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A3G (APOBEC3G) has broad antiviral activity against retroviruses and hepatitis B virus. However, the role of IFNs in regulating A3G during innate immunity has not been established. In this study, we show that the A3G gene is uniquely regulated by IFNs in a cell type-dependent manner. A3G was up-regulated by IFN-α in liver cells and macrophages, but not in T lymphoid cells or epithelial 293T cells. In contrast, other IFN-α-stimulated genes such as dsRNA-activated protein kinase were induced in all these cells, suggesting additional cellular factors may regulate IFN-α-induced A3G expression. Consistent with this idea, IFN-α-mediated induction of A3G, but not other IFN-α-stimulated genes, was potently inhibited by the drug Rotterlin, through a mechanism independent of STAT1 activation. The canonical IFN-α-mediated pathway of gene transcription requires both STAT1 and STAT2. Surprisingly, induction of A3G was STAT1 independent, but STAT2 dependent in liver cells. However, STAT1 signaling was functional and required for IFN-γ induction of A3G in these cells. Our results indicate that A3G may participate in antiviral cellular defenses through a novel IFN-mediated signaling pathway.

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STAT1-Independent Cell Type-Specific Regulation of Antiviral APOBEC3G by IFN-α

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APOBEC3G (A3G) is a member of the APOBEC family of proteins with homologous cytidine deaminase domains (1). Although its cellular target(s) is not known, A3G is a potent antiviral protein that can suppress HIV type 1 (HIV-1),³ hepatitis B virus (HBV), and endogenous retro-elements (2–8). The antiviral mechanism of A3G against HIV-1 has been well studied. In the absence of the viral Vif protein, the A3G cytidine deaminase converts cytidines to uridines in single-stranded viral cDNA during reverse transcription, resulting in lethal hypermutation of the virus genome (9–13). The HIV-1 Vif protein, however, can degrade A3G through a proteasome-dependent pathway (14–20) involving the Cullin5-containing E3 ubiquitin ligase (14). By targeting A3G for degradation in the virus-producing cell, Vif is able to prevent A3G molecules from incorporating into virions, where they would otherwise be carried into the newly infected cell to inhibit productive infection. Although HBV is a DNA virus, it replicates through reverse transcription and can likewise be targeted by A3G (21, 22).

Although many studies have focused on the antiviral effects and posttranslational regulation of A3G, little is known about how it is transcriptionally regulated. It is not known whether A3G may be induced in vivo in response to inflammation or cytokines, and whether A3G participates in IFN-mediated host defenses is unclear. One previous study found that A3G was induced by PMA, but not induced by IFNs in an immortalized CD4+ T cell line H9 (23). However, recent studies reported that A3G could be induced by IFNs in liver cells (24, 25) and macrophages (26). The effects of IFNs on A3G transcription in primary CD4+ T cells have not been reported. In the present study, we surveyed the ability of IFNs to up-regulate A3G in various cells, including various primary cells (hepatocytes, macrophages, and CD4+ T cells) and cell lines (liver carcinoma cells, H9 cells, and 293T cells). All of these cells, except T cells, up-regulated A3G mRNA in response to IFN-α. Only liver cells consistently responded to IFN-γ.

IFNs are capable of inducing hundreds of genes, some of which may have the potential to affect the viral life cycle. Therefore, understanding how A3G might be uniquely regulated would help to distinguish its role in antiviral defense from that of other IFN-stimulated genes (ISG). IFN-α and IFN-γ signal through separate type I and type II IFN receptors, respectively, resulting in phosphorylation of the associated Jak. (For reviews of Jak/STAT signaling pathways, see references 27–32). The result is a cascade of signaling events through the STAT family of proteins to induce transcriptional activation of ISG. The classical models assert that IFN-α induces transcription through a STAT1:STAT2 heterodimer in complex with the IFN regulatory factor (IRF)-9 cofactor, which binds to an IFN-stimulated response element (ISRE) in the promoter of IFN-α-responsive genes. The IFN-γ pathway uses the STAT1 homodimer without a cofactor, which binds to the γ-activated sequence in gene promoters. Many ISG are inducible by either IFN-α or IFN-γ exclusively, but some genes (e.g., low molecular mass polypeptide (LMP2)) are inducible by both cytokines (29, 33). In our study, we showed that IFN-α uses a novel STAT1-independent pathway to up-regulate A3G, along with certain other ISG, in liver cells. In addition, transcriptional activation of A3G was uniquely sensitive to the drug Rotterlin in liver cells through a mechanism independent of STAT1 activation. Although STAT1-independent pathways of IFN-γ transcriptional activation are known, to our knowledge this will be the first report of a STAT1-independent pathway of IFN-α-mediated transcriptional activation of antiviral genes. Therefore, our results demonstrate that A3G

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3 Abbreviations used in this paper: HIV-1, HIV type 1; HBV, hepatitis B virus; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISGF3, ISG factor 3; ISRE, IFN-stimulated response element; LMP, low molecular mass polypeptide; PKC, protein kinase C; PKR, dsRNA-activated protein kinase; qRT-PCR, quantitative real-time RT-PCR; siRNA, small interfering RNA.
may exert its antiviral activities through a novel IFN-mediated signaling pathway.

Materials and Methods

Cells

Freshly isolated primary hepatocytes from anonymous donors were obtained from BD Biosciences. Hepatocytes were received within 24–48 h of isolation as adherent cultures in 6-well plates in Hepatostim medium (BD Biosciences) and used immediately for induction studies. Hep3B, HepG2 (American Type Culture Collection), and HuH7 (a gift from C. Rice, The Rockefeller University, New York, NY) are hepatocellular carcinoma cell lines. The 293T cells were obtained from American Type Culture Collection. The U3A are STAT1-negative and U6a are STAT2-negative cells derived from the parental 2TGH cells (a gift from G. Stark, Cleveland Clinic Foundation, Cleveland, OH). All cell lines were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS. Primary CD4+ T cells were purified from freshly isolated PBMC by incubation with CD4-conjugated magnetic microbeads (Miltenyi Biotec), according to the manufacturer’s instructions, and cultured in RPMI 1640 with 10% FBS. To obtain macrophages, freshly isolated PBMC were plated in 6-well plates overnight at 2 × 10^5 cells/ml in RPMI 1640 with 10% FBS, after which nonadherent cells were removed and the medium was replaced every 2 days. Macrophages, differentiated via adherence to the plastic, were used on day 12 after isolation for induction studies.

Cytokines, Abs, and inhibitors

All cytokines and inhibitors were obtained from EMD Biosciences. IFN-α and IFN-γ were dissolved in PBS with 0.5% BSA (control medium) and stored in single-use aliquots at −70°C. Unless otherwise stated, IFN-α was used at 1000 IU/ml and IFN-γ at 10 IU/ml. In IFN induction experiments, cells are treated with equal volumes of either IFN or control medium. The following inhibitors were dissolved in DMSO, stored in aliquots at −20°C, and used at indicated final concentrations: Jak inhibitor I (5 μM), Go6983 (1 μM), GFI0203X (5 μM), Rottlerin (6 μM), and actinomycin D (5 μg/ml). In experiments using these inhibitors, equivalent volumes of inhibitor or DMSO control were used. Final concentrations of DMSO were <0.5%. Anti-A3G, from W. Greene (University of California, San Francisco, CA) was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, (16). All other Abs were commercially available: anti-STAT1, anti-STAT3, and anti-pSTAT1 Tyr705 (Santa Cruz Biotechnology); anti-STAT2, anti-pSTAT3 Tyr705, and anti-pSTAT1 Ser723 (BioSource International); anti-protein kinase C (PKC)-δ (Cell Signaling Technology); anti-IRF-9 (BD Biosciences); and anti-ribosome p19 (ImmuNoVision).

Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was performed according to standard protocols (34). Briefly, total RNA from cells was isolated using the RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions, including an on-column DNase digestion step using the RNase-free DNase Set (Qiagen). One-fifth of the RNA was reverse transcribed using random primers and the MultiScribe Reverse Transcriptase (Applied Biosystems). The cDNA was amplified using TaqMan Universal PCR master mix (Applied Biosystems) and an ABI Prism 7000 sequence detection system (Applied Biosystems). The primers/probe sets were pre-designed TaqMan gene expression assays specific for A3G, dsRNA-activated protein kinase (PKR), IRF-1, ISG15, MX1, and LMP2 (assay identification numbers: Hs00224151_m1, Hs00169345_m1, Hs02233608_m1, Hs00192713_m1, Hs00182073_m1, and Hs00160610_m1, respectively). Amplification of target genes was normalized using amplification levels of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and β-actin as endogenous controls. Gene expression was calculated using the 2^(-ΔΔCT) comparative method, as previously described (34). Gene expression is expressed as fold inductions of a gene measured in IFN-treated samples relative to samples treated with control medium (PBS 0.5% BSA). A >2-fold induction is considered positive. In experiments in which inhibitors were used, relative gene expression in IFN-treated samples was measured against control-treated samples that were pretreated with the same inhibitor or control. In experiments with small interfering RNA (siRNA)-transfected cells, relative gene expression in IFN-treated samples was measured against control-treated samples that were transfected with the same siRNA. In experiments in which inhibitors were used, relative gene expression in IFN-treated samples was measured against control-treated samples that were pretreated with the same inhibitor or control. In experiments with small interfering RNA (siRNA)-transfected cells, relative gene expression in IFN-treated samples was measured against control-treated samples that were transfected with the same siRNA.

Immunoblot assays

In preparation for immunoblot assays, cells were lysed in PhosphaSafe extraction buffer (Novagen), diluted in 1X loading buffer (0.08 M Tris (pH 6.8), with 2.0% SDS, 10% glycerol, 0.1 M DTT, and 0.2% bromphenol blue), and boiled for 5 min. Equal amounts of cell lysates were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes using a semidry apparatus (Bio-Rad). Membranes were probed with various primary Abs against the proteins of interest; secondary Abs were alkaline phosphatase-conjugated anti-rabbit IgG and anti-human IgG (Jackson ImmunoResearch Laboratories). Staining was conducted with 5-bromo-4-chloro-3-indolyl phosphate and NBT solutions prepared from chemicals obtained from Sigma-Aldrich.

RNA interference

RNA interference against STAT1, STAT2, IRF-9, STAT3, and PKC-δ was conducted using SMARTPool siRNAs, which are predesigned pools of four duplexed siRNAs targeting a single gene (Dharmacon). Cells were transfected with siRNA pools at a total final concentration of 100 nM using Lipofectamine 2000 (Invitrogen Life Technologies). siCONTROL nontargeting siRNA 2 (Dharmacon) was used as a control. Ninety-six hours after transfection, IFNs were added to cells for a period of 8 h before processing for mRNA detection by qRT-PCR.

Results

Induction of A3G by IFN is donor and cell-type dependent

Recent studies reported that A3G is inducible by IFN-α in liver cells (24, 25) and macrophages (26), but not in immortalized CD4+ T cells (23). The effects of IFNs on A3G transcription in primary CD4+ T cells and other cell types such as epithelial cells have not been reported. In the present study, we tested the ability of IFNs to up-regulate A3G in various primary cells (hepatocytes, macrophages, and CD4+ T cells) and cell lines (liver carcinoma cells, 293T cells, and H9 cells). Using a qRT-PCR method, we observed that A3G was up-regulated by both IFN-α and IFN-γ in primary hepatocytes from one donor (85) through a time course of up to 24 h (Fig. 1A) and followed a similar kinetic to that of PKR (Fig. 1B), a known IFN-α-inducible gene. IFN-γ also induced A3G (Fig. 1A) in the hepatocytes, but to a lesser extent. IFN-induced expression of A3G protein in primary hepatocytes was also demonstrated by immunoblotting (Fig. 1C). This result is consistent with the previous studies that reported on IFN-α induction of A3G in primary hepatocytes (24). We asked whether there are differences in A3G expression among multiple individuals, and more importantly, whether there are quantitative differences in the levels of A3G after IFN-α treatment. Therefore, we compared the A3G expression levels in control primary hepatocytes and cells treated with IFN-α from four donors (78, 79, 80, and 85). The comparative ΔACT method was used to calculate relative expression using β-actin as a normalizing gene, and the lowest A3G-expressing cell sample in the group (control-treated hepatocytes from donor 78) was set as the calibrator with an arbitrary unit of 1. A3G expression in other cells was expressed as relative fold units above the calibrator. Some variation in A3G expression can be noted between the untreated cells, but a more marked variation can be observed after IFN treatment (Fig. 1D). The range of A3G mRNA expressed was between 9- and 38-fold above the calibrator after IFN-α treatment. In contrast, the range of PKR mRNA expressed was between 6- and 13-fold over the calibrator after IFN-α treatment (Fig. 1E).

Although IFN-α could induce A3G mRNA (Fig. 2A) and A3G protein (Fig. 2C) as well as PKR (Fig. 2B) in primary macrophages from donor 1, A3G could not be induced in primary CD4+ T cells (Fig. 2D) from the same donor, while PKR (Fig. 2E) was positively induced. We consistently observed that A3G was not up-regulated by IFN in primary CD4+ T cells or whole PMBC from several other donors (data not shown). IFN-γ did not significantly induce A3G in macrophages from this particular donor (Fig. 2A).

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PKR mRNA were measured by qRT-PCR using the comparative CT method. Although IFN-γ could not induce A3G in the H9 T cell line (Fig. 2F), but that it could in liver cell lines Hep3B, HepG2, and Huh7 (Fig. 2G). Although IFN-α was generally more effective for inducing A3G in primary hepatocytes (Fig. 1, A and D), IFN-γ was more effective for inducing A3G in two of the liver cell lines (Hep3B and HepG2) (Fig. 2H). As positive controls for IFN stimulation, we detected other known ISG such as PKR, which was mainly induced by IFN-α, and IRF-1, which was primarily induced by IFN-γ (Fig. 2, G and H).

Taken together, these results indicate that A3G regulation by IFNs is cell-type and donor dependent. The observed individual variation in IFN responsiveness in primary hepatocytes may account for differences in viral pathogenesis and clinical outcomes in HBV infection.

**A3G is uniquely regulated by a Rottlerin-sensitive target in Hep3B liver cells**

PMA induces A3G transcription in T cells through PKC-αβ activation (23). The PKC pathway also has been linked to the IFN pathway of transcription. The PKC-δ isoform, in particular, was reported to be the kinase responsible for phosphorylating STAT1 at Ser727, a modification reportedly required for full transcriptional activation of ISG (35). We observed that preincubation of Hep3B cells with Rottlerin, reported to be a specific inhibitor of PKC-δ, for 1 h before addition of IFN-α inhibited IFN-mediated induction of A3G (Fig. 3A). However, induction of other IFN-α-responsive genes such as PKR (Fig. 3B) and ISG15 (Fig. 3C) was not affected by Rottlerin. General PKC inhibitors (Go6983 and GF109203X) did not inhibit A3G induction by IFN-α in Hep3B cells (Fig. 3A), suggesting that none of the PKC isoforms participates in IFN-α signaling in liver cells. To investigate whether PKC-δ was actually involved in the specific transcriptional activation of A3G, we transfected Hep3B cells with PKC-δ gene-specific siRNA (Fig. 3D). Despite the fact that PKC-δ was dramatically reduced in these cells, induction of neither A3G, PKR, nor ISG15 genes by IFN-α was affected, when compared with that in cells treated with control siRNA (Fig. 3E). STAT1 was constitutively phosphorylated at Ser727 in Hep3B cells, but this phosphorylation was enhanced by IFN-α treatment (Fig. 3F). Rottlerin also did not reduce the enhanced phosphorylation of STAT1 at Ser727 by IFN-α (Fig. 3F). Therefore, the specific inhibition of IFN-α-induced A3G mRNA expression by Rottlerin is apparently independent of PKC-δ and STAT1 activation.

To examine whether Rottlerin might affect the stability of A3G mRNA after IFN-α induction, we treated Hep3B cells with IFN-α or control medium for 8 h, and then added Rottlerin or DMSO to the cultures with actinomycin D to stop transcription (Fig. 3G). A3G mRNA levels were then measured in the IFN-α-treated samples relative to the control samples at times up to 12 h after addition of Rottlerin or DMSO. The level of A3G mRNA was expressed as a relative percentage of the amount obtained after the 8-h incubation with IFN-α (time 0 in Fig. 3G), which was set at...
A3G mRNA stability was not different between cells treated with Rottlerin or DMSO. Therefore, it is likely that Rottlerin interfered with IFN-\(\alpha\)/H9251-induced A3G (Fig. 3A). IFN-\(\alpha\)/H9251 induction of A3G is independent of STAT1, but dependent on STAT2 in Hep3B liver cells. Our data to date have suggested a unique pathway of IFN induction of A3G. Therefore, we examined whether the induction of A3G by IFNs was indeed dependent on the classical Jak/STAT pathways that mediate transcriptional regulation of other well-known antiviral genes (27–31). The importance of STAT1 and STAT2 is well established in the literature for the IFN-\(\alpha\)/H9251 pathway of transcription. Therefore, we transfected Hep3B cells with a non-targeting control siRNA or siRNA specific for STAT1 or STAT2. After 4 days, we treated the cells with either control medium, IFN-\(\alpha\), IFN-\(\gamma\), or control medium for 8 h, and then cells were collected to measure fold inductions of A3G or PKR by qRT-PCR. STAT1 knockdown did not block the IFN-\(\alpha\)-mediated induction of A3G (Fig. 4B) or of certain other ISGs such as PKR (Fig. 4C), ISG15 (Fig. 4D), or MX1 (Fig. 4E). However, induction of LMP2 by IFN-\(\alpha\) was significantly inhibited by the STAT1 knockdown (Fig. 4F), suggesting that STAT1 activation by IFN-\(\alpha\) was required for some genes in these liver cells. Induction of A3G, PKR, ISG15, or Mx1 by IFN-\(\alpha\) was blocked by STAT2 knockdown (Fig. 4, B–F), indicating that STAT2, but not STAT1, remains critical for the IFN-\(\alpha\) induction of these genes in liver cells.

To study whether STAT1 was indeed activated in these cells, we treated the Hep3B liver cells with a general inhibitor of Jak, Jak inhibitor I, for 1 h before induction with IFNs for 8 h in the continuous presence of the drug. Phosphorylation of STAT1 at Tyr701 by Jak is required for dimerization and transcriptional activation. STAT1 Tyr701 phosphorylation was induced by IFN-\(\alpha\) and was blocked by Jak inhibitor I (Fig. 4G). Inhibition of the Jak potently blocked the induction of both A3G (Fig. 4H) and PKR (Fig. 4I) by IFN-\(\alpha\), indicating that A3G required Jak for transcriptional activation. Our data suggest that despite a functional mechanism of Jak-dependent activation of the STAT1 pathway in Hep3B cells,
STAT1 itself was not required for IFN-α/H9251-induced A3G expression. This observation was not specific to Hep3B cells, as we have also observed STAT1-independent, IFN-α/H9251-induced A3G expression in another liver cell line, Huh7 (data not shown).

IFN-α induction of A3G is dependent on STAT1 in Hep3B liver cells

We next investigated whether STAT1 is functional and required for the IFN-γ pathway. Unlike the IFN-α pathway of transcription, the IFN-γ pathway uses a STAT1 homodimer that forms upon activation. We treated Hep3B liver cells with a general inhibitor of Jak, Jak inhibitor I (Jak inhibitor I) or Rottlerin (Rot) for 1 h before induction with IFN-α/H9251 or control medium for 8 h. Relative mRNA expression of A3G, PKR, and ISG15 was measured by qRT-PCR. Induction of genes by IFNs is expressed as a fold induction of IFN-treated cells relative to control-treated cells. D, Hep3B cells were transfected with control or PKC-δ siRNA for 4 days, and then treated with IFN-α or control medium for 8 h. The cells were then harvested to measure PKC-δ protein or ribosome p19 as a loading control. E, One-half of the cells treated in D were collected for measurement of A3G, PKR, and ISG15 mRNA induction by qRT-PCR. F, Hep3B cells were treated with DMSO, Jak Inhibitor I (Jak), or Rottlerin (Rot) for 1 h. The cells were then treated with IFN-α for 1 h before collection for immunoblot analysis. Equal amounts of cell lysates were run in separate SDS-PAGE gels and transferred to three separate nitrocellulose membranes. Phosphorylated STAT1 proteins were detected using Abs against phospho-STAT1 Ser727 and phospho-STAT1 Tyr701 proteins. Total STAT1 protein was detected in the third membrane as a total protein-loading control. G, Hep3B cells were treated with IFN-α for 8 h, and then Rottlerin or DMSO control was added to the cultures with actinomycin D (transcription inhibitor). A3G induction was measured in the IFN-α-treated samples over the control samples at times 0, 4, 8, and 12 h postaddition of Rottlerin or DMSO. Relative mRNA stability of A3G was expressed as a relative percentage of the amount obtained after the 8-h incubation with IFN-α (time 0 in Fig. 2G), which was set at 100%.

STAT1-INDEPENDENT REGULATION OF A3G BY IFN-α

FIGURE 3. A3G is uniquely regulated by a Rottlerin-sensitive target in Hep3B liver cells. A–C, Hep3B cells were treated with DMSO, Go6983, GF109203X (GFX), Jak Inhibitor I (Jak Inh I), or Rottlerin (Rot) for 1 h before treatment with IFN-α or control medium for 8 h. Relative mRNA expression of A3G, PKR, and ISG15 was measured by qRT-PCR. Induction of genes by IFNs is expressed as a fold induction of IFN-treated cells relative to control-treated cells. D, Hep3B cells were transfected with control or PKC-δ siRNA for 4 days, and then treated with IFN-α or control medium for 8 h. The cells were then harvested to measure PKC-δ protein or ribosome p19 as a loading control. E, One-half of the cells treated in D were collected for measurement of A3G, PKR, and ISG15 mRNA induction by qRT-PCR. F, Hep3B cells were treated with DMSO, Jak Inhibitor I (Jak), or Rottlerin (Rot) for 1 h. The cells were then treated with IFN-α for 1 h before collection for immunoblot analysis. Equal amounts of cell lysates were run in separate SDS-PAGE gels and transferred to three separate nitrocellulose membranes. Phosphorylated STAT1 proteins were detected using Abs against phospho-STAT1 Ser727 and phospho-STAT1 Tyr701 proteins. Total STAT1 protein was detected in the third membrane as a total protein-loading control. G, Hep3B cells were treated with IFN-α for 8 h, and then Rottlerin or DMSO control was added to the cultures with actinomycin D (transcription inhibitor). A3G induction was measured in the IFN-α-treated samples over the control samples at times 0, 4, 8, and 12 h postaddition of Rottlerin or DMSO. Relative mRNA stability of A3G was expressed as a relative percentage of the amount obtained after the 8-h incubation with IFN-α (time 0 in Fig. 2G), which was set at 100%.
IRF-9 is required for IFN-α induction of A3G and other ISG in Hep3B cells

It has been shown previously that STAT2 and IRF-9 can weakly bind DNA and mediate transcription in the absence of STAT1 (36, 37). Therefore, we tested the requirement for IRF-9 in the IFN-α induction of A3G and other ISG in Hep3B cells. We transfected Hep3B cells with control, IRF-9, IRF-9 plus STAT1, or IRF-9 plus STAT2 siRNA for 4 days, and then treated the cells with control medium or IFN-α for 8 h. Equal amounts of cell lysates were run on separate SDS-PAGE gels to detect pSTAT1 Tyr701 or total STAT1 proteins. After 96 h, cells transfected with each type of siRNA were treated with IFN-α, IFN-γ, or control medium for 8 h. Protein expression of STAT1, STAT2, and ribosome p19 control was detected by immunoblotting, but highly induced by IFN-α control medium was added to the culture for 8 h. Fold induction of A3G or PKR by IFN-α over control cells was measured by qRT-PCR. Error bars, SDs of fold inductions from triplicate cell samples.

STAT1 is required for both the IFN-α and IFN-γ induction of ISG in 293T cells

To determine whether our observation of the STAT1-independent IFN-α-induced gene expression was unique to liver cells, we tested whether STAT1 was required in 293T cells for the IFN-α pathway of transcription. We transfected 293T cells with control, STAT1, or STAT2 siRNA for 4 days, and then treated the cells with either IFN-α or IFN-γ for 8 h. Both STAT1 and STAT2 proteins were efficiently and specifically reduced (Fig. 7B). Although A3G was not inducible in this cell line (Fig. 7B), we observed that IFN-α induction of other ISG such as ISG15 (Fig. 7C), MX1 (Fig. 7D), and LMP2 (Fig. 7E) was inhibited by both STAT1 and STAT2 siRNA. As observed in Hep3B cells (Fig. 5E), IFN-γ induction of IRF-1 (Fig. 7F) as well as LMP2 (Fig. 7G) was inhibited specifically by STAT1 siRNA and not STAT2 siRNA in 293T cells. Therefore, STAT1 was required for both the IFN-α and IFN-γ induction of ISG in 293T cells, consistent with the classical model of IFN-signaling complexes.
qRT-PCR to measure induction of A3G and IRF-1 by IFN-α/H9253 SDs of fold inductions from triplicate cell samples.

inhibitor I (Jak Inh I) or DMSO for 1 h, and then IFN-α treatment of hepatocytes from four individual donors (Fig. 1D). It is known that the abilities of spontaneous clearance of viral infection after HBV exposure differ significantly among various subjects (42–44). Such variations in A3G protein expression and IFN inducibility in liver cells may contribute to differences in response to treatment of HBV infections and different courses of viral pathogenesis in infected individuals.

There are several unique and novel contributions of this study, however, to both the understanding of A3G transcriptional regulation and the knowledge of IFN signaling pathways. First, we demonstrated that transcriptional up-regulation of A3G by IFN-α was differentiated from other ISG and affected specifically by the drug Rottlerin in Hep3B liver cells through a mechanism independent of PKC-δ and STAT1 activation (Fig. 3). Rottlerin has been reported as a PKC-δ-specific inhibitor and used widely to implicate the role of PKC-δ in various processes, including the tyrosine phosphorylation of STAT1 (35). However, one study found that Rottlerin potently inhibited multiple kinases in vitro, but had little effect on PKC-δ (45). It is possible that PKC-δ may be inhibited by Rottlerin in vivo through an indirect mechanism (46), but our data suggest that PKC-δ was not involved in the IFN-α induction of A3G. Further study will be required to identify the target of Rottlerin responsible for the specific IFN regulation of A3G.

Second, we observed novel STAT1-independent IFN-α induction of A3G and other ISG in liver cells. The canonical models of IFN signaling pathways specify that the ISG factor 3 (ISGF3) complex consisting of STAT1, STAT2, and IRF-9 is important for gene transcription in response to IFN-α. In our study, we used siRNA against these three transcripts in Hep3B cells to show that only STAT2 and IRF-9, but not STAT1, were necessary for induction of A3G and other genes by IFN-α. It may be argued that complete knockdown of STAT1 was not achieved, and therefore residual proteins may be sufficient to mediate transcription. However, STAT1 shutdown was sufficient to inhibit the IFN-γ pathway of gene transcription (Fig. 5) in liver cells. Furthermore, similar levels of protein reduction were achieved with STAT1 and STAT2 siRNA (Fig. 4A), and STAT2 siRNA was able to inhibit the IFN-α-mediated induction of ISG (Fig. 4, B–F). Finally, the same siRNA strategy was used in 293T cells to block STAT1-dependent gene transcription in that cell line (Fig. 7).

At first glance, it may not be surprising that we found that STAT2 and IRF-9 were required for induction of ISG in the Hep3B cell line. It has been demonstrated that STAT2 can homodimerize and mediate gene transcription in conjunction with IRF-9 in the absence of STAT1. However, it was argued that the DNA-binding affinity of such a complex was weak (36) and that H9 cells are relatively resistant to certain IFN-induced responses (38–41), we observed normal IFN-α-induced PKR expression in H9 cells as well as primary CD4+ T lymphocytes (Fig. 2, E and F). This observation suggests at least some IFN-α-mediated signaling pathways are functional in these T cells. It remains to be discovered why A3G could not be induced in T cells, although it is possible that a factor required for specific induction of A3G is absent in T cells. Liver cells and macrophages are primary targets of lentiviruses and HBV, respectively. It is therefore plausible that up-regulation of A3G by IFNs could contribute to innate in vivo antiviral defenses against HBV in liver cells and lentiviruses in macrophages.

Although two recent studies had also reported that A3G could be induced by IFN-α in primary hepatocytes (24, 25), our study offered a quantitative comparative analysis of A3G induction by both IFN-α and IFN-γ in multiple primary hepatocytes. We found that there was a wide variation in the level of A3G expressed after IFN-α treatment of hepatocytes from four individual donors (Fig. 1D). It is known that the abilities of spontaneous clearance of viral infection after HBV exposure differ significantly among various subjects (42–44). Such variations in A3G protein expression and IFN inducibility in liver cells may contribute to differences in response to treatment of HBV infections and different courses of viral pathogenesis in infected individuals.

The functional ISRE is not within the proximal 135 bp 5′ of the first exon of the A3G gene

It was published previously that the ISRE of the A3G gene was located with a 135-bp fragment from the 5′ region before the first exon of the A3G gene (24). We therefore wanted to confirm this result by cloning the identical fragment as published into the pGL2-basic luciferase reporter plasmid (pGL2-135). This fragment is identical with that cloned into p127+/−7, as published in Tanaka et al. (24) (confirmed by sequencing). However, no basal promoter activity nor IFN-α-inducible activity was observed when we tested the construct in HepG2 cells as in the original study (Fig. 8). The positive control plasmid showed high promoter activity, indicating that the cells were transfectable and the assay was functional. Therefore, the functional ISRE is not within this 135-bp fragment and remains to be identified.

Discussion

A3G has potent antiviral activity against diverse retroviruses (3–8) and HBV (21, 22), and can be classified among other well-known ISGs, such as PKR, ISG15, and MX1, which mediate a variety of antiviral effects. IFN induction of A3G is cell-type dependent, unlike other ISG, such as PKR, which can be stimulated in all cell types. A3G can be induced by IFN in liver cells, both primary hepatocytes and liver cell lines, and primary macrophages, but not in primary CD4+ T cells or H9 cells. Although it may be argued
STAT1 provided additional stability to increase the trans activation of the trimeric ISGF3 complex. That study was performed in STAT1-negative cells (U3A) derived from the parental 2fGTH epithelial cells. In fact, we have tested induction of A3G and other genes by IFN-α/H9251 and IFN-γ/H9253 in 2fTGH, U3A, and U6A (STAT2-negative) cells. A3G was only minimally induced to 3-fold in the parental cells and not induced in the STAT1- and STAT2-negative cells (data not shown). PKR was not induced in either the STAT1-negative or STAT2-negative cells by IFN-α, and IRF-1 was not induced in the STAT1-negative cells by IFN-γ, as expected from the classical models (data not shown). We had obtained similar results in 293T cells treated with STAT1 or STAT2 siRNA (Fig. 6).

The unique observation in the present study, however, was that reduction of STAT1 by siRNA did not even partially reduce the transcriptional activation of certain ISG by IFN-α/H9251 in the liver Hep3B cells (Fig. 4). Therefore, our results suggest that a fully functional STAT1-independent IFN-α pathway is most likely functioning either in parallel with the classically described ISGF3 complex or in the absence of STAT1 (31). We believe that the STAT1 independence of gene induction by IFN-α is unique to liver cells because the phenomenon of STAT1-independent transcription was observed specifically in Hep3B cells (Fig. 4) and Huh7 cells (data not shown), but not in 293T cells (Fig. 6) or 2fTGH cells (data not shown). Future studies will be needed to address whether IFN-α may also up-regulate ISG in primary hepatocytes via a STAT1-independent pathway as well. Although STAT-independent IFN-γ pathways of transcriptional activation are known (47, 48), to our knowledge this is the first report of an IFN-α-independent pathway of up-regulating antiviral genes.

Because signaling through Jak (Figs. 4, H and I, and 5, B and C), STAT2 (Fig. 4), and IRF-9 (Fig. 6) is essential for ISG induction in these cells, our study opens questions as to which additional molecules may mediate this pathway. There are seven members of the STAT family of proteins that become activated by dimerization through Src homology 2 interactions upon tyrosine phosphorylation by Jak. Most of the STAT proteins can homodimerize, and it is possible that STAT2 may be acting as a homodimer in complex with IRF-9 to mediate transcription in the liver cells. However, STAT1 is known to heterodimerize with STAT2 and STAT3 (31) in response to IFN. Furthermore, STAT3 is activated (phosphorylated at Tyr705) in response to IFN-α in liver cells (49) as well as macrophages and T cells (data not shown), although its role in gene transcription is unknown. If the alternative pathway to the classical STAT1:STAT2:IRF-9 complex were a STAT3:STAT2:IRF-9 complex, then we would observe an effect on IFN induction of ISG if both STAT1 and STAT3 were reduced by siRNA. However, we did not observe a role for STAT3 in induction of A3G or other ISG because their up-regulation was not affected by STAT3 siRNA or STAT1 plus STAT3 siRNA treatment (data not shown).
Although primary hepatocytes from STAT1 genetic knockout mice have been shown to display enhanced STAT3 signaling (phosphorylation at Tyr705) in response to IFN-α/H9253 (50), we did not observe enhanced STAT3 signaling by IFN-α/H9251 in STAT1 siRNA-treated Hep3B cells (data not shown). Therefore, STAT3 is most likely not involved in an alternative pathway of STAT1-independent IFN-α/H9251-mediated transcription in liver cells.

Because STAT2 and IRF-9 are required for A3G induction by IFN-α/H9251, and STAT2 and IRF-9 have been shown to bind DNA without STAT1 (36), it is likely that this transcriptional complex binds to some version of an ISRE. The various STAT hetero/homo-dimers bind to an 8- to 10-bp inverted repeated element with a variable ISRE sequence with the consensus sequence of 5′-N4–6 AA-3′ (51). A recent study by Tanaka et al. (24) had reported on the identification of the ISRE in the 5′ region before the first exon of the A3G gene. However, we did not observe basal promoter activity nor IFN-α/H9251-inducible activity when the construct was tested as in the original study (Fig. 8). We believe that the elements responsible for the A3G gene up-regulation in response to IFNs remain to be identified, as well as the core promoter region providing basal promoter activity in the absence of IFN stimulation.

The classical Jak/STAT mechanisms of IFN transcription are well described, but cell type-specific signaling pathways are not clearly understood. We summarize our findings with a proposed model of STAT1-independent IFN-α pathway of induction of A3G and other ISG in liver cells (Fig. 9). The alternative STAT1-independent pathway may occur in parallel with the classical Jak/STAT pathway or is activated in the absence of STAT1. Because the STAT1-independent transcriptional complex requires both STAT2 and IRF-9, which mediate binding to DNA, it may bind to the same ISRE as used by the ISGF3 complex of the classical pathway. However, the promoter of the A3G gene remains to be identified.

**FIGURE 8.** The functional ISRE is not within the proximal 135 bp 5′ of the first exon of the A3G gene. The 135-bp fragment from the 5′ region before the first exon of the A3G gene was cloned into the pGL2-basic luciferase reporter plasmid (pGL2-135). This fragment is identical with that cloned into p127/7, as published in Tanaka et al. (24). HepG2 cells were cotransfected with p5V-β-galactosidase and either the pGL2-basic (no promoter), pGL2-135, or positive control plasmid (pGL2 control containing SV40 promoter) for 24 h. The cells were then treated with or without IFN-α (1000 IU/ml) for 9 h. Luciferase activity was measured by using the Luciferase Assay System (Promega) and the Multilabel Counter (WALLAC1420), and normalized by measuring β-galactosidase activity using the β-Galactosidase Enzyme Assay System (Promega). The luciferase activity is shown as units relative to the activity from cells transfected with pGL2-basic (without IFN) set at 1 unit. Error bars, SD of triplicate samples.
characterized (Fig. 8), and its ISRE may or may not be the same as that of other ISG. As more data accumulate on the transcriptome of A3G and other ISG, the ISRE of the A3G gene has yet to be identified, and therefore may or may not be like the ISRE of other ISG.

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Disclosures
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References


