Interferon Signaling Is Dependent on Specific Tyrosines Located within the Intracellular Domain of IFNAR2c

EXPRESSION OF IFNAR2c TYROSINE MUTANTS IN U5A CELLS

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Type I interferons (IFNs) are cytokines that play a central role in mediating antiviral, antiproliferative, and immunomodulatory activities in virtually all cells. These activities are entirely dependent on the interaction of IFNs with their particular cell surface receptor. In this report, we identify two specific tyrosine residues located within the cytoplasmic domain of IFNAR2c that are obligatory for IFN-dependent signaling. Various IFNAR2c tyrosine mutants were expressed in a human lung fibrosarcoma cell line lacking IFNAR2c (USA). Stable clones expressing these mutants were analyzed for their ability to induce STAT1 and STAT2 activation, ISGF3 transcriptional complex formation, gene expression, and cell growth regulation in response to stimulation with type I IFNs. The replacement of all seven cytoplasmic tyrosine residues of IFNAR2c with phenylalanine resulted in a receptor unable to respond to IFN stimulation. Substitution of single tyrosines at amino acid residue 269, 316, 318, 337, or 512 with phenylalanine had no effect on IFN-dependent signaling, suggesting that no single tyrosine is essential for IFN receptor-mediated signaling. In addition, IFNAR2c retaining five proximal tyrosine residues (269, 306, 316, 318, and 337) or either two distal tyrosine residues (411 or 512) continued to be responsive to IFN stimulation. Surprisingly, the presence of only a single tyrosine at either position 337 or 512 was sufficient to restore a complete IFN response. These results indicate that IFN-dependent signaling proceeds through the redundant usage of two tyrosine residues in the cytoplasmic domain of IFNAR2c.

Many cell surface receptors contain tyrosine residues that are phosphorylated upon ligand-induced receptor activation. These phosphorylated tyrosines are specifically recognized by Src homology domains (SH2 or SH3) that are present in many transcription factors, phosphatases, and other mediators of downstream signaling (1). The binding of SH2 or SH3 domains to phosphorylated tyrosines is, in many cases, an absolute requirement for the activation of transcription factors (2). In addition, these phosphorylated tyrosine residues are essential mediators of the interactions of specific phosphatases with certain cell surface receptors (3–7). For some receptors, the mutation or deletion of specific tyrosines completely abrogates ligand-dependent signaling, whereas other cell surface receptors, such as the erythropoietin receptor, do not appear to depend upon any one particular tyrosine but instead are capable of utilizing any one of several tyrosines for signaling (8). Overall, such observations demonstrate the importance of tyrosine residues in signaling and suggest that in certain pathways particular tyrosine residues can mediate the activation of specific transcription factors that may in turn induce the expression of a distinct subset of genes.

One example of how intracellular tyrosines are utilized in signaling is the type I interferon (IFN)1 pathway. Human type I IFNs (α, β, and ω) have been shown to induce the expression of a large number of genes involved in regulating a variety of important biological responses, including antiviral, antiproliferative, and immunomodulatory activities. The mechanisms by which type I IFNs initiate such a broad spectrum of biological activities is only beginning to emerge. Type I IFN-dependent signaling requires both type I IFN receptor chains, IFNAR1 (human type I interferon α receptor chain 1) and IFNAR2c (human type I interferon α receptor chain 2) (9–12). Binding of type I IFNs induces the assembly of these receptor chains, which leads to the phosphorylation of tyrosine residues located in the intracellular domain of each receptor chain. These tyrosine phosphorylation events are thought to be carried out by the Janus kinases TYK2 and JAK1, which are themselves activated by tyrosine phosphorylation (13, 14). IFN receptor activation also leads to the phosphorylation and activation of the transcription factors STAT1 and STAT2. Additional transcription factors, such as STAT3 (15), phosphatidylinositol 3-kinase (16), mitogen-activated protein kinase (17), and CrkL/STAT5 (18, 19), also become activated in response to IFN. It is clear that phosphorylation of the cytoplasmic tyrosines of IFNAR1 and IFNAR2c is involved in type I IFN signaling; however, the role that specific tyrosines play in this signal transduction pathway is unclear.

It is thought that the phosphorylation of tyrosine residues on IFNAR1 and IFNAR2c plays a critical role in the activation of STAT1 or STAT2. The proposed STAT2-binding site on IFNAR1 includes two phosphorylated residues, Tyr506 and Tyr510 (20). STAT-binding sites on IFNAR2c have also been proposed from studies using IFNAR2c truncation mutants expressed in human (21) or mouse cell lines (22). In human cells, truncation of the cytoplasmic domain of IFNAR2c to amino acid

1 The abbreviations used are: IFN, type I interferon; ISGF3, IFN-stimulated gene factor 3; STAT, signal transducer and activator of transcription; FACS, fluorescence-activated cell sorting.

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417 produces a receptor that is unable to mediate the phosphorylation of STAT1 or STAT2. Expression of a specific tyrosine mutant of human IFNAR2c in mouse L929 cells suggests that the tyrosine phosphorylation of at least one of the proximal tyrosines (Tyr269, Tyr306, Tyr316, Tyr318, and Tyr337) of IFNAR2c is required but not sufficient by itself, for STAT2 activation (22). Thus, in both human and mouse cells, IFN-dependent signaling requires both the presence of specific tyrosines within the cytoplasmic domains of the receptor chains and the presence of STATs competent to interact with these domains.

To examine the role of tyrosines present in the cytoplasmic domain of IFNAR2c, we have expressed tyrosine mutants of IFNAR2c in U5A cells, which do not express IFNAR2c, and have analyzed their ability to respond to IFN (23). This approach has allowed us to analyze, for the first time, the functional importance of specific tyrosine residues of IFNAR2c in a human cell background.

MATERIALS AND METHODS

Cell Lines and Reagents—All cell lines, unless otherwise noted, were purchased from the American Type Culture Collection (Rockville, MD). Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin and streptomycin (Invitrogen), and incubated at 37 °C in 5% CO2. U5A cells were obtained as previously described (21). Human IFNα1b (specific activity 2.6 × 10⁸ units/mg), IFNβ2 (specific activity 3.0 × 10⁸ units/mg), and IFNAR2c antisera were obtained as previously described (24). Antibodies against STAT1, STAT2, and phosphotyrosine were purchased from Santa Cruz Biotechnology. Phycoerythrin-conjugated anti-mouse Fab antibodies were purchased from Dako A/S (Glostrup, Denmark).

Selection of U5A Cell Lines Stably Expressing IFNAR2c Tyrosine Mutants—The mutants analyzed in this study are shown in Fig. 1. A vector expressing IFNAR2c and a form of IFNAR2c in which all the intracellular tyrosines were substituted with phenylalanine, pZFNpROrLYF (7F) (21, 22), was kindly provided by Dr. Oscar Colamuni (Department of Pharmacology, University of Chicago, Chicago, IL). The 7F construct was subjected to site-directed mutagenesis to add back single tyrosines creating plasmids F269Y, F306Y, F316Y, F318Y, and F337Y. The 7F plasmid was also used as a PCR template and cloned into the vector pCDEF1, kindly provided by Dr. Jerome A. Canger (Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Piscataway, NJ) using HindIII and SpeI. This construct was then mutagenized using the QuikChange site-directed mutagenesis kit (Stratagene) to add back single tyrosines at positions 411 and 512. A double tyrosine add-back at residues 411 and 512 (45Y5) was also constructed in this vector. Plasmids containing single tyrosine to phenylalanine substitutions (Y269F, Y316F, Y318F, Y337F, and Y512F) were kindly provided by Dr. M. Rubinstein (Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot, Israel). The Y512F plasmid was mutagenized to create the double tyrosine knockout at residues 411 and 512 (45F5). USA cells (1 × 10⁶ cells/5-mm well) were transfected using Superfect (Qiagen) as previously described (21). Multiple stable cell populations for each IFNAR2c mutant were selected in growth medium containing G418 (1 mg/ml). After drug selection, cell populations were selected for single clones expressing IFNAR2c by fluorescence-activated cell sorting (FACS) and cloned by limiting dilution (FACS Vantage™ SE, Becton Dickinson Bioscience, San Jose, CA). For FACS analysis, 1 × 10⁶ cells were incubated with a purified monoclonal antibody (3D11), recognizing the extracellular domain of IFNAR2c, for 1 h at 4 °C, washed with ice-cold phosphate-buffered saline and then incubated with a phycoerythrin-conjugated anti-mouse Fab antibody (Dako, Glostrup, Denmark) for 1 h at 4 °C. The resulting individual clones were then screened by FACS, and only clones having a FACS signal equal to or greater than the parental HT1080 cells were picked and expanded. IFNAR2c expression for all clones was further confirmed by using a radioligand binding assay (see below).

Immunoprecipitation and Immunoblotting—STAT1, STAT2, and IFNAR2c were immunoprecipitated from each mutant cell line as previously described (21). Briefly, 1 × 10⁷ cells were solubilized in lysis buffer (20 mM Tris-HCl, pH 7.5, 1% Nonidet P-40 (v/v), 150 mM sodium chloride, 1 mM EDTA, 2.5% glycerol (v/v), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 5.0 μg/ml trypsin inhibitor) overnight at 4 °C, and insoluble material was removed by centrifugation. For immunoprecipitation, the indicated antibodies were added to each sample, incubated overnight, mixed with Protein-G agarose (Roche Molecular Biochemicals), and resolved by SDS-PAGE (10% Tris-glycine). The proteins were transferred to polyvinylidene difluoride membranes (Pro-Blot, Applied Biosystems, Inc.), and the membrane was placed in blocking buffer (20 mM Tris-HCl, pH 7.5, 0.1% Tween 20 (v/v), 150 mM sodium chloride, 1 mM EDTA, 1.0 mM sodium fluoride, 1.0 mM sodium orthovanadate, 1.0 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 5.0 μg/ml trypsin inhibitor) overnight at room temperature, incubated with the appropriate antibody (1:1000 dilution), and washed in blocking buffer. For immunoprecipitation, the membrane was incubated with a specific horse radish peroxidase-conjugated secondary antibody (1:1000 dilution) (BIOSOURCE International) for 1 h, washed in blocking buffer, and developed using a chemiluminescent detection method (Pierce). To reprobe immunoblots, membranes were incubated overnight in 0.1% sodium citrate, pH 3.0, washed in blocking buffer, and probed again with the appropriate antibody.

Ligand Binding Assay—EZ-Label™ human IFNα2 (PBL Biomedical Laboratories) was labeled with γ32P-labeled ATP as previously described (21). Radiolabeled IFNα2 (278 ps; specific activity of 60–62 Ci/mg) was added to 5 × 10⁵ cells and allowed to bind for 90 min at room temperature before the unbound IFN was washed away with cell culture medium. Nonspecific binding was determined by adding a 100-fold excess of unlabeled IFNβ1b to compete for binding during the 90-min incubation. The cells were lysed in phosphate-buffered saline containing 1% SDS, and the radioactivity was determined by scintillation counting.

Electrophoretic Mobility Shift Assay—Gel shift assays were performed using the γ32P-labeled double-stranded IFN-stimulated response elements oligonucleotide 5’-GCTCCAGTTCCGTTCCCTTCCGAGG-3’ and its complement (25). Briefly, the cells were stimulated with IFNα2 (1000 units/10⁶ cells) or IFNβ1b (1000 units/10⁶ cells) for 30 min, and the cell pellets were collected and processed for electrophoretic mobility shift assay as previously described (25). The resulting DNA-binding complexes were separated by electrophoresis through a 6% polyacrylamide gel and analyzed by autoradiography.

RNA Protection Assay—The cells were stimulated with either human IFNα2 (1000 units/10⁶ cells) or IFNβ1b (1000 units/10⁶ cells) for 17 h before RNA was isolated, and gene expression was analyzed by an RNase protection assay as previously described (21, 26).

Cell Growth Assay—The cells were seeded (5 × 10⁵ cells/well) in a 6-well 35-mm cell culture plate and allowed to attach overnight. Complete medium alone or containing IFNβ1b (1500 units/10⁶ cells) or IFNβ1b (1000 units/10⁶ cells) was added every day for the duration of the experiment. Cell growth was determined using the Alamar Blue method as previously described (27). Alamar Blue™ (BIOSOURCE International) dye reduction was measured after incubation with cells for 40 min at 37 °C using a Millipore fluorescence measurement system Cyberflour 2300 (λ emission = 590; λ excitation = 530).

RESULTS

The cell surface expression of IFNAR2c for all the stable IFNAR2c tyrosine mutant clones (Fig. 1) was confirmed by FACS (Fig. 2A) and radioligand binding (Fig. 2B). FACS and radioligand binding analysis correlated closely with each other (Fig. 2) and demonstrated that the cell surface expression levels of IFNAR2c on all the clones analyzed was equal to or greater than that observed in the parental HT 1080 cell line. The only cell line in which IFNAR2c could not be detected on the cell surface was the USA cell line that was previously shown to lack IFNAR2c (Fig. 2).

Using these clones, we analyzed STAT1 and STAT2 activation by phosphotyrosine immunoblotting after 15 min of stimulation with either IFNα2 or IFNβ1b (1000 units/10⁶ cells). Cell lines in which only a single tyrosine had been substituted with phenylalanine, STAT1 and STAT2 activation were efficiently induced by both IFNα2 and IFNβ1b (Fig. 3A). IFN-dependent STAT activation was also observed in the two cluster mutants of IFNAR2c, one having both the distal tyrosines at amino acid positions 411 and 512 substituted to phenylalanine (45F5) and the other having all five proximal tyrosines (Tyr269, Tyr306, Tyr316, Tyr318, and Tyr337) substituted by phe-
genes using an RNase protection assay. The I-TAC, ISG 54, and TRAIL genes are preferentially induced by IFN-β1b, as compared with various IFNα species, whereas the ISG 6-16 gene was induced by either IFN (21, 26). Briefly, 1 × 10⁶ cells were stimulated with 1000 units/ml of either IFN-β1b or IFN-α2, cells were harvested, RNA was isolated, and the RNase protection assay was performed as described under “Materials and Methods.” Induction of I-TAC, ISG 54, ISG 6-16, and TRAIL by IFN appeared to be unaffected by any of the single tyrosine-to-phenylalanine substitutions (Fig. 5A). As expected, both the U5A cells and the 7F clone did not demonstrate IFN-mediated gene expression, whereas the 4F5F and 4Y5Y cluster mutants were fully functional (Fig. 5B). Furthermore, single tyrosine add-backs, except for 337Y and 512Y, failed to exhibit IFN-inducible expression of I-TAC, ISG 54, and TRAIL. Surprisingly, IFN-dependent stimulation of the 337Y and 512Y clones resulted in the full expression of all of the genes analyzed. IFN-inducible gene expression in 337Y and 512Y was at levels equal to or greater than those observed for the parental HT1080 cells (Fig. 5C). It is also of interest to note that the

**FIG. 1.** IFNAR2c tyrosine mutants expressed in U5A cells. Schematic representation of the cytoplasmic domain of IFNAR2c tyrosine mutants expressed in U5A cells. The seven cytoplasmic tyrosines of IFNAR2c were specifically mutagenized to produce 16 different mutants that were stably expressed in U5A cells. The residue number for each of the seven tyrosines is indicated followed by the single-letter amino acid code for that residue. U5A cells expressing the wild type IFNAR2c (R2c) and a form of IFNAR2c in which all the tyrosines have been replaced with phenylalanine (7F) are shown. Single tyrosine-to-phenylalanine substitutions at residues 269, 316, 318, 337, and 512 are shown along with the double tyrosine-to-phenylalanine substitution mutant having only the distal tyrosines, Tyr411 and Tyr512, replaced with phenylalanine (4F5F). The single tyrosine add-back clones are shown in the lower half of the figure along with the double add-back clone where all the tyrosines were replaced with phenylalanine except for Tyr411 and Tyr512 (4Y5Y).

narylalanine (4Y5Y) (Fig. 3B). However, STAT activation was not observed when all seven tyrosines were absent (clone 7F) (Fig. 3B). Next, IFNAR2c mutants in which each of the seven tyrosines were individually added back were assessed for their ability to induce STAT activation in response to IFN stimulation (Fig. 3C). Surprisingly, a single tyrosine at either amino acid position 337 or 512 (clones F337Y or F512Y, respectively) was sufficient to mediate IFN-dependent activation of STAT1 and STAT2. All of the remaining single tyrosine add-back clones (269Y, 306Y, 316Y, 318Y, and 411Y) were unable to sustain any detectable IFN-dependent STAT activation.

After receptor activation, STAT1 and STAT2 are phosphorylated and form a complex with the DNA-binding protein p48 to make up the IFN-stimulated growth factor complex, ISGF3. The ISGF3 transcription factor then translocates to the nucleus and binds to IFN-stimulated response elements, located within the promoter regions of IFN-responsive genes, to induce gene expression. Therefore, it was important to determine whether the IFN-dependent tyrosine phosphorylation of STAT1 and STAT2, mediated by the various IFNAR2c tyrosine mutants, resulted in the assembly of a functional ISGF3 (Fig. 4). Cell lines expressing IFNAR2c tyrosine mutants were stimulated with 1000 units/10⁶ cells IFN-β1b or IFN-α2 for 30 min, cells were harvested, and ISGF3 complex formation was determined as described under “Materials and Methods.” In all cases, formation of an ISGF3 complex was detected in IFNAR2c tyrosine mutants that were previously shown (Fig. 3) to be capable of inducing tyrosine phosphorylation of STAT1 and STAT2 in response to IFN stimulation.

IFN stimulation results in the expression of a plethora of genes that mediate a variety of important biological effects. To further characterize IFNAR2c tyrosine mutants, we investigated the differential expression of a number of IFN-inducible genes using an RNase protection assay. The I-TAC, ISG 54, and

**FIG. 2.** IFNAR2c tyrosine mutants are expressed on the surface of U5A cells. A, FACS analysis of U5A cells expressing IFNAR2c tyrosine mutants. IFNAR2c was detected on the surface of U5A cells expressing IFNAR2c tyrosine mutants using a specific monoclonal antibody (2D7D1) that recognizes IFNAR2c as described under “Materials and Methods.” Shown on the y axis is the normalized geometric mean of the fluorescent signal. The geometric means were normalized to the geometric mean of the fluorescence signal observed for U5A cells. The data are representative of the means (±15%) derived from multiple experiments (n ≥ 2). B, radioligand binding of phosphorylated IFN-α2 to U5A cells expressing IFNAR2c tyrosine mutants. Ligand binding was performed as described under “Materials and Methods.” Ligand binding is presented on the y axis as the fold increase in binding over U5A cells. All values were corrected for nonspecific binding (100-fold excess unla belled IFN-α2). The values were calculated from a representative mean (±15%) derived from experiments performed in quadruplicate. HT1080, parental cell line; U5A, U5A cells alone. All other cell lines are as described for Fig. 1.
expression of ISG 6-16, especially in response to IFNβ1b, was not completely lost in the single tyrosine add-back clones 269Y, 306Y, 316Y, and 318Y (Fig. 5). The relative levels of gene expression did vary somewhat among the different IFNAR2c tyrosine mutants with the highest level observed in a cell line expressing wild type IFNAR2c (Fig. 5). Finally, the preferential induction of I-TAC, ISG 54, and TRAIL by IFNβ1b that was observed in the parental HT1080 cell line was still present in both the 337Y and 512Y clones.

IFNs have potent antiproliferative effects on many different cell types. Therefore, to evaluate the IFNAR2c mutants in terms of a functional response, we measured the IFN-dependent antiproliferative effects for each clone (Fig. 6). The results are expressed as the percentages of growth compared with untreated controls. Cell growth rates were measured for up to 6 days using Alamar Blue, as previously described (27). As expected, in U5A cells expressing wild type IFNAR2c, all the single tyrosine knock-out mutants and the proximal and distal cluster mutants were able to express clear antiproliferative effects in response to IFNβ1b stimulation (Fig. 6). Among the single tyrosine add-backs, only the 337Y and 512Y IFNAR2c clones were able to elicit an antiproliferative effect in response to IFN stimulation. The magnitude of the effect was equal to or greater than that observed for HT1080 or U5A cells expressing wild type IFNAR2c (R2c). This result further illustrates the importance of Tyr337 and Tyr512 in IFN-dependent signaling and extends this observation to include their roles in the mediation of important biological effects such as antiproliferative responses.

DISCUSSION

The regulation of many cellular activities is mediated through the control of phosphorylation and dephosphorylation events. Many different cytokine and growth factor receptors contain specific tyrosine residues within their cytoplasmic domains that are required for signaling. In the case of the type I IFN receptor, tyrosine phosphorylation events have been shown to involve the JAK-STAT signaling pathway (28). The role of phosphorylation and dephosphorylation events in type I IFN signaling appears to be tightly controlled by the interplay of a complex system of different tyrosine kinases and phosphatases, which is not yet understood.

In terms of IFN signaling, STAT1 and STAT2 play a major role in type I IFN signaling, although STAT3 (15), STAT4 (29), and STAT5 (19) can also be activated in response to IFN in some cells. In all cases, ligand-dependent activation of STATs require that they become phosphorylated on specific tyrosines located in the C-terminal portions of the proteins. The phosphorylation of these tyrosines involves the receptor-associated Janus kinases JAK1 and TYK2, which themselves become activated by tyrosine phosphorylation. Activation of JAK1 and TYK2 requires the heterodimeric assembly of the two type I IFN receptor chains, IFNAR1 and IFNAR2c. It is thought that one of the first phosphorylation events carried out by activated Janus kinases is the specific phosphorylation of tyrosine residues on IFNAR1 and IFNAR2c.

Because it is known that the Janus kinases can phosphorylate various STATs, other mechanisms must be involved that confer signaling specificity. One of these mechanisms may involve the specific phosphorylation of either IFN receptor chain. Indeed, a number of studies have identified important tyrosines located within the cytoplasmic domain of many cytokine and growth factor receptors. It has been shown that these tyrosines can mediate the specific docking of adapter proteins, STATs, and phosphatases to the receptors (30). Indeed, specific tyrosines in IFNAR1 have been suggested to be involved in the regulation of IFNAR1-associated phosphatase activity (31) as well as STAT2 binding (20). Further studies have suggested that STAT2 and STAT1 preassociate with IFNAR2c and that phosphorylation of Tyr466 on IFNAR1 acts as an additional docking site and/or activation step for STAT2 that leads to STAT1 activation and the subsequent formation of a STAT1-STAT2 heterodimer (32). Recent studies using mutants of IFNAR2c having a truncated (21, 22) or substituted (20, 33–35) cytoplasmic domain lend support to the hypothesis that the cytoplasmic tyrosines of IFNAR2c play a role in the activation
of STAT1 and STAT2. Furthermore, such studies suggest that tyrosines located in the cytoplasmic domain of IFNAR2c play an important role in STAT activation leading to IFN-mediated gene activation and antiproliferative effects. This assumption is supported by recent studies carried out using mouse L929 cells expressing a tyrosine mutant of human IFNAR2c in which it was shown that tyrosines in the cytoplasmic domain of IFNAR2c are important for IFN-dependent activation of ISGF3 (22). However, other studies demonstrate that when a form of human IFNAR1, in which all the tyrosines are mutated to phenylalanines, was expressed in a mouse background, it retained its ability to respond to human IFN (36). This observation calls into question the essential role of IFNAR1 tyrosines in IFN signaling and explains the complicated nature of analyzing the effects of human receptor chains expressed in a mouse cell background.

A summary of the functional properties of all the IFNAR2c tyrosine mutants analyzed in the study is presented in Table I. We have shown that replacing all of the cytoplasmic tyrosines of IFNAR2c with phenylalanines results in a receptor unable to induce JAK-STAT-mediated signaling in response to IFN stimulation, thereby demonstrating the general importance of these tyrosines in type I IFN-dependent signaling. Tyrosine residues within the cytoplasmic domain of IFNAR2c are physically clustered into two groups, both proximal (Tyr269, Tyr306, Tyr316, Tyr318, and Tyr377) and distal (Tyr411 and Tyr512) to the cell membrane. Of the seven cytoplasmic tyrosines of IFNAR2c, Tyr306 and Tyr411 are not conserved across species and are therefore not likely to be crucial to signaling. Complete tyrosine-to-phenylalanine substitution of either tyrosine cluster (4F5F or 4Y5Y) resulted in clones fully responsive to IFN

FIG. 5. IFN-dependent gene induction in U5A cells expressing IFNAR2c tyrosine mutants. IFN-dependent gene expression of U5A cells expressing tyrosine mutants of IFNAR2c was determined using RNase protection. The relative expression levels of I-TAC, ISG 54, TRAIL, and ISG 6-16 were determined for all mutant cell lines. The cells were left untreated (0) or stimulated with IFNα2 (α) or IFNβ1b (β) (1000 units/10⁶ cells) for 17 h, harvested, and processed for RNase protection analysis as described under “Materials and Methods.” The figure shows an autoradiogram derived from one representative experiment. The relative expression levels of β-actin were included as a control. The location of each gene is indicated. HT1080, parental cell line; U5A, U5A cells. All other cell lines are as described for Fig. 1.

FIG. 6. Antiproliferative activities of U5A cells expressing IFNAR2c tyrosine mutants in response to IFNβ1b stimulation. Cell growth was measured in all mutants cell lines in the absence or presence of IFNβ1b (1000 units/ml) as described under “Materials and Methods.” Cell growth was determined using the Alamar Blue™ method and expressed on the y axis as a percentage of cell growth relative to untreated cells. The data represent the mean values (n = 3), and variations between replicates were less than 15%. Similar results were obtained in a 6-day assay (data not shown). HT1080, parental cell line; U5A, U5A cells. All other cell lines are as described for Fig. 1.
that no single tyrosine is absolutely essential for IFN-dependent STAT activation, gene regulation, and biological effects.

When single IFNAR2c tyrosine add-backs were analyzed for their ability to mediate IFN-dependent signaling, Tyr$^{337}$ and Tyr$^{512}$ were both capable of mediating signaling on their own. Surprisingly, only the individual substitution of these two tyrosines was able to restore STAT1 and STAT2 activation, ISGF3 complex formation, gene expression, and antiproliferative responses in U5A cells. Detailed studies are ongoing to determine the roles of Tyr$^{337}$ and Tyr$^{512}$ in IFN-dependent antiviral responses.

Previous studies using IFNAR2 truncation mutants expressed in U5A cells suggested that the distal region of IFNAR2c (amino acids 462–515) was not necessary for receptor function. However, truncation of IFNAR2c at amino acid residues 417 or 346 resulted in a completely inactive receptor (21). Both of these mutants contain Tyr$^{357}$, which we have now shown is sufficient to mediate signaling in the context of an otherwise functional receptor. This apparent discrepancy could be explained by the fact that a deletion of the cytoplasmic domain of IFNAR2c might disrupt the tertiary structure around Tyr$^{337}$ in such a way that it is no longer able to play a role in IFN-dependent signaling.

It is interesting to note that the induction by IFN$\gamma$ of the ISG 6-16 gene could be observed at low levels in some IFNAR2c mutants having only a single tyrosine. These clones show no detectable STAT1/STAT2 activation, ISGF3 formation, antiproliferative response, or ISG 54, TRAIL, or I-TAC ligand induction. STAT activation, gene regulation, and biological effects.

Table I: Functional characteristics of U5A cell lines expressing IFNAR2c tyrosine mutations

| Cell line | U5A | R2C | 7F | 269F | 316F | 318F | 337F | 512F | 4F | 5F | 269Y | 306Y | 316Y | 318Y | 337Y | 411Y | 512Y | 4YY |
|-----------|-----|-----|---|------|------|------|------|------|----|----|------|------|------|------|------|------|------|------|
| Ligand binding | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| STAT1/STAT2 activation | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| ISGF3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Gene expression | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Growth inhibition | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

Functional signaling in U5A cells expressing IFNAR2c mutants

The cell lines are defined as shown in Fig. 1. Specific ligand binding was performed using radiolabeled IFNα. Clones that bind ligand equal to or greater than HT1080 cells (+) or fail to bind IFN at all (−) are shown. STAT1 and STAT2 activation was determined by phosphotyrosine immunoblotting where a plus sign (+) indicates that both phosphorylated STAT1 and STAT2 were detected after IFN-stimulation and a minus sign (−) means that no STAT activation was detected. IFN-dependent ISGF3 formation was determined by electrophoretic mobility shift assay. In this case, a plus sign (+) designates when an ISGF3 complex was detected after IFN$\gamma$ or IFN$\gamma$ stimulation, whereas a negative sign (−) indicates no detectable complex formation. An RNase protection assay was used to detect the presence of four IFN-inducible genes: I-TAC, ISG 54, ISG 6–16, and TRAIL ligand. Cell lines in which the IFN-dependent induction of these genes was detected (+) or not (−) are presented. Cell lines in which the induction of I-TAC, ISG 54, and TRAIL ligand by IFN$\gamma$ were not detected but a small induction of ISG 6–16 was observed are also included (+/−). An antiproliferative response to IFN$\gamma$ was observed in a number of cell lines (+); however, other cell lines were unable to elicit an antiproliferative effect in response to IFN$\gamma$ (−).

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