Interferons and cytokines modulate gene expression via a simple, direct signaling pathway containing receptors, JAK tyrosine kinases, and STAT transcription factors. The interferon-α pathway is a model for these cascades. Two receptors, IFNaR1 and IFNaR2, associate exclusively in a constitutive manner with two JAK proteins, TYK2 and JAK1, respectively. Defining the molecular interface between JAK proteins and their receptors is critical to understanding the signaling pathway and may contribute to the development of novel therapeutics. This report defines the IFNaR1 interaction domain on TYK2. In vitro binding studies demonstrate that the amino-terminal half of TYK2, which is ∼600 amino acids long and contains JAK homology (JH) domains 3–7, comprises the maximal binding domain for IFNaR1. A fragment containing amino acids 171–601 (JH3–6) also binds IFNaR1, but with reduced affinity. Glutathione S-transferase-TYK2 fusion proteins approximating either the JH6 or JH3 domain affinity-precipitate IFNaR1, suggesting that these are major sites of interaction within the larger binding domain. TYK2 amino acids 1–601 act in a dominant manner to inhibit the transcription of an interferon-α-dependent reporter gene, presumably by displacing endogenous TYK2 from the receptor. This same fragment inhibits interferon-α-dependent tyrosine phosphorylation of TYK2, STAT1, and STAT2.

Tyrosine kinase activation is a common mechanism for triggering eukaryotic signaling pathways. Receptor-type tyrosine kinases, which have extracellular ligand-binding domains and intracellular kinase domains, are activated following ligand-induced dimerization or oligomerization (1–3). The ligands for these receptors include a variety of growth factors, hormones, and cytokines. The interferon/cytokine receptor family of signaling molecules, most notably the JAK proteins, have a unique structure among non-receptor tyrosine kinases. In the case of the interferon-α (IFN-α) receptor, two subunits have been identified (IFNaR1 (7) and IFNaR2 (8)), and these are known to bind exclusively to two JAK kinases (TYK2 and JAK1, respectively) (8–10). Following IFN-α binding, the JAK proteins are activated (11–14), and two STAT proteins (STAT1 and STAT2) are tyrosine-phosphorylated, heterodimerize, translocate to the nucleus, and stimulate transcription of genes containing the interferon-stimulated gene response element (ISRE) (15). A variety of studies demonstrate that both TYK2 and JAK1 are required for IFN-α signaling (14, 16, 17).

The JAK kinases have a unique structure among non-receptor tyrosine kinases. In addition to a carboxyl-terminal tyrosine kinase domain, these proteins contain a set of six other regions of homology known as JAK homology (JH) domains (18), which are common to all members (JAK1 (19), JAK2 (18), JAK3 (20), TYK2 (21), and the Drosophila hopscotch gene (22)). Adjacent to the bona fide kinase domain is a kinase-like domain, which apparently lacks catalytic activity and has no clear function. The amino-terminal half of the JAK proteins is composed of five shared domains (JH3–7), which are not found in any other known proteins. Neither Src homology 2 or 3 nor pleckstrin homology domains are present in the JAK proteins.

The interferon/cytokine receptor cytoplasmic tails function as multipurpose docking proteins. In addition to sites for the binding of JAK tyrosine kinases, these receptors often contain tyrosine residues that are inducibly phosphorylated following ligand binding. Some of these phosphorylated sites bind Src homology 2 domains of signaling molecules, most notably the STAT transcription factors (4). For example, we have identified a binding site for STAT2 centered around tyrosine 466 of the IFNaR1 subunit of the interferon-α receptor (23). We have previously characterized the TYK2-binding site on IFNaR1 in detail (9, 11, 24). The minimal binding domain is an ∼33-amino acid juxtamembrane region, overlapping the box 1 and box 2 sequences of IFNaR1 (24). Nearly every phylogenetically conserved residue in the region is required for binding. However, the proline-rich box 1 sequence, which is not well conserved in IFNaR1, appears to play only a minor role in the TYK2-IFNaR1 interaction. In contrast, it has been reported that box 1 is a critical determinant of the binding domain in the cytoplasmic portion of other cytokine receptors (25, 26). Our results have also demonstrated that the TYK2-IFNaR1 interaction is absolutely required for signaling (24). In this report, we complement our previous characterization of the TYK2 interaction domain on IFNaR1 by identifying the regions of TYK2 that bind to IFNaR1. Our results show that most, if not

1 The abbreviations used are: IFN-α, interferon-α; ISRE, interferon-stimulated gene response element; JH, JAK homology; mAb, monoclonal antibody; GST, glutathione S-transferase; HA, influenza hemagglutinin epitope.
all, of the amino-terminal half of TYK2 is required for this interaction.

**MATERIALS AND METHODS**

**Reagents**—Interferon-α2 was a gift of J. Seipinwall (Hoffmann-La Roche). Polyclonal rabbit antisera against TYK2 has been described previously (11). The following monoclonal antibodies (mAbs) were obtained from commercial suppliers: 4G10, against phosphotyrosine (Upstate Biotechnology, Inc.); anti-glutathione S-transferase (GST) (Santa Cruz Biotechnology, Inc.), and anti-TYK2 (Transduction Laboratories). A mAb against the influenza hemagglutinin epitope (HA) (27) was from J. Kitajewski (Columbia University, New York, NY), a polyclonal antibody against STAT1 and STAT2 (15) were provided by C. Schindler (Columbia University). All DNA-manipulating enzymes were purchased from New England Biolabs Inc.

**Plasmid Constructs**—Plasmids encoding GST-TYK2 fusion proteins were prepared by digesting the TYK2 cDNA with the appropriate restriction enzymes (illustrated in Fig. 3), bluntting the ends as required, and ligating into the Smal site of pGEX-1, -2T, or -3X (Pharmacia Biotech Inc.). GST-IFNaR1 fusion proteins have been described previously (9, 24). To express TYK2 in mammalian cells, the full-length cDNA (21) and subclones, prepared by restriction digestion as illustrated in Fig. 1, were blunt to allow ligation into a version of the pMT2T expression vector (28) containing Pet, EcoRI, NotI, and SalI cloning sites. Stop codons in all three reading frames are contained in the expression vector. In the case of the two double truncation constructs (which span amino acids 171–601 and 263–601, respectively, as illustrated in Fig. 1), a cassette containing an HA tag and the 15 amino acids found at the amino terminus of TYK2 was amplified by polymerase chain reaction from a previously described construct (29) and ligated upstream of the corresponding TYK2 fragments. The CD4-IFNaR1 chimera has been previously described (30).

**Cell Culture, Infection, and Transfection**—U2OS cells were from American Type Culture Collection, and 293T cells were from H. Young (Columbia University). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine (293T) or 1.7% d-glucose (U2OS) from a newly confluent plate the day prior to transfection, and subsequently transfected as described (31) with calcium phosphate precipitates containing 10 μg of plasmid DNA/10-cm dish (see below for cotransfection protocol for luciferase assay). Two days post-transfection, cells were lysed as described previously (29). For the reporter gene experiments, U2OS cells were transfected with 5 μg of the β-galactosidase expression plasmid, 5 μg of the ISRE-luciferase plasmid, and 10 μg of the expression vector (16).

**Binding Assays**—Lysates from transfected cells were incubated at 4 °C with GST fusion proteins, purified as described (23), already bound to the glutathione beads (Sigma) for 2–4 h. The complexes were recovered by centrifugation, washed three to four times, and then subjected to SDS-polyacrylamide gel electrophoresis in preparation for immunoblotting (see below).

**Immunoprecipitation and Immunoblotting**—Cells were lysed in 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 2 mM phenylmethylsulfonyl fluoride, 0.2 unit/ml aprotinin, 1 mM sodium orthovanadate, 100 mM NaF, and 5 mM ZnCl2, and the proteins of interest were immunoprecipitated with a 1:50 to 1:200 dilution of the appropriate antibody, a 1:10,000 dilution of the appropriate peroxidase antibody, a 1:50 to 1:200 dilution of the appropriate antibody, and 5 μg of the isre-luciferase plasmid, and 10 μg of the expression vector (16).

**Results**

**Truncations Demarcate the IFNaR1-binding Domain on TYK2**—We have previously demonstrated that the TYK2-IFNaR1 interaction can be faithfully reproduced in vitro (9, 24). Therefore, to characterize the region of TYK2 required for binding to IFNaR1, we performed a series of analogous binding assays. Fig. 1 illustrates the TYK2 constructs used in the first set of binding experiments. These fragments were transiently expressed at high levels in 293T cells; the lysates were affinity-precipitated with GST fusion proteins containing all or part of the cytoplasmic domain of IFNaR1; and the resulting complexes were immunoblotted. Fig. 2A shows that a set of progressive carboxyl-terminal truncations were expressed at similar levels, but only the truncations terminating at amino acids 601 and 876 were precipitated as efficiently as full-length TYK2. The faint band observed in the other lanes was also seen in control experiments employing GST alone as an affinity precipitation reagent (data not shown). Probing the lower part of the same immunoblot with an anti-GST mAb demonstrated equal recovery of the fusion protein in each lane (data not shown).

Assuming that residue 601 is the carboxyl terminus of the interaction domain, a second binding experiment was performed with fragments spanning residues 171–601 and 263–601. Since these constructs delete a portion of the region recognized by the anti-TYK2 mAb used to immunoblot in Fig. 2A, an HA epitope tag was incorporated into the constructs. Fig. 2B shows equivalent levels of expression for the constructs, whereas only construct 171–601 was affinity-precipitated by the GST-IFNaR1 cytoplasmic domain. Affinity precipitation with GST alone revealed no detectable binding (data not shown). Construct 171–601 appears to be bind less than full-length TYK2 (Fig. 2B), whereas construct 1–601 appears to bind more avidly than full-length TYK2 (Fig. 2A). Because these two constructs cannot be immunoblotted with the same antibody, we have not compared them directly.

To demonstrate the specificity of the interaction, a subset of the lysates utilized in Fig. 2A were precipitated with three separate GST-IFNaR1 fusion proteins: one containing the entire cytoplasmic domain of IFNaR1 (amino acids 466–557; used in Figs. 2, A and B), a second containing the juxtamembrane half of the cytoplasmic domain (amino acids 466–511) that includes...
the entire TYK2-binding domain (9), and a third containing a mutant version of the same juxtamembrane region (denoted 466–511* in Fig. 2C) that does not bind TYK2 in vitro or in vivo (24). In the latter case, amino acids 504–506 (IEE) have been replaced with three alanines. Fig. 2C shows that both full-length TYK2 (FL) and amino acids 1–601 bind to the entire cytoplasmic domain and the juxtamembrane portion, but not to the mutant. The construct containing amino acids 1–449 does not bind any of the three fusion proteins. As observed in Fig. 2A, construct 1–601 appears to bind IFNaR1 more strongly than full-length TYK2.

**Fig. 3. Binding of GST-TYK2 fusion proteins to IFNaR1.** A, schematic of GST-TYK2 fusion protein constructs. The diagram follows the format of Fig. 1. Restriction site abbreviations are as follows: Sp, SpI; P, PvuII; A, ApaI; S, SalI; X, XhoI; N, NaeI; E, EcoRI. Constructs displaying strong binding are indicated (+). B, in vitro binding of GST-TYK2 fusion proteins to the CD4-IFNaR1 chimera. Lysates from bacteria expressing fusion proteins containing the indicated TYK2 amino acids were used to affinity-precipitate a CD4-IFNaR1 chimera expressed in 293T cells. The complexes were immunoblotted and probed with the anti-CD4 mAb (upper panel). Lane 15 contained only crude CD4-IFNaR1 lysate (~10% of the total used in each affinity precipitation reaction). The original filter was stripped and reprobed with the anti-GST mAb (lower panel). The position of each fusion protein is indicated by an arrow. The slower migrating bands are assumed to be degradation products.

**Fig. 2. In vitro binding of TYK2 truncation constructs to IFNaR1.** A, carboxyl-terminal truncations. Lysates from 293T cells expressing the indicated constructs (see Fig. 1; FL is the full-length construct spanning residues 1–1187) were immunoblotted either prior to (left panel) or following (right panel) affinity precipitation (Ppt) with a GST fusion protein containing the entire cytoplasmic domain of IFNaR1. Approximately 10% of the total lysate was used for the blot in the left panel, and 90% was used for affinity precipitation (right panel). The immunoblots were probed with the anti-TYK2 mAb. The identity of the higher molecular weight bands in some of the lanes containing carboxy-terminal truncation constructs is not known. B, double truncation constructs. The binding experiment was performed exactly as described for A, except that the resulting blots were probed with the anti-HA mAb. C, specificity of the TYK2-IFNaR1 in vitro interaction. Lysates were immunoblotted either prior to (first panel) or following (second through fourth panels) affinity precipitation with a GST-IFNaR1 fusion protein containing the entire cytoplasmic domain of IFNaR1 (amino acids 466–557), a fusion protein containing the TYK2-binding domain (amino acids 466–511), or a fusion protein containing a mutant version of the binding domain that does not bind TYK2 (amino acids 466–511*). Approximately 10 times more lysate was used for each affinity precipitation relative to the blot in the first panel. Blots were probed with the anti-TYK2 mAb.
the CD4 extracellular domains are dimerized by antibody cross-linking (30). We employed the chimera because CD4 antisera is a sensitive reagent for immunoblotting (33).

Four of the GST-TYK2 fusion proteins (indicated with plus signs in Fig. 3A) displayed strong binding to the CD4-IFNaR1 chimera. Two contained regions of TYK2 corresponding to single JH domains (JH6 and JH13) (Fig. 3B, lanes 4 and 7). In contrast, constructs encoding the JH4 and JH5 domains showed very little binding (lanes 5 and 6). Constructs that spanned amino acids 1–591 and 53–591 (lanes 10 and 11) also displayed strong binding, consistent with the data in Fig. 2. The fusion proteins encoding these longer constructs were expressed poorly, suggesting that when binding is normalized for protein content, these constructs bind IFNaR1 with significantly greater affinity than the single JH domains. Four other constructs displayed weak binding, despite adequate levels of expression (lanes 8, 9, 12, and 13). With the exception of one (amino acids 53–127, corresponding approximately to JH7), these all overlapped the JH3 or JH6 domain.

**Amino-terminal Fragments of TYK2 Inhibit IFN-α-dependent Reporter Gene Transcription**—To demonstrate that the interaction domain identified in vitro is relevant in vivo, we assayed the ability of various TYK2 fragments to inhibit IFN-α-dependent reporter gene activity. Previously, we have shown that overexpression of kinase-deficient TYK2 in U2OS cells can block the IFN-α-dependent expression of a luciferase gene under the control of an ISRE and a minimal promoter (16). Presumably, this dominant-negative effect is caused by the displacement of endogenous TYK2 from the receptor by exogenous, overexpressed, kinase-deficient TYK2, thereby preventing receptor-mediated TYK2 activation and subsequent STAT phosphorylation.

Fig. 4 shows that TYK2 truncations corresponding to the IFNaR1-binding domain can also inhibit IFN-α-dependent reporter gene activity in U2OS cells. Specifically, carboxyl-terminal truncation constructs spanning amino acids 1–876 and 1–601 inhibited reporter gene activity relative to wild-type TYK2, whereas further truncation toward the amino terminus was ineffective. The extent of inhibition (~50–60%) is quantitatively similar to that observed with the previously reported kinase-deficient TYK2 construct (16). A dominant inhibitory effect was not observed, however, for the double truncation spanning amino acids 171–601, although this construct binds in vitro (Fig. 2) and partially blocks STAT activation in vivo (see below).

**Amino-terminal Fragments of TYK2 Inhibit Tyrosine Phosphorylation of TYK2 and STAT Proteins**—We hypothesized that the inhibition of ISRE-controlled luciferase activity resulted from displacement of endogenous TYK2 from the receptor. As a consequence of such displacement, tyrosine phosphorylation of TYK2 and both STAT1 and STAT2 should be inhibited. To test this, 293T cells were transfected with a subset of the constructs used in Fig. 4, and tyrosine phosphorylation was assayed. Because 293T cells can be transfected at very high efficiencies (>90%), an effector gene can be overexpressed in nearly every cell in a given culture. Thus, it is possible to determine the dominant inhibitory effect of the products of such transfected effector genes on endogenous proteins, as we have demonstrated previously (16, 23, 24). We did not employ 293T cells in the reporter gene induction experiments in Fig. 4, however, because E1A overexpression has been shown to inhibit STAT2 transactivation (34).

As expected, IFN-α induces tyrosine phosphorylation of STAT1 in 293T cells transfected with vector alone (Fig. 5A). In contrast, transfection of either the kinase-deficient TYK2 construct (K930I) or the construct containing amino acids 1–601 substantially blocks STAT1 phosphorylation. The construct containing residues 1–449, which does not bind IFNaR1 in vitro (Fig. 2), has no effect on STAT1 phosphorylation. Control blots demonstrate equal recovery of STAT1 in each lane and approximately equal expression of the appropriately sized TYK2 constructs in each set of transfectants. In Fig. 5B, two double truncation constructs were tested in a similar experiment. Under conditions where construct 1–601 strongly inhibited STAT1 tyrosine phosphorylation, a construct spanning amino acids 171–601 was partially effective, whereas a construct spanning residues 263–601 was ineffective. Thus, the dominant-negative effect of the TYK2 truncation constructs correlates closely with the in vitro binding activity seen in Fig. 2. Finally, Fig. 5C shows that tyrosine phosphorylation of TYK2 and STAT2 is also effectively blocked in cells overexpressing construct 1–601.

**DISCUSSION**

Since cytokines regulate the growth, differentiation, and effector function of a wide variety of cells, characterizing the interface between cytokine receptors and Jak family kinases is important for understanding the biology of these cells and may eventually aid in the design of new drugs. Toward these ends, we have begun to define the interaction domains on both IFNaR1 (9, 11, 24) and TYK2. This report demonstrates that the TYK2 domain spans the amino-terminal half of the protein. Specifically, in vitro binding of TYK2 truncation constructs to a GST fusion protein encoding the cytoplasmic domain of IFNaR1 reveals that maximal binding requires amino acids 1–601 of TYK2 and that weaker binding can still be detected with a construct encoding amino acids 171–601 (Fig. 2, A and
TABLE 1. Properties of the JH Domains

<table>
<thead>
<tr>
<th>Domain</th>
<th>Binding Characteristics</th>
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<tbody>
<tr>
<td>JH1</td>
<td>Weak binding</td>
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<tr>
<td>JH2</td>
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<td>JH3</td>
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<td>JH7</td>
<td>Strong binding</td>
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These constructs correspond to the JH3–7 and JH3–6 domains of TYK2, respectively. Binding of the fragment containing amino acids 1–601 to the IFNaR1 cytoplasmic domain was disrupted by IFNaR1 mutations previously shown to abolish the ability of IFNaR1 to bind TYK2 and to mediate cell signaling (24) (Fig. 2C). A second set of in vitro binding studies (Fig. 3), using GST fusion proteins corresponding to TYK2 JH domains, indicate that the JH3 and JH6 domains are the major binding sites within the IFNaR1-binding domain. Although variable levels of fusion protein expression compromise our ability to precisely quantitate binding, the data indicate that the intervening domains (JH4 and JH5) bind the IFNaR1 cytoplasmic domain poorly. Longer constructs containing most or all of the JH3–7 domains appeared to bind IFNaR1 more avidly than the single JH3 and JH6 domains. Finally, two sets of dominant inhibitory experiments (Figs. 4 and 5) demonstrate that overexpression of the binding domain within the amino-terminal half of TYK2 effectively blocks both ISRE-driven reporter gene expression and tyrosine phosphorylation of TYK2, STAT1, and STAT2, presumably by displacing endogenous TYK2 protein from the receptor complex in the transfected cells. Interestingly, the TYK2 construct corresponding to amino acids 171–601 can dominantly interfere with TYK2 and STAT tyrosine phosphorylation (Fig. 5B), but is ineffective in blocking reporter gene expression (Fig. 4). This suggests that under the transfection conditions employed, submaximal STAT phosphorylation might nonetheless be sufficient to induce maximal levels of reporter gene transcription. However, these results might also reflect differences in the two cell lines employed. Overall, our data show that the binding of IFNaR1 to TYK2 requires a 601-amino acid region of TYK2 containing the JH3–7 domains, and within this region, the JH3 and JH6 domains appear to be particularly important.

A number of cytokine receptor-binding domains within JAK family kinases have now been characterized. Chen et al. (35) have recently reviewed these data. In the case of JAK2, which associates with a wide variety of receptors, binding to the growth hormone receptor appears to require the entire amino-terminal half of the protein (JH3–7) (25). In contrast, binding to the granulocyte-macrophage colony-stimulating factor receptor requires only the JH6 and JH7 domains of JAK2, although this binding is quite weak (36). JAK3 association with the common y-chain of the interleukin-2 receptor appears to be also mainly mediated by the JH6 and JH7 domains, although the JH3–5 domains appear to also play a role (35). Including our data on the TYK2-IFNaR1 interaction, there are two examples of two different classes of binding sites: one involving primarily the JH6 and JH7 domains and a second requiring the JH3–7 domains.

Assuming that all cytokine receptor-JAK complexes are similar in overall topology, Chen et al. (35) have attempted to reconcile the existing data by proposing the existence of two domains that mediate receptor binding by a given JAK protein. One domain would be in the JH6 and JH7 region, and a second would be somewhere in the JH3–5 region. One of these domains may act as a generic JAK-binding site, whereas the other could provide specificity for a given receptor. The pattern of binding observed for different receptor-JAK pairs would then reflect variation in the relative affinity of these two sites. In cases where the affinity of the two sites is similar for a receptor, then binding will appear to require most or all of the JH3–7 domains. In this scenario, the TYK2-IFNaR1 interaction would fall into this category. Our observation that the entire JH3–7 region is required for strong binding but that the individual JH3 and JH6 domains show some independent binding would be consistent with such a model. Characterization of additional receptor-JAK pairs, identification of the specific JAK residues required for binding, and the eventual solution of the three-dimensional structure of one or more receptor-JAK complexes will be needed to prove this model.

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IFNAR1-binding Domain on TYK2

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