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Specific Contribution of Tyk2 JH Regions to the Binding and the Expression of the Interferon α/β Receptor Component IFNAR1*

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Cytokine signaling involves the activation of the Janus kinase (JAK) family of tyrosine kinases. These enzymes are physically associated with cytokine receptor components. Here, we sought to define the molecular basis of the interaction between Tyk2 and IFNAR1, a component of the interferon α/β receptor, by delimiting a minimal IFNAR1 binding region in the Tyk2 protein. Using an *in vitro* assay system, we narrowed down the interaction domain to a region comprising the JH7 and part of the JH6 homology boxes (amino acids 22–221). When expressed in Tyk2-negative cells, the JH7-6 region was unable to stabilize IFNAR1 protein levels, a critical function that we previously attributed to the N region (amino acids 1–591) of Tyk2. Moreover, substitution of the JH7-JH6 domain in JAK1 with that of Tyk2 did not restore IFNAR1 level nor interferon α signaling in Tyk2-negative cells. Thus, the major interaction surface lies within JH7-6, but additional JH regions (JH5-4-3) contribute in a specific manner to the *in vivo* assembly of Tyk2 and IFNAR1. Evidence is also provided of the lack of specificity of the Tyk2 kinase-like and tyrosine kinase domains in interferon α/β receptor signaling.

The Janus kinase (JAK)¹ family of non-receptor tyrosine kinases consists of four mammalian proteins (Tyk2, JAK1, JAK2, and JAK3) that play a critical role in initiating signaling cascades of a large number of cytokine receptors (1, 2). All JAK proteins possess a carboxyl-terminal tyrosine kinase (TK) catalytic domain, a central kinase-like (KL) domain, and a large amino-terminal (N) region, which has been subdivided into five JAK homology regions (JH7 to JH3) based on sequence conservation (3). The specific and noncovalent association of these

kinases to the intracellular region of cytokine receptors governs their activation upon ligand binding (2). We are interested in understanding the mode of action and specific roles of Tyk2, which is activated, together with another JAK family member, by the type I interferons (IFN) (several α and one β subtypes), by interleukin (IL) 6, IL-10, and IL-12 (4–9).

The IFN- α/β receptor is present at low numbers on the surface of all cell types and consists of two transmembrane proteins called IFNAR1 and IFNAR2 (10, 11). The IFNAR2 gene generates several alternatively spliced forms, but only the product harboring a long intracytoplasmic domain (IFNAR2c) is part of a functional IFN- α/β receptor (12). Whereas the stoichiometry and spatial organization of these components within the receptor complex are unknown, the epitopes on the IFN molecule contacting IFNAR1 and IFNAR2 are being identified (13). High affinity binding of IFN- α/β to the receptor results in tyrosine phosphorylation and enzymatic activation of the associated JAK1 and Tyk2 in a defined temporal order, which is thought to result from the topology of each kinase within the complex (14, 15). Studies of kinase-deficient mutant cell lines showed that in the absence of either kinase, high affinity IFN- α binding is impaired, demonstrating a structural role of these enzymes in the formation of functional receptors (4, 16, 17). Our recent *in vivo* studies of deleted forms of Tyk2 expressed in Tyk2-deficient 11,1 cells have highlighted distinct functions of the protein toward the expression and the binding activity of the receptor complex. Each function appears to be contributed by a different domain adding more complexity to the receptor-kinase complex. The N region, previously defined as the amino-terminal 591 residues and comprising the JH7 to JH3 regions (Fig. 2), maintains the steady-state level of the IFNAR1 protein in the cell. The kinase-like domain contributes to the formation of high affinity receptor binding sites, and the tyrosine kinase domain is essential for optimal binding and signaling function (14, 18, 19).

That Tyk2 interacts physically with IFNAR1 was suggested by co-immunoprecipitation of the two endogenous proteins from cell extracts and by retention of baculovirus-expressed Tyk2 by a fusion protein bearing the cytoplasmic domain of IFNAR1 (20, 21). It was shown that the 45 membrane-proximal amino acids of IFNAR1 were necessary and sufficient for this interaction and that Ala substitution of three critical residues (Ile-Ile-Glu) disrupted this interaction (22). Because *in vivo* co-immunoprecipitation studies in reconstituted Tyk2-negative cells are hampered by the modulation of IFNAR1 level by Tyk2 itself (19), an *in vitro* system was used here to identify the molecular determinant(s) of Tyk2 which govern its interaction with IFNAR1. Using carboxyl- and amino-terminal deletion mutants of N, we delimited a region including JH7 and part of JH6 that is sufficient to bind recombinant IFNAR1. However, this region alone could not sustain IFNAR1 in Tyk2-negative cells, showing a requirement for additional JH boxes (JH5-4-3).

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¹ The abbreviations used are: JAK, Janus kinase; KL, kinase-like; TK, tyrosine kinase; IFN, interferon; IFNAR, interferon α receptor; GST, glutathione S-transferase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; HAT, hypoxanthine/aminopterin/thymidine; STAT, signal transducer activator of transcription; VSV-G, vesicular stomatitis virus glycoprotein; kb, kilobase pair(s); WT, wild-type; IL, interleukin; PCR, polymerase chain reaction.

Thus, to address the specificity, if any, of this additional region, a number of Tyk2/JAK1 chimeric constructs were generated. Of four chimeras bearing different JH domains of Tyk2 fused to JAK1, only one could rescue IFNAR1 protein levels and IFN- α signaling. This functional chimera contains the entire N region of Tyk2 fused to the KL and TK domains of JAK1. These results will be discussed in the light of recent reports on other JAK/receptor pairs.

MATERIALS AND METHODS

Plasmids

Histidine-tagged Constructs—All constructs were made in pQE-based expression vectors (Qiagen). To generate plasmid pHis-N-(1–451), an *SphI* fragment was released from plasmid bs-Tyk2 (human Tyk2 cDNA in pBluescript) and cloned into pQE10. pHis-N-(1–451) encodes p53 (Fig. 2) containing the amino-terminal sequence Met-Arg-Gly-Ser-(His)₆-Thr-Asp-Pro-Arg fused to Tyk2 amino acids 1–451 and the carboxyl-terminal Arg-Arg-Pro-Ala-Ala-Lys-Leu-Asn sequence. pHis-N-(1–591) was obtained by inserting a 140-base pair *SacII/EcoRI* fragment spanning amino acids 452–591 into the *SacII-HindIII* blunt-ended pHis-N-(1–451) DNA. pHis-N-(1–591) encodes p69 (Fig. 2) with the (His)₆ sequence (see above) fused to Tyk2 amino acids 1–591 and the carboxyl-terminal Ile-Glu-Leu-Ala sequence. pHis-N-(1–385), pHis-N-(1–369), pHis-N-(1–314), and pHis-N-(1–220) were generated by digesting pHis-N-(1–451) with *PstI* and either *PmlI*, *SacII*, *DraIII*, or *NaeI*, blunting the ends and re-circularizing each product. Due to the cloning procedure, the resulting proteins contain between 3 and 5 extra carboxyl-terminal amino acids. The three amino-terminal deletion mutants were derived from pHis-N-(1–385) (p46 in Fig. 2). A fragment encoding amino acids 22–385 of Tyk2 was amplified by PCR using primers with appropriate restriction sites and was ligated into *BamHI-SphI*-digested pHis-N-(1–385) to generate pHis-N-(22–385). The resulting protein ($\Delta 21$ in Fig. 2) contains the amino-terminal His sequence (see above) fused to Tyk2 amino acids 22–385 and to Ala-Lys-Leu-Asn. pHis-N-(1–385) was digested with *StuI* and *HindIII*, the resulting 1.1-kb fragment was ligated to the *HincII-HindIII*-digested pQE10 vector to generate pHis-N-(28–385). The resulting protein ($\Delta 27$ in Fig. 2) contains the amino-terminal His sequence fused to Tyk2 amino acids 28–385 of Tyk2 and Ala-Lys-Leu-Asn. pHis-N-(1–385) was digested with *PvuII* and *HindIII*, and the resulting 1-kb fragment was ligated to the *HincII-HindIII*-digested pQE11 vector to generate pHis-N-(54–385). The protein ($\Delta 53$ in Fig. 2) contains the amino-terminal His sequence fused to Tyk2 amino acids 54–385 and Ala-Lys-Leu-Asn.

GST Fusion Constructs—The cytoplasmic domain of the human IFNAR1 (amino acids 458 to 557) cloned into the bacterial pGEX-2T expression vector was provided by L. Ling (Biogen Inc., Boston, MA). The Ile-Ile-Glu \rightarrow Ala-Ala-Ala mutant (amino acids 504–506) was obtained by two-step PCR-mediated mutagenesis. The GST-Ik β β chain construct was a gift from R. Weil.

Eucaryotic Expression Constructs—All final expression vectors were cloned into the pRc/CMVneo vector (Invitrogen). To generate $\Delta 1$ –21, a *SalI* fragment of bs-Tyk2 was substituted with a *SalI* PCR fragment spanning Tyk2 amino acids 22–170. The plasmid $\Delta 1$ –51 has been described elsewhere (19). Full-length human JAK1 cDNA (a gift of A. Wilks) was cloned into bs (bs-JAK1), and an oligonucleotide encoding an epitope of the vesicular stomatitis virus glycoprotein (VSV-G) was added at the 3' end. bs-T-(1–62)-J encodes amino acids 1–62 of Tyk2 fused to amino acids 55–1142 of JAK1. It was generated by amplifying a fragment spanning Tyk2 amino acids 1–62 with a *NotI* site at the 5' end and a *SphI* site at the 3' end and ligating it into *NotI-SphI*-digested bs-JAK1. bs-T-(1–275)-J encodes Tyk2 amino acids 1–275 fused to amino acids 275–1142 of JAK1. It was generated by amplifying a fragment spanning amino acids 1–275 of Tyk2 fused to amino acids 275–502 of JAK1, to which a *NotI* site was added at the 5' end and which included a single *RsrII* site at the 3' end. The resulting *NotI-RsrII* fragment was ligated to *NotI-RsrII*-digested bs-JAK1. bs-T-(1–518)-J encodes Tyk2 amino acids 1–518 fused to JAK1 amino acids 500–1142. It was generated by digesting bs-T-(1–275)-J with *RsrII* and partially with *XhoI* and ligating it to an *XhoI-RsrII* PCR fragment spanning amino acids 271–502 of Tyk2. bs-T-(1–581)-J encodes Tyk2 amino acids 1–581 fused to amino acids 564–1142 of JAK1. It was generated by digesting bs-T-(1–518)-J with *SphI* and *NsiI* and ligating it with a *SphI-NsiI*-digested PCR fragment encoding amino acids 496–581 of Tyk2 fused to amino acids 564–761 of JAK1. The sequences of the oligonucleotides used for PCRs are available upon request. All chimeric constructs contain 20 nucleotides of 5'-untranslated Tyk2 sequences

and the VSV-G epitope at the 3' end. After sequencing (370A DNA Sequencer, Applied Biosystems), all resulting cDNAs were introduced into the pRc/CMV vector.

Protein Purification and Interaction Assay

Histidine-tagged proteins were expressed in bacteria, purified on nickel-nitrilotriacetic acid (Ni²⁺-NTA) agarose beads according to the manufacturer's protocol (Qiagen), eluted, and dialyzed against 50 mM Tris-HCl, pH 8, 100 mM KCl, 5 mM MgCl₂, 20% glycerol, and 0.1% Nonidet P-40. GST fusion proteins were affinity-purified on glutathione-Sepharose (Amersham Pharmacia Biotech), eluted with 50 mM Tris-HCl, pH 8, 10% glycerol, 10 mM glutathione, and stored at -80°C . Approximately 10 to 100 pmol of each freshly purified recombinant protein were incubated in 1 \times phosphate-buffered saline for 60 min at 4 $^{\circ}\text{C}$ in a total volume of 1 ml. Fifty μl of glutathione-Sepharose beads as a 50% slurry were added. Beads were pelleted and washed four times in 1 \times phosphate-buffered saline, 0.5 M NaCl. Bound proteins were boiled in Laemmli sample buffer, separated by SDS-PAGE, and analyzed by Coomassie staining or immunoblotting with the appropriate antibody. Whenever Ni²⁺-NTA agarose beads were used, binding was performed in 10 mM HEPES-NaOH, pH 7.4, 100 mM KCl, 1 mM MgCl₂, 0.1% Triton X-100, and 2 mM imidazole.

Cell Culture

The mutant cell line 11,1 (also called U1A) has been described (16). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 250 $\mu\text{g/ml}$ hygromycin. Calcium-phosphate/DNA transfection and selection in G418 (450 $\mu\text{g/ml}$) was performed as described (18). Cell survival in hypoxanthine/aminopterin/thymidine (HAT) or in 6-thioguanine (6TG) containing media was assayed in the presence of different concentrations of IFN- $\alpha 2$ (human recombinant IFN- $\alpha 2b$, kindly provided by D. Gewert, Wellcome). Binding experiments with ¹²⁵I-IFN- $\alpha 2c$ were performed as described (19).

Antibodies, Immunoprecipitation, and Immunoblotting

The GST mAb used in Western blot was kindly provided by Hybridolab (Institut Pasteur). The histidine-tag mAb was purchased (Dianova). Tyk2 antibodies used for immunoprecipitation and Western blotting (R5 and T10–2) have been described (19). Anti-IFNAR1 mAb EA12 and GB8 were from Biogen Inc. The anti-phosphotyrosine 4G10 mAb was from Upstate Biotechnology, Inc. The VSV-tag mAb was purchased from Sigma. Preparation of cell extracts, immunoprecipitations, and Western blot analysis were performed as described previously (14).

RESULTS

In Vitro Interaction of N and IFNAR1cyt—An *in vitro* interaction assay was set up to identify an IFNAR1 binding domain within the first 591 amino acids (the N region) of Tyk2. Histidine-tagged N was expressed in *Escherichia coli* and purified as a 69-kDa protein (p69) on Ni²⁺-NTA-agarose beads. The cytoplasmic domain of IFNAR1 was expressed as GST fusion protein (GST-IFNAR1cyt) and purified by glutathione-Sepharose affinity chromatography. The two proteins were co-incubated, and the complex retained on Ni²⁺-NTA-agarose beads was analyzed by SDS-PAGE and Coomassie staining. GST-IFNAR1cyt was retained on beads in the presence, but not in the absence, of p69 (Fig. 1A, lanes 2 and 3).

In subsequent experiments, proteins retained on beads were analyzed by Western blotting with the Tyk2 or the GST mAbs. In Fig. 1B, p69 was incubated with either the GST-IFNAR1cyt protein, a mutated version of it in which residues 504 to 506 (Ile-Ile-Glu) were replaced by alanines (22), or with a control fusion protein (GST-Ik β). The material retained on glutathione-Sepharose beads was analyzed with Tyk2 mAb. p69 bound to GST-IFNAR1cyt and to a much lesser extent to the mutated version, albeit it did not bind to GST-Ik β (Fig. 1B, lanes 1–3). To confirm this interaction, the reverse experiment was performed, where Ni²⁺-NTA-agarose beads were used to pull down the complex and the GST mAb was used for blotting: GST-IFNAR1cyt was able to interact with p69, but not with a control His-MxA protein (Fig. 1C). Taken together, these results show

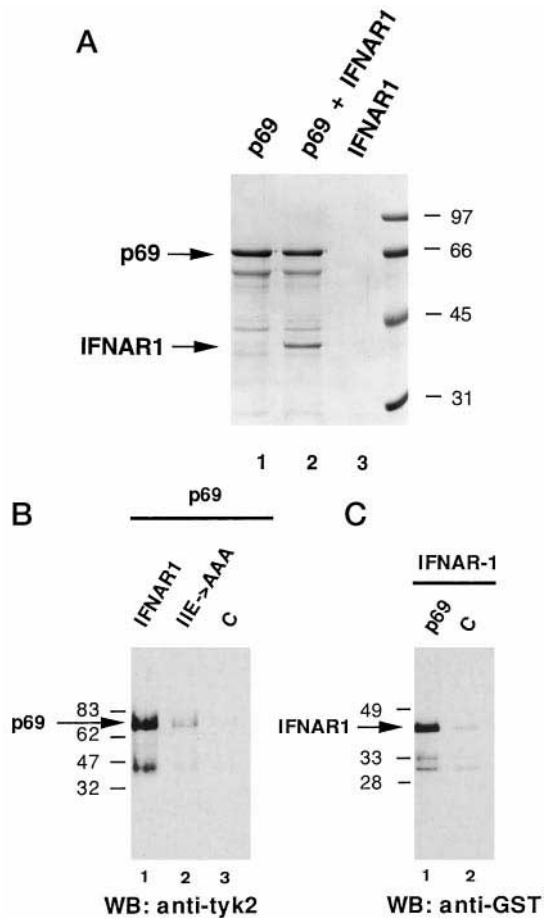


FIG. 1. *In vitro* interaction of His-N with GST-IFNAR1cyt. A, His-tagged N encoding Tyk2 amino acids 1–591 (p69) was incubated without (*lane 1*) or with a GST fusion protein (IFNAR1) containing the cytoplasmic domain of IFNAR1 (*lane 2*). Proteins bound to nickel beads were separated on SDS-PAGE and visualized by Coomassie staining. Molecular size markers are on the *right*. The 60-kDa species is a contaminant that binds consistently to the resin (*lanes 1* and *2*) and is not recognized by Tyk2 antibodies (see *B*). B, His-tagged N (p69) was incubated with wild-type GST-IFNAR1cyt (*lane 1*), with a mutated version of GST-IFNAR1cyt (IIE → AAA, *lane 2*) or with C, a control GST-Ik β fusion (*lane 3*). Proteins retained on glutathione-Sepharose were analyzed by Western blotting using Tyk2 mAb. The 40-kDa band reacting with the antibody (*lane 1*) was microsequenced and found to be a carboxyl-terminal degradation product of p69. C, GST-IFNAR1cyt (IFNAR1) was incubated with His-tagged N (p69) or with a control His-tagged MxA protein (C), and proteins retained on nickel beads were analyzed by Western blotting using a GST mAb.

that the N region of Tyk2 interacts specifically with the cytoplasmic domain of IFNAR1 *in vitro*. This interaction is impaired by alanine substitution of three IFNAR1 residues that were previously shown to be critical for optimal Tyk2 binding (22).

The JH7-6 Region of N Interacts with IFNAR1cyt *In Vitro*—To initially delimit an IFNAR1 binding domain within N, five carboxyl-terminal truncated versions of His-N were generated and named according to their apparent molecular weights (Fig. 2). Each protein was tested for its ability to bind GST-IFNAR1cyt. A glutathione-Sepharose-based interaction assay was performed, and the bound material was analyzed by Western blotting with the Tyk2 mAb or, for the three shorter versions, lacking the Tyk2 epitope, with a commercial His-tag mAb (Fig. 3). All five carboxyl-terminal deleted versions of p69 retained the ability to interact with GST-IFNAR1cyt (Fig. 3, *lanes 1, 3, 5, 7, 9, and 11*), whereas they did not interact with the control GST-Ik β fusion protein (Fig. 3, *lanes 2, 4, 6, 8, 10,*

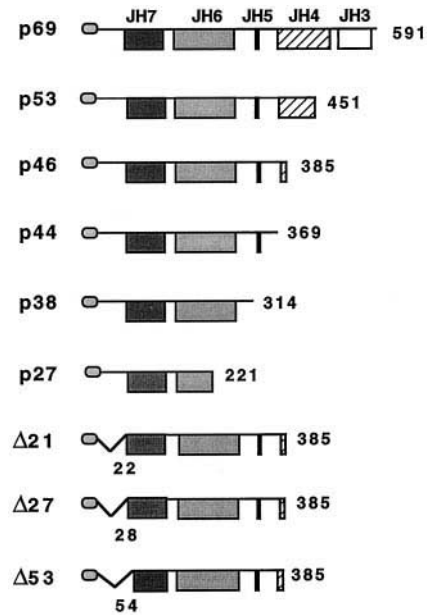


FIG. 2. Schematic diagram of His-N (p69) and derived carboxyl- and amino-terminal deletion mutants. Carboxyl-terminal deletion constructs were named according to their apparent molecular weights. Three amino-terminal deletions ($\Delta 21$, $\Delta 27$, $\Delta 53$) were derived from p46. Boxes indicate JAK homology regions (JH). The (His) $_6$ -containing tag is indicated at the amino terminus of each construct.

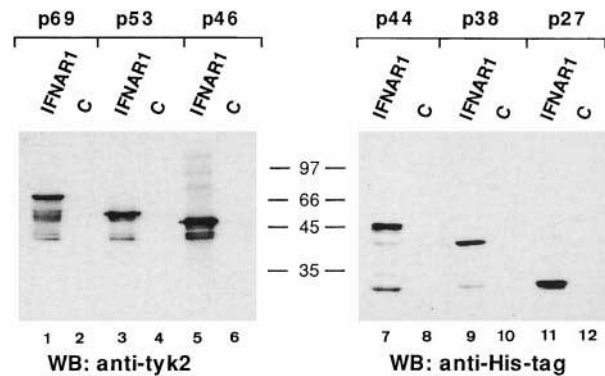


FIG. 3. Ability of carboxyl-terminal deleted forms of His-N to bind GST-IFNAR1cyt. The indicated His-tagged proteins were incubated with GST-IFNAR1cyt (IFNAR1) or with a GST-Ik β fusion control (C). Proteins retained on glutathione-Sepharose were analyzed by Western blot with the Tyk2 mAb (*left panel*) or with an antibody specific for the His-tag (*right panel*).

and 12). This result was confirmed upon using the Ni $^{2+}$ -NTA resin-based assay (Fig. 4A). Thus, the amino-terminal 221 residues of N, comprising JH7 and part of JH6, retain the ability to interact with IFNAR1cyt.

To delimit the amino-terminal boundary of this binding domain, three amino-terminal deletions were generated from the p46 protein, lacking 21, 27, and 53 amino acids, respectively ($\Delta 21$, $\Delta 27$, and $\Delta 53$ in Fig. 2). The purified proteins were tested for their ability to interact with the wild-type or the mutant GST-IFNAR1cyt in pull-down experiments using either of the two resins. $\Delta 27$ and $\Delta 53$ did not interact with GST-IFNAR1cyt (Fig. 4A, *lanes 5* and *7* and Fig. 4B, *lane 2*), whereas, as shown above, p46 and p27 bound specifically to wild-type GST-IFNAR1cyt (Fig. 4A, *lanes 1* and *3*). On the other hand, the $\Delta 21$ fusion protein retained IFNAR1 binding capacity (Fig. 4B, *lane 1*). These results demonstrate that the first 21 amino acids are dispensable for the *in vitro* binding of N to IFNAR1 and that the binding domain boundary is situated between residues 22 and 28.

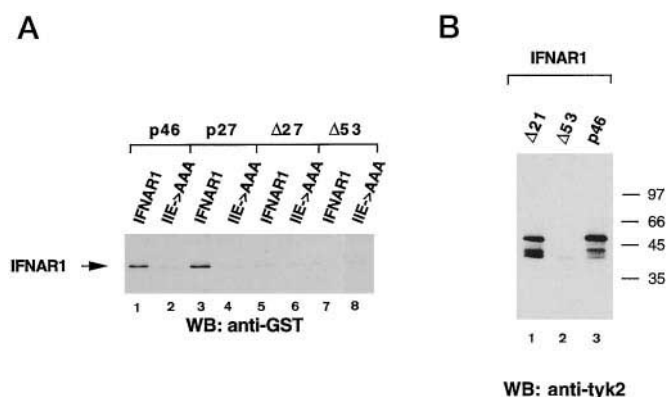


FIG. 4. Ability of amino-terminal deleted forms of His-N to bind GST-IFNAR1cyt. *A*, the indicated His-tagged proteins were incubated with the wild-type (IFNAR1) or the mutated (IIE → AAA) version of GST-IFNAR1cyt. Proteins retained on nickel beads were analyzed by Western blot with GST antibody. *B*, the indicated His-tagged proteins were incubated with GST-IFNAR1cyt. Proteins retained on glutathione-Sepharose were analyzed by Western blotting with the Tyk2 mAb.

Tyk2 Lacking Amino Acids 1–21 Is Able to Restore Signaling in 11,1 Cells—To correlate the *in vitro* binding results (Fig. 4) with the function of the native protein in cells, we stably expressed in Tyk2-negative cells a mutant form derived from the full-length Tyk2 and lacking amino acids 1–21 (Δ 1–21). Two independent *neo*^r transfectants were studied. As a measure of *in vivo* Tyk2 function, we first analyzed the levels of endogenous IFNAR1. The Δ 1–21 clones were compared with WT cells expressing wild-type Tyk2, to Δ 1–51 cells expressing a Tyk2 deleted of residues 1–51 (19), and to 11,1 cells. As can be seen in Fig. 5A, unlike Δ 1–51, expression of Δ 1–21 restored IFNAR1. In light of the different *in vitro* IFNAR1cyt interaction capacity of Δ 21 and Δ 53, this result is likely to reflect the different ability of the two deletion mutants to physically interact with IFNAR1 *in vivo*. We also measured the IFN- α binding activity of Δ 1–21 expressing cells, using WT and 11,1 cells as controls. As shown in Fig. 5B, iodinated IFN- α 2 bound similarly to WT and to Δ 1–21 expressing cells, whereas it did not bind to 11,1 cells or to Δ 1–51 expressing cells (see also Fig. 5 in Ref. 19). Furthermore, no difference in the sensitivity to IFN- α could be measured between the Δ 1–21 clones and WT cells upon testing their phenotype in HAT or 6TG media (18) (data not shown). Thus, we conclude that the amino-terminal 21 residues of Tyk2 are dispensable not only for the *in vitro* binding of N to IFNAR1 but also for the *in vivo* structural and signaling functions of Tyk2 through the IFN- α/β receptor.

Tyk2 JH5-4-3 Are Specifically Required to Sustain IFNAR1—The recombinant p27 protein, lacking JH5-4-3, was as effective as full-length p69 in complexing with IFNAR1 *in vitro* (Figs. 3 and 4B). On the other hand, previous work showed that deletion of JH4 and JH3 abrogated the ability of N to sustain endogenous IFNAR1 in 11,1 cells (Fig. 4 in Ref. 19). Altogether, these results indicate that the JH5-4-3 segment plays an essential function in the *in vivo* assembly of Tyk2 with IFNAR1. We therefore addressed the question of the specificity of this segment by generating chimeric constructs in which amino-terminal portions of Tyk2 were swapped into JAK1 (Fig. 6A). In each chimera, the fusion was made within a stretch of identical residues so as to maintain the integrity of highly conserved regions (see “Materials and Methods”). Wild-type Tyk2, wild-type JAK1, and the four chimeras were independently transfected into 11,1 cells. The level of exogenous protein was measured in *neo*^r clones by Western blotting with an antibody specific for the carboxyl-terminal VSV-G epitope tag present in each construct (data not shown). Clones expressing comparable

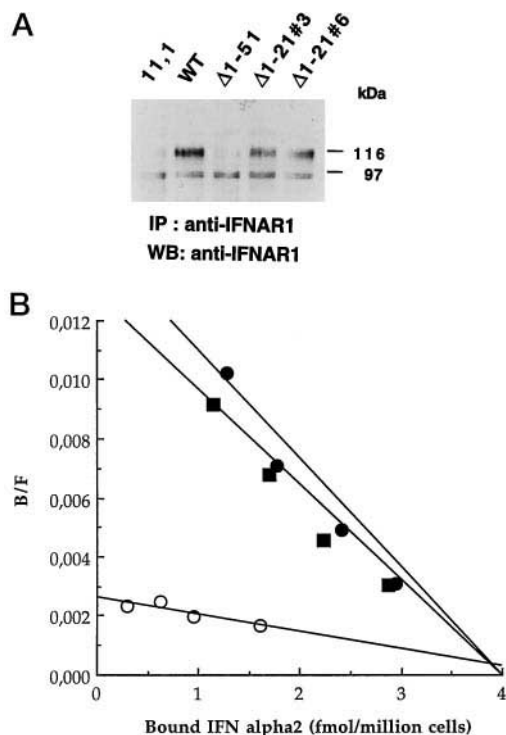


FIG. 5. Level of IFNAR1 and IFN- α binding in 11,1 cells and stable derivatives. 11,1 cells and *neo*^r clones expressing comparable levels of wild-type Tyk2 (WT), of mutant Δ 1–51, or of mutant Δ 1–21 (two clones) were analyzed. *A*, post-nuclear lysates (1 mg) were immunoprecipitated with the IFNAR1 mAb EA12, fractionated on SDS-7% polyacrylamide gel, and blotted with IFNAR1 mAb GB8. *B*, Scatchard plot of direct binding of ¹²⁵I-labeled IFN- α 2c at 37 °C. ●, WT; ○, 11,1; ■, Δ 1–21.

levels of exogenous protein were chosen, and their level of IFNAR1 was analyzed. As can be seen in Fig. 6B, only in cells expressing wild-type Tyk2 or the T-(1–581)-J chimera was the level of IFNAR1 restored. Particularly significant is the lack of IFNAR1 rescuing by the T-(1–518)-J chimera which bears not only the JH7-6 interaction domain defined *in vitro*, but also the Tyk2 JH5-4 segment. These results demonstrate that the JH regions of JAK1 cannot functionally substitute for the corresponding JH regions of Tyk2 and that regions besides JH7-6 are required for the *in vivo* interaction of Tyk2 with IFNAR1.

Having shown that the T-(1–581)-J chimera was competent in restoring IFNAR1, we assessed the function of the receptor-kinase complex in these cells by studying activation and signaling. The basal and IFN- α -induced phosphorylation levels of the T-(1–518)-J and T-(1–581)-J chimeras were compared. Two clones expressing the same chimera were studied and, as they behaved identically, results are shown for one. The T-(1–581)-J chimera, but not the T-(1–518)-J, was inducibly phosphorylated to a level comparable with Tyk2 in WT cells (Fig. 7A, upper panel). Basal phosphorylation of both chimeras was detectable upon longer blot exposure (data not shown). In parallel, immunoprecipitations with antibodies specific for the JAK1 TK domain were performed to analyze the phosphorylation level of the endogenous JAK1 protein in these cells. As expected, the JAK1 antibodies reacted with the chimeric constructs as well (Fig. 7B, lower panel). Induced phosphorylation of endogenous JAK1 was comparable in cells expressing wild-type Tyk2 or the T-(1–581)-J chimera, whereas no activation occurred in cells expressing the T-(1–518)-J chimera (Fig. 7B, upper panel). The IFN- α sensitivity of T-(1–581)-J-expressing cells was measured in media containing HAT or 6TG and IFN- α and was found to be comparable with WT cells (data not shown). The antiviral response against vesicular stomatitis

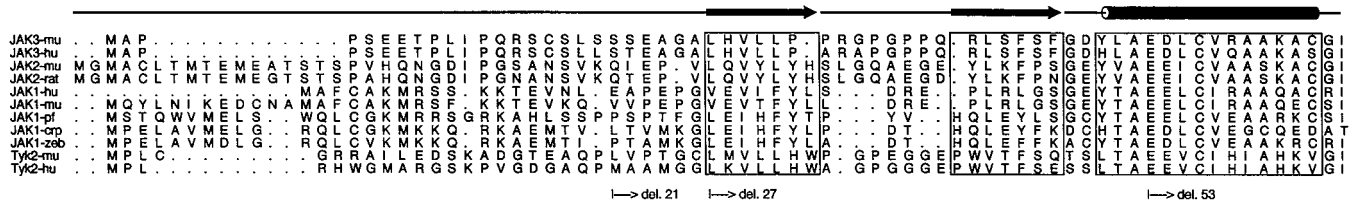


Fig. 8. Alignment of the amino-terminal ends of mammalian JAKs. Secondary structure predictions were performed using the PHD (EMBL, Heidelberg) program. The *small arrows* indicate the position of the first Tyk2 residue in the His-tagged constructs $\Delta 21$, $\Delta 27$, and $\Delta 53$.

the $\gamma R1$ receptor protein was independent from the presence of the interacting JAK1. Thus, it appears that the requirements for JH regions other than JH7-6 vary in different receptor/JAK systems and this may relate to the variation in the affinities of the interaction between given partners.

In the present study, we show that, despite its ability to associate with IFNAR1 *in vitro*, the JH7-6 domain of Tyk2 is not sufficient to rescue IFNAR1 levels when tested in Tyk2-negative cells, indicating a critical requirement for the JH5-4-3 regions for this function. Moreover, our analysis of the Tyk2/JAK1 chimeras (Fig. 6) clearly showed that these regions from JAK1 could not functionally replace the Tyk2 ones, indicating strict specificity. These results suggest that JH5-3 is involved in the IFNAR1 stabilizing function. The mechanism by which this occurs is unknown (19), and no other examples of such phenomenon have been described to date. We cannot exclude the possibility that the JH5-3 region augments the affinity of the interaction of the JH7-6 binding domain with IFNAR1 or that it interacts with another component of the receptor complex. Whatever the mechanism, this contribution would be negligible in the *in vitro* system and become critical in the cellular environment. A study was recently reported on the IFNAR1 binding region of Tyk2 (27) which showed, in accordance with our *in vivo* data, that an intact N region was required for maximal binding to IFNAR1. This group also used lysates from bacteria expressing GST-Tyk2 fusions to pull down a CD4-IFNAR1 chimera transfected into 293T cells and proposed the existence of two weak binding sites centered around the JH6 and the JH3 boxes, respectively. We have no evidence of such weak interaction domains, and this discrepancy could be due to the different stringencies of the two experimental approaches. More sensitive methods will be required to define the relative contribution, if any, of independent JH boxes.

Our analysis of the chimeric Tyk2/JAK1 proteins also provides some clues about the specificity of the KL and the TK domains. Full restoration of function (gene induction and antiviral protection) by the T-(1-581)-J chimera demonstrates the interchangeability of these domains for Tyk2 function in cellular responses to IFN- α . Similar conclusions were reached for a JAK1/JAK2 chimera in the IFN- γ response (25). Although the function of the KL domain is not yet known, we have previously shown that, in Tyk2, it contributes to the ligand binding activity of the receptor (18, 19). Given the rescuing capacity of the T-(1-581)-J chimera, which contains the KL domain of JAK1, we conclude that this function of KL is not Tyk2-specific.

IFN- α/β -induced activation of the JAKs is thought to involve trans-phosphorylation of regulatory tyrosine(s) located in the activation loop of the TK domain. This event takes place in a specific temporal order (JAK1 \rightarrow Tyk2) with JAK1 playing the prominent role (14, 15). We previously proposed that this order may relate more to spatial constraints and stoichiometry of the receptor-kinase complex than to properties intrinsic to each kinase. Our present finding that endogenous JAK1 appears phosphorylated equally well when juxtaposed to the T-(1-581)-J chimera or to Tyk2 (Fig. 7B) provides further evidence of the lack of specificity of these kinases. To fully demonstrate

this, it will be necessary to investigate whether the reciprocal chimera, bearing the N region from JAK1 fused to the kinase domains of Tyk2, can restore IFN- α responses in JAK1-negative cells. In this regard, it has been reported that a chimera bearing the N region of JAK1 fused to the kinase domains of JAK2 can sustain substantial though incomplete IFN- α -induced gene expression and antiviral protection to EMC virus (25). Whether other biological responses to IFN- α , not measurable in our system, could specifically require Tyk2 kinase domains cannot be ruled out.

The analysis of the IFNAR1/Tyk2 pair points to a variant, *i.e.* the expression level and the intrinsic stability of endogenous cytokine receptor subunit, which could be critical for some receptor-kinase complexes and/or in some cell types. It will be interesting to investigate whether the Tyk2 domains defined here for the assembly with IFNAR1 overlap with those involved in the interaction with other Tyk2-activating cytokine receptors, such as the IL-12 or the IL-10 receptors (28, 29). Similarly, it remains to be seen whether any specificity in the function of the kinase domains of Tyk2 could be revealed through the study of these two other receptor-kinase complexes.

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