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Distinct Domains of the Protein Tyrosine Kinase *tyk2* Required for Binding of Interferon- α/β and for Signal Transduction*

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***tyk2* belongs to the JAK family of nonreceptor protein tyrosine kinases recently found implicated in signaling through a large number of cytokine receptors. These proteins are characterized by a large amino-terminal region and two tandemly arranged kinase domains, a kinase-like and a tyrosine kinase domain. Genetic and biochemical evidence supports the requirement for *tyk2* in interferon- α/β binding and signaling. To study the role of the distinct domains of *tyk2*, constructs lacking one or both kinase domains were stably transfected in recipient cells lacking the endogenous protein. Removal of either or both kinase domains resulted in loss of the *in vitro* kinase activity. The mutant form truncated of the tyrosine kinase domain was found to reconstitute binding of interferon- α and partial signaling. While no contribution of this protein toward interferon- β binding was evident, increased signaling could be measured. The mutant form lacking both kinase domains did not exhibit any detectable activity. Altogether, these results show that a sequential deletion of domains engenders a sequential loss of function and that the different domains of *tyk2* have distinct functions, all essential for full interferon- α and - β binding and signaling.**

The JAK family of protein tyrosine kinases presently consists of four members, *tyk2*, *JAK1*, *JAK2*, and *JAK3* (1–6). These 120–130-kDa proteins are characterized by the presence of (i) a carboxyl kinase domain, (ii) an adjacent kinase-like domain, and (iii) five conserved domains extending toward the amino terminus and the absence of SH2 or SH3 domains (reviewed in Refs. 1 and 2). The COOH-terminal kinase domain (or TK¹ domain) contains all the conserved residues associated

with tyrosine kinases (7). The kinase-like domain (or KL domain) contains the subdomains shared by protein kinases, but lacks several residues thought to be essential for protein kinase activity, and thus it would not be predicted to exhibit kinase activity. The function of this domain has yet to be established. The remaining five blocks of homology exhibit varying degrees of conservation among the family members and their functional role is to be elucidated. It is likely that some of them might be responsible for the association of the JAKs with members of the cytokine receptor superfamily. Recent studies have in fact established the involvement of these enzymes in signaling through interferon (IFN) receptors and a large number of cytokine/growth factor receptors (1, 2, 4, 5). Physical interaction between JAKs and the membrane-proximal region of some of these receptor chains has also been demonstrated (reviewed in Ref. 2) (8–14).

As most cytokine receptors, the IFN- α/β receptor has a multichain structure composed of the chain IFNAR1, interacting with at least another membrane component (15, 16). Although no formal evidence exists yet, it is likely that the IFN- α/β binding protein of 51 kDa recently identified by Novick *et al.* (17) represents a component of the receptor unit possibly interacting with IFNAR1. The signaling cascade initiated by binding of IFN- α/β to its receptor relies on the activity of *tyk2* and *JAK1*, as was first demonstrated through the study of the IFN-resistant cell lines 11,1 (or U1A) and U4A, deficient in one or the other of these protein tyrosine kinases (18, 19). The absence of either protein prevents high affinity binding of IFN- α , phosphorylation of the downstream transcriptional components (p113 or STAT2 and p91/p84 or STAT1 α/β) and activation of inducible genes (20). Thus, complementation of 11,1 cells with a cosmid or the cDNA encoding *tyk2* was shown to restore IFN- α/β responsiveness (18, 21), whereas complementation of mutant U4A with *JAK1* cDNA restored both IFN- α/β and IFN- γ signaling (19). In an initial study of the *tyk2*-deficient cell line 11,1 we found a residual sensitivity of these cells to IFN- β , that suggested the existence of a minor IFN- β -specific pathway and possibly a different utilization of *tyk2* by the two IFN species (22, 23).

We have recently reported on the biochemical characterization of *tyk2* as a 134-kDa protein mostly cytosolic, with a minor membrane-associated fraction. The protein is transiently phosphorylated on tyrosine in response to IFN- α/β treatment and possesses an inducible tyrosine kinase activity measurable *in vitro* (21). To understand the role of the kinase domains of *tyk2*, here we have constructed deleted forms of the protein lacking either or both kinase domains and stably expressed them in the *tyk2*-deficient cell line. The binding of IFN- α and IFN- β and the signaling activities induced in these transfectants were analyzed and compared.

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¹ The abbreviations used are: TK, tyrosine kinase; KL, kinase-like; IFN, interferon; IFNAR, interferon- α receptor; STAT, signal transducer and activator of transcription; HAT, hypoxanthine/aminopterin/thymidine; VSV-G, vesicular stomatitis virus Glycoprotein; ISGF3, interferon-stimulated gene factor 3; kb, kilobase pair(s).

MATERIALS AND METHODS

Cell Culture and Transfection—Parental 2fTGH cells, mutants 11,1, U1B, and U1C were previously described (22, 24). Cells were propagated in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum and hygromycin (250 μ g/ml). Plasmid DNA transfections of 11,1 cells were carried out using calcium phosphate as described previously (22). Two days after transfection, cells were seeded 1:10 in medium containing 450 μ g/ml G418 (Sigma). Resistant colonies were ring-cloned 2 weeks later and expanded. Survival in hypoxanthine/aminopterin/thymidine (HAT) medium was assayed in the presence of different concentrations of IFN. Wellferon is a highly purified mixture of human IFN- α subtypes (10⁸ IU/mg of protein, Wellcome Research Laboratories) (25). Recombinant human IFN- α 8, purified to homogeneity, was a gift from CIBA-GEIGY (Basel, Switzerland). Purified recombinant human IFN- β at 250 μ g/ml was a gift from Biogen Inc. (Boston, MA).

***tyk2* Deletion Constructs**—All constructs were made in the pRc/CMV expression vector (Invitrogen). In this vector, the cDNA of interest is under the control of the cytomegalovirus immediate-early promoter. All plasmids were tagged at their 3' end with an oligonucleotide encoding an epitope of the vesicular stomatitis virus (VSV-G) as previously described (21). A *Sall* fragment, comprising nucleotides 777-4146 of *tyk2* cDNA in plasmid H9S (21) was subcloned into the phagemid vector pBluescript KS⁺ (Stratagene) to facilitate subsequent deletion constructs (b.s.3.3 B-Sal). To delete the tyrosine kinase domain, a 2.1-kb *EcoRI-Eco47III* fragment (nucleotides 777-2950) from b.s.3.3 B-Sal was cloned into the *EcoRI-SmaI* site of a plasmid containing the VSV-G epitope sequence. The new plasmid was digested with *Sall* and *XbaI*, the 2.2-kb fragment was purified, and, together with an 850-base pair fragment comprising the 5'-untranslated sequence and nucleotides 1-777 of *tyk2* cDNA, it was ligated to the *HindIII-XbaI*-digested vector. The final construct, Δ TK, encodes a protein containing amino acids 1-895 of *tyk2*. To delete the kinase-like domain, b.s.3.3 B-Sal was digested with *AatII* and *EcoRV*, treated with DNA polymerase to form blunt ends and religated. The resulting plasmid was digested with *EcoRI*, blunted as before and then digested with *SpeI*. The *SpeI* site is in the vector portion of the plasmid. The 3.9-kb band was isolated and ligated to a 1.3-kb *SpeI-PvuII* fragment (nucleotides 777-2040) of b.s.3.3 B-Sal. A 2.2-kb *Sall-XbaI* fragment was prepared from this plasmid, and a three-part ligation, with the 850-base pair *HindIII-Sall* fragment and the *HindIII-XbaI* vector band as described for Δ TK, was performed. The final construct generates a protein, Δ KL, in which amino acids 594-877 have been deleted, and an additional Tyr has been inserted after amino acid 593. To generate a protein deleted of both kinase domains, a 2.1-kb *HindIII-Eco47III* fragment from plasmid Δ KL was purified and ligated to the VSV-G plasmid digested with *HindIII-SmaI*. The resulting plasmid was digested with *HindIII* and *XbaI*, and the subsequent 2.2-kb fragment was ligated to the *HindIII-XbaI*-digested vector. This construct encodes a protein, N, that contains amino acids 1-591, followed by a three-amino acid insertion of Ile-Glu-Phe and finally amino acids 878-895 of *tyk2*. All final plasmids were sequenced across the junctions of the deletions to confirm that the coding region remained in frame. Sequence analysis was performed by the dideoxy chain termination method using a Sequenase kit (U. S. Biochemical Corp.) and double-stranded plasmid DNA as template.

Northern Analysis—Total RNA was extracted from subconfluent cultures with guanidinium thiocyanate (26). Ten micrograms of RNA per sample were fractionated in 1.2% agarose gels containing 2.2 M formaldehyde, transferred to Hybond-N membrane (Amersham Corp.), and hybridized under standard conditions (26) to specific cDNA probes.

Binding Experiments with ¹²⁵I-Labeled IFN—IFN- α 8 and IFN- β were labeled with ¹²⁵I (Amersham Corp., IMS 30). IFN- α 8 was labeled to a specific radioactivity of 25 Bq/fmol of monomeric IFN and stored at -80 °C at a concentration of 20 nM, as described previously (27). The labeling procedure was slightly modified for IFN- β , using a protein/iodine ratio 2.5 times higher than for IFN- α 8, to give a specific radioactivity of 10 Bq/fmol of monomeric IFN at 50 nM. Cell binding was carried out on subconfluent monolayer cultures (1.5 \times 10⁵ cells/cm²). Each point is an average from three replicate cultures, each containing 1.5 \times 10⁶ cells. Binding measured at 37 °C was terminated by placing the cultures on ice and aspirating the supernatant. The cultures were then washed three times with equivalent volumes of ice-cold medium containing 1% of fetal calf serum, detached in trypsin and EDTA for transfer to a γ counter (Kontron, counting efficiency 75%). Conversion of radioactive counts to molecules of IFN was as described previously (28). Binding at 4 °C was carried out by equilibrating the cultures on ice for 30 min before adding labeled IFN and terminated as above by

aspiration and washing. As incubation times were necessarily longer at 4 °C than at 37 °C, the culture medium was buffered by addition of Hepes to 50 mM. After several hours at 4 °C, cells detach easily, and extreme care was exercised during the washing. Binding results are presented as receptor-specific with background (estimated from cultures treated with labeled IFN in the presence of a 200-fold molar excess of unlabeled IFN) subtracted. Backgrounds were typically at 0.05% of the input radioactivity.

Immunoblotting and Immunoprecipitation—Immunoblots and immunoprecipitations were done as described elsewhere (21). For immunoprecipitation with anti-91C antibody, cells were lysed in Nonidet P-40-containing lysis buffer (21) containing 300 mM NaCl. p91 immunoblots were blocked with 5% bovine serum albumin (fraction V, 96-99% albumin) in 1 \times TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) buffer. Tyrosine phosphorylation of p91 was detected with a mixture of PY20 (ICN) and 4G10 (Upstate Biotechnology, Inc.) antibodies. Antisera to p113 and p91 (a gift of C. Schindler) were used at a 1:1,000 and 1:500 dilution respectively for immunoprecipitation and at a 1:10,000 dilution for immunoblotting. Affinity-purified anti-JAK1 antibodies (a gift of A. Ziemiecki) were used at a 1:250 dilution for immunoprecipitation and at a 1:2,000 dilution for immunoblotting. An ECL Western blotting detection system (Amersham Corp.) was used according to the manufacturer's instructions.

In Vitro Kinase Assay—The *in vitro* kinase assay was performed as previously reported (21). Gel was transferred to Hybond-C Super membrane (Amersham Corp.), and phosphorylated proteins were visualized by autoradiography.

RESULTS

Biological Activity of *tyk2* Deletion Mutants—To carry out a structure-function analysis of *tyk2*, we generated three deleted cDNA forms lacking one or both kinase domains. The proteins encoded by these constructs are schematically depicted in Fig. 1A. The mutant protein designated Δ TK is a truncated *tyk2* form lacking the tyrosine kinase domain. The Δ KL protein lacks the kinase-like domain but retains an intact tyrosine kinase domain, whereas the protein designated N corresponds to the NH₂-terminal region. The wild-type and the deleted cDNAs were cloned in the pRc/CMV eukaryotic expression vector, which contains the neomycin-resistance marker. Mutant 11,1 cells were transfected with the four constructs, selected in G418, and independent neo^r clones arising from each transfection were analyzed for the presence of *tyk2* by immunoblot. All transfectants analyzed expressed *tyk2* forms of the predicted size, at levels ranging from 0.5- to 10-fold the endogenous *tyk2* level present in parental 2fTGH cells (21). Minor *tyk2* forms of higher mobility were routinely detected in *tyk2*-overexpressing cells (Fig. 1B) and might result from *in vivo* degradation. Four representative clones (Fig. 1B) expressing almost comparable levels of protein (approximately 5-fold the endogenous *tyk2* level in 2fTGH cells) were chosen for further studies.

We tested the ability of the four neo^r transfectants to grow in the selective medium (HAT) in the presence of different concentrations of IFN- α . This medium allows survival of 11,1 derivatives which have reverted to IFN sensitivity (22) and constitutes our biological assay for *tyk2* activity. Wild-type *tyk2*-expressing cells showed IFN-dependent growth with as little as 10 IU/ml IFN- α (Wellferon, a mixture of purified human α subtypes). Δ TK-expressing cells survived in HAT medium only when a higher concentration of IFN- α was used. In contrast, 11,1 cells and Δ KL- and N-expressing cells failed to grow at any given concentration of IFN- α tested (Fig. 2A). To rule out the possibility that this behavior was unique to 11,1 derivatives, we generated Δ TK and Δ KL neo^r transfectants from cell lines U1B and U1C, two independent mutants of the same complementation group as 11,1 (24). The IFN responsiveness of each transfectant gave comparable results (data not shown).

We next tested the sensitivities of the 11,1 transfectants to recombinant IFN- α 8 and - β . These results are summarized in

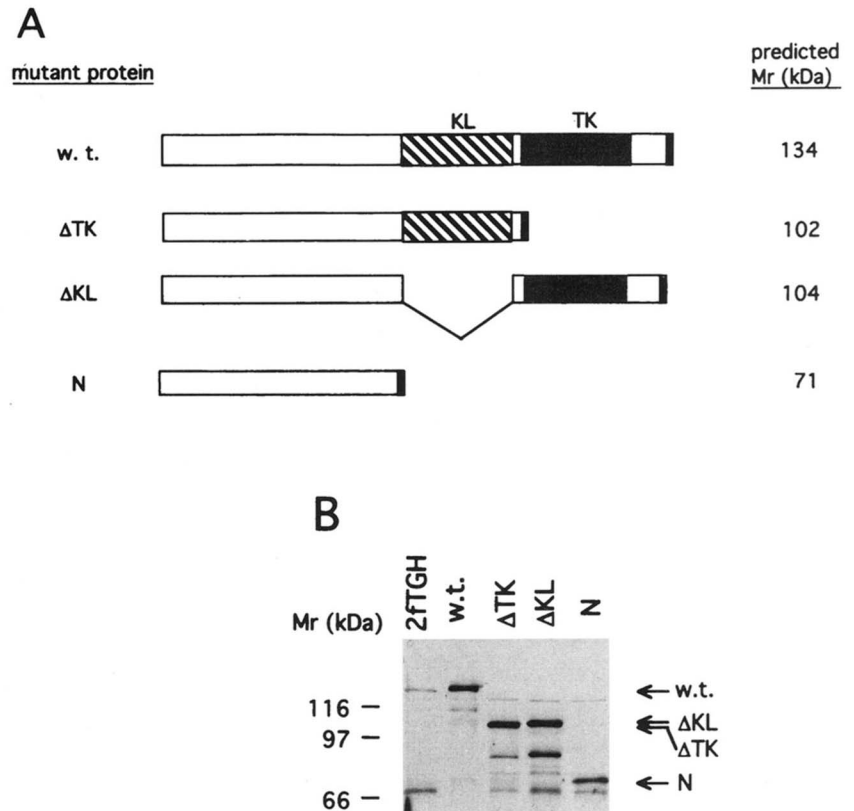


FIG. 1. Structure and expression of the deleted tyk2 proteins. *A*, Schematic representation of the tyk2 deleted forms. Names of the proteins are on the left and their predicted molecular masses in kilodaltons (*kDa*) are on the right. *w.t.*, wild-type; *KL*, kinase-like domain; *TK*, tyrosine kinase domain; *N*, NH₂-terminal region. The striped box at the C termini corresponds to the VSV-G epitope. *B*, anti-tyk2 immunoblot to compare the level of protein in 2fTGH cells and in clones transfected with the indicated cDNAs. Fifteen micrograms of total protein obtained from crude cell extracts were loaded on a SDS-7% polyacrylamide gel. Minor forms of higher mobility were routinely detected in cells overexpressing tyk2.

Fig. 2*B*. The specific activity of IFN- β was higher than that of IFN- α 8 on all clones tested. There was a 30–100-fold difference in the sensitivities of Δ TK-expressing cells and 11,1 to either IFN. Interestingly, the sensitivity of Δ TK-expressing cells to IFN- α 8 attained the level of sensitivity of 11,1 cells to IFN- β . The analysis of the Δ KL- and N-expressing clones gave results essentially identical to 11,1. These data indicate that both tyk2 kinase domains are required to restore a wild-type response to both IFN species. The truncated Δ TK form can, however, partially reconstitute sensitivity to both IFNs.

As a measure of the response of the transfectants to IFN- α , we studied the transcriptional induction of the endogenous IFN-responsive 6-16 gene by Northern blot. Analysis of total RNA from wild-type tyk2-expressing cells treated for 4 h showed accumulation of the 6-16 transcript with as little as 10 IU/ml IFN- α (Fig. 3). A comparable level of transcript accumulated in Δ TK-expressing cells treated with 5000 IU/ml IFN- α . In contrast, Δ KL-expressing cells failed to induce the 6-16 message, behaving as mutant 11,1. These results confirm the fine correlation between the transcriptional activation of the 6-16 gene promoter by IFN and the ability of the cells to grow in HAT medium. The steady-state mRNA level of three other IFN-responsive genes (ISG-54, GBP, and IRF-1) was analyzed in the various cell lines. None of these transcripts accumulated in cells expressing the deleted tyk2 forms, treated with up to 5000 IU/ml IFN- α (data not shown).

Δ TK Contributes to the Affinity of Binding of IFN- α —Previous work had suggested that tyk2 exerts an effect on receptor-mediated uptake of IFN- α , *i.e.* mutant 11,1 cells lacking tyk2 showed reduced uptake of labeled IFN- α 2 (22), whereas complementing the defect by tyk2 transfection restored binding as well as signaling (18). Furthermore, 11,1 cells are known to be partially responsive to IFN- β (Fig. 2*B*) (22, 23). To complete these observations, we studied the kinetics of uptake of radiolabeled IFN- β and IFN- α 8 in the mutant cell line and in its derivatives (Fig. 4). The kinetic results shown here are for a

single concentration of IFN (\sim 300 pM), but the relative form of the curves was similar across the dose range 50–500 pM. Since essentially identical curves were obtained from Δ KL- and N-expressing cells and 11,1 cells, only data from 11,1 were represented in Fig. 4, *A* and *B*. Cells expressing wild-type tyk2 showed the characteristic binding dynamics for IFN- β seen in parental 2fTGH cells (Fig. 4*A*). While a similar level of uptake was attained in both 11,1 cells and Δ TK-expressing cells, there was no down phase as in parental cells. Similar results were obtained with IFN- α 8 (Fig. 4*B*) except that 11,1 cells showed a much reduced uptake than Δ TK-expressing cells.

To investigate this further and to eliminate the dynamic aspects of cellular uptake, we compared the binding of IFN- α 8 and IFN- β at 4 °C on the partially sensitive Δ TK-expressing cells and on the IFN-insensitive Δ KL-expressing cells. The results, in the form of Scatchard graphs, are shown in Fig. 4*C*. The binding of IFN- β was nearly the same on the two cell lines, showing that, for IFN- β , receptor expression appears to be unaffected by the status of tyk2. The binding of IFN- α 8 on the Δ TK-expressing cells was greater and shows a Scatchard plot with a slope 2.4 times greater than that obtained on the Δ KL-expressing cells. This suggests that the mutant protein Δ TK contributes to the affinity of binding of IFN- α over the concentration range used. The Scatchard coefficient gave K_d equivalent to 0.54 nM and 0.62 nM for IFN- β , 0.87 nM and $>$ 2.1 nM for IFN- α 8, on Δ TK- and Δ KL-expressing cells, respectively (the K_d of 2.1 nM falls outside the range of experimental points). The linear regressions extrapolated to infinite ligand concentration meet the intercept at values close enough to suggest that the main reason for the reduced uptake of IFN- α 8 on Δ KL cells is a lower binding affinity.

Deletion of Either Kinase Domain Affects Phosphorylation of tyk2—It has been previously reported that tyrosine phosphorylation of tyk2 occurs within min of IFN- α/β treatment (11, 19, 21). We therefore investigated the *in vivo* phosphorylation state of deleted tyk2 forms in the transfected clones. Cells were

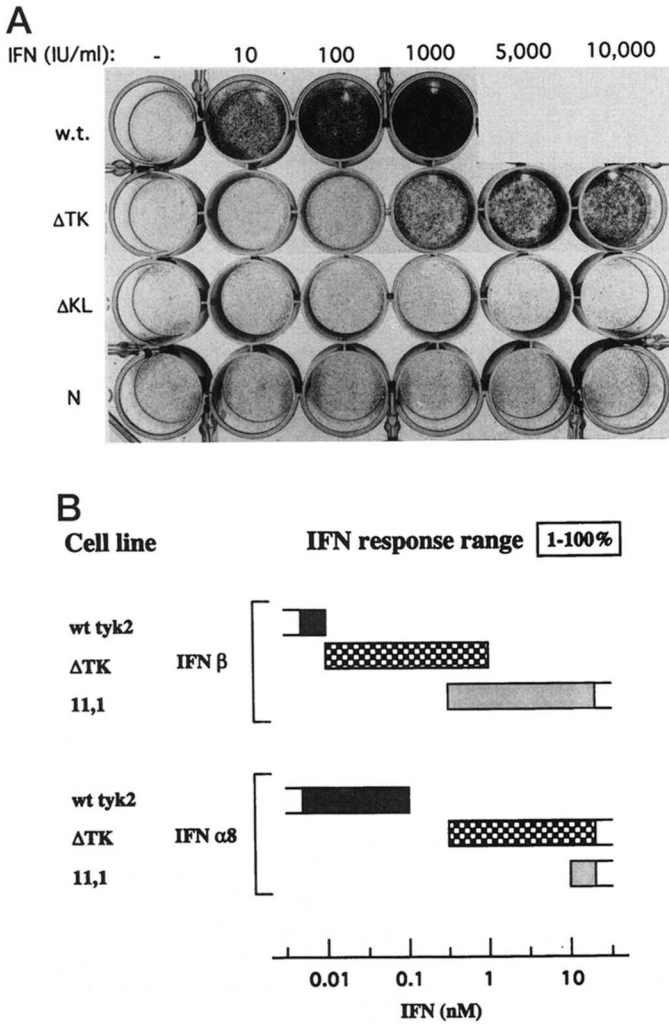


FIG. 2. Biological assay of the activity of wild-type and deleted tyk2 forms. *A*, The sensitivity to IFN- α of the indicated cell lines was tested by seeding 3×10^4 cells/well in a 24-well plate in HAT medium without or with the indicated concentrations of IFN- α (Wellferon). After 6–7 days, cells were fixed and stained. *B*, comparison of the dose-response range for IFN- α 8 and IFN- β of mutant 11,1 cells and its derivative transfectants expressing wild-type tyk2 or the deleted Δ TK form. The IFN response was assayed as described in *A*. After fixation, staining intensity was visually compared for the three cell lines. Boxes are closed on the left side at $\approx 1\%$ growth response, and on the right side at $\approx 100\%$ growth response. A half-log difference is regarded as significant. 1 nM corresponds approximately to 4000 IU/ml. The same profile of sensitivity was obtained for the 11,1 cell line and the clones expressing Δ TK and N.

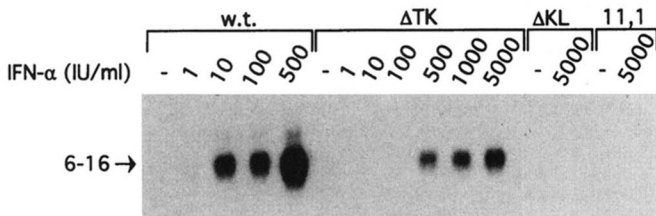


FIG. 3. Induction of the 6–16 mRNA by IFN- α in cells expressing wild-type or deleted tyk2 forms. Northern transfers of 10 μ g of total RNA from untreated cells (–) or cells treated with the indicated amounts of IFN- α (Wellferon) for 4 h were hybridized to a 6-16-specific cDNA probe. Comparable signals in each lane were obtained with a β -actin probe (data not shown).

left untreated or treated with IFN- α for 5 min and tyk2 immunoprecipitates were analyzed by blotting with antibodies against phosphotyrosine and, after stripping, with anti-tyk2

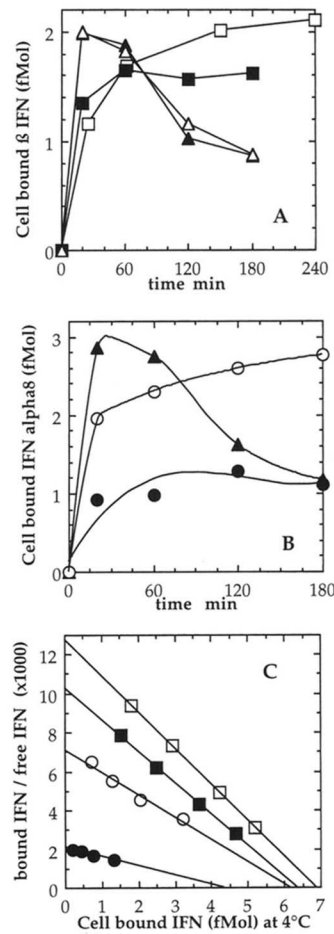


FIG. 4. Binding of human IFN- α 8 and IFN- β to cells expressing wild-type or deleted tyk2 forms. *A*, Kinetics of uptake of 125 I-labeled human IFN- β (280 pM) at 37 °C by \blacktriangle , 2fTGH cells; \blacksquare , 11,1 cells; \triangle , wild-type tyk2-expressing cells; and \square , Δ TK-expressing cells. Values are femtomoles of IFN taken up per million cells. *B*, kinetics of uptake of 125 I-labeled human IFN- α 8 (350 pM) at 37 °C by \bullet , 11,1 cells; \blacktriangle , wild-type tyk2-expressing cells; \circ , Δ TK-expressing cells. Values are femtomoles of IFN taken up per million cells. *C*, Scatchard plots of direct binding of 125 I-labeled human IFN- α 8 (\bullet , \circ) and IFN- β (\blacksquare , \square) for 6 h at 4 °C to \circ , \square , Δ TK-expressing cells; \bullet , \blacksquare , Δ KL-expressing cells. Values are for femtomoles of IFN bound per million cells.

antibodies. In response to IFN- α , wild-type tyk2 was rapidly phosphorylated on tyrosine above a basal level of phosphorylation (Fig. 5A, lanes 1 and 2, upper panel; see also Fig. 3 in Ref. 21). The N and Δ TK mutant forms were not phosphorylated (Fig. 5A, lanes 4 and 8). Conversely, the Δ KL mutant protein showed an IFN-independent phosphorylation (Fig. 5A, lanes 5 and 6). Comparable levels of protein were present in all extracts analyzed (Fig. 5A, lower panel).

IFN- α induces tyk2 kinase activity and this can be measured *in vitro* in an anti-tyk2 immunocomplex (21). To determine whether the deletions had an effect on the kinase activity of the protein, *in vitro* kinase assays were performed on the wild-type tyk2 and the Δ TK and Δ KL mutant forms. As shown in Fig. 5B, wild-type tyk2 exhibited increased autophosphorylating activity upon IFN- α treatment. In contrast, Δ TK and Δ KL did not exhibit autophosphorylating activity. A similar result was obtained when the two deletion constructs were co-expressed in 11,1 cells (data not shown). To investigate the ability of these proteins to phosphorylate an exogenous substrate, the *in vitro* kinase assay was performed in the presence of acid-denatured enolase. Upon IFN- α treatment, enolase was markedly phosphorylated by wild-type tyk2 (Fig. 5B, lane 2). In contrast, the

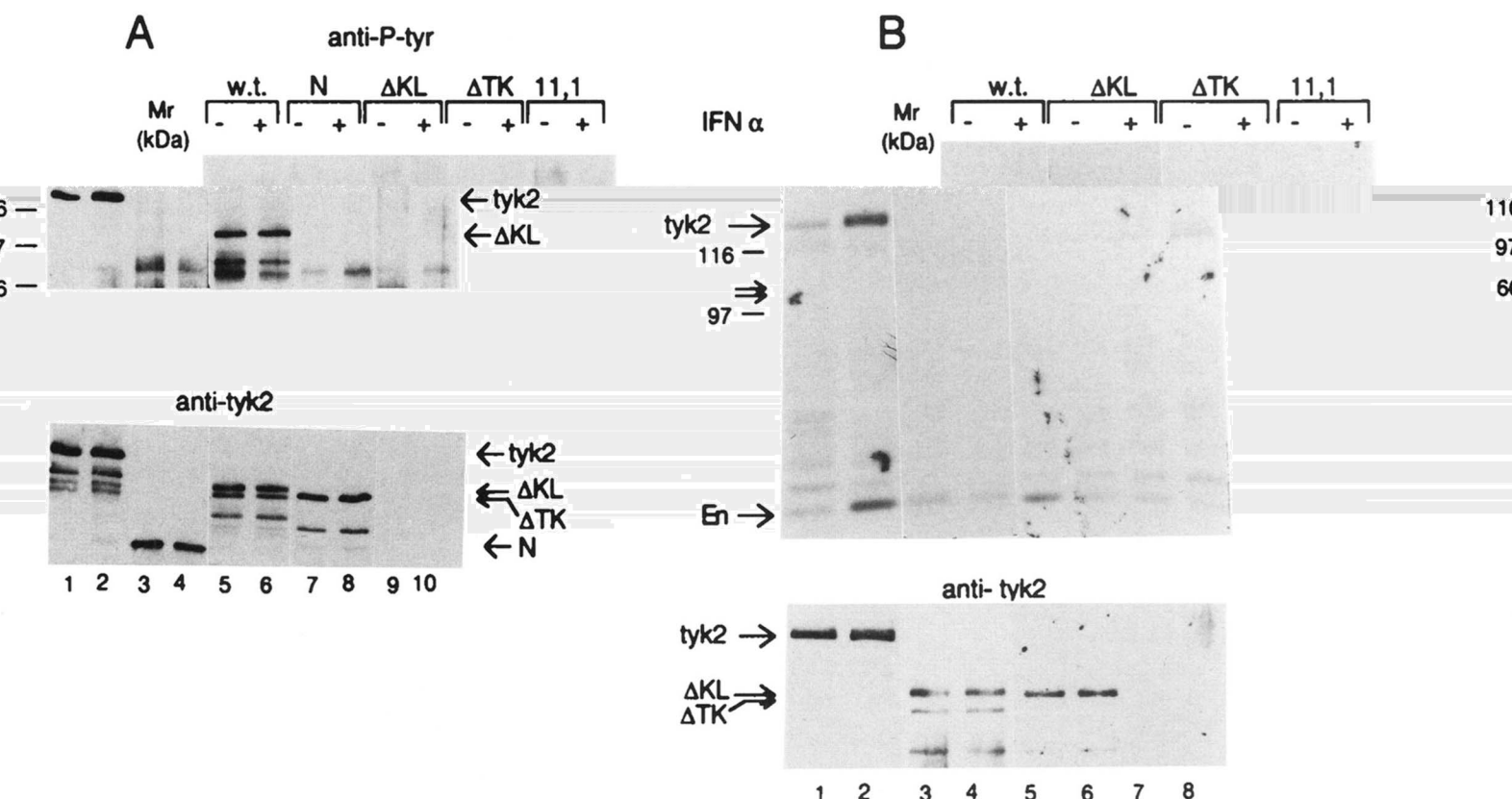


Fig. 5. *In vivo* and *in vitro* tyrosine phosphorylation of *tyk2* in response to IFN- α in wild-type and deleted *tyk2*-expressing cells. Anti-*tyk2* immunoprecipitates from wild-type, deleted *tyk2*-expressing, and mutant 11,1 cells (5×10^6 cells) were immuno-blotted with either anti-phosphotyrosine 4G10 (upper panel) or anti-*tyk2* (lower panel) antibody. IFN treatment (1000 IU/ml of Wellferon) was as follows: lanes 1, 3, and 9, no treatment; lanes 2, 4, 6, 8, and 10, 5 min with IFN- α . The position of wild-type and mutant *tyk2* forms is marked by an arrow. Faster migrating bands are routinely detected in cells overexpressing *tyk2*. **B**, anti-*tyk2* immunoprecipitates from post-nuclear lysates (600 μ g) of untreated or IFN- α treated (1000 IU/ml Wellferon) wild-type, 11,1, Δ KL, and Δ TK-expressing cells were assayed in an *in vitro* kinase assay. Acid-denatured enolase was added to the reaction as an exogenous substrate. Kinase reaction products were fractionated on a 7% SDS-acrylamide gel electrophoresis and transferred to Hybond-C super membrane. Following autoradiography, the filter was probed with anti-*tyk2* (bottom panel) antibodies. The expected positions of *tyk2*, enolase (En), and mutant Δ KL and Δ TK forms are marked.

ogenous substrate was not phosphorylated in Δ KL and Δ TK-expressing cells treated with IFN (Fig. 5B, lanes 4 and 6). The abundance of the immunoprecipitated proteins was visualized by blotting the filter with anti-*tyk2* antibodies (Fig. 5B, lower panel). These results indicate that both kinase domains appear necessary for the enzymatic activity of the protein since deletion of either domain generates a kinase-inactive protein.

No Detectable Activated JAK1, p91, and p113 in Cells Expressing *tyk2* Deletion Mutants—To try to uncover the mechanism underlying the low IFN- α signaling observed in Δ TK-expressing cells, we analyzed the phosphorylation state of components known to be involved in the IFN- α / β signaling pathway. In wild-type cells, JAK1 is phosphorylated on tyrosine after IFN- α treatment, whereas no phosphorylation is detected in cells lacking *tyk2* (19, 29, 30). We therefore examined the phosphorylation of JAK1 in cells expressing deleted *tyk2* forms (Fig. 6A). No phosphorylated JAK1 was detected in cells expressing the deleted *tyk2* forms even after long treatment with IFN (Fig. 6A, lanes 4 and 6). The protein levels were comparable in all cell lines tested (Fig. 6A, bottom panel).

We next investigated whether p113 and p91, components of transcriptional complex ISGF3, were phosphorylated upon IFN- α treatment of cells expressing the *tyk2* deletion mutants. Phosphorylated p113 was detected in wild-type *tyk2*-expressing cells after 20 min of IFN- α treatment (Fig. 6B, lanes 2), whereas no phosphorylated p113 was detected in cells expressing the deleted *tyk2* forms (Fig. 6B, lanes 4 and 6). Similarly, neither phosphorylated p91 nor p113-p91 complex (Fig. 6C,

lane 2) were observed in cells expressing mutant *tyk2* forms (Fig. 6C). Comparable levels of proteins were present in all extracts (Fig. 6, B and C, bottom panels). We were not successful at detecting phosphorylated JAK1, p91, or p113 in the Δ TK-expressing cells even when different experimental conditions, such as increased dose and time of IFN treatment or increased amount of cell extract immunoprecipitated, were assayed.

DISCUSSION

In this study we have examined the effects of deletions on the activity of *tyk2* expressed in an IFN-resistant cell line lacking the endogenous protein. Deletions of one or both kinase domains were found to abrogate *tyk2* catalytic activity when measured *in vitro* for autophosphorylation and phosphorylation of an exogenous substrate. The absence of autophosphorylation could simply be consequent to the removal of major phosphorylation sites. However, the mutant Δ KL protein retains two contiguous tyrosine residues, recently identified as targets of *in vitro* IFN-induced autophosphorylation in the wild-type *tyk2*. These sites (Tyr₁₀₅₄-Tyr₁₀₅₅) map in the TK domain, between subdomains VII and VIII, and, as shown for other protein tyrosine kinases, their phosphorylation parallels activation of the enzyme.² Thus, it appears that, when juxtaposed to the NH₂-terminal region, the TK domain of *tyk2* is not active. This could either reflect a conformational alteration of

² L. Velazquez, R. McKendry, S. Pellegrini, unpublished observation.

FIG. 5. *In vivo* and *in vitro* tyrosine phosphorylation of *tyk2* in response to IFN- α in wild-type and deleted *tyk2*-expressing cells. **A**, Anti-*tyk2* immunoprecipitates from wild-type, deleted *tyk2*-expressing, and mutant 11,1 cells (5×10^6 cells) were immuno-blotted with either anti-phosphotyrosine 4G10 (upper panel) or anti-*tyk2* (lower panel) antibody. IFN treatment (1000 IU/ml of Wellferon) was as follows: lanes 1, 3, and 9, no treatment; lanes 2, 4, 6, 8, and 10, 5 min with IFN- α . The position of wild-type and mutant *tyk2* forms is marked by an arrow. Faster migrating bands are routinely detected in cells overexpressing *tyk2*. **B**, anti-*tyk2* immunoprecipitates from post-nuclear lysates (600 μ g) of untreated or IFN- α treated (1000 IU/ml Wellferon) wild-type, 11,1, Δ KL, and Δ TK-expressing cells were assayed in an *in vitro* kinase assay. Acid-denatured enolase was added to the reaction as an exogenous substrate. Kinase reaction products were fractionated on a 7% SDS-acrylamide gel electrophoresis and transferred to Hybond-C super membrane. Following autoradiography, the filter was probed with anti-*tyk2* (bottom panel) antibodies. The expected positions of *tyk2*, enolase (En), and mutant Δ KL and Δ TK forms are marked.

exogenous substrate was not phosphorylated in Δ KL and Δ TK-expressing cells treated with IFN (Fig. 5B, lanes 4 and 6). The abundance of the immunoprecipitated proteins was visualized by blotting the filter with anti-*tyk2* antibodies (Fig. 5B, lower panel). These results indicate that both kinase domains appear necessary for the enzymatic activity of the protein since deletion of either domain generates a kinase-inactive protein.

No Detectable Activated JAK1, p91, and p113 in Cells Expressing *tyk2* Deletion Mutants—To try to uncover the mechanism underlying the low IFN- α signaling observed in Δ TK-expressing cells, we analyzed the phosphorylation state of components known to be involved in the IFN- α / β signaling pathway. In wild-type cells, JAK1 is phosphorylated on tyrosine after IFN- α treatment, whereas no phosphorylation is detected in cells lacking *tyk2* (19, 29, 30). We therefore examined the phosphorylation of JAK1 in cells expressing deleted *tyk2* forms (Fig. 6A). No phosphorylated JAK1 was detected in cells expressing the deleted *tyk2* forms even after long treatment with IFN (Fig. 6A, lanes 4 and 6). The protein levels were comparable in all cell lines tested (Fig. 6A, bottom panel).

We next investigated whether p113 and p91, components of transcriptional complex ISGF3, were phosphorylated upon IFN- α treatment of cells expressing the *tyk2* deletion mutants. Phosphorylated p113 was detected in wild-type *tyk2*-expressing cells after 20 min of IFN- α treatment (Fig. 6B, lanes 2), whereas no phosphorylated p113 was detected in cells expressing the deleted *tyk2* forms (Fig. 6B, lanes 4 and 6). Similarly, neither phosphorylated p91 nor p113-p91 complex (Fig. 6C,

lane 2) were observed in cells expressing mutant *tyk2* forms (Fig. 6C). Comparable levels of proteins were present in all extracts (Fig. 6, B and C, bottom panels). We were not successful at detecting phosphorylated JAK1, p91, or p113 in the Δ TK-expressing cells even when different experimental conditions, such as increased dose and time of IFN treatment or increased amount of cell extract immunoprecipitated, were assayed.

DISCUSSION

In this study we have examined the effects of deletions on the activity of *tyk2* expressed in an IFN-resistant cell line lacking the endogenous protein. Deletions of one or both kinase domains were found to abrogate *tyk2* catalytic activity when measured *in vitro* for autophosphorylation and phosphorylation of an exogenous substrate. The absence of autophosphorylation could simply be consequent to the removal of major phosphorylation sites. However, the mutant Δ KL protein retains two contiguous tyrosine residues, recently identified as targets of *in vitro* IFN-induced autophosphorylation in the wild-type *tyk2*. These sites (Tyr₁₀₅₄-Tyr₁₀₅₅) map in the TK domain, between subdomains VII and VIII, and, as shown for other protein tyrosine kinases, their phosphorylation parallels activation of the enzyme.² Thus, it appears that, when juxtaposed to the NH₂-terminal region, the TK domain of *tyk2* is not active. This could either reflect a conformational alteration of

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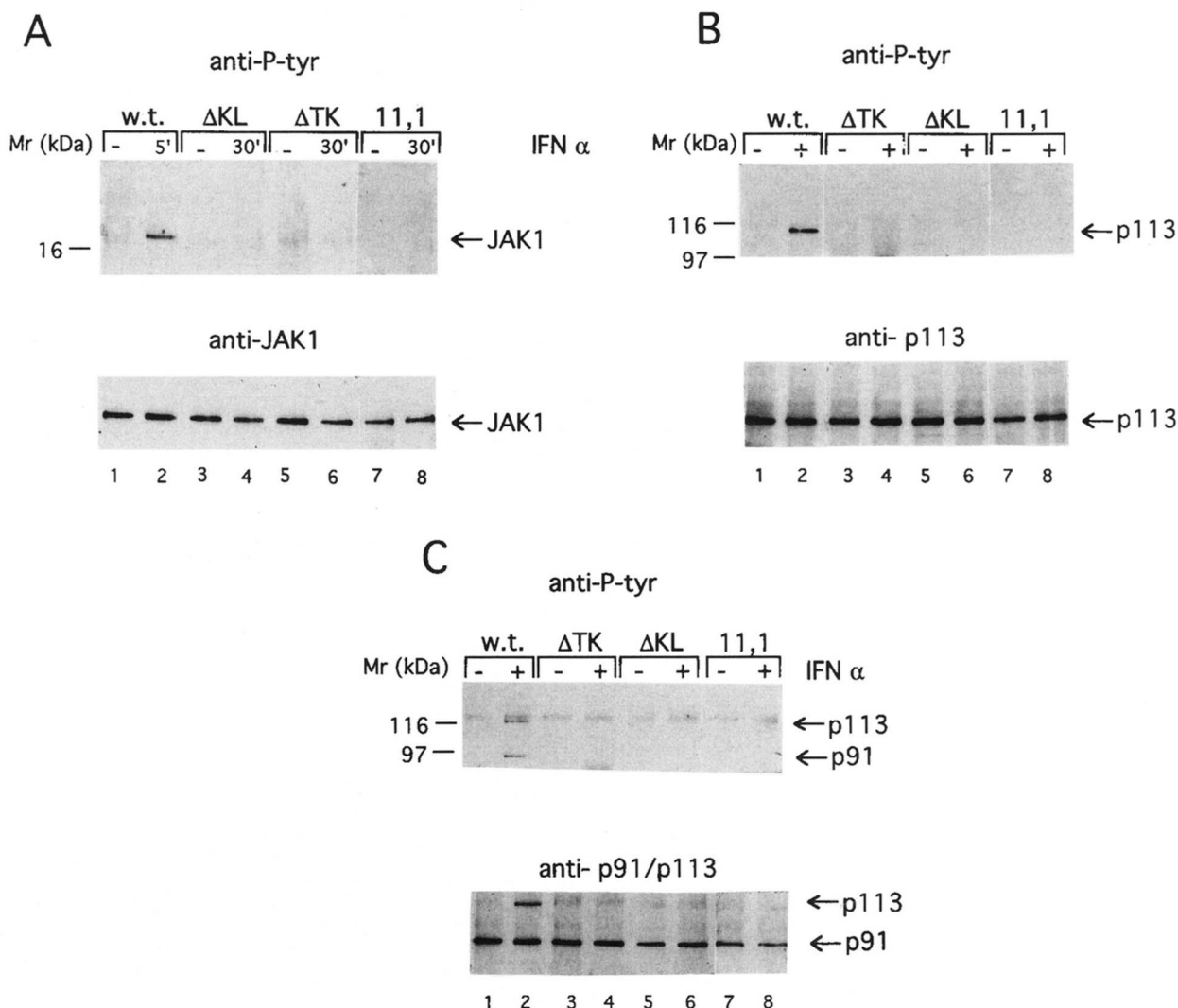


FIG. 6. Tyrosine phosphorylation of JAK1, p113, and p91 in cells expressing the wild-type and the deleted *tyk2* forms. *A*, cells were left untreated (–, lanes 1, 3, 5, and 7) or were treated (+, lanes 2, 4, 6, and 8) for the indicated times (minutes) with IFN- α (1000 IU/ml Wellferon). Cell lysates were immunoprecipitated with anti-JAK1 and immunoblotted with anti-phosphotyrosine (upper panel) or anti-JAK1 (lower panel) antibodies. *B* and *C*, cells were left untreated (–, lanes 1, 3, 5, and 7) or were treated (+, lanes 2, 4, 6, and 8) for 20 min with IFN- α (1000 IU/ml of Wellferon). Cell lysates were immunoprecipitated with anti-p113 (*B*) or anti-p91C (*C*) antibodies, followed by immunoblotting with anti-phosphotyrosine 4G10 (*B*, upper panel) or a mixture of anti-phosphotyrosine 4G10 and PY20 antibodies (*C*, upper panel). The blots were stripped and reprobbed with anti-p113 or/and anti-p91 as indicated (lower panels). The positions of JAK1, p113, and p91 are marked.

the protein caused by the deletion, or be a consequence of the excision of a domain playing an essential role in the activity of the enzyme. Despite the fact that the Δ KL mutant protein is catalytically inactive, it is phosphorylated *in vivo*, independently of the presence of IFN (Fig. 5A). When expressed in IFN-treated cells containing endogenous *tyk2*, the mutant form Δ KL showed no evidence of phosphorylation above the basal level (data not shown), suggesting that IFN-induced transphosphorylation between the wild-type and the mutant protein cannot take place. Although at this time the site and the functional significance of this IFN-independent phosphorylation is unknown, these observations argue for the existence of another cellular protein tyrosine kinase capable of phosphorylating Δ KL. *Tyk2* deleted of its TK domain (Δ TK) is catalytically inactive, indicating that the KL domain lacks intrinsic kinase activity. Indeed, no activity of the bacterially expressed JAK1 kinase-like domain could be detected for a number of exogenous substrates (31).

One fundamental question regarding *tyk2* and the other JAK family members concerns their mode of regulation which might differ from that of cytoplasmic protein tyrosine kinases pos-

sessing an SH2 domain and a single kinase domain (32), resembling more that of other bifunctional proteins bearing a kinase-related domain (33–35). One such protein is the cell surface receptor for atrial natriuretic peptide containing in its intracellular region a kinase-like domain next to a guanylyl cyclase catalytic domain. Deletion of the kinase domain constitutively activates the cyclase (33, 36). Thus, a negative regulatory role has been proposed for this kinase-like domain, repressing the guanylyl cyclase in absence of atrial natriuretic peptide. This is not the case for *tyk2*, since deletion of its KL domain does not create a constitutively active enzyme. It is likely that a more complex mode of regulation involving both kinase domains exists for the JAK family.

To study the biological activity of *tyk2* mutant forms, we have taken advantage of a unique property of 11,1 cells in which the expression of the marker guanine phosphoribosyl transferase and the consequent resistance to HAT is dependent on IFN and *tyk2* (18). Thus, we have found that the function of *tyk2* is completely abrogated by removal of the KL domain or of both kinase domains: cells expressing the mutant *tyk2* forms Δ KL and N behaved essentially as cells lacking *tyk2*. Con-

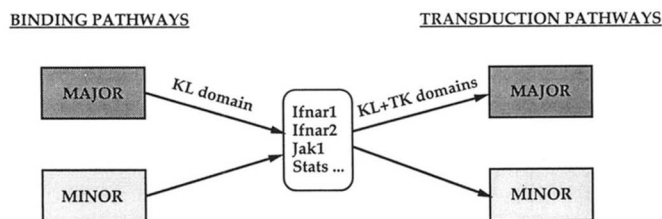


FIG. 7. Tyk2 in the receptor-mediated pathways induced by IFN- α and - β . The central box contains all known and unknown components, other than tyk2, involved in signal transduction. *Ifnar1* and *Ifnar2* are ligand binding proteins (43). Both binding pathways are deemed to be open to IFN- β , whereas IFN- α is restricted to the major binding pathway.

versely, expression of the Δ TK protein resulted in a partial reconstitution of sensitivity toward IFN- α and in an increased sensitivity to IFN- β (Fig. 2). This effect could be appreciated only when transfectants were expanded in G418, prior to testing their IFN responsiveness. When transfectants were directly seeded in HAT, no survivors could be isolated in up to 10^4 IU/ml IFN, probably due to the poor colony-forming ability of these cells.

Our binding studies showed that the capacity for receptor-mediated IFN- α uptake is partially restored by the Δ TK mutant protein, probably through an increase in binding affinity (Fig. 4). The inability of the N and the Δ KL proteins to perform this function suggests that the KL domain in its proper conformation contributes toward the binding affinity of the receptor unit and indeed may be in contact with some component of that unit. In relation to this, tyk2 has been recently found to physically associate with the chain IFNAR of the receptor complex, independently of the presence of the ligand (11).³ Despite an increase in the affinity of binding of IFN- α on Δ TK-expressing cells, there are dynamic aspects of the interaction that have not been reconstituted, and that must require the TK domain. One hypothesis awaiting further investigation is that ligand-induced tyk2 kinase activity could play a role in post-binding events such as the internalization rate of the receptor unit, as proposed for the intrinsic kinase activity of epidermal growth factor and insulin receptors (37–39).

The contribution of the Δ TK protein toward ligand binding is much less evident for IFN- β than for IFN- α . While there is a partial reconstitution of sensitivity towards IFN- β with the Δ TK protein, the early receptor binding events appear to be unaffected. Full reconstitution requires the tyrosine kinase domain. These data suggest that tyk2 has both structural and enzymatic functions in the IFN- α/β response, with a more direct structural relationship with IFN- α than with IFN- β , and this has been schematized in Fig. 7. In wild-type cells, the different elements are obviously well integrated. From the study of mutant cell lines, we would suggest that IFN- β can utilize a minor or tyk2-independent binding pathway(s), an option not available to IFN- α . Further work will be needed to identify elements unique to the IFN- β response and the recent report of a protein that can be coprecipitated with one ligand binding protein and phosphorylated only following IFN- β treatment goes into that direction (40). If the apparently greater flexibility of IFN- β is a reflection of its evolutionary seniority (41), then the close dependence of IFN- α and tyk2 may have its origin in the diversification of the mammalian IFNs.

We have not yet identified the signaling components involved in the minor pathway(s). Phosphorylated JAK1 or STATs could not be detected in Δ TK-expressing cells in re-

sponse to IFN- α , possibly due to the low number of molecules activated and the limited sensitivity of this assay. Recently, it has been shown that IFN- α/β induce tyrosine phosphorylation of the p95^{vav} proto-oncogene in hematopoietic cell lines, suggesting that *vav* may be involved in an alternative IFN- α/β signaling pathway in addition to the ISGF3 pathway (42). It will be interesting to determine if signaling proteins similar to *vav* are activated in response to IFN- α/β in cells other than hematopoietic cell lines, as it may provide a hint of a possible alternative IFN signaling pathway.

Certainly, more work will be needed to understand how tyk2 and its deleted Δ TK form contribute to receptor function. In parallel, the study of the JAK1-deficient U4A mutant cell line, whose IFN- α binding activity is impaired (19, 20), should help to clarify the role of JAK1 in receptor function. In relation to this, recently Novick *et al.* (17) have shown that the IFN- α/β -binding chain that they have identified can be co-precipitated with JAK1. Altogether, our studies support a model in which heterodimerization of receptor chains and consequent juxtaposition and activation of associated JAK1 and tyk2 all contribute to the generation or stabilization of ligand-induced, conformationally correct, and productive receptor units.

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