STAT-1 and c-Fos interaction in nitric oxide synthase-2 gene activation

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gene activation.

INTERFERON-\gamma (IFN-\gamma) induces gene expression through activation of specific members of the Janus kinase 
(JAK) family, which in turn phosphorylate signal transducer and activator of transcription 1 (STAT-1; see Refs. 14 and 15). The phosphorylated STAT-1 mol- 
ecules form homodimers, translocate to the nucleus, and bind to \gamma-activated sites (GAS) in the 5'-flanking 
regions of genes, such as the nitric oxide synthase-2 
(NOS2) gene. The importance of STAT-1 for IFN sig- 
naling is clearly demonstrated by STAT-1-deficient mice, which fail to respond to IFNs and are conse- 
quently highly sensitive to microbial infection, which is 
the result of lack of induction of downstream target 
genes, such as NOS2 (5, 29, 32).

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plexes, comprised of homodimers of STAT-3, heterodimers of STAT-3 and STAT-1, and homodimers of STAT-1 (31, 46). STAT proteins demonstrate cooperative DNA binding not only with other STAT family members (e.g., STAT-1/STAT-2 and STAT-1/STAT-3; see Refs. 10, 14, and 16) but also with other proteins and transcription factors, including transcriptional activator specificity protein (SP)-1 and CCAAT enhancer binding protein (22, 23). Recently, physical association between STAT-3 and c-Jun on the α2-macroglobulin enhancer element has been shown to yield maximal enhancer functions (48).

Based upon this knowledge, we hypothesized that cooperative interaction between AP-1 and STAT-1 pathways may be important in the activation of IFN-γ-activated genes, such as NOS2. Here, we show that c-Fos rapidly interacts with STAT-1 after IFN-γ activation and the c-Fos/STAT-1 complex binds to the GAS element in close proximity to AP-1 sites located in a 665-bp region at 4.9 kb upstream of the transcription start site. Taken together, our findings support a physical interaction between c-Fos and STAT-1 and suggest a role for c-Fos and STAT-1 in transcriptional activation of NOS2 gene.

MATERIALS AND METHODS

NOS2 luciferase reporter constructs. A series of deletion constructs containing the NOS2 gene 5′-flanking region cloned into the pGL3-3-basic luciferase reporter gene vector (Promega, Madison, WI), and named pGL3–8296, -7196, -6251, -5574, -4909, -4711, -3137, and -336, or the pGL3–6251, -5574, -4909, -4711, -4060, -3137, and -336, or the pGL3–5060, -3137, and -336. Combinations of these constructs yielded the 5′-flanking NOS2 promoter that had undergone oligonucleotide-directed mutagenesis of AP-1-binding sites used in experiments (3, 26). All constructs were subjected to digestion with restriction enzymes and sequence analysis to verify the 5′-end of the insert.

Cell culture. HAEC were isolated from bronchoscopic brushing of the airway, or from surgical specimens of tracheas and main-stem bronchi, and cultured as previously described (11, 41). Primary cultures of passage 0–3 were used in experiments. The epithelial nature of primary and cultured cells was confirmed by immunocytochemical staining, as previously described (12). A549 cells, an epithelial cell line derived from lung adenocarcinoma, were cultured in MEM (Invitrogen, Carlsbad, CA) with 10% heat-inactivated FCS, or 24 h before cytokine stimulation with 1% FCS. A549 cells were maintained in DMEM (Invitrogen) with 10% FCS. Human IFN-γ was a gift from Genentech (South San Francisco, CA) or was purchased from R&D Systems (Minneapolis, MN). Recombinant human interleukin (IL)-1β and tumor necrosis factor (TNF)-α were purchased from Biosource (Camarillo, CA).

Transient transfection and luciferase assay. With the use of Lipofectamine Reagent (Invitrogen), 40% confluent HAEC and 30% confluent A549 cells in six-well plates were transfected with various NOS2 luciferase reporter constructs. Transfections were performed using equal amounts of DNA, as previously described (3). After adding the DNA with Lipofectamine to each well and incubating for 4 h for HAEC and 10 h for A549, the medium was replaced with normal growth medium for HAEC and MEM with 1% FCS for A549 cells. After (24 h) transfection, cells were exposed to cytokine mixture (CK). Later (24 h), cells were washed in PBS, harvested after the addition of 250 μl of 1× Passive Lysis Buffer, freeze-thawed two times, and centrifuged (12,000 g, 2 min). Supernatants were assayed for luciferase activity using the Dual-Luciferase Reporter Assay (Promega) in which luciferase activities are normalized by dual (Renilla) luciferase assay (Promega). In separate experiments, pCMV-β-Gal (Invitrogen) was used to determine the percentage of cells transfected. Relative luciferase activity is reported as
means of values from more than three independent experiments, each performed in triplicate.

The antisense phosphorothioated oligodeoxynucleotide (5'-CCGAGAACATCATCGTGGCG-3') was directed against the translation initiation site of c-Fos mRNA (40). Corresponding sense oligodeoxynucleotide (5'-CGCCACGATGATGTTCTCGG-3') was used as a control. With the use of Lipofectamine reagent, A549 cells were cotransfected with a 8,296-bp full-

![Graph A](image1)

![Graph B](image2)

Fig. 2. Effect of CK or IFN-γ on activity of human NOS2 promoter constructs and effect of mutation of the activator protein (AP)-1-binding sites in the NOS2 gene in A549 cells and HAEC. Serial-deletion luciferase constructs of the 5'-flanking region of human NOS2 gene and 8,296-bp full-length promoter with mutation of the AP-1 sites (mAP-1) fused to luciferase were transfected in A549 (A) or HAEC (B) and then incubated in the presence and absence of CK or IFN-γ alone. Data are expressed as the degree of induction compared with unstimulated cells (n ≥ 3 experiments).
length NOS2 promoter and the c-Fos antisense phosphorothioated oligodeoxynucleotide or sense oligodeoxynucleotide and then incubated in the presence or absence of CK for 6 or 12 h. Cells were harvested, and supernatants were assayed for luciferase activity.

With the use of Lipofectamine Reagent (Invitrogen), 293T cells or A549 cells were transfected with 2 µg DNA containing various expression vectors, HA-tagged STAT-1 (HA-STAT-1; see Ref. 34), HA-tagged TAK1 (HA-TAK1; see Ref. 37), or pRSV-c-Fos (33). The medium was replaced with 1% FCS after 10 h transfection. After transfection (24 h), cells were exposed to CK for 24 h, and then cells were washed in PBS and harvested.

EMSA. Whole cell extract (WCE) was prepared as previously described (11, 41). For nuclear extract, the cell suspensions were centrifuged and resuspended in 0.4 ml ice-cold buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, and 1 mM DTT] by gentle pipetting in a yellow tip, and then cells were allowed to swell on ice for 15 min. Subsequently, 25 µl of 10% solution of Nonidet P-40 was added, vigorously vortexed for 10 s, and then spun for 30 s in a microfuge. The nuclear pellet was resuspended in 50 µl ice-cold buffer [20 mM HEPES (pH 7.9), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 1 mM DTT].

The duplex oligonucleotides used in EMSA (Table 1) were synthesized by Operon (Alameda, CA) and then end-labeled with [γ[^32]P]ATP by polynucleotide kinase (11, 41). For binding reactions, cell extract was incubated in 20 µl total reaction volume containing 20 mM HEPES (pH 7.9), 5% glycerol, 50 mM NaCl, 5 mM DTT, 0.1 mM EDTA, 100 µg/ml BSA, and 2 µg polyoxyinosin-polyoxyctydilic acid (Amersham, Arlington Heights, IL) for 15 min at room temperature. The [^32]P-labeled oligonucleotide (2 × 10^6 counts/min) was added to the reaction mixture and incubated for 20 min at room temperature. The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.25 M buffer containing 12.5 mM Tris, 12.5 mM borate, and 0.5 mM EDTA. The gels were dried and analyzed by autoradiography. To demonstrate specificity of binding, competition was performed by adding unlabeled wild-type and mutated oligonucleotide at a 100-fold molar excess of[^32]P-labeled oligonucleotide probe in the binding reaction. To specifically identify AP-1, GAS binding-factor, and NF-κB proteins in binding complexes, 4 µg rabbit anti-c-Fos, FosB, Fra-1, Fra-2, c-Jun and JunD, STAT-3, STAT-5, p50 or p65 polyclonal antibody (Ab; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-STAT-1 polyclonal Ab (11, 13), or nonimmune rabbit IgG (Biosciences, Saco, ME) was added to the binding reaction mix and incubated for 30 min at room temperature before adding the [^32]P-labeled oligonucleotide. Antibodies used in experiments include anti-c-Fos Ab (rabbit polyclonal Ab against domain of c-Fos p62 of human origin; Santa Cruz Biotechnology), and c-Fos(2) Ab (rabbit polyclonal Ab against the amino terminus of c-Fos p62 of human origin; Santa Cruz Biotechnology).

Immunoprecipitation and Western blot analysis. Extracts were prepared by lysing the cells in ice-cold buffer containing 50 mM Tris (pH 7.9), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, 10% glycerol, 1 mM PMSF, 5 µg/ml leupeptin, 10 µg/ml pepstatin A, 200 µM NaOV, and 20 µg/ml lysed.
HAEC and A549 cells were transiently transfected with constructs containing various segments of the proximal 5'-flanking region of the human NOS2 gene driving luciferase expression. After transfection, cells were exposed for 24 h to 10,000 U/ml IFN-γ or CK containing 10,000 U/ml IFN-γ, 0.5 ng/ml IL-1β, and 10 ng/ml TNF-α or were left untreated. Transfection of pGL3-basic reporter constructs lacking promoter served as a negative control. Efficiency of Lipofectamine transfection in HAEC determined by β-galactosidase expression plasmid (pCMV-β-Gal) was 5 ± 2% cells/high-power field, whereas 15 ± 5% A549 cells were transfected. As previously shown (3, 26), the NOS2 promoter (regions containing 5,574 bp or greater up to 8,296 bp) was activated by CK in A549 cells, whereas CK inducibility of constructs in A549 cells was lost with the 4,909-bp or shorter regions of the 5' flanking promoter region construct (Fig. 2A). In contrast, full-length or deletion constructs of the NOS2 promoter were not activated by IFN-γ alone in A549 cells (degree of induction compared with unstimulated A549 cells of the 5,574-bp promoter construct: CK induced 1.4 ± 0.2; n ≥ 3). Similar to A549 cells, induction of the NOS2 promoter by CK in HAEC also occurred with 5,574-bp or longer constructs (Fig. 2B). However, IFN-γ alone activated the NOS2 promoter (regions containing 5,574 bp or greater up to 8,296 bp) in HAEC (degree of induction compared with unstimulated HAEC of the 5,574-bp promoter construct: CK induced 2.4 ± 0.3, IFN-γ induced 2.5 ± 0.2; n ≥ 3). CK or IFN-γ inducibility of constructs in HAEC was lost with the 4,909-bp or shorter regions of the 5'-flanking promoter.
region construct, indicating that the 665-bp region upstream of the 4,909-bp fragment contained important transcription regulatory elements (degree of induction compared with unstimulated HAEC of the 4,909-bp promoter construct: CK induced 1.2 ± 0.3, IFN-γ induced 1.4 ± 0.3; n = 3). In HAEC incubated with CK or IFN-γ alone, the luciferase activities increased at 24 h and were highest at 48 h (Fig. 3). Thus IFN-γ or CK similarly induced the NOS2 promoter in HAEC but not in A549 cells. Yet the important cytokine-responsive elements for IFN-γ or CK activation of the promoter in both A549 cells and HAEC were located within the 665-bp region between −5,574 and −4,909 bp.

Effect of mutation in AP-1-binding sequence of NOS2 gene in HAEC and A549 cells. Previous studies have demonstrated that the GAS element in NOS2 promoter is essential for IFN-γ activation (8, 9, 45). However, prior work has also emphasized the importance of AP-1-binding sites in activation of NOS2 promoter in response to cytokine combinations (3, 26). Multiple cytokine binding sites are present in the nucleotide sequence from −5,574 to −4,909 bp of the NOS2 promoter (Fig. 4). Based on the consensus sequence TTN$_5$AA (14), this region contains two putative GAS, two consensus-binding sites (TGANTCA) for AP-1 (3, 35), and two consensus-binding sites (GGGRNW-YYCC) for NF-κB (3, 39).

Fig. 6. NOS2 GAS binding activity in HAEC and A549 cells treated with IFN-γ. A: WCE from nonstimulated HAEC (lanes 1) or HAEC stimulated with IFN-γ for 30 min (lanes 2–10) was analyzed by EMSA using oligonucleotide bearing the sequence of the NOS2 GAS sequence motif. The specificity of the binding complex (arrow a) was assessed by the addition of a 100-fold molar excess of unlabeled wild-type GAS oligonucleotides (lane 3) before incubation with the labeled probe. Anti-c-Fos, c-Jun, STAT-1, STAT-3, STAT-5, or NF-κB p65 polyclonal Ab was added to binding reactions to identify proteins in the binding complex. IFN-γ led to binding activity (arrow a) that was supershifted by anti-STAT-1 (lane 7, arrow b) or anti-c-Fos Ab (lane 5, arrow c). Similar results were obtained in 3 separate experiments. B: WCE from HAEC stimulated with CK for 30 min (lanes 1–3), IFN-γ for 30 min (lanes 4–6), or TNF-α for 30 min (lanes 7–9) was analyzed by EMSA using the NOS2 GAS. CK or IFN-γ led to a prominent binding complex (arrow). STAT-1 and c-Fos were both present in the complex, as shown by supershift with anti-STAT-1 (lanes 3 and 6) and anti-c-Fos Ab (lanes 2 and 5). Similar results were obtained in a minimum of 3 separate experiments.
To determine whether AP-1 sites are important in induction of NOS2 in HAEC by IFN-γ alone, 8,296-bp full-length NOS2 promoter bearing three-base mutations in the AP-1-binding sites termed mAP-1 (26) was studied. HAEC and A549 cells transfected with mutated NOS2 promoter (mAP-1) were exposed to CK or IFN-γ for 24 h. As previously shown, A549 cells transfected with the AP-1 mutated construct had a marked decrease in luciferase activity with CK (Fig. 2A; see Ref. 26). Compared with the wild-type promoter construct, HAEC transfected with mutant AP-1 constructs had decreased induction by CK and, unexpectedly, by IFN-γ (Fig. 2B). These results suggested that CK or IFN-γ induction of the human NOS2 promoter requires AP-1 regulatory region(s).

STAT-1 and c-Fos interaction in IFN-γ induced NOS2 GAS activation. To determine which transcription factors bind to the GAS in the region from -5,574 to -4,909 bp of the NOS2 promoter, DNA-protein interactions were investigated by EMSA using extracts

Fig. 7. Identification of the NOS2 AP-1-binding proteins in A549 and HAEC by EMSA. A: EMSA of WCE from nonstimulated A549 (lane 1) or A549 stimulated with CK for 3 h (lanes 2–8) was analyzed using the radiolabeled NOS2 AP-1u oligonucleotide. Antibodies were added to reactions to identify binding proteins as indicated. Anti-c-Fos (lane 3), Fra-2 (lane 6), c-Jun (lane 7), or JunD (lane 8) led to significant supershift of the complex. No supershift was detected with FosB or Fra-1. The autoradiograph is representative of 2 independent experiments. B: EMSA of WCE from nonstimulated HAEC (lane 1) or HAEC stimulated with CK for 3 h (lanes 2–7) using radiolabeled AP-1u sequence. The specificity of the binding complex (arrow) was assessed by the addition of a 100-fold molar excess of unlabeled wild-type (lane 4) or mutant AP-1u (lane 3) oligonucleotide before the labeled probe. Anti-c-Fos, c-Jun, or STAT-1 polyclonal Ab was added to reactions to identify binding proteins. Anti-c-Fos (lane 5), anti-c-Jun (lane 6), and perhaps anti-STAT-1 (lane 7) led to supershift of the complex. The autoradiographs are representative of 3 experiments. C: NOS2 AP-1u and AP-1d binding activation in A549 cells by EMSA. WCE from nonstimulated A549 (lanes 1 and 4) or A549 stimulated with CK for 3 h (lanes 2–3 and 5–6) was analyzed by EMSA using radiolabeled oligonucleotide AP-1u or downstream (d) AP-1. Anti-c-Fos Ab was added to reactions to identify binding protein.
from HAEC or A549 cells after exposure to IFN-γ for 30 min. With the use of oligonucleotides bearing both the sequences of upstream AP-1 (AP-1u) and GAS elements in the NOS2 promoter (Table 1), WCE from HAEC had binding activity at baseline that increased by exposure of cells to IFN-γ (Fig. 5). Antibodies to c-Fos or c-Jun produced supershift of the complex, indicating that these proteins are present in the DNA-protein complexes. With the use of an oligonucleotide only bearing the sequence of the GAS element in the NOS2 promoter (Table 1), DNA binding activity was present in HAEC WCE (Fig. 6A) or A549 cell nuclear extract (data not shown) exposed to IFN-γ but not in nonstimulated cells. Antibodies against c-Fos or STAT-1 supershifted DNA-protein bands, indicating that both proteins are present in the binding complex. On the other hand, antibodies against STAT-3, STAT-5, c-Jun (Fig. 6), FosB, Fra-1, Fra-2, or JunD (data not shown) did not produce a supershift of the binding complex, indicating that no other members of the AP-1 complex (FosB, Fra-1, Fra-2, c-Jun, or JunD) interact with STAT-1. NF-κB binding sites overlap GAS in the NOS2 promoter. To determine whether NF-κB binding occurs in this region, EMSA was performed on WCE of TNF-α-stimulated A549 cells using an oligonucleotide containing the NOS2 NF-κB binding sequence that overlaps GAS (Table 1). WCE from CK- or IFN-γ-exposed cells contained binding activity to GAS that was the result of STAT-1 and AP-1 binding but not the result of NF-κB (Fig. 6B). Notably, competitive binding of STAT-1 and NF-κB has been demonstrated in the region of the GAS site such that STAT-1 precludes NF-κB binding (8).

To investigate AP-1 activation and which transcription factors bind to the AP-1 site, EMSA was also performed using oligonucleotide containing only the AP-1u binding sequence (Table 1). Basal AP-1 binding activity in nonstimulated A549 was low and significantly increased by exposure of cells to CK for 3 h (Fig. 7A). Anti-c-Fos, Fra-2, c-Jun, or JunD led to significant supershift of the complex. No supershift was detected with FosB or Fra-1 (Fig. 7A). WCE from HAEC had basal binding activity that was not appreciably increased by exposure to CK (Fig. 1B) or IFN-γ (data not shown). Antibodies to c-Fos or c-Jun supershifted the complex, indicating that c-Fos and c-Jun are activated and bind to the AP-1 element even in the absence of cytokine stimulation in HAEC. This basal AP-1 activation may explain, in part, why IFN-γ alone is sufficient to activate NOS2 in HAEC, whereas multiple cytokines are required in A549 cells. With the use of an oligonucleotide containing only the downstream AP-1 (AP-1d) sequence, binding activity determined by EMSA was much weaker with AP-1d than with AP-1u (Fig. 7C). This indicated that the AP-1u is more relevant to promoter activation.
Effect of c-Fos antisense phosphorothioated oligodeoxynucleotide on CK induction of human NOS2 promoter. To further test whether c-Fos is important in induction of NOS2, A549 cells were cotransfected with the 8,296-bp full-length NOS2 promoter and the c-fos antisense phosphorothioated oligodeoxynucleotide. Compared with the sense oligodeoxynucleotide and no oligo, CK induction of the NOS2 promoter was decreased significantly in A549 cells exposed to CK for 6 or 12 h ($P < 0.05$; Fig. 8), which indicates the essential role of c-Fos in NOS2 induction. However, STAT-1 and c-Fos overexpression did not produce any significant increase in NOS2 expression in A549 and 293T cells (data not shown).

Interaction between c-Fos and STAT-1 proteins. In the context that STAT-1 and c-Fos are present in the binding complexes with the GAS sites, we investigated the interactive binding of c-Fos and STAT-1, and whether c-Fos interaction with STAT-1 requires DNA-binding, using coimmunoprecipitation and Western blot analysis. The cell lysate of HAEC exposed to IFN-γ (400 μg total protein) was immunoprecipitated with an anti-c-Fos polyclonal Ab. The immunocomplex was resolved on an 8% SDS-polyacrylamide gel, and the immunoblot was probed with an anti-STAT-1 polyclonal Ab. Cell lysates of HAEC and A549 cells immunoprecipitated with anti-STAT-1 polyclonal Ab and probed with anti-STAT-1 polyclonal Ab served as a positive control, and the STAT-1-deficient fibrosarcoma cell line, U9A, served as a negative control. The time course (0–24 h) and the dose response to IFN-γ were evaluated. STAT-1 coimmunoprecipitated with c-Fos by 30 min after IFN-γ exposure (Fig. 9A). An IFN-γ dose of 100 U/ml was necessary to detect STAT-1 coimmunoprecipitation with c-Fos (Fig. 9B). Lack of coimmunoprecipitation at baseline suggests that STAT-1 activation/phosphorylation is required for c-Fos/STAT-1 interaction.

To further confirm the interactive binding of c-Fos and STAT-1, 293T cells were transfected with an expression construct for HA-STAT-1 (34) or as a control with the expression construct or HA-TAK1, a protein inhibitor of Jak 2 kinase, inhibits cytokine-induced NOS2 expression in a dose-dependent manner in A549 cells (8). These data suggest that the JAK-STAT pathway is involved in regulating cytokine or IFN-γ-induced NOS2 expression in lung epithelial cells. Furthermore, cotransfection with the dominant-negative STAT-1 expression vector significantly inhibits cytokine-induced NOS2 reporter expression (8), implicating STAT-1 as a positive regulator of NOS gene transcription in these cells.

DISCUSSION

In this study, we demonstrate the physical interaction between c-Fos and STAT-1, which participate in NOS2 gene transcriptional activation after IFN-γ activation. Fos and Jun family proteins usually function as dimeric transcription factors that bind to AP-1 regulatory elements [TGA(C/G)/TCA] in the promoter of numerous genes, including NOS2 (2, 35, 43). Jun proteins can form stable homodimers or heterodimers with Fos proteins, but Fos proteins do not form stable homodimers. Fos-Jun heterodimers bind DNA more stably than Jun homodimers (2, 28, 35, 43). Thus c-Fos heterodimerization with Jun family members enhances association of Jun proteins to DNA. Although other Fos family members may be capable of substituting functionally for c-Fos in c-Fos-deficient mice, our studies show that only c-Fos physically associates with STAT-1 after IFN-γ activation (17). Notably, AP-1 is activated in unstimulated HAEC in culture, with no appreciable increase in activation after stimulation with CK or IFN-γ. This suggests that basal AP-1 activation is sufficient to allow subsequent activation of NOS2 gene expression by IFN-γ alone in the HAEC; a unique feature of these cells (12, 42).

Previous studies indicate that genistein, a tyrosine kinase inhibitor of the JAK-STAT-1 pathway, abolishes induction of NOS2 by IFN-γ in airway epithelial cells (11), and tyrophostin A25, a pharmacological inhibitor of Jak 2 kinase, inhibits cytokine-induced NOS2 expression in a dose-dependent manner in A549 cells (8). These data suggest that the JAK-STAT pathway is involved in regulating cytokine or IFN-γ-induced NOS2 expression in lung epithelial cells. Furthermore, cotransfection with the dominant-negative STAT-1 expression vector significantly inhibits cytokine-induced NOS2 reporter expression (8), implicating STAT-1 as a positive regulator of NOS gene transcription in these cells.

Cooperative DNA binding of proteins usually involves regions in close proximity, which functionally represent a composite regulatory element (2, 48). In this study, the 100-bp region encompassing the GAS and AP-1 site of NOS2 promoter may serve as composite binding elements. These closely located sites support that heterodimeric c-Fos interaction with STAT-1 in binding complexes on DNA elements is important for maximal gene activation. Experimental support of this is provided by the decreased IFN-γ inducibility of the NOS2 promoter containing mutated AP-1 sites. Definitive evidence of physical association between c-Fos and STAT-1 is provided by coimmunoprecipitation of endogenously expressed or exogenously expressed...
factors from cells after exposure to IFN-γ. We speculate that STAT-1 binding may be facilitated on the GAS element through interaction with c-Fos, i.e., STAT-1 may more firmly associate with GAS, in part through interaction with c-Fos. In support of this concept, previous studies have shown that low-affinity and low-specificity Smad-family DNA binding proteins rely on interactions with other DNA-binding proteins, including Jun, to target them to specific regulatory DNA elements (2). Similarly, a previous study of IFN-γ induction of intercellular adhesion molecule-1 in primary human airway cells has shown that STAT-1 activation and DNA binding require the transcriptional activator SP-1 (23). Furthermore, transcription factor TFII-I can form protein-protein complexes with STAT-1, STAT-3, and serum response factor, which enhances the response of the c-fos promoter (19). Taken together, pairing of STAT-1 and c-Fos to the promoter provides maximal activation of NOS2 expression in cells.

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