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# Phosphorylated interferon-α receptor 1 subunit (IFNaR1) acts as a docking site for the latent form of the 113 kDa STAT2 protein

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**Interferon-α (IFNα) induces rapid tyrosine phosphorylation of its receptors, two JAK kinases and three STAT transcription factors. One kinase, p135$^{tyk2}$, is complexed with the IFNaR1 receptor, and may catalyze some of these phosphorylation events. We demonstrate that, *in vitro*, p135$^{tyk2}$ phosphorylates two tyrosines on IFNaR1. A phosphopeptide corresponding to the major phosphorylation site (Tyr466) binds STAT2, but not STAT1, in an SH2-dependent manner. Furthermore, only latent, non-phosphorylated STAT2 interacts with this phosphopeptide. When this phosphopeptide is introduced into permeabilized cells, the IFNα-dependent tyrosine phosphorylation of both STATs is blocked. Finally, mutant versions of IFNaR1, in which Tyr466 is changed to phenylalanine, can act in a dominant negative manner to inhibit phosphorylation of STAT2. These observations are consistent with a model in which IFNaR1 mediates the interaction between JAK kinases and the STAT transcription factors.**

*Keywords*: interferon/signal transduction/transcription factor/tyrosine kinase

## Introduction

The molecular events in the signaling pathways triggered by cytokines are rapidly being cataloged. It appears that in most cases, a major pathway is composed of three components: multi-subunit receptors, JAK family tyrosine kinases and STAT (signal transducer/activator of transcription) family proteins. These act together in a relatively simple and direct signaling cascade capable of activating gene transcription (reviewed in Darnell *et al.*, 1994; Schindler and Darnell, 1995). In the case of interferon-α (IFNα), three STATs were originally identified as proteins which specifically bound an IFNα-stimulated gene response element (ISRE) found upstream of many IFNα-responsive genes (Kessler *et al.*, 1990; Schindler *et al.*, 1992a). All contain SH2 domains, suggesting they bind to tyrosine-phosphorylated proteins (Fu, 1992). Subsequently, it was found that these three proteins, the 113 kDa product of the STAT2 gene and the 91 and 84 kDa products of the STAT1 gene, are all rapidly tyrosine phosphorylated following IFNα treatment (Fu, 1992; Schindler *et al.*, 1992b). The phosphorylated STATs form heterodimeric complexes composed of either p113$^{STAT2}$ and p91$^{STAT1}$ or p113$^{STAT2}$ and p84$^{STAT1}$ and translocate to the nucleus (Müller *et al.*, 1993b).

A parallel investigation identified components in the IFNα signaling pathway by complementing mutants which were unable to activate an IFN-responsive reporter gene (Pellegrini *et al.*, 1989). A previously described JAK family tyrosine kinase, p135$^{tyk2}$ (Firmbach-Kraft *et al.*, 1990), was linked to the IFNα pathway by this method (Velazquez *et al.*, 1992). Subsequent biochemical studies showed that p135$^{tyk2}$ is rapidly activated and phosphorylated after IFNα treatment, and that it forms a stable complex with the IFNα receptor subunit 1 (IFNaR1, also known as the α-subunit) (Velazquez *et al.*, 1992; Barbieri *et al.*, 1994; Colamonici *et al.*, 1994a,b). Analysis of other signaling-deficient mutants verified the requirement for STAT1 (Müller *et al.*, 1993b) and STAT2 (Leung *et al.*, 1995) and demonstrated that the previously described JAK-1 tyrosine kinase (Wilks *et al.*, 1991) was also needed (summarized in Hunter, 1993; Müller *et al.*, 1993a).

These data suggested a simple and direct model for IFNα signal transduction in which JAK-1 and/or tyk2 phosphorylated the STATs (Darnell *et al.*, 1994). There are three other known members of this family of non-receptor kinases: JAK-2, JAK-3 and the *Drosophila* homolog known as *hopscotch* (Harpur *et al.*, 1992; Cance *et al.*, 1993; Binari and Perrimon, 1994; Kawamura *et al.*, 1994; Takakashi and Shirasawa, 1994; Witthuhn *et al.*, 1994). All known mammalian JAK kinases have been shown to be associated with one or more cytokine receptors, and activated in response to ligand binding (cataloged in Schindler and Darnell, 1995). The JAK kinases share a distinctive structure, including a kinase-like domain immediately N-terminal to the *bone fide* tyrosine kinase domain (Wilks *et al.*, 1991). No function has been ascribed to this kinase-like domain, nor to five other regions of JAK family homology (Harpur *et al.*, 1992) found in the N-terminal half of all these proteins. At present, there is no direct evidence indicating that the tyk2 or JAK-1 kinases associate with, or phosphorylate, STAT1 or STAT2.

The IFNα receptor consists of at least two subunits (IFNaR1 and IFNaR2) (Uzé *et al.*, 1995), apparently encoded by at least two separate genes (Uzé *et al.*, 1990; Novick *et al.*, 1994) localized within a 400 kb region of chromosome 21 (Soh *et al.*, 1994). Like other cytokine receptors, these two proteins are rapidly phosphorylated on tyrosine in response to IFNα (Platanias and Colamonici, 1992; Abramovich *et al.*, 1994; Constantinescu *et al.*, 1994; Platanias *et al.*, 1994). IFNaR1 can be phosphorylated *in vitro* by JAK family kinases (Colamonici *et al.*,

1994a), but it is not known which kinase(s) serve this role *in vivo*. The α-subunit of the IFNγ receptor (IFNγRα) is also tyrosine phosphorylated in response to ligand (Greenlund *et al.*, 1994). Further, in this case, a tyrosine residue is part of a site within the receptor which is required for signaling (Farrar *et al.*, 1992). A phosphopeptide corresponding to the region surrounding this tyrosine residue binds the $p91^{STAT1}$ protein (Greenlund *et al.*, 1994), suggesting that phosphorylated IFNγRα may act as a docking site for the STAT1 protein, thereby facilitating its phosphorylation. In this report, we show that a similar interaction occurs between $p113^{STAT2}$ and phosphorylated IFNaR1 and, further, that the formation of this complex requires a STAT2 protein which is latent (unphosphorylated) and contains a functional SH2 domain. Our data support a model in which a single site on the IFNaR1 subunit specifies the phosphorylation and dimerization of STAT1 and STAT2.

## Results

### *JAK family kinases phosphorylate STATs in a promiscuous manner*

The IFNα pathway utilizes the tyk2 and JAK-1 kinases and the STAT1 and STAT2 proteins, while the IFNγ pathway uses the JAK-1 and JAK-2 kinases and the STAT1 protein. These correlations suggested that STAT2 might be a specific substrate for the $p135^{tyk2}$ kinase, but not a substrate for JAK-1 or JAK-2. To test this hypothesis, we prepared recombinant baculoviruses encoding $p91^{STAT1}$ and $p113^{STAT2}$, and co-infected Sf9 cells with one of these two viruses and a virus encoding one of the JAK kinases. Lysates from the infected cells were immunoprecipitated with antibodies recognizing the appropriate STAT protein, and then immunoblotted with an antibody against phosphotyrosine. Figure 1 (top panels) shows that all three wild-type JAK kinases (JAK-1, JAK-2 and tyk2; lanes labeled J1, J2 and WT, respectively) were able to phosphorylate both STAT proteins, while the kinase-deficient form of tyk2 (Colamonici *et al.*, 1994b) (lanes labeled K) was not. Portions of the same lysate were immunoprecipitated with anti-JAK antibodies, and then immunoblotted with an anti-phosphotyrosine antibody, indicating that the extent of STAT phosphorylation was generally proportional to the degree of autophosphorylation for a particular kinase (Figure 1, bottom panels), and suggesting a correlation with kinase activity. Control blots (Figure 1, middle panels) demonstrate that STAT expression was similar in all cases.

Some substrate preference is seen in Figure 1. Specifically, $p113^{STAT2}$ is phosphorylated more efficiently by JAK-2, while $p91^{STAT1}$ is phosphorylated more efficiently by JAK-1, even after normalizing for the relative kinase activity. In addition, the phosphorylated STAT proteins can form STAT1–STAT2 heterodimers (Gupta *et al.*, 1996), strongly implying that the STATs are phosphorylated on the physiologically relevant site. STAT phosphorylation in these co-infected cells is absolutely dependent on the presence of a functional SH2 domain, as arginine → lysine mutations in the critical residue presumed to be involved in SH2 phosphotyrosine binding ($R_{602}$ in STAT1 and $R_{601}$ in STAT2) abolished tyrosine phosphorylation by any of the JAK kinases (Gupta *et al.*, 1996). Further, JAK-1 is

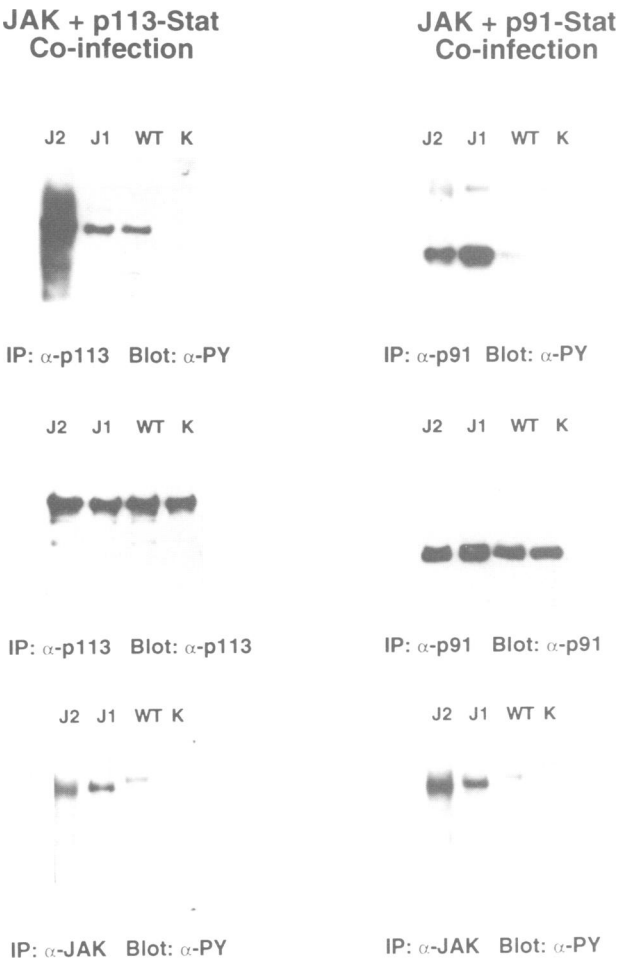


**Fig. 1.** Phosphorylation of STAT1 and STAT2 by JAK family kinases. Sf9 insect cells were co-infected, at high multiplicity, with a baculovirus encoding one of three JAK family kinases [either JAK-1 (J1), JAK-2 (J2), wild-type tyk2 (WT) or a kinase-deficient tyk2 containing a $K_{930}I$ mutation in the putative ATP binding site (K)], as indicated above each lane, plus a baculovirus encoding either the 91 kDa STAT1 (p91-STAT, panels on the right) or the 113 kDa STAT2 (p113-STAT, panels on the left) proteins. Two days later, cells lysates (corresponding to ~$6\times10^6$ infected cells) were prepared, immunoprecipitated with the antibodies indicated below each panel, subjected to SDS–PAGE and immunoblotted with the antibodies indicated below each panel.

co-immunoprecipitated by antibodies against both STAT1 and STAT2 from the co-infected cells (Figure 1, top panels).

Immunoprecipitates of baculovirus-encoded JAK kinases were also used to catalyze *in vitro* phosphorylation of glutathione-S-transferase (GST) fusion proteins containing the STAT1 and STAT2 SH2 domains and phosphorylation sites. Although activity was relatively weak, once again all three JAKs phosphorylated both STATs (data not shown). We conclude that in these experimental settings, the JAK kinases do not display the specificity observed *in vivo*, suggesting that under physiologic conditions another component of the signaling cascade is required.

### *IFNaR1 is phosphorylated in vitro at two sites by $p135^{tyk2}$*

To test the hypothesis that phosphorylated IFNaR1 is the component which acts to confer specificity in the IFNα

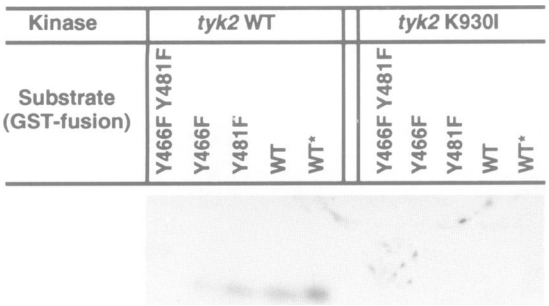


**Fig. 2.** *In vitro* phosphorylation of GST–IFNα receptor fusion proteins by the *tyk2* kinase. GST fusion proteins containing a portion of the cytoplasmic domain of IFNaR1 were partially purified by affinity chromatography on glutathione–agarose beads, eluted with excess glutathione and then used as a substrate in *in vitro* kinase assays (in the presence of [$\gamma$-$^{32}$P]ATP) catalyzed by anti-*tyk2* immunoprecipitates, prepared from ~$1\times10^7$ Sf9 cells previously infected with recombinant baculoviruses encoding either the wild-type *tyk2* kinase (*tyk2* WT, left panel) or a kinase-deficient form of the *tyk2* kinase (*tyk2* K930I, right panel). The fusion protein was recovered on glutathione–agarose beads, subjected to SDS–PAGE and autoradiographed. The GST fusion labeled WT* contains residues 465–511 of the IFNaR1, while all of the other fusions contain residues 462–486. The latter set of fusions encode the wild-type sequence (WT) or mutant versions containing tyrosine (Y) to phenylalanine (F) mutations in the indicated residue(s).

pathway, we first sought to identify the tyrosine residues which are phosphorylated on this protein. Previously, we showed that three different JAK family kinases could phosphorylate *in vitro* a GST fusion protein containing the cytoplasmic portion of the IFNaR1 protein (Colamonici *et al.*, 1994a). Only the juxtamembrane half of this domain was phosphorylated, suggesting specificity with respect to the receptor protein. Since this half of the protein contains two tyrosines ($Y_{466}$ and $Y_{481}$), we employed the same *in vitro* kinase assay to determine if one or both sites are phosphorylated. Site-specific mutagenesis was used to create tyrosine → phenylalanine mutations at each site, as well as the double mutant. Figure 2 shows that both GST fusions containing single tyrosine mutations are phosphorylated *in vitro* by immunoprecipitates from Sf9 cells overexpressing the wild-type, but not a kinase-inactive, form of the p135$^{tyk2}$ kinase. The $Y_{466}$ site appears to be more extensively phosphorylated than the $Y_{481}$ site. As expected, the double mutant is not phosphorylated at all, indicating that the kinase activity is specific for receptor sequences within the fusion protein. Similar amounts of fusion protein were detected in each lane; in addition, the level of tyk2 autokinase activity was similar in all samples incubated with the wild-type kinase (data not shown).

### A phosphopeptide containing the IFNaR1 sequence surrounding Tyr466 binds STAT2

To determine if the phosphorylated version of either $Y_{466}$ or $Y_{481}$ can act as a docking site for a STAT protein, we synthesized biotinylated phosphopeptides corresponding to the sequences surrounding these two sites. These peptides, along with a set of control phosphopeptides, were bound to strepavidin beads and then incubated with insect cell lysates containing either STAT1 or STAT2 protein. The washed complexes were immunoblotted with the appropriate anti-STAT antibodies. Figure 3A demonstrates that STAT2 binds strongly and specifically to the phosphopeptide corresponding to the region surrounding $Y_{466}$. STAT1 does not bind to this peptide, but does bind to a phosphopeptide corresponding to the $Y_{440}$ of the IFNγ receptor α-subunit, as shown previously (Greenlund *et al.*, 1994).

We observed weak binding of p113$^{STAT2}$ to the phosphopeptide corresponding to the region surrounding $Y_{481}$, as evidenced by the faint band in Figure 3A. This binding has been observed frequently in repeat experiments (data not shown), suggesting that it represents bone fide binding and not simply an artifact. In addition to the controls shown, phosphopeptides corresponding to the two tyrosines in the IFNaR1 subunit (positions 527 and 538) which were not phosphorylated *in vitro* (Colamonici *et al.*, 1994a), have been tested and failed to bind either p113$^{STAT2}$ or p91$^{STAT1}$ (data not shown). Finally, Figure 3B shows that the non-phosphorylated version of the peptide corresponding to the region surrounding $Y_{466}$ also failed to bind to p113$^{STAT2}$.

### The STAT2–IFNaR1 interaction requires a functional SH2 domain

To further test the hypothesis that p113$^{STAT2}$ binds to the phosphorylated $Y_{466}$ of the IFNaR1 via a phosphotyrosine–SH2 interaction, we produced GST fusion proteins containing either wild-type or mutant SH2 domains of the STAT1 $R_{602}K$ and STAT2 $R_{601}K$ proteins described above. Structural analysis of the src SH2 domain has demonstrated that the equivalent arginine residue is located in the phosphotyrosine binding pocket and interacts directly with the negatively charged oxygens on the phosphate moiety (Waksman *et al.*, 1993). Binding experiments similar to those described in Figure 3 were performed, substituting the GST fusion proteins for the recombinant baculoviral-produced STAT proteins. Figure 4A shows that the phosphopeptide corresponding to the region surrounding $Y_{466}$ binds to the wild-type STAT2 SH2 domain (lane labeled S2), but not the $R_{601}K$ mutant version (lane labeled S2*). As expected from the results in Figure 3, neither wild-type (S1) nor mutant (S1*) SH2 domains derived from the p91$^{STAT1}$ protein bind the IFNaR1 $Y_{466}$ phosphopeptide.

The findings illustrated in Figure 4A were confirmed with intact p113$^{STAT2}$, as seen in Figure 4B and C. In this case, baculoviral-encoded wild-type and $R_{601}K$ mutant p113$^{STAT2}$ proteins and the phosphopeptide corresponding to the sequences surrounding IFNaR1 $Y_{466}$ were employed in binding assays identical to those shown in Figure 3. As seen in Figure 4B, the site corresponding to $Y_{466}$ bound the wild-type, but not the mutant, form of STAT2. Figure 4C shows that similar amounts of wild-type and mutant protein were used in the binding assays.

### Tyrosine-phosphorylated STAT proteins do not bind receptor docking sites

Next, we sought to determine if the phosphorylated form of p113$^{STAT2}$ would also bind to the $Y_{466}$ phosphopeptide. We took advantage of the observation (Figure 1) that STAT proteins could be tyrosine-phosphorylated by any of the JAK kinases when Sf9 cells were co-infected with baculoviruses expressing these proteins. Lysates were prepared from cells infected with STAT-producing viruses

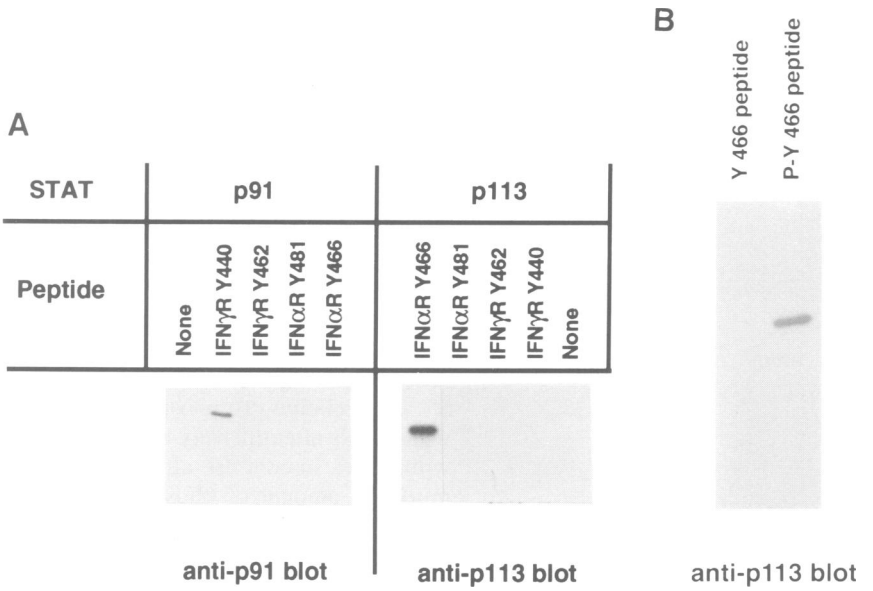


**Fig. 3.** $p113^{STAT2}$ binds a phosphopeptide corresponding to the sequence surrounding Tyr466 of IFNaR1. Biotinylated peptides were bound to strepavidin-linked beads, and then incubated with lysates derived from Sf9 cells infected with recombinant baculoviruses encoding either STAT1 (p91) or STAT2 (p113). The STAT–peptide-bead complexes were washed, subjected to SDS–PAGE, and then immunoblotted with appropriate anti-STAT antibodies, as indicated. (**A**) The peptides used are indicated (using the single letter amino acid code) as follows: IFNγR Y440, TSFGY[$PO_4$]DKPHVLV; IFNγR Y462, SLIGY[$PO_4$]RPTEDSKEFS; IFNαR Y466, RCINY[$PO_4$]VFFPSLKPSS; IFNαR Y481, SIDEY[$PO_4$]FSEQPLKNLL. The samples in the lanes marked 'None' were prepared by incubating strepavidin beads alone with the appropriate STAT lysates. (**B**) The peptides used are indicated: Y 466 peptide, RCINYVFFPSLKPSS (unphosphorylated peptide) and P-Y 466 peptide, RCINY[$PO_4$]VFFPSLKPSS [identical to IFNαR Y466 in (A)].

alone, or with STAT-producing viruses and a virus encoding the JAK-1 kinase. In some cases the various lysates were mixed together before carrying out the binding assays. The lysates and lysate mixtures were then subjected to the same binding assay shown in Figure 3, using either the $Y_{466}$ phosphopeptide derived from the IFNaR1 (IFNαR) or the $Y_{440}$ phosphopeptide derived from the IFNγR α-subunit (IFNγR). Figure 5A demonstrates that only reaction mixtures which contained unphosphorylated $p113^{STAT2}$ bound the IFNaR1 $Y_{466}$ phosphopeptide. The data in Figure 5B indicate that there is also no interaction between phosphorylated $p91^{STAT1}$ and its docking site on the IFNγRα.

The phosphorylated STAT protein preparations might fail to bind the appropriate phosphopeptide because the STAT SH2 domains are already bound to STAT phosphotyrosines, forming homodimers. In the case of the IFNγRα and STAT1, the avidity of the STAT1 homodimer has been shown to be greater than the avidity of STAT1 for IFNγRα (Greenlund *et al.*, 1995). Alternatively, the STATs may be bound, via SH2 domains, to JAK-1. This latter assertion is supported by the finding that some JAK-1 co-immunoprecipitates with $p113^{STAT2}$ from co-infected cell lysates (Figure 1, upper left panel), as well as the fact that the STAT phosphorylation observed in Figure 1 requires an intact SH2 domain (Gupta *et al.*, 1996). Interestingly, Figure 5 also shows that binding of the unphosphorylated STATs is not appreciably reduced by the presence of phosphorylated STAT protein, implying that the phosphorylated STAT proteins produced in this system form stable complexes, which do not disassociate at an appreciable rate, at least as measured under our binding conditions.

### *The IFNaR1 Tyr466 phosphopeptide inhibits IFNα-mediated activation of both STAT2 and STAT1*

To test the physiologic relevance of our findings, we permeabilized HeLa cells with streptolysin O (Ahnert-Hilger *et al.*, 1989) and introduced phosphopeptides corresponding to the sequences surrounding the four tyrosine residues in the IFNaR1 subunit or, as a control, a non-phosphorylated peptide corresponding to the $Y_{466}$ site. The permeabilized cells were treated with IFNα, and then assayed for STAT tyrosine phosphorylation and the formation of the characteristic electrophoretic mobility shift complex known as ISGF-3, which is formed from STAT1, STAT2 and a 48 kDa DNA binding protein in response to IFNα (Fu *et al.*, 1992; Schindler *et al.*, 1992a; Veals *et al.*, 1992). If the phosphopeptide corresponding to the putative docking site at $Y_{466}$ binds to the SH2 domain of latent STAT2 protein under these conditions, it should specifically block the access of $p113^{STAT2}$ to the receptor and prevent both tyrosine phosphorylation and DNA binding.

Following treatment, portions of each cell lysate were immunoprecipitated separately with anti-STAT2 (anti-p113 IP) and anti-STAT1 (anti-p91 IP) antibodies and then immunoblotted with an anti-phosphotyrosine antibody (anti-PY blot). Figure 6A and B (lanes 6 versus 7, top portions) demonstrates that, as expected (Schindler *et al.*, 1992b), IFNα treatment of mock-permeabilized cells induces tyrosine phosphorylation of both $p113^{STAT2}$ and $p91^{STAT1}$. The detection of co-immunoprecipitating phosphorylated STAT1 or STAT2 indicates that the STATs form a heterodimeric complex, again as previously observed (Schindler *et al.*, 1992b). Introduction of the $Y_{466}$ phosphopeptide into permeabilized cells before IFNα treatment

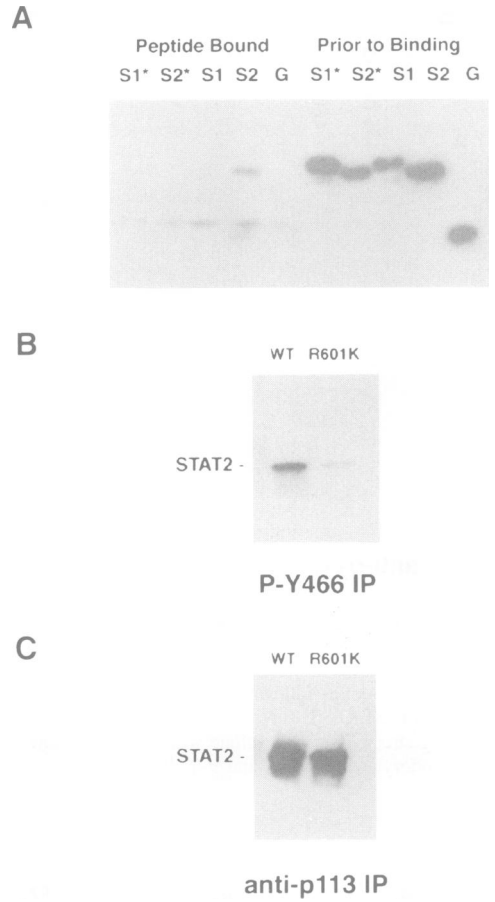


**Fig. 4.** Binding of STAT2 to Tyr466 of IFNaR1 requires an intact SH2 domain. (**A**) Left side: GST or GST fusion proteins containing the SH2 domain of wild-type $p113^{STAT2}$ (S2), wild-type $p91^{STAT1}$ (S1) or versions containing an arginine → lysine point mutation (S2* and S1*, respectively) were partially purified by affinity chromatography on glutathione–agarose beads, eluted with excess glutathione and then incubated with a strepavidin-bound biotinylated phosphopeptide (RCINY[$PO_4$]-VFFPSLKPSS) corresponding to the region surrounding $Y_{466}$ of the IFNaR1 subunit. After washing, the complexes were subjected to SDS–PAGE, and then immunoblotted with an anti-GST antibody. Right side: identical aliquots of either GST or the indicated GST fusion proteins used in the left panel were directly subjected to SDS–PAGE, and immunoblotted with an anti-GST antibody. (**B**) Binding experiments were performed as in Figure 3, using the $Y_{466}$ phosphopeptide to precipitate lysates from cells infected with baculoviruses encoding either wild-type (STAT2 WT) or mutant (STAT2 R601K) $p113^{STAT2}$ followed by immunoblotting with anti-STAT2 antibodies. (**C**) Aliquots from the lysates used in (B) were immunoprecipitated and immunoblotted with anti-STAT2 antibodies.

significantly reduces the IFNα-induced phosphorylation of both $p113^{STAT2}$ and $p91^{STAT1}$, while the non-phosphorylated version of the same peptide had no effect. The phosphorylated peptides corresponding to the other tyrosine residues on IFNaR1 were also ineffective in attenuating the STAT phosphotyrosine signals. In replicate experiments we have sometimes observed weak inhibition by the $Y_{481}$ phosphopeptide (data not shown), consistent with its weak ability to bind STAT2 (see Figure 3). When the filters used for anti-phosphotyrosine blotting were stripped and re-probed with appropriate anti-STAT antibodies, signals of approximately equal intensity were detected in all lanes (Figure 6A and B, bottom portion). In addition, the $Y_{466}$ phosphopeptide did not inhibit IFNα-induced tyrosine phosphorylation of tyk2 (data not shown), consistent with the notion that the STAT docking events occur downstream of the JAK kinase activation events.

The ability of a STAT2 binding peptide to inhibit phosphorylation of both STATs confirms recent genetic studies indicating that in the IFNα pathway STAT phosphorylation occurs sequentially (Leung *et al.*, 1995). Figure 6 shows that less than an equimolar amount of phosphorylated STAT1 is recovered from STAT2 immunoprecipitates (compare STAT1 and STAT2 in Figure 6A). In contrast, STAT1 immunoprecipitates contain an approximately equimolar amount of phosphorylated STAT2 (Figure 6B). This observation suggests that an excess of phosphorylated STAT2, relative to phosphorylated STAT1, is present within cells after this very brief (3 min) period of treatment with IFNα. In contrast, after longer treatment periods (15 min), the amount of phosphorylated STAT1 and STAT2 are approximately equal (see, for example, lane 2 in Figure 7).

Inhibiting the phosphorylation of both STATs should block formation of the ISGF-3 gel shift complex (Levy *et al.*, 1989), which has been shown to contain phosphorylated $p91^{STAT1}$ and $p113^{STAT2}$ (Fu *et al.*, 1992; Schindler *et al.*, 1992a). Figure 6C shows that this is indeed the case. Aliquots of the same lysates used in Figure 6A and B were incubated with a radiolabeled ISRE probe and subjected to electrophoretic mobility shift analysis (EMSA). Lysates from the cells incubated with the $Y_{466}$ IFNaR1 phosphopeptide did not induce a detectable shift of the ISRE probe, while lysates from cells incubated with phosphopeptides corresponding to the other tyrosine residues on IFNaR1, or the unphosphorylated $Y_{466}$ peptide, did.

### *Overexpression of IFNaR1 $Y_{466}F$ mutants block IFNα-dependent STAT phosphorylation*

The data in Figures 3–6 depend on phosphopeptide–STAT2 binding to support our proposal that phosphorylated $Y_{466}$ is a docking site for STAT2, and forms an obligatory intermediate required for STAT2 activation. To confirm these binding experiments independently, we constructed tyrosine → phenylalanine mutants of epitope-tagged versions of the IFNaR1 subunit, and tested the ability of these mutant proteins to block STAT tyrosine phosphorylation. Specifically, the wild-type, $Y_{466}F$, $Y_{481}F$ and $Y_{466}FY_{481}F$ versions of the IFNaR1 gene were transfected into 293T cells (Pear *et al.*, 1993). Two days later, the transfected cultures were divided into two parts and one-half of the cells were treated with IFNα. Cell lysates were prepared, immunoprecipitated with antibody against STAT2 and then immunoblotted with an anti-phosphotyrosine antibody.

Figure 7A shows that in cells transfected with the wild-type IFNaR1 gene, IFNα induced tyrosine phosphorylation of STAT1 and STAT2 (lanes 1 and 2). A similar degree of STAT phosphorylation is observed in untransfected cells (data not shown). However, when cells were transfected with either the $Y_{466}F$ mutant (lanes 3 and 4) or the double mutant (lanes 5 and 6), the extent of IFNα-dependent STAT phosphorylation is significantly reduced. The double mutant appears to be somewhat more effective than the $Y_{466}F$ single mutant, as evidenced by the faint STAT2 band visible in lane 4 of panel A. The $Y_{481}F$ mutant has a slight effect relative to the wild-type (lanes

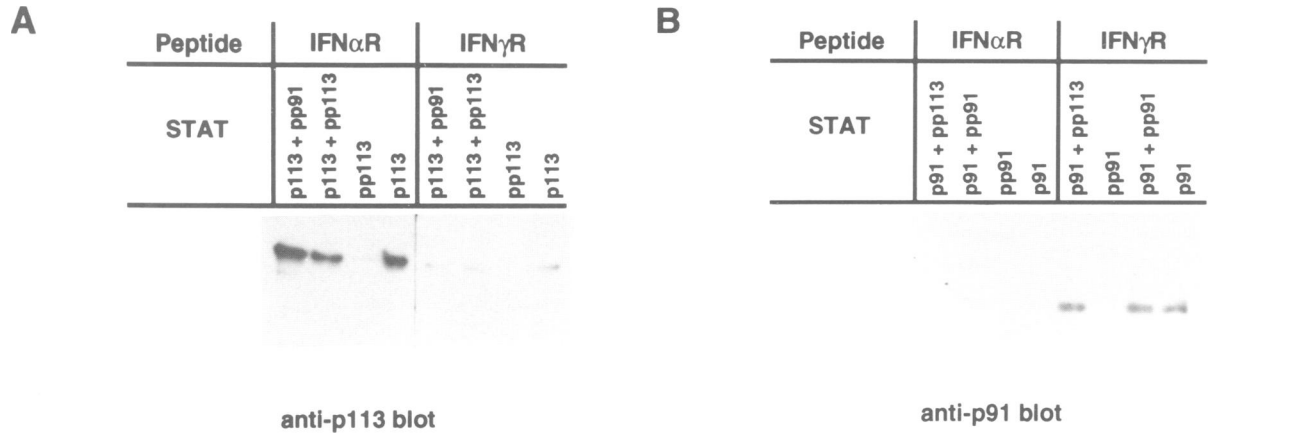


**Fig. 5.** Phosphorylated STAT proteins do not bind to phosphopeptides corresponding to receptor docking sites. Sf9 insect cells were infected with recombinant baculoviruses encoding either $p113^{STAT2}$ or $p91^{STAT1}$ or, alternatively, co-infected with one or the other of these STAT baculoviruses and a baculovirus encoding the JAK-1 kinase, producing phosphorylated STAT protein. Lysates from the infected cells, or mixtures of lysates from separately infected cells, were used in an *in vitro* binding assay as outlined in Figure 3. (**A**) Lysates or mixtures of lysates containing various STAT proteins are indicated as follows: p113 + pp91, a mixture of a lysate obtained from Sf9 cells infected with $p113^{STAT2}$ alone and a lysate obtained from cells infected with both $p91^{STAT1}$ and JAK-1; p113 + pp113, a mixture of a lysate obtained from Sf9 cells infected with $p113^{STAT2}$ alone and a lysate obtained from cells infected with both $p113^{STAT2}$ and JAK-1; pp113, a lysate obtained from Sf9 cells infected with both $p113^{STAT2}$ and JAK-1; p113, a lysate obtained from Sf9 cells infected with $p113^{STAT2}$ alone. The indicated STAT lysate, or mixture, was incubated with a either a phosphopeptide (RCINY[$PO_4$]VFFPSLKPSS) corresponding to the region surrounding $Y_{466}$ of the IFNaR1 subunit (IFNαR), or a phosphopeptide (TSFGY[$PO_4$]DKPHVLV) corresponding to the region surrounding $Y_{440}$ of the IFNγRα subunit (IFNγR), subjected to SDS–PAGE, and then immunoblotted with an anti-STAT2 antibody. (**B**) Lysates or mixtures of lysates containing various STAT proteins are indicated as follows: p91 + pp113, a mixture of a lysate obtained from Sf9 cells infected with $p91^{STAT1}$ alone and a lysate obtained from cells infected with both $p113^{STAT2}$ and JAK-1; p91 + pp91, a mixture of a lysate obtained from Sf9 cells infected with $p91^{STAT1}$ alone and a lysate obtained from cells infected with both $p91^{STAT1}$ and JAK-1; pp91, a lysate obtained from Sf9 cells infected with both $p91^{STAT1}$ and JAK-1; p91, a lysate obtained from Sf9 cells infected with $p91^{STAT1}$ alone. The indicated STAT lysate, or mixture, was incubated with the same two peptides used in (A), subjected to SDS–PAGE, and then immunoblotted with an anti-STAT1 antibody.

7 and 8). These latter two observations are in agreement with the fact that the $Y_{481}$ phosphopeptide can bind STAT2 weakly. Control blots demonstrate that equal amounts of STAT2 were present on the original filter (Figure 7B) and that equal amounts of epitope-tagged receptor protein were expressed in the transfected cells (Figure 7C). Thus, overexpression of receptor constructs which cannot be phosphorylated at the STAT2 binding site inhibits STAT phosphorylation.

## Discussion

With the recent identification of the major components which constitute the interferon-α signaling cascade, interest has focused on understanding the detailed molecular interactions among three types of proteins: receptors, JAK family kinases and STAT family transcription factors. Similar pathways are triggered by many cytokines, suggesting a critical role for these families of molecules in controlling normal growth and development and in mediating a wide spectrum of human disease. In particular, a major unsolved question concerns the mechanism of STAT phosphorylation. The recent discovery that the IFNγRα subunit contains a critical tyrosine residue which, when phosphorylated, binds STAT1, encouraged us to look for a similar STAT2 docking site on the phosphorylated IFNaR1 subunit.

The data presented here demonstrate that the JAK family kinases phosphorylate STATs in a relatively non-specific fashion (Figure 1). We investigated the hypothesis that the IFNα receptor acts to mediate specific JAK–STAT interactions, and found that IFNaR1 $Y_{466}$, which is the major site of *in vitro* phosphorylation by the $p135^{tyk2}$ kinase (Figure 2), is a STAT2 binding site. Importantly, this STAT2–IFNaR1 interaction has all the hallmarks of a phosphotyrosine–SH2 type interaction. Specifically, only phosphorylated peptides mediate the binding (Figure 3), and an intact, functional SH2 domain is both necessary and sufficient for binding (Figure 4). Since it is unlikely that the baculovirus-infected insect cell lysates contain other proteins involved in the IFNα signaling pathway, our data strongly imply a direct interaction between phosphorylated $Y_{466}$ and $p113^{STAT2}$.

Preparations of STAT2 which have been phosphorylated by co-infecting insect cells with baculoviruses encoding STAT2 and JAK-1 fail to bind to the receptor docking site (Figure 5). In all likelihood, this *in vivo* phosphorylation of STAT2 leads to the formation of either STAT2 homodimers, or other STAT SH2–phosphotyrosine complexes, which prevent the STAT2 SH2 domain from binding to the $Y_{466}$ phosphorylated peptide. This may mimic the physiologic situation, suggesting that STAT1–STAT2 heterodimers are more stable than the STAT–IFNaR1 complex. The formation of STAT dimers might drive the release of STAT complexes from the receptor docking site. Thus, these data are the first demonstration of direct binding between these two molecules, and the first demonstration that such binding requires a functional SH2 domain as well as the latent, non-phosphorylated version of $p113^{STAT2}$.

Additionally, the $Y_{466}$ phosphopeptide efficiently blocks the phosphorylation of STAT1 and STAT2 as well as ISGF-3 formation *in vivo* (Figure 6), suggesting that the molecular interactions we have observed *in vitro* are

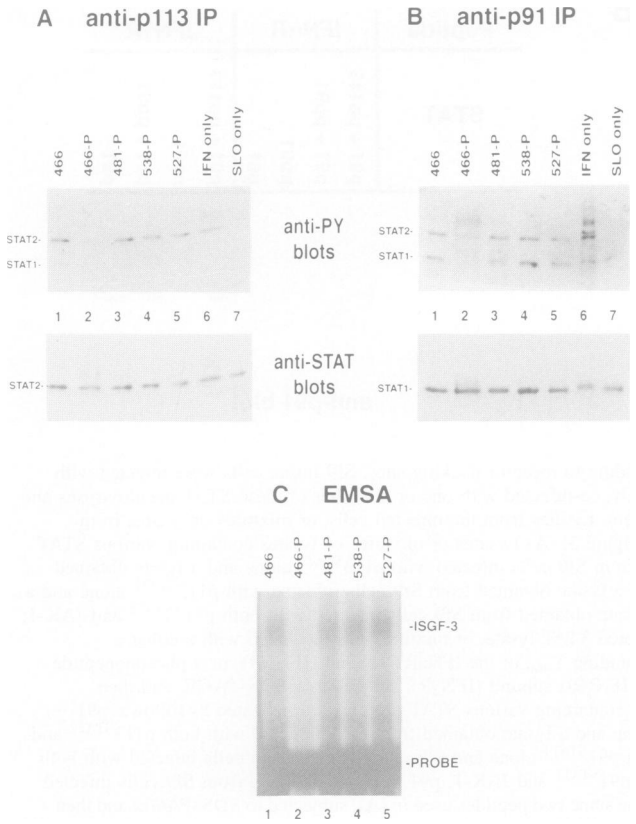


**Fig. 6.** The IFNaR1 $Y_{466}$ phosphopeptide inhibits STAT activation in permeabilized HeLa cells. Aliquots of HeLa cells were permeabilized with streptolysin O, incubated in the presence of the specified peptide (lanes 1–6 only) and then either treated with IFNα (lanes 1–5) or left untreated (lane 7, marked 'SLO only'). Lysates were prepared, and divided into three portions. (**A** and **B**) Two equal-sized aliquots (corresponding to ~$3\times10^6$ cells) were immunoprecipitated separately with either anti-STAT2 (anti-p113 IP)(A) or anti-STAT1 (anti-p91 IP) (B) antibodies and then subjected to SDS–PAGE and immunoblotting with anti-phosphotyrosine antibody [upper portion of both (A) and (B)]. The filters were stripped and re-probed with the appropriate anti-STAT antibodies [anti-STAT2 for (A) and anti-STAT1 for (B)]. These exposures are shown in the bottom portion of each panel. The lanes are derived from samples containing the following peptides: lane 1 (466), RCINYVFFPSLKPSS (unphosphorylated peptide); lane 2 (466-P), RCINY[$PO_4$]VFFPSLKPSS; lane 3 (481-P), SIDEY[$PO_4$]-FSEQPLKNLL; lane 4 (538-P), DSENY[$PO_4$]SNEDESE; lane 5 (527-P), HDKKY[$PO_4$]SSQTSQD. Samples corresponding to lanes 6 and 7 did not contain peptide. The position of the STAT1 and STAT2 proteins are identified for each blot. The slight difference in migration of the STAT bands in lane 6 of the upper portion of (B) resulted from loading a larger volume in this particular lane. The origin of the apparently artifactual slower migrating bands in this same lane is unknown. (**C**) The remaining aliquot of the lysate from the samples corresponding to lanes 1–5 was incubated with a radiolabeled double-stranded IFNα-stimulated gene response element (ISRE) DNA probe, subjected to non-denaturing SDS–PAGE and autoradiographed. The lanes are labeled and numbered as in (A) and (B). The position of ISGF-3 and the DNA probe are indicated.

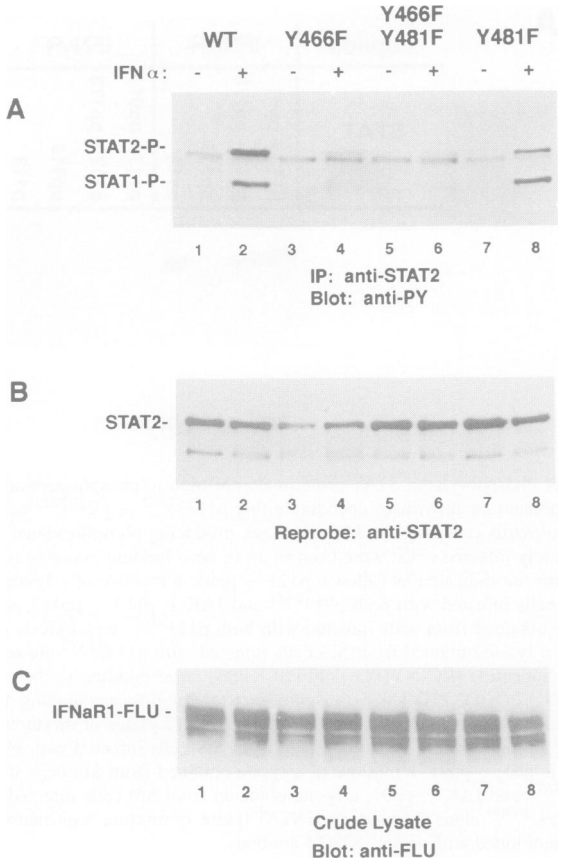


**Fig. 7.** Tyrosine → phenylalanine IFNaR1 mutants inhibit IFNα-dependent tyrosine phosphorylation of STAT1 and STAT2. Approximately $3\times10^7$ 293T cells were transfected with the indicated constructs, and then either left untreated (odd-numbered lanes), or treated with 5000 U/ml of IFNα2 for 15 min (even-numbered lanes). (**A**) Lysates were prepared, and most of the lysate was immunoprecipitated with an anti-STAT2 antibody and then subjected to SDS–PAGE and immunoblotting with an anti-phosphotyrosine antibody (anti-PY). The blot shows protein from cells transfected with the wild-type (WT) construct in lanes 1 and 2; the Tyr466 mutant (Y466F) construct in lanes 3 and 4; the Tyr481 mutant (Y481F) in lanes 5 and 6; and the Tyr466/481 double mutant (Y466F Y481F) in lanes 7 and 8. The band which migrates slightly faster than STAT2 and is present in all lanes is apparently artifactual. (**B**) The filter in (A) was stripped and reprobed with an anti-STAT2 antibody. The lower molecular weight band seen in this panel is artifactual. (**C**) A small portion of the crude lysate was subjected directly to SDS–PAGE and immunoblotting with an anti-HA epitope antibody (anti-FLU). The lanes correspond to those on the gel depicted in (A). The positions of STAT2 and epitope-tagged IFNaR1 (IFNaR1-FLU) are indicated.

physiologically relevant to this signaling pathway. Our observation that this phosphopeptide blocks the tyrosine phosphorylation of both STATs independently confirms the recent finding that STAT1 is not phosphorylated in response to IFNα in mutant cell lines which are deficient in STAT2 (Leung *et al.*, 1995), and is consistent with the suggestion by these authors that phosphorylated STAT2 may be acting as a docking site for STAT1. These data are also consistent with the idea that the phosphorylated IFNaR1–STAT2–STAT1 trimeric complex subsequently isomerizes to release a STAT1–STAT2 heterodimer containing two reciprocal SH2–phosphotyrosine contacts. As previously noted (Leung *et al.*, 1995), this mechanism ensures the exclusive formation of the heterodimeric STAT complex in response to IFNα. Finally, mutant versions of the IFNaR1 subunit in which Tyr466 has been changed to phenylalanine block IFNα-dependent tyrosine phosphorylation of STAT2 (Figure 7). It is likely that this effect involves a dominant negative mechanism in which the exogenous mutant version of IFNaR1 displaces the endogenous wild-type version from the functional receptor complex and thereby prevents $Y_{466}$ phosphorylation and subsequent STAT binding. This complements the peptide

binding studies by employing a distinct experimental methodology to demonstrate that $Y_{466}$ is required for STAT phosphorylation, the critical event in IFNα signaling.

Most importantly, our data directly identify the region surrounding Tyr466 as the STAT2 binding site on the IFNaR1 subunit. Combined with our previous identification of a tyk2 binding site on IFNaR1 (Colamonici *et al.*, 1994a,b), it is now clear that the cytoplasmic domain of this subunit of the receptor serves a critical role in mediating the IFNα signal transduction pathway, by acting as a multi-purpose docking site. Our prior studies demonstrated that the tyk2 binding site spans the juxtamembrane half of the IFNaR1 cytoplasmic domain (residues 465–511) (Colamonici *et al.*, 1994a). More recent analyses (H.Yan, J.T.E.Lim and J.J.Krolewski, unpublished results) indicate that the critical residues are located between amino acids 479 and 511. Assuming that the STAT2 binding site, like other SH2 binding sites, involves only a few residues C-terminal to the tyrosine, then our data would place the STAT2 and tyk2 contact sites only about 10 residues apart along the primary sequence, suggesting the possibility of an intimate interaction between enzyme and substrates. Presumably, a complex of four closely apposed, tyrosine-phosphorylated proteins (IFNaR1, tyk2, STAT1 and STAT2) exists transiently, just before the formation of the STAT heterodimer, which ultimately imparts specificity to this signaling pathway. However, identification of the IFNaR1 binding site on tyk2, as well as detailed structural studies will be required to obtain a comprehensive picture of these molecular interactions.

With the present report, two STAT2 SH2 binding sites are now known: IFNaR1 $Y_{466}$ and STAT1 $Y_{701}$ (Schindler *et al.*, 1992b; Gupta *et al.*, 1996). The $Y_{466}$ site (YVFFP) is quite similar to those bound by group 3 SH2 domains, as it contains hydrophobic residues in the positions C-terminal to the tyrosine residue (Songyang *et al.*, 1993). The $Y_{701}$ site (YIKTE) is not closely related, although it does contain a hydrophobic residue adjacent to the tyrosine. Thus, we cannot establish a consensus STAT2 SH2 binding site. The lack of similarity between the two sites also suggests that the affinity of the STAT2 SH2 domain will be different for the phosphorylated versions of these two sites. In fact, a recent study characterizing the interaction of $p91^{STAT1}$ with a completely analogous pair of binding sites, on the IFNγRα and on STAT1 itself, has demonstrated a significantly higher affinity of the STAT1 SH2 domain for the receptor site, compared with the STAT1 $Y_{701}$ site (Greenlund *et al.*, 1995). However, the STAT1 homodimer, held together by two SH2–phosphotyrosine interactions, is more stable than the STAT1–IFNγ complex (Greenlund *et al.*, 1995). We propose that a similar scenario exists for STAT2 and IFNaR1.

Our data complement and extend recent reports which have utilized chimeric molecules to probe the mechanism of STAT phosphorylation. In one study (Stahl *et al.*, 1995), mutation of critical tyrosine residues on chimeric receptor molecules effectively abolished STAT phosphorylation. Furthermore, a five amino acid motif surrounding such a tyrosine could function as a modular STAT binding site, when appended to a receptor which was otherwise unable to mediate STAT activation. Both structural studies (Waksman *et al.*, 1993) and phosphopeptide binding studies (Songyang *et al.*, 1993, 1994) indicate that SH2

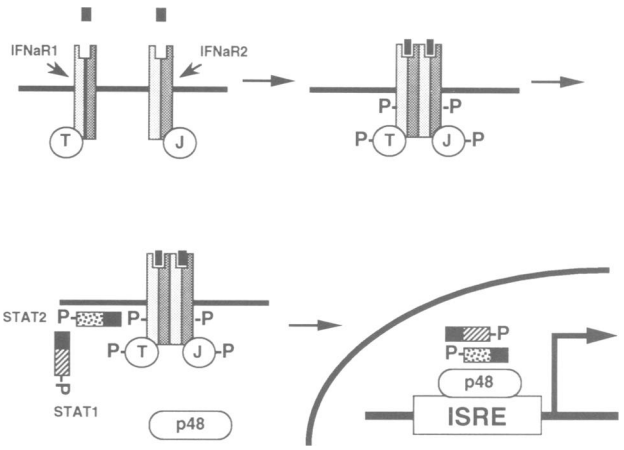


**Fig. 8.** Model for the IFNα signaling pathway. In the proposed model, ligand binding results in the formation of a heterodimeric receptor–JAK complex containing two transmembrane ligand binding subunits (IFNaR1 and IFNaR2, also known as the α- and β-subunits, respectively) and the tyk2 (T) and JAK-1 (J) tyrosine kinases. Although the diagram depicts two receptors forming a simple heterodimer, the stoichiometry of the complex is unknown, and may include additional receptor molecules. Subsequently the JAK family kinases are activated, *trans*-phosphorylate each other, and then phosphorylate the receptor subunits. Phosphorylation of IFNaR1 on Tyr466 recruits latent (unphosphorylated) STAT2 to the receptor, where it then presumably becomes a substrate for one or the other of the JAK kinases. The phosphorylated STAT2, bound to IFNaR1, recruits latent STAT1, which is also presumed to be subsequently phosphorylated by one of the JAKs. The cytoplasmic domain of IFNaR2 functions to bind JAK-1, but it is unclear what function, if any, tyrosine phosphorylation of this subunit serves. The STAT1–STAT2 heterodimer containing a single SH2–phosphotyrosine contact is released, isomerizes into a form containing two SH2–phosphotyrosine contacts and then moves to the nucleus, along with p48, where it binds to and activates the IFNα-stimulated gene response element (ISRE) upstream of IFNα-regulated genes. Our model differs from that previously proposed by Leung *et al.* (1995) mainly in that we have now identified the major STAT2 binding site as $Y_{466}$ on the IFNaR1 subunit.

domains bind to similar short motifs surrounding phosphorylated tyrosine residues. These chimeric receptor experiments convincingly implicate receptor tyrosines in the process of selective STAT phosphorylation, but do not eliminate the possibility that an intervening adaptor molecule is involved. In a second study (Heim *et al.*, 1995), a STAT1-derived SH2 domain conferred IFNγ responsiveness to STAT2, when this chimera was introduced into a STAT1-deficient cell line. This indicated that STAT specificity is determined by an SH2–phosphotyrosine interaction; presumably, in this case with the site previously identified on the IFNγRα. In contrast, a STAT1 chimera containing a STAT2 SH2 domain was not activated by IFNα when the construct was introduced into STAT2-deficient cells. The reasons for this seemingly contradictory observation are unknown; however, unpublished data cited by Heim *et al.* (1995) indicate that the SH2 domain is required for STAT2 function since $R_{601}K$ mutants, like those used in Figure 4, abrogate the ability of STAT2 constructs to complement STAT2-deficient cells. Thus, our results confirm this observation by providing direct documentation that the SH2 domain is required for the docking of $p113^{STAT2}$ to the IFNα receptor.

Figure 8 summarizes our current model for the IFNα signaling pathway. Similar models have been proposed

for other cytokines (Hou *et al.*, 1994; Greenlund *et al.*, 1995; Pawson, 1995), and for IFNα (Leung *et al.*, 1995). The legend describes the sequence of molecular events believed to culminate in the stimulation of IFNα-dependent gene transcription. As reviewed in the Introduction, multiple lines of experimentation have demonstrated that the three sets of components (receptors, kinases and STATs) illustrated are required for signaling, although it is not certain that these components are entirely sufficient. For example, it has not been demonstrated that the two known receptor subunits will fully reconstitute signaling. Nonetheless, a major interest in this signaling pathway now lies in verifying that the critical protein–protein interactions predicted by the model do indeed occur in response to the binding of IFNα.

One of these interactions—stable and direct binding of JAK family kinases to receptor subunits—has been unequivocally demonstrated for IFNaR1 and tyk2, as outlined above. Co-immunoprecipitation of IFNaR2 and JAK-1 (Novick *et al.*, 1994) suggests that these two molecules interact similarly, although definitive evidence is still lacking. It is also not known precisely how the receptors become phosphorylated. Genetic evidence indicates that tyk2 and JAK-1 *trans*-phosphorylate each other (Müller *et al.*, 1993a), and it is believed that this event activates both kinases. Our *in vitro* data suggest that for IFNaR1 either JAK kinase will suffice (Colamonici *et al.*, 1994a) and that $Y_{466}$ is the major site of phosphorylation. Recently, we have observed that dimerization of IFNaR1 activates the tyk2 kinase and induces the phosphorylation of $Y_{466}$, suggesting that tyk2 is the *in vivo* IFNaR1 kinase and that the STAT2 binding site is phosphorylated *in vivo* (H.Yan, J.T.E.Lim and J.J.Krolewski, unpublished results). The last interaction in the proposed signal pathway sequence, STAT–STAT SH2–phosphotyrosine-dependent dimerization, is also well characterized. It has been previously shown that $p91^{STAT1}$ forms an SH2-dependent homodimer in response to IFNγ (Shuai *et al.*, 1994). Recent studies have now shown that the IFNα-dependent STAT1–STAT2 heterodimer is formed in a similar manner (Gupta *et al.*, 1996).

This report strongly supports a key sequence of interactions proposed to occur between the two steps outlined above: (i) phosphorylation of $Y_{466}$; (ii) SH2-dependent binding of STAT2 to $Y_{466}$; (iii) subsequent phosphorylation of STAT2; and (iv) recruitment of the SH2 domain of STAT1 to form a trimeric complex. Furthermore, our findings support the prediction that phosphorylation of STAT1 drives the concerted heterodimerization and release of the STAT1–STAT2 complex from the receptor. The identity of the tyrosine kinase responsible for phosphorylating either STAT remains unknown. The co-localization of the STAT and tyk2 binding sites on IFNaR1 hints that tyk2 is the STAT kinase, but it is certainly possible that JAK-1, or an as yet unidentified kinase serves this role, with or without the assistance of tyk2. Additionally, the finding that the STAT phosphorylation observed in the experiments depicted in Figure 1 is SH2-dependent could indicate a role for SH2-mediated JAK–STAT interactions in this pathway (Gupta *et al.*, 1996). Detailed structural studies, along with functional studies aimed at reconstituting the relevant phosphorylation events, will be required to further delineate the precise mechanism of STAT phosphorylation.

## Materials and methods

### Antibodies

Polyclonal rabbit antisera against tyk2 (Colamonici *et al.*, 1994b) and STAT1 and STAT2 (Schindler *et al.*, 1992b) have been described previously. Monoclonal antibodies against GST (Santa Cruz Biotechnology, Inc.) and phosphotyrosine (Upstate Biotechnology, Inc.) were obtained commercially. Polyclonal rabbit antisera against JAK-1 and JAK-2 were a gift of James Ihle and Fred Quelle (St Jude's Children's Hospital, Memphis, TN) and the monoclonal antibody against the influenza HA epitope was a gift of Jan Kitajewski (Columbia University, College of Physicians and Surgeons).

### Peptides

Peptides were synthesized as described previously (Greenlund *et al.*, 1995). The following biotinylated peptides were used in both the binding assays and permeabilized cell assays: unphosphorylated IFNaR1 $Y_{466}$, RCINYVFFPSLKPSS; phosphorylated IFNaR1 $Y_{466}$, RCINY-[$PO_4$]VFFPSLKPSS; phosphorylated IFNaR1 $Y_{481}$, SIDEY[$PO_4$]FSEQ-PLKNLL; phosphorylated IFNaR1 $Y_{527}$, HDKKY[$PO_4$]SSQTSQD; phosphorylated IFNaR1 $Y_{538}$, DSENY[$PO_4$]SNEDESE; phosphorylated IFNγRα $Y_{440}$, TSFGY[$PO_4$]DKPHVLV; phosphorylated IFNγRα $Y_{462}$, SLIGY[$PO_4$]RPTEDSKEFS.

### DNA constructs

To prepare baculoviruses encoding wild-type STAT proteins, we first used PCR to modify the 5′ end of the $p91^{STAT1}$ cDNA (Schindler *et al.*, 1992a). A novel fragment containing a *Bam*HI site 9 bp upstream of the ATG and extending to the unique *Nco*I site was amplified and substituted for the original *Bam*HI–*Nco*I fragment. Next, the original plasmid containing the $p113^{STAT2}$ cDNA (Fu *et al.*, 1992) and the modified $p91^{STAT1}$ cDNA were digested with *Eco*RI or *Not*I–*Eco*RV, respectively, and ligated into compatible sites in the pVL1393 baculovirus shuttle vector (InVitrogen). Plasmids encoding STAT proteins with inactivating mutations in the SH2 domain (STAT1 $R_{602}K$ and STAT2 $R_{601}K$) were prepared by substituting restriction fragments encoding the appropriate mutant residue into the wild-type STAT cDNAs, and then cloning these mutant cDNAs into pVL1393, as described above. The mutant restriction fragments (*Stu*I–*Hin*dIII and *Hin*dIII–*Sma*I, from the STAT2 and STAT1 cDNAs, respectively) were derived from plasmids constructed as described elsewhere (Gupta *et al.*, 1996).

The DNAs encoding GST–STAT SH2 fusion proteins (spanning amino acids 569–710 for STAT1 and 567–697 for STAT2) were prepared by PCR, incorporating flanking *Bam*HI restriction sites for subsequent cloning into pGEX-3X (Pharmacia). The STAT cDNAs described above were used as templates. The DNAs encoding GST–IFNaR1 fusion proteins (spanning either amino acids 465–511 or 462–486) were prepared by PCR, incorporating flanking *Bam*HI and *Eco*RI restriction sites for subsequent cloning into pGEX-3. An IFNaR1 cDNA was used as a template (Uzé *et al.*, 1990). Phenylalanine mutations in the IFNaR1–GST fusion proteins were introduced by point mutation in the appropriate PCR primer(s).

To prepare full-length epitope-tagged versions of the IFNaR1 gene (Uzé *et al.*, 1990), we first modified the 3′ end of the gene to incorporate a single copy of the influenza HA epitope (Kolodziej and Young, 1991) at the extreme C-terminus. PCR was employed, using a 5′ primer within the gene and a 3′ primer encoding the C-terminus, the epitope sequence, a new termination codon and an *Xho*I site. This product was cloned and then excised as an *Xmn*I–*Xho*I fragment and substituted into the IFNaR1 gene construct. To create the various tyrosine to phenylalanine mutations, single step or overlap (Horton *et al.*, 1990) PCR was used to introduce point mutations into a fragment covering the region between the *Nsi*I and *Mfe*I sites of the IFNaR1 cDNA. Again, the products were cloned and transferred back into the full-length gene. The resulting constructs were cloned into the *Eco*RI site of the pMT2T eukaryotic expression vector (Sambrook *et al.*, 1989).

Oligonucleotides were obtained from Ransom Hill Biosciences. Sequencing of the PCR fragments, as well as the mutant restriction fragments used to construct the STAT SH2 mutants, using a Sequenase™ kit (USB), and appropriate oligonucleotide primers, confirmed that these DNAs contained only the appropriate mutation.

### Cell culture and transfection

Sf9 cell culture and baculovirus infection was performed as described (O'Reilly *et al.*, 1992; Colamonici *et al.*, 1994b). To generate baculoviruses encoding STAT proteins, the shuttle vectors described above were co-transfected with a mutant (BaculoGold™) version of the AcNPV baculovirus strain (PharMingen), as previously described (Colamonici *et al.*, 1994b). In the case of co-infection, each virus was added at an m.o.i. of 5–10. HeLa S3 cells were grown as described (Colamonici *et al.*, 1994b). The 293T cell line was grown and transfected as previously described (Davis *et al.*, 1995).

### Cell permeabilization

HeLa S3 cells were permeabilized with streptolysin O (Murex Diagnostics) (Ahnert-Hilger *et al.*, 1989). In brief, $1\times10^7$ cells were pelleted and resuspended in phosphate-buffered saline. Streptolysin O was added to a final concentration of 0.6 U/ml, the cells were incubated on ice for 10 min to allow binding of the toxin, pelleted, resuspended in 78 mM KCl, 4 mM $MgCl_2$, 50 mM HEPES, pH 7.2, 2 mM DTT, incubated at 37°C for 6 min to allow pore formation, and finally incubated on ice for 40 min with or without exogenous peptide (7 μM final concentration). The permeabilized cells were then treated with or without 20 000 U/ml IFNα2 (Hoffman–LaRoche) at 37°C for 3 min, and the cultures were subsequently processed for immunoprecipitation, as described below.

### Immunoprecipitation and immunoblotting

Cells were lysed in 1% NP-40, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 2 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin, 1 mM sodium orthovanadate, 100 mM NaF, 5 mM $ZnCl_2$ and then the proteins of interest were immunoprecipitated with a 1:50 to 1:200 dilution of the appropriate anti-JAK or anti-STAT antibodies, for 2–4 h on ice. The immunoprecipitates were collected on protein A beads, boiled, fractionated on an SDS–polyacrylamide gel, and transferred electrophoretically to a nitrocellulose membrane. The membrane was sequentially reacted with a 1:1000 to 1:3000 dilution of the primary antibody, a 1:10 000 dilution of the appropriate peroxidase-conjugated anti-IgG secondary antibody (Sigma) and LumiGlo™ chemiluminescence reagent (Kirkegaard and Perry Laboratories Inc.), and then briefly exposed to film.

### GST fusion proteins

Cultures (200 ml) of *Escherichia coli* DH5α containing IFNaR1 or STAT sequences in the pGEX expression vector were induced with 0.1 mM isothiogalactopyranoside for 3–5 h. In the case of the IFNaR1 fusions, the bacteria were pelleted, resuspended in phosphate-buffered saline, 1% Triton X-100, 100 mM EDTA, sonicated, and centrifuged to remove debris. Soluble protein was recovered on glutathione–agarose beads (Sigma), washed and eluted with glutathione. Small portions were used directly in the *in vitro* kinase assay. In the case of the STAT SH2 fusions, the pelleted bacteria were resuspended in 100 mM NaCl, 10 mM Tris–HCl, pH 8, 1 mM EDTA, sonicated and then centrifuged to recover inclusion bodies containing insoluble protein. The inclusion bodies were solubilized with Sarkosyl (1% final concentration), which was subsequently removed by mixed micelle formation using a 10-fold excess of Triton X-100. Portions of this preparation were bound to glutathione–agarose beads for use, as needed, in the binding assays.

### Immune complex kinase assays

Immunoprecipitates of JAK kinases were prepared from baculovirus-infected cell lysates, and used to catalyze the incorporation of [$\gamma$-$^{32}$P]ATP into the soluble GST–IFNaR1 or GST–STAT–SH2 fusion proteins, as described previously (Colamonici *et al.*, 1994b). The soluble GST fusion proteins were recovered on glutathione beads and the immunoprecipitated tyk2 protein was recovered on protein A beads. These were separately washed, fractionated on an SDS–polyacrylamide gel, fixed and autoradiographed.

### Peptide binding of baculoviral proteins

Sf9 cells infected with STAT baculoviruses were lysed in the same buffer used for preparing immunoprecipitates. Biotinylated peptides (~50 pmol) were bound to strepavidin-linked beads (Pierce), washed and then incubated with aliquots of lysates (corresponding to ~$2\times10^5$ infected cells) containing baculovirus-expressed STAT proteins, in a total volume of 0.5 ml of 20 mM HEPES, pH 7.4, 300 mM NaCl, 0.5% NP-40, 0.5 mM DTT. After 2 h rotating at 4°C, the complexes were washed, boiled, fractionated on an SDS–polyacrylamide gel, and immunoblotted with anti-STAT antibodies, as described above.

### Electrophoretic mobility shift assay

A double-stranded $^{32}$P-labeled DNA probe corresponding to the interferon-stimulated gene 15 ISRE was incubated with an aliquot of lysate from $2\times10^5$ permeabilized HeLa cells, fractionated on a non-denaturing polyacrylamide gel, and autoradiographed, as previously described (Levy *et al.*, 1988; Schindler *et al.*, 1992a).

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