Role of the Intracellular Domain of the Human Type I Interferon Receptor 2 Chain (IFNAR2c) in Interferon Signaling

EXPRESSION OF IFNAR2c TRUNCATION MUTANTS IN U5A CELLS

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A human cell line (U5A) lacking the type I interferon (IFN) receptor chain 2 (IFNAR2c) was used to determine the role of the IFNAR2c cytoplasmic domain in regulating IFN-dependent STAT activation, interferon-stimulated gene factor 3 (ISGF3) and c-sis-inducible factor (SIF) complex formation, gene expression, and antiproliferative effects. A panel of U5A cells expressing truncation mutants of IFNAR2c on their cell surface were generated for study. Janus kinase (JAK) activation was detected in all mutant cell lines; however, STAT1 and STAT2 activation was observed only in U5A cells expressing full-length IFNAR2c and IFNAR2c truncated at residue 462 (R2.462). IFNAR2c mutants truncated at residues 417 (R2.417) and 346 (R2.346) or IFNAR2c mutant lacking tyrosine residues in its cytoplasmic domain (R2.Y-F) render the receptor inactive. A similar pattern was observed for IFN-inducible STAT activation, STAT complex formation, and STAT-DNA binding. Consistent with these data, IFN-inducible gene expression was ablated in U5A, R2.Y-F, R2.417, and R2.346 cell lines. The implications are that tyrosine phosphorylation and the 462–417 region of IFNAR2c are independently obligatory for receptor activation. In addition, the distal 53 amino acids of the intracellular domain of IFNAR2c are not required for IFN-receptor mediated STAT activation, ISGF3 or SIF complex formation, induction of gene expression, and inhibition of thymidine incorporation. These data demonstrate for the first time that both tyrosine phosphorylation and a specific domain of IFNAR2c are required in human cells for IFN-dependent coupling of JAK activation to STAT phosphorylation, gene induction, and antiproliferative effects. In addition, human and murine cells appear to require different regions of the cytoplasmic domain of IFNAR2c for regulation of IFN responses.

Type I interferons (IFNs), IFNα, IFNβ, and IFNω are required for the induction of antiviral responses in a variety of animal species (1). Type I IFNs also elicit important antiproliferative effects in a number of cell lines and play a major role in mediating immunomodulatory activity (2). Cellular responses to type I IFNs require the interaction of type I IFNs with their cognate receptor, which is composed of two receptor subunits, IFNAR1 and IFNAR2c (also designated α and βL, respectively). Once activated, the type I IFN receptor initiates signaling events, which culminate in the induction of a broad spectrum of IFN-responsive genes (2, 3). One of the major signaling events coupled to receptor stimulation is the activation of signal transducers and activators of transcription (STATs). IFN-activated STAT transcription complexes include heterodimers of STAT1 and STAT2 along with a DNA-binding protein of 48 kDa present in the cell cytoplasm (3). This IFN-stimulated gene factor 3 (ISGF3) binds to IFN-sensitive response elements present in the promoter regions of IFN-inducible genes and initiates gene expression (2, 3).

The mechanism by which activation of the type I IFN receptor leads to STAT activation and gene expression is unclear. It is known that early stages of signaling require IFN-induced receptor heterodimerization of both receptor chains (2, 3). However, the mechanism by which STATs and other regulatory proteins interact with the human type I IFN receptor is unclear, despite some emerging evidence of receptor interactive domains.

A proposed STAT2 binding site on IFNAR1 has been suggested which includes two phosphorylated tyrosines, Tyr<sup>466</sup> and Tyr<sup>481</sup>, in which an SH2 domain within STAT1 and STAT2 mediates the binding of STAT2 to these sites. For one of these sites, critical residues include not only Tyr<sup>466</sup> but also valine +1 and serine +5 carboxyl-terminal to tyrosine 466 (4). STAT binding sites on IFNAR2c have also been proposed from in vitro results using glutathione S-transferase-IFNAR2c<sup>444-462</sup> “pull-down” experiments, where both STAT1 and STAT2 have been shown to pre-associate with IFNAR2c, in a manner independent of receptor phosphorylation and dimerization (5). This pre-association entails the binding of STAT2 in the absence of STAT1. More recent studies, using mouse L929 cells, have mapped this constitutive binding site for STAT2 to amino acids 404–462 of IFNAR2c (6). In addition to this constitutive site on IFNAR2c, tyrosine phosphorylation of the proximal tyrosines (tyrosines 269, 306, 316, 318, and 333) of IFNAR2c is required, however, it is insufficient by itself, for efficient STAT2 activation (6). Therefore, stimulation of mouse cells expressing human IFNAR2c containing only the proximal tyrosines of IFNAR2c or the constitutive docking site with human IFNα2 results in STAT tyrosine phosphorylation, ISGF3 formation, but no antiviral response. Thus, in this case, efficient STAT2 activation...
requires both constitutive and phosphotyrosine-dependent binding sites on the receptor. To further define the role of IFNAR2c in IFN signaling, we have chosen to express mutated forms of IFNAR2c having specific modifications in its intracellular domain, in a human cell line, U5A, which lacks IFNAR2c (7). In this way, one can directly measure the effects of such mutants on a variety of IFN-inducible responses, in a human cell that contains complementary Janus kinases and STAT proteins in a background devoid of heterologous receptor chains.

MATERIALS AND METHODS

Cell Lines and Reagents—All cell lines were purchased from American Type Culture Collection (ATCC) and grown at 37 °C in 5% CO2. HT1080 or U5A cells (provided by Drs. Ian Kerr and George Stark) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum, l-glutamine, and 5% penicillin and streptomycin (Life Technologies, Inc.). U5A cells were obtained as described previously (8). Human IFNγib (specific activity = 2.5 × 107 units/mg) was produced as described previously (9) and IFNα2b (1000 units/106 cells) for 17 h; whole cell pellets collected and processed for TaqMan analysis as described previously (15). For RNase protection assays of gene expression, cells were stimulated and harvested as described previously (16).

IFN-induced receptor assembly likely leads to conformational changes in the receptor (10). Production of IFNAR2c mutants was demonstrated in transfected U5A cells and multiple cell lines of each clone were assayed with similar results. Initially, integration of cDNA encoding full-length IFNAR2c (7). Therefore, U5A cells provide a human cell line in which mutant forms of IFNAR2c can be used to determine the role of this receptor in a human cell background. Using this approach, intracellular truncation mutants of IFNAR2c were stably expressed in U5A cells and multiple cell lines of each clone were analyzed with similar results. Initially, integration of cDNA encoding IFNAR2c mutants was demonstrated in transfected U5A cells by polymerase chain reaction using primer sets spanning introns. Positive clones were further expanded and tested for their ability to bind type I IFN.

Immunoprecipitation and Immunoblotting—Cell lines expressing IFNAR2c truncation mutants—U5A cells (1 × 106 cells/well) were transfected with the corresponding plasmid using Superfectin (11). Plasmids containing a neomycin selection marker, IFNAR2c truncations, and the full intracellular tyrosine to phenylalanine substitutions were constructed as described previously (6, 12). Multiple stable cell lines for each IFNAR2c mutant were selected in media containing G-418 (1.0 mg/ml). After selection, individual clones were picked and expanded; an integration of each mutant DNA was determined by polymerase chain reaction using primer sets spanning introns. Positive clones were further expanded and tested for their ability to bind type I IFN.

Immunoprecipitation and Immunoblotting—Cell lines expressing IFNAR2c truncation mutants (1 × 106 cells) were solubilized in lysis buffer (20 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40 (v/v), 150 mM sodium chloride, 1 mM EDTA, 2.5% glycerol (v/v), 1.0 mM sodium fluoride, 1.0 mM sodium orthovanadate, 1.0 mM phenylmethysulfonyl fluoride, 0.5 μg/ml leupeptin, and 50 μg/ml trypsin inhibitor) for 30 min at 4 °C and insoluble material 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% Novex gels). Proteins were transferred to polyvinylidene difluoride filters (Bio-Rad) and incubated in blocking buffer (20 mM Tris-HCl, pH 7.5, containing 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 phenylmethysulfonyl fluoride, 0.5 μg/ml leupeptin, and 50 μg/ml trypsin inhibitor) overnight at 4 °C, incubated with the appropriate antibody and washed in blocking buffer. Following washing, the membrane was incubated with a specific secondary antibody coupled to horseradish peroxidase for 1 h, washed three times in blocking buffer, and developed using a chemiluminescent detection method (Pierce). To reprobe immunoblots, membranes were incubated overnight in 0.01 M sodium citrate, pH 3.0, washed in blocking buffer, and reprobed with the appropriate antibody.

Ligand Binding Assay—A phosphorylated form of IFNα2 was used, and ligand binding assays were performed as described previously (10). Ligands were phosphorylated (specific activities of 60–62 Ci/mmol) as described previously (9) and IFNα2b (1000 units/106 cells) for 17 h; whole cell pellets collected and processed for EMSA as described previously (14). Reaction mixtures were separated by electrophoresis through a 6% polyacrylamide gel and analyzed by autoradiography (14).

TaqMan® and RNase Protection Assay—Cells were stimulated with human IFNα2 (1000 units/106 cells), IFNβib (1000 units/106 cells), or IFNγ (1000 units/106 cells) for 17 h; whole cell pellets collected and processed for TaqMan analysis as described previously (15). For RNase protection assays of gene expression, cells were stimulated and harvested as described previously (16).

RESULTS

U5A is a human lung fibrosarcoma cell line that lacks IFNAR2c but expresses IFNAR1. Sensitivity to type I IFNs can be restored in U5A cells upon transfection with a plasmid encoding full-length IFNAR2c (7). Therefore, U5A cells provide a human cell line in which mutant forms of IFNAR2c can be used to determine the role of this receptor in a human cell background. Using this approach, intracellular truncation mutants of IFNAR2c were stably expressed in U5A cells and multiple cell lines of each clone were analyzed with similar results. Initially, integration of cDNA encoding IFNAR2c mutants was demonstrated in transfected U5A cells by polymerase chain reaction (data not shown). For clones of interest, receptor number and binding affinities were then directly determined (Table I). High affinity binding of type I IFNs to the receptor requires both IFNAR1 and IFNAR2c (10, 20). Such IFN-inducible activation of IFNAR1 results in the specific activation of the Janus kinases, TYK2 and JAK1, and the subsequent phosphorylation of both receptor chains. The assembly of both receptor chains initiates activation of these kinases in an IFN-dependent manner. Earlier work has demonstrated a specific association between IFNAR1 and TYK2 and IFNAR2c and JAK1 (18, 23–26). Accordingly, to confirm that the various truncations or mutations to IFNAR2c had no effect on TYK2-IFNAR1 function, we examined the extent of IFN-inducible TYK2 activation in trans-
FIG. 1. Activation of TYK2 and JAK1 kinases in U5A cells expressing IFNAR2c mutants. A, cells were either untreated with IFN as a negative control (1) or stimulated (2, 3) with IFNα1b (1000 units/10^6 cells) for 15 min, after which cell lysates were prepared and subjected to immunoprecipitation using either a nonspecific (2) or TYK2-specific antibody (3) as described under “Materials and Methods.” Immunoprecipitates were then resolved by SDS-PAGE and subjected to immunoblotting using an anti-phosphotyrosine-specific antibody (p-tyr). The resultant membrane was then stripped and reprobed with a TYK2-specific antibody. B, cells were stimulated with IFNβ1b (1000 units/10^6 cells) for 15 min, cell lysates were prepared, and JAK1 was immunoprecipitated as described under “Materials and Methods,” then resolved by SDS-PAGE and subjected to immunoblotting. Detection of activated JAK1 was performed using an “activation-dependent” dual phosphospecific antibody as described under “Materials and Methods.” Figure shows parental cell line (HT1080), parental cell line (HT1080), U5A cells expressing full-length IFNAR2c (R2c), truncation mutants (R2.462, R2.417, R2.346), and full intracellular domain tyrosine to phenylalanine substitution (R2.Y-F).

FIG. 2. STAT1 and STAT2 activation in U5A cells expressing IFNAR2c truncation mutants. Cells were left untreated (0) or stimulated with IFNα2 (a) or IFNβ1b (b) (1000 units/10^6 cells) for 15 min, solubilized in lysis buffer, and STAT1 and STAT2 immunoprecipitated. Following immunoprecipitation, tyrosine phosphorylation of STAT1 and STAT2 was detected using a phosphotyrosine-specific antibody (p-tyr). After detection of phosphotyrosine, the membrane was stripped and reprobed with STAT1 and STAT2 antibodies (Blot; STAT1/2). Figure shows parental cell line (HT1080), U5A cells alone (U5A), U5A cells expressing R2.515 (R2c), IFNAR2c truncation mutants (R2.462, R2.417, R2.346), and a full intracellular tyrosine to phenylalanine substituted IFNAR2c (R2.Y-F). IP, immunoprecipitating antibody. Blot, immunoblotting antibody.

FIG. 3. ISGF3 complex formation and EMSA in U5A cells expressing IFNAR2c truncation mutants. Cells were left untreated (0) or stimulated with IFNα2 (a) or IFNβ1b (b) (1000 units/10^6 cells) for 15 min, A, ISGF3 gel shift analyses were performed as described under “Materials and Methods.” B, EMSA using m67SIE probes were performed as described under “Materials and Methods.” Figure shows parental cell line (HT1080), U5A cells expressing full-length IFNAR2c (R2c), IFNAR2c truncation mutants (R2.462, R2.417, R2.346), and a full intracellular tyrosine to phenylalanine substituted IFNAR2c (R2.Y-F).
IFN Signaling in U5A Cells Expressing IFNAR2c Mutants

**TABLE II**

| Cell line | βR1 | ISG 54 | ISG 6–16 | IFNβ | IFNα | IFNγ |
|-----------|-----|--------|----------|------|------|------|
| HT 1080   | 1.54| 0.155  | 1.00     | 11.7 | 6.93 | 1.0  |
| R2c       | 17.7| 22.3   | 1        | 10.6 | 10.7 | 1    |
| R2.462    | 9.7 | 1.35   | 1        | 10.6 | 10.7 | 1    |
| R2.417    | <0.01| <0.01 | 1        | <2.0 | <2.0 | 1    |
| R2.346    | <0.01| <0.01 | 1        | <2.0 | <2.0 | 1    |
| R2.Y-F    | <0.01| <0.01 | 1        | <2.0 | <2.0 | 1    |

In all cases for which gene expression could be measured, both IFNα2 and IFNβ1b were capable of inducing gene expression, although some variation in gene expression levels was observed depending on whether IFNα2 or IFNβ1b was used. As expected, differential expression of βR1 and ISG-54 was observed in HT1080 cells and to some extent in the R2c and R2.462 cell lines. Consistent with the gel shift data, IFNα- and IFNβ-dependent gene expression was absent in cells expressing IFNAR2c truncation mutants R2.417 or R2.346 and IFNAR2c lacking intracellular tyrosine residues (R2.Y-F).

**DISCUSSION**

Studies using identical mutant human IFNAR2c variants expressed in mouse L-929 cells have been reported previously.

In a recent report, it has been suggested that IFN-inducible STAT complex formation and DNA binding does not necessarily correlate with IFN-inducible transcriptional activation (29). Apparently, following STAT-DNA binding, there is an obligatory event, which is p38-dependent, which is required for transactivation and transcription (29). Accordingly, we undertook experiments to determine whether any of the mutations introduced into IFNAR2c in the transfectants affected IFN-inducible gene induction. Specifically, IFN-inducible gene expression for known IFN-responsive genes was examined by TaqMan® analysis (15) and RNase protection assays (16). As shown in Table II, IFN-inducible βR1, ISG-54, and ISG-6–16 gene expression was observed in the HT1080, R2c, and R2.462 cell lines but not in U5A cells expressing the R2.417, R2.346, and R2.Y-F IFNAR2c mutants. A similar pattern of gene expression was observed using RNase protection assays (data not shown).

In all cases for which gene expression could be measured, both IFNα2 and IFNβ1b were capable of inducing gene expression, although some variation in gene expression levels was observed depending on whether IFNα2 or IFNβ1b was used. As expected, differential expression of βR1 and ISG-54 was observed in HT1080 cells and to some extent in the R2c and R2.462 cell lines. Consistent with the gel shift data, IFNα- and IFNβ-dependent gene expression was absent in cells expressing IFNAR2c truncation mutants R2.417 or R2.346 and IFNAR2c lacking intracellular tyrosine residues (R2.Y-F).

IFN-dependent gene expression leads to a number of important cellular responses such as control of cell growth. Therefore, we examined the effects of the various IFNAR2c mutants on IFN-dependent growth inhibitory effects as measured by short term [3H]thymidine incorporation. Consistent with STAT activation and gene expression studies, IFN-inducible growth inhibition was not observed in the U5A, R2.417, R2.346, and R2.Y-F transfectant cell lines (Fig. 4). However, IFN stimulation of U5A cells expressing IFNAR2c (R2c) or the R2.462 mutant did result in a strong inhibition of [3H]thymidine incorporation (Fig. 4). A similar pattern of antiproliferative effects was observed over a 4–5-day period (data not shown).
(6, 12). Such cells simultaneously express a heterologous combination of both mouse and human type I IFN receptor chains in which the specific response to human IFNs is generally dependent on the absolute species specificity of type I IFNs. Using this approach, two IFN-regulatory regions of IFNAR2c have been reported. These include an IFNβ response region (IBR) located between residues 417–462 (30) and a distal negative regulatory domain (31). Furthermore, a STAT2 binding site was mapped to the 404–462 region of IFNAR2c using glutathione S-transferase fusion proteins encoding different regions of the intracellular domain (6).

The existence of an IFNβ response region (IBR) or distal negative regulatory domain was not observed in the current study in human cells due to a complete loss of receptor function in IFNAR2c mutants truncated at residue 417. This is in contrast to results obtained when identical IFNAR2c truncation mutants were stably expressed in mouse L929 cells. In this study in human cells, the distal tyrosine at residue 512 (Tyr512) is not present as substrates for activated JAKs. The lack of any STAT phosphorylation, there are no tyrosine residues within signaling events such as STAT activation. In addition, it is likely that, even though in U5A cells expressing similar IFNAR2c truncation mutants, TYK2 and JAK1 activation occurs in response to type I IFNs, the inability of R2.417, R2.346, and R2.Y-F to induce STAT phosphorylation is due to the inability of these mutants to bind STATs or correctly present receptor activation. Clearly, our data demonstrate for the first time that differences exist in the manner in which the human and murine IFNAR2c influences IFN-dependent STAT activation. It will now be necessary to determine which phosphotyrosine residues in IFNAR2c are critical for IFN signaling and what role they play in regulating differential type I IFN signaling and gene expression in human cells.

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