Role of Interferon Regulatory Factor 1 in Induction of Nitric Oxide Synthase
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Summary
Interferon-γ (IFN-γ) interacts synergistically with bacterial lipopolysaccharide (LPS) to induce transcription of iNOS, the isoform of nitric oxide synthase whose activity is independent of elevated Ca\(^{2+}\) and exogenous calmodulin. To define a cis-acting element mediating IFN-γ-dependent synergy, we made deletions in iNOS promoter constructs fused to reporter genes, transfected RAW 264.7 macrophages, and treated the cells with IFN-γ and/or LPS. This analysis implicated the region from positions -951 to -911, a cluster of four enhancer elements known to bind IFN-γ-responsive transcription factors, including an interferon regulatory factor binding site (IRF-E) at nucleotides -913 to -923. Site-specific substitution of two conserved nucleotides within IRF-E in the context of the full-length iNOS promoter ablated IFN-γ's contribution to synergistic enhancement of transcription. Electromobility shift assays performed with a probe containing IRF-E revealed the existence of a complex in nuclei of RAW 264.7 macrophages that was present only after treatment with IFN-γ, which reacted specifically with anti-IRF-1 immunoglobulin G and which included a species migrating at 40-45 kD, consistent with the apparent molecular weight of murine IRF-1. Thus, the synergistic contribution of IFN-γ to transcription of iNOS in RAW 264.7 macrophages requires that IRF-1 bind to IRF-E in the iNOS promoter. In conjunction with the work of Kamijo et al. (Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, J. Gerecitano, D. Shapiro, J. Le, K. S. Im, T. Kimura, S. Green et al. 1994. Science [Wash. DC]. 263:1612), these findings identify iNOS as the first gene that requires IRF-1 for IFN-γ-dependent transcriptional regulation.

Nitric oxide (NO)\(^1\), a radical gas, acts as an intercellular messenger in most mammalian organs, participating in vascular homeostasis, neurotransmission, and defense against infectious agents (1). Three isoforms of NO synthase (NOS) have been cloned (2-4). The isoform expressed in the widest variety of cell types is termed iNOS because its activity is independent of elevated Ca\(^{2+}\) or exogenous calmodulin (4, 5). Activity of iNOS has been associated with tissue damage in arthritis, nephritis, insulitis, and septic shock (6-10), leading to interest in factors that control its expression.

Expression of iNOS generally requires exposure of cells to immune or inflammatory stimuli (1). This inductive phenomenon was first demonstrated with mouse peritoneal macrophages treated with bacterial LPS, in which NO production was enhanced synergistically by the addition of IFN-γ (11, 12). Synergistic enhancement of NO production has subsequently been demonstrated in many types of myeloid, mesenchymal, and epithelial cells exposed to two or more stimuli (13). That expression of iNOS is regulated largely at the transcriptional level has been formally demonstrated in mouse macrophages (4), where it has also been shown that the synergistic interaction between LPS and IFN-γ in the induction of iNOS is itself largely transcriptional (14).

Cloning of the promoter of the murine iNOS gene (15, 16) has opened a molecular route to the analysis of iNOS induction. During induction of iNOS in mouse macrophages by LPS, a protein complex containing nuclear factor for κ L chain in B lymphocytes (NF-κB) heterodimers (p50/c-Rel and p50/RelA) binds to the downstream NF-κB site, NF-κB1 (17). On the other hand, the promoter region upstream from -722 is necessary for IFN-γ to enhance synergistically the induction of iNOS by LPS (15, 16). This upstream region contains four copies of an IFN-γ response element (γ-IRE), two copies of the γ-activated site (GAS), and two copies of the IFN-stimulated response element (ISRE), designated

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\(^1\) Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; GAS, γ-activated site; iNOS, nitric oxide synthase whose activity is independent of exogenous calmodulin and elevation of intracellular \([Ca^{2+}]\) above the level in resting cells, the expression of which is usually inducible; IRF, interferon regulatory factor; IRF-E, IRF-binding element; ISRE, interferon-stimulated response element, downstream/upstream; NF-κB, nuclear factor for κ light chain in B lymphocytes; NO, nitric oxide.
ISRE\textsubscript{u} for upstream and ISRE\textsubscript{d} for downstream. Finally, the complementary nucleotide sequence of the ISRE\textsubscript{d} core (Fig. 1A) closely matches a consensus sequence termed IFN regulatory factor element (IRF-E) (18).

IRF-1 is an IRF-E binding protein (14) whose physiologic importance has been a matter of controversy (19–24). CD8\textsuperscript{+} lymphocytes were decreased in mice with a targeted disruption of the IRF-1 gene (25). However, no specific gene was identified whose expression was directly affected by the lack of IRF-1. Recently, Kamijo et al. (26) discovered that macrophages from IRF-1\textsuperscript{-/-} mice failed to accumulate iNOS mRNA after stimulation with LPS in the presence or absence of IFN-\gamma. However, it was not clear if IRF-1 controlled expression of iNOS directly or via the regulation of another gene.

The present study establishes that the IRF-E is required for IFN-\gamma to exert synergistic induction of iNOS transcription, and identifies IRF-1 as a major protein in IFN-\gamma-treated RAW 264.7 macrophages that binds directly to this element in the iNOS promoter.

**Materials and Methods**

**Cell Culture.** The macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM l-glutamine, and 200 \( \mu \)g/ml each of penicillin and streptomycin.

**Reagents.** Recombinant IFN-\gamma was a gift of Genentech (South San Francisco, CA). LPS (from *Escherichia coli* O113:B4) was from Sigma Chemical Co. (St. Louis, MO). Poly(dI-dC)-poly(dI-dC) was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Isotopes were from Amersham Corp. (Arlington Heights, IL). Purified rabbit IgG against purified recombinant murine IRF-1 was prepared by Teruaki Nomura and Heinz Ruffner and was kindly provided by Dr. Luiz Reis (University of Zurich, Zurich, Switzerland). Rabbit antiserum against human STAT91, a kind gift of K. Shuai and J. Darnell (The Rockefeller University, New York, NY), cross-reacts with mouse STAT91 (Shuai, K., personal communication).

**Plasmids.** Plasmid p10.5iNOS-CAT was constructed by deleting the upstream region of the iNOS promoter (–1588 to –976) from the plasmid pIiNOS-CAT (10) after a HindlII site was created at position –975 by PCR mutagenesis. A 2-bp substitution (AA to GG, Fig. 1B) was introduced into the IRF-E of the iNOS promoter cloned in plasmid pUP1 (15) by oligonucleotide-directed mutagenesis with single-stranded DNA. The Sall promoter fragment containing the generated mutation was subcloned into the pCAT-Basic vector (Promega Corp., Madison, WI) to form the plasmid pI(\textit{IRF}^*) (15). Two dual-reporter gene constructs, p1-lac and p1(\textit{IRF}^*)-lac, were obtained by inserting the BglII-BamHI fragment of pGL2-control vector (Promega Corp.), containing a SV40 promoter, a luciferase reporter gene, a splicing segment, and a polyadenylation signal, at the BamHI site of the plasmids pliNOS-CAT and pI(\textit{IRF}^*)

**Transient Transfection and Reporter Gene Assays.** Plasmid preparations were used only if their content of LPS was calculated to be <25 pg/ml at the concentrations used for transfection, based on assay of undiluted stocks by the chromogenic Limulus assay (Whittaker Biomolecular, Walkersville, MD). RAW 264.7 cells were transfected and chloramphenicol acetyltransferase (CAT) activity assayed by TLC as described (15). The luciferase assay was carried out as described by the manufacturer (Promega) and used to normalize the results of the CAT assay.

**Oligonucleotides and Probes.** Single-stranded oligonucleotides

![Figure 1](image-url)
Results

The Region between −975 to −722 is Important for IFN-γ's Contribution to Induction of the iNOS Promoter. Comparison of pliNOS-CAT (containing the full-length iNOS promoter region −1588 to +161) and p3iNOS-CAT (−721 to +161) revealed that the upstream portion (−1588 to −722) of the iNOS promoter contributed to the synergistic action of IFN-γ with LPS for induction of the iNOS gene (17). More CAT constructs were derived from pliNOS-CAT by making progressive deletions from the 5' end up to −976. These constructs were transfected into RAW 264.7 cells and CAT activity was measured 15 h after exposure to the cells to IFN-γ, LPS, both, or neither. p10.5iNOS-CAT, in which the promoter was deleted to −975, displayed activity indistinguishable from pliNOS-CAT. That is, IFN-γ alone induced no CAT activity, but IFN-γ contributed synergistically to the CAT activity induced by LPS (data not shown). Thus, the region −975 to −722 contains elements important for the synergistic induction of iNOS by IFN-γ and LPS.

Analysis of the promoter sequence in the region −973 to −722 revealed the presence of five potential sites for binding of known IFN-γ-activated transcription factors. Four of these—one GAS, one IRF-E, and two ISRE (ISREa and ISREd) are clustered within the region −951 to −911 (Fig. 1A). A independent deletion analysis that was not presented was said to implicate the region −1029 to −913 (16).

A Promoter Construct Bearing a Site-specific Mutation in IRF-E Fails to Support Synergistic Activation by LPS and IFN-γ. The induction of iNOS mRNA by IFN-γ and LPS is blocked by inhibition of protein synthesis (28-30). Therefore, among the candidate sites for IFN-γ action identified above, we focused on IRF-E, because it is the only one for which the IFN-γ–induced intranuclear mobilization of binding factors is believed to depend on new protein synthesis. To test the role of the iNOS IRF-E, a site-specific mutation of two adjacent nucleotides in IRF-E was generated in pliNOS-CAT to generate the plasmid pl(IFEm) (Fig. 1B). The positions mutated are conserved among known IRF-1s (18). A similar substitution in ISRE, an element closely related in sequence to IRF-E but which binds transcription factors distinct from IRF-1, also led to its functional inactivation (31). To determine the effect of this mutation without a confounding effect of possible differences in the efficiency of transfection between the wild-type and mutant promoter constructs, we subcloned into both pliNOS-CAT and pl(IFEm) an independent
reporter gene encoding luciferase under the control of the constitutively active SV40 promoter, giving rise to the plasmids p1-luc and p1(\textit{IRFm})-luc. Transfection of RAW 264.7 macrophages with the latter plasmid preserved inducibility of LPS, but the synergistic contribution of IFN-\(\gamma\) was abrogated. In fact, a modest suppressive effect of IFN-\(\gamma\) on LPS-induced CAT expression was revealed (Fig. 2).

\textit{IFN-\(\gamma\) Induces the Appearance of Nuclear Proteins that Bind the IRF-E in the iNOS Promoter.} We next performed EMSAs with extracts of nuclei isolated from RAW 264.7 cells after 2 h of treatment with various stimuli. With a 48-bp probe containing four putative binding sites for IFN-activated transcription factors (Fig. 1A), two constitutive complexes were found in untreated cells (designated A and C in Fig. 3A). Nuclear extracts from IFN-\(\gamma\)-treated cells contained an additional complex, termed B (Fig. 3A). Complexes A and B were preserved but complex C was lost in EMSAs performed with a shorter probe consisting mostly of the IRF-E/ISRE\(_d\) sites. Reciprocally, complex C was preserved whereas complexes A and B were lost in EMSAs performed with a probe consisting mostly of the GAS/ISRE\(_u\) sites (Fig. 3A). These results suggested that constitutively active nuclear protein(s) bind both sets of sites, but that IFN-\(\gamma\) activates the binding of protein(s) specifically to the IRF-E or ISRE\(_d\) site to form complex B (Fig. 3A). Competition assays demonstrated that complexes A, B, and C each bound specifically in the pattern described above (Fig. 3B). Moreover, IFN-\(\gamma\)-inducible complex B was not bound to the probe containing the mutant IRF-E/ISRE\(_d\) site (Fig. 3A).

\textit{The IFN-\(\gamma\)-induced Promoter-binding Complex Contains IRF-1 Protein.} Nuclear extracts from IFN-\(\gamma\)-treated cells were treated with specific antibodies against the two known transcription factors whose activity is regulated by IFN-\(\gamma\) and that bind to GAS, ISRE, or IRF-E sites in the promoters.

![Figure 3](https://example.com/figure3.png)

\textbf{Figure 3.} Binding of proteins from nuclei of RAW 264.7 cells to iNOS promoter fragments. (A) The binding reaction was performed with nuclear extracts from cells treated for 2 h with or without the indicated stimuli at the concentrations shown in Fig. 2. The reaction was carried out with oligonucleotide probes consisting chiefly of all four binding sites (lanes 1-4), IRF-E/ISRE\(_d\) (lanes 5-8), GAS/ISRE\(_u\) (lanes 9-12) or mutant IRF-E\(^m\)/ISRE\(_{d\,m}\) (lanes 13-16). Positions of complexes A, B, and C are indicated by arrows. (B) The binding reaction was carried out in the absence (lanes 1-4) or presence of a 250-fold molar excess of oligonucleotide competitors containing IRF-E/ISRE\(_d\) (lanes 5 and 6) or GAS/ISRE\(_u\) (lanes 7 and 8) binding sites. The radiolabeled probe containing all four sites was used for the reaction. Cells were treated as in A.
Identification of IKF-1 in complex b. Nuclear extracts were incubated in the absence (lanes 1–4) or presence of preimmune IgG (lane 7 and 8) or specific anti-IKF-1 IgG (lanes 5, 6, 9, and 10). IgG was added in a final dilution of 1:10 (750 ng/reaction) or 1:250 (30 ng/reaction). In lanes 6, 8, and 10 IgG was added 60 min before the oligonucleotide, whereas in lanes 5, 7, and 9 IgG was added 30 min after the oligonucleotide.

of other genes, namely, STAT91 (32) and IRF-1 (19). Results were similar whether antibodies were added before or after nuclear proteins were allowed to bind to the oligonucleotide probes. Immune serum against STAT91 did not interfere with the formation of complexes a, b, or c (data not shown). In contrast, when the extracts were treated with anti-IRF-1 IgG, complex b disappeared. In its place, when relatively high amounts of IgG were used (750 ng/reaction), radioactive material was retained in the well (Fig. 4, lanes 5 and 6), whereas if 25-fold less IgG was used, radioactive material was distributed between the well and upper part of the gel lane. Preimmune IgG at the same concentrations had no effect. This specific “supershift” demonstrated that complex b contains IRF-1 protein. UV cross-linking of the IFN-γ-inducible complex b to the radiolabeled 48-nt probe revealed a heavily labeled protein migrating in SDS-PAGE at 40–45 kD, together with two faintly labeled species migrating at ~105 and 205 kD (Fig. 5).

Discussion

Many cell types have the capacity to produce large amounts of NO after exposure to immune or inflammatory stimuli. This may reflect a beneficial role of iNOS in mediating some of the antiviral actions of IFN-γ (33). However, the same enzyme is demonstrably autotoxic (6–10). Thus, its expression is likely to be highly regulated. Indeed, iNOS is regulated in the rate of its transcription (14–17), the stability and rate of translation of its mRNA, and the stability of the protein (34), as well as by undefined, nondegradative posttranslational means (35). The focus here on synergistic induction of transcription of iNOS does not exclude that the same stimuli may interact synergistically at posttranscriptional levels as well.

Induction of iNOS by LPS in RAW 264.7 cells involves the binding of NF-κB heterodimers p50/RelA and p50/RelA to the NF-κB site at −85 to −75 in the iNOS promoter (17). Here we have marshalled complementary evidence with regard to the action of IFN-γ in the same macrophage-like cell line. However, the extent of the synergistic interaction between LPS and IFN-γ in transfected cells was restricted (Fig. 2; and ref. 11, see Fig. 3) compared to what is seen with untransfected cells (14). Whereas we are not certain what causes this restriction, we emphasize that it is extremely difficult to prepare plasmid DNA for transfection from E. coli host cells without trace contamination by LPS. If mouse macrophages are pretreated with LPS in the picogram per milliliter concentration range, they express markedly decreased levels of iNOS mRNA when subsequently exposed to IFN-γ (36). Thus, preexposure to traces of LPS at the time of transfection may have blunted the subsequent inductive response. Within the limitations of a transfection-based reporter gene model, the evidence presented here establishes that the IKF-E at −913 to −923 and its binding of IKF-1 are critical molecular determinants of the action of IFN-γ on the iNOS promoter.

IKF-1 is activated by viral infection, double-stranded RNA, IFN-α, and IFN-γ (20, 37), and serves as a transcriptional activator for type I IFN genes as well as for a number of IFN-inducible genes, such as MHC class I (e.g., H-2Kb) and 2′-5′ oligoadenylate synthase (23, 24). However, all these genes are also regulated in part if not predominantly by other transcription factors, such as interferon-stimulated gene factor 3 (ISGF3) and gamma-activating factor (GAF), as such genes are induced normally in fibroblasts from IKF-1−/− mice (25). In contrast, the inability of macrophages from these animals
demonstrated that IRF-1 plays a critical physiologic role in the induction of iNOS. Kamijo et al. (26) showed further that a nucleotide element from the iNOS promoter could compete with the binding of recombinant IRF-1 to an idealized IRF-E concatemer. These authors, however, studied neither the IRF-1 promoter itself, nor transcriptional control proteins from nuclei of IFN-γ-treated cells. The present work establishes that IFN-γ mobilizes intranuclear IRF-1, that IRF-1 binds to the IRF-E in the iNOS promoter, and that these events are necessary for IFN-γ to enhance the iNOS promoter's transcriptional activity. Together with the results of Kamijo et al. (26), our study points to iNOS as the first specific gene for whose direct transcriptional regulation by IFN-γ, IRF-1 is found to be indispensable.

However, the role of IRF-1 in induction of iNOS may not be limited to its interaction with IRF-E in the iNOS promoter. In the studies of Kamijo et al. (26), IRF-1"promoters failed to express iNOS not only when stimulated with IFN-γ, but also when stimulated with LPS alone. Thus, IRF-1 is likely to participate, directly or indirectly, in the cycloheximide-sensitive assembly of the LPS-activated transcription factor complex on NF-κB which we have shown is involved in LPS-induced transcription of iNOS (17). For example, IRF-1 may regulate transcription of the unidentified 115-kD protein that participates in the formation of this complex (17).

Site-specific mutation of IRF-E within the context of the full-length iNOS promoter/enhancer region demonstrated the importance of this site in the response of iNOS to IFN-γ. In addition, mutation of IRF-E revealed the action of a silencer, in that LPS-induced expression of reporter gene activity was even higher than with the wild-type promoter. A likely candidate for the silencer element is IRF-E itself, and candidate inhibitory factors binding thereto are IRF-2 (38) and IFN consensus sequence binding protein (39), which act as constitutive repressors in other systems. Moreover, mutation of IRF-E converted IFN-γ's action on transcription from stimulatory to suppressive, suggesting the operation of an IFN-γ-activated silencing mechanism operating elsewhere than via wild-type IRF-E.

The inference that IRF-E supported the IFN-γ-induced binding of nuclear proteins was confirmed by EMSA analysis. The DNA probe containing IRF-E/ISRE sites formed two distinct complexes with nuclear proteins, A and B (Fig. 3 A). Both complexes were specific in that their formation was prevented by excess unlabeled oligonucleotide. While complex A was constitutive, complex B appeared only after exposure of the cells to IFN-γ. The substitution of two adjacent nucleotides conserved in the IRF-E prevented the formation of complex B, corroborating the functional analysis of the full-length promoter/enhancer with the mutation. A similar band pattern was reported with the IRF-E from the promoter of the H-2Kb MHC class I gene, whose IRF-E conforms to the iNOS IRF-E in 10/11 nucleotides (40). Likewise, a 2-bp substitution in the H-2Kd IRF-E blocked formation of the IFN-γ-inducible complex (although different positions were substituted than in iNOS), and IRF-1 (termed IBPi) was identified in the IFN-γ-inducible complex (40).

In the iNOS promoter, even though both complexes A and B formed on the same site (IRF-E/ISREs), we found no higher order complex corresponding to A and B, implying a competition between two binding factors. This interpretation is consistent with the observation that treatment with LPS and IFN-γ usually led to an increase in complex B at the expense of complex A (Fig. 3 A, compare lanes 2 and 4, or 6 and 8). Complex C formed constitutively on GAS/ISREs, reminiscent of the results of in vivo footprinting analysis of the overlapping GAS/ISRE sites in the promoter of guanylate binding protein (41). Complex C did not make a joint complex with A and/or B (Fig. 3). Thus, the binding of complexes, A, B, and C each appeared to be mutually exclusive.

Two observations supported the conclusion that complex C contained IRF-1—a supershift in EMSA with specific anti-IRF-1 antibody (Fig. 4), and UV-cross-linking of the 48-bp IRF-E-containing probe to complex B protein(s) (Fig. 5). The protein predominantly cross-linked to the labeled DNA migrated with an apparent molecular mass of 40–45 kD. The molecular mass of mouse IRF-1 deduced from its cDNA is 37.3 kD, but recombinant mouse IRF-1 migrated at 48 kD in SDS-PAGE (42). The two cross-linked polypeptides we observed migrating at ~105 and ~205 kD are reminiscent of the two species of >97 kD that copurified with human ISGF2 (22). These may be additional components of complex B whose contact with DNA was more limited than that of IRF-1. Alternatively, the higher relative molecular mass species may represent cross-linked oligomers of IRF-1 or contaminants of the binding reaction.

The identification of IRF-E as critical to the impact of IFN-γ on the iNOS promoter does not exclude a possible role of candidate IFN-γ-response sites downstream of position −975, including seven ISREs, three GAS sites, and an X box (15). To determine whether IRF-E alone mediates the positive effects of IFN-γ on the iNOS promoter would require mutating or deleting each of these elements.

Now that sites in the iNOS promoter have been identified for the transcriptional effects of both LPS and IFN-γ, it may be possible to define the precise mechanism of their interaction. For example, one or more proteins in the NF-κB-containing complex forming at −85 to −76, may make contact with protein(s) in the IRF-1-containing complex at −913 to −923. Such an interaction between NF-κB and ISGF2 (IRF-1) underlies the synergistic activation of the promoter of the HLA-B7 MHC class I gene by TNF and IFN-γ (43). However, whereas the NF-κB site and the IRF-E on the HLA-B7 promoter are only 10 bp apart, the cognate sites in the iNOS promoter are separated by 828 bp. The binding of the iNOS promoter that would presumably be required for interaction of NF-κB and IRF-E-binding proteins may be facilitated by the intervening regions with potential Z-DNA conformation (15).

The benign phenotype of uninfected mice with lifelong IRF-1 deficiency (25) raises the possibility that a temporary state of functional inactivation of IRF-1 might be tolerable.
in uninfected individuals with inflammatory disorders. Inhibition of IRF-1 binding would be expected to block expression of iNOS without interfering with expression of the neural and endothelial isoforms of NOS. Development of IRF-1 blockers could complement the screening for iNOS-specific substrate analog inhibitors, an approach that has yet to bear fruit.

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