Resistance to IFN-α–Induced Apoptosis Is Linked to a Loss of STAT2

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Abstract

Type I IFNs (IFN-α/β) are pleitropic cytokines widely used in the treatment of certain malignancies, hepatitis B and C, and multiple sclerosis. IFN resistance is a challenging clinical problem to overcome. Hence, understanding the molecular mechanism by which IFN immunotherapy ceases to be effective is of translational importance. In this study, we report that continuous IFN-α stimulation of the human Jurkat variant H123 led to resistance to type I IFN–induced apoptosis due to a loss of signal transducers and activators of transcription (STAT)2 expression. The apoptotic effects of IFN-α were hampered as STAT2-deficient cells were defective in activating the mitochondrial-dependent death pathway and ISGF3-mediated gene activation. Reconstitution of STAT2 restored the apoptotic effects of IFN-α as measured by the loss of mitochondrial membrane potential, cytochrome c release from mitochondria, caspase activation, and ultimately cell death. Nuclear localization of STAT2 was a critical event as retention of tyrosine-phosphorylated STAT2 in the cytosol was not sufficient to activate apoptosis. Furthermore, silencing STAT2 gene expression in Saos2 and A375S.2 tumor cell lines significantly reduced the apoptotic capacity of IFN-α. Altogether, we show that STAT2 is a critical mediator in the activation of type I IFN–induced apoptosis. More importantly, defects in the expression or nuclear localization of STAT2 could lessen the efficacy of type I IFN immunotherapy.

Introduction

Type I IFNs (IFN-α/β) are therapeutic cytokines used in the treatment of several forms of cancer (1–3), chronic hepatitis B and C (4, 5), and multiple sclerosis (6). Yet, the beneficial effects of IFNs may be limited as these diseases may already be resistant to type I IFN immunotherapy or patients develop resistance during the course of therapy. This has been reported in patients with chronic myelogenous leukemia (7), melanoma (8, 9), and hepatitis C (10). A clear understanding of the molecular mechanism of IFN resistance remains obscure. A reduced or loss of signal transducers and activators of transcription (STAT)1 and Janus kinase (JAK)1 (11) expression and STAT5 overexpression (12) have been implicated in IFN insensitivity. Other studies report increased free circulating type I IFN receptors in patients with adenocarcinoma (14) and epigenetic silencing of genes that are essential for the biological actions of IFNs (15) as contributors to IFN resistance.

Type I IFNs are a family of pleitropic cytokines that induce their antiproliferative, apoptotic, and antiviral effects through activation of receptor-associated JAK1 and TYK2 (16). STAT1 and STAT2 are recruited to the IFN-α/β receptor and become tyrosine phosphorylated by JAKs. Activated STATs bind to each other as STAT1 homodimers or STAT1/STAT2 heterodimers that when bound to IRF9 form the IFN-stimulated gene factor–3 (ISGF3) complex. Both STAT complexes translocate to the nucleus and initiate transcription of IFN-stimulated genes (ISG). STAT1 homodimers recognize and bind to a γ-activated region (17), whereas the ISGF3 complex binds to an IFN-stimulated response element (ISRE) region in the promoter of ISGs (18).

STAT2 is one of seven members of the STAT family of transcription factors (19). In marked contrast to other STATs that can be activated by multiple growth factors and cytokines, only type I IFNs have been found to cause the activation of STAT2 (20). STAT2 is an integral component in the IFN-induced activation of the JAK-STAT signaling pathway. This is supported by the observation that the antiproliferative effects of IFN-α are impaired in STAT2-deficient human fibrosarcoma U6A cells and mouse T cells (21–23). Additionally, studies of STAT2 null mice provide direct evidence to support that STAT2 plays a vital role in type I IFNs signaling and consequently in viral...
immunity and cancer (24, 25). More importantly, these studies showed that STAT2 is not always required for STAT1 activation. In spite of that, the molecular mechanism of cell growth inhibition and apoptosis mediated by type I IFN and the role STAT2 plays in this response are less studied.

We recently reported on the characterization of a type I IFN–resistant H123 clone that arose after sustained stimulation with IFN-α. Biochemical and genetic analysis revealed that this clone had acquired a mutation that changed a conserved proline 630 to leucine in the SH2 domain of STAT2 and led to impaired IFN-α signaling (26). In contrast, introduction of a tyrosine to phenylalanine mutation, adjacent to proline 630, enhanced the antiproliferative effects of IFNs due to activation of apoptosis (27). These findings indicated the presence of a regulatory switch in STAT2. In this study, we identify STAT2 as a critical mediator of type I IFN–induced apoptosis. A loss of STAT2 results in impaired activation of the mitochondrial death–dependent pathway but not that of STAT1-mediated responses triggered by type I IFNs. Most importantly, nuclear localization of tyrosine-phosphorylated STAT2 is a key event for the onset of apoptosis. Altogether, our data hint that a loss of STAT2 function may reduce the efficacy of type I IFN immunotherapy.

Materials and Methods

Cells and Cell Culture Reagents

H123 cells, a variant of the human leukemic Jurkat cell line, mutant variants derived from H123 (28), and the human osteosarcoma SAOS2 were maintained in RPMI 1640. Human melanoma A375.S2 cells were maintained in DMEM. Both media were supplemented with 10% fetal bovine serum containing 2.2 g of GlutaMax-1, 10 U/mL penicillin G sodium, and 10 μg/mL streptomycin sulfate (Invitrogen Corp) at 37°C in a 5% CO₂ atmosphere. Stable clones of STAT2-deficient H123 cells reconstituted with wild-type or various mutant forms of STAT2 were maintained in G418 at 1 mg/mL. Recombinant human IFN-α-2a (specific activity, 2 × 10⁷ U/mL) was purchased from PeproTech, Inc. CellTiter 96 Aqueous One Solution Reagent was from Promega.

Site-Directed Mutagenesis

Flag-tagged STAT2 construct in pcDNA3, kindly provided by C. Horvath (Northwestern University, Evanston, IL), was used as template DNA. STAT2 mutagenesis was done with the QuikChange XL Site-Directed Mutagenesis kit (Stratagene) using the following primers synthesized by Integrated DNA Technologies:

- STAT2 Y690F 5′-CAGGAACGGAGGAATTCCTGAACACAGGCTC-3′
- STAT2 R409A 5′-CTGGAGCAAAGCTTACGTCGTCAGCAGGAAGG-3′
- STAT2 K415A 5′-GGTTGATTACGAGCCGCGCAAAATAAGG-3′

Mutagenesis was confirmed by sequencing the entire STAT2 sequence.

Transfections

H123 STAT2-deficient cells were transfected with 10 μg of either STAT2-flag tag, STAT2 R409A:K415A, or STAT2 Y690F plasmid DNA by using a 2-mm cuvette and a Bio-Rad electroporator set at 140 V, 1,000 μF Capacitance, ∞ Ω Resistance. Twenty-four hours later, cells were maintained in a complete medium containing 1 mg/mL of G418, and 2 wk later, individual clones were then selected by limiting dilution.

Apoptosis Assay

STAT2-deficient and STAT2-reconstituted H123 cells were left untreated or treated with either 3,000 U/mL IFN-α or TRAIL for the indicated times at 37°C. Cells were harvested, washed in 1× PBS, and stained in 50 μL of binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂] containing 2 μL of Annexin V-FITC (Pharmingen) and 2 μL of PI (50 μg/mL; Sigma). Cells were then incubated for 10 min at room temperature, were protected from light, followed by the addition of 400 μL of binding buffer, and were analyzed by FACS-cant (Becton Dickinson). Data were collected for 10,000 cells, stored and analyzed using CellQuest software (Becton Dickinson).

Flow Cytometry

Cells were incubated for 30 min on ice in staining buffer (PBS solution containing 0.2% NaN₃ and 2% FCS) containing phycoerythrin-labeled anti-human MHC class I ABC or isotype-matched control antibodies. Surface expression of this marker was analyzed using a FACSScan TM cytometer (Becton Dickinson) and was further analyzed using CellQuest software (Becton Dickinson).

Proliferation Assay

Cells were seeded in triplicate in flat-bottomed 96-well plates at a concentration of 5 × 10⁴ cells in 50 μL volume per well. Fifty microliters of medium or IFN-α were added...
to each well and cells were incubated at 37°C for 72 h. Wells without cells containing 100 μL of medium served as background controls. Cell proliferation was assessed by MTS assay. Briefly, 20 μL of CellTiter 96-Aqueous One Solution Reagent were added to each well and the plate was incubated at 37°C for 1 to 4 h. Absorbance was measured at 490 nm using a VictorTM 1420 multilabel counter (Perkin-Elmer). Background values were first subtracted from each well before proceeding with data analysis.

**Preparation of Cell Extracts**

Following treatment, cells were collected, washed in 1× PBS, and lysed in ice-cold lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.5% Triton X-100, and 1 mmol/L sodium orthovanadate, 200 μmol/L phenylmethylsulfonyl fluoride] supplemented with protease inhibitors (Sigma). After centrifugation at 4°C for 10 min, supernatants were collected and protein concentration was measured by standard Bio-Rad Bradford protein assay.

**Western Blot Analysis**

Cell extracts were boiled with laemmli buffer and then resolved by 10% SDS-PAGE (Invitrogen). Proteins were transferred to a polyvinylidene difluoride membrane. The membranes were blocked for 30 min with blocker casein in TBS (Pierce) and then incubated with the specified antibodies. Following washing, immunoblots were incubated with the corresponding IgG isotype secondary antibody (1:10,000; Zymed) for 30 min. Membranes were washed with TBS (Pierce) and developed by chemiluminescence using the enhanced chemiluminescence Western blotting system (Pierce) as described previously (26).

**RNA Isolation and RNase Protection Assay**

Cells were either left untreated or stimulated with IFN-α for the indicated times. RNA was isolated with RNAzol B (Tel-Test, Inc.) according to the manufacturer’s instructions. RNA concentration was determined by 260/280 ratios.

Antisense RNA probes were synthesized by *in vitro* transcription using T7 or SP6 RNA polymerase (Life Technologies-Bethesda Research Laboratories) and [α-32P]-UTP (ICN; ref. 28). Ten micrograms of RNA and 32P-labeled riboprobes were incubated overnight in a hybridization buffer [containing 80% formamide, 40 mmol/L PIPES (pH 6.7), 400 mmol/L NaCl, and 1 mmol/L EDTA] at 56°C followed by digestion with T1 RNase (Life Technologies-Bethesda Research Laboratories) for 1 h at 37°C, phenol extraction, and ethanol precipitation. Protected RNA fragments were solubilized in RNA loading buffer [98% formamide, 10 mmol/L EDTA (pH 8.0), bromophenol blue, and xylene cyanole], boiled for 2 min, and resolved by electrophoresis on a 4.5% polyacrylamide-urea gel.

**Quantitative RT-PCR**

Five micrograms of total RNA were reverse transcribed to generate cDNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative RT-PCR primers were obtained from Applied Biosystems. Briefly, cDNA was mixed with Taqman 2X PCR master mix (Applied Biosystems), using primers with FAM reporter dyes, and quantitative PCRs were done using the 7300 Real-time PCR system (Applied Biosystems). Samples were amplified using the following PCR variables: 55°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), 95°C (40 cycles) for 30 s, and 60°C for 1 min. Messenger RNA quantification was normalized by multiplexing with 18S-VIC primers.

**Electrophoretic Mobility Shift Assays**

Synthetic double-stranded oligonucleotide corresponding to the ISRE of the ISG15 promoter was used as a DNA probe. Probe was end labeled with [γ-32P]-ATP using T4 polynucleotide kinase (Cell Signaling) as described previously (27). The DNA-protein complexes were subjected to electrophoresis on a 4.7% polyacrylamide gel and visualized by autoradiography.

**Mitochondrial Membrane Potential**

Loss of mitochondrial membrane integrity was measured by using the fluorescent dye JC-1 (Invitrogen). Cells were left untreated or treated with IFN-α for the indicated times. Cells were stained with 2.5 μmol/L JC-1 resuspended in PBS, incubated for 20 min at 37°C, washed, and immediately analyzed by flow cytometry. A loss in mitochondrial membrane potential was determined by a decrease in green/red double fluorescence to an increase in green single fluorescence.

**Confocal Microscopy Analysis**

To measure cytochrome c release, following treatment, cells were incubated with 50 mmol/L Mitotracker Red CMXRos for 15 min and then washed thrice with 1× PBS. Samples were cytospun to glass slides at 300 rpm in a Cytospin (Thermo Scientific), fixed in 4% paraformaldehyde for 10 min at room temperature, and were washed again. Cells were then permeabilized with 0.2% Triton X-100 for 5 min before being placed in blocking solution (2% goat serum, 2 mg/mL bovine serum albumin in PBS). Slides were incubated with anti-cytochrome c antibody (1:200 in blocking solution) or anti-Flag antibody (1:200 in blocking solution) overnight at 4°C. The slides were washed with blocking buffer and were incubated for 1 h at room temperature with a FITC-labeled mouse IgG isotype (1:200 in blocking solution, Alexis Biochemicals). After several washes with blocking buffer, the slides were mounted with VectaMount (Vector Laboratories, Inc.). To visualize STAT2 localization, permeabilized cells were incubated with anti-STAT2 antibody (1:200 in blocking solution). Confocal images were acquired using a Zeiss LSM510 Meta NLO confocal laser-scanning microscope (Carl Zeiss).
Measurement of Caspase-3 Activation

Caspase-3 activation was measured using an EnzoLyte AMC Caspase-3 Assay Fluorimetric kit (AnaSpec) following the manufacturer instructions. Briefly, 1 × 10^5 cells were plated in triplicate in a flat-bottomed 96-well plate. Cells were stimulated with or without IFN-α. After incubation, caspase-3 substrate was added to each well. Plates were incubated for 30 min at room temperature. Fluorescence intensity was measured in a Victor™ 1420 multilabel counter (Perkin-Elmer), at Ex/Em of 354 nm/442 nm.

STAT2 and IRF-1 shRNA Constructs and Lentiviral Infection

STAT2 and IRF-1 shRNA constructs cloned in pLKO.1 puromycin vector were obtained from Open Biosystems. Each construct contained a 21-bp sequence targeting specific regions of these genes. Lentivirus production and infection were performed as instructed by the manufacturer. Gene silencing was confirmed by Western blot analysis.

Results

Development of Type I IFN Apoptosis Resistance in H123 Tumor Cells

Several reports have shown that type I IFNs induce apoptosis in some but not all tumor cell lines (29). We previously characterized the human leukemic H123 Jurkat cell line as cells that were isolated for having a defect in T-cell receptor signaling that inexplicably gained sensitivity to the apoptotic effects of IFN-α (28). To begin elucidating the signaling mediators involved in the promotion of apoptosis by IFN-α, we used H123 cells to explore this process. H123 cells were maintained under selection pressure in IFN-α for 2 months. Each week, surviving cells were recovered and recultured with fresh IFN-α. This approach generated multiple individual clones that survived the apoptotic effects of both type I IFNs, IFN-α and IFN-β. We chose to study one of these IFN-resistant clones for further analysis. In response to IFN-α stimulation, parental H123 cells were dramatically growth inhibited as early as 12 hours (Fig. 1A, left). In contrast, the IFN-resistant clone showed no decrease in cell proliferation during the course of 72 hours even when high-dose IFN-α was used (Fig. 1A, right). Furthermore, although >60% of the parental H123 cells underwent apoptosis with IFN-α treatment (Fig. 1B, top), no such effect took place in the IFN-resistant clone (Fig. 1B, bottom). Thus, these results show that we had isolated an H123 variant that is resistant to the antigrowth effects of type IFNs.

Type I IFN Apoptosis-Resistant H123 Does Not Express STAT2

To help explain why the H123 IFN-resistant line had lost its ability to undergo apoptosis with IFN-α stimulation, we examined the expression of critical signaling components of the JAK/STAT pathway. Western blot analysis revealed that protein levels of JAK1, TYK2, STAT1, and STAT3 remained unchanged between parental and IFN-resistant H123 cells (Fig. 2A). However, when STAT2 expression was analyzed, a loss of STAT2 was detected in the IFN-resistant H123 clone (Fig. 2B). A loss of STAT2 protein was confirmed by Western blot analysis using two distinct antibodies that recognize the NH₂ terminus (Fig. 2B, left) or COOH terminus of STAT2 (Fig. 2B, right). We excluded the possibility that during selection, the IFN-resistant cells now expressed a truncated form of STAT2 because no other protein bands were detected. This observation was further tested by assaying the IFN-α-induced formation of the ISGF3 complex in parental and IFN-resistant H123 cells. Electrophoretic mobility shift assay using an ISRE oligonucleotide probe showed that binding of ISGF3 to DNA was detected only in parental H123 cells (Fig. 2C). Formation of a different protein complex that is able to bind the ISRE in the IFN-resistant H123 cells was not detected, thereby showing the absence of a truncated version of STAT2. Interestingly, despite a loss of STAT2 protein, reverse transcription-PCR (RT-PCR) and Northern blot analysis showed no discernible changes in the expression of STAT2 mRNA (data not shown). Moreover, STAT2 mRNA was sequenced, and although a few single base substitutions were detected, these produced silent mutations. To verify that STAT2 was not targeted for degradation, treatment of IFN-resistant H123 cells with two different proteasome inhibitors did not restore STAT2 protein expression (Fig. 2D). Thus, these results showed that we have isolated a STAT2-deficient clone.

STAT2-Deficient H123 Cells Remain Responsive to Type I FNs and Susceptible to TRAIL-Induced Apoptosis

Because our IFN-resistant cells now referred to as STAT2-deficient H123 had lost their capacity to undergo apoptosis in response to IFN-α, it was important to establish whether these cells retained any responsiveness to IFN-α. As shown in Fig. 3A, IFN-α stimulation of either parental or STAT2-deficient H123 cells resulted in similar levels of tyrosine-phosphorylated STAT1 (Fig. 3A). IFN-α-induced responses were evaluated by measuring increases in the expression of IRF-1, a STAT1-regulated gene (Fig. 3B). Following 48 hours of IFN-α stimulation, IRF-1 levels were unchanged in parental and STAT2-deficient H123 cells. Surface expression of MHC class I (A, B, and C) molecules was also measured as transactivation of this gene is induced by IFN-α and mediated by STAT1 and IRF-1 (30, 31). Flow cytometric analysis showed that upregulation of MHC class I protein was detected in STAT2-deficient H123 cells (Fig. 3C). However, the level of MHC class I was slightly higher in the parental H123 cells. In response to IFN-α, the fold change expression levels in parental and STAT2-deficient H123 cells as determined by mean fluorescent intensity was 2.1 and 1.7, respectively. Moreover, lack of STAT2 protein did not impair the ability of these cells to respond to other apoptotic stimuli. Indeed, treatment of STAT2-deficient H123 cells
with TRAIL induced the activation of apoptosis to similar levels detected with parental H123 cells (Fig. 3D). These results show that, with the exception of induction of apoptosis, other type I IFN–regulated effects are not severely impaired in the absence of STAT2.

Reconstitution and Sufficient Levels of STAT2 Are Required to Mediate IFN-α–Induced Apoptosis

If STAT2 is an integral component in type I IFN–induced apoptosis, re-expression of STAT2 in STAT2-deficient H123 cells should restore this effect. To test this premise, a mammalian expression vector encoding flag-tagged wild-type STAT2 was stably expressed in these cells. Individual clones expressing STAT2 levels similar to that of parental H123 cells were selected for analysis. Additionally, clones expressing varied amounts of STAT2 were chosen to determine if the amount of STAT2 was critical in the activation of IFN-α–induced apoptosis (Fig. 4A, right). STAT2 reconstitution restored IFN-α–induced activation of apoptosis (Fig. 4A, left). Of interest was the finding that an optimal amount of STAT2 protein was necessary for IFN-α to maximally mediate cell death.

The next question we asked was if a deficiency in STAT2 altered the kinetics of IFN-α–induced STAT1 activation and ISGF3-mediated gene expression because based on our results (Fig. 3B) and contrary to earlier published work (19), we found STAT2 not to be required in the sequential activation of STAT1. Western blot analysis showed that STAT1 activation was virtually undistinguishable between parental and STAT2-deficient H123 cells (Fig. 4B).

We then measured IFN-α–mediated gene activation by RNase protection assay in the same STAT2 reconstituted clones, with each expressing different amounts of STAT2. Figure 4C shows that in the absence of STAT2, expression of ISGF3-dependent genes, such as IFI-6-16 and IFI-15K, was impaired and reintroduction of STAT2 restored transcription of these ISGs. It is important to note that in STAT2-reconstituted clones expressing low levels of STAT2, there was a delay in ISG expression (compare clones 20 and 30 to clone 36 and parental H123 cells).
These results indicate that an adequate level of STAT2 protein was required for maximal expression of ISGF3-driven ISGs and activation of type I IFN–induced apoptosis.

**STAT2 Is Essential in IFN-α–Induced Activation of the Mitochondrial Death Pathway**

We showed previously that IFN-α–induced apoptosis in H123 cells occurred through the activation of mitochondrial and caspase-dependent death pathways (28). The hallmark of this signaling process entails (a) loss of mitochondrial membrane potential, (b) release of cytochrome c from the mitochondria, and (c) activation of caspases. To determine whether STAT2 played a role in the IFN-α–induced activation of the mitochondrial-dependent death pathway, we first directly measured changes in mitochondrial membrane potential. Parental H123, STAT2-deficient H123, and STAT2-deficient H123 reconstituted with STAT2 were treated with IFN-α for 24 or 48 hours followed by incubation with the fluorescent dye JC-1 (Fig. 5A). Changes in fluorescence were analyzed by flow cytometry. IFN-α treatment of parental H123 cells and STAT2-deficient H123 cells reconstituted with STAT2 resulted in an increase in JC-1 fluorescence, an indication of loss of mitochondrial membrane potential. In contrast, a change in the mitochondrial membrane potential in STAT2-deficient H123 cells was not detected after 48 hours of IFN-α treatment. Next, we examined defects in cytochrome c release in the various H123 cell lines after treatment with IFN-α for 48 hours. As shown in Fig. 5B, in untreated cells, cytochrome c (green) was localized in the mitochondria (red) as shown by the overlay of the two colors (yellow). In contrast, IFN-α treatment resulted in the

**FIGURE 2.** Type I IFN–resistant H123 cells show loss of STAT2 protein. Whole-cell extracts of parental and IFN-resistant H123 cells were prepared and analyzed by Western blot analysis for expression of (A) JAK1, TYK2, STAT1, and STAT3 and (B) STAT2 by using antibodies against the NH2-terminus or COOH-terminus end of STAT2. C. Nuclear extracts from parental and IFN-resistant H123 cells were analyzed by electrophoretic mobility shift assay using an ISRE oligonucleotide. D. Cells were pretreated overnight with the indicated doses of proteosome inhibitors and STAT2 protein expression was assessed by Western blot analysis.
translocation of cytochrome c to the cytosol away from the mitochondria in parental and STAT2-reconstituted H123 cells, but not in STAT2-deficient H123 cells. Furthermore, measurement of caspase-3 activation after IFN-α treatment of H123 cells also revealed a dependence on STAT2 to drive this event (Fig. 5C). All these results point to STAT2 as an integral mediator in type I IFN-induced activation of the mitochondrial-dependent death pathway.

Requirement of Tyrosine Phosphorylation and Nuclear Localization of STAT2 for IFN-α–Induced Apoptosis

We explored the possibility that STAT2 may have other activities in addition to gene transcription as that of an adaptor molecule. To test this hypothesis, we constructed two expression vectors each encoding a distinct STAT2 mutation. Tyrosine (Y)-690 was changed to phenylalanine.
(Y690F) to produce a transcriptionally inactive protein. Mutation of the tyrosine phosphorylation site prevents STAT2 from dimerizing with STAT1 and their translocation as a complex to the nucleus (32). In contrast, arginine-(R)-409 and lysine-(K415) located in the nuclear localization signal were changed to alanine (R409A, K415A). This STAT2 mutant can be tyrosine phosphorylated, associate with STAT1, but does not import to the nucleus (33). Both mutants helped us assess whether in response to IFN-α, the transcriptional activity of STAT2 was essential to promote apoptosis. We stably reconstituted STAT2-deficient cells with each STAT2 mutant and evaluated their competence against STAT2-deficient H123-reconstituted wild-type STAT2 cells to activate apoptosis. Consistent with published reports, following IFN-α treatment, both STAT2 Y690F and STAT2 R409A,K415A (21, 33) failed to localize to the nucleus, thus resulting in impaired gene transcription (Fig. 6A and B). Interestingly, IFN-α activated STAT2 (STAT2 R409A:K415A) localized in the cytosol was not sufficient to promote apoptosis (Fig. 6C). These results clearly indicate that tyrosine phosphorylation and nuclear localization of STAT2 are two sequential events required for type I IFNs to induce apoptosis. More importantly, detection of activated STAT2 in the cytosol may be wrongly associated with an intact IFN response.

**STAT2 Gene Silencing Impairs Type I IFN–Induced Apoptosis**

To show a role of STAT2 in the activation of apoptosis, we silenced the expression of STAT2 in other cell lines that are susceptible to the apoptotic effects of IFN-α. The following tumor cell lines A375.S2 and SAOS2 were stably transduced with lentiviral vectors expressing mock or specific STAT2 shRNAs. Silencing of STAT2 abrogated type I IFN-induced apoptosis by ~50% (Fig. 7A). In addition to STAT2, the expression of apoptotic genes driven by

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**FIGURE 4.** Reconstitution of STAT2 rescues type I IFN–induced apoptosis and ISGF3-driven gene activation. A. Percent apoptosis was measured by dually staining cells with Annexin V and PI following IFN-α stimulation. STAT2 protein levels in reconstituted clones were assessed by Western blot analysis. Actin served as a control for equal protein loading (right, bottom). Quantitative analysis of STAT2 levels in reconstituted cell lines relative to H123 cells (right). B. Cells were treated with 1,000 U/mL IFN-α for the indicated times. Western blot analysis was done with phosphotyrosine-specific antibodies against STAT2 and STAT1. Membranes were reprobed with antibodies against total STAT2 and STAT1 antibodies to verify for equal protein loading. Levels of pSTAT2 to STAT2 were quantified. C. Total RNA was extracted and expression of IFI-8-16 and IFI-15K RNAs was determined by RNase protection assay. Actin RNA served as an internal control to normalize for equal loading.
STAT1 homodimers may be involved in promoting apoptosis by type I IFNs. Although IFN-α–mediated upregulation of IRF-1 protein was not observed in parental H123 cells, nevertheless, it was important to determine whether IRF-1 played a role in promoting apoptosis of H123 cells. Lentiviral silencing of IRF-1 in H123 cells, however, did not protect from the killing effects of IFN-α (Fig. 7B). This result suggests the existence of other STAT2-regulated apoptotic signaling mediators.

**Discussion**

In the present study, we showed that persistent exposure to IFN-α led to the development of a STAT2-deficient cell line that is resistant to type I IFN–induced apoptosis. An unexpected finding was to discover that a lack of STAT2 was not associated with gene methylation, lack of STAT2 mRNA transcripts, or mutations that altered its reading frame. Although we do not have an explanation for the lack of STAT2 protein in the presence of mRNA, gene silencing by microRNAs remains a possibility. Another important finding was that STAT2 mutants that do not impair STAT1 activation, but fail to dimerize with STAT1 or localize to the nucleus, do not rescue IFN-α–induced apoptosis. Thus, our results lead us to speculate that perhaps a subset of patients receiving long-term IFN treatment may experience resistance to type I IFN therapy due to defects in STAT2 function or expression.

The role of STAT1 and STAT2 in IFN resistance remains unclear. Work by Chawla-Sarkar et al. (34) showed that defects in STAT1 and STAT2 expression are infrequent in melanoma cell lines and tumor samples, and this did not correlate with IFN resistance. In a different study, Lesinski et al. (35) reported that the expression levels of STAT1 and STAT2 and their distribution in cytosolic and nuclear compartments in malignant melanoma cells did not always correlate with a favorable response to IFN-α adjuvant therapy. However, a few melanoma-resistant cell lines and tumor samples used in these studies were analyzed for STAT2 defects. In contrast, Zhou et al. (36) reported that IFN-α treatment of patients with carcinoid tumors led to increased levels of STAT1 and STAT2 in their tumors, which correlated with stable disease or objective response. Whereas Mischiati et al. (37) showed that STAT2 expression was lost in 67% of 15 metastatic melanoma lesions. However, in this study, no correlation between IFN-α resistance and loss of STAT2 was made.

Understanding the role of STAT2 in mediating type I IFN–induced apoptosis has been daunting. Before the isolation of the STAT2-deficient H123, there were two STAT2-deficient cell lines, in which STAT2 expression was restored. Yet STAT2 reconstitution did not make these cell lines susceptible to IFN-α–induced apoptosis. In our studies, we show a requirement for STAT2 in type I IFN–induced apoptosis as reconstitution of STAT2 rescued the apoptotic activity of IFN-α. We observed that a threshold of STAT2 protein was necessary for IFN-α–induced apoptosis. In fact, low expression of STAT2 protein resulted in delayed ISG expression and without or little induction of apoptosis. Furthermore, by silencing STAT2 expression in Saos2 and A375.S2 tumor cells, we show that STAT2 is a critical apoptotic mediator. However, these cells were not fully protected from the lethal effects of IFN-α, thus indicating a requirement of additional apoptotic mediators.

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*Our unpublished results.*
**IRF-1** is a gene regulated by IFN-activated STAT1 and is known for its role in the activation of IFN-induced apoptosis (38). We found STAT1 activation in H123 cells to be independent of STAT2. Our result supports a previous observation in which STAT1 activation is intact in STAT2-deficient mouse macrophages (39). Next, we found IRF-1 not to be upregulated by IFN-α in parental and STAT2-deficient H123. In addition, IRF-1 gene silencing in H123 cells conferred no significant protection against the killing effects of IFN-α. This observation implies that IRF-1 does not always mediate the apoptotic effects of type I IFNs. Recently, a small subset of IFN-inducible, STAT2-dependent apoptotic genes were identified on chromosome 22 (40). Therefore, it is likely that the expression of these genes require transcriptionally competent STAT2 for mediating type I IFN-induced apoptosis. Consistent with what has been reported about the mode of action of type I IFNs (41), earlier characterization of the H123 cell line showed that IFN-α activated the intrinsic mitochondrial-dependent death pathway (28). In this same model, we now show that alterations in mitochondrial membrane potential, cytochrome c release from mitochondria to the cytosol, and caspase activation, all of which are characteristics of activation of the intrinsic apoptotic pathway are events regulated by STAT2. In multiple myeloma, members of the Bcl-2 family such as Bax and Bak have been implicated in mediating type I IFN–induced apoptosis (42, 43). However, we did not detect activation of these molecules in H123 cells, possibly due to cell type differences. Collectively, these findings strongly suggest the existence of additional signaling modules that are involved in the activation of apoptosis.

**FIGURE 6.** Nuclear localization of activated STAT2 is required for ISGF3-mediated gene expression and induction of type I IFN-induced apoptosis. STAT2-deficient H123 cells reconstituted with various forms of STAT2 were left either untreated or treated with IFN-α (3,000 U/mL) for the indicated times. A. Subcellular distribution of STAT2 was analyzed by confocal immunofluorescence microscopy. B. Gene activation of ISGs was assessed by quantitative RT-PCR. Figure shown is representative of three experiments with similar results. C. Apoptosis was measured by dually staining of cells with Annexin V and PI. Columns, mean from three independent experiments; bars, SEM.
Work by Panaretakis et al. (44) showed an alternative mechanism wherein IFN-α–induced apoptosis is mitochondrial dependent but in a transcriptionally, JAK/STAT signaling–independent manner. However, our work seems to contradict these findings because transcriptionally active STAT2 localized to the nucleus was essential for the activation of apoptosis by type IFNs. In addition, tyrosine-phosphorylated STAT2 when retained in the cytoplasm is insufficient to mediate apoptosis. This discrepancy could lie on the nature of distinct cell types used for these studies. For instance IFN-α treatment of H123 cells does not activate c-Jun-NH₂-kinase, and pharmacologic inhibition of phosphoinositide 3-kinase activity does not protect cells from undergoing apoptosis. Quite the contrary, the presence of these inhibitors in combination with IFN-α accelerate the onset of apoptosis.⁵

In summary, our findings have clinical relevance as a subset of patients who are or become resistant during the course of IFN therapy may be experiencing a loss of STAT2 expression or function. Most importantly, IFN-α responses measured either in the tumor or blood of patients must be interpreted carefully as tyrosine-phosphorylated STAT2 may not always be indicative of intact STAT2 function and favorable IFN responses. Further studies are warranted to establish a link between a loss of STAT2 function and type I IFN resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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