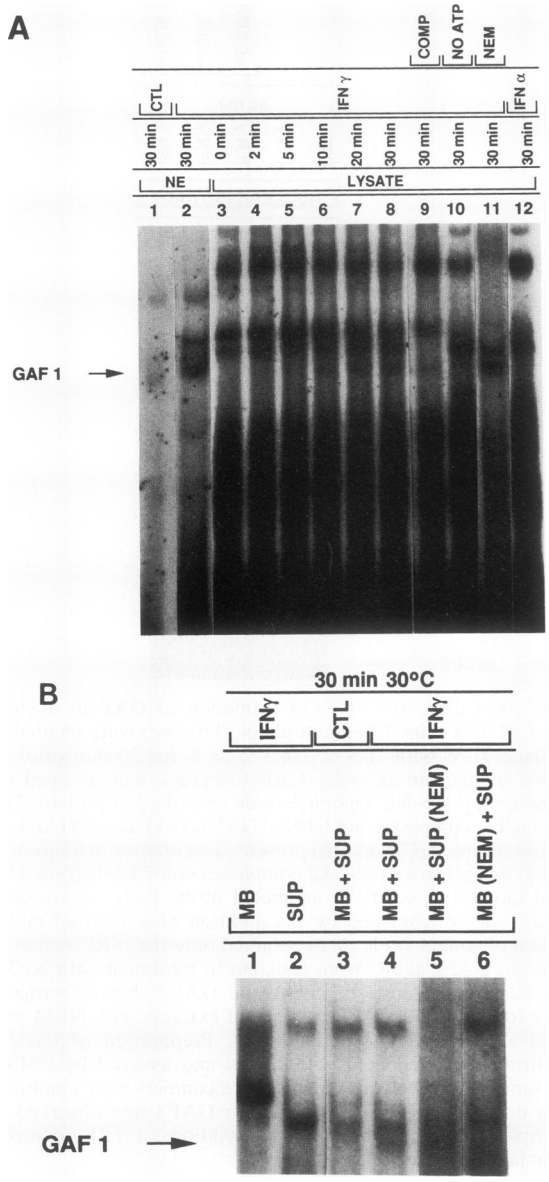


FIG. 2. (A) GAF1 formation is induced by IFN-γ in fibroblast homogenates. BUD-8 cells (20 confluent 150-cm$^2$ plates) were trypsinized and homogenized in reaction buffer. Aliquots (50 μl) were incubated at 30°C for the times (in minutes) indicated over the lanes either alone or with 10 ng of IFN-γ, 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.

4). In addition, the ISRE of ISG15 did not inhibit formation of any of the GAF complexes (data not shown). These findings corroborated those reported by Decker et al. (8), who employed an exonuclease protection assay to detect GAF interaction with GAS, using a similar DNA sequence to characterize the IFN-γ-induced stop site. *N*-ethylmaleimide (NEM) treatment of nuclear extracts prepared from IFN-γ-treated cells did not affect the formation of GAF1, while the formation of GAF2 was eliminated and that of GAF3 was partially affected (lane 5). Since ISGF3 is NEM sensitive (20), this observation served as an independent confirmation that GAF1 and probably GAF3 are not ISGF3.

To investigate the mechanisms by which IFN-γ activated the formation of GAF, we designed an in vitro system in which activation of the transcription factor(s) was duplicated in fibroblast homogenates. Cells were collected and homogenized in reaction buffer under conditions where greater than 99% of the cells were lysed. The cell homogenate was then incubated with IFN-γ at 30°C, the reaction was stopped, and formation of GAF was assayed by an EMSA with the GBP probe (Fig. 2A). Although we have been unable to observe IFN-γ-induced formation of GAF2 or GAF3 under these conditions, GAF1 can clearly be seen in the fibroblast homogenate incubated for as little as 5 min with IFN-γ (lanes 3 versus 5). Maximal induction occurred within 20 min. The rates of GAF1 formation parallel that reported to occur in vivo (8). IFN-γ induction of GAF1 was ligand specific, in that IFN-α did not induce GAF1 formation (lane 12). The complex was similar to that defined as GAF1 in nuclear extracts, in that excess unlabeled oligonucleotide inhibited the formation of the complex (lane 9) and in that it is NEM resistant (lane 11). Because ATP was required for activation of GAF1 formation, it was unlikely that the effects being observed are due to residual intact cells (lane 10). In addition, the nonhydrolyzable ATP analog adenylyl-(β,γ-methylene)-diphosphonate (AMP-PCP) could not substitute for ATP in the reaction solution (data not shown).

To obtain more information concerning IFN-γ activation of GAF1, each homogenate was fractionated into a pellet by centrifugation (18,000 × *g*) and the remaining SUP. The SUP and membrane-enriched pellet (MB) were then incubated with IFN-γ either alone or together at 30°C (Fig. 2B). Although neither the MB fraction nor the SUP alone supported IFN-γ activation of GAF1, incubation of the fractions together allowed signaling to proceed (lanes 1 and lane 2 versus 4). Treatment of the SUP fraction with NEM prior to incubation with the MB fraction and IFN-γ had no effect on the activation of GAF1, while treatment of the MB fraction with NEM prior to the addition of IFN-γ prevented the formation of GAF1 (lanes 5 and 6). This result indicated that although the GAF1 complex is NEM resistant, the IFN-γ-mediated signaling which permits activation of GAF1 does contain an NEM-sensitive factor and that this NEM-sensitive factor was associated with the membrane.

The fact that activation of GAF1 in cell homogenates required ATP and a NEM-sensitive component is consistent

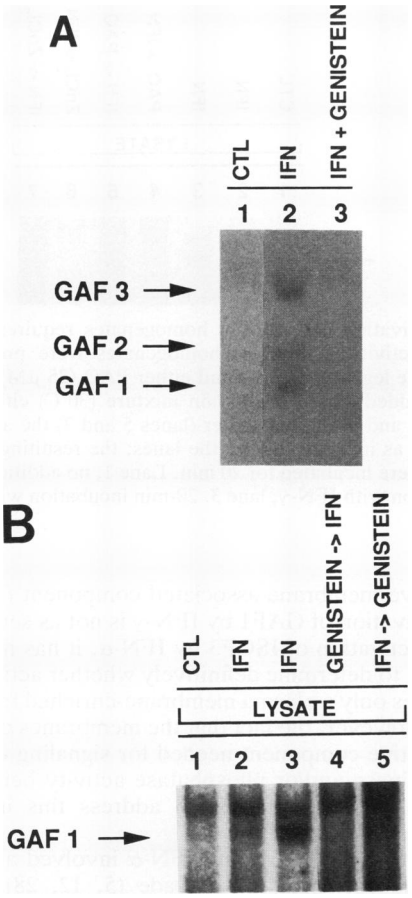


FIG. 3. (A) Activation of GAF formation by IFN-γ is inhibited by genistein in BUD-8 cells. Confluent plates of cells were incubated with genistein 30 min prior to the addition of IFN-γ for 30 min. Nuclear extracts were prepared, and GAF formation was assayed by EMSA as described in the legend to Fig. 1. Lane 1, no addition; lane 2, 30-min incubation with IFN-γ; lane 3, incubation with genistein (30 μg/ml) for 30 min and then with IFN-γ for 30 min. (B) IFN-γ-induced GAF1 formation is inhibited in BUD-8 homogenates by genistein. Homogenates were prepared from cells as described in the legend to Fig. 2. Genistein (50 μg/ml) was then added to aliquots (50 μl) either 5 min before or 5 min after the addition of IFN-γ and incubated at 30°C. Lane 1, no additions; lane 2, 5-min incubation with IFN-γ; lane 3, 20-min incubation with IFN-γ; lane 4, incubation with genistein for 5 min before the addition of IFN-γ for 20 min; lane 5, incubation with genistein for 5 min after the addition of IFN-γ for 20 min. CTL, control.

with a protein kinase-dependent reaction. Removal of the nonhydrolyzable GTP analog GTP-γ-S and $Ca^{2+}$ from the reaction buffer or the addition of guanosine-5′-*O*-(2-thiodiphosphate) (GDP-β-S) (which inhibits G-protein-mediated signaling systems) or the $Ca^{2+}$ chelator EGTA did not alter IFN-γ-induced GAF1 formation (data not shown). These data were consistent with an IFN-γ-mediated signaling mechanism which did not require a guanine nucleotide-binding protein or a $Ca^{2+}$-activated protein kinase.

Recent evidence has indicated that IFN-α activation of ISGF3 requires both a membrane-associated tyrosine kinase and phosphatase (5). In light of the fact that activation of GAF had several properties in common with ISGF3 activation, we investigated whether formation of this transcription complex in vivo and in vitro was mediated by a tyrosine 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 phosphorylated tyrosine residues in the formation of the transcription complex were performed. Nuclear extracts from IFN-γ-treated fibroblasts were incubated with a monoclonal antiphosphotyrosine 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 IgG1 had no effect (lane 2), suggesting that these transcription complexes contained tyrosine phosphate. To determine whether phosphorylated 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

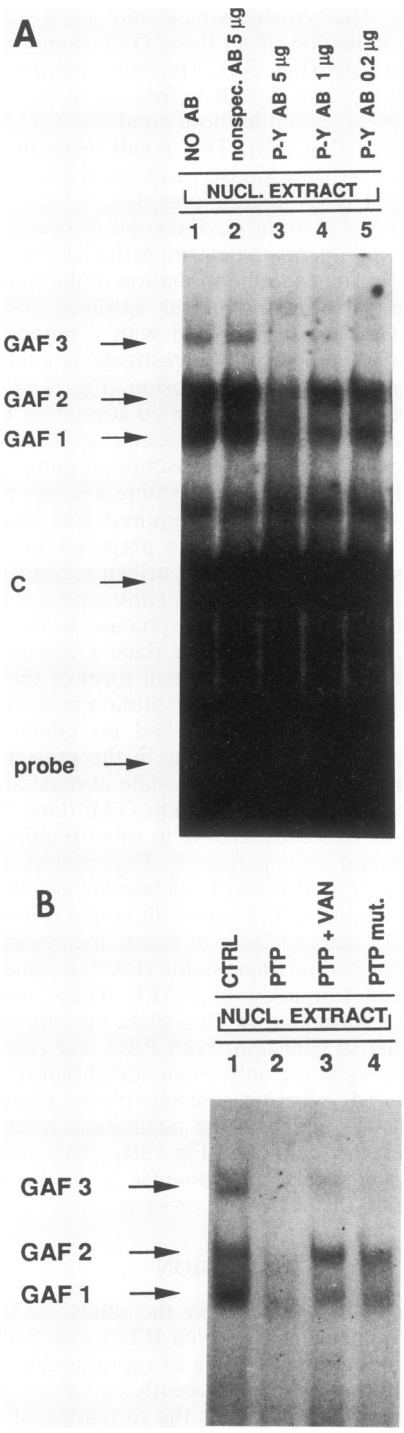


FIG. 4. Phosphorylated tyrosine is necessary for assembly of the GAF transcription complex. (A) Antiphosphotyrosine antibodies prevent formation of GAF1, -2, and -3. Nuclear extracts were prepared from cells incubated with IFN-γ for 30 min. Antiphosphotyrosine antibodies were incubated with extracts for 3 h at 4°C prior to EMSA using a GAF probe. Lane 1, no IFN-γ; lane 2, IFN-γ and a nonspecific antibody; lanes 3 to 5, IFN-γ and different concentrations of a monoclonal phosphotyrosine antibody. Abbreviations: AB, antibody; nonspec., nonspecific; P-Y AB, antiphosphotyrosine antibody; NUCL., nuclear. (B) Recombinant protein tyrosine phosphatase prevents GAF formation. Nuclear extracts, as described above for Fig. 4A, were incubated at 30°C for 30 min with no additions (lane 1), with the native enzyme alone (1 μg) (lane 2) or 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.

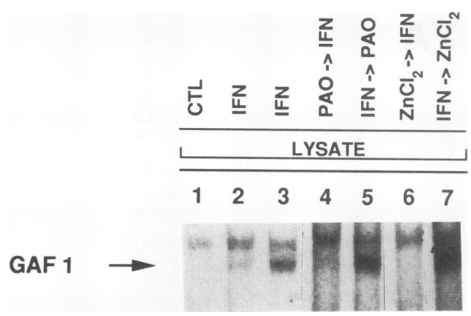


FIG. 5. Activation of GAF1 in homogenates requires tyrosine phosphatase activity. Fibroblast homogenates were prepared as described in the legend to Fig. 2 and either PAO (25 μM) or $ZnCl_2$ (1 mM) was added to the incubation mixture (30°C) either 5 min before (lanes 4 and 6) or 5 min after (lanes 5 and 7) the addition of IFN-γ (10 ng), as indicated above the lanes; the resulting mixtures (lanes 3 to 7) were incubated for 20 min. Lane 1, no addition; lane 2, 5-min incubation with IFN-γ; lane 3, 20-min incubation with IFN-γ. CTL, control.

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 as yet 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. Dephosphorylation 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

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 coimmunoprecipitate two proteins (11). Experiments to determine whether this coimmunoprecipitate 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 γ.1 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

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