An Interferon-γ-activated Site (GAS) Is Necessary for Full Expression of the Mouse iNOS Gene in Response to Interferon-γ and Lipopolysaccharide*

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Mouse macrophages can be stimulated by interferon (IFN)-γ and bacterial lipopolysaccharide (LPS) to produce nitric oxide (NO) as the result of expression of the inducible NO synthase (iNOS; EC 1.14.13.39) gene. The iNOS gene promoter contains a candidate γ-interferon-activated site (GAS). In transfection studies reported here, it was demonstrated that a luciferase reporter gene construct, containing four synthetic copies of the iNOS GAS, was inducible when transfected macrophages were stimulated with either IFN-γ, LPS, or a combination of the two. Consistent with this finding were other transfection analyses, which showed that responsiveness of the intact iNOS promoter to these same agents was significantly reduced when two conserved nucleotide positions within the GAS were mutated. Oligonucleotide probes, which mimicked the iNOS GAS, formed a complex with proteins that appeared in the nuclei of IFN-γ or IFN-γ + LPS-treated macrophages within 30 min of stimulation, as shown by electrophoretic mobility shift assay. LPS alone also caused the appearance of a nuclear protein capable of binding the iNOS GAS-containing oligonucleotide; however, in contrast to binding induced by IFN-γ, approximately 2 h of stimulation with LPS were required. The protein bound to the iNOS GAS-containing oligonucleotide reacted specifically with an antibody raised against Stat1α, regardless of the stimulus used. These data collectively support the conclusion that binding of Stat1α to the iNOS promoter’s GAS is required for optimal induction of the iNOS gene by IFN-γ and LPS.

Nitric oxide (NO), a short-lived, free radical gas, is a multifunctional effector in many physiological processes. Its biological effects include vasorelaxation (1), inhibition of platelet aggregation (2), neurotransmission (3), as well as microbial and tumor cell killing (4). In mammalian cells, production of NO from the substrate, L-arginine, is catalyzed by NO synthase (NOS) (5). Three related genes encode isoforms of NOS in different tissues. Neuronal and endothelial NOS are both constitutively expressed and each is dependent upon Ca2+/calmodulin for activity (6, 7). Mouse macrophages, and many other cell types, express an inducible isoform of NOS (iNOS) that accumulates after cell activation by stimuli such as interferon-γ (IFN-γ) plus bacterial lipopolysaccharide (LPS). This latter isoform functions independently of intracellular Ca2+ (8), because it has calmodulin bound as an integral subunit. In addition to the beneficial functions noted above, NO production has been associated with tissue damage (9) and septic shock (10). Given the wide spectrum of positive and negative effects that NO has, study of iNOS gene expression is of particular importance.

The mouse iNOS promoter has been extensively characterized (11, 12) and is known to contain two transcriptional regulatory regions, an enhancer and a basal promoter, within which a number of response elements have been localized. Those known to be active include κB sites located both in the enhancer (13, 14) and basal promoter (15–18); two juxtaposed enhancer-linked IFN-stimulated response elements (ISREs), the distal one of which is a strong activator (16, 17), while the proximal one is a weak activator of transcription (16) and an octamer element in the basal promoter (14). The candidate γ-interferon-activated site (GAS), located in the enhancer (nucleotide positions −942 to −934), has been reported in one study to be nonfunctional in regulating iNOS gene expression (17). An extensive evaluation of this finding has not been established, however.

GAS elements are known to bind homodimers of a phosphorylated form of the 91-kDa transcription factor, Stat1α (STAT), which is a latent cytoplasmic protein. Treatment of cells with IFN-γ causes tyrosine phosphorylation of Stat1α by the IFN-γ receptor-associated Janus kinases 1 and 2. Subsequently, phosphorylated Stat1α forms homodimers and translocates into the nucleus where it induces transcription of GAS-containing genes. Other STAT proteins, e.g. Stat3–6, which are induced by growth factors and cytokines other than IFN-γ, bind to DNA elements that are closely related to the GAS (20, 21).

The present study demonstrates that the candidate iNOS GAS is, indeed, necessary for optimal IFN-γ, LPS-, and IFN-γ

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‡ The abbreviations used are: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; GAS, interferon-γ-activated site; IFN, interferon; IRF-1, IFN regulatory factor-1; ISGF-3, IFN-stimulated gene factor-3; ISRE, IFN-stimulated response element; LPS, bacterial lipopolysaccharide; STAT, signal transducer and activator of transcription; EMSA, electrophoretic mobility shift assay; NF-IL6, nuclear factor of the interleukin-6 gene promoter.

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The present study demonstrates that the candidate iNOS GAS is, indeed, necessary for optimal IFN-γ, LPS-, and IFN-γ
+ LPS-mediated induction of the iNOS promoter. Furthermore, Stat1α, unlike Stats 3–6, is able to bind the iNOS GAS in IFN-γ-, LPS-, or IFN-γ + LPS-stimulated RAW 264.7 mouse macrophages. These data provide evidence for the direct involvement of Stat1α in the transcriptional induction of the mouse iNOS gene.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The macrophage-like cell line RAW 264.7 (American Type Culture Collection no. TIB-71, Rockville, MD) was used in all studies. Cells of this line were cultured in spinner flasks containing 25 ml Hepes-buffered RPMI 1640 medium (Sigma, which contained 10% (v/v) Fetal Clone I bovine serum product (HyClone Laboratories, Logan, UT), 2 mM glutamine (ICN Biomedicals, Inc., Irvine, CA), 100 μg/ml streptomycin (Sigma), and 100 units/ml penicillin (APOTHECON, Princeton, NJ). The lipid A-rich fraction II of LPS was phenol-extracted from Escherichia coli O111:B4 and obtained from List Biological Laboratories, Inc. (Campbell, CA). Reconstituted mouse IFN-γ (1.27 × 10^6 antiviral units/mg) was provided by Schering-Plough through the American Cancer Society (Atlanta, GA). Endotoxin was undetectable in all culture media and reagents, at a sensitivity of 50 pg/ml, as determined by the Limulus amebocyte assay (Associates of Cape Cod, Woods Hole, MA). Restriction enzymes and T4 polynucleotide kinase were obtained from New England BioLabs (Beverly, MA). [γ-32P]ATP (specific activity, 4,500 mCi/mmol) was from ICN Biomedicals, Inc.

Generation of Plasmid Constructs—The plasmid pGHL/H2 (12), containing the full-length iNOS promoter cloned into the pGCL2 Basic luciferase reporter gene vector (Promega, Madison, WI), was used for oligonucleotide-directed mutagenesis of the GAS and ISRE using the Unique Site Elimination (U.S.E.) mutagenesis protocol from Pharmacia Biotech Inc., according to the manufacturer’s instructions. After mutagenesis, both the enhancer and basal promoter of each construct were sequenced to confirm that the appropriate mutations were isolated and that no ectopic mutations within other iNOS functional elements had been introduced.

Four copies of the iNOS gene’s GAS and two copies of the interferon regulatory factor-1 (IRF-1) GAS gene (both synthesized by Integrated DNA Technologies, Coralville, IA) were each cloned upstream of a thymidine kinase basal promoter linked to the luciferase reporter gene of pGCL2-Basic. The identity of each construct was confirmed by DNA sequence analysis. All plasmid DNA was purified by two sequential CsCl/ethidium-bromide equilibrium centrifugations.

Transfection and Transient Expression of Luciferase Reporter Gene Constructs—Each construct (3 pmol) was transfected into RAW 264.7 cells by electroporation (12, 22). A human growth hormone expression vector, pXGH5, was cotransfected (0.6 pmol/transfection) as an internal control of transfection efficiency. Cells from three electroporations were pooled to eliminate differences between individual transfections and the mixture then equally divided among 12 wells of a 24-well cluster plate (Corning Costar Corp., Cambridge, MA). They were then allowed to adhere to the substrate for 2 h before the medium was changed. After 72 h (which were needed to reduce background levels of luminescence), the medium was again changed and triplicate cultures were incubated for 8 h with medium alone or medium that contained either 100 units/ml IFN-γ, 100 ng/ml LPS, or a combination of the two stimuli. The cells were then assayed for luciferase activity and human growth hormone production, as described previously (12). The results of human growth hormone assays showed that there was no significant difference in the relative efficiency of transfection between constructs (data not shown), consistent with earlier reports (12, 13, 22). All data are reported as fold induction, which was calculated by dividing the relative light units of each stimulated culture with that of the corresponding unstimulated control culture and averaging three independent experiments. The average is reported along with the standard error of the mean.

EMSA Analysis—Each pair of complementary single-stranded oligonucleotides (Life Technologies, Inc., Custom Primers) was end-labeled by T4 polynucleotide kinase and annealed, as described by Muroi et al. (23). Unlabeled, annealed oligonucleotides were used as cold competitor for sequence-specific DNA binding. The iNOS GAS construct, which contained four tandem copies of the GAS, was transcribed using T7 RNA polymerase. The antisense strand was end-labeled with [γ-32P]ATP and purified by ethanol precipitation. The probes were electrophoresed through a 4% native polyacrylamide gel (30 mA, 3 h) and transferred to a Hybond-XL membrane (Amersham). The membrane was pre-electrophoresed at 350 V at 4°C until the current fell to about 7 mA. Twenty-five μl of each reaction mixture were then loaded, and the gel was electrophoresed for 2 h and 15 min.

RESULTS

The iNOS GAS Can Direct Expression of an Unrelated Promoter in Response to Either IFN-γ, LPS, or IFN-γ + LPS—Given that the iNOS GAS has been described as nonfunctional (17), a simple, direct test of its activity was sought to assess the validity of this earlier observation. The approach chosen was to test expression of a linked reporter gene using synthetic promoters. Two luciferase reporter-gene constructs were made. One of these contained four tandem copies of the iNOS GAS and the second, two tandem copies of the IRF-1 GAS as a positive control. Each was linked to a synthetic thymidine kinase (TK) basal promoter. RAW 264.7 macrophages were transfected with each of these reporter constructs, stimulated with either IFN-γ, LPS, or the combination of IFN-γ + LPS, and assayed for luciferase activity (Table I). The four iNOS GAS copies mediated an approximate 3-fold increase in luciferase reporter gene activity when transfected cells were stimulated with IFN-γ, a 1.6-fold increase when stimulated with LPS, and more than a 3-fold increase when stimulated with IFN-γ + LPS. These results strongly suggested that the iNOS GAS is functional in response to each of these three stimuli. The inductive capacity of the IRF-1 GAS was greater than that of the iNOS GAS. This was evidenced by the fact that the IRF-1 GAS construct, which had only two elements driving the TK promoter, compared with four in the iNOS GAS construct, yielded more than twice the fold induction when either IFN-γ alone or IFN-γ + LPS were used as stimuli. By contrast, there was no significant difference (p = 0.27 by Student’s t test) between the fold induction observed with the two constructs using LPS alone as a stimulus.

Mutation of the iNOS GAS Causes a Reduction in iNOS Promoter Activity Induced by Either IFN-γ, LPS, or IFN-γ + LPS—To determine whether the iNOS GAS is functional in the environment of the iNOS promoter, mutational analyses were undertaken. Three constructs were made (one containing the
wild-type iNOS promoter, a second containing the iNOS promoter bearing a two-nucleotide mutation in the GAS, and a third containing the iNOS promoter bearing a two-nucleotide mutation in the strong, distal ISRE) and transfected into RAW 264.7 macrophages. This was followed by stimulation of cell cultures with IFN-γ, LPS, or IFN-γ + LPS. As shown in Fig. 1, RAW 264.7 macrophages transfected with a construct containing the GAS mutation showed a significant 2–3-fold decrease in luciferase production, depending upon the stimulus, when compared with cells transfected with the wild-type construct. The reporter construct containing the mutated strong, distal ISRE also showed decreased responsiveness to these mediators. The extent of reduction was found to be equivalent to that seen for the mutated GAS. This not only indicated that the GAS is functional in regulating mouse iNOS gene expression, but also that it is as important as the strong, distal ISRE.

**IFN-γ, LPS, and IFN-γ + LPS Induce Binding of a Nuclear Protein to the iNOS GAS**—In view of the results reported above, it was predicted that the iNOS GAS should bind one or more nuclear proteins after cellular stimulation with IFN-γ, LPS, or a combination of the two. To test this prediction, electrophoretic mobility shift assays were performed using nuclear extracts from RAW 264.7 macrophages. By using a 15-base pair probe that contained the iNOS GAS, a specific binding complex (denoted by the arrowhead in Fig. 2, A–C) was detected in stimulated, but not unstimulated, cells (Fig. 2, A–C, compare lanes 2 with lanes 1, respectively). The specificity of this binding complex was demonstrated by the fact that unlabeled iNOS GAS oligonucleotide could compete effectively for binding (Fig. 2, A–C, lanes 3), while an unrelated NF-IL6 oligonucleotide could not (Fig. 2, A–C, lanes 5). Additional confirmation of specificity was obtained by using an oligonucleotide in this competition study that contained a mutated iNOS GAS sequence (the same two mutations introduced into the luciferase reporter-gene construct used for mutational analysis, above); this oligonucleotide also did not compete for binding (Fig. 2, A–C, lanes 4). In addition to the specific complex, we also detected nonspecific binding complexes both in unstimulated and stimulated cells (Fig. 2, A–C, indicated by the bracket).

In RAW 264.7 macrophages treated with either IFN-γ or IFN-γ + LPS, the appearance of the specific binding complex was rapid, occurring within 30 min of stimulation (Fig. 2, A and C). However, in LPS-stimulated macrophages this binding complex was not observed at 30 min (data not shown) but, rather, required approximately 2 h of stimulation before it became detectable (Fig. 2B). Furthermore, the relative amount of binding activity induced by LPS was greatly reduced, compared with that stimulated by IFN-γ or IFN-γ + LPS, as indicated by the fact that more nuclear extract, and longer exposure times were required to achieve the autoradiographic result shown in Fig. 2B (refer to the legend in Fig. 2).

**Fig. 1. Mutational analysis of the mouse iNOS GAS.** Two conserved nucleotides each, in both the iNOS promoter GAS and ISRE, were mutated by oligonucleotide-directed mutagenesis. Luciferase reporter-gene constructs, containing either wild-type or mutated iNOS promoters, were transfected into RAW 264.7 cells which were subsequently treated as indicated under “Experimental Procedures.” The thick bar represents the iNOS gene 5'-flanking region and a portion of the iNOS transcription unit. The arrows represent the transcriptional start points. GASm and ISREs indicate the identities and approximate locations of the mutations being investigated. Numerical values were calculated as described under “Experimental Procedures.”

**Fig. 2. Binding of nuclear proteins to the iNOS GAS.** Cell cultures were stimulated and EMSA binding reactions were performed as indicated under “Experimental Procedures.” A radiolabeled, 15-base pair oligonucleotide containing the iNOS GAS was used in all reactions. Panel A, RAW 264.7 macrophages were treated with IFN-γ for 30 min prior to preparation of nuclear extract. Ten µg of nuclear extract were used in each lane. Lane 1, nuclear extract of cells cultured in medium alone; lane 2, nuclear extract of an IFN-γ-treated culture; lanes 3–5, nuclear extract of an IFN-γ-treated culture premixed with an excess of unlabeled: GAS oligonucleotide (lane 3); mutated GAS oligonucleotide (lane 4); or NF-IL6 oligonucleotide (lane 5). The dried polyacrylamide gel was exposed to x-ray film for approximately 48 h. Panel B, lanes 1–5 were similar to those in panel A except that, in lanes 2–5, LPS was used to stimulate the cell culture for 2 h prior to the preparation of nuclear extract. Twenty µg of nuclear extract were used in each lane. The dried polyacrylamide gel was exposed to x-ray film for approximately 7 days. Panel C, lanes 1–5 were similar to those in panel A, except that, in lanes 2–5, IFN-γ + LPS was used to stimulate the cell culture for 30 min prior to the preparation of nuclear extract. Ten µg of nuclear extract were used in each lane. The dried polyacrylamide gel was exposed to x-ray film for approximately 48 h. In each panel, a specific binding complex is indicated by the solid arrowhead. Nonspecific binding complexes, i.e., those that could not be competed by unlabeled oligonucleotide, are represented by a bracket.

The iNOS GAS Binding Complex Contains Stat1α Regardless of Stimulus—To identify the nuclear protein(s) that specifically bound to the iNOS GAS, we next performed antibody supershift assays. Antibodies specific for each of five members of the STAT family of transcription factors (Stat1, Stat3–6), which are known to be activated either by IFN or by other cytokines or hormones (26–30) and known to bind sequences related to the GAS, were used in these supershift assays. Antibody against Stat1α was the only one able to supershift the specific binding complex, and it could do so in assays of nuclear extracts from cells stimulated with either of the two stimuli, or by the combination of both (Fig. 3, A–C, indicated by the open arrowhead). It was concluded from these results that the specific binding complex identified by EMSA contains Stat1α.

**DISCUSSION**

The results presented are important because they show that the enhancer-linked GAS is, in fact, active in the induction of...
transcription of the mouse macrophage iNOS gene in response to LPS + IFN-γ. In addition, it has been shown here that the transcription factor Stat1α binds to this GAS and that such binding occurs regardless of whether RAW 264.7 cells are stimulated with IFN-γ or LPS. Finally, the results shown here indicate that while both IFN-γ and LPS treatment of mouse macrophages cause the binding of Stat1α to the iNOS gene, there is a temporal difference between the two.

Mouse macrophages can be induced by the combination IFN-γ + LPS to produce NO even at concentrations of these two mediators that, by themselves, are ineffective. In other words, the two mediators function synergistically (31). This would seem to suggest that IFN-γ and LPS induce separate, complementary signaling pathways that are both required for optimal expression of the iNOS system. However, at high concentrations of LPS alone (i.e. greater than 10 ng/ml), iNOS gene expression and NO production occur in a dose-dependent manner. Upon first consideration, this would seem inconsistent with the hypothesis that IFN-γ and LPS generate independent signals. It has recently been demonstrated, however, that high levels of LPS also induce mouse macrophages to produce IFN-β, and that autocrine/paracrine stimulation by this mediator is critical to the LPS-mediated production of NO (32, 33).

The delayed induction of Stat1α binding by LPS alone, compared with IFN-γ alone or IFN-γ + LPS, is consistent with the fact that autocrine/paracrine IFN-β plays a role in LPS-mediated activation of macrophages for NO production. The likely reason that lower GAS binding activity is generated by IFN-β is that most of the tyrosine-phosphorylated Stat1α produced by this stimulus is sequestered into the transcription factor ISGF-3, although small amounts of Stat1α homodimers are detectable in type-I IFN-stimulated cells (34). ISGF-3 binds to a subset of ISREs that differ in sequence from the iNOS ISRE. IFN-γ, by contrast, induces predominantly GAS-binding homodimers of Stat1α (19, 20). Thus, we propose that the lower level of iNOS promoter induction caused by LPS (and autocrine/paracrine IFN-β) versus IFN-γ + LPS is likely the result of lower availability of Stat1α homodimers generated by LPS which, in turn, are needed to bind the GAS maximally. It should be noted, however, that other autocrine/paracrine factors produced by LPS-stimulated mouse macrophages, such as IL-6 and IL-10, also cause the tyrosine-phosphorylation of Stat1α (reviewed in Finbloom and Lerner (20)). It is possible, therefore, that these mediators could be the source of a portion of the GAS binding activity seen in LPS stimulated RAW 264.7 macrophages.

In addition to a direct role for Stat1α in iNOS gene activation, this transcription factor can also act indirectly. For example, it is required for induction of the IRF-1 gene (35), which encodes the IRF-1 transcription factor that binds the two juxtaposed ISREs in the iNOS promoter’s enhancer. In IRF-1 knockout mice, IFN-γ + LPS induces virtually no iNOS mRNA accumulation or NO production. This is in spite of the fact that all other transcription factors necessary for iNOS gene induction (i.e. NF-κB, Stat1α, and octamer binding factors) are ostensibly available to interact with cis-regulatory elements of the iNOS gene. In work reported elsewhere (13), we have similarly shown that mutation of the enhancer-linked κB element of the iNOS promoter abolishes responsiveness of the enhancer not just to LPS, but to added IFN-γ as well. This is in spite of the fact that NF-κB itself is not activated by IFN-γ. The targeted disruption of the Stat1 gene also abolishes macrophage NO production in response to IFN-γ + LPS (36). However, it cannot be concluded from this result whether the effect is attributable directly to the absence of Stat1α or, indirectly to a relative lack of IRF-1.

Taken together, the above observations suggest that transcription factors that interact with the iNOS enhancer must do so cooperatively. Stated another way, iNOS gene expression appears to require the simultaneous presence of all transcription factors that bind its enhancer; when all are present transcription is enhanced, and when any one of them is absent transcription either remains off or is greatly reduced. This is consistent with the observation that the expression of iNOS in mouse macrophages is population-based (37, 38). Increases in iNOS expression are achieved by increasing the number of cells within the population that express the iNOS gene and, therefore, iNOS protein, indicating that expression of the iNOS system in individual cells is either fully on or off. This suggests that in the absence of maximal stimulation, such as by the combination of IFN-γ + LPS, at least one transcription factor controlling iNOS gene expression may be limiting. By this reasoning, full expression of iNOS protein would only be achieved by those cells in the population that exceed threshold levels of the limiting factor(s). Stat1α may be that factor because increasing numbers of cells, in a given population, can be induced to express the iNOS gene when increasing concentrations of either IFN-β (37) or IFN-γ (38) is added to LPS-stimulated cell cultures.

In conclusion, a minimum of six, cis-acting regulatory elements are now known to control regulation of the mouse iNOS gene, two ISREs (16, 17), two κB elements (13–15), an octamer element (14), and as shown here, a GAS. These are bound by transcription factors either activated or induced by LPS plus one of the interferons. The picture that emerges is one of complex regulation of the mouse iNOS gene, the product of which can be both beneficial (4, 18) and detrimental (9, 10) to the host. Although we do not yet understand the manner in which the transcription factors necessary for gene induction...
interact on the iNOS promoter, there are several indications that they must do so cooperatively. Identification of the various transcription factors involved and discovery of how they interact with one another on and, perhaps off, the iNOS promoter is essential to understanding transcriptional control of the mouse iNOS gene and, therefore, regulation of NO production.

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