A Mutation in the SH2 Domain of STAT2 Prolongs Tyrosine Phosphorylation of STAT1 and Promotes Type I IFN-induced Apoptosis

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Type I interferons (IFN- α/β) induce apoptosis in certain tumor cell lines but not others. Here we describe a mutation in STAT2 that confers an apoptotic effect in tumor cells in response to type I IFNs. This mutation was introduced in a conserved motif, PYTK, located in the STAT SH2 domain, which is shared by STAT1, STAT2, and STAT3. To test whether the tyrosine in this motif might be phosphorylated and affect signaling, Y631 of STAT2 was mutated to phenylalanine (Y631F). Although it was determined that Y631 was not phosphorylated, the Y631F mutation conferred sustained signaling and induction of IFN-stimulated genes. This prolonged IFN response was associated with sustained tyrosine phosphorylation of STAT1 and STAT2 and their mutual association as heterodimers, which resulted from resistance to dephosphorylation by the nuclear tyrosine phosphatase TcPTP. Finally, cells bearing the Y631F mutation in STAT2 underwent apoptosis after IFN- α stimulation compared with wild-type STAT2. Therefore, this mutation reveals that a prolonged response to IFN- α could account for one difference between tumor cell lines that undergo IFN- α -induced apoptosis compared with those that display an antiproliferative response but do not die.

INTRODUCTION

The antiviral, antiproliferative, and immunomodulatory properties of type I interferons (IFN α , β , and ω) have facilitated the introduction of this cytokine as adjuvant therapy for the treatment of certain forms of cancer and viral infections (Kjaergard *et al.*, 2002; Bleumer *et al.*, 2003; Stoutenburg *et al.*, 2004). The antiproliferative effects of IFNs are unpredictable, as tumor cells can be either growth inhibited with or without the induction of apoptosis (Sangfelt *et al.*, 1997;

Sandoval *et al.*, 2004). The mechanisms that dictate IFN-induced apoptosis and how the signal transducers and activators of transcription (STAT) are involved in this process have not yet been fully elucidated. Type I IFNs initiate a chain of signaling events by interacting with their cognate receptor, leading to receptor dimerization and activation of the Janus kinase (JAK)/STAT pathway (Stark *et al.*, 1998). JAK1 and TYK2 tyrosine kinases phosphorylate STATs, which then dimerize via Src-homology 2 (SH2) domain-phosphotyrosyl interactions and subsequently translocate to the nucleus where they bind DNA sequences found in the promoters of IFN responsive genes (Stark *et al.*, 1998). Phosphorylated STAT1 and STAT2 heterodimers associate with the DNA-binding protein p48/ISGF3 γ /IFN regulatory factor (IRF9) to form the IFN-stimulated gene factor 3 (ISGF3) complex that binds the IFN-stimulated response element (ISRE; Reid *et al.*, 1989; Qureshi *et al.*, 1995; Horvath *et al.*, 1996). Type I IFNs also induce the formation of STAT1 homodimers, which bind the gamma responsive region (GRR) or gamma activation sequence (GAS; Decker *et al.*, 1991; Schindler *et al.*, 1992), which together with ISGF3 drive transcription of IFN-stimulated genes (ISG) that are responsible for the biological effects of IFNs.

Our current understanding of the contribution of STATs to the antiproliferative effects of type I IFN and the role of specific domain(s) or amino acid(s) within the STATs responsible for their function have come from several lines of in vivo and in vitro evidence. Mice deficient in STAT1,

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Abbreviations used: GRR, gamma responsive region; IFN, interferon; ISGF3, interferon-stimulated gene factor 3; ISRE, interferon-stimulated responsive element; SH2, Src-homology-2; STAT, signal transducers and activators of transcription; TcPTP, T-cell protein tyrosine phosphatase.

STAT2, or IRF9 have impaired antitumor and/or antiviral immunity (Kimura *et al.*, 1996; Meraz *et al.*, 1996; Park *et al.*, 2000). Cell lines deficient in STAT1, STAT2, or IRF9 are resistant to the antiviral and antiproliferative effects of type I IFNs (John *et al.*, 1991; Leung *et al.*, 1995; Bromberg *et al.*, 1996). Mutation of STAT1 or STAT2 in the conserved arginine in the SH2 domain or mutation in the conserved tyrosine phosphorylation site abrogates dimerization or nuclear translocation (Gupta *et al.*, 1996; Li *et al.*, 1997; Mowen and David, 1998). Although a mutation in the conserved serine 727 found in the transactivation domain (TAD) of STAT1, as well as in STAT3 and STAT4, impairs transcriptional activity (Wen and Darnell, 1997; Zhu *et al.*, 1997; Morinobu *et al.*, 2002), a mutation in the N-terminal domain of STAT1 (F77A) impairs its oligomerization, tyrosine dephosphorylation, and gene transcription (Meyer *et al.*, 2004). In contrast, forced dimerization of STAT1 by mutation of two conserved cysteine residues within the SH2 domain renders cells more responsive to the apoptotic effects of IFNs (Sironi and Ouchi, 2004).

In the absence of a crystal structure for STAT2, our only approach to study the function of this protein is to rely on mutational analysis of conserved regions found among the STATs. We previously characterized a type I IFN apoptotic-resistant cell line that allowed the identification of a conserved proline in the SH2 domain that when mutated to leucine (P630L) in STAT2 impairs the activation of IFN-inducible ISRE-dependent gene transcription (Gamero *et al.*, 2004). In this study, we expanded our early observations and examined the conserved amino acids surrounding P630 in the SH2 domain of STAT2. In this study we have identified and analyzed a conserved PYTK motif in the SH2 domain of STAT2 by site-directed mutagenesis. This approach exposed Y631 as an important residue when mutated to phenylalanine. In response to IFN- α stimulation, this STAT2 Y631F mutant demonstrates that the maintenance of signaling events such as sustained STAT1 and STAT2 tyrosine phosphorylation, their persistence in the nucleus, and prolonged activation of the ISGF3 complex can change the antiproliferative effects of type I IFNs to one of apoptosis.

MATERIALS AND METHODS

Cells and Cell Culture Reagents

The human fibrosarcoma cell line 2FTGH, and variants from this cell line: U1A (TYK2^{-/-}), U2A (IRF9^{-/-}), U3A (STAT1^{-/-}), U4A (JAK1^{-/-}), and U6A (STAT2^{-/-}), a gift from G. Stark (Cleveland Clinic Foundation, Cleveland, OH) were cultured in high glucose DMEM containing 10% fetal calf serum, 1% Glutamax, 1% penicillin, and streptomycin (Invitrogen, Carlsbad, CA) at 37°C. 2FTGH variants were transfected with 5 μ g of STAT2 or STAT2 mutants using Metafectene reagent (Biontex Laboratories, Munich, Germany). Stably expressing clones were maintained with 500 μ g/ml G418. Recombinant human IFN- α 2a and IFN- γ (specific activity 2×10^7 U/ml) were purchased from PeproTech (Rock Hill, NJ). Recombinant human IFN- β was a generous gift from BiogenIdec (Cambridge, MA).

STAT2 Site-directed Mutagenesis

A flag-tagged STAT2 construct in pcDNA3, kindly provided by C. Horvath (Northwestern University, Evanston IL), was used as template DNA. STAT2 mutagenesis was performed with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the following primers synthesized by Integrated DNA Technologies (Coralville, IA): P630L: 5'-CATCTACTCTGTGCAACTGTACACGAAGGAGGTGC-3' and 5'-GCACCTCCTTCGTGTACAGTTGCACAG AGTAGATG-3'; Y631F: 5'-CTCTGTGCAACCGTTTCACGAAGGAGGTGC-3' and 5'-GCACCTCCTTCGTGAACGGTTCACAGAG-3'; T632A: 5'-TCTGTGCAACCGTACGCGAAGGAGGTGCTGC-3' and 5'-GCAGACCTCCTTCGCGTACGGTTCACAGAG-3'; and K633A: 5'-GTGCAACCGTACACGGCGAGGTGCTGCAGTC-3' and 5'-GACTGCAGCACCTCCGCCGTGACGGTTCAC-3'.

Mutagenesis was confirmed by sequencing the entire STAT2 SH2 sequence.

Preparation of Cell Extracts

Nuclear extracts were prepared as previously described (Petricoin *et al.*, 1994). For whole-cell extracts, cells were resuspended in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 10 mM β -glycerophosphate, 1 mM sodium orthovanadate, 25 mM NaF, 0.5 mM dithiothreitol, and 200 μ M phenylmethylsulfonyl fluoride [PMSF]) supplemented with a complete protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Nutley, NJ). After centrifugation at 4°C for 10 min, supernatants were collected and protein concentrations measured by standard Bio-Rad Bradford protein assay (Richmond, CA).

Cell Proliferation Assay

Cells were seeded in flat-bottom 96-well plates at a concentration of 1×10^3 cells in 100 μ l volume/well. Cells were stimulated with or without IFN- α at the indicated concentrations and incubated for 3 d at 37°C. Cell proliferation was assessed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt] assay (Promega, Madison, WI) according to the manufacturer's instructions.

Measurement of Apoptosis

Cells were either untreated or stimulated with IFN- α for the indicated times. Cells were then harvested, resuspended in 50 μ l of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂), containing 2 μ l of annexin V-fluorescein isothiocyanate (FITC; BD Pharmingen, San Diego, CA) and 2 μ l of propidium iodide at 50 μ g/ml (Sigma-Aldrich, St. Louis, MO), and incubated for 10 min at room temperature. Cells were collected ungated (10,000 events) by two-color flow cytometry using FACScan™ (Becton-Dickinson, San Jose, CA) to discriminate between apoptotic and late apoptotic/necrotic cells. Data were then analyzed using CellQuest (Becton Dickinson).

Confocal Microscopy

To monitor nuclear translocation of STATs, cells were plated in a six-well plate on a glass coverslip, washed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and then washed again in PBS. Cells were permeabilized with 0.2% Triton-X 100 in PBS for 5 min, followed by incubation with primary antibody diluted in blocking solution (2% goat serum, 2 mg/ml BSA in PBS) at 4°C overnight. The slides were washed with blocking buffer and then incubated for 1 h with FITC- and tetramethylrhodamine isothiocyanate (TRITC)-labeled secondary antibodies (Alexis Biochemicals, San Diego, CA). After several washes with PBS, the slides were counterstained with DAPI Vectashield mounting media (Vector Laboratories, Burlingame, CA). To visualize apoptotic cells, cells were plated in a 12-well plate at 20% confluency. After IFN stimulation, cells were washed twice with PBS and incubated for 15 min at room temperature in the dark with 500 μ l of annexin-V-FITC (BD Pharmingen) diluted 1:50 with binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂). For nuclear staining, cells were counterstained with 5 μ g/ml Hoechst dye (Sigma-Aldrich). Cells were examined using a Zeiss LSM 510 NLO inverted confocal laser scanning microscope equipped with a Plan-Neofluar 100 $\times$ /1.3 NA oil objective (Carl Zeiss, Jena, Germany). Images were viewed and overlaid using the Zeiss LSM Image Browser software.

Immunoprecipitation and Western Blot Analysis

Nuclear extracts were subjected to immunoprecipitation with anti-STAT2 antibody (C20, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h followed by the addition of protein G-Sepharose beads (Amersham Biosciences, Piscataway, NJ) for another 2 h at 4°C. Immunoprecipitates, nuclear extracts, or whole-cell extracts were then separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with antibodies specific to phospho-STAT1 Y701 (Cell Signaling Technology, Beverly, MA), phospho-STAT2 Y690 (Upstate Biotechnology, Charlottesville, VA), STAT1 (a gift from A. Larner, Cleveland Clinic Foundation, Cleveland, OH), STAT2, and Flag epitope (Sigma-Aldrich). Anti-actin and anti-RPA70 antibodies were purchased from Abcam (Cambridge, MA) and used as an internal control to monitor for equal loading and purity of nuclear extract samples, respectively. Membranes were developed by chemiluminescence using the ECL Western blotting system (Pierce, Rockford, IL) as previously described (Gamero *et al.*, 2004).

Electrophoretic Mobility Shift Assays

Synthetic double-stranded oligonucleotides corresponding to the ISRE of the ISG15 promoter and the GRR sequence of the Fc γ RI promoter were used as DNA probes. Each probe was end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase (Cell Signaling) as previously described (Gamero *et al.*, 2004). The DNA-protein complexes were subjected to electrophoresis on a 4.7% polyacrylamide gel and visualized by autoradiography.

RNA Preparation and Quantitative RT-PCR

Cells were either left untreated or stimulated with IFN- α for the indicated times. RNA was isolated with RNazol B (Tel-Test, Friendswood, TX) accord-

ing to the manufacturer's instructions. Five micrograms of total RNA was reverse transcribed to generate cDNA using Superscript II reverse transcriptase (Invitrogen). Quantification RT-PCR (qRT-PCR) primers for IFIT2, IFI 6-16, and TRAIL were obtained from Applied Biosystems (Foster City, CA). Briefly, cDNA was mixed with Taqman 2× PCR master mix (Applied Biosystems), using primers with FAM reporter dyes, and qPCR reactions were performed 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 (expression was measured at this point by the instrument). Each mRNA quantification was normalized by multiplexing with 18S-VIC primers.

Pulse Chase Tyrosine Dephosphorylation

Cells were stimulated with IFN- α for 30 min and washed once with complete DMEM medium to remove cytokine. Cells then were treated for various times with 500 nM staurosporine (Sigma-Aldrich) resuspended in complete DMEM medium to stop further tyrosine phosphorylation. Whole-cell extracts were prepared and analyzed by Western blotting with phospho-STAT1 Y701 antibody.

TcPTP In Vitro Dephosphorylation Assay

To examine defects in the dephosphorylation of STAT1 by the STAT1 protein tyrosine phosphatase TcPTP (T-cell protein tyrosine phosphatase; Millipore, Billerica, MA), nuclear extracts were subjected to immunoprecipitation with anti-STAT1 antibody or normal rabbit serum as irrelevant control and protein G-Sepharose beads (Amersham Biosciences) at 4°C. Immunoprecipitates were washed twice with lysis buffer without sodium orthovanadate and once with protein tyrosine phosphatase (PTP) buffer (25 mM Tris, pH 7.5, 0.5 mg/ml BSA, 10 mM DTT, complete protease inhibitor cocktail tablet, and 1 μ M PMSF). Immunoprecipitates were then incubated for 1 h at 30°C with various concentrations of recombinant human TcPTP enzyme in a volume of 30 μ l of PTP buffer. Samples were resolved by 10% SDS-PAGE and tyrosine dephosphorylation of STATs was analyzed by Western blotting with antiphospho-STAT1 Y701 and antiphospho-STAT2 Y690 antibody.

RESULTS

Characterization of a Conserved Motif in the SH2 Domain of STATs

We recently identified proline 630 (P630) as a conserved residue in the SH2 domain of STATs that is critical for transcriptional function (Gamero *et al.*, 2004). To identify additional amino acids that might be important for STAT function, sequence homology analysis revealed a conserved set of residues not previously characterized in the SH2 domain of human STAT1, STAT2, and STAT3 adjacent to P630 that we termed "PYTK" motif (Figure 1, A and B). This motif is also conserved in other mammalian species. To study whether this motif contributed to the function of STAT2, each amino acid in the PYTK motif was mutated as follows: proline to leucine (P630L), tyrosine to phenylalanine (Y631F), threonine to alanine (T632A), or lysine to alanine (K633A), by site-directed mutagenesis. Expression vectors encoding flag-tagged wild-type and STAT2 mutants were introduced singly by stable transfection into U6A cells, a variant of the human fibrosarcoma cell line, 2fTGH, which does not express STAT2. As shown in Figure 1C (bottom panel), all transfectants expressed adequate levels of STAT2 protein. The panel of U6A cell lines was then stimulated with IFN- α , and the antiproliferative response was assessed on day 3. The effects of IFN- γ , a type II IFN, was also tested in this experiment as this cytokine has been reported to activate STAT2 in certain cell types, albeit weakly (Matsumoto *et al.*, 1999). As expected, both IFN- α and - γ failed to induce an antiproliferative response in U6A cells deficient in wild-type STAT2 (Figure 1C). In response to IFN- γ , all U6A lines expressing wild-type STAT2 or various STAT2 mutants showed similar levels of antiproliferative activity ranging from 25 to 40% (Figure 1C). As previously reported, U6A cells expressing STAT2 P630L were unresponsive to the antiproliferative effects of IFN- α (Gamero *et al.*, 2004). In addition, the STAT2 T632A and STAT2 K633A mutants had no effect on IFN- α -induced

Figure 1. The PYTK motif within the SH2 domain of STAT1, STAT2 and STAT3. (A) The conserved motif PYTK (boxed) is conserved in STAT1, STAT2 and STAT3 (B) Domains of human STAT2. The position of PYTK motif located within the SH2 domain is marked. C-C indicates coil-coil domain; DBD, DNA-binding domain; SH2, Src-homology-2, and TAD, transactivation domain. (C) Panel of U6A cells expressing STAT2 with single amino acid substitutions were stimulated with or without IFN- α (3000 U/ml) or IFN- γ (10 ng/ml) and (%) growth inhibition was measured on day 3 by MTS assay. Results are shown as mean $\pm$ SD. Lower panel shows immunoblot analysis with anti-STAT2 and anti-actin antibodies. STAT2 expression levels were normalized against actin using densitometry and compared relative to U6A WT STAT2 expression. This is a representative experiment of three independent experiments.

growth inhibition compared with that of wild-type STAT2 (~25%). In contrast, expression of STAT2 Y631F rendered the cells highly susceptible to the antiproliferative effects of IFN- α ranging from 45 to 77%. To rule out the possibility that this was a consequence of a single clonal effect, we analyzed a panel of six independent U6A clones stably expressing STAT2 Y631F and found similar enhanced IFN- α responsiveness effects (Figure 1C and data not shown). These results suggested that within this PYTK conserved motif, Y631 is functionally important to type I IFN, but not to type II IFN signaling.

STAT2 Y631F Promotes IFN- α -induced Apoptosis

To determine whether the robust IFN- α -mediated antiproliferative response observed with STAT2 Y631F was dose-dependent, U6A cells expressing wild-type STAT2 or STAT2 Y631F were stimulated with either 500 or 3000 U/ml IFN- α , respectively, and the antiproliferative activity was measured on day 3. As shown in Figure 2A, U6A cells expressing STAT2 Y631F displayed greater growth inhibition in a dose-dependent manner (60 and 80%) as opposed to U6A cells expressing wild-type STAT2 cells (15 and 25%). Light mi-

Figure 2. STAT2 Y631F stimulates the induction of apoptosis by type I IFNs. (A) U6A (STAT2^{-/-}) cells or U6A reconstituted with wild-type STAT2 or STAT2 Y631F were stimulated with either 500 or 3000 U/ml IFN- α and growth inhibition was measured on day 3. (B) Same as in A except cells were stimulated with 3000 U/ml IFN- α and dually stained with Annexin V (x-axis) and propidium iodide (y-axis) and analyzed by flow cytometry. (C) Confocal microscopy of U6A cells reconstituted with wild-type STAT2 (top row) or STAT2 Y631F (bottom row) stimulated with or without IFN- α (3000 U/ml), IFN- β (3000 U/ml), or IFN- γ (10 ng/ml). (D) Same as in (C) except cells were pretreated for 2 h with the caspase inhibitor ZVAD (50 μ M) before treatment with IFN- α (top row). Light field microscopy of cells shows morphological changes of apoptosis (bottom row). These experiments were repeated at least three times with similar results. Data are shown as mean $\pm$ SD.

croscopic analysis of IFN- α -stimulated U6A cells expressing STAT2 Y631F revealed morphological changes characteristic of apoptosis (Figure 2D, middle bottom). To confirm whether expression of STAT2 Y631F led to IFN- α -induced apoptosis, we dually stained cells with Annexin V and propidium iodide (Figure 2B). Unstimulated U6A cells expressing either wild-type STAT2 or STAT2 Y631F showed low levels of apoptosis (10 and 13%, respectively; Figure 2B). In contrast, after treatment with IFN- α for 72 h, a greater

proportion of U6A cells expressing STAT2 Y631F underwent apoptosis, whereas U6A cells expressing wild-type STAT2 did not show an increase in cell death (compare 66 vs. 10%, respectively). Similar results were obtained with six other U6A clones stably expressing STAT2 Y631F (data not shown).

To examine whether other IFNs, such as IFN- β (another type I IFN) and IFN- γ (type II), conferred similar apoptotic effects as those observed with IFN- α -stimulated U6A cells expressing STAT2 Y631F, confocal microscopic analysis was performed by staining cells with Annexin V-FITC and Hoechst 33342 dye. Although IFN- γ treatment of U6A cells expressing STAT2 Y631F did not induce apoptosis, treatment with either IFN- α or IFN- β induced cells to undergo apoptosis (Figure 2C). In contrast, expression of wild-type STAT2 did not result in an apoptotic response to either type I IFN(- α / β) or type II IFN (- γ) (Figure 2C). The contribution of the caspase pathway in the apoptotic process triggered by IFN- α was confirmed as pretreatment of U6A cells expressing STAT2 Y631F with the pan-caspase inhibitor ZVAD completely abrogated the apoptotic effects of IFN- α , with cells retaining intact cellular morphology (Figure 2D).

STAT2 Y631F Promotes Prolonged Activation and Nuclear Accumulation of STAT1 after IFN- α Stimulation

To determine whether the STAT2 Y631F mutation caused alterations in IFN- α signal transduction, nuclear extracts were prepared to examine the kinetics of activation of STAT1 and STAT2. IFN- α treatment of U6A cells expressing STAT2 Y631F led to sustained phosphorylation of STAT1-Y701 and STAT2-Y690 that was detectable at 8 h after stimulation (Figure 3A, right). In contrast, tyrosine phosphorylation of STAT1 in IFN- α -treated U6A cells expressing wild-type STAT2 was barely detectable at 8 h, whereas STAT2 remained tyrosine phosphorylated (Figure 3A, left). It should be noted that mutation to the Y631 residue in STAT2 did not induce spontaneous tyrosine phosphorylation of either STAT1 or STAT2 in unstimulated cells. To confirm these findings, nuclear localization of STAT1 and STAT2 was examined by confocal microscopy. As shown in Figure 3B in U6A cells expressing STAT2 Y631F after 5 h of IFN- α stimulation, STAT1 and STAT2 remained localized to the nucleus while in U6A cells expressing wild-type STAT2, STAT1 returned to the cytoplasm. Collectively, these findings suggested that in response to IFN- α , STAT2 Y631F alters the normal tyrosine dephosphorylation of STAT1.

Given that the expression of STAT2 Y631F resulted in the prolonged IFN- α -mediated tyrosine phosphorylation of STAT1, it was important to determine whether this effect was attributed to STAT2 Y631F having an enhanced avidity for STAT1. Therefore, the association between nuclear STAT1 and STAT2 was analyzed by coimmunoprecipitation assays. STAT2 immunoprecipitates showed that unlike U6A cells expressing wild-type STAT2, there was a substantial amount of STAT1 in complex with STAT2 in the nucleus of U6A cells expressing STAT2 Y631F after 5 h of IFN- α stimulation (Figure 3C). Furthermore, electrophoretic mobility shift assay (EMSA) analysis showed that the kinetics of the heterotrimeric ISGF3 transcription complex binding to the ISRE was also prolonged in U6A cells expressing STAT2 Y631F. In contrast STAT1 homodimers binding to the GRR was the same in both U6A cells expressing either wild-type STAT2 or STAT2 Y631F (Figure 3D). These findings, therefore, indicated that in response to IFN- α stimulation, STAT2 Y631F prolongs its association with STAT1 and its activation, does not impair the formation of STAT1 homodimers, but delays STAT1 nuclear export.

Figure 3. STAT2 Y631F prolongs STAT1 activation. Nuclear extracts were prepared from cells incubated with or without IFN- α for the indicated times. (A) Nuclear extracts were resolved by SDS-PAGE and immunoblot analysis was performed with antibodies against phosphoSTAT1-Y701 and phosphoSTAT2-Y690. Immunoblots were reprobed with RPA70 antibody to control for both equal loading of protein and purity of the nuclear extracts (lower panels). (B) Subcellular distribution of STAT1 and STAT2 was analyzed by confocal immunofluorescence microscopy. Cells were permeabilized and stained with anti-STAT1 (magenta), anti-Flag (STAT2, green), and counterstained with DAPI. STAT1 and STAT2 overlay is depicted as white. (C) Nuclear extracts were immunoprecipitated with anti-STAT1 (top panel) anti-phospho-STAT2 (middle panel) or anti-STAT2 (bottom panel) antibodies to ensure equal levels of immunoprecipitated proteins. Immunoprecipitation with IgG or beads alone were used as nonspecific negative controls. (D) Nuclear extracts were assayed by EMSA for formation of ISGF3 and STAT1 homodimers with an ISRE and GRR probe, respectively.

STAT2 Y631F Promotes Sustained IFN- α -stimulated Gene Transcription

As predicted, prolonged activation of STAT1 and STAT2 in U6A cells expressing STAT2 Y631F translated into early and/or prolonged expression of ISRE-dependent ISG: IFIT2, IFI 6-16, and TRAIL, compared with U6A cells expressing wild-type STAT2 (Figure 4). In contrast, when IFN- α -induced expression of suppressors of cytokine signaling (SOCS)1, 2, and 3 was examined, no difference in the induction of these genes was seen in U6A cells

Figure 4. STAT2 Y631F augments ISGF3-mediated gene expression. Total RNA was isolated and qRT-PCR was performed in triplicate wells using specific primers to the indicated genes. 18S primers were included as an internal control to normalize for equal amount of cDNA. This is a representative experiment of three that were performed.

expressing either STAT2 or STAT2 Y631F (data not shown). It is also noteworthy that mass spectrometric analysis showed that STAT2 Y631 is not phosphorylated in response to IFN- α (data not shown).

Impaired STAT1 Tyrosine Dephosphorylation in the Presence of STAT2 Y631F

Prolonged activation of STAT1 and STAT2 was assumed to be due to a defect in the rate of tyrosine dephosphorylation, given that the levels of STAT were similar for both cell lines expressing either wild-type STAT2 and STAT2 Y631F. To further examine this possibility, cells were treated with IFN- α for 30 min followed by a pulse chase with the kinase inhibitor staurosporine (STS) to block further increases in STAT1 tyrosine phosphorylation. As shown in Figure 5A, a defect in the rate of STAT1 tyrosine dephosphorylation was evident in U6A cells expressing STAT2 Y631F compared with those expressing wild-type STAT2, where tyrosine dephosphorylation of STAT1 was complete after 1 h of treatment with STS (Figure 5A, compare lane 4 vs. lane 10). The concentration of STS used in this experiment was adequate as tyrosine phosphorylation of STAT1 was blocked in cells treated with STS and IFN- α simultaneously (Figure 5A, lane 6).

To determine whether STAT1 was protected from tyrosine dephosphorylation when associated with STAT2 Y631F, we treated U6A cells expressing either wild-type STAT2 or STAT2 Y631F with or without IFN- α for 30 min. STAT1 was immunoprecipitated from nuclear extracts to evaluate its resistance to tyrosine dephosphorylation in the presence of increasing amounts of the STAT1 nuclear protein tyrosine phosphatase TcPTP. Figure 5B shows that STAT1 is less susceptible to the enzymatic dephosphorylation activity of TcPTP in the presence of STAT2 Y631F as 2 μ g of this enzyme was not sufficient to tyrosine dephosphorylate STAT1 to a level comparable to that observed in cells expressing wild-type STAT2. The reciprocal experiment was

Figure 5. STAT2 Y631F inhibits STAT1 tyrosine dephosphorylation. (A) Cells were stimulated with IFN- α (1000 U/ml) for 30 min followed by pulse chase with 500 ng/ml staurosporine (STS) for the indicated times. Whole-cell extracts were prepared and immunoblots were probed with an antibody against phosphoSTAT1-Y701. Membrane was reprobed for total STAT1 (bottom panel) to ensure equal loading of protein. (B) Same as in A except, STAT1 was immunoprecipitated from nuclear extracts and then incubated with increasing amounts of the protein tyrosine phosphatase TcPTP. IgG immunoprecipitates are shown as irrelevant negative controls.

performed by evaluating STAT2 immunoprecipitates and similar results were obtained. These results indicate that STAT1 and STAT2 Y631F remain phosphorylated as heterodimers longer because they are resistant to the enzymatic effects of tyrosine phosphatases after IFN- α treatment.

STAT2 Y631F-mediated IFN- α -induced Apoptosis is JAK/STAT Pathway Dependent

To gain insight into the contribution of the JAK/STAT pathway in the induction of apoptosis by type I IFNs, STAT2 Y631F was stably expressed in variants of the 2fTGH cell line that do not express either JAK1, TYK2, STAT1, or IRF9. Given that IFN- α treatment of cells not only leads to the formation of STAT1 homodimers, but also to STAT1/STAT2 heterodimers or STAT1/STAT2 in association with IRF9, this approach allowed the identification of which STAT complex is driving apoptosis in the presence of STAT2 Y631F. A panel of 2fTGH variants stably expressing STAT Y631F was stimulated with IFN- α for 3 d, and the antiproliferative and apoptotic effects of this cytokine were assayed in parallel. As shown in Table 1, in the absence of JAK1, TYK2, or STAT1, the antiproliferative effects of IFN- α were lost. Reconstitution of STAT2 Y631F in STAT2-deficient cells led to the induction of apoptosis (~60%). Interestingly, expression of STAT2 Y631F in the absence of IRF9 restored the antiproliferative effects of IFN- α (~30%), but it was not sufficient to promote apoptosis. It is worth noting that expression of STAT2 Y631F in parental 2fTGH cells, which express endogenous levels of STAT2, rendered these cells susceptible to the apoptotic effects of IFN- α (data not shown). These data, therefore, indicate that expression of STAT2 Y631F has a dominant effect. More importantly, this mutation allowed cells to switch their response from one of antiproliferative to the induction of apoptosis.

Table 1. Induction of apoptosis by IFN- α via STAT2 Y631 is JAK/STAT dependent

Transfected with STAT2 Y631F	IFN- α -mediated growth inhibition	IFN- α -mediated apoptosis
U1A (TYK2 ^{-/-})	—	—
U2A (IRF9 ^{-/-})	+	—
U3A (STAT1 ^{-/-})	—	—
U4A (JAK1 ^{-/-})	—	—
U6A (STAT2 ^{-/-})	+	+

Variants of the 2fTGH cell line that lack expression of TYK2 (U1A), JAK1 (U4A), STAT1 (U3A), STAT2 (U6A), or IRF9 (U2A) were stably transfected with STAT2 Y631F. Cells were incubated in the absence or presence of IFN- α (3000 U/ml). Percent growth inhibition and apoptosis were assessed on day 3 by MTS, and annexin V plus propidium iodide staining was assessed on day 2. Experiments were repeated at least three times with similar results.

DISCUSSION

In this study, we are the first to report the existence of a novel PYTK motif for proline/tyrosine/threonine/lysine in a distinct region of the SH2 domain of STAT2, which is shared by STAT1 and STAT3 (Figure 1). Mutational analysis of this motif in STAT2 exposed a tyrosine residue at position 631 that when mutated to phenylalanine (STAT2 Y631F) dramatically augmented the antiproliferative effects of type I IFNs and switched their mode of action to the promotion of apoptosis. This apoptotic effect was not due to increased levels of total STAT2 protein. In addition, this process was caspase-dependent and exclusively induced by type I, but not by type II IFNs.

Mutational analysis of specific amino acids conserved within the domains of STATs has yielded insight into how functionally relevant they are. For instance, mutation of a conserved arginine in the SH2 domain of STATs revealed the importance of STAT tyrosine phosphorylation and dimerization for their nuclear import (Li *et al.*, 1997; Mowen and David, 1998). A different study showed that the DNA-binding domain of STAT2 is functional, as two amino acid substitutions (V453I, V454I) affected IFN- α -mediated biological responses that were STAT2 dependent, but ISGF3 independent (Brierley and Fish, 2005). A conserved serine residue found in the TAD of STAT1, STAT3, STAT4, and STAT5 must be phosphorylated to maximally drive gene expression (Wen and Darnell, 1997; Zhu *et al.*, 1997; Morinobu *et al.*, 2002; Xue *et al.*, 2002; Pilz *et al.*, 2003).

The molecular mechanism(s) underlying the antiproliferative responses induced by IFNs is unpredictable in tumor cells as this process can occur in the absence or presence of apoptosis (Sangfelt *et al.*, 1997; Sandoval *et al.*, 2004). We questioned whether the JAK/STAT pathway contributed to programming tumor cells to undergo apoptosis. To address this question, cells were maintained in the continuous presence of IFN- α to develop resistance to the apoptotic effects of this cytokine (Gamero *et al.*, 2004). Individual clones with defects in the activation of the JAK/STAT signaling pathway were selected, which led to the identification of a conserved proline residue in the SH2 domain of STATs. Type I IFNs signaling was impaired when STAT2 P630 was mutated to leucine due to its weak tyrosine phosphorylation and inability to associate with STAT1. In this study, by contrast, mutation of the adjacent amino acid, Y631, to phenylalanine (Y631F) produced a form of STAT2 that not

only retained STAT1 in the nucleus as heterodimers, but also rendered cells more responsive to IFN- α by programming them to undergo apoptosis.

Mass spectrometry analysis revealed that Y631 does not appear to be tyrosine phosphorylated. Nevertheless, biochemical characterization of the Y631F STAT2 mutation has yielded important information regarding certain signaling events that must be sustained, as in the case of ISGF3 activation, for type I IFNs to program tumor cells to die when their inherent response is to be growth arrested. One recent study showed that when two amino acid substitutions are made in the SH2 domain of STAT1 (STAT1-C), allowing STAT1 to spontaneously form homodimers, cells become hyperresponsive to IFN- γ and undergo apoptosis (Sironi and Ouchi, 2004). In another study, the same STAT1 mutant rendered cells more responsive to the antiviral effects of IFNs (Zhang *et al.*, 2005). Unlike STAT1-C, STAT2 Y631 affected tyrosine phosphorylation of STAT1 without enhancing the antiproliferative effects of IFN- γ (Figure 1C). Interestingly, when inactive, unlike STAT1, STAT2 dynamically shuttles between the cytoplasm and nucleus because of its strong intrinsic nuclear export rate (Banninger and Reich, 2004). After IFN- α stimulation, nuclear export is abolished and STAT2 subsequently accumulates in the nucleus. However, mutations introduced to the characterized nuclear export signal not only impair STAT2 shuttling back to the cytoplasm, but also prolonged STAT2 accumulation in the nucleus can have toxic effects on cells (Banninger and Reich, 2004; Frahm *et al.*, 2006).

The induction of apoptosis by IFN- α , mediated via STAT2 Y631F, was primarily dependent on the JAK/STAT pathway. Notably in the absence of IRF9, expression of STAT2 Y631F restored the antiproliferative effects of IFN α ; however, IRF9 remains essential in the promotion of apoptosis. This result comes as no surprise because there are reports that STAT1/STAT2 heterodimers in the absence of IRF9 bind to a distinct DNA element, termed palindromic IFN response element (pIRE), which is similar to the consensus GAS element that drives expression of specific ISGs, such as IRF1 (Li *et al.*, 1996; Ghislain *et al.*, 2001).

Our results provide the first evidence of the existence of a regulatory apoptotic switch in the SH2 domain of STAT2 wherein the JAK/STAT pathway is essential, and the ISGF3 heterotrimeric complex comprised of STAT1/STAT2 Y631F/IRF9 and not STAT1 homodimers is responsible for the apoptotic effects mediated by type I IFNs. Moreover, the presence of STAT2 Y631 increased IFN responsiveness; as it impaired STAT1 tyrosine dephosphorylation by protecting STAT1 from the enzymatic activity of TcPTP, thus allowing ISGF3 to bind longer to DNA, which presumably, activates the expression of genes required for the induction of apoptosis. Despite the lack of IFN- α -inducible tyrosine phosphorylation of STAT2 Y631, it remains to be determined whether mutations introduced in the PYTK motif prevent or facilitate STAT interaction with other proteins. We are currently characterizing STAT1 and STAT3 carrying mutations in the PYTK motif for alterations in function.

The signaling mechanisms or molecules involved in programming tumor cells to undergo apoptosis in response to type I IFNs have not yet been fully elucidated. Understanding the functional significance of the conserved motifs found in STATs is important as they influence type I IFN signal transduction pathways. Thus far, our results strongly indicate that specific amino acid residues in the SH2 domain of STATs exist that can dictate whether or not IFNs will favor an apoptotic response in tumor cells. This information has clinical ramifications as we poorly understand why only a

subset of cancer patients responds to IFN immunotherapy. Our data, however, suggest that if mutations arise in the STAT conserved motif, IFN responses may be altered. Hence we are currently screening tumor cell lines for mutations within this motif. Given that the clinical success of IFN- α as adjuvant therapy is variable, our basic findings provide a rationale for the development of therapeutic drugs that target STAT2 to improve the antitumor and antiviral efficacy of type I IFNs.

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