A Conserved IFN-α Receptor Tyrosine Motif Directs the Biological Response to Type I IFNs[1]

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Mammalian type I IFNs (IFN-Is) mediate their potent biological activities through an evolutionarily conserved IFN-α receptor (IFNAR), consisting of IFNAR1 and IFNAR2. These two chains direct the rapid activation of two founding members of the STAT family of transcription factors, STAT1 and STAT2. To understand how IFN-Is direct the recruitment and activation of STATs, a series of mutant murine IFNAR1 and IFNAR2 receptors were generated and evaluated in *IFNAR1* and *IFNAR2* knockout cells. These studies reveal that a single conserved IFNAR2 tyrosine, Y^{510}, plays a critical role in directing the IFN-I-dependent activation of STAT1 and STAT2, both in murine fibroblasts and macrophages. A second IFNAR2 tyrosine, Y^{335}, plays a more minor role. Likewise, $Y^{510} > Y^{335}$ play a critical role in the induction of genes and antiviral activity traditionally associated with IFN-Is. ***The Journal of Immunology,*** **2008, 180: 5483–5489.**

Interferons, first recognized for their potent antiviral activity 50 years ago, can be divided into three major classes, type I, II, and III (1–3). Of these, type I IFNs (IFN-Is[3]; e.g., IFN-α and IFN-β) are most abundant in number, distribution and expression. Moreover, they are highly conserved among mammals in both structure and function. Characterization of the ability of IFN-α to induce genes and antiviral response led to the identification of the JAK-STAT signaling cascade (4, 5). Subsequent studies determined that four JAKs and seven STATs mediate the biological response for all ~50 members of the four-helix bundle family of cytokines (5).

The IFN-I receptor (IFN-α receptor; IFNAR) consists of two chains, IFNAR1 and IFNAR2, which are associated with Tyk2 and JAK1, respectively (3, 5). Gene targeting studies have revealed that both receptor chains and JAK1 are critical for biological response (6–8). In contrast to humans however, loss of Tyk2 in mice is only associated with modest defects in IFN-I response (9, 10). Despite an important role in the elucidation of JAK-STAT signaling, insight into how IFNAR directs STAT-dependent biological response lags behind that of most other cytokine receptors, where conserved phosphorylated receptor tyrosine motifs have been shown to direct the recruitment and activation of specific STATs, as well as other signaling molecules (e.g., suppressor of cytokine signaling (SOCS) and phosphatases) (11–16). Although studies on IFN-I response have highlighted tissue specific differences in IFN-I-dependent STAT activation (17), the mechanism by which this is achieved has not been elucidated. Rather, despite strong conservation of biological response (5, 18), studies have implicated differing tyrosines, as well as nontyrosine motifs, in STAT recruitment and activation (19–25).

Once activated, STAT1 and STAT2 form two important transcription factors, ISGF-3 ($STAT1^+STAT2^+IRF9$) and STAT1 homodimers (5). ISGF-3 directs the expression of IFN-I-stimulated response element (ISRE) driven genes and the IFN-I autocrine loop, whereas STAT1 homodimers direct the expression of GAS (γ-IFN activation site) driven genes, which IFN-γ also induces through the formation of STAT1 homodimers. IFN-Is are also known to promote the activation of other STATs, especially STAT3 and STAT4 (26, 27). However, a compelling role for STAT3 signaling in IFN-I response has not been elucidated and STAT4's role may be restricted to lymphocytes (data not shown) (26, 28).

We set out to test the hypothesis that, as is the case with all other cytokine receptors (11, 15, 29), evolutionarily conserved tyrosine motifs direct the IFN-I-dependent activation of STATs and the ensuing biological response. To this end, five of seven conserved and one of three nonconserved IFNAR tyrosines were mutated and evaluated in *IFNAR1[−/−]* and *IFNAR2[−/−]* murine embryonic fibroblasts (MEFs), respectively. Only two IFNAR2 tyrosines were required for IFN-I response in fibroblasts and macrophages, Y^{510} and Y^{335}, with Y^{510} exhibiting a considerably more significant role than Y^{335}. This included IFN-I-dependent activation of STAT1, STAT2, and STAT3, as well as the expression of target genes and antiviral activity. Consistent with an important role in the recruitment and activation of STATs, a phosphorylated Y^{510} peptide effectively competed for binding to STAT1, and likely STAT2. These studies not only establish an important role for Y^{510} in murine IFN-I response, but also raise the intriguing question as to what role the remaining conserved tyrosine motifs may play in IFN-I biology.

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Received for publication December 4, 2007. Accepted for publication February 7, 2008.

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[1] This work was supported by National Institutes of Health Grants AI 058211 (to W.Z., C.L., and C.S.) and 5T32 AI07525 (to C.P.), the National Multiple Sclerosis Society (Award RG 3237), and the Immunology Training Program (5T32 GM 07367 to C.P.).

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[3] Abbreviations used in this paper: IFN-I, type I IFN; IFNAR, IFN-α receptor; SOCS, suppressor of cytokine signaling; ISRE, IFN-I-stimulated response element; GAS, γ-IFN activation site; MEF, murine embryonic fibroblast; BMM, bone marrow macrophages; VSV, vesicular stomatitis virus; pMIG, MSCV-IRES-GFP; Q-PCR, quantitative-PCR.

Table I. *Sequence of oligonucleotides and peptides*

Cloning		
R1	Forward	5′AGATCTAGCAGGAGGAGAATGTGAGCCG3′
	Reverse	5′TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGCATAGAAGTGCTGGCTCTGTCC3′
$R1^{\Delta 471}$	Forward	5′CAAATGTCCAGACTACGCACTGTGTC3′
	Reverse	5′TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCACGCGTAATACTGCGGGGAGGCTTGAGT3′
$R1^{\Delta 511}$	Forward	5′AGATCTAGCAGGAGGAGAATGTGAGCCG3′
	Reverse	5′TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCACGCGTAGGCGCGTGCTTTACTTCTAC3′
R2	Forward	5′GCAGATCTCCACCATGCGTTCACGATGCACCGTC3′
	Reverse	5′TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTCTCATGATGTAGCCGTCCC3′
$R2^{\Delta 334}$	Forward	5′GCAGATCTCCACCATGCGTTCACGATGCACCGTC3′
	Reverse	5′TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGCCCGTGACACTTGCTGTGG3′
$R2^{\Delta 372}$	Forward	5′GCAGATCTCCACCATGCGTTCACGATGCACCGTC3′
	Reverse	5′TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCACGCGTAGCTTCATCAGATTCCTCAGC3′
$R2^{\Delta 503}$	Forward	5′GCAGATCTCCACCATGCGTTCACGATGCACCGTC3′
	Reverse	5′TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCACGCGTATCGGAATCTGAGGTGTCTGA3′
Mutagenesis		
$R1^{Y455F}$	Forward	5′GGAGCGTCTGGAAATTCCTGTGTCATGTGTGCTTC3′
	Reverse	5′GAAGCACACATGACACAGGAATTTCCAGACGCTCC3′
$R1^{Y518F}$	Forward	5′GACCTCAGGAAGTTCAGCTCACAGACC3′
	Reverse	5′GGTCTGTGAGCTGAACTTCCTGAGGTC3′
$R1^{Y529F}$	Forward	5′GACTCGGGCAACTTTTCCAACGAAGAG3′
	Reverse	5′CTCTTCGTTGGAAAAGTTGCCCGAGTC3′
$R2^{Y268F}$	Forward	5′CGTAATGCTGAAACGGATTGGCTTCATATGCCTAAAAGACAATTTGCCC3′
	Reverse	5′GGGCAAATTGTCTTTTAGGCATATGAAGCCAATCCGTTTCAGCATTACG3′
$R2^{Y335F}$	Forward	5′CACAGCAAGTGTCACTGGCTTCACCATGCATGAACTGAC3′
	Reverse	5′GTCAGTTCATGCATGGTGAAGCCAGTGACACTTGCTGTG3′
$R2^{Y510F}$	Forward	5′GATGTGGGGGACGGCTTCATCATGAGAGGTAAG3′
	Reverse	5′CTTACCTCTCATGATGAAGCCGTCCCCCACATC3′
Q-PCR		
β-actin	Forward	5′GCT CCT CCT GAG CGC AAG T3′
	Reverse	5′TCG TCA TAC TCC TGC TTG CTG AT3′
OAS-1	Forward	5′CTG CCA GCC TTT GAT GTC CT3′
	Reverse	5′TGA AGC AGG TAG AGA ACT CGC C3′
MxA-1	Forward	5′AAG ATG GTC CAA ACT GCC TTC G3′
	Reverse	5′GCC TTG GTC TTC TCT TTC TCA GC3′
EMSA		
OAS ISRE†		5′agct TCTGAG GAAAC GAAAC CAACAG 3′
IRF1 GAS†		5′gatc GATTT CCCCG AAAT 3′
Peptides		
$p\text{-St1}^{Y701}$		DPKRTG-pY-IKTELI
$R2^{Y510}$		ADVGDGYIMRGKP
$p\text{-R2}^{Y510}$		ADVGDG-pY-IMRGKP

† Palindromic oligonucleotides are self-annealed to generate double-stranded probe.

Materials and Methods

Mice

IFNAR1[−/−], *IFNAR2[−/−]*, and wild-type (WT) mice were on a pure 129/SVE background and housed under specific pathogen-free conditions (6, 7, 30). Columbia and Monash University Institutional Animal Care and Use Committee approved the murine studies.

Cell culture

HEK-293T and Vero cells (American Type Culture Collection) were cultured in DMEM (Invitrogen Life Technologies), supplemented with 10% FCS (HyClone), and penicillin/streptomycin (Invitrogen Life Technologies). Primary murine embryonic fibroblasts were prepared from E12.5 day embryos as previously described (17). Immortalized MEFs were generated through continuous passage of subconfluent primary murine embryonic fibroblasts in DMEM, 10% FCS, and penicillin/streptomycin. Murine cells were either stimulated with a bifunctional, chimeric, human IFN-$\alpha_{A/D}$ (1000 U/ml; PBL), murine IFN-α_1 (50 U/ml) (31), or murine IFN-γ (66 U/ml; PBL), as indicated. For transient expression studies, DNA was transfected into MEFs with FuGENE 6 (Roche), and then evaluated 1 day later for IFN-α-dependent induction of an ISRE-driven luciferase reporter (Stratagene), as previously described (7). For retroviral mediated transduction, MEFs or primary bone marrow macrophages (BMMs) were infected with high titer retroviral stocks, as previously reported (32). For antiviral response, IFN-$\alpha_{A/D}$ treated MEFs were infected with vesicular stomatitis virus (VSV). Twenty-four hours later, viral yield in the supernatant was determined by Vero cell plaque assay on, as previously reported (32).

DNA and cloning

DNA modifying enzymes were from New England Biolabs, unless otherwise noted. Murine IFNAR1 and IFNAR2 cDNAs were a gift from R. Schreiber (Washington University, St. Louis MO) (7). IFNAR1 was cloned into the BstX1 site in pEF-BOS and *Bgl*II–*Xho*I sites of pMIG (MSCV-IRES-GFP), as previously reported (7, 32). IFNAR2 cDNA was cloned into the *Bgl*II–*Eco*RI sites of pMIG. In most cases, this entailed amplifying cDNA by high fidelity PCR (PFU; Stratagene) with a 3′ primer that included a V5 epitope tag (GKPIPNPLLGLDST) and a stop codon (see Table I for primers). Deletion mutants were generated by an analogous PCR-based strategy with primers listed in Table I. Point mutants were prepared by site directed mutagenesis (Quickchange kit; Stratagene) of IFNAR1 and IFNAR2 cDNAs cloned into the *Kpn*I-*Xba*I sites pBluescript II sk+ and confirmed by sequencing. They were then moved into pMIG or pEF-BOS as detailed above.

RNA

Total RNA was prepared from MEFs by Trizol (Invitrogen Life Technologies) extraction. Five micrograms of total RNA was treated with RQ1 DNase (Promega) and then reverse transcribed with SuperScript II (Invitrogen Life Technologies), as previously reported (16, 32). The cDNA was quantitatively amplified in an ABI Prism 7700 with SYBR green master mix (Applied Biosystems) and MxA-1- and OAS-1-specific primers (see Table I). Gene expression was normalized to a β-actin control. For each primer set, control values and standard curves were generated by plotting log of DNA concentration vs critical amplification threshold (CT) from 1/5 serial dilutions with SDS1.9.1 software (Applied Biosystems).

Biochemical studies

Whole cell and nuclear extracts were prepared from IFN-treated cells and evaluated by immunoblotting or EMSA, as previously reported (16, 17). Abs for immunoblotting included: STAT1 (33); STAT2 (17); STAT3 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA); phosphotyrosine-STAT1 (no. 9171S; Cell Signaling Technology); phosphotyrosine-STAT2 (no. 07-224; Upstate Group); phosphotyrosine-STAT3 (no. 9171S; Cell Signaling Technology); β-actin (sc-1616; Santa Cruz Biotechnology); and V5

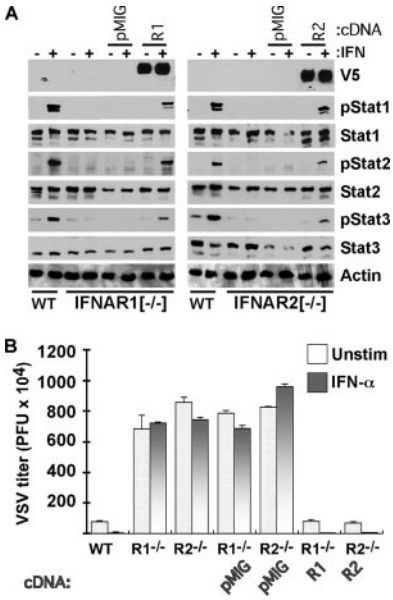

FIGURE 1. IFN-α-dependent responses in *IFNAR1[−/−]* and *IFNAR2[−/−]* MEFs. *A*, IFN-$\alpha_{A/D}$ (1000 U/ml; 30 min)-dependent STAT1, STAT2, and STAT3 activation was evaluated by phosphoimmunoblotting in *IFNAR1[−/−]* and *IFNAR2[−/−]* MEFs ectopically expressing IFNAR1 (R1) or IFNAR2 (R2) cDNAs, respectively. Control studies included WT MEFs, infection with empty vector (pMIG), as well as immunoblotting for total STAT1, STAT2, STAT3, V5, and actin. This analysis is representative of at least three independent experiments. *B*, The antiviral activity of *IFNAR1[−/−]* and *IFNAR2[−/−]* MEFs ectopically expressing IFNAR1 (R1) and IFNAR2 (R2) cDNAs, respectively, was evaluated by viral yield assay. Cells were treated with IFN-$\alpha_{A/D}$ (1000 U/ml, 16 h) before infection with VSV (2 h; multiplicity of infection [MOI] = 1.0). Twenty-four hours later, 500 μl of supernatant was serially diluted (10×) and evaluated for PFU/ml on Vero cell monolayers. The analysis is representative of at least three independent experiments, including cytopathic effect assays.

(Mouse Monoclonal IgG2a; Invitrogen Life Technologies). EMSAs were conducted with either an IRF-1 GAS or OAS ISRE probe (see Table I) (16, 17). Peptides and phosphotyrosine peptides, corresponding to critical tyrosine motifs in STAT1 (i.e., Y^{701}) and IFNAR2 (i.e., Y^{510}; Table I; GenScript), were dissolved in water, preincubated with IFN-stimulated cellular extracts (15 min., 0°C), and then with either an ISRE or GAS probe (15 min, 22°C). For surface biotinylation studies, cells were washed with ice-cold PBS, treated with sulfo-NHS-LC-Biotin (1.5 mg/ml, 30 min, 4°C; EZ-Link, Pierce Biotechnology), washed, lysed, and then incubated with NeutrAvidin agarose (20 μg of lysate with 100 μl beads; 16 h, 4°C; Pierce Biotechnology). The complexes were then washed, eluted, and immunoblotted for V5.

Results

A role for IFNAR1 and IFNAR2 in the biological response to IFN-α

Characterization of *IFNAR1* knockout mice has underscored the critical role this receptor chain plays in mediating biological responses to IFN-Is (6, 18, 34). More limited studies on *IFNAR2[−/−]* mice have supported a similar essential role for this receptor chain in IFN-I response as well (7, 8, 23). To develop an assay system to functionally evaluate mutant IFNAR chains, MEFs were prepared from available IFNAR1 and IFNAR2 knockout mice. As anticipated, both *IFNAR1[−/−]* and *IFNAR2[−/−]* MEFs were defective in IFN-α-dependent activation of STAT1, STAT2, and STAT3 (see Fig. 1*A*), as well as the potent antiviral response observed in WT cells (Fig. 1*B*). Moreover, knockout MEFs exhibited far higher basal (i.e., in the absence of exogenous IFN-α) yields of virus when infected with VSV than WT cells, owing to the activity of the IFN-I autocrine loop. In contrast, ectopic retroviral directed expression of IFNAR1 and IFNAR2 cDNAs restored IFN-α-dependent STAT activation and antiviral activity in *IFNAR1[−/−]* and *IFNAR2[−/−]* MEFs, respectively. Notably, empty retroviral vector controls failed to restore IFN-I response. Similar results were observed when IFNAR1 and IFNAR2 cDNAs were ectopically expressed in *IFNAR1[−/−]* and *IFNAR2[−/−]* leukocytes (data not shown; see also Fig. 4*B*). These studies illustrate that the ectopic expression of IFNAR cDNAs in IFNAR knockout MEFs restores the biological response to near WT levels, validating this approach to study the function of IFNAR mutants.

Sequence comparison of the murine and human IFNAR cytoplasmic domains identified several conserved tyrosine and nontyrosine motifs, reflecting a highly conserved biological response to IFN-Is. Subsequent studies focused on the conserved IFNAR tyrosine motifs, because of the important role they play in directing STAT recruitment and activation for all well-characterized cytokine receptors (35). This included Y^{518} and Y^{529} in IFNAR1, as well as Y^{268}, Y^{335}, and Y^{510} in IFNAR2 (see Fig. 2). A series of deletion and point mutants were prepared for each receptor chain (Fig. 2) and then cloned into the retroviral expression vector pMIG, as above (Fig. 2). A V5 carboxy terminal epitope tag was also included to compare receptor expression levels. The epitope tag was also exploited to demonstrate robust localization to the cell membrane through cell surface biotinylation (data not shown).

All IFNAR1 mutants restore biological response to IFN-α

Extensive analysis of *IFNAR1[−/−]* mice have underscored the critical role this chain plays in mediating biological responses to

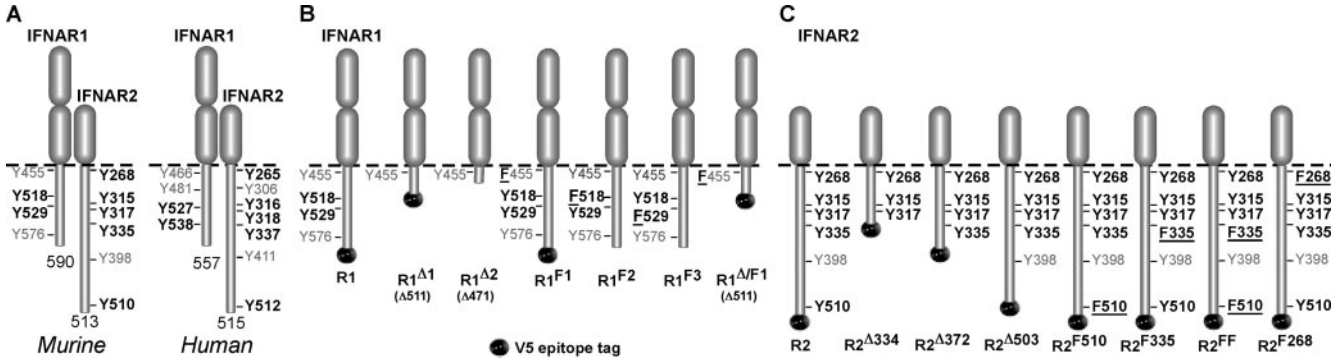

FIGURE 2. Diagram of WT and mutant IFNAR components. *A*, Comparison of tyrosine motifs in the human and murine IFNAR chains. *B*, Diagram of V5 epitope tagged IFNAR1 mutants used in this study. *C*, Diagram of V5 epitope tagged IFNAR2 mutants used in this study.

IFN-Is (5, 6, 18). IFNAR1 features a conserved set of tandem tyrosines, Y^{518} and Y^{529} (i.e., RK-Y^{518}SSQTSQDSGN-Y^{529}SNEEE), as well as membrane-proximal domains variably implicated in Tyk2 recruitment/activation (i.e., a box1 motif-^{461}FPPLKP^{466}; and a binding domain- ^{474}FSEPPSKNLVLLTAEEHTERCFIIE^{498}, (36, 37); see Fig. 2). IFNAR1 also features two nonconserved tyrosines at Y^{455} and Y^{576}. Of note, Y^{455}, which is analogous to membrane proximal Y^{466} in human IFNAR1, has previously been implicated in STAT recruitment (19, 20). To quickly identify which IFNAR1 regions contribute to IFN-I response, the IFNAR1$^{\Delta 511}$ deletion mutant was generated. Unexpectedly, IFNAR1$^{\Delta 511}$ was as effective as WT IFNAR1 in restoring biological response to IFN-α (see Fig. 3). This included activation of STAT1, STAT2, and STAT3, as well as induction of two ISRE target genes (i.e., *MxA-1* and *OAS-1*; Fig. 3*B*). Likewise, IFNAR1$^{\Delta 511}$ supported WT IFN-α-dependent antiviral responses (Fig. 3*D*). To exclude a role for Y^{455}, two additional mutants were prepared, IFNAR1^{Y455F} and IFNAR1$^{Y455F/\Delta 511}$, both of which exhibited normal patterns of STAT activation. Consistent with this, IFNAR1 point mutants (i.e., IFNAR1^{Y455F}, IFNAR1^{Y518F}, and IFNAR1^{Y529F}) directed a robust IFN-α-dependent induction of an ISRE-driven luciferase reporter in *IFNAR1[−/−]* MEFs (Fig. 3*C*). Analogous to IFNAR1$^{\Delta 511}$, the IFNAR1$^{\Delta 517}$ deletion mutant used in the reporter assay was fully functional. However, IFNAR1$^{\Delta 471}$ failed to drive luciferase expression, suggesting that residues 474–498 are important for Tyk2 binding/activation (36, 37). These studies support a model where IFNAR1 plays an important role in ligand binding and Tyk2 recruitment (3). However, neither Y^{455} nor residues distal to amino acid 511 appear to significantly contribute to the biological responses traditionally associated with IFN-Is. This includes IFN-I-dependent STAT3 activation that had previously been ascribed to Y^{527}, because it features a canonical STAT3 recruitment motif (i.e., YXXQ) (11, 38).

IFNAR2 mutants are defective in their biological response to IFN-α

IFNAR2, with its larger cytoplasmic tail, features a larger number of conserved domains, including tyrosines (Y^{268}, Y^{335}, and Y^{510}), a tyrosine doublet motif (NKKKRLWN-Y^{315}D-Y^{317}EDGSDSD), as well as two nontyrosine motifs (membrane proximal ^{287}WI IPERSPS^{295}, implicated in JAK1 recruitment; and ^{421}GDNIIFNV NLNSVFLRVLHD ED^{442}). Murine Y^{398}, human Y^{306}, and human Y^{411} are not conserved. To quickly identify which murine IFNAR2 regions contribute to IFN-I response, three receptor deletion mutants were prepared, IFNAR2$^{\Delta 334}$, IFNAR2$^{\Delta 372}$, and IFNAR2$^{\Delta 503}$ (see Fig. 2). None of these mutants restored IFN-I-dependent STAT activation in *IFNAR2[−/−]* MEFs (Fig. 4*A*), raising the possibility that a domain distal to 503, or both a proximal and distal domain, might be critical for signaling. Attention was first directed toward Y^{335}, Y^{510}, and Y^{268}, previously implicated in STAT activation ((19, 23); see Fig. 2). Single and one double IFNAR point mutants were prepared. IFNAR2^{Y268F} restored IFN-α-dependent STAT activation to WT levels and thus served as a positive control. IFNAR2^{Y335F} exhibited effective IFN-α-dependent activation of STAT1 and STAT3, but reduced STAT2 activation (Fig. 4*A*). Notably, cells expressing IFNAR2^{Y510F} and IFNAR2$^{Y335F/Y510F}$ were severely impaired in STAT activation. More careful dose response studies in *IFNAR2[−/−]* bone marrow derived macrophages also revealed defective STAT activation with IFNAR2^{Y510F} and IFNAR2$^{Y335F/Y510F}$ (Fig. 4*B*). There was also a modest reduction of STAT1 and STAT2 activation in IFNAR2^{Y335F} macrophages.

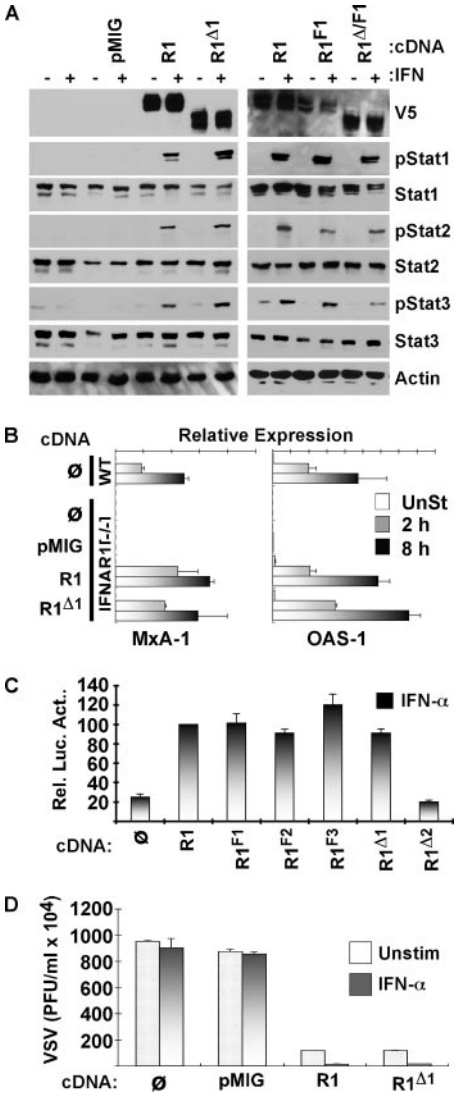

FIGURE 3. Functional characterization of IFNAR1 mutants. *A*, IFN-$\alpha_{A/D}$ (1000 U/ml; 30 min)-dependent STAT1, STAT2, and STAT3 activation in WT, as well as *IFNAR1[−/−]* MEFs ectopically expressing IFNAR1 (R1), IFNAR1$^{\Delta 511}$ (R1$^{\Delta}$), IFNAR1^{Y455F} (R1^{F}), or IFNAR1$^{Y455F/\Delta 511}$ (R1$^{\Delta/F}$) were evaluated by phospho-immunoblotting, as in Fig. 1. This analysis is representative of at least three independent experiments. *B*, IFN-$\alpha_{A/D}$ (1000 U/ml)-dependent *MxA-1* and *OAS-1* expression was evaluated by Q-PCR (t = 0, 25 and 8 h) in MEFs from *A*. This analysis is representative of three independent experiments. *C*, IFN-α_1-dependent expression of ISRE-Luciferase reporter (ISRE-Luc; Stratagene) transiently coexpressed with either IFNAR1 (R1), IFNAR1^{Y455F} (R1^{F1}), IFNAR1^{Y518F} (R1^{F2}), IFNAR1^{Y529F} (R1^{F3}), IFNAR1$^{\Delta 517}$ (R1$^{\Delta 1}$), or IFNAR1$^{\Delta 471}$ (R1$^{\Delta 2}$) in *IFNAR1[−/−]* MEFs. Cells were cotransfected, in triplicate, with 30 ng of ISRE-Luc, 100 ng of TK-Renilla, and 0.3 ng of IFNAR1 mutant receptors (adjusted to a total of 500 ng with pEF-BOS). The following day, cells were stimulated with IFN-α_1 (50 U/ml; 7 h) and evaluated for relative luciferase activity (Rel. Luc. Act.). Data was normalized to renilla activity and presented as fold induction (IFN-treated/unstimulated). The response to R1 transfected cells was set to 100%. Results are representative of three independent experiments. *D*, The IFN-I dependent antiviral response in MEFs from *A* was determined by viral yield assay, as in Fig. 1. The analysis is representative of three independent experiments.

Next, a set of biochemical studies were undertaken to determine whether Y^{510} plays an important role in the IFN-α-dependent recruitment and activation of these STATs. Specifically, the ability

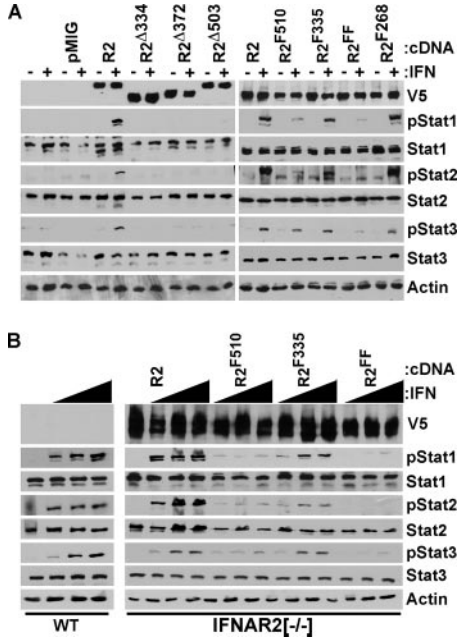

FIGURE 4. Functional characterization of IFNAR2 mutants. *A*, IFN-$\alpha_{A/D}$ (1000 U/ml; 30 min)-dependent activation of STAT1, STAT2, and STAT3 in WT and *IFNAR2[−/−]* MEFs ectopically expressing IFNAR2 (R2), IFNAR2$^{\Delta 334}$ (R2$^{\Delta 334}$), IFNAR2$^{\Delta 372}$ (R2$^{\Delta 372}$), IFNAR2$^{\Delta 503}$ (R2$^{\Delta 503}$), IFNAR2^{Y510F} (R2^{F510}), IFNAR2^{Y355F} (R2^{F355}), IFNAR2$^{Y355F/Y510F}$ (R2^{FF}), or IFNAR2^{Y268F} (R2^{F268}) was evaluated as in Fig. 1. *B*, IFN-$\alpha_{A/D}$ (increasing doses of 0, 100, 500, and 1000 U/ml; 30 min)-dependent activation of STAT1, STAT2, and STAT3 in day 7 WT and *IFNAR2[−/−]* bone marrow-derived macrophages ectopically expressing IFNAR2^{Y510F} (R2^{F510}), IFNAR2^{Y355F} (R2^{F355}), and IFNAR2$^{Y355F/Y510F}$ (R2^{FF}), as in Fig. 1. BMMs were evaluated 16 h after transduction, and this analysis is representative of three independent experiments.

of phospho-Y^{510} to compete for association with STAT1 and STAT2 was evaluated by DNA binding assay, previously exploited to identify STAT recruitment motifs in other cytokine receptors (15, 39, 40). An analogous pair of peptides, centered on STAT1-Y^{701}, served as a control, because previous studies had illustrated that p-St1^{Y701} competed for binding to the SH2 domains of STAT1 and STAT2 (15, 41, 42). Consistent with these studies, addition of p-St1-Y^{701}, but not the unphosphorylated peptide, impaired both the GAS (i.e., STAT1:STAT1) and ISRE (i.e., ISGF-3) DNA binding activity of IFN-α-stimulated extracts, and in a dose-dependent manner (see Fig. 5). Intriguingly, phospho-Y^{510}, but not the unphosphorylated peptide, was at least equally effective in disrupting the GAS DNA binding complex. Although in contrast to p-St1-Y^{701}, it was more effective in disrupting the GAS binding

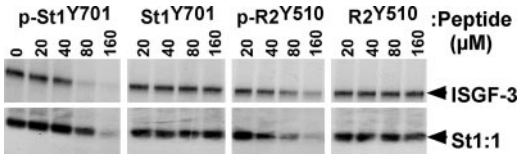

FIGURE 5. STAT1:STAT1 and ISGF-3 DNA binding inhibition assay. Tyrosine phosphorylated and native peptides were added at increasing concentrations (0, 20, 40, 80, and 160 μM, as indicated) to IFN-$\alpha_{A/D}$ (1000 U/ml; 30 min) WT MEF extracts and evaluated for ISGF-3 (*upper panels*) or STAT1:STAT1 (*lower panels*) DNA binding activity with a radio-labeled ISRE or GAS probe, respectively. Peptides included those centered on Y^{510} of IFNAR2 (R2Y^{510} and p-R2Y^{510}) and Y^{701} of STAT1 (St1Y^{701} and p-St1Y^{701}).

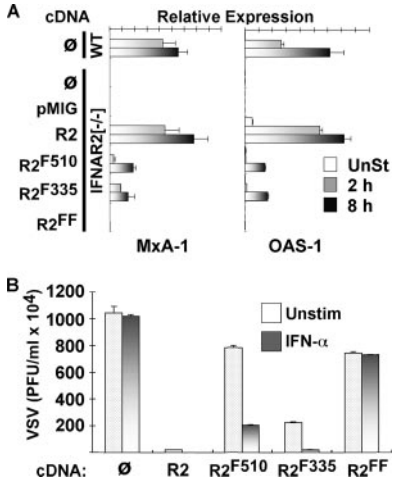

FIGURE 6. Evaluation of mutant IFNAR2 biological function. *A*, IFN-$\alpha_{A/D}$ (1000 U/ml)-dependent expression of MxA-1 and OAS-1 was evaluated by Q-PCR in WT and *IFNAR2[−/−]* MEFs from Fig. 4, as outlined in Fig. 1. This analysis is representative of three independent experiments. *B*, The antiviral response of MEFs in Fig. 4 was determined by viral yield assay, as in Fig. 1. The analysis is representative of three independent experiments.

complex than the ISRE binding complex. These studies illustrate that p-R2-Y^{510} is actively recognized by the SH2 domain of STAT1 and possibly STAT2, supporting an important role in the recruitment to IFNAR2.

A final set of studies determined whether defects in IFN-α-stimulated STAT activation correlated with defects in target gene expression and antiviral activity. Quantitative-PCR (Q-PCR) analysis of IFNAR2^{Y335F} and IFNAR2^{Y510F} MEFs revealed significant, but defective, IFN-α-dependent MxA-1 and OAS-1 expression. There was, however, no target gene expression in IFNAR2$^{Y335F/Y510F}$ MEFs (Fig. 6*A*). Although the marked reduction in gene expression observed with IFNAR2^{Y510F} and IFNAR2$^{Y335F/Y510F}$ paralleled the loss in STAT activation, this was not the case with IFNAR2^{Y335F}, where a defect in gene expression was more apparent than changes in STAT activation. However, IFNAR2^{Y335F}, featuring a functional Y^{510}, restored antiviral activity to levels observed with IFNAR2. IFNAR2^{Y510F} (with a functional Y^{335}) only partially restored the response to exogenous IFN-α and did not rescue the autocrine response to IFN-Is (Fig. 6*B*). These data underscore an essential role for Y^{510} in the biological response to IFN-Is and suggest a secondary role for Y^{335}.

Discussion

The emergence, during vertebrate evolution, of distinct receptors for IFN-Is corresponds with the emergence of the characteristic cluster of single exon IFN-Is found in humans, mice, and other mammals (1). Consistent with this, the biological response to IFN-Is, including STAT activation, expression of target genes, and antiviral response, is highly conserved between humans and mice (3, 5). To evaluate the unique activation of STAT2 by IFN-Is, its gene was targeted for deletion (17). Curiously, the ability of IFN-Is to activate STATs and direct a biological response was fully abrogated in STAT2-deficient fibroblasts. Yet, IFN-I-stimulated *STAT2[−/−]* leukocytes retained the ability to activate STAT1 and drive the expression of GAS-driven target genes (16, 17). These observations highlighted intriguing differences in the ability of IFNAR to activate STAT1 in fibroblasts and leukocytes. They also underscored the need to develop a better mechanistic understanding of how IFNAR directs the recruitment and activation of STATs.

To this end, we set out to characterize IFNAR through targeted mutagenesis. We hypothesized, as in the case with other cytokine receptors (11–16), that one or likely more conserved tyrosine motifs play a critical role in the recruitment and activation of STATs. To avoid potential concerns over the expression of an endogenous IFN-α receptor, mutant receptors were evaluated in cells isolated from *IFNAR1* and *IFNAR2* knockout mice (6–8). As previously reported, the biological response to IFN-α was found to be dependent on IFNAR1 but not on any sequences distal to residue 511, including its two conserved tyrosine motifs (Fig. 2). Moreover, mutant IFNAR1 receptors activated STATs with WT kinetics (data not shown). These studies are consistent with the notion that IFNAR1's primary role in IFN-I response is to sanction ligand binding and direct Tyk2 recruitment to the receptor complex (3).

Analogous studies on IFNAR2 underscored an important role in signal transduction. IFNAR2 deletion mutants revealed that a domain distal to residue 503 is critical for the biological response to IFN-Is. Mutation of the only distal tyrosine, Y^{510}, demonstrated that it played a critical role in the IFN-α-dependent activation of STATs, especially in primary cells. Mutation of a second, more proximal tyrosine, Y^{335}, led to a modest reduction in the activation of STAT1 and STAT3 and a more significant reduction in the activation of STAT2, contrasting observations made in an IFNAR2-deficient human tumor line (23). Mutation of both Y^{335} and Y^{510} ($IFNAR2^{Y335F/Y510F}$) completely abrogated IFN-α-dependent STAT activation. Despite significant differences in their capacity to direct STAT activation, both $IFNAR2^{Y335F}$ and $IFNAR2^{Y510F}$ exhibited significant reductions in their ability to induce the expression of MxA-1 and OAS-1 in response to IFN-α. Nonetheless, Y^{510} appeared to play a considerably more important role in the antiviral response to IFN-α. These observations underscore the critical role Y^{510} plays in IFN-α-dependent STAT activation and the ensuing antiviral response in murine tissues. Consistent with this, phospho-Y^{510} peptide effectively competed for association with STAT1, and possibly STAT2, suggesting it serves to directly recruit these STATs to the receptor complex. The more minor role Y^{335} plays in murine IFN-I response suggests that it either regulates an alternate signaling pathway (e.g., PI3 kinase, SOCS or a phosphatase) (28), or serves as a secondary/backup site for STAT activation. Four lines of evidence support the latter model suggesting Y^{335} directs a low level of STAT activation (i.e., below the limits of detection), which is not sufficient for many biological responses to ectopic IFN-Is, but may be important during autocrine IFN-I stimulation. First, STAT activation and biological response are only fully abrogated in $IFNAR2^{Y335F/Y510F}$ expressing cells. Second, similarities in the Y^{335} (GYTMH) and Y^{510} (GYIMR) tyrosine motifs suggest they are recognized by the same SH2 domain (e.g., STAT1, STAT2, and STAT3) (41, 43). Third, human Y^{337}, which corresponds to murine Y^{335}, directs robust STAT activation (23, 44). Fourth, lack of kinetic differences in the ability of $IFNAR2^{Y335F}$ and $IFNAR2^{Y510F}$ to activate STATs argues against a specific role for Y^{335} in directing the activity of negative regulators (e.g., SOCS and phosphatases; data not shown).

Although these studies underscore the important role $Y^{510} > Y^{335}$ play in directing the IFN-I-dependent recruitment and activation of STATs, they do not exclude a potential role for additional enabling regulatory events, like acetylation, which has recently been reported to facilitate signaling through a distinct IFNAR2 motif (25). Future studies will determine whether CREB-binding protein recruitment motifs, acetylation sites, and/or Y^{335} are required to direct the full biological response to murine IFN-Is in vivo. These studies may also finally shed light on why STAT2 is required for the IFN-α-dependent activation of STAT1 in fibroblasts, yet not in macrophages (17).

Disclosures

The authors have no financial conflict of interest.

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