Identification of a Novel Conserved Motif in the STAT Family That Is Required for Tyrosine Phosphorylation* 

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The rapid transcriptional activation of cellular genes by either type 1 interferons (IFNα/β) or type 2 interferon (IFNγ) is responsible for many of the pleiotropic effects of these cytokines, including their antiviral, antitumor, and immunomodulatory activities. Interferon-stimulated gene expression is mediated by transcription factors termed Stats, which upon being tyrosine-phosphorylated, translocate to the nucleus and bind enhancers of interferon-activated genes. We have recently characterized a new Jurkat cell variant, named H123, where IFNα stimulates programmed cell death. H123 clones that are resistant to the apoptotic actions of IFNα have been selected. One of these clones (Clone 8) is defective in its responses to IFNα with regard to activation of genes that require tyrosine phosphorylation of Stat2. Stimulation of Clone 8 cells with IFNα induces normal tyrosine phosphorylation of Stat1 and Stat3. Sequencing of Stat2 RNA reveals a substitution of proline 630 located within the Src homology 2 domain of Stat2 to leucine (P630L). Pro-630 and its adjacent amino acids are conserved in all Stat family members but are absent in other proteins that contain Src homology 2 domains. Expression of Stat2 P630L in cells inhibits IFNα-stimulated gene expression. These results not only define a critical motif in Stat2 required for its transcriptional activity, but they also provide evidence that resistance to type 1 IFNs can be mediated by mutations in Stat2 as well as those previously described for Stat1.

Identification of components required for interferon-mediated expression of immediate early genes was greatly facilitated by selection of cells unresponsive to interferons (IFN)1 (1). Variants of the HT1080 fibrosarcoma cell line that do not respond to IFNα were isolated after multiple rounds of exposure to the frameshift mutagen ICR191 (2). These variants fail to express proteins encoding Jaks or Stats and have been used extensively to define important domains required for the structure and function of these proteins. Other domains known to be essential for the function of Jaks and Stats have been gleaned from the identification of mutant proteins that result in specific diseases. For instance, point mutations in Jak3 that render the kinase inactive were found in several patients with severe combined immunodeficiency (3, 4). Many of these mutations occur in the N-terminal region of the protein, which aided in defining its “FERM” domain (4). Melanomas and malignant breast cancer cell lines have been reported to lack expression of Stat1, Stat2, and p48 (5). It is unclear whether there is a mutation in the gene or some other event that makes the expression of this transcription factor undetectable. A single point mutation in Stat1 (leucine 706 to serine) renders individuals susceptible to infection with mycobacteria (6). Leucine 706 has been shown to play a crucial role in Stat1 homodimerization (6). Furthermore, constitutively activated Stat3 and Stat5 have been observed in a number of hematopoietic malignancies, including T cell leukemias (7–10). Mutations in Jaks or Stats associated with constitutive tyrosine phosphorylation of Stat3 and Stat5 have not yet been identified in these patients. However, it is known that in some patients there is constitutive tyrosine phosphorylation of Jak3 (10).

In cells expressing mutations in components of the Jak/Stat pathway, it remains unclear what if any selective pressure might be present to allow such mutant cells to expand. One mechanism by which mutations could arise is as a result of excessive amounts of a given cytokine or a constitutively activated cytokine receptor. This has been reported to occur in thanatophoric dysplasia type II dwarfism where Stat1 is constitutively activated due to the expression of an activated fibroblast growth factor receptor (11). Treatment of patients with type I IFNs is used therapeutically in a variety of malignancies. However, prolonged and repeated exposure to this cytokine often results in IFN resistance, suggesting that cell variants might be selected containing mutations in components of the Jak/Stat signaling pathway. To test this possibility we have taken advantage of a Jurkat cell variant (H123) obtained by chemical mutagenesis. Although parental Jurkat cells show no IFNα-mediated apoptosis, H123 cells undergo massive death when exposed to this cytokine.2 To determine what signaling events are required for IFNα-stimulated apoptosis of H123 cells, we selected H123 cells that survive when exposed for extended periods to IFNα. One IFNα-resistant variant that we have identified from this screen (Clone 8) has a point mutation in the SH2 domain of Stat2. The characterization of this Stat2 variant not only provides us with important information concerning an uncharacterized domain in Stats, but it also demonstrates that mutations in cytokine signaling can be readily obtained by selective pressure of cytokine exposure as opposed to chemical mutagenesis. Selection of such variants

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‡ The abbreviations used are: IFN, interferon; Stat, signal transducer and activator of transcription; ISG, interferon-stimulated gene; ISGF3, interferon-stimulated gene factor 3; ISRE, interferon-stimulated response element; GRR, γ response element; EMSA, electrophoretic mobility shift assay; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline; SH, Src homology.

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can provide important information on the regulation of these proteins.

MATERIALS AND METHODS

Cells and Cell Culture Reagents—H123- and Clone 8-derived Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen) containing 2 mM L-glutamine, penicillin, and streptomycin (Invitrogen). The human fibrosarcoma 2TGH mutant cell line deficient in Stat2 (U6A) (a gift from G. Stark, The Cleveland Foundation) and 293T cells were cultured in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics. Stable lines of U6A cells reconstituted with wild-type Stat2-P630L were generated by transfection with Superfect reagent. Cells were maintained in the presence of 450 μg/ml G418. Recombinant human IFN-α-2a was a gift from Hoffmann-LaRoche.

RNA Protection Assays—RNA protection assays were performed as described previously (12, 13). Briefly, total RNA was isolated with RNazol B (Tel-Test Inc.). Antisense [γ-32P]ATP-labeled RNA probes were synthesized by in vitro transcription using T7 or SP6 RNA polymerase (New England Biolabs, Inc.). 10 μg of RNA and 25-32P-labeled probes were incubated in hybridization buffer (80% formamide, 40 mM Pipes (14), 400 mM NaCl, and 1 mM EDTA) overnight at 56°C followed by digestion with T1 RNAse (Ambion) for 1 h at 37°C (15). After phenol/chloroform extraction and ethanol precipitation, protected RNA fragments were solubilized and subjected to electrophoresis on a 4.5% polyacrylamide-urea gel.

Preparation of Cell Extracts—Cells were lysed by vigorously vortexing for 20 s in 20 mM Hepes, 25 mM NaCl, 10 mM KCl, 1 mM MgCl2, 20% glycerol, 0.1% Nonidet P-40, 10 mM β-glycerophosphate, 1 mM orthovanadate, 25 mM NaF, 200 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol. After centrifugation (14,000 g, 10 min) at 4°C, supernatants were collected as cytoplasmic extracts. Nuclear extracts were prepared by resuspension of the crude nuclei in nuclear extraction buffer (20 mM Hepes, 300 mM NaCl, 10 mM KCl, 1 mM MgCl2, 20% glycerol, 0.1% Nonidet P-40, 10 mM β-glycerophosphate, 1 mM orthovanadate, 25 mM NaF, 200 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) at 4°C for 30 min, and the supernatants were collected as nuclear extracts after centrifugation at 4°C for 10 min. For whole cell extracts, cells were lysed in whole cell extraction buffer (20 mM Hepes, 300 mM NaCl, 10 mM KCl, 1 mM MgCl2, 20% glycerol, 1% Nonidet P-40, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 25 mM NaF, 200 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) supplemented with a protease inhibitor mixture (Complete; Roche Applied Science). After centrifugation at 4°C for 10 min, supernatants were collected. Protein concentration was measured by the Bio-Rad protein assay.

RT-PCR Analysis and Generation of Stat2 P360L Construct—Total RNA from Clone 5 cells was prepared as indicated above and used to synthesize cDNA using the cDNA synthesis kit (Invitrogen). The entire Stat2 was PCR-amplified using the following primers 5’-ATGGCCGACTGTGGGAAATGTCGCCGACGCCAAAGGCAGGTCCGAC-3’ and 5’-CTAGAATCTGACAGAGGTGGATCAGTGCCCACAAC3’. Cloning into PGEM-T-Easy vector (Promega) followed by DNA sequencing. The FLAG-tagged Stat2 construct in pCDNA3 (kindly provided by C. Horvath, Mount Sinai School of Medicine) was used as template DNA. Amino acid substitution of proline 630 to leucine was generated by PCR-based site-directed mutagenesis (Stratagene) following the manufacturer’s instructions using the oligonucleotides 5’-CATCCTACTGGTCTCGAAGTCGACAGGGAGGTTCCGAC-3’ and 5’-GCACCTCTTCGTTGGTTACAGTTACAGAGTATGGATTACAG-3’. Mutagenesis was confirmed by sequencing the entire Stat2 coding sequence.

Electrophoretic Mobility Shift Assays (EMSAs)—Synthetic double-stranded oligonucleotides corresponding to the ISRE of the ISG15 promoter (5’-GATCCCTCGCCGGAGAGCCAGCAACCTGAGCC-3’) and the IFN-γ response region (GR) sequence of Fc-γRI promoter (5’-AATTTAGCTTGGGTTGAGATGATGATGATGATG-3’) were used as probes. They were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs, Inc.). Each 40-μl reaction mixture contained 5 ng of labeled oligonucleotides, poly(dI-dC) (Amerham, England), and equal amounts of protein in ISRE binding buffer (25 mM HEPEs, pH 7.0, 50 mM KCl, 1.25 mM MgCl2, 0.125 mM EDTA, 0.625 mM dithiothreitol, 5% Ficoll, 0.025% Nonidet P-40) or GR binding buffer (25 mM HEPEs, pH 7.4, 125 mM KCl, 125 mM NaCl, 8.25 mM MgCl2, 12.5% glycerol). The mixture was incubated at 25°C for 30 min and then left on ice for 30 min. The DNA-protein complexes were subjected to electrophoresis on a 4.7% polyacrylamide gel in 0.25× Tris-borate EDTA buffer (0.9 T Tris-borate, 0.002 M EDTA) at 285 V for 2.5 h and visualized by autoradiography (12).

RESULTS

To isolate H123 cells that were resistant to the apoptotic actions of type 1 interferons, cells were incubated with 1,000 units/ml of IFNα for 4 h. To remove dead cells, the cultures were ficolled, and surviving cells were cultured again in the presence of IFNα for 7 days. This selection process was repeated a total of 6 times followed by expansion of cells in the absence of IFNα. Single colonies were further tested to ensure that exposure to IFNα did not induce cell death (Fig. 1A). Although incubation of H123 cells with IFNα for 48 h caused 45% of cells to be annexin V-positive, there was no change in the number of annexin V-positive cells in Clone 8 cells exposed to IFNα. Clone 8 cells maintained their resistance to IFNα-induced apoptosis when cultured in the absence of the cytokine for months (data not shown). To determine whether Clone 8 cells showed any responsiveness to IFNα, we examined expression of major histocompatibility complex class I protein (Fig. 1B). Incubation of either H123 or Clone 8 cells with IFNα resulted in similar enhanced expression levels of major histocompatibility complex class I, indicating that at least some of the responses to IFNα are intact in Clone 8 cells.

IFNα-activated expression of IFN-α-stimulated genes (ISGs) requires expression and activation of Jak1 and Tyk2 that results in tyrosine phosphorylation of Stat1 and Stat2. To determine whether the amount of these proteins was altered in Clone 8...
cells, cell extracts were prepared from H123 and Clone 8 cells, and Western blots were probed with antisera against Stat1, Stat2, Jak1, and Tyk2 (Fig. 2A). Although concentrations of Stat1, Jak1, and Tyk2 were the same in H123 and Clone 8 cells, the amount of Stat2 in these cells was clearly diminished.

The fact that the concentrations of Stat2 were decreased in Clone 8 cells suggested that there could be a defect in IFN-α-stimulated Stat2-dependent gene expression in these cells. Examination of IFN-α-stimulated Stat1 tyrosine phosphorylation by electrophoretic mobility shift assays with an oligonucleotide that binds tyrosine-phosphorylated Stat1 (GRR) showed approximately the same amount of activated Stat1 in H123 and Clone 8 cells, the amount of Stat2 in these cells was clearly diminished.

The fact that the concentrations of Stat2 were decreased in Clone 8 cells suggested that there could be a defect in IFN-α-stimulated Stat2-dependent gene expression in these cells. Examination of IFN-α-stimulated Stat1 tyrosine phosphorylation by electrophoretic mobility shift assays with an oligonucleotide that binds tyrosine-phosphorylated Stat1 (GRR) showed approximately the same amount of activated Stat1 in H123 and Clone 8 cells (Fig. 2B, upper panel). However, IFN-α-stimulated formation of the heterotrimeric transcription complex ISGF3 as assayed by EMSA using an oligonucleotide (ISRE) that binds tyrosine-phosphorylated Stat1, Stat2, and IRF9 was not detectable in nuclear extracts prepared from IFN-α-treated Clone 8 cells (Fig. 2B, lower panel). These results suggest that RNAs whose expression is dependent upon IFN-α-stimulated formation of ISGF3 binding to an ISRE are absent in Clone 8 cells. This is indeed the case; IFN-α-stimulated expression of ISG54, ISG15, and 6–16 RNAs is not observed in Clone 8 cells after various times of incubation with IFN-α (Fig. 2C). As seen previously, IFN-α-induced expression of these RNAs is very robust in H123 cells (lanes 1–3) as well as in wild type Jurkat cells (data not shown).

To directly examine whether there are changes in IFN-α-stimulated tyrosine phosphorylation of Stats in H123 compared with Clone 8 cells, cell extracts were prepared from untreated and IFN-α-treated cells. Immunoblots were probed with specific antiserum that recognizes only the tyrosine-phosphorylated forms of Stat1, -2, or -3 (Fig. 3A). Although IFN-α treatment of both Clone 8 and H123 cells caused equal amounts of tyrosine-phosphorylated Stat1 and Stat3 (Fig. 3A), IFN-α-
stimulated tyrosine phosphorylation of Stat2 was present but extremely weak in Clone 8 cells.

Previous studies have indicated that when cells are exposed to IFN/\textit{H9251}, an association between Stat1 and Stat2 can be detected in immunoprecipitates using either Stat1 or Stat2 antibodies (16). We are unable to detect any association between Stat1 and Stat2 in IFN/\textit{H9251}-treated Clone 8 cells (Fig. 3 B, lanes 3 and 4). However, the association of these proteins is easily detectable in H123 cells incubated with IFN/\textit{H9251} (lanes 1 and 2).

We also examined whether nuclear translocation of Stat2 occurs in IFN/\textit{H9251}-treated Clone 8 cells. H123 and Clone 8 cells were left untreated or incubated for 30 min with IFN/\textit{H9251} prior to fixation and staining for Stat1 and Stat2 (Fig. 3C). As expected, IFN/\textit{H9251}-stimulated nuclear accumulation of Stat1 was readily detectable in both H123 and Clone 8 cells. In H123 cells we could also see IFN/\textit{H9251}-dependent nuclear translocation of Stat2 using antibodies against Stat2 or tyrosine-phosphorylated Stat2. In contrast, IFN/\textit{H9251}-stimulated nuclear translocation of Stat2 in Clone 8 cells was dramatically reduced. In some experiments there appeared to be a very modest IFN/\textit{H9251}-induced nuclear accumulation of tyrosine-phosphorylated Stat2, whereas in other experiments the staining was not above background.

Although the level of tyrosine-phosphorylated Stat2 is diminished in Clone 8 cells compared with H123 cells, Clone 8 cells also express significantly less Stat2 protein. We have not been able to detect any IFN/\textit{H9251}-stimulated ISG expression in Clone 8 cells. This may be because of an insufficient amount of tyrosine-phosphorylated Stat2 in Clone 8 cells or because of tyrosine-phosphorylated Stat2 that is not functional. To distinguish between these two possibilities, we incubated both H123

![Fig. 2. Clone 8 cells display a defect in IFN/\textit{H9251}-stimulated ISGF3-driven responses. A, whole cell extracts from unstimulated cells were resolved by SDS-PAGE, and immunoblot analysis was performed with antibodies against Stat1, Stat2, Jak1, and Tyk2 proteins. Immunoblots were reprobed with actin antibody to control for equal loading of protein (lower panel). B, nuclear extracts from cells incubated with or without IFN/\textit{H9251} for 30 min were assayed for Stat1 homodimer or ISGF3 formation by EMSA with a GRR (top panel) or ISRE probe (bottom panel), respectively. C, H123 and Clone 8 Jurkat cells were left untreated or stimulated with IFN/\textit{H9251} for the indicated times. Total RNA was isolated and expression of ISG54, ISG56, and 6–16 RNAs was determined by RNase protection assay. Actin RNA was used as an internal control to normalize for equal amounts of RNA used in each hybridization.](image)

![Fig. 3. Clone 8 cells have impaired tyrosine phosphorylation and nuclear translocation of Stat2. H123 or Clone 8 Jurkat cells were left untreated or stimulated with IFN/\textit{H9251} for 30 min. A, whole cell extracts were prepared, resolved by SDS-PAGE, and immunoblot analysis was performed with phospho-specific antibodies against Stat1, -2, and -3 antibodies. Membranes were reprobed with antibodies against Stat1, -2, and -3 to verify equal loading of protein. B, whole cell extracts were immunoprecipitated with anti-Stat1 antisera. The immunoprecipitates were separated by SDS-PAGE, and immunoblots were probed with anti-Stat2 (upper panel) and anti-phosphorytrosine Stat1 antibodies (middle panel). The membrane was reprobed with anti-Stat1 antibody to ensure equal levels of immunoprecipitated Stat1 (lower panel). C, subcellular distribution of Stat1 and Stat2 was analyzed by immunofluorescence microscopy. Cells were permeabilized and stained with anti-Stat1, anti-Stat2, or anti-phosphorytrosine Stat2 antibodies and counterstained with 4',6-diamidino-2-phenylindole.](image)
and Clone 8 cells with different concentrations of IFNα and examined tyrosine phosphorylation of Stat2 after 30 min and ISG RNA expression after 6 h. These incubation times have been shown to be optimal for analysis of these two events (17). Incubation of cells with 10 units/ml of IFNα caused tyrosine phosphorylation of Stat2 in H123 cells, which was about the same amount as that seen in Clone 8 cells incubated with 100 units/ml (Fig. 4A). At 10 units/ml, IFNα-stimulated expression of the 6–16 RNA was readily detectable, whereas induction of ISG54 and ISG56 was weak (Fig. 4B). The differences in the expression of these RNAs may be due to a variety of reasons, including the fact that the half-lives of ISG54 and ISG56 are very short compared with 6–16, which is very stable (data not shown). Incubation of Clone 8 cells with 1000 units/ml of IFNα causes a slightly larger amount of tyrosine-phosphorylated Stat2 than 100 units/ml and is between that observed with 10 and 100 units/ml of IFNα in H123 cells. Incubation of H123 cells with 100 units/ml of IFNα induced maximal accumulation of ISG RNAs, whereas neither 10 nor 100 units/ml of IFNα stimulated ISG RNA expression in Clone 8 cells. IFNα-stimulated tyrosine phosphorylation of Stat1 was approximately the same in both cell lines. These results strongly suggest that not only is there diminished tyrosine phosphorylation of Stat2 in Clone 8 cells incubated with IFNα, but there is likely a defect in the protein because the amount of tyrosine-phosphorylated Stat2 detected in Clone 8 cells clearly induces ISG expression in H123 cells but not in Clone 8 cells. The fact that we cannot detect ISGF3 formation or an association of Stat1 and Stat2 in Clone 8 cells treated with IFNα supports this hypothesis.

To determine whether there is a mutation in the Stat2 expressed in Clone 8 cells, RNA was isolated and RT-PCR was used to obtain Stat2 cDNAs. Four individual clones were sequenced, and a single point mutation (Cys to Thr) was detected in the coding region (Fig. 5A). This mutation causes a substitution of proline 630 to leucine (P630L). Sequence homology alignment shows that Pro-630 is present in all mammalian Stat proteins as well as in Drosophila Stat (see Fig. 5B). Several of the amino acids adjacent to Pro-630 are also conserved in other Stat family members, suggesting its importance in Stat structure and/or activity. It is notable that the crystal structure of Stat1 indicates that the hydrophobic side chains of Val-641 and Ala-642 are located in the dimer interface of Stat1 (18).

To verify that the defect in IFNα-stimulated ISRE-dependent gene expression could be restored in Clone 8 cells by expression of Stat2, ISRE reporter assays were performed. Clone 8 cells were transfected with an ISRE-luciferase reporter construct and increasing concentrations of wild type Stat2 plasmid DNA. Cells were left untreated or stimulated with IFNα for 6 h and harvested for determination of firefly luciferase activity in cell extracts. Values were normalized against Renilla luciferase activity and shown as fold induction with respect to untreated samples. Results are shown as mean ± S.D. of triplicate samples.
Novel Conserved Motif Required for Tyrosine Phosphorylation

**Fig. 6.** Stat2 P630L is poorly activated and fails to associate with Stat1. U6A (Stat2−/−) cells stably expressing FLAG-tagged wild type Stat2 or Stat2 P630L were left untreated or stimulated with IFNα for 30 min. A, whole cell extracts were prepared, resolved by SDS-PAGE, and analyzed by immunoblot analysis with antibodies against phosphotyrosine Stat1, phosphotyrosine Stat2, FLAG, or Stat1. B, same as in panel A except that cell extracts were immunoprecipitated with either anti-FLAG (upper panel) or anti-Stat2 (lower panel) antibody. Immunoprecipitates were resolved by SDS-PAGE, and immunoblots were probed with antibodies against anti-phosphotyrosine Stat2 or Stat1. Membranes were reprobed with anti-FLAG or anti-Stat2 antibodies to ensure equal levels of immunoprecipitated proteins.

These results confirm that the IFNα signaling defect in Clone 8 cells can be restored with expression of Stat2.

To examine the function of Stat2 P630L in the absence of endogenous wild type Stat2, we expressed either FLAG-tagged wild type or Stat2 P630L in Stat2-null 2fTGH human fibrosarcoma cells (U6A). Pools of cells that stably express Stat2 protein were selected and incubated with or without IFNα for 30 min. Whole cell extracts were immunoblotted with phosphospecific Stat1 and Stat2 antibodies (Fig. 6A). Similar to Clone 8 cells, IFNα-stimulated tyrosine phosphorylation of Stat2 in U6A cells that express Stat2 P630L was greatly diminished compared with cells expressing wild type Stat2 protein (Fig. 6A). IFNα-stimulated tyrosine phosphorylation of Stat1 has also been shown to be diminished in U6A cells, probably because Stat2 provides a docking site for Stat1 to effectively engage Jak1 and Tyk2 when they are bound to the receptor (19). Interestingly, whereas IFNα-stimulated tyrosine phosphorylation of Stat1 is enhanced in U6A cells expressing wild type Stat2, in cells expressing Stat2 P630L the level of tyrosine-phosphorylated Stat1 remains about the same as cells that do not express Stat2 (Fig. 6, compare lanes 2, 4, and 6). Consistent with Clone 8 cells treated with IFNα (see Fig. 3B), we are also unable to detect any association between Stat1 and Stat2 in IFNα-treated U6A cells reconstituted with Stat2 P630L (Fig. 6B, lane 2 compared with lane 4, lower panel).

We have examined the ability of Stat2 P630L to restore IFNα-stimulated transcription in U6A cells (Fig. 7A). U6A cells stably expressing wild type or Stat2 P630L were transfected with an ISRE luciferase reporter and a cytomegalovirus-driven Renilla luciferase plasmid to normalize for transfection efficiency. Cells were incubated with or without IFNα for 6 h, and extracts were assayed for luciferase activity. Transfection of cells with wild type Stat2 permitted IFNα to induce luciferase activity about 10-fold compared with untreated cells. This degree of IFNα-stimulated ISRE reporter activity has been previously observed (20, 21). Transfection of Stat2 P630L is unable to restore an IFNα-stimulated ISRE-dependent gene expression. To determine whether Stat2 P630L functions to block the actions of wild type Stat2, we have expressed Stat2 P630L with either wild type Stat2 or by itself in H123 cells or in 293T cells. In all cellular contexts, transfection of increasing amounts of Stat2 P630L plasmid blocked IFNα-stimulated ISRE luciferase reporter activity. These results suggest that the Stat2 P630L not only is ineffective as a transcriptional activator, but its expression can inhibit the actions of wild type Stat2.

**Fig. 7.** Stat2 P630L mutant inhibits IFNα-stimulated ISRE-driven transcriptional activity. U6A (Stat2−/−) (A), 293T (B), or H123 Jurkat (C) cells were transiently transfected with a 5× ISRE luciferase reporter construct and increasing concentrations of Stat2 P630L plasmid DNA. Cells were left untreated or stimulated with IFNα for 6 h and harvested for determination of firefly luciferase activity in cell extracts. Values were normalized against Renilla luciferase activity and shown as fold induction with respect to untreated samples. Results are shown as mean ± S.D. of triplicate samples.

**DISCUSSION**

Chemical mutagenesis and selection of cells lines that do not express Jaks or Stats have contributed greatly to our knowledge of the components of the Jak/Stat pathway required for activation of ISGs by interferons. Using these cell lines, it has been possible to identify domains within Jaks and Stats required for their function (1). The determination of individual residues that are important for the function of these proteins has been obtained through examination of sequences that are conserved between members of the family as well as between species. In addition, the crystal structure of the Stats has provided useful information concerning important residues for Stat function (18). However, the number of conserved regions within the Jak and Stat family of proteins are numerous, and analysis of each conserved residue by point mutations and
expression in null cells is a daunting task. The other approach to determine important amino acids and/or domains required for the function of proteins is the examination of diseases that result from mutations in known signaling molecules. Point mutations in Jak3 in humans cause severe combined immunodeficiency, and analysis of a variety of these naturally occurring mutations has provided important information concerning the structure-function of Jak3 as well as other members of the family (3, 4). So far, three mutations in Stat1 have been described in humans that are associated with impairment of immunity to mycobacterial and viral infection (6). One mutation (L706S) is an amino acid that is required for Stat1 homodimerization, whereas the other mutations (L600P and a two-nucleotide deletion in exon 20 that generates a premature stop codon at position 603) both reside within the SH2 domain (6).

Although identification of naturally occurring mutants of Jaks and Stats that are associated with diseases has been very informative in terms of understanding the functions of these proteins, these mutations are very rare, limiting the information that can be obtained from this approach. To define both physiologically and structurally important mutants in the Jaks and Stats, we have used a selection strategy to isolate IFNα-resistant cells, which normally do not survive in the presence of this cytokine. Using this approach we hoped to isolate IFNα-resistant variants that will provide us with information concerning critical previously unrecognized residues that are important for Jak/Stat function. One IFNα-resistant clone obtained from this screen expresses a Stat2 protein with an amino acid substitution in proline 630 N-terminal to the Pro-630 are also highly conserved (Fig. 5). Furthermore, the hydrophobic side chains of two of these residues, alanine 641 and valine 642, pack at the interface of Stat1 homodimers. The fact that we are unable to detect Stat1/Stat2 dimers by either EMSA or communoprecipitation suggests that Pro-630 is conserved in all mammalian Stats as well as in Drosophila Stat. One amino acid N-terminal and three amino acids C-terminal to the Pro-630 are also highly conserved (Fig. 5).

The lack of tyrosine phosphorylation and nuclear translocation of Stat1 is also observed with a mutation in the conserved arginine 602 in the SH2 domain of Stat1 (18, 22). Interestingly, examination of the crystal structure of Stat1 also shows that the conserved proline 630 in the SH2 domain is in close proximity to arginine 602. It is possible that both amino acid residues might be required to form a high affinity binding site for the phosphorylated tyrosyl residue in Stats for dimerization. Although Pro-630 and the surrounding conserved amino acids are located in the SH2 domains of Stat proteins, we have not identified this sequence in the domains of any other SH2 domain-containing proteins.

There are presently a number of malignancies that are being treated with type I IFNs, including chronic myelogenous leukemia, non-Hodgkin’s lymphomas, and melanoma. Clinical resistance to IFNα occurs in all of these malignancies. Now that we have identified a mutation in Stat2 that was selected by chronic exposure of the human leukemic Jurkat variant line to IFNα, we are in a position to determine whether this mutation might be also seen in patients who develop resistance to IFNα as a result of extended therapy with this cytokine.

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