Stat2 Binding to the Interferon-α Receptor 2 Subunit Is Not Required for Interferon-α Signaling*

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Vinh-Phuc Nguyen‡‡, Abu Z. M. Saleh‡, Allison E. Arch‡, Hai Yan‡, Flavia Piazza‡, John Kim‡, and John J. Krolewski‡‡‡‡

From the ‡Department of Pathology and the **Chao Family Comprehensive Cancer Center, University of California, Irvine, Irvine, California 92697, the †Johns Hopkins Oncology Center, Johns Hopkins University, Baltimore, Maryland 21231, and the ‡Department of Pathology, Columbia University, College of Physicians and Surgeons, New York, New York 10032

The interferon-α (IFNα) receptor consists of two subunits, the IFNα receptor 1 (IFNaR1) and 2 (IFNaR2) chains. Following ligand binding, IFNaR1 is phosphorylated on tyrosine 466, and this site recruits Stat2 via its SH2 domain. In contrast, IFNaR2 binds Stat2 constitutively. In this study we have characterized the Stat2-IFNaR2 interaction and examined its role in IFNα signaling. Stat2 binds the major IFNaR2 protein but not a variant containing a shorter cytoplasmic domain. The interaction does not require a STAT SH2 domain. Both tyrosine-phosphorylated and non-phosphorylated Stat2 bind IFNaR2 in vitro; however, relatively little phosphorylated Stat2 associates with IFNaR2 in vivo. In vitro binding assays defined IFNaR2 residues 418–444 as the minimal interaction domain and site-specific mutation of conserved acidic residues within this domain disrupted in vitro and in vivo binding. An IFNaR2 construct carrying these mutations was either (i) overexpressed in 293T cells or (ii) used to complement IFNaR2-deficient U5A cells. Unexpectedly, the activity of an IFNaR2-dependent reporter gene was not reduced but, instead, was enhanced up to 2-fold. This suggests that this particular IFNaR2-Stat2 interaction is not required for IFNα signaling, but might act to negatively inhibit signaling. Finally, a doubly truncated recombinant fragment of Stat2, spanning residues 136–702, associated with IFNaR2 in vitro, indicating that the interaction with IFNaR2 is direct and occurs in a central region of Stat2 marked by a hydrophobic core.

Type I interferons (IFNs), including multiple interferon-α (IFNα) isoforms and interferon-β (IFNβ), bind a common receptor complex consisting of two subunits, interferon-α receptor 1 (IFNaR1) and interferon-α receptor 2 (IFNaR2). IFNaR1 displays low affinity for most human IFNα isoforms and appears to be primarily a signal transducing subunit (1, 2). The other subunit, IFNaR2, mediates both ligand binding and signaling (3–5). It is expressed as three variants: a soluble receptor (5), a short transmembrane form (5), and a long transmembrane form believed to be the physiologically relevant receptor (3, 4). The short and long transmembrane forms are referred to as IFNaR2-1 and IFNaR2-2, respectively. IFNaR2-1 is usually expressed at lower levels than IFNaR2-2 and may exert a dominant negative effect on IFNα signaling, although its precise role is unclear (6).

IFNa receptor subunits lack intrinsic enzymatic activity, instead relying on members of the Janus tyrosine kinase (JAK) family to transduce signals. Genetic complementation experiments have linked two JAKs, Tyk2 and Jak1, to IFNα signaling (7, 8), and biochemical studies subsequently demonstrated constitutive and direct association of these JAKs with the IFNaR1 and IFNaR2 subunits, respectively (9–12). Two members of the signal transducer and activator of transcription (STAT) family, Stat1 and Stat2, have also been implicated (13, 14). Signaling begins with IFNα binding, triggering receptor oligomerization and juxtaposing associated JAKs. A series of auto- and/or transphosphorylations result in Jak1 and Tyk2 activation (8, 11, 15) and phosphorylation of tyrosine residue 466 on IFNaR1 (10, 12, 16). Once phosphorylated, this tyrosine recruits Stat2 in an SH2-dependent manner (10, 17). Activated Tyk2 or Jak1 phosphorylate Stat2 on tyrosine that, in turn, recruits Stat1 to the receptor (10, 18). Following Stat1 phosphorylation, the two STATs subsequently heterodimerize via SH2-phosphotyrosine interactions (19, 20). With the p48/IRF9 protein, Stat1-Stat2 heterodimers form the interferon-stimulated gene factor 3 complex (14, 21, 22), which binds to the interferon-stimulated responsive element (ISRE) to direct transcription.

Latent STATs were initially believed to exist as monomers in the cytoplasm, but recent studies indicate that they exist primarily in high molecular weight complexes (23, 24). In this regard, constitutive association of Stat2 with IFNaR2 has been reported, and termed “pre-docking” (25). Although it has been suggested that this interaction facilitates the subsequent SH2 domain-dependent Stat2 recruitment, the biological significance of this association remains unclear. Therefore, we sought to delineate the Stat2 interaction domain on IFNaR2 and investigate its role in IFNα signaling. We have found that the minimal Stat2 binding region is between residues 418 and 444 of IFNaR2, although a larger domain (residues 340–462) is required for maximal binding. Mutation of conserved acidic amino acids corresponding to residues 435–438 disrupts IFNaR2 binding to Stat2. Importantly, expression of these mutated IFNaR2 constructs in two separate cell systems dem-
onstrates that ISRE-driven reporter gene activity is increased relative to cells expressing the wild-type receptor. Thus, this interaction is dispensable for effective IFNα signaling and might function in the negative regulation of such signaling.

MATERIALS AND METHODS

Reagents, Viruses, and Cells—The following antibodies were used: 4G10, against phosphotyrosine (Upstate Biotechnology); SC-135, against glutathione S-transferase (GST) (Santa Cruz Biotechnology); SC-248, against the influenza virus hemagglutinin (HA) epitope (Santa Cruz Biotechnology) (26); H15, against the polyhistidine tag epitope (Santa Cruz Biotechnology); T20220, against Tyk2 (BD Transduction Laboratories); J24320, against Jak1 (BD Transduction Laboratories); polyclonal rabbit antisera against the carboxyl terminus of Tyk2 (11); polyclonal rabbit antisera against Stat2 (C. Schindler, Columbia University, College of Physicians and Surgeons, New York, NY) (14); and AC-15 against β-actin (Sigma). Recombinant IFNγ was from M. Brunda (Hoffmann-La Roche, Nutley, NJ). Baculoviruses encoding Stat1, Stat2, and Jak2 (12) were used to infect S9 cells (Invitrogen) (27). Four human cell lines, embryonic kidney 293T cells (H. Young, Columbia University, College of Physicians and Surgeons, New York, NY), osteogenic sarcoma U2OS cells (ATCC, Manassas, VA), IFNγR2-deficient 2fTG cells expressing Stat1 (4), and 2fTG cells, the parental line for U5A cells, containing wild-type IFNγR2 (the latter two lines are from G. Stark, Lerner Research Institute, Cleveland Clinic Foundation), were all maintained as adherent cultures in Dulbecco's modified Eagle's medium plus 10% heat-inactivated fetal calf serum.

Expression Constructs—Fragments of the IFNγR2 cytoplasmic domain were generated by PCR and were cloned, sequenced, and transfected into an appropriate pGEX vector (Amersham Biosciences) to encode GST-IFNγR2 fusion proteins. To create site-specific mutants the overlapping PCR technique (28) was used to generate DNA fragments (spanning residues 376–462) with alanine substitutions at positions 435–438 (mutant 1, DDED to AAAA), and 440–443 (mutant 2, DDLE to AAAA) of IFNγR2, respectively. The full-length IFNγR2 cDNA and the corresponding 435–438 mutant were cloned into pMT2T for expression in human cells (29). A DNA fragment flanked by XhoI sites, encoding amino acids 136–702 of Stat2, was PCR-amplified using Pfu polymerase (Stratagene), cloned, sequenced, and then transferred into pET11b (Novagen) for expression in bacteria. Truncated Stat2 constructs (corresponding to residues 1–293, 1–323, and 123–517, respectively) were generated using convenient restriction sites and cloned into pET11b. The remaining cytoplasmic tails left untruncated expression constructs were first subjected to non-saturating exposures of immunoblot autoradiographs of treated and untreated controls to normalize expression. The luciferase gene construct is under the control of an ISRE from the ISG-15 gene (31).

Recombinant Proteins—Escherichia coli DH5α, containing the appropriate pGEX-IFNγR2 construct, was grown at 37 °C to log phase and induced at 30 °C with 0.1 mM isothioligalactopyranoside (IPTG) for 3–4 h. Pelleted bacteria, resuspended in STE (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) containing 150 μg/ml of lysozyme and 1 mM phenylmethylsulfonyl fluoride, were sonicated. Inclusion bodies were pelleted and solubilized in Sarkosyl (32) and stored at −80 °C. Fractions of these crude extracts were diluted into medium containing 400 μg/ml G418 (Invitrogen). Individual clones were propagated and screened by immunoblotting cytoplasmic lysates with an anti-HA antibody to identify lines expressing IFNγR2 proteins. Stable derivative cell lines were periodically cultured in the presence of G418 and monitored by immunoblotting with anti-HA antibodies to ensure the continued expression of the exogenous IFNγR2 constructs.

Immunoprecipitation and Immunoblotting—Nearly confluent cultures were lysed in TBE/5% Nonidet P-40 plus 0.2 mM phenylmethylsulfonyl fluoride. Nuclei, debris, and cell membranes were removed by centrifugation to yield a mainly cytoplasmic protein extract. In some cases portions of these crude extracts were mixed with sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose (Osmobion), blocked with 5% nonfat milk or 3% bovine serum albumin, and sequentially probed with an appropriate primary antibody, followed by a secondary antibody linked to horseradish peroxidase. Bands were visualized by chemiluminescence (Pierce Super Signal Substrate). In other cases proteins were first immunoprecipitated by incubating the cytoplasmic lysates with appropriate antibodies and protein A-Sepharose beads (Sigma). The antibody–protein complexes were collected by a secondary antibody linked to horseradish peroxidase. Results were analyzed by an unpaired Student's t-test using a Macintosh computer (Apple).
cytoplasmic domain of IFNaR2-2, IFNaR2-1, or IFNaR1 were incubated with a human Stat2-containing SF9 cell extract, and complexes were immunoblotted with anti-Stat2 antisemur. A strong interaction was detected with GST-IFNaR2-2 (Fig. 1A, lane 5), but not with GST, GST-IFNaR1, or GST-IFNaR2-1 (Fig. 1A, lanes 2–4), essentially as previously observed (25, 33). We did not observe consistent binding of baculovirus-produced Stat1 and GST-IFNaR2-2 (data not shown). Next, to investigate whether phosphorylated Stat2 can bind IFNaR2-2, we cofected SF9 cells with recombinant baculoviruses encoding Jak1 and Stat2. Under these conditions Stat2 is tyrosine-phosphorylated in a manner similar to that seen following IFNα treatment (10, 34). Employing the same in vitro binding assay, we observed a strong association between phosphorylated Stat2 and IFNaR2-2 (Fig. 1B, lane 3).

Docking of Stat2 to IFNaR1 requires phosphorylation of tyrosine residue 466 on IFNaR1 as well as an intact Stat2 SH2 domain (10). Because it is unlikely that the bacterially expressed GST fusions employed in Fig. 1 are tyrosine-phosphorylated, it is similarly unlikely that IFNaR2 phosphorylation is required for the interaction with Stat2. Thus, we anticipated that the Stat2 SH2 domain would also be superfluous for the Stat2-IFNaR2-2 interaction. As expected, a strong signal was observed (Fig. 1C, lane 3) when the in vitro binding assay was performed with lysate from cells infected with a Stat2 baculovirus bearing an inactive SH2 domain mutation (R601K) (10).

Mapping the Minimal Stat2 Binding Domain on IFNaR2—Fig. 1 indicates that Stat2 specifically interacts with IFNaR2-2. Therefore, we employed the IFNaR2-2 subunit, referred to hereafter as IFNaR2 for the remainder of our studies. To delineate the Stat2 binding domain on IFNaR2, a panel of GST fusion proteins encoding portions of the cytoplasmic domain was used in similar in vitro binding assays (Fig. 2A). Truncation of IFNaR2 from the carboxyl terminus to residue 444 (Fig. 2B, lane 4), or from the amino terminus to residue 340 (Fig. 2B, lane 7) did not affect binding. However, a doubly truncated construct (340–444) displayed some reduction in binding (Fig. 3, compare lanes 4 and 3). Further truncation from the amino terminus revealed that a 25-amino acid fragment (418–444) of IFNaR2 bound Stat2, albeit at reduced efficiency (Fig. 2B, lane 11). These data suggest that residues in this small region are critical for the interaction. Alignment of human and murine IFNaR2 revealed substantial homology, including a block (435–438; DDED) containing four acidic residues (Fig. 3A). To test the role of these acidic residues two mutants were created by substituting alanine for either these four amino acids (designated mutant 1), or for another, non-conserved block of mainly acidic amino acids (mutant 2). Stat2 binding to IFNaR2 was diminished when alanine substitution was made at positions 435–438 (Fig. 3B, compare lanes 7 and 6) but not when similar changes were made at the non-conserved residues (Fig. 3B, lane 8).

Mutation of Acidic Residues Disrupts Stat2 Binding to IFNaR2 in Vivo—The in vitro binding data prompted us to examine if mutation of residues 435–438 also decreased binding in vivo. Constructs encoding Stat2 and HA-tagged wild-type (wt) or mutant 1 (m1) IFNaR2 proteins were transfected alone or in combination into 293T cells. Co-immunoprecipitation of Stat2 and IFNaR2 was significantly reduced in cells expressing m1 IFNaR2 (Fig. 4A, compare lanes 5 and 6), indicating that acidic residues at positions 435–438 of IFNaR2 are critical for association both in vitro and in vivo.

Effect of IFNs on STAT Phosphorylation and Stat2-IFNaR2 Interaction—As seen in Figs. 1–4, the Stat2-IFNaR2 association appears to be constitutive and therefore ligand-independent. However, to investigate the effect of IFNα treatment on the interaction, transfected cells overexpressing Stat2 and HA-tagged wt or m1 IFNaR2 were treated with IFNα. Co-immunoprecipitation analysis indicates that Stat2 association with wt IFNaR2 increases following ligand binding (Fig. 5A, compare lanes 4 and 3). However, in other experiments we observed little or no increase (Fig. 5E, compare lanes 3 and 4), suggesting that IFNα treatment has a slight effect on the amount of Stat2 bound to IFNaR2. Stat2 binding to the m1 IFNaR2 construct was minimally detectable following IFNα treatment (Fig. 5A, lane 6) but, as seen in Fig. 4, was greatly reduced compared with wt IFNaR2 (Fig. 5A, compare lanes 4 and 6).

Both phosphorylated and non-phosphorylated Stat2 bound GST-IFNaR2 in vitro (Fig. 1, A and B). To determine whether this occurs in vivo cells transiently co-expressing Stat2 and wt IFNaR2 were again treated with IFNα. Part of each extract was immunoprecipitated with anti-HA antibody, and co-immunoprecipitating tyrosine-phosphorylated Stat2 was detected by immunoblotting with an anti-phosphotyrosine antibody (Fig. 5D, lanes 1–4). The remainder of each extract was immunoprecipitated with anti-Stat2 antisemur and immunoblotted with an anti-phosphotyrosine antibody (Fig. 5D, lanes 5–8). A small fraction of tyrosine-phosphorylated Stat2 associated with IFNaR2 (Fig. 5D, compare lanes 4 and 8). Reprobing the immunoblot in Fig. 5D with anti-Stat2 antisemur indicates that, as expected, only a portion of the Stat2 protein binds the receptor (Fig. 5E, compare lanes 3 and 4 with lanes 7 and 8). Factoring in the relative recovery of Stat2 revealed by comparing Fig. 5, D and E, it appears that phosphorylated Stat2 does not preferentially associate with IFNaR2.

Overexpression of Mutant 1 IFNaR2 Increases Stat2 Phosphorylation—To determine whether expression of m1 IFNaR2 affects IFNα signaling, we transiently expressed this mutant receptor in 293T cells and measured tyrosine phosphorylation of endogenous Stat2. We reasoned that expressing the mutant receptor at high levels would increase the likelihood that receptor complexes containing both m1 IFNaR2 and IFNaR1 will form, allowing us to determine the effect of this mutation on various aspects of JAK-STAT signaling. Endogenous Stat2 was
phosphorylated after IFNα stimulation (Fig. 6A, lanes 2, 4, and 6), as expected. Moreover, its phosphorylation increased in m1 IFNαR2-expressing cells relative to those expressing wt IFNαR2 or those transfected with empty vector (Fig. 6A, compare lane 6 to lanes 4 or 2, respectively). The reason for the increase in Stat2 tyrosine phosphorylation in cells transfected with the wt IFNαR2 construct, relative to vector-transfected cultures, is not known. It is possible that the level of IFNαR1 exceeds that of IFNαR2. Therefore, overexpression of IFNαR2 might increase the level of functional receptor heterodimer.
thereby increasing the responsiveness of the transfected cells. To gain further insight, kinetic experiments were carried out, extending IFNα/H9251 stimulation to 60 min. Stat2 phosphorylation appeared to rise to a higher level and sustain that level for a longer period of time in cells expressing m1 IFNαR2 compared with counterparts expressing wt IFNαR2 (Fig. 6C, compare lanes 2 and 3 with 5 and 6). Note that the effect of m1 IFNαR2 on Stat2 phosphorylation (panel C) may be underestimated in this experiment as these transfectants apparently expressed lower levels of m1 IFNαR2 relative to the wild type (Fig. 6E).

We performed experiments similar to those shown in Fig. 6, A and B, to examine the IFNα-induced tyrosine phosphorylation of Jak1, the tyrosine kinase associated with the IFNαR2 subunit. There was no discernable difference in the level of induced Jak1 tyrosine phosphorylation in cells transfected with m1 IFNαR2 relative to those receiving wt IFNαR2 or vector (data...
not shown). Although IFNaR2 has been reported by others to be tyrosine-phosphorylated in response to IFNo treatment (35–37), we did not detect tyrosine phosphorylation of transfected wt or m1 IFNaR2 (data not shown). In this regard it remains possible that our experiments may lack the sensitivity to detect IFNaR2 tyrosine phosphorylation, that the level of phosphorylation is low in this particular cell line, or that the signal from phosphorylated IFNaR2 is diluted by IFNaR2 molecules that are not part of the functional receptor complex. Finally, IFNo-induced tyrosine phosphorylation of the Tyk2 tyrosine kinase, which associates with IFNaR1, was not different in cells transfected with wt or m1 IFNaR2 (data not shown).

**Disruption of the Stat2-IFNaR2 Interaction Culminates in Increased IFNo-dependent Transcription**—Next, we examined ISRE-dependent reporter gene activity (31, 38). Adenovirus-transformed 293T cells cannot be used in such assays because viral E1A binds p300/CFB, a Stat1- and Stat2-interacting transcriptional coactivator, blocking IFNo-mediated transcription (39, 40). Therefore, U2OS cells were co-transfected with an ISRE-driven luciferase reporter, a constitutive β-galactosidase plasmid, and constructs encoding wt or m1 IFNaR2. In cells transfected with empty vector, IFNo increased luciferase activity 7-fold (Fig. 7, left column). Similar treatment resulted in a 12-fold induction in wt IFNaR2-expressing cells (Fig. 7, middle column). This increase, relative to vector-transfected cultures, is similar to the effect seen in Fig. 6, and again its cause is unknown. Transcriptional activity was enhanced 18-fold in the presence of the m1 IFNaR2 subunit (Fig. 7, right column), statistically higher than in cells expressing either the wild-type receptor or empty vector. These results demonstrate that signaling is enhanced when the Stat2 interaction with IFNaR2 is severely impaired.

**Complementation of IFNaR2-deficient Cells with Mutant 1 IFNaR2 Also Enhances IFNo-dependent Transcription**—One potential shortcoming of the data in Fig. 7 is the possibility that the endogenous, wild-type IFNaR2 receptor subunit is still playing a role in the signaling events occurring in those cells overexpressing m1 IFNaR2. To address this issue, and to provide independent confirmation of the physiologic function of the Stat2 binding site on IFNaR2, the wild-type and mutant forms of IFNaR2 were introduced into the U5A cell line, which does not express IFNaR2 (4). Stable transfectants were expanded and assayed for expression of IFNaR2, using an anti-HA antibody. Fig. 8A shows the characterization of a single clone expressing wild-type IFNaR2 and two independent clones expressing m1 IFNaR2. Because we were unable to identify wt and m1 clones expressing exactly the same amount of protein, we examined two separate clones, expressing higher and lower amounts, respectively, of m1 IFNaR2 (relative to a single wild-type-expressing clone), to evaluate potential dosage effects.

Next, we performed a reporter gene assay, essentially identical to the one shown in Fig. 7. Fig. 8B confirms our results in
the U2OS overexpression system. In general, the level of IFNα-dependent reporter gene induction is lower, compared with U2OS cells, in the U5A cells complemented with wt IFNaR2 as well as the parental 2fTGH cells, which also express the wild-type gene. The difference in induced reporter gene activity between these two lines is not significant. However, both U5A clones expressing m1 IFNaR2 display a statistically significant enhancement of IFNα-dependent reporter gene activity relative to U5A cells complemented with wt IFNaR2. The degree of enhancement is not proportional to the level of m1 IFNaR2 protein expression (compare Fig. 8, A and B).

We did not observe a consistent difference in the level of Stat2 tyrosine phosphorylation among the various U5A transfectants (data not shown), in contrast to our observations in the 293T overexpression system. However, both of the U5A clones complemented with m1 IFNaR2 express higher levels of Stat2 protein relative to the clone complemented with wt IFNaR2 (Fig. 8C). Densitometry of the data shown in Fig. 8C, as well as data from a replicate experiment performed on a separate set of cell extracts (data not shown), revealed an average increase of about 3.5-fold for Stat2 protein levels when normalized to β-actin protein levels.

**Mapping the IFNaR2-interacting Domain on Stat2**—To map the IFNaR2-interacting domain on Stat2, we generated truncated Stat2 derivatives and tested them in an *in vitro* assay similar to that employed in Figs. 1–3. Using STAT chimeras created by exchanging homologous sequences between Stat1 and Stat2, Li et al. (25) implicated a domain encompassing Stat2 residues 295–315 in the binding to IFNaR2. However, although we observed strong binding to full-length Stat2 (Figs. 1–3), we were unable to detect binding to constructs spanning residues 1–293, 1–323, and 123–517, respectively (Fig. 9A and data not shown). The construct spanning residues 136–702 was designed on the basis of proteolytic digestion and crystallographic data suggesting that both Stat1 and Stat3 form central core structures held together by hydrophobic interactions, which are topologically distinct from the extreme amino and carboxyl terminal domains (41, 42). A strong association was readily detected between this double-truncated Stat2 and wild-type GST-IFNaR2 (376–462), but not with an IFNaR2 mutant 1 fusion (Fig. 9B, compare lanes 3 and 4), indicating that amino and carboxyl termini are dispensable. In addition, because both recombinant proteins employed in Fig. 9B were purified from bacteria, the data indicate that Stat2 and IFNaR2 almost certainly bind directly to one another in eukaryotic cells without any intervening proteins.

**DISCUSSION**

SH2 domain-mediated binding of Stat2 to tyrosine-phosphorylated IFNaR1 is well established as a critical protein-protein interaction required for signaling in response to IFNa (10, 17, 25). In this study we have investigated a second interaction between Stat2 and the IFNa receptor complex; the constitutive, SH2-independent interaction between Stat2 and IFNaR2. Our experimental approach consisted of two parts. First, we characterized binding between Stat2 and IFNaR2. In *in vitro* binding between Stat2 and IFNaR2 was previously reported by Li et al. (25) and subsequently mapped to residues 404–462 of IFNaR2 (33). We confirmed these data (Fig. 1) and then narrowed the Stat2 binding domain to a 26-residue region, spanning amino acids 418–444 (Fig. 2). Within this domain, conserved acidic residues are crucial for Stat2-binding because their mutation severely diminishes the Stat2-IFNaR2 interaction both *in vitro* and *in vivo* (Figs. 3, 4, and 5A). A search for homologous sequences has revealed no similarity to known proteins, suggesting this Stat2 binding site may be unique to IFNaR2. The IFNaR2 interaction domain on Stat2 has not been as clearly defined. Neither Stat2 tyrosine phosphorylation nor an intact SH2 domain are required for the Stat2-IFNaR2 interaction (Fig. 1). Using Stat1-Stat2 chimeric proteins, Li et al. (25) have shown that a sequence spanning residues 295–315 of Stat2 is necessary for binding to IFNaR2. Although we observed an *in vitro* interaction using a Stat2 construct spanning residues 136–702 (Fig. 9), we were unable to detect binding to shorter fragments of Stat2 overlapping the region identified by Li and colleagues. Thus, residues 295–315 of Stat2, although necessary, may not be sufficient to mediate binding, and additional portions of Stat2 may be required. Alternately, because the central portion of Stat2 (residues 136–702) is apparently held together by a single, contiguous hydrophobic core (41, 42), proteins encoding fragments of this core may not be folded properly. To resolve this issue, we are currently mutating a core Stat2 construct to identify residues critical for this association.

Second, we investigated the physiologic effects of disrupting the Stat2-IFNaR2 interaction. The most important and consistent result of these studies is the finding that the m1 IFNaR2 construct, when overexpressed in 293T cells or when used to complement IFNaR2-deficient U5A cells, does not impair IFNα-dependent induction of an ISRE-linked reporter gene (Figs. 7 and 8). These data indicate that the Stat2-IFNaR2 interaction is not required for signaling. The relatively small but reproducible enhancement of IFNα-dependent reporter gene activity observed in these two independent experiments suggests that under physiologic conditions the Stat2 binding site on the IFNaR2 subunit might negatively regulate IFNα signaling. Because we are uncertain of the molecular mechanism regulating this inhibitory effect, we consider this latter conclusion to be speculative at this time. When m1 IFNaR2 was overex-
pressed in 293T cells Stat2 tyrosine phosphorylation was enhanced (Fig. 6). However, the effect, although reproducible, was quite modest. Furthermore, in the US5 complementation experiments we did not observe a reproducible effect on Stat2 phosphorylation. Instead, we saw a definite increase in the level of total Stat2 protein (Fig. 8C). Thus, it is possible that the mechanism involved might modulate either Stat2 phosphorylation and/or stability. Clearly, a variety of other mechanisms that ultimately lead to an increase in ISRE-mediated gene transcription could be operable here, and this remains an area for future investigation.

Li et al. (25) correlated tyrosine phosphorylation and reporter gene activity with in vitro binding to IFNaR2 by various Stat1–2 chimeras. They concluded that the Stat2-IFNaR2 interaction is required for the IFNα signaling and suggested that Stat2 predocks on IFNaR2 prior to its ligand-dependent recruitment to phosphorylated tyrosine 466 on IFNaR1. This model is at odds with our conclusions. Although differences in the experimental systems make direct comparisons difficult, we believe it is likely that the chimeric STAT constructs are not recruited via tyrosine 466, and an alternative mechanism is presumably utilized. The IFNaR2-Stat2 interaction has been invoked as such an alternate, and we cannot exclude this possibility. However, it should be noted that none of these studies employed specific mutations within the IFNaR2 cytoplasmic domain to directly address the hypothesis that the Stat2 interaction with residues 340–462 of IFNaR2 is required for signaling. Additionally, the relevance of these experimental scenarios to the physiologic situation for IFNα, where IFNaR1 and IFNaR2 contribute to the receptor complex, remains to be determined.

It has been proposed that an unidentified tyrosine phosphatase binds IFNaR2 near the region we have identified as a Stat2 binding site (47). Although it is conceivable that the Stat2-IFNaR2 interaction modulates the activity of such a phosphatase, we believe that this is unlikely for two reasons. First, deletions removing the residues 418–444 of IFNaR2 do not affect that activity of the phosphatase (47), even though this region is critical for Stat2 binding to IFNaR2 (Figs. 2 and 3). In addition, we do not see any change in the tyrosine phosphorylation of the receptor-associated JAK kinases (data not shown).

Finally, it should be noted that the in vivo binding results shown in Fig. 5 also explicitly fail to support the idea that IFNaR2 mediates an obligatory docking and/or predocking step in the process of Stat2 activation. First, triggering the signaling cascade by treating cells with IFNα does not decrease the amount of Stat2 that is bound to the IFNaR2 subunit as might be expected if this site served a predocking function. In fact, we observed some increase in the amount of Stat2 binding to IFNaR2 following IFNα treatment (Fig. 5). Second, very light tyrosine-phosphorylated Stat2 associates with IFNaR2 following IFNα treatment, suggesting that this site is not acting as a functional docking site in and of itself. Furthermore, we have recently observed that recombinant Stat2 binds IFNaR2 more avidly than it binds IFNaR1 which is tyrosine-phosphorylated on residue 465. These data are inconsistent with the idea that tyrosine-phosphorylated IFNaR1 recruits Stat2 from an interaction site on IFNaR2.

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