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Synergistic Transcriptional Activation of Indoleamine Dioxygenase by IFN- γ and Tumor Necrosis Factor- α

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Abstract

Interferon- γ (IFN- γ)-induced indoleamine 2,3-dioxygenase (IDO) activity inhibits the growth of susceptible intracellular pathogens by catalyzing the oxidative cleavage of the indole ring of L-tryptophan and depleting pools of the essential amino acid. Tumor necrosis factor- α (TNF- α) synergistically enhances the IDO activity induced by IFN- γ at the level of transcription in human epithelial cells. The purpose of this study was to characterize the molecular mechanisms responsible for synergistic gene expression in response to IFN- γ and TNF- α . It was found that IFN- γ -induced mechanisms, such as the binding of Stat1 to gamma activation sequences (GAS) and IFN regulatory factor-1 (IRF-1) to IFN-stimulated response elements (ISREs), are more highly activated following treatment with IFN and TNF- α . This enhanced signal transduction may be due to the increase in IFN- γ receptor (IFNGR) expression following combined cytokine stimulation and is a likely contributor to the synergy. Additionally, the contribution of a third previously uncharacterized GAS element that forms a complex with Stat1 was investigated using a plasmid reporter system that controls for copy number. When the GAS-3 sequence was included in the regulatory region, gene expression was significantly increased relative to a region containing the mutated GAS-3. This suggests that GAS-3 is transcriptionally active and contributes to IFN- γ -induced regulation of the IDO gene.

INTRODUCTION

INTERFERON- γ (IFN- γ) SECRETED BY ACTIVATED Th1 and natural killer (NK) γ cells, induces production of antimicrobial enzymes, such as nitric oxide synthase (NO) (1) and indoleamine 2,3-dioxygenase (IDO). (2,3) IDO catalyzes the oxidative cleavage of the indole ring of L-tryptophan, converting it to N-formylkynurenine. (4) This activity depletes intracellular and extracellular pools of the essential amino acid, thereby restricting growth of susceptible pathogens, such as *Chlamydia* spp. (2,5,6) and *Toxoplasma gondii* (7,8) *in vitro*.

Molecular mechanisms responsible for IFN- γ -mediated IDO induction have been well characterized in human fibroblasts. (9–11) The region upstream of the IDO transcriptional start site contains numerous regulatory elements (Fig. 1), several of which have been characterized as important in IFN- γ responsiveness. There are two distantly separated IFN-stimulated response elements (ISRE-1 and ISRE-2), with the latter closest to the start of transcription. Two previously identified gamma activation sequences (GAS-1 and GAS-2) also reside close to each other just downstream of ISRE-1. Studies using reporter constructs have shown ISRE-1 and the nearby GAS-2 site in the IDO promoter are critical to IFN- γ responsiveness. (10) These elements interact with IFN regulatory factor-1 (IRF-1) and signal transducer and activator of transcription 1 α (Stat1 α), respectively, the former requiring new protein synthesis. (9) GAS-1 has been shown through mutational studies to be inessential in conferring responsiveness to IFN- γ . (9) ISRE-2, which also interacts with IRF-1, has also been shown to be important and

is a stronger transcriptional enhancer than ISRE-1. In a chloramphenicol acetyltransferase (CAT) reporter vector with an IDO promoter cloned upstream, deletion of ISRE-2 causes a greater decrease in activity than does deletion of ISRE-1.(11)

Studies using reporter constructs have been effective in characterizing regulatory regions important in conferring responsiveness to IFN- γ , yet differences between chromosomal and plasmid-associated copies of the promoter have been reported.(12) Thus, it is possible that the complete regulatory region has not been characterized. A third GAS element (GAS-3) exists at -1249 (Fig. 1), upstream of what was identified as an IFN- γ -responsive region, but its effect on IDO regulation has not been explored. This sequence could account for some of the observed differences in IFN- γ responsiveness with chromosomal and plasmid-associated copies of the promoter.

Although IDO is primarily an IFN- γ -induced gene, other inflammatory mediators that alone are ineffective in inducing IDO activity enhance the IFN- γ -mediated induction. Tumor necrosis factor- α (TNF- α), likely to be present within an infectious environment, synergistically enhances the IDO activity induced by IFN- γ in human macrophages(13) and epithelial cells.(14) In human macrophages, this increase has been correlated with enhanced IDO mRNA expression.(13) Furthermore, a green fluorescent protein (GFP) reporter construct transfected into human epithelial cells has shown increased transcriptional activity of the IDO promoter following treatment with combined IFN- γ and TNF- α (14) Increased activation of IFN- γ -induced signal transduction pathways, with the potential for cooperative interaction with elements of the TNF- α signal transduction pathway, may be responsible for the observed synergy.

It is important to identify the complete IDO regulatory region and fully understand regulation within an infectious context. The aim of this study was to identify molecular mechanisms at the transcriptional level that are responsible for the observed synergistic IDO expression in response to IFN- γ and TNF- α . This identification includes evaluation of a potential role for the third and previously uncharacterized GAS element in transcription of IDO.

MATERIALS AND METHODS

Cytokines and antibodies

Recombinant IFN- γ (specific activity 1.6×10^7 U/ml protein) was provided by Biogen (Cambridge, MA). Recombinant human TNF- α (50% effective dose [ED₅₀], the concentration of cytokine required for 50% maximal response = 0.02–0.05 ng/ml) was purchased from R&D Systems (Minneapolis, MN).

Polyclonal rabbit antihuman Stat1 (catalog 06–501) and antiphospho-Stat1 (Y701) (catalog 07–307) IgGs were purchased from Upstate Biotechnology (Lake Placid, NY). Goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (sc-2047), rabbit polyclonal antihuman IRF-1 (sc-497) IgG, and mouse monoclonal antihuman Stat1 (sc-417) IgG1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antirabbit IgG alkaline phosphatase-conjugated secondary antibody (A-9919) and phycoerythrin (PE)-conjugated goat antirabbit IgG F(ab')₂ was purchased from Sigma Chemical Company (St. Louis, MO). Rabbit polyclonal antihuman IFN- γ receptor (IFNGR) α chain IgG was purchased from Research Diagnostic, Inc. (Flanders, NJ). Y12 antibody is directed against a family of constitutively expressed human splicing factors (generous gift of Dr. Eileen Bridge, Miami University).

Primers

Oligonucleotide primers were synthesized on a MilliGen 7500 DNA synthesizer (Millipore, Bedford, MA) or purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Primers used in generation of reporter constructs were designed for insertion of the GAS-3 site or a mutated GAS-3 site and included unannealed nucleotides in the first round of amplification. The oligonucleotide sequences with the GAS-3 site underlined and individual base substitutions in boldface are as follows; GASinsIDO forward primer, 5'-GCGCATTAAATTCCTTGAACTGATTCCCCAAAGTATTAGCCTCATGAATCATGTAGTCATA-3'; MutGASinsIDO forward primer, 5'-GCGCATTAAAT**ATAGTTGA**ACTGATTCCCCAAAGTATTA GCCTCATGAATCATGTAGTCATA-3'; IDOprom reverse primer, 5'-GCAGGGCTAGCTCTTGTAGTCTGCTCCTCTGGTGCC-3'.

Other primer pairs were used to quantify specific mRNAs as follows: IDOcDNA primers, 5'-CCTGACTTATGAGAACATGGAGCT-3' and 5'-ATACACCAGACCGTCTGATAGCTG-3'; β -actin primers, 5'-GCACCACACCTTCTACAATGAG-3' and 5'-ATAGCACAGCCTGGATAGCAAC-3'; Stat1 primers, 5'-ATCACATTACATGGGTGGAG-3' and 5'-AAACTCCTCAGGAGACATGG-3'.

Cell culture

The HeLa 229 human epithelial cell line, obtained from American Type Culture Collection (Rockville, MD) was maintained in complete medium: Eagle's minimum essential medium (EMEM, BioWhittaker, Walkersville, MD) supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone, Logan, UT), 10 μ g/ml gentamicin sulfate (Sigma), and 100 μ g/ml streptomycin sulfate (Sigma) at 37°C in 5% CO₂.

Determination of IDO activity

HeLa cells (10⁵ cells/ml) were cultivated in 24-well tissue culture plates at a final volume of 1 ml in complete medium alone or medium supplemented with IFN- γ (0.1–30 ng/ml) with or without TNF- α (5 ng/ml) for 48 h at 37°C to induce IDO activity. Following incubation, the medium was replaced with 0.4 ml Hank's balanced salt solution (HBSS) containing 5-³H-tryptophan (1 μ Ci/ml) and a 25- μ M unlabeled tryptophan carrier. As a control to determine the background level of tryptophan decyclization, a triplicate set of wells received radiolabeled tryptophan containing HBSS. Culture plates were incubated another 4 h, and supernatants were collected. Samples were analyzed by reverse-phase high-performance liquid chromatography (HPLC) as described previously.(14)

Reverse transcription-PCR

HeLa cell monolayers in 60-mm plates were cultivated in medium alone or medium containing IFN- γ (10 ng/ml), TNF- α (5 ng/ml), or combined IFN- γ and TNF- α for 4 h or 24 h. Total cellular RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RNA (1 μ g) was used for first-strand synthesis in a 20- μ l reaction containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 0.5 mM dNTPs, 10 mM DTT, RNasin (20 U), 25 μ M random hexanucleotides, and 200 U SuperScript II (Invitrogen, Carlsbad, CA). Following incubation at 42°C for 50 min, the reaction was terminated by heating at 70°C for 15 min.

cDNA (2 μ l) was amplified in a 100- μ l PCR reaction containing 2.5 U *Taq* polymerase, 50 mM KCl, 10 mM Tris, pH 9.0, 2 mM MgCl₂, 0.4 mM dNTPs, 1 μ l α ³²P-deoxyadenosinetriphosphate (specific activity 800 Ci/mmol) (Perkin-Elmer, Boston, MA), and

0.2 μ M forward and reverse primers of IDO or Stat1 and β -actin. PCR amplification was performed in a Perkin-Elmer thermal cycler (Norwalk, CT) according to the following protocol: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min for 26 cycles, followed by a final 5-min extension at 72°C. PCR products (20 μ l) were separated electrophoretically on 8% polyacrylamide. Images were detected by scanning with a STORM[®] 860 PhosphorImager (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ). Band intensities were analyzed using ImageQuant[®] 5.2 software (Molecular Dynamics), and radioactivity in the IDO or Stat1 specific band was normalized to that of the β -actin band.

Preparation of nuclear extracts

HeLa cell monolayers in 100-mm plates were cultivated in medium alone or medium containing IFN- γ (10 ng/ml), TNF- α (5 ng/ml), or combined IFN- γ and TNF- α for 4 h, allowing sufficient time for production of IRF-1. Cells were harvested, and extracts were prepared as described previously,(15) with the following modifications. Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT) was substituted for hypotonic buffer, and nuclei were lysed in buffer C (20 mM HEPES, pH 7.9, 25% [v/v] glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 M EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 0.5 mM DTT). Nuclear fractions were also dialyzed in 200 volumes of buffer C. Nuclear fractions were aliquoted and stored at -70°C until used in immunoblots or binding assays. Protein concentrations were determined using a Bradford assay kit (Pierce Chemical Co., Rockford, IL).

Immunoblot analysis

Nuclear extracts were mixed in an equal volume of sample buffer (125 mM Tris, pH 6.8, 5% [w/v] SDS, 100 mM DTT, 25% [v/v] glycerol, and 0.1% [w/v] bromophenol blue), separated on denaturing 12% polyacrylamide, and transferred to nitrocellulose for probing. Y12 antibody was used in addition to antibodies specific for transcriptional activators to normalize nuclear protein. Because Y12 is specific for a family of constitutively expressed splicing factors unchanged by cytokine treatments, changes in transcriptional activators per nucleus could be assessed. ECF substrate (Amersham Biosciences) was applied for protein visualization. Images were detected by scanning with a STORM 860 PhosphorImager, and band intensities were analyzed using ImageQuant 5.2 software. Expression values are the ratio of transcription factor/splicing factor (29-kDa band).

Electrophoretic mobility shift assays (EMSA)

Synthetic 20-mer probes were created by annealing and end-labeling with α^{32} P-deoxycytidine triphosphate (specific activity 3000 Ci/mmol) (Perkin-Elmer) using the Klenow fragment of DNA polymerase I (5 U) (Takara Biomedicals, Otsu, Shiga, Japan) in a 15- μ l reaction mixture (10 mM Tris-HCl, pH 7.5, 7 mM MgCl₂, 0.1 mM DTT, H₂O, 25 mM each of dATP, dGTP, and dTTP). Radiolabeled products were purified on a G25 Sephadex column (Roche Diagnostics Corp., Indianapolis, IN). Normalized extract volumes were incubated 20 min at ambient temperature with radiolabeled probes (1 pmol), binding bufer (100 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10 mM DTT, 10 mM EDTA, 50% [v/v] glycerol, and H₂O), 1 or 6 μ g poly [dL-dC], 0.5 μ g yeast tRNA, and H₂O to an appropriate volume. For competition reactions, a 50-fold excess of unlabeled probe was included. For supershifting reactions, 1 or 2 μ g isotype control or specific polyclonal antibody was incubated with nuclear extracts for 1 h at ambient temperature prior to use in the binding assay. Reactions were separated on 5.5% polyacrylamide, low-ionic strength gels at 15 V/cm for 6 h at 4°C. Images were detected by scanning with STORM 860 PhosphorImager and quantified using ImageQuant 5.2 software.

Generation of reporter vector

The 1245-bp promoter region of the human IDO gene within the IDOp/EGFP reporter vector (14) was amplified by PCR according to standard protocols using the primers designed to insert GAS-3. The PCR product was ligated into the *Ase*I and *Nhe*I sites of the EGFP-C1 vector (Clontech, Palo Alto, CA), replacing the CMV IE promoter and creating GASinsIDOp/EGFP-C1 and mutGASinsIDOp/EGFP-C1. T4 DNA ligase (400 U) (New England BioLabs, Beverly, MA) was added to 10 μ l reaction mixture (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 μ g/ml bovine serum albumin [BSA], and H₂O) containing varied ratios of insert and vector. Ligation reactions (5 μ l) were transformed by standard techniques into *Escherichia coli* DH5 α made competent with RbCl. Transformants were selected by growth on Luria agar supplemented with kanamycin (30 μ g/ml) (Fisher Scientific, Pittsburgh, PA) at 37°C. Insertion of the IDO promoter was confirmed by restriction digestion and PCR amplification of plasmid purified using a minicolumn (Qiagen Inc., Santa Clarita, CA). Presence of the GAS-3 site was confirmed by sequencing on ABI Prism™ 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) in a reaction containing 500 ng plasmid template, 3.2 pmol forward primer, 3.5 μ l Terminator Ready Reaction Mix (PE Applied Biosystems), and H₂O to a final volume of 20 μ l.

Transfection and assessment of promoter activity in transfectants

The GASinsIDOp/EGFP-C1 and mutGASinsIDOp/EGFP-C1 constructs were purified using a maxicolumn (Qiagen) and transfected into HeLa cells using an Effectene transfection kit (Qiagen). HeLa cells (8×10^4 cells/well) were seeded into 24-well plates and cotransfected with 100 ng GASinsIDOp/EGFP-C1 or mutGASinsIDOp/EGFP-C1 and 100 ng DsRed-C1 (Clontech) to monitor copy number. Additionally, 200 ng of a nonfluorescent control plasmid, pcDNA (Invitrogen) was transfected to assess the effect of transfection on background fluorescence. Culture medium was replaced 18–24 h after transfection with medium alone or medium supplemented with cytokines. Following 48 h incubation, the cultures were harvested with EDTA/trypsin (200 μ l), pelleted, and resuspended in phosphate-buffered saline (PBS) (500 μ l) for analysis using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Enhanced GFP and DsRed2 were excited using an argon laser tuned to 488 nm. Green fluorescence was recorded in the FL-1 emission channel containing a 530-nm filter, and red fluorescence was recorded in the FL-2 emission channel containing a 580-nm filter. Cells expressing both red and green fluorescent proteins were selected, and CELLQuest software (Becton Dickinson) was used for analysis of the data. For assessing the effect of mutation within GAS-3, three replicates of GASinsIDOp/EGFP-C1 transfections and three replicates of mutGASinsIDOp/EGFP-C1 transfections for each cytokine treatment were analyzed. Three independent experiments were performed. The data were pooled, and a two-way analysis of variance (ANOVA) was used to assess the statistical significance of the differences in GFP expression.

Analysis of IFN- γ receptor expression

HeLa cells (10^6 cells/ml) were cultivated with IFN- γ (10 ng/ml), TNF- α (5 ng/ml), combined IFN- γ and TNF- α , or medium alone for 4 h at 37°C. The medium was replaced with 0.2% EDTA in PBS (w/v), and the cells were collected by gentle scraping with a rubber policeman. After fixing the cells in 1% p-formaldehyde for 20 min, the cells were washed and incubated with anti-IFNGR antibody for 45 min and PE-conjugated secondary antibody for 45 minutes and analyzed for receptor expression by flow cytometry.

RESULTS

Synergistic induction of IFN- γ -induced IDO

IFN- γ -induced IDO activity in a dose-dependent manner (Fig. 2, top). Following stimulation of HeLa cells for 48 h, specific tryptophan catabolism was detectable when stimulated with IFN- γ alone at 10 ng/ml, and much greater IDO activity was induced at 30 ng/ml. Furthermore, this activity was enhanced synergistically by TNF- α (Fig. 2, top). (13,14) To confirm that synergistically increased IDO activity in HeLa cells correspond to changes in IDO mRNA concentration, IDO mRNA was analyzed by RT-PCR at 24 h posttreatment, during the linear phase of induction (Fig. 2, top, inset). Although no IDO mRNA was detected in cells treated with medium or TNF- α (5 ng/ml) alone, it was detected in cells stimulated with IFN- γ . Furthermore, TNF- α increased the amount of IDO mRNA detected in IFN- γ -treated cells. When quantified IDO band intensities were normalized, TNF- α treatment increased the ratio of IDO/ β -actin from 1.0 to 1.5.

To correlate increases in IDO activity with a transcriptional reporter system containing the GFP coding sequence under regulation of the IDO promoter, (14) cytokine stimulation of GFP production was assessed by flow cytometry in parallel (Fig. 2 bottom) to measurements of IDO activity. Although TNF- α alone had no effect, it increased the mean fluorescence intensity of IFN- γ -treated cells at each IFN- γ concentration tested. Because this is a transcriptional construct lacking any IDO translational IDO signals, it suggests the synergy to be at the level of transcription.

IFN- γ and TNF- α increase translocation of IFN- γ -induced transcriptional activators to nucleus

Because IDO is induced primarily by IFN- γ , there may be increased translocation of IFN- γ -induced activators to the nucleus following exposure to combined IFN- γ and TNF- α treatment. Because IRF-1 is important in IDO regulation (9,10,11) and must be synthesized following IFN- γ treatment, nuclear extracts were prepared as described from HeLa cells left untreated or exposed to IFN- γ , TNF- α , or combined IFN- γ and TNF- α for 4 h. Concentrations that showed clear and reproducible synergy below saturable transcriptional activity were based on data from a previously published study. (14) Nuclear extracts were subjected to immunoblot analysis, and the band intensities were quantified.

The activated, tyrosine-phosphorylated form of Stat1 protein was present in the nucleus after treatment with IFN- γ , and its concentration was increased 5-fold following treatment with IFN- γ and TNF- α (Fig. 3A). To determine if the increase in nuclear phosphorylated Stat1 in cells treated with a combination of IFN- γ and TNF- α was due to increased synthesis of Stat1, RT-PCR was used to quantify Stat1 mRNA levels (Fig. 3B). IFN- γ alone increased the ratio of Stat1/ β -actin mRNA 7.5-fold. The addition of TNF- α had no effect on that increase. This suggests that the TNF- α -enhanced increase in nuclear phosphorylated Stat1 was not due to changes in Stat1 gene expression. IRF-1 also was present in the nucleus following treatment with IFN- γ and increased >2-fold following the addition of TNF- α (Fig. 3C). These results indicate that IFN- γ -induced transcription factors are more abundant in the nucleus following treatment with combined IFN- γ and TNF- α than with either IFN- γ or TNF- α alone. Furthermore, the increase in activated Stat1 appears to be the result of enhanced Stat1 phosphorylation and not of changes in Stat1 mRNA concentration.

Increased binding of IRF-1 to ISREs following treatment with IFN- γ and TNF- α

EMSA was performed to assess the binding of Stat1 and IRF-1 to specific enhancer elements within the regulatory region of IDO (Fig. 1). Radiolabeled probes (20-mer) comprising the binding sequence and flanking nucleotides were incubated with nuclear extracts and subjected

to electrophoresis in 5.5% acrylamide, low ionic strength gels. IRF-1 binds ISRE (consensus AGTTTCNNTTTNCC/T), two of which exist upstream of IDO (Fig. 1). Although both the ISRE-1 and ISRE-2 probes were shifted, albeit weakly, by the nuclear extract from IFN- γ -treated cells, band intensity was much stronger with the nuclear extract from cells treated with combined IFN- γ and TNF- α (Fig. 4A). Furthermore, when comparing band intensities of the two sequences, IRF-1 appeared to have a stronger affinity to the ISRE-2 probe.

A representative supershift and competition experiment demonstrated specificity for both the ISRE-2 probe and the bound complex. Similar results were obtained with the ISRE-1 probe. The addition of IRF-1-specific polyclonal antibody resulted in a higher molecular weight complex, but isotype control antibody did not impede migration of the ISRE-2 bound complex (Fig. 4B), confirming the identity of the bound protein. Including a 50-fold excess of unlabeled ISRE-2 eliminated most of the binding to the radiolabeled probe (Fig. 4C, cold), whereas a 50-fold excess of unlabeled irrelevant probe had no effect on formation of the binding complex (Fig. 4C, irr). Consistent with immunoblot data showing an increase in IRF-1 in the nucleus following exposure to IFN- γ and TNF- α , there was increased binding to the ISRE sites upstream of IDO.

Increased binding of Stat1 to GAS following treatment with IFN- γ and TNF- α

Phosphorylated Stat1 dimers bind the palindromic GAS (consensus TTNCNNNAA), and strong binding was exhibited to both the GAS-2 and GAS-3 probes with IFN- γ -treated extracts (Fig. 5A). Consistent with an increase of Stat1 in the nucleus in response to combined IFN- γ and TNF- α (Fig. 3A), there was increased binding to the GAS probes (Fig. 5A). Specificity for the GAS probes and the bound complex was again demonstrated by supershift and competition experiments. A representative experiment with the GAS-2 probe is shown in Figure 5, and similar results were obtained with the GAS-3 probe. Two different antibodies were used to identify Stat1. The complex was supershifted by antibody that binds outside the DNA-binding domain (Fig 5B, shift), but antibody recognition in the DNA-binding domain blocked binding to the GAS-2 probe (Fig 5B, block). Isotype control antibody did not further restrict migration of the Stat1 complex (Fig 5B). A 50-fold excess of unlabeled GAS-2 probe as a competitor eliminated binding to the radiolabeled probe (Fig. 5B, cold), but a 50-fold excess of unlabeled irrelevant probe had no effect (Fig. 5B, irr). Thus, both GAS-2 and GAS-3 bind Stat1 specifically.

Functional role of GAS-3

Because Stat1 forms a complex with the previously uncharacterized GAS-3, this enhancer element may be involved in transcriptional activation of IDO. To examine this possibility, bases in GAS-3 critical to interaction with Stat1 were mutated. These mutations prevented formation of a binding complex (Fig. 6A). To quantify the effect of GAS-3 on IDO regulation, the region including the GAS-3 sequence or a mutant GAS-3 sequence was cloned upstream of the coding sequence for GFP. Both GASinsIDOp/EGFP-C1 and mutGASinsIDOp/EGFP-C1 were cotransfected into HeLa cells with DsRed-C1 for monitoring plasmid copy number. The DsRed-C1 construct expresses red fluorescent protein constitutively from a CMV IE promoter. Following an appropriate incubation time to allow for uptake of the plasmids, the cells were treated in triplicate with medium alone or medium supplemented with IFN- γ (10 ng/ml), TNF (5 ng/ml), or IFN- γ and TNF- α combined for 48 h. Red and green fluorescence was quantified by flow cytometry and assessed for statistical significance by a two-way ANOVA. Three independent experiments comparing GASins-IDOp/EGFP-C1 with mutGASinsIDOp/EGFP-C1 revealed 26% greater GFP expression in IFN- γ -treated cells when the native sequence was present than when the mutated sequence was used ($p < 0.05$) (Fig. 6B). Likewise, 15% greater GFP expression was observed in cells containing the native sequence with IFN-

γ and TNF- α ($p < 0.05$). Thus, the GAS-3 element contributed to transcriptional activation from the IDO promoter.

TNF- α increases IFNGR surface expression

Tyrosine residues in the cytoplasmic tail of the IFNGR γ -chain become phosphorylated by Janus kinases in response to coupling of the receptor and ligand.(16) These tyrosine residues then serve as docking sites for Stat1 phosphorylation by Janus kinases.(17) Inasmuch as TNF- α increased Stat1 phosphorylation without affecting Stat1 mRNA concentration (Fig. 3), it is possible that the TNF- α -enhanced IFN- γ signaling may originate from greater recruitment of Stat1 as a result of increased IFNGR expression. To explore this possibility, cells were treated with medium alone or medium supplemented with IFN- γ (10 ng/ml), TNF- α (5 ng/ml), or IFN- γ and TNF- α combined for 4 h and then analyzed for IFNGR expression by flow cytometry. Although a slight increase in surface expression was observed with TNF- α alone (Fig. 7), this increase was not statistically significant. However, a substantial and highly significant ($p < 0.003$) increase was observed following treatment with IFN- γ and TNF- α . This suggests the possibility that the synergistic increase in Stat1 activation could be a direct result of enhanced interaction between IFN- γ and its receptor because of greater receptor density on the cell surface.

DISCUSSION

IDO is an important immune effector mechanism for the elimination of specific intracellular pathogens.(2,5–8) IFN- γ is the major inducer of IDO activity,(4) and its effect can be upregulated by a number of proinflammatory mediators, including lipopolysaccharide (LPS), interleukin-1 (IL-1), and TNF- α .(13,18) Although the mechanism of this synergy has not yet been defined, previous studies in human macrophages showed that increases in IDO activity had a concomitant increase in IDO mRNA. Furthermore, in cells transfected with a GFP reporter regulated by the IDO promoter, TNF- α and IL-1 upregulated IFN- γ -induced GFP. (14) In this study, changes in IDO activity in epithelial cells were found to correlate with increased promoter activity and increased IDO mRNA concentration, suggesting that the proinflammatory mediators enhance transcriptional activation of the IDO gene. To further characterize the mechanism responsible for the synergy, changes in specific transcription factors and their interaction with regulatory elements in the IDO regulatory region have been explored. Although IFN- γ and TNF- α have independent signal transduction systems, each of which activates distinct transcription factors, the results here suggest that more highly activated IFN- γ -induced signal transduction mechanisms underlie the synergistic induction of IDO activity by IFN- γ and TNF- α . Both IFN- γ -inducible transcriptional activators Stat1 and IRF-1 were more abundant in the nucleus following treatment with combined IFN- γ and TNF- α than with either cytokine alone. The synergistic increase in phosphorylated Stat1 was not the result of enhanced transcription of Stat1 mRNA. TNF- α did not increase the amount of Stat1 mRNA found in IFN- γ -treated cells. This suggests the possibility that the increase in Stat1 activation was at the level of the IFNGR. When examined, a nearly 2-fold increase in receptor expression was observed in response to combined IFN- γ and TNF- α treatment. Thus, it seems likely that an enhanced clustering of IFNGRs in response to ligand binding could mediate a stronger signaling response demonstrated by increased Stat1 phosphorylation.

Increased IRF-1 in the nucleus is consistent with a synergistic increase in IRF-1 mRNA concentration reported in other cell types after exposure to IFN- γ and TNF- α .(19) This increase in nuclear IRF-1 may be a direct consequence of the increase in activated Stat1 in the nucleus. Stat1 translocates to the nucleus within minutes of IFN- γ binding(16) and can bind to the GAS enhancer upstream of the IRF-1 coding region.(19) In this way, an increase of Stat1 in the

nucleus can upregulate expression of IRF-1, and this again illustrates the importance of the IFNGR increase and subsequent Stat1 phosphorylation.

Although the increased presence of transcription factors within the nucleus correlates with synergistic IDO induction, they must also bind to specific enhancer elements present in the IDO regulatory region. EMSA revealed binding of Stat1 and IRF-1 to specific enhancer elements upstream of IDO (Fig. 1), and the binding, in correlation with immunoblot data, is increased in response to treatment with combined IFN- γ and TNF- α . IRF-1 appeared to have a higher affinity for the ISRE-2 site, which is consistent with previously published data showing the ISRE-2 site to be a stronger transcriptional enhancer.(11) This was not a result of isotope intensity or exposure time. Both probes were labeled on the same day with isotope stock of the same specific activity and separated electrophoretically on the same gel. The additional nucleotide that separates the two core recognition sequences (GAAA)(20) in the ISRE-1 site may account for the reduced affinity.

There are three GAS sites upstream of IDO. GAS-1 has been shown previously to be inactive (10) and is supported by the absence of protein binding to the GAS-1 probe by nuclear extracts from IFN- γ -treated cells (data not shown). GAS-2 is critical in IFN- γ -mediated IDO induction, and this is the first characterization of GAS-3. From these results, it is clear that Stat1 becomes more abundant in the nucleus with the combination of IFN- γ and TNF- α , resulting in increased binding to GAS-2 and GAS-3 sites upstream of IDO. In addition, GAS-3 has been shown to have a role in transcriptional activation. When the GAS-3 site is present upstream of the GFP coding region, there is a significant increase in gene expression over that obtained with a mutated GAS-3 that abrogates binding. The absence of this site may contribute to the differences seen in previous comparisons of chromosomal and plasmid-associated copies of the IDO regulatory region.(12) When an IFN- γ -resistant cell line defective in Stat1 expression was cotransfected with pIDO-CAT(11) and a Stat1 expression vector, change in IDO activity in the cells did not correlate with change in CAT activity,(12) suggesting differences in regulation between the promoters on the plasmid and on the chromosome. The regulatory region cloned upstream of the CAT reporter did not contain the GAS-3 enhancer, which is present upstream of the IDO coding region on the chromosome.

In as much as IDO is an immunologic defense mechanism that would be induced in response to cytokines produced as a result of infection, it is important to elucidate the molecular mechanisms involved in IDO induction within an infectious context. IFN- γ and TNF- α are proinflammatory cytokines, and the synergy in IDO expression exhibited between them is likely to occur within the infectious environment. Although *Chlamydia* is inhibited by IDO activity *in vitro*, persistence of chlamydial infections worldwide suggests that the pathogen possesses a means of avoiding the otherwise effective IDO activity *in vivo*. To address how *Chlamydia* may evade an IDO response, it is necessary to fully characterize IDO regulation. This study has shown that an increase in the activation and binding of IRF-1 and Stat1 to ISRE and GAS sites, respectively, contributes to the synergy. It is not yet clear whether increased IFN- γ -induced signal transduction is solely responsible for the synergy, and experiments are underway to identify other potential mechanisms mediated by TNF- α . Although no NF- κ B consensus site is present within the cloned IDO regulatory region, one has been identified along with a GAS site upstream of the IRF-1 gene. As NF- κ B activation, translocation to the nucleus, and subsequent binding within the IRF-1 promoter have been demonstrated following TNF- α treatment,(19) NF- κ B may help mediate the increase in IRF-1 reported in this study.

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FIG. 1.

The indoleamine 2,3-dioxygenase (IDO) regulatory region. A scaled diagram of the 1300 nucleotides upstream of the IDO transcriptional start site is presented. Previously characterized ISREs and GAS-3 are identified by black boxes. A region comprising 700 nt determined to be unresponsive to IFN- γ is represented by the slanted lines.

FIG. 2.

Synergistic IDO induction. Triplicate samples of HeLa cells were treated in parallel with increasing concentrations of IFN- γ (0.1–30 ng/ml, squares) alone or combined with TNF- α (5 ng/ml, circles) for 48 h. **(Top)** One set of samples was analyzed for IDO activity by HPLC. **(Bottom)** The other set was analyzed for GFP by flow cytometry. The results (mean $\pm$ SD) from one of three similar experiments are presented. **(Inset)** IDO mRNA amounts were assessed by RT-PCR following 24 h treatment with medium alone (med), IFN- γ at 10 ng/ml (IFN), TNF- α at 5 ng/ml (TNF), or combined IFN- γ and TNF- α (IFN + TNF) in culture medium. β -actin mRNA served as an internal control for normalization.

FIG. 3.

Effect of TNF- α on IFN-mediated translocation of transcriptional activators. HeLa cells were cultivated with medium alone (med), IFN- γ at 10 ng/ml (IFN), TNF- α at 5 ng/ml (TNF), or combined IFN- γ and TNF- α (IFN + TNF) in culture medium for 4 h. (A) Nuclear extracts were prepared as described, separated on denaturing 12% polyacrylamide, transferred to nitrocellulose, and probed antiphosphorylated Stat1. To normalize for nuclear protein concentration, gels were also probed with Y12 antibody specific for constitutively expressed splicing factors. (B) Total RNA was isolated from 4-h cultures, and Stat1 mRNA amounts were assessed by RT-PCR. β -actin mRNA served as an internal control for normalization. (C) results obtained from nuclear extracts following probing with anti-IRF-1.

FIG. 4.

Effect of TNF- α on IFN-mediated binding of IRF-1 to ISREs. **(A)** Nuclear extracts from cells cultivated with medium alone (med), IFN- γ at 10 ng/ml (IFN), TNF- α at 5 ng/ml (TNF), or combined IFN- γ and TNF- α (IFN + TNF) were prepared as described, incubated for 20 min with synthetic probes for ISRE-1 or ISRE-2, separated on 5.5% polyacryl-amide, and subjected to phosphorimaging. The IRF-1-specific migration of the band is indicated by an arrow. **(B)** For supershifting reactions, nuclear extracts were incubated with 1 μ g specific polyclonal antibody (anti IRF-1) or isotype control (iso Ab) for 1 h at ambient temperature prior to incubation with the ISRE-2 probe. The supershifted complex is indicated by an arrow. **(C)** For competition reactions, a 50-fold excess of unlabeled ISRE-2 probe (cold) or irrelevant probe (irr) was included.

FIG. 5.

Effect of $\text{TNF-}\alpha$ on IFN-mediated binding of Stat1 to GAS. **(A)** Nuclear extracts from cells cultivated with medium alone (med), IFN- γ at 10 ng/ml (IFN), $\text{TNF-}\alpha$ at 5 ng/ml (TNF), or combined IFN- γ and $\text{TNF-}\alpha$ (IFN + TNF) were prepared as described, incubated for 20 min with synthetic probes for GAS-2 or GAS-3, separated on 5.5% polyacrylamide, and subjected to phosphorimaging. The Stat1-specific migration of the band is indicated by an arrow. **(B)** For supershifting reactions, nuclear extracts were incubated with 2 μg specific polyclonal antibody (shift or block) or isotype control (iso Ab) for 1 h at ambient temperature prior to incubation with the GAS-2 probe. The supershifted complex is indicated by an arrow. For competition reactions, a 50-fold excess of irrelevant probe (irr) or unlabeled GAS-2 probe (cold) was included.

FIG. 6.

GAS-3 contributes to IFN-mediated IDO induction. **(A)** Nuclear extracts from cells cultivated with IFN- γ at 10 ng/ml in medium were prepared as described, incubated for 20 min with synthetic probes for GAS-3 (GAS-3, TTCCTTGAA) or mutated GAS-3 (GAS-3mut, ATAGTTGAA). (Base changes in the mutated sequence are underlined.) **(B)** HeLa cells were cotransfected in triplicate with GASinsIDOp/EGFP-C1 (GAS-3) or mutGASinsIDOp/EGFP-C1 (GAS-3mut) reporter vectors and DsRed-C1. Following 18–24 h for plasmid uptake, transfected samples were cultivated with medium alone, IFN- γ at 10 ng/ml, TNF- α at 5 ng/ml, or IFN- γ and TNF- α for 48 h and analyzed for fluorescent protein expression by flow cytometry. Fold increase in GFP expression was calculated by comparing the mean fluorescence intensity of cytokine-stimulated cells relative to that of unstimulated cells. The results (fold increase $\pm$ SD) from three experiments are presented.

FIG. 7.

Effect of $\text{TNF-}\alpha$ on IFNGR expression. Triplicate plates of HeLa cells (10^6) were cultivated with medium alone (med), IFN- γ at 10 ng/ml (IFN), $\text{TNF-}\alpha$ at 5 ng/ml (TNF), or combined IFN- γ and $\text{TNF-}\alpha$ (IFN + TNF) in culture medium for 4 h. Cells were analyzed for IFNGR expression by flow cytometry. The results (mean fluorescence intensity $\pm$ SD) from one of three similar experiments are presented.