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Interferon-α-dependent Activation of Tyk2 Requires Phosphorylation of Positive Regulatory Tyrosines by Another Kinase*

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Tyk2 and J AK1, members of the J akus kinase (J AK) family of protein tyrosine kinases, are required for interferon-α/β binding and signaling. Both enzymes are associated with the interferon-α/β receptor, and upon ligand binding, they undergo tyrosine phosphorylation and activation in an interdependent manner. To identify residues involved in Tyk2 regulation and to understand the basis of the interdependence of Tyk2 and J AK1, six mutated versions of Tyk2 bearing single or multiple point mutations in the tyrosine kinase domain were studied in a cell line lacking endogenous Tyk2. The Y1054F/Y1055F substitutions in the putative activation loop prevented ligand-dependent activation of Tyk2, without abolishing its catalytic potential. The K930R mutation in the ATP binding site generated a kinase-negative protein, which however, still became phosphorylated upon interferon-α treatment. The Y1054F/Y1055F substitutions in this kinase-negative Tyk2 abolished the induced phosphorylation. These results indicate that Tyk2 is activated by phosphorylation on Tyr-1054 and/or Tyr-1055 and that this phosphorylation requires another kinase, most likely J AK1. While the Tyk2 forms mutated on Tyr-1054 and Tyr-1055 or on Lys-930 allowed some inducible gene expression, the combination of the three point mutations totally abolished signaling.

Tyk2 (1) belongs to the J AK1 family of intracellular protein-tyrosine kinases (PTKs), which presently has three other mammalian members, J AK1 (2), J AK2 (3, 4), and J AK3 (5, 6), and a Drosophila homologue, hopscotch (7). These PTKs of relative molecular mass in the range of 120–130 kDa are unique in having two tandemly arranged kinase domains: a carboxy-terminal, functional tyrosine kinase (TK) domain and an adjacent kinase-like (KL) domain of unknown function (see Fig. 1). No other recognizable protein motifs (SH2, SH3, etc.) are present, although there are five further regions of sequence homology conserved among the family members (2).

The J AKs play a crucial role in signaling through cytokine receptors, as was first demonstrated by their ability to complement the genetic defect of mutant cell lines unresponsive to interferons (IFN): it was later found that ligand binding to almost all known cytokine receptors induces the tyrosine phosphorylation of one or more J AK family members. The physical association of these enzymes with a number of receptor chains was also demonstrated, and deletions or mutations abolishing this interaction were found to inactivate signaling (for recent reviews, see Refs. 8 and 9).

One of the best characterized cytokine signaling pathways is the one triggered by IFN-α/β. The involvement in this pathway of two J AKs, Tyk2 and J AK1, was well established through the study of the IFN-α unresponsive human mutant cell lines 11, 1 and U4, lacking Tyk2 and J AK1, respectively (10, 11). Both kinases have been shown to physically interact with components of the IFN-α/β receptor complex (12): Tyk2 is associated with IFNAR1 (13, 14), and J AK1 appears to associate with the recently identified long form of IFNAR2, called IFNAR2-L or βL subunit (15–17). Moreover, a functional receptor requires the presence of the two kinases, as shown by the IFN-α binding defect of 11, 1 and U4 cells (11, 18). We have recently reported that both the amino-terminal region and the KL domain of Tyk2 are required to restore high affinity binding sites for IFN-α in 11, 1 cells, but that there are some dynamic characteristics of the binding which require the TK domain (19). Whether the presence of the TK domain or rather the enzymatic activity of Tyk2 is required to reconstitute a fully active receptor/ligand complex is unknown.

IFN-α/β treatment induces tyrosine phosphorylation and enzymatic activation of both J AK1 and Tyk2 (11, 20). Once activated, the J AKs initiate the IFN-α/β signaling cascade, whose known steps are the phosphorylation of the receptor chains (21–23), the recruitment, activation by tyrosine phosphorylation, homodimerization, and translocation of STAT2 and STAT1 to the nucleus where, through a 48-kDa subunit, they bind to conserved DNA elements within the promoter of IFN-responsive genes inducing their transcription (9).

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1 The abbreviations used are: J AK, Janus kinase; TK, tyrosine kinase; PTK, protein tyrosine kinase; KL, kinase-like; IFN, interferon; IFNAR, interferon-α receptor; STAT, signal transducer activator of transcription; GST, glutathione S-transferase; HAT, hypoxanthine/aminopterin/thymidine; wt, wild type; PAGE, polyacrylamide gel electrophoresis; ndf, neomycin-resistant.
IFN-α-dependent Phosphorylation of Tyk2 by Another Kinase

Stimulation of the activity of the JAKs as measured in vitro correlates, in the intact cell, with an increase in their level of tyrosine phosphorylation, suggesting, as for most receptor and nonreceptor protein kinases, that the activity of these enzymes is modulated by their relative level of phosphorylation. Several details remain, however, to be clarified: 1) the identification of the tyrosine residues critical for kinase activation/inhibition; 2) the precise nature of the activating step, whether a conformational change and/or the juxtaposition of the kinases brought about by the ligand-driven receptor chain clustering; and 3) the spatial and temporal order of events and the precise role of each individual JAK protein. To address some of these questions, we have generated and studied six mutant forms of Tyk2 bearing single or multiple mutations in the TK domain. All mutant forms have been stably expressed in the Tyk2-deficient 11.1 cell line and the IFN-α response analyzed. Here we show that Tyk2 enzymatic activity is regulated by phosphorylation at Tyr-1054 and/or Tyr-1055 in the activation loop and that, in response to IFN-α in the intact cell, this event is dependent on the induced activity of another kinase, most likely JAK1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The mutant cell line 11.1 (also called U1A) was described previously (18). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum and 250 μg/ml hygromycin. Plasmid DNA transfections were carried out using calcium phosphate as reported previously (18). Two days after transfection, cells were seeded at 0.4–4 × 10^5 cells/90-mm dish, in the presence of 450 μg/ml hygromycin. Plasmid DNA transfections were carried out using calcium phosphate as reported previously (18). Two days after transfection, cells were seeded at 0.4–4 × 10^5 cells/90-mm dish, in the presence of 450 μg/ml hygromycin. Plasmid DNA transfections were carried out using calcium phosphate as reported previously (18). Two days after transfection, cells were seeded at 0.4–4 × 10^5 cells/90-mm dish, in the presence of 450 μg/ml hygromycin.

RESULTS

Four single point mutations and one double mutation were generated within the TK domain or the carboxyl-terminal tail of Tyk2 (Fig. 1). In mutant K930R the invariant lysine of the ATP binding site within subdomain II of the TK domain (28) was substituted with an arginine. In the other four mutants, tyrosine residues were replaced with phenylalanines. Tyrrosines at positions 1054 and 1055, replaced in mutant Y1054F/Y1055F, lie in the so-called activation loop, which in most PTKs contains well established sites of regulatory phosphorylation (28, 29). Tyr-1068 (mutant Y1068F) is in subdomain III, a putative binding site for the p85 subunit of the phosphatidylinositol 3-kinase, and in the carboxyl-terminal tail of Tyk2 (Fig. 1). In mutant Y1054F/Y1055F, Y1068F, Y1145F, and Y1176F were obtained by oligonucleotide-mediated site-directed mutagenesis of a Tyk2 cDNA PlsI fragment subcloned into a bacteriophage M13 vector (26). The T10-2 monoclonal antibody (Hybridolab, Institut Pasteur) was used for detection in Western blot. Both antibodies were raised against a GST fusion protein containing amino acids 289–451 of human Tyk2. The JAK1 antiserum was a gift of A. Ziemek. The anti-phosphotyrosine 4G10 antibody was purchased from Upstate Biotechnology, Inc. Preparation of cellular extracts, immunoprecipitations, and immunoblotting were carried out essentially as described previously (13, 20). For anti-JAK1 immunoprecipitations, cells were lysed in radioimmune precipitation buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS). An ECL Western blotting detection system (Amersham Corp.) was used according to the manufacturer’s instructions.

In Vitro Kinase Assay—For the in vitro kinase assay, anti-Tyk2 immunoprecipitates were washed three times with ice-cold lysis buffer containing 400 mM NaCl, once in kinase buffer (50 mM Hepes, pH 7.4, 10 mM MgCl₂), and resuspended in kinase buffer containing 10 μCi of [(γ-³²P)ATP (3000 Ci/mmol, Amersham Corp.). 1 μg of a GST fusion protein containing the entire cytoplasmic region of human IFNAR1 (GST-IFNAR1cyt) was added to the reaction as an exogenous substrate. The GST-IFNAR1cyt construct was a gift from L. Ling. Phosphorylated products were resolved on a 7% SDS-PAGE gel, transferred to Hybond-C Super membrane (Amersham Corp.), visualized by autoradiography, and quantitated by a PhosphorImager (Molecular Dynamics).

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different point mutations on the in vivo phosphorylation state and the enzymatic activity of Tyk2. Cells expressing the wt or the mutated proteins were left untreated or treated with IFN-α and Tyk2 immunoprecipitates were either analyzed by blotting with antibodies against phosphotyrosine or subjected to an in vitro kinase assay. The level of phosphorylation and the kinase activity of wt Tyk2 and mutants Y1145F, Y1176F (Fig. 2), and Y1054F/Y1055F (data not shown) were all comparably increased in response to IFN-α. Although in this experiment the in vivo phosphorylation of mutant Y1054F/Y1055F is barely detectable in noninduced cells, further analysis confirmed a basal level of phosphorylation comparable to the wt protein.

In contrast, the in vivo phosphorylation state of the Y1054F/Y1055F mutant protein was unchanged by IFN-α addition (Fig. 3A, lanes 3 and 4). In vitro, the basal autophosphorylation and the phosphorylation of an exogenous substrate, here a GST fusion protein containing the cytoplasmic region of IFNAR1 (GST-IFNARcyt) (33), could not be enhanced by IFN-α treatment (Fig. 3B, lanes 3 and 4). Similar results were obtained for two other Y1054F/Y1055F transfectants expressing different amounts of the mutated protein (data not shown). These data suggest that phosphorylation of Tyr-1054 and/or Tyr-1055 occurs upon IFN-α treatment and is responsible for the enzymatic activation of wt Tyk2. Quantitation of the data presented in Fig. 3B showed that the auto- and substrate-phosphorylation activity of mutant Y1054F/Y1055F was half of the activity of the wt protein in noninduced cells.

The K930R mutant, bearing a mutation in the ATP binding site, is catalytically inactive (Fig. 3B, lanes 5 and 6). This protein is not basally phosphorylated, while the wt and the Y1054F/Y1055F proteins are (Fig. 3A), suggesting that auto-phosphorylation is responsible for the basal level of phosphorylation. On the other hand, the K930R protein becomes phosphorylated upon IFN treatment while the phosphorylation of the Y1054F/Y1055F protein does not change (Fig. 3A), indicating that another kinase is responsible, at least in part, for the IFN-induced phosphorylation. To confirm that this induced phosphorylation occurs on tyrosines 1054 and/or 1055, we constructed a triple Tyk2 mutant, containing the K930R and the Y1054F/Y1055F substitutions. Clones expressing levels of the K930R/Y1054F/Y1055F protein comparable to that of the other transfectants were analyzed for the in vivo phosphorylation state of the protein. As predicted, no basal nor induced phosphorylation of this mutant form could be detected (Fig. 4).

Mutant Y1054F/Y1055F and Mutant K930R Sustain Reduced Signaling—We next addressed the question of whether these mutated proteins were able to sustain a response to IFN-α. We tested the ability of all transfectants to grow in HAT selective medium in the presence of increasing concentrations of IFN-α. As reported previously, this medium constitutes a sensitive biological assay for IFN-α signaling (18, 19). Whereas wt Tyk2-expressing cells grew with 100 IU/ml IFN-α, cells expressing the Y1054F/Y1055F or the K930R mutant required at least a 25-fold higher concentration (Fig. 5A). Unexpectedly, none of the four clones analyzed expressing the K930R/Y1054F/Y1055F mutant could survive at any dose of IFN-α. Transfectants expressing Y1068F, Y1145F, and Y1176F exhibited a wild-type behavior (data not shown).

As a measure of the IFN-α response in the Y1054F/Y1055F and in the K930R transfectants, we also studied the transcriptional induction of the endogenous IFN responsive 6-16 gene by Northern blot. A dose-response analysis of the accumulation of the 6-16 transcript in wt-, Y1054F/Y1055F- and K930R-expressing cells is shown in Fig. 5B. Accumulation of the 6-16 transcript was detectable in Y1054F/Y1055F and K930R transfectants after IFN-α treatment, but strongly reduced when compared with wt Tyk2-expressing cells. Quantitation of this and other similar experiments showed that in K930R and Y1054F/Y1055F transfectants treated with 1000 IU/ml IFN-α, the level of 6-16 mRNA is approximately one-third of the level
in cells expressing the wt protein treated with 10 IU/ml. This was confirmed for another inducible mRNA, guanylate-binding protein. As expected from their lack of survival in HAT plus IFN-α medium, the K930R/Y1054F/Y1055F transfectants did not induce gene expression (data not shown).

Weak Induced JAK1 Phosphorylation Detectable in Y1054F/Y1055F Transfectants—

It has been previously suggested that a functional interdependence exists between Tyk2 and JAK1 (11, 19). Therefore we monitored the in vivo phosphorylation of JAK1 in the K930R and the Y1054F/Y1055F transfectants. For each mutant, four neo' clones were analyzed. No evident change in the basal phosphorylation state of JAK1 was observed when comparing the various transfectants and no detectable enhancement of phosphorylation occurred in K930R- and K930R/Y1054F/Y1055F-expressing cells in response to IFN. On the other hand, in Y1054F/Y1055F-expressing cells, induced JAK1 phosphorylation was seen, although reduced compared to wt cells (Fig. 6). These results suggest that, upon ligand binding, the basal kinase activity exhibited by the Y1054F/Y1055F mutant contributes to JAK1 phosphorylation.

**DISCUSSION**

In this study we have analyzed the role of TK domain tyrosine residues in Tyk2 activation and signaling. We have found that single replacement of Tyr-1068, Tyr-1145, or Tyr-1176 with phenylalanine did not affect the phosphorylation state and the enzymatic activity of the protein. Furthermore, no change in the IFN-induced gene expression was observed in these transfectants compared with wt transfectants. These results suggest that such tyrosine residues are not targets of regulatory phosphorylation. The finding that substitution of Tyr-1176 in the carboxyl tail of Tyk2 does not affect the regulation of Tyk2 is a posteriori not surprising considering that the equivalent phosphorylated Tyr-527 in pp60c-src represses enzyme activity by intramolecularly interacting with the SH2 domain (34), and no such domain is present in Tyk2. Furthermore, no tyrosine equivalent to Tyr-1176 in Tyk2 is found in other members of the JAK family.

**FIG. 3.** In vivo phosphorylation state and in vitro kinase activity of mutants Y1054F/Y1055F (F1054-55) and K930R (R930). A, in vivo tyrosine phosphorylation. Anti-phosphotyrosine (upper panel) and anti-Tyk2 (lower panel) immunoblottings were performed as in Fig. 2. B, in vitro kinase assay. The assay was essentially performed as in Fig. 2, with the addition to the reaction of 1 μg of GST-IFNAR1cyt as an exogenous substrate. In the lower panel the filter was probed with anti-Tyk2 antibody. Minor Tyk2 forms of higher mobility were routinely detected and probably result from in vivo degradation.

**FIG. 4.** In vivo phosphorylation of the K930R/Y1054F/Y1055F (F1054-55/R930) mutant. Anti-phosphotyrosine (upper panel) and anti-Tyk2 (lower panel) immunoblotting were performed as in Fig. 2.
yalanine in Tyk2 reduces basal catalytic activity and, more importantly, abolishes the IFN-dependent activation of the protein (Fig. 3). Tyr-1054 and Tyr-1055 lie within the so-called “activation loop” of subdomain VII, a region conserved among individual members of different protein kinase families (28, 29) and absent in the KL domain of JAKs. Virtually all known PTKs, with the exception of the Csk family, contain from one to three tyrosine residues in this loop and their phosphorylation positively regulates enzymatic activity. The recent determination of the crystal structure of the inactive insulin receptor kinase domain has shown that the activation loop, unphosphorylated, is engaged in the active site and precludes the access of ATP. Phosphorylation in trans of Tyr-1162, one of three well established sites of ligand-induced autophosphorylation present in this loop, would disengage it from the active site, stabilize the enzyme in an active conformation, and allow access of ATP and substrate (35, 36). From our data it appears clear that an absolute requisite for ligand-induced autophosphorylation present in this loop, would disengage it from the active site, stabilize the enzyme in an active conformation, and allow access of ATP and substrate (35, 36). From our data it appears clear that an absolute requisite for ligand-induced autophosphorylation present in this loop, would disengage it from the active site, stabilize the enzyme in an active conformation, and allow access of ATP and substrate (35, 36). From our data it appears clear that an absolute requisite for ligand-induced autophosphorylation present in this loop, would disengage it from the active site, stabilize the enzyme in an active conformation, and allow access of ATP and substrate (35, 36).

Our data show that in vivo another kinase is responsible (at least in part) for the IFN induced Tyr1054 and/or Tyr1055 phosphorylation. In fact, the kinase inactive K930R protein can be phosphorylated, albeit weakly, upon IFN addition, while the Y1054F/Y1055F (and the triple mutant) cannot. The finding that the induced phosphorylation of K930R is weaker than that of the wt protein (Fig. 3A) can be ascribed to lack of autophosphorylation and/or impaired trans-phosphorylation by the other kinase. While it would be rather difficult to evaluate the relative contribution of each of these two events, the absence of Tyk2 phosphorylation in the presence of a kinase inactive JAK1 (see below) suggests an order of events whereby the initiating step toward Tyk2 phosphorylation, and activation, is JAK1-mediated.

Interestingly, for a number of other PTKs, phosphorylation of tyrosine residues in the activation loop has been shown to rely on a distinct kinase. Focal adhesion kinase and ZAP-70 are intracellular tyrosine kinases, evolutionarily close to Tyk2, possessing two equivalent adjacent tyrosines. Maximal kinase activity of focal adhesion kinase requires phosphorylation of these two residues, most likely by a recruited Src family enzyme (37). In ZAP-70, a Syk family member required for T cell development and T cell antigen receptor function, mutation of one of the two adjacent tyrosines abolishes further phosphorylation events and receptor function. As for focal adhesion kinase, phosphorylation of this residue appears to depend on

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Fig. 5. Biological activity of Y1054F/Y1055F (F1054-55) and K903R (R930) mutants. A, the sensitivity to IFN-α of the mutant- and wt Tyk2-expressing cell lines was compared by seeding 6 × 10⁴ cells/well in six-well plates in HAT medium without or with the indicated concentrations of IFN-α. After 10 days, cells were fixed and stained. B, induction of the 6-16 mRNA by IFN-α. Northern transfers of 10 μg of total RNA from untreated cells (–) or cells treated with the indicated amounts of IFN-α for 4 h were hybridized to a 6-16-specific cDNA probe (upper panel) or, as a loading control, with a β-actin probe (lower panel).
with a regulatory tyrosine phosphatase. It is conceivable that the basal level of Tyk2 phosphorylation in the cell is the balanced result of spontaneous autoactivation and dephosphorylation by an interacting phosphatase and that deletion of critical domains or overexpression could displace this equilibrium (41, 42).

Sequential Activation and Cooperativity of the j AKs in the IFN-α/β System—Tyk2-deficient cells have impaired IFN-α binding (reduced uptake of labeled IFN), which can be fully reconstituted by transfection of wt Tyk2 (18, 19). This and the finding that transfection of an inactive Tyk2 deleted of its TK domain (ΔTK) partially restores receptor-mediated IFN uptake highlighted a structural role of Tyk2 in receptor complex formation (19). Though an increase in binding affinity was observed, some dynamic aspects of productive binding (in particular the down phase) were not reconstituted. The uptake of labeled IFN-α is comparable in the K930R, Y1054F/Y1055F, and ΔTK transfectants, both in dynamics and affinity (data not shown), suggesting that it is not the physical presence of the TK domain, but rather its ligand-dependent activation, that contributes to the down phase of productive binding, which probably involves changes in stoichiometry, aggregation of binding sites, and a subsequent internalization of the complex (12, 43). In spite of a similar IFN-α uptake ability, inducible gene expression is 10-fold higher in the Y1054F/Y1055F and K930R transfectants than in ΔTK-expressing cells (data not shown), suggesting that the TK domain contributes to the response. While the Y1054F/Y1055F mutant would contribute to JAK1 activation through its residual kinase activity, the function contributed by the kinase inactive mutant is more difficult to explain, as induced JAK1 phosphorylation is undetectable in these cells. We cannot totally exclude the possibility that the Lys to Arg substitution present in the K930R does not totally abolish kinase activity, as was reported for a Ser/Thr kinase bearing the same mutation in the ATP binding site (44). On the other hand, kinase active JAK1 appears to be an essential prerequisite to Tyk2 activation and signaling, as was recently reported by I. M. Kerr and collaborators. They have expressed a kinase inactive form of JAK1 in a cell line deficient in endogenous JAK1 (11). Such transfectants do not respond to IFN-α (45) nor can induced Tyk2 phosphorylation be detected. Altogether these results highlight the nonequivalence of the two kinases in the immediate activation steps of the cascade and suggest a temporal order of events whereby ligand binding to the multimeric receptor leads to JAK1-driven Tyk2 activation through trans-phosphorylation on Tyr-1054 and/or Tyr-1055. Activated Tyk2 might then potentiate signaling through auto-phosphorylation and positive feedback onto JAK1. We think that the nonequivalence of Tyk2 and JAK1 in this system is not an intrinsic feature of the enzymes, but rather is dictated by the properties of the receptor chain to which each kinase is physically associated. The individual role of each receptor chain, the stoichiometry of the receptor/JAK complex, and possible changes upon ligand binding are not well known at the present time (12). Tyk2 physically interacts with the membrane-proximal region of IFNAR1 and it is assumed that JAK1 physically interacts with the recently identified receptor chain IFNAR2−2 or βL. Assuming that these are unique or strongly preferred receptor/kinase interactions, the behavior of each enzyme (i.e., their spatial and temporal activation) will depend on the specific ligand binding properties of the receptor subunit to which it is bound.

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2 M. C. Gauzzi and S. Pellegrini, unpublished observation.

3 J. Briscoe, N. Rogers, and I. M. Kerr, personal communication.
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