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Molecular Characterization of an Alpha Interferon Receptor 1 Subunit (IFNaR1) Domain Required for TYK2 Binding and Signal Transduction

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Binding of alpha interferon (IFNα) to its receptors induces rapid tyrosine phosphorylation of the receptor subunits IFNaR1 and IFNaR2, the TYK2 and JAK1 tyrosine kinases, and the Stat1 and Stat2 transcription factors. Previous studies have demonstrated that TYK2 directly and specifically binds to and tyrosine phosphorylates IFNaR1 in vitro. We now report a detailed analysis of the TYK2 binding domain on the IFNaR1 subunit. First, we used an in vitro binding assay to identify the TYK2 binding motif in IFNaR1 as well as the critical residues within this region. The most striking feature is the importance of a number of hydrophobic and acidic residues. A minor role is also ascribed to a region resembling the proline-rich "box 1" sequence. In addition, mutations which disrupt in vitro binding also disrupt the coimmunoprecipitation of the receptor and TYK2. We also provide direct evidence that the binding region is both necessary and sufficient to activate TYK2 in vivo. Specifically, mutations in the binding domain act in a dominant-negative fashion to inhibit the IFNα-induced tyrosine phosphorylation of TYK2 and Stat2. Further, introduction of dimerized glutathione *S*-transferase–IFNaR1 fusion proteins into permeabilized cells is sufficient to induce phosphorylation of TYK2 and the receptor, confirming the role of the binding domain in IFNα signal transduction. These studies provide clues to the sequences determining the specificity of the association between JAK family tyrosine kinases and cytokine receptors as well as the functional role of these kinases in cytokine signal transduction.

A number of polypeptide hormones initiate signal transduction by binding to their cognate receptors and stimulating intracellular protein tyrosine kinase activity (43). Although cytokine receptors do not possess intrinsic tyrosine kinase activity, rapid tyrosine phosphorylation occurs in response to cytokine treatment of cells (reviewed in references 10, 22, 40, and 46). In a number of cases, JAK family tyrosine kinases are found associated with cytokine receptors and become activated following ligand binding (3, 8, 54). The JAKs are a structurally distinct subfamily of nonreceptor tyrosine kinases composed of TYK2 (14), JAK1 (53), JAK2 (19), JAK3 (6, 23, 39, 45, 55), and the gene product of the *Drosophila hopscotch* locus (5). These kinases are proteins of 115 to 135 kDa that contain a bona fide carboxy-terminal kinase domain along with an adjacent kinase-like domain and lack both SH2 and SH3 domains. The kinase-like domain contains a number of the sequence motifs characteristic of protein kinases but lacks some of the conserved amino acids thought to be essential for protein kinase activity (18). The amino-terminal half of the JAK kinases contains five additional regions of homology and a relatively unique region present at the extreme amino terminus of the protein (19, 55). Some of these amino-terminal domains are apparently involved in the association of the JAKs with members of the cytokine receptor superfamily (47, 58). However, there are no reports of a small discrete domain within any of the JAK kinases that is sufficient for mediating the interaction with a particular cytokine receptor.

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Analysis of the structure of the JAK kinase binding domains on various cytokine receptors has been more revealing. For example, JAK1 binds to the α subunit of the gamma interferon (IFNγ) receptor (21) and to the β subunit of the interleukin-2 (IL-2) receptor complex (28, 38). JAK2 associates with the growth hormone receptor (3, 51), the erythropoietin receptor (47, 54), and the β_c chain of the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor (37). JAK3 binds to the IL-2 γ subunit (28, 38). The gp130 subunit of the IL-6 receptor family can associate with either JAK1, JAK2, or TYK2 but not JAK3 (27, 31, 32, 44, 47). The β subunit of the leukemia inhibitory factor (LIF) receptor, which is closely related to gp130, also binds multiple JAKs (44). In almost every case, the JAK kinases bind to the membrane-proximal region of the cytoplasmic domain of the receptor. Two short, conserved motifs (box 1 and box 2) are found in the membrane-proximal regions of a number of these receptors (30). Both of these "boxes" have been shown to be required for induction of proliferative signals by many cytokines. The box 1 motif, which is a proline-rich sequence usually preceded by hydrophobic amino acids, appears to be required for many of the JAK-receptor associations already described (31, 37, 47, 51).

In the case of IFNα, two JAK family members, JAK1 and TYK2, were linked to the pathway by genetic complementation experiments (29, 52). Subsequent biochemical studies showed that TYK2 and JAK1 are rapidly activated and tyrosine phosphorylated after IFNα treatment (4, 8, 29). The activated TYK2 and JAK1 kinases are believed to mediate the tyrosine phosphorylation of the Stat1 and Stat2 transcriptional factors. Once activated, Stat1 and Stat2 form a complex and translocate, with the p48 transcription factor, into the nucleus and promote specific gene transcription (15, 41, 42). Two IFNα

receptor subunits, IFNaR1 and IFNaR2 (also known as the α and β subunits, respectively), have been cloned (13, 33, 49; reviewed in reference 50). Like other cytokine receptors, these two proteins are rapidly phosphorylated on tyrosine in response to IFNα (1, 9, 35, 36).

Our previous data demonstrated a direct and specific interaction between IFNaR1 and TYK2 both in vivo (8) and in vitro (7). In in vitro experiments, IFNaR1 fusion proteins containing the cytoplasmic domain of the receptor bound TYK2 but not JAK1 nor JAK2 proteins expressed in insect cells. More recently, we have also demonstrated a direct and specific in vitro association between the alternatively spliced, longer form of IFNaR2 and JAK1 (12a). We have also shown that tyrosine residues in the cytoplasmic domain of IFNaR1 can be phosphorylated by TYK2 and that the major phosphorylation site acts as a docking site for the SH2 domain of Stat2 (56). The TYK2 binding region and Stat2 docking site are both restricted to the membrane-proximal region, which is only about 46 amino acids in length. Thus, structure-function studies on the TYK2-IFNaR1 interaction should, in combination with studies on other cytokine receptor-JAK kinase pairs, provide a detailed picture of the molecular interactions which control the initial steps of IFNα signaling. In this report, we begin this process by localizing the minimal TYK2 binding motif in the IFNaR1 cytoplasmic domain, identifying the specific amino acids required for binding, and demonstrating that these residues are also required for TYK2-IFNaR1 binding in vivo. Further, two independent experimental approaches demonstrate that the binding domain is both necessary and sufficient to initiate IFNα signaling.

MATERIALS AND METHODS

Peptides, antibodies, and interferon. Peptides were synthesized as described elsewhere (17). Two biotinylated peptides were synthesized: wild-type IFNaR1(479–511), DEYFSEQPLKNLLLSTSEEQIEKCFIIENISTI, and mutant IFNaR1(479–511), DEYFSEQPLKNLLLSTSEEQIEKCFAAANISTI. Polyclonal rabbit antiserum against TYK2 has been described previously (8). The following monoclonal antibodies 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 monoclonal antibody against the influenza virus hemagglutinin epitope (FLU) (24) was obtained from J. Kitajewski (College of Physicians and Surgeons, Columbia University). A polyclonal rabbit antiserum against CD4 (12) was provided by R. Sweet (Smith-Kline-Beecham Laboratories). A polyclonal rabbit antiserum against Stat2 (42) was provided by C. Schindler (College of Physicians and Surgeons, Columbia University). IFNα2 was provided by J. Sepinwall and P. Sorter (Hoffmann-La Roche).

Cell culture and transfection. Sf9 cell culture and infection with recombinant baculoviruses were performed as described before (7, 8, 34). Human HeLa S3 cells were grown as described previously (7). Human 293T cells were grown and transfected with calcium phosphate DNA precipitates, as described previously (11). In some cases, transfected 293T cell cultures were reseeded on the day following transfection and treated with 10 μg of tunicamycin (Sigma) per ml for up to 24 h.

DNA constructs. GST-IFNaR1 fusion proteins (spanning amino acids 465 to 557, 465 to 510, or 511 to 557) were described previously (7). GST-IFNaR1 fusion proteins (spanning amino acids 460 to 486, 479 to 511, 486 to 511, or 479 to 501) were prepared by PCR, incorporating flanking *Bam*HI and *Eco*RI restriction sites for subsequent cloning into pGEX vectors. An IFNaR1 cDNA was used as a template (49). The overlapping PCR technique (20) was used to generate similar PCR fragments encoding alanine substitution mutations in the IFNaR1. Oligonucleotides were obtained from Ransom Hill Biosciences. The accuracy of PCR-generated DNAs was confirmed by dideoxy sequencing with a Sequenase kit (US Biochemicals). FLU-tagged IFNaR1 constructs have been described previously (56). Construction of the CD4-IFNaR1 chimeras will be described elsewhere (25).

GST fusion proteins. Cultures of *Escherichia coli* DH5α containing IFNaR1 sequences in the pGEX expression vector were induced with 0.1 mM isothiogalactopyranoside for 3 h. The bacteria were pelleted, resuspended in phosphate buffered saline (PBS)–1% Triton X-100–100 mM EDTA, sonicated, and centrifuged to remove debris. Soluble protein was recovered on glutathione-agarose beads (Sigma) and washed with the lysis buffer. For binding experiments, the washed GST fusion protein-bead complexes were added directly to cell lysates. For the cell permeabilization experiments, the purified GST fusion proteins were eluted with glutathione. The eluent, containing 10 mM glutathione in 50 mM Tris (pH 8.0), was added directly to the permeabilized cell preparation.

Cell permeabilization. HeLa S3 cells were permeabilized with streptolysin O (Murex Diagnostics) as described before (2, 57). In brief, 10^7 cells were pelleted and resuspended in PBS. Streptolysin O was added to a final concentration of 0.6 U/ml, and the cells were incubated on ice for 10 min, pelleted, resuspended in 78 mM KCl–4 mM MgCl–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.2)–2 mM dithiothreitol (DTT), incubated at 37°C for 6 min, and finally incubated on ice for 30 min with or without exogenous GST-IFNaR1 proteins (3 μM final concentration). The permeabilized cells were then processed for immunoprecipitation.

Immunoprecipitation and immunoblotting. The procedures used have been described in detail before (7, 8, 56). In brief, cells were washed with PBS and then lysed in a buffer containing 1% Nonidet P-40 (Sigma). The nuclei and membranes were removed by centrifugation, and the clarified lysate was used for immunoprecipitation or in some cases applied directly to the gel. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose (MSI), and probed with the appropriate primary and secondary antibodies as described previously (7). The anti-CD4 and anti-FLU primary antibodies were used at a 1:2,000 dilution. LumiGlo chemiluminescence reagents (Kirkegaard and Perry Laboratories, Inc.) were used for visualization.

Peptide binding. Biotinylated peptides (50 pmol) were bound to streptavidin-linked beads (Pierce), washed, and then incubated for 2 h with lysate (corresponding to 2×10^5 infected Sf9 cells) containing baculovirus-expressed TYK2 protein, in a total volume of 0.5 ml of 20 mM HEPES (pH 7.4)–300 mM NaCl–0.5% Nonidet P-40–0.5 mM DTT. The complexes were washed, boiled, fractionated by SDS-PAGE, and immunoblotted with anti-TYK2 antibody as described previously (56).

FACS. For fluorescence-activated cell sorting (FACS), transfected 293T cells (5×10^5) were washed twice with PBS and resuspended in 100 μl of PBS containing either 5 μl of phycoerythrin (PE)-conjugated anti-CD4 (Caltag Laboratories) or 5 μl of isotype-matched control anti-immunoglobulin G2a (IgG2a). Cells were incubated with the staining antibodies for 30 min at room temperature and then washed three times with PBS. Analysis of the stained cells was performed on a Becton-Dickinson FACScan, using Cell Quest software, according to the manufacturer's specifications.

RESULTS

Definition of the minimal IFNaR1 cytoplasmic region required for TYK2 binding. We have previously reported that IFNaR1 specifically and directly binds to the TYK2 tyrosine kinase and that this binding is constitutive (7). To further characterize this interaction, we generated a panel of GST fusion proteins containing portions of the IFNaR1 cytoplasmic domain (for a schematic depiction, see Fig. 2). The GST-IFNaR1 truncated fusion proteins were bound to glutathione-agarose beads, and the resulting complexes were incubated with lysate from TYK2 recombinant baculovirus-infected Sf9 cells. The precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-TYK2 antibody. Figure 1A shows that the TYK2 binding domain is restricted to a 46-amino-acid juxtamembrane region, spanning residues 465 to 511, beginning eight amino acids carboxy terminal to the transmembrane domain (compare lanes 3 and 4) as reported previously (7). Amino-terminal truncation of this 46-amino-acid binding domain progressively decreases binding (compare lane 4 with lanes 6 and 8). Thus, a 25-amino-acid region between residues 486 and 511 still retains detectable binding activity. Further removal of the 10 amino acids at the carboxy terminus of the 25-amino-acid fragment completely eliminates binding (compare lanes 6 and 7). Taken together, these data suggest that the proximal half of the IFNaR1 cytoplasmic domain is required for maximal binding but that most of the critical interactions occur in the 25-amino-acid region between amino acids 486 and 511. We have also confirmed our previous results indicating that the IFNaR1-TYK2 interaction is specific by showing that baculovirus-expressed JAK1 does not bind to the GST-IFNaR1 fusion protein spanning residues 465 to 511 (data not shown).

Identification of specific amino acids required for TYK2 binding. In order to identify the amino acids which are critical

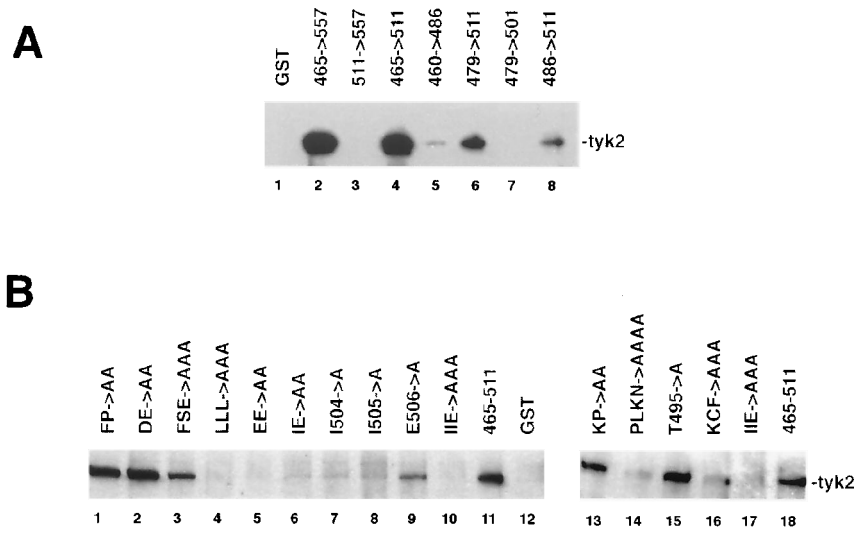

FIG. 1. In vitro binding of GST-IFNaR1 fusion proteins defines the TYK2 binding site. (A) Binding of baculovirus-encoded TYK2 to truncated GST-IFNaR1 fusion proteins. GST and GST fusion proteins containing various segments of the IFNaR1 cytoplasmic domain (as indicated by the positions of the amino acids of the full-size protein) were bound to glutathione-agarose beads and used to precipitate baculovirus-expressed TYK2, and the resulting complexes were then immunoblotted with anti-TYK2 antibody. The position of the 135-kDa TYK2 protein is indicated. (B) Modified alanine scan. With the PCR-based technique described in Materials and Methods, the indicated amino acids were changed to alanine. GST fusion proteins containing IFNaR1 residues 465 to 511 with the appropriate mutations, the wild-type sequence (465 to 511), or GST alone were prepared and used in an in vitro binding assay as described for panel A. The diagram in Fig. 2 identifies the positions of the mutations in the IFNaR1 cytoplasmic domain. Lanes 1 to 12 and 13 to 18 represent separate experiments; each contains an internal positive control (465 to 511). The position of the 135-kDa TYK2 protein is indicated. In both panels, the lower portion of each immunoblot was probed separately with an anti-GST antibody to verify that approximately equal amounts of fusion protein were present in each binding reaction (data not shown).

for TYK2-IFNaR1 association, we applied a modified alanine scan technique, changing the indicated amino acids (Fig. 2) to alanine to produce GST fusion proteins containing mutated IFNaR1 (spanning amino acids 465 to 511). We concentrated on those residues which are either identical or similar in the human, murine (48), and bovine (26) homologs of IFNaR1. In vitro binding to baculovirus-expressed TYK2 protein was again determined. Most of the conserved amino acids between residues 481 and 511 are required for TYK2 binding, while those immediately upstream are apparently dispensable (Fig. 1B). Thus, the region identified by site-specific mutagenesis is almost identical to the minimal binding domain defined in Fig. 1A. A number of hydrophobic amino acids, mainly isoleucine and leucine, and the acidic amino acids aspartic and glutamic acid are important for binding. In Fig. 3, HeLa cell lysates were used in place of the baculovirus-expressed TYK2 lysates in the in vitro binding experiments to confirm that mammalian TYK2 protein binds to IFNaR1 in the same pattern as the insect cell protein.

In order to exclude the possibility that the GST moiety affects in vitro binding and to confirm that amino acids 479 to 511 are sufficient for binding, we repeated the in vitro binding experiment with synthetic peptides in place of GST fusion proteins. A biotinylated peptide containing IFNaR1 residues 479 to 511 as well as a mutant version in which residues 504 to 506 (IIE) were replaced by three alanines were bound to streptavidin-linked beads, washed, and then incubated with baculovirus-expressed TYK2. The complexes were immunoblotted with an anti-TYK2 antibody, and as shown in Fig. 4, the wild-type but not the mutant peptide binds to TYK2.

Key residues in the TYK2 binding sites are required for TYK2-IFNaR1 association in vivo. To test if the sequences involved in TYK2 binding in vitro are required for TYK2 association in vivo, CD4-IFNaR1 chimeric molecules composed of the extracellular domain of CD4 fused to the intracellular domain of IFNaR1 were constructed (Fig. 5A). Mutant chimeras were constructed by using restriction fragment cassettes from the GST fusion constructs shown in Fig. 1 and 3. Following transient transfection into 293T cells, FACS analysis demonstrated that the chimeras were equally well expressed on the cell surface (Fig. 5B). Cells were lysed and immunoprecipitated with anti-TYK2 antiserum, and the precipitates were immunoblotted with an anti-CD4 antibody. Only the chimera containing the wild-type intracellular domain of IFNaR1 can be coimmunoprecipitated with anti-TYK2 antiserum (upper half of Fig. 5C).

Surprisingly, when we immunoblotted crude lysates from the transfected cells with an anti-CD4 antibody, we observed that the mutants which failed to coimmunoprecipitate with TYK2 all migrated slower than the wild-type IFNaR1 (lower half of Fig. 5C). Further, a mutation (KP→AA) which does not affect binding (Fig. 1 and 3) produces a protein which migrates with the same apparent molecular weight as the wild-type protein (data not shown). Thus, we suspected that the inability of TYK2 to associate with the CD4-IFNaR1 chimera results in a posttranslational modification of the receptor. Treatment of cells with tunicamycin, which inhibits N-linked glycosylation, resulted in an apparent decrease in molecular weight for both the wild-type and mutant proteins, as expected. However, the mutants which affected binding all still migrated more slowly than wild-type CD4-IFNaR1 (data not shown). Thus, O-linked glycosylation or some other posttranslational modification may be altering the apparent mobility of those chimeras which do not bind TYK2.

IFNaR1 containing a mutant binding motif inhibits TYK2 and Stat tyrosine phosphorylation in response to IFNα treatment. To test the physiological relevance of our findings, we constructed genes encoding an epitope-tagged version of IFNaR1 as well as the mutant version with residues 504 to 506 (IIE) replaced by alanines and transiently transfected 293T cells with these constructs. After 2 days, cells were either treated with IFNα or left untreated and lysed, and a small

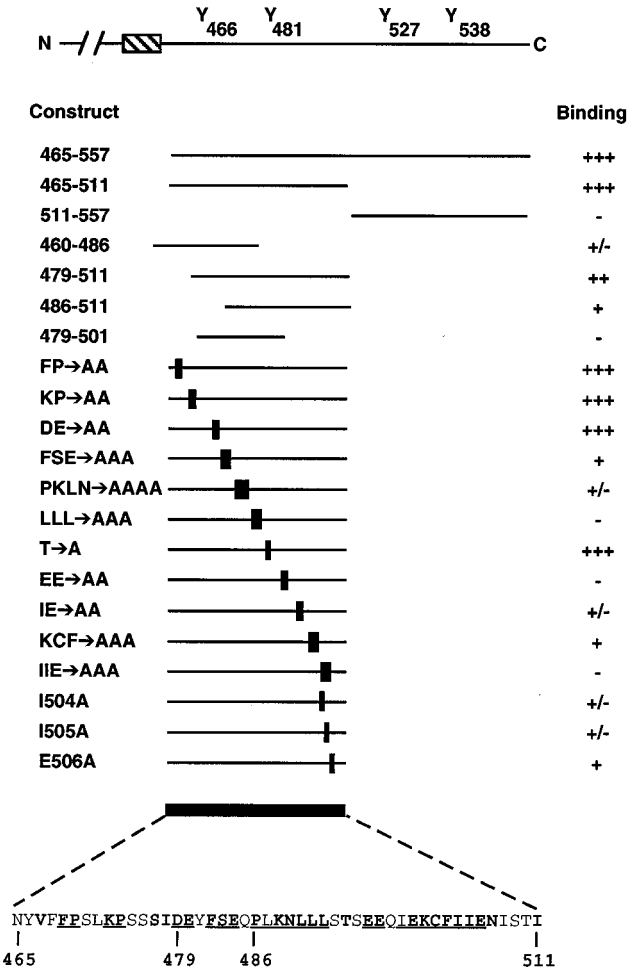

FIG. 2. Summary of TYK2 binding activity of various GST-IFNaR1 mutants. The figure summarizes the locations and binding activities of the truncation and site-specific mutants analyzed in Fig. 1. The top line illustrates the IFNaR1 protein, identifying the amino (N) and carboxy (C) termini, the transmembrane domain (cross-hatched box), and the relative positions (and amino acid numbers) of the four tyrosine residues in this domain. The central portion of the figure diagrams the portion of the receptor subunit contained in each construct, along with the designation (left-hand column) and binding activity (right-hand column) for each construct. The relative binding activity (− to +++) is based on visual assessment of band intensities in multiple separate experiments. Solid rectangles mark the position of alanine residues in each particular site-specific mutant. The lower portion of the figure shows the sequence of the maximal binding domain (residues 465 to 511). Residues which are identical or similar in bovine, murine, and human IFNaR1 are indicated in boldface type. The amino acid clusters which were changed to alanine are underlined. For the purposes of this figure, the following groups of amino acids are considered to be similar: V, L, and I; D and E; and K and R.

amount of each lysate was immunoblotted with an antibody against the epitope tag. This blot (data not shown) demonstrated similar levels of expression for the IFNaR1 constructs in all transfected cultures. In addition, the mutant and wild-type proteins are identical in size, in contrast to the chimeric molecules examined in Fig. 5. The remainder of each lysate was immunoprecipitated with anti-TYK2 antiserum and then assayed for tyrosine phosphorylation by immunoblotting with an antiphosphotyrosine antibody. IFNα-dependent tyrosine phosphorylation of TYK2 has been shown to correlate with activation of the kinase, as measured in immune complex reactions (4). We reasoned that overexpressed mutant IFNaR1 could act in a dominant-negative fashion to inhibit IFNα signaling transduction. As expected, TYK2 was tyrosine phosphorylated in response to IFNα in cells transfected with wild-type IFNaR1 (Fig. 6A, lanes 3 and 4) or with vector alone (lanes 1 and 2). However, IFNα-dependent TYK2 tyrosine phosphorylation was significantly inhibited in cells transfected with the mutant IFNaR1 (lanes 5 and 6). Reprobing of this filter with an anti-TYK2 antiserum confirmed that the protein was recovered with similar efficiency in all samples (Fig. 6B).

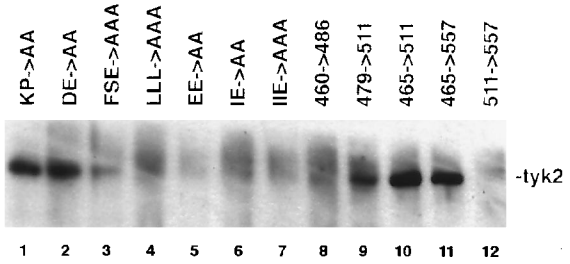

FIG. 3. TYK2 from HeLa cells binds to GST-IFNaR1 fusion proteins. A subset of the GST-IFNaR1 fusions used in Fig. 1, containing either truncated IFNaR1 or IFNaR1(465–511) with the indicated amino acids changed to alanine, were used to precipitate TYK2 from HeLa cell lysates, and the resulting complexes were immunoblotted with anti-TYK2 antibody, as in Fig. 1. The lower portion of the immunoblot was probed separately with an anti-GST antibody to verify that approximately equal amounts of fusion protein were present in each binding reaction (data not shown). The diagram in Fig. 2 identifies the positions of the constructs employed. The position of the 135-kDa TYK2 protein is indicated.

A similar transfection experiment was carried out, followed by immunoprecipitation with an anti-Stat2 antibody and antiphosphotyrosine immunoblot analysis. Figure 6C shows that the IFNaR1 protein containing a mutant TYK2 binding site but not the wild-type construct inhibited the IFNα-dependent phosphorylation of Stat2 and Stat1 (the latter coimmunoprecipitates with the former in treated cells). Figure 6D confirms that similar amounts of Stat2 protein were present on the blot filter.

Dimerized GST-IFNaR1(465–511) induces TYK2 and receptor tyrosine phosphorylation. To further demonstrate the biological role of the IFNaR1 TYK2 binding domain, we permeabilized HeLa cells with streptolysin O and introduced wild-type and mutant GST-IFNaR1 fusion proteins into the

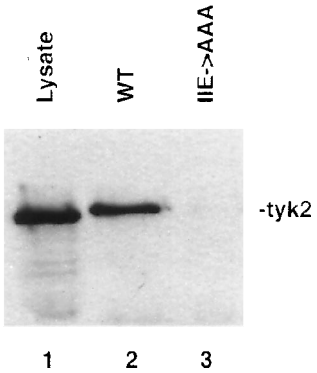

FIG. 4. A 33-amino-acid IFNaR1 peptide is sufficient to bind TYK2. Biotinylated peptides corresponding to IFNaR1 residues 479 to 511 (WT, lane 2; DEYFSEQPLKNL LLSTSEEQIEKCFIIENISTI) and a mutant version (IIE→AAA, lane 3; DEYFSEQPLKNLLLSTSEEQ IEKCFAAANISTI) were bound separately to strepavidin-linked beads and used to precipitate lysate containing baculovirus-expressed TYK2, and the resulting complexes were then immunoblotted with an anti-TYK2 antibody. Lysate from infected cells, corresponding to 20% of that used in lanes 2 and 3, was coelectrophoresed (lane 1).

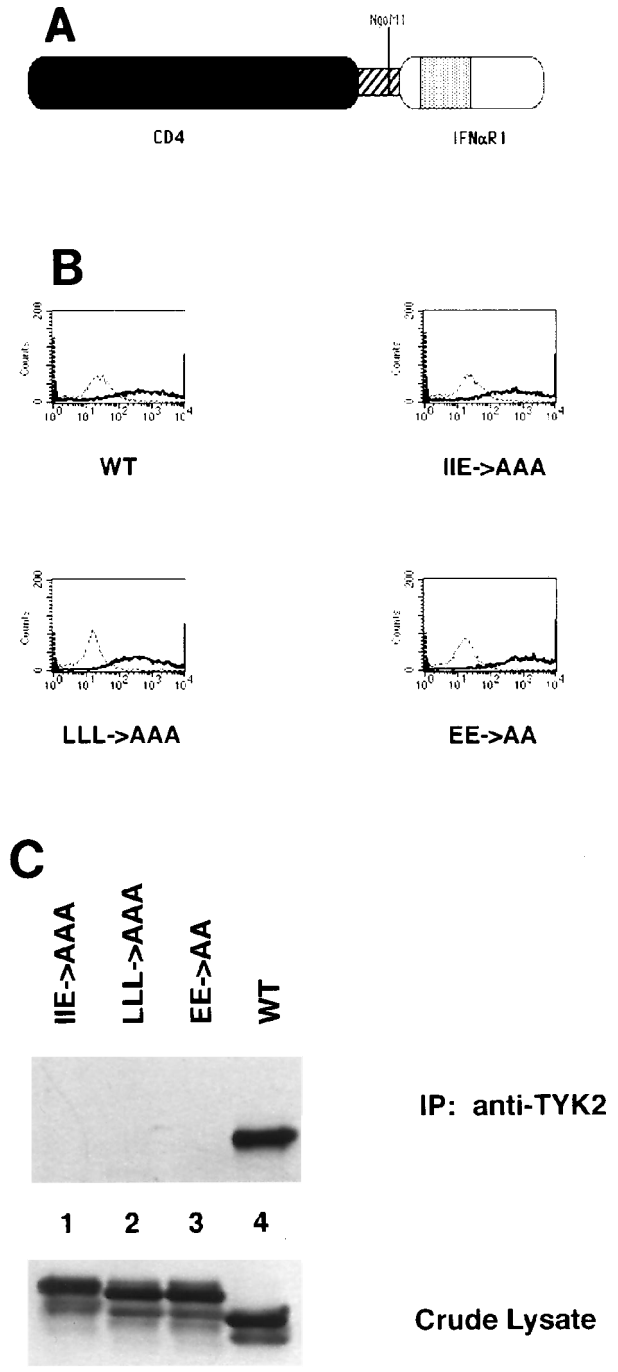

FIG. 5. Wild-type but not mutant CD4-IFNaR1 chimeras coimmunoprecipitate with TYK2. (A) Chimeric CD4-IFNaR1 structure. The sequences encoding the extracellular domain (black) as well as the amino-terminal half of the transmembrane domain (cross-hatched) of the CD4 gene were fused at an *Ngo*MI site to the sequences encoding the carboxy-terminal half of the IFNaR1 gene as well as the sequences encoding the cytoplasmic domain (white). The stippled gray box indicates the position of the maximal TYK2 binding site (residues 465 to 511). (B) FACS analysis of transfectants. Aliquots of transfected 293T cells were washed with PBS and then incubated with PE-conjugated monoclonal antibodies. FACS histograms, in which events (counts) are plotted against relative fluorescence intensity for each transfectant stained with either anti-CD4 (thick line) or anti-IgG2a (thin line) antibodies, are shown. Analysis of untransfected cells and of cells transfected with the vector alone showed an anti-CD4 curve which was identical to the anti-IgG2a curve seen in the histograms depicted. The diagrams are labeled with the terminology used in Fig. 2. Note that all of the chimeras contain the entire IFNaR1 cytoplasmic domain and not just the 465 to 511 domain. (C) Coimmunoprecipitation of CD4-IFNaR1 by anti-TYK2 antiserum. (Upper panel) Cells transfected with chimeric CD4-IFNaR1 molecules including either wild-type or mutant IFNaR1 cytoplasmic domains (as indicated) were lysed and immunoprecipitated (IP) by anti-TYK2 antibody, and the resulting complexes were immunoblotted with an anti-CD4 antibody. (Lower panel) A portion of the corresponding lysate for each of the samples analyzed in the upper panel was immunoblotted with an anti-CD4 antibody.

permeabilized cells. Following cell lysis, protein complexes were recovered with either anti-TYK2 antiserum or glutathione beads and immunoblotted with an antiphosphotyrosine antibody. Recently, we have demonstrated that GST fusion proteins containing the cytoplasmic domain of IFNaR1 can mimic receptor dimerization in permeabilized cells and activate TYK2, presumably because these fusion proteins exist as dimers in solution (57). Figure 7A confirms our previous observation by showing that wild-type GST-IFNaR1(465–511) is as effective as IFNα treatment in triggering the tyrosine phosphorylation of TYK2 (compare lanes 4 to 6). Introduction of the KP→AA mutant (lane 3), which displays wild-type TYK2 binding activity in vitro, into permeabilized cells also activates TYK2, while the IIE→AAA and LLL→AAA mutants, which do not bind TYK2 in vitro, had little or no effect (lanes 1 and 2). The filter shown in Fig. 7A was reprobed with an anti-TYK2 antibody to demonstrate that similar amounts of TYK2 were present in each lane of the filter (Fig. 7B).

Lysates from the permeabilized cultures analyzed in lanes 1 to 4 of Fig. 7A were also precipitated with glutathione beads and immunoblotted with an antiphosphotyrosine antibody. As shown in Fig. 7C, the two GST-IFNaR1 fusion proteins which activated TYK2 were tyrosine phosphorylated (lanes 3 and 4). In contrast, the IIE→AAA and LLL→AAA mutants did not induce any detectable phosphorylation (lanes 1 and 2). Figure 7D confirms that approximately equal amounts of the fusion proteins were recovered in each lane of Fig. 7C. Finally, when the upper portion of the filter shown in Fig. 7C was reprobed with an anti-TYK2 antibody, TYK2 coimmunoprecipitation with GST-IFNaR1(wild type) and GST-IFNaR1(KP→AA) was detected (Fig. 7E). These coimmunoprecipitation results correlate completely with those in Fig. 1. Thus, these data suggest that TYK2 can directly tyrosine phosphorylate IFNaR1 and that this activity depends on an intact TYK2 binding site on IFNaR1.

DISCUSSION

An elegant series of genetic and biochemical studies have identified the major components of the IFNα signaling pathway and provided an outline of how these proteins interact to stimulate specific gene transcription. The subsequent discovery that many other cytokines stimulate analogous JAK-Stat signaling pathways emphasizes the value of the IFNα pathway as a model for cytokine signaling. Rapid progress has been made in cataloging the association of the four mammalian JAK kinases with various members of the cytokine receptor family. Typically, a given receptor binds a specific kinase; gp130 and the β subunit of the LIF receptor are apparently notable exceptions in that they can bind multiple JAK kinases (31, 44). While there are a variety of different patterns of JAK association with cytokine receptor complexes (22), in all cases the net effect of ligand binding is believed to be dimerization or oligomerization of either one or two JAK kinases, thereby activating the kinase(s) and initiating signaling. Thus, the mechanism is similar to that employed by the receptor-type tyrosine kinases (43).

A number of critical molecular details remain to be determined, however, to fully understand how the JAK-Stat path-

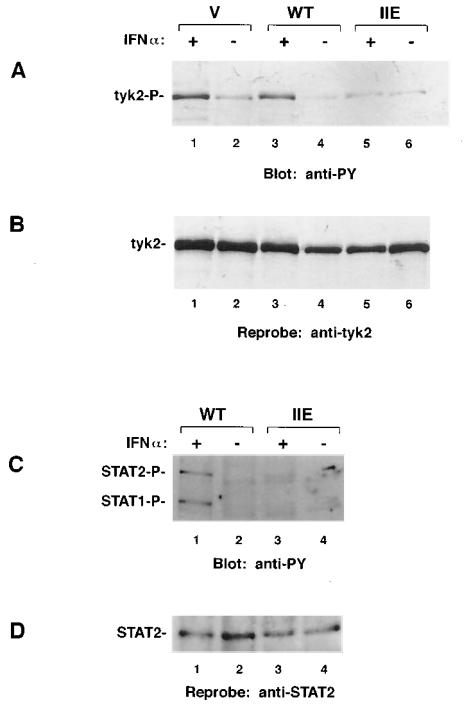

FIG. 6. Mutant IFNaR1 acts in a dominant-negative fashion to suppress IFNα-dependent tyrosine phosphorylation of TYK2, Stat1, and Stat2. (A) Cells transfected with cloning vector (V, lanes 1 and 2) or constructs encoding wild-type IFNaR1 (WT, lanes 3 and 4) or a TYK2 binding site mutant of IFNaR1 (IIE, lanes 5 and 6) were either treated for 15 min with 5,000 U of IFNα2 per ml (lanes +) or left untreated (lanes −), lysed, and immunoprecipitated with an anti-TYK2 antibody, and the resulting complexes were immunoblotted with an antiphosphotyrosine antibody. The position of phosphorylated TYK2 is indicated. (B) The filter shown in panel A was stripped and reprobed with a monoclonal anti-TYK2 antibody. The position of TYK2 is indicated. (C) Cells transfected with constructs encoding wild-type IFNaR1 (WT, lanes 1 and 2) or a TYK2 binding site mutant of IFNaR1 (IIE, lanes 3 and 4) were either treated for 15 min with 5,000 U of IFNα2 per ml (lanes +) or left untreated (lanes −), lysed, and immunoprecipitated with an anti-Stat2 antibody, and the resulting complexes were immunoblotted with an antiphosphotyrosine antibody. The positions of phosphorylated Stat2 and Stat1 are indicated. (D) The filter shown in panel C was stripped and reprobed with an anti-Stat2 antibody. The position of Stat2 is indicated.

ways operate. Most importantly, there is still no conclusive evidence that the JAK kinases directly phosphorylate the Stats. Our recent data demonstrate that tyrosine residue 466 on the IFNaR1 subunit of the receptor is an obligatory docking site for the SH2 domain of latent (unphosphorylated) Stat2 and suggest that this docking facilitates the phosphorylation of Stat2 by either the TYK2 or JAK1 tyrosine kinase (56). Since our preliminary studies (7) suggested that the TYK2 but not the JAK1 or the JAK2 kinases bind directly to the IFNaR1 subunit in a region that overlaps the Stat2 docking site, we have carried out a detailed characterization of this binding site to further our understanding of the molecular interactions required for the tyrosine phosphorylation of the JAK kinases and Stat transcription factors.

In this report, we demonstrate that a 33-amino-acid domain between residues 479 and 511 of IFNaR1 is entirely sufficient to mediate the binding of TYK2 to the receptor (Fig. 4). As we have previously argued (7), since the binding experiments were performed with insect cell lysates, which are unlikely to contain proteins related to other components of the IFNα signaling pathway, the binding is probably direct. This region is only 12 residues distal to the Stat2 binding site. A slightly shorter region (486 to 511) still retains low-level binding activity in vitro (Fig. 1). Within this minimal region, almost all of the phylogenetically conserved amino acid residues were required for binding, at least in the context of our assay protocol, in which two to four adjacent residues were converted as a group to alanine.

We also tested three of the alanine substitution mutations that prevent in vitro binding and found that all disrupted the ability of TYK2 to coimmunoprecipitate with a chimeric receptor containing the entire cytoplasmic domain of IFNaR1 (Fig. 5). A mutation that did not affect in vitro binding did not prevent coimmunoprecipitation. Thus, in vitro binding seems

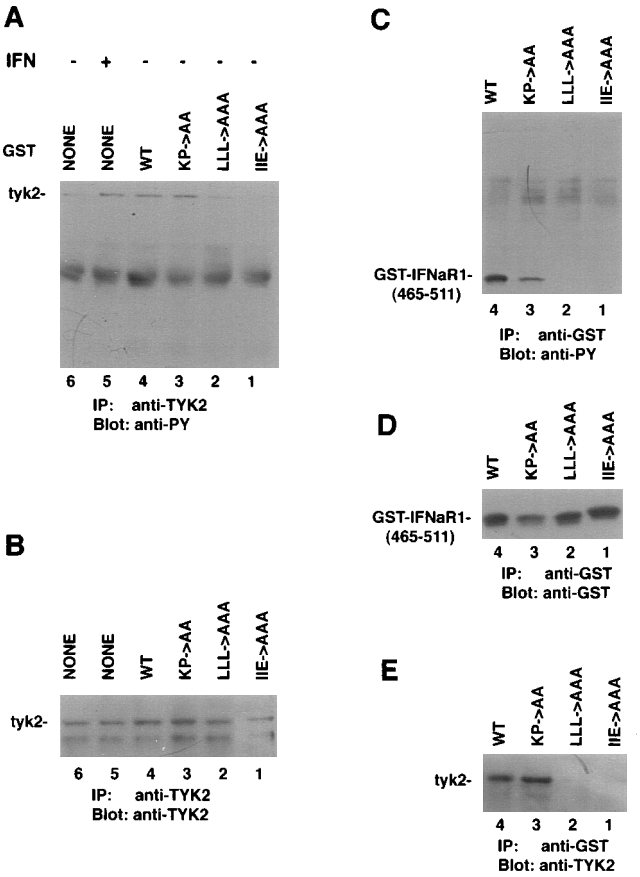

FIG. 7. The TYK2 binding domain is required for TYK2 activation and IFNaR1 phosphorylation in permeabilized cells. (A and B) Antiphosphotyrosine blot of permeabilized cells following the introduction of GST-IFNaR1 proteins. Aliquots of HeLa cells were permeabilized with streptolysin O and incubated in the presence of the specified GST-IFNaR1(465–511) protein (lanes 1 to 4) and the absence of IFNα2 (lanes −). Mock-permeabilized HeLa cells were either treated for 5 min with 20,000 U of IFNα2 per ml (lane 5) or left untreated (lane 6). Lysates were prepared and divided into two equal portions. (A) One aliquot was immunoprecipated with an anti-TYK2 antibody, and the resulting complexes were immunoblotted with an antiphosphotyrosine antibody. (B) The filter from panel A was stripped and reprobed with a monoclonal anti-TYK2 antibody. The position of TYK2 is indicated for each panel. (C to E) The remaining aliquot of the lysate prepared from the cells used in panel A, lanes 1 to 4, was precipitated with glutathione beads, and the resulting complexes were immunoblotted with an antiphosphotyrosine antibody (C). The position of the phosphorylated GST-IFNaR1 proteins is indicated. The filter used in panel C was then cut into two parts and stripped. The upper portion was reprobed with an anti-GST monoclonal antibody (panel D), and the lower portion was reprobed with an anti-TYK2 monoclonal antibody (panel E). In panels A and C, the coimmunoprecipitating tyrosine phosphoproteins (GST-IFNaR1 and TYK2, respectively) are not detected because of the relatively short exposure times. These bands are discernible upon overexposure of the filters (data not shown).

to accurately mirror the situation in live cells. When the protein levels of the chimeric receptors employed in the coimmunoprecipitation studies were examined by blotting, we observed that those mutants which did not bind TYK2 showed an altered mobility, with an apparent molecular mass that is ~10 kDa larger than either the wild type or a mutant which does not affect TYK2 binding (Fig. 5C). The molecular basis of this altered mobility is unclear, but it is likely a result of some posttranslational modification. While it is conceivable that the altered modification of the mutant receptors prevents binding of TYK2, we favor the idea that the IFNaR1 mutations disrupt TYK2 binding, with altered modification occurring as a secondary phenomenon. Our view is supported by the following observations: (i) the mutant proteins are expressed at wild-type levels; (ii) they are all found at similar levels on the cell surface; (iii) there is complete concordance between the failure to bind in vivo and in vitro; and (iv) the same mutations in the full-length IFNaR1 proteins (used in the experiments shown in Fig. 6) do not produce an alteration in mobility.

Maximal binding is achieved with a GST fusion protein spanning residues 465 to 511 (Fig. 1 and 3). One of the key differences between the minimal and maximal binding sites is the presence of a proline-containing region (FPSLKP) which bears a resemblance to box 1 sequences (7). Conversion of the two proline residues in this region to alanine does not produce a detectable alteration in the in vitro TYK2 binding activity of the 465 to 511 domain (Fig. 1 and 3). Thus, the box 1-like sequences in the IFNaR1 subunit appear to play only a minor role in TYK2 binding. In contrast, the box 1 region has been implicated in JAK kinase binding for a number of cytokine receptors (31, 37, 47, 51). In the cases examined in most detail, the box 1 sequence was shown to be necessary for JAK association in mammalian cells after being either deleted (47), removed by truncation (31), or eliminated by converting four prolines to alanine (51). Mutation of any single proline residue in the gp130 box 1 sequence has either no effect or only a modest effect on JAK2 binding (47). Thus, it appears that the role of the box 1 sequence is apparent only when a substantial alteration that may well disrupt the conformation of upstream or downstream sequences is made in the protein sequence.

In no case was the box 1 site alone shown to be sufficient for JAK binding (47), although in the case of gp130, a 26-amino-acid juxtamembrane region including a box 1 sequence was sufficient for JAK kinase coimmunoprecipitation (31). Interestingly, the JAK1 binding site in the α subunit of the IFNγ receptor does contain a critical proline residue (17), but there is no obvious box 1 type sequence anywhere in the cytoplasmic domain of this subunit. However, the GM-CSF receptor, which also binds JAK1, does contain a box 1 sequence which is required for binding (37). Finally, the murine and bovine IFNaR1 proteins both contain additional proline residues relative to the human homolog in the box 1-like region (26, 48). Apparently these prolines are not required for binding. Thus, it appears that a proline-rich sequence is not universally critical to the structure of a generic JAK kinase binding site. Whether the box 1 sequences will prove to be the most important and/or only critical motif within the JAK kinase binding domains of other cytokine receptors awaits a more comprehensive definition and mutational analysis of these regions.

The data presented here rigorously define a minimal region of a cytokine receptor that is sufficient for JAK kinase binding. Furthermore, this report is the first systematic analysis of the residues required for binding within this region. Many of the critical residues contain either hydrophobic or acidic side chains (see summary in Fig. 2). As described in the introduction, box 2 is a second conserved domain found in the membrane-proximal region of many cytokine receptors. While the IFNaR1 subunit does not have a region that clearly resembles box 2, it may be noteworthy that one of the amino acid clusters which is critical for TYK2 binding (IIE, corresponding to positions 504 to 506) is found at the same relative position within the box 2 sequences of gp130 (VVE), the LIF receptor β subunit (VLE), and the G-CSF receptor (VLE) (16, 30). Again, systematic mutagenesis studies of these other cytokine receptors will be required to verify the importance of this short motif.

In addition to comprehensively defining the structural requirements for TYK2 binding in vitro and in vivo, our studies also demonstrate that the binding domain is necessary for IFNα signal transduction and that it appears to be wholly sufficient for the first steps of the process. Specifically, when a mutation which prevents TYK2-IFNaR1 binding is incorporated into the full-length receptor and introduced into cells, the resulting protein acts in a dominant negative fashion to inhibit the initial events in IFNα signaling (Fig. 6). Previously, genetic complementation experiments established that TYK2 is required for Stat-dependent gene expression (52). Subsequent biochemical experiments demonstrated that IFNα treatment induces both tyrosine phosphorylation and activation of the kinase (4, 8). These data refine our understanding of the role of the TYK2 kinase in the pathway by demonstrating that TYK2 must be physically associated with the receptor in order for both TYK2 and Stat2 tyrosine phosphorylation events to occur.

The data in Fig. 7 demonstrate that the IFNaR1 domain spanning residues 465 to 511 is sufficient to mediate the dimerization-induced activation of the TYK2 kinase and to promote tyrosine phosphorylation of the receptor itself, further supporting the critical role for this domain in initiating signal transduction. We previously demonstrated that the IFNaR1 sequence itself is not capable of dimerization but that instead the process is mediated by the GST moiety (57). It is probable that kinase and receptor phosphorylations are the very first events that follow ligand binding. The stoichiometry of the IFNα receptor complex remains unknown, and it is therefore unclear if homodimerization of IFNaR1 is a physiological event. In fact, genetic complementation studies suggest that the TYK2 and JAK1 kinases probably *trans* phosphorylate (29), and this is likely to be the key event in activating these two kinases. Similarly, our experiments cannot determine if TYK2 is the kinase which directly phosphorylates IFNaR1 under physiologic circumstances. However, we believe that the net effect of GST-induced dimerization—activation of IFNaR1-bound TYK2—approximates the outcome of ligand-mediated receptor clustering and supports our contention that residues 465 to 511 are sufficient to mediate TYK2 activation.

In conclusion, the present study establishes the structural characteristics and functional role of the TYK2 binding site on the IFNaR1 subunit of the IFNα receptor. Although the present study clearly demonstrates that TYK2 must bind IFNaR1 for productive signaling, the immediate substrate(s) of this kinase remains uncertain. Specifically, it remains to be determined if TYK2 directly phosphorylates JAK1, IFNaR1, and/or the Stats. Further investigation, including characterizing the interaction between IFNaR2 and the JAK1 kinase and determining if and how these two receptor-kinase pairs can reconstitute signaling, will be required to fully understand how the JAK kinases initiate signaling in response to this prototypical cytokine.

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