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Two Rare Disease-Associated Tyk2 Variants Are Catalytically Impaired but Signaling Competent

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Tyk2 belongs to the Janus protein tyrosine kinase family and is involved in signaling of immunoregulatory cytokines (type I and III IFNs, IL-6, IL-10, and IL-12 families) via its interaction with shared receptor subunits. Depending on the receptor complex, Tyk2 is coactivated with either Jak1 or Jak2, but a detailed molecular characterization of the interplay between the two enzymes is missing. In human populations, the Tyk2 gene presents high levels of genetic diversity with >100 nonsynonymous variants being detected. In this study, we characterized two rare Tyk2 variants, I684A and P1104A, which have been associated with susceptibility to autoimmune disease. Specifically, we measured their in vitro catalytic activity and their ability to mediate Stat activation in fibroblasts and genotyped B cell lines. Both variants were found to be catalytically impaired but rescued signaling in response to IFN-α/β, IL-6, and IL-10. These data, coupled with functional study of an engineered Jak1 P1084A, support a model of nonhierarchical activation of Janus kinases in which one catalytically competent Jak is sufficient for signaling provided that its partner behaves as proper scaffold, even if inactive. Through the analysis of IFN-α and IFN-γ signaling in cells with different Jak1 P1084A levels, we also illustrate a context in which a hypomorphic Jak can hamper signaling in a cytokine-specific manner. Given the multitude of Tyk2-activating cytokines, the cell context–dependent requirement for Tyk2 and the catalytic defect of the two disease-associated variants studied in this paper, we predict that these alleles are functionally significant in complex immune disorders. The Journal of Immunology, 2013, 190: 000–000.

Tyk2 is one of the four Janus (Jak) tyrosine kinases and is involved in signaling of cytokines, such as type I and type III IFN, proinflammatory cytokines IL-6, -12, -22, -23, and -26, and the anti-inflammatory IL-10. Thus, Tyk2 is an essential enzyme centralizing inputs from cytokines that shape the immune response. Signaling defects to some of these cytokines have been documented in human and murine Tyk2-deficient cells (1–4). To date, Tyk2 deficiency in two patients has been associated with different clinical pictures, one more complex with features of hyper-IgE syndrome, skewed Th2 lymphocyte polarization, and susceptibility to numerous pathogens and the second with disseminated bacillus Calmette-Guérin infection, neurobrucellosis, and herpes zoster infection (4, 5). A role of Tyk2 in the pathogenesis of autoimmune and inflammatory diseases was suggested from the phenotype of the B10. Q/J strain of mice with a Tyk2 missense mutation (6–8). Studies in null murine models showed that the impact of Tyk2 in cytokine responses and defense to pathogens varies among cell types and infectious contexts (reviewed in Ref. 9). Tyk2 participates in the protective antitumor response, and Tyk2 null mice were shown to have increased predisposition to tumor formation due to a compromised immune surveillance (10).

The survey of sequence databases from International Consortia (dbSNP, 1000 Genomes) reveals a high number of single nucleotide polymorphisms (SNPs) in the Tyk2 gene. Many SNPs are in the coding region, and >100 nonsynonymous variants have been annotated. Genome-wide association studies and more targeted candidate approaches have shown strong linkage of Tyk2 haplotypes or individual SNPs to systemic and organ-specific autoimmune diseases, namely systemic lupus erythematosus (SLE) (11–14), multiple sclerosis (MS) (15–19), Crohn’s disease (20–22), psoriasis (23), type 1 diabetes (24), endometriosis-related infertility (25), and primary biliary cirrhosis (26) (Table I). Interestingly, one rare MS-associated variant (rs34536443) is considered to be protective, whereas another (rs55762744) was recently identified as a risk allele at the risk allele (17). rs1270356, initially reported to be protective in SLE (13), was not confirmed in a replication study (12) and instead found to be a risk allele in Crohn’s disease (20, 22). A frequent variant (rs2304256) has been shown to be protective in SLE, Crohn’s, and type 1 diabetes in European populations (Table I). Tyk2 allelic variants may predispose to tumor formation. Using a computational approach to distinguish polymorphisms from cancer-associated mutations, Kaminker et al. (27) identified the Tyk2 rs34536443 variant as associated to increased cancer risk. rs34536443 was also identified in a high-throughput sequencing approach used to find tyrosine kinase–encoding gene variants potentially relevant in de novo acute myeloid leukemia (28). Altogether, these studies point to Tyk2 as one of the genetic determinants in some complex diseases.

The activation of Tyk2 by immunoregulatory cytokines relies upon its ability to bind specifically to one of the two components of heterodimeric receptors. Whether coactivated with Jak1 or Jak2, Tyk2 is always found associated to the low-affinity receptor subunit (i.e., IFNAR1, IL-12Rβ1, and IL-10R2), which is devoid of essential phospho-Tyr–based Stat recruitment motifs. Early studies of the type I IFN (IFN-α/β) receptor (IFNAR1 and IFNAR2) demonstrated that the structural integrity of the receptor/Tyk2/
Jak1 complex is essential for signaling, because cells lacking either of the two Jaks or expressing only the receptor-binding domain are unresponsive to IFN. The large N-terminal region of Tyk2 (FERM and Src homology 2-like domains) interacts with the membrane-proximal portion of IFNAR1. Phospho-transfer activity requires the regulatory JH2 (kinase-like, pseudokinase) domain and the catalytic tyrosine kinase JH1 domain (29–31). In addition, Tyk2 has a noncatalytic scaffolding function that ensures plasma membrane stability of IFNAR1 (32). As is the case for the other Jaks, little is known of the connectivity and interplay between the various domains of Tyk2 in the basal state. Also, the molecular details that allow cytokine-induced activation of the receptor-associated kinases are not well defined. It is unclear whether heterodimerizing Jaks play equivalent roles or whether one exerts a dominant catalytic (initiator) role and the other a subordinate role that may be essential in some cellular contexts. Related to this is the question of whether each Jak activates unique signaling steps, or both are equally needed to downstream signals. These issues are of clear relevance to the development of specific inhibitors as therapeutics and are addressed in this study.

We studied the functional impact of two Tyk2 disease-associated variants. We demonstrate that Tyk2 I684S (rs12720356) and Tyk2 P1104A (rs34536443) variants are catalytically impaired. However, in contrast to the canonical ATP-binding mutant Tyk2 K930R, these variants were as competent as Tyk2 wild-type (WT) in reconstituting IFN-α-induced Stat signaling in Tyk2-null cells. These data were corroborated by analyses of IFN-α, IL-6, and IL-10 signaling in EBV-B lymphocytes from genotyped individuals. Thus, these two rare Tyk2 variants are hypomorphic alleles for which catalytic deficiency can be compensated by the Jak partner, at least in some cellular contexts and for some cytokines.

Materials and Methods

Plasmid constructs

Tyk2 WT has been described as pRc-Tyk2 (30). Tyk2 P1104A and Tyk2 I684S, in pRc-CMV and pRES vectors respectively, were generated by standard PCR technique. To obtain Tyk2 V678F/P1104A, a 1-kb SgrAI-XbaI fragment spanning the P1104A mutation was prepared from Tyk2 P1104A in the pRc-CMV vector and swapped into pRc-Tyk2 (33). The human Jak1 P1084A and K908E mutants were generated by site-directed mutagenesis in pBS-Jak1 and recloned into pRc/CMV-Jak1 (31) with AfeI/HindIII. All new plasmids were verified by sequencing. All expression constructs in pBS-Jak1 and recloned into pRc/CMV-Jak1 (31) with AfeI/HindIII. All new plasmids were verified by sequencing. All expression constructs in pBS-Jak1 and recloned into pRc/CMV vector and swapped into pRc-Tyk2 (33). The human Jak1 P1084A and K908E mutants were generated by site-directed mutagenesis in pBS-Jak1 and recloned into pRc/CMV-Jak1 (31) with AfeI/Xbal. All new plasmids were verified by sequencing. All expression constructs, except Tyk2 I684S, have a C-ter vesicular stomatitis virus glycoprotein (VSV-G) epitope tag.

Cells and transfection

EBV-transformed B cell lines were obtained from Coriell Cell Repositories (Camden, NJ) and the Centre de Ressources Biologiques of Réseau Français d’Etude Générale sur la Sclérose en Plaques. Genotyping for rs12720356 and rs34536443 confirmed the presence of the specific polymorphism in the EBV-B lines. Cells were cultured in RPMI 1640 and 10% heat-inactivated FCS, and before cytokine stimulation and protein analysis, cells were serum starved for 2 h. The IFN-α-unresponsive human fibrovascular 11,1 (U1A, Tyk2-deficient) cells and U4C (Jak1-deficient) cells were cultured in DMEM and 10% heat-inactivated FCS. Transfections were performed with FuGENE6 (Roche Applied Science). The 11,1 cells were transfected with pRc-CMV–based plasmids for stable expression of Tyk2 WT or mutants, and clones were selected in 400 μg/ml G418. U4C cells were cotransfected with pSV2-puro and pRc-CMV-Jak1 WT, Jak1 P1084A, and Jak1 K908E. PuroR clones were selected in 400 ng/ml puromycin. IFN-α2 was a gift from D. Gewert (Wellcome Research Laboratories). Recombinant human IL-10 was from PeproTech France. IL-6 was a gift from Merck Serono.

Western blot analysis and Abs

Cells were lysed in modified RIPA buffer (34). A total of 30 μg proteins was separated by 7% SDS-PAGE and analyzed by Western blot. Membranes were cut horizontally according to molecular size markers, and stripes were incubated with different Abs. Immunoblots were analyzed by ECL with the ECL Western blotting Reagent (Pierce) or the more sensitive Western Lightning Chemiluminescence Reagent Plus (PerkinElmer). For reprobing, blots were stripped in 0.2 M glycine (pH 2.5) for 30 min at room temperature. The following Abs were used, unless otherwise indicated in figure legends: Tyk2 mAb T10–2 (Hybridolab, Institut Pasteur); anti-Jak1–phospho-YY1022/23 (BioSource International, Invitrogen); anti-Stat1, anti-Stat2, anti-Stat3–phospho-Y689, anti-STAT3, anti-Jak1, and anti-phosphotyrosine mAb 4G10 (Millipore); and anti-Stat1–phospho–Y701, anti-Stat3–phospho–Y705, and anti-Tyk2–phospho–YY1054/55 (Cell Signaling Technology, Beverly, MA).

In vitro kinase assay

Cells were lysed in 50 mM Tris (pH 6.8), 0.5% Nonidet P-40, 200 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM sodium vanadate, 1 mM sodium fluoride, 10 mM PMPSF, 3 μg/ml aprotinin, 3 μg/ml leupeptin, and 3 μg/ml pepstatin. Tyk2 and Jak1 were immunoprecipitated from 2 μg lysate using noncommercial R5-17 polyclonal Abs or affinity-purified anti–VSV-G polyclonal Abs (a gift from M. Arpin, Institut Curie). Immunocomplexes were washed three times in buffer 1 (50 mM Tris [pH 7.6], 400 mM NaCl, 0.5% Triton X-100, and 1 mM EDTA), once in buffer 2 (50 mM Tris [pH 6.8] and 200 mM NaCl), and once in kinase buffer (50 mM HEPES [pH 7.6] and 10 mM MgCl2). The kinase reaction was carried out in 50 mM HEPES [pH 7.6], 10 mM MgCl2, 0.6 μg recombinant Stat3 (SignalChem), and with or without 30 μM ATP at 30°C for 5 min in a total volume of 30 μl. The reaction was terminated by boiling in Laemmli buffer. Half of the sample was loaded for SDS-PAGE, transferred to a nitrocellulose membrane, and phosphorylated proteins were detected by using ECL detection reagents (Western Lightning; PerkinElmer).

Results

Tyk2 I684S is catalytically impaired but relays cytokine signaling

rs12720356 refers to a rare-occurring nonsynonymous variant that maps in exon 15 of the Tyk2 gene and causes replacement of the amino acid Ile at position 684 with a serine (I684S) (Table I). The
Ile residue is located in the JH2 domain and is conserved across species in Tyk2, Jak1, and Jak2 (Fig. 1A, 1B). A threonine residue is present in Jak3 orthologs and Drosophila Hopscotch. To functionally analyze this allelic form, we studied EBV-B-transformed lines established from individuals genotyped for rs12720356. Two lines (referred as AA1 and AA2 in this paper) are homozygote for the ancestral A allele, two are homozygote for the derived C allele (CC1 and CC2), and four are heterozygote (AC1 to AC4). The comparable level of Tyk2 expression in the eight cell lines excluded an effect of the mutation on stability (Fig. 2A). First, we compared the autophosphorylation ability of the AA (Tyk2 I684) and the CC variants (Tyk2 S684). For this, the protein was immunoprecipitated from homozygote cells and subjected to in vitro kinase assay in the presence or absence of added ATP. The reaction product was analyzed by immunoblotting to phospho-tyrosine 4G10 mAb and phospho-Tyk2 Abs specific to Tyr1054–1055 in the activation loop. As shown in Fig. 2B (left panel), in the absence of ATP, a weak phospho-Tyk2 band was detected with 4G10 in both samples, suggesting comparable basal phosphorylation in cells. When ATP was added to the reaction, the intensity of the phospho-Tyk2 reactive band strongly increased in the AA sample (Tyk2 I684), indicating activity. No activity, however, was detected in the CC sample (Tyk2 S684).

We then assessed the in vitro activity of Tyk2 immunopurified from cells stimulated with IFN-α. AA2 and CC2 lines were used as they express comparable surface levels of IFNAR1 and IFNAR2 (data not shown). Upon ATP addition, a 3- to 4-fold increase in phospho-Tyk2 was observed for IFN-α–treated AA2 cells but not for CC2 (Fig. 2B, right panel). Overall, these data indicate that, in vitro, Tyk2 S684 has impaired basal and IFN-α–induced autophosphorylation activity. Nonetheless, in cells, the variant is phosphorylated to the same extent as the ancestral form.

Next, we compared the activation of Stat proteins in the EBV-B lines stimulated with cytokines that activate Tyk2. As shown in Fig. 2C, the extent of phosphorylation of Tyk2, Stat1, and Stat2 was comparable in AA2 and CC2 cells stimulated with nonsaturating doses of IFN-α. Activation of Stat3 by IL-6 and IL-10 was measured in two AA lines and two CC lines and found to be roughly comparable (Fig. 2D, 2E). Of note, the low constitutive level of phospho-Stat3 in these cells may be due to secrete autocrine-acting cytokine(s) (35).

To exclude potential effects from other Tyk2 polymorphisms, we engineered the I684S substitution in the Tyk2 cDNA and derived stable transfectants in Tyk2-deficient 11.1 cells. The in vitro catalytic activity of immunopurified Tyk2 WT and Tyk2 I684S was compared as described above. rStat3 was added to the reaction as exogenous substrate, and the reaction products were immunoblotted with 4G10 mAb to reveal phosphotyrosines. Fig. 3A shows that ATP-dependent autophosphorylation and substrate phosphorylation are impaired in the case of Tyk2 I684S. Of note, when immunopurified from IFN-α–stimulated cells, Tyk2 I684S was phosphorylated on the activation loop to the same extent as the WT protein (Fig. 3A, lanes 3 and 7). Next, we compared IFN-α–induced Stat1/2/3 phosphorylation in WT and I684S-expressing cells. No consistent differences were found in the extent of Stat activation, even in the context of a clone expressing a lower level of Tyk2 I684S (cl.2, Fig. 3B). These results reinforce the conclusions drawn from the analysis of EBV-B cell lines that replacement of Ile684 with a serine abrogates Tyk2 kinase activity without affecting cytokine-induced signaling.

Tyk2 P1104A is catalytically impaired but relays Stat signaling
rs34536443 refers to a rare nonsynonymous Tyk2 variant that maps in exon 23 of the Tyk2 gene and is found only in heterozygote state (Table I). At the protein level, this polymorphism causes alanine substitution of a highly conserved proline residue (Pro1104), which is located in a small Jak-specific insertion in the JH1 domain (Fig. 1A, 1C). To analyze whether this substitution affects Tyk2 function, we engineered the Pro to Ala mutation in Tyk2 cDNA. Stable WT and P1104A-expressing clones were derived from Tyk2-deficient cells to assess in vitro activity in the presence of rStat3 as exogenous substrate (Fig. 4A). The reaction products were immunoblotted with phospho-Tyk2–specific Abs and 4G10 mAb. Addition of ATP to the WT sample led to rec-Stat3 phosphorylation and Tyk2 autophosphorylation (Fig. 4A, lanes 1–4). On the contrary, Tyk2 P1104A was totally impaired in exogenous substrate phosphorylation (lanes 5–8). In IFN-stimulated cells, the mutant was phosphorylated and possessed in vitro a weak auto-phosphorylation activity targeted to tyrosines other than Tyr1054–55 in the activation loop (Fig. 4A, lanes 7 and 8, 4G10 detection). In comparison, Tyk2 K930R, mutated in the conserved lysine of the ATP binding pocket, was not phosphorylated neither in cells nor in vitro (Fig. 4B). Next, we compared Stat activation in cells expressing Tyk2 WT, P1104A, and K930R that were stimulated with IFN-α. As shown in Fig. 4C, the phosphorylation profiles were comparable in WT and P1104A-expressing cells, whereas signaling was severely hampered in K930R-expressing cells. In accordance with these data, the analysis of EBV-B cells prepared from these heterozygote rs34536443 individuals did not reveal defect in IFN-α signaling (data not shown).

To gain more insight into the impact of the Pro to Ala mutation on enzymatic activity, we asked whether this mutation would perturb the hyperactivity of Tyk2 V678F. This designed mutant is catalytically hyperactive in vitro and basally highly phosphorylated on the activation loop and thus mimics the gain-of-function pathogenic Jak2 V617F (33). We generated the V678F/P1104A double mutant (VF/PA), which was expressed to roughly equivalent level as Tyk2 WT, K930R, P1104A, and V678F in 293T cells (Fig. 5A). As expected, Tyk2 V678F was hyperphosphorylated on the activation loop. Remarkably, the double VF/PA mutant was ~10-fold less phosphorylated than V678F. Moreover, in Tyk2...
V678F-expressing cells, endogenous Stat3 was highly phosphorylated (33), but this was not the case in VF/PA-expressing cells (Fig. 5A). Next, we measured the catalytic potential of the double VF/PA mutant in vitro (Fig. 5B). As expected, Tyk2 V678F exhibited a robust activity. Conversely, the VF/PA mutant was impaired in both ATP-dependent autophosphorylation and substrate phosphorylation. Overall, these data showed that the catalytic gain-of-function effect exerted by phenylalanine at position 678 is abrogated by the Pro1104-to-Ala substitution. Thus, the Pro-to-Ala substitution has a strong negative and dominant impact on phospho-transfer activity.

Jak1 P1084A is catalytically impaired but relays Stat signaling

The natural Tyk2 I684S and P1104A variants were found to be functionally impaired in vitro and yet inducibly phosphorylated in cells and competent to rescue signaling. These data supported the view that the kinase activity of Tyk2 is dispensable or subordinate with respect to that of Jak1 (30, 36). To test this model and gain more mechanistic insight on the specific contribution of Tyk2 and Jak1, we engineered the corresponding Jak1 mutant (Jak1 P1084A) and stably expressed it in Jak1-null U4C cells. Clones expressing Jak1 WT exhibited ATP-dependent (basal and IFN-induced) activity detected with phospho-Tyr1022–1023–specific Abs and 4G10 (Fig. 6A, lanes 1–4). Conversely, Jak1 P1084A, although phosphorylated in IFN-stimulated cells, failed to autophosphorylate in vitro on the activation loop (Fig. 6A, lanes 7 and 8). A weak ATP-dependent phospho-Jak1 P1084A band was detected with 4G10 in IFN-stimulated samples (Fig. 6A, lanes 7 and 8; 4G10), similar to that shown for Tyk2 P1104A (Fig. 4A). IFN-induced Stat1/2/3 activation was restored in Jak1 P1084A-expressing cells but not in Jak1 K908E-expressing cells (Fig. 6B).

In conclusion, the Pro-to-Ala substitution in both Tyk2 and Jak1 strongly impacted catalytic activity but did not affect their ability to rescue IFN-α signaling. Thus, these data did not support the notion that Tyk2 is catalytically subordinate with respect to Jak1, but rather suggested a model of catalytic equivalence of the two enzymes and the possibility of their reciprocal compensation.

Pro-to-Ala mutants of Tyk2 and Jak1 are substrates for trans phosphorylation

A question raised by the above data concerned the remarkably different behavior between the Pro-to-Ala mutants (Tyk2 P1104A and Jak1 P1084A) and the ATP-binding mutants (Tyk2 K930R and Jak1 K908E). Although all four mutants were inactive in vitro, only the Pro-to-Ala mutants were inducibly phosphorylated on the

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**FIGURE 2.** Functional analyses of Tyk2 variants in rs12720356 genotyped EBV-B cells. (A) Tyk2 expression level in eight EBV-transformed B cell lines from rs12720356 genotyped individuals. Individuals AA1 and AA2 carry two ancestral A alleles; individuals CC1 and CC2 carry two derived C alleles; four individuals, AC1 to AC4, are heterozygotes. Cell lysates (30 μg) were immunoblotted with anti-Tyk2 and anti-Akt as loading control. (B) Left panel: basal in vitro kinase (IVK) activity of Tyk2 from unstimulated EBV-B cells of homozygote AA individual (Tyk2 I684) and CC individual (Tyk2 S684). Tyk2 was immunoprecipitated (IP) and subjected to an in vitro kinase reaction for 5 min at 30°C in the presence (+) or absence (−) of 30 μM ATP. Phosphorylated proteins in the reaction were revealed by successive immunoblotting with 4G10, anti–phospho-Tyr1022–1023 specific to the activation loop (Tyk2-P-Y), and anti-Tyk2. Right panel: kinase activity of Tyk2 from the indicated EBV-B cells after stimulation for 15 min with 50 pM of IFN-α. Samples were processed as described above. (C) IFN-α–induced Jak/Stat activation in EBV-B cells from one AA individual (Tyk2 I684) and one CC individual (Tyk2 S684). Cells were stimulated with increasing doses of IFN-α for 15 min. The level of tyrosine-phosphorylated Tyk2, Stat1, and Stat2 was analyzed by Western blot with phospho-specific Abs. The membrane was reprobed for Tyk2 and total Stat3 levels. A low level of phospho-Stats was reproducibly detected in nonstimulated cells. (D) Stat3 phosphorylation levels in the indicated EBV-B cells stimulated with IL-6 for 15 min. Tyk2 levels shown as loading control. (E) Stat3 phosphorylation levels in the indicated EBV-B cells stimulated with IL-10 for 15 min. Stat3 levels shown as loading control.
We reasoned that this may reflect the competence of the protein to be transphosphorylated (i.e., to act as substrate of the Jak partner). To test this, we performed mixed kinase assays in vitro to compare the ability of Tyk2 P1104A and Tyk2 K930R to act as substrates for Jak1. Lysates of Jak1-expressing cells were mixed with lysates of Tyk2 P1104A or Tyk2 K930R-expressing cells. Tagged Jak proteins were coimmunopurified to be assayed in vitro. As shown in Fig. 6C, addition of ATP led to strong phosphorylation of Tyk2 P1104A but not of Tyk2 K930R, indicating that, of the two catalytically impaired mutants, only Tyk2 P1104A is a suitable substrate for Jak1.

In another set of experiments, we assessed whether Jak1 P1084A was a good substrate for Tyk2 WT. For this, Jak1 P1084A-expressing cells were stably transfected with Tyk2 WT or Tyk2 P1104A. Tagged proteins were coimmunopurified and analyzed in vitro. As shown in Fig. 6D, Jak1 P1084A was phosphorylated only when copurified with Tyk2 WT (lanes 1 and 2). Conversely, when the two Pro to Ala mutants were copurified, no phosphorylated products were detected (lanes 3 and 4). These data con

**FIGURE 3.** Functional analyses of the engineered Tyk2 I684S mutant stably expressed in Tyk2-deficient cells. (A) In vitro kinase (IVK) activity of Tyk2 WT and Tyk2 I684S. Tyk2 was immunoprecipitated (IP) from cells nonstimulated or stimulated with IFN-α (500 pM, 15 min) and subjected to an in vitro kinase reaction in the presence or absence of 30 μM ATP for 5 min at 30°C. rStat3 (0.6 μg) was added to the reaction as exogenous substrate. Phosphorylated proteins in the reaction were revealed by sequential blotting with anti–phospho-Tyr mAb 4G10, anti–phospho-Tyk2, and anti-Tyk2. (B) IFN-α–induced Jak/Stat activation in cells expressing Tyk2 WT or Tyk2 I684S. One WT clone and two I684S-expressing clones were stimulated with the indicated doses of IFN-α for 15 min. The phosphorylation level of the indicated proteins was analyzed by immunoblotting with phospho-tyrosine–specific Abs. The membranes were reprobed for total protein level. Total Tyk2, Stat1, Stat2, and Stat3 are shown. Note that IS cl.2 expresses less Tyk2 (~70%) with respect to the other clones.

**FIGURE 4.** Functional analyses of the engineered Tyk2 P1104A mutant. (A) In vitro kinase (IVK) activity of Tyk2 WT and Tyk2 P1104A. NeoR clones were derived from Tyk2-deficient 11.1 cells. Tyk2 was immunoprecipitated (IP) with anti-Tyk2 from nonstimulated or IFN-α–stimulated cells and subjected to in vitro kinase reaction in the presence or absence of 30 μM ATP for 5 min at 30°C. rStat3 (0.6 μg) was added to the reaction as exogenous substrate. Phosphorylated proteins were revealed by blotting the reaction products with 4G10 and anti–phospho-Tyk2. Total levels of Tyk2 are shown. (B) In vitro kinase (IVK) activity of Tyk2 WT and Tyk2 K930R. Analysis as in (A). (C) IFN-α–induced Jak/Stat activation. Clones expressing Tyk2 WT, Tyk2 P1104A, or Tyk2 K930R were stimulated with the indicated doses of IFN-α for 15 min. Phosphorylation levels of the indicated proteins were analyzed by Western blot with phospho-tyrosine–specific Abs. The membranes were reprobed for total Tyk2 and Stat3 levels.
firmed the catalytic impairment of the Pro-to-Ala mutants and demonstrated that they are suitable substrates for the juxtaposed WT partner.

**Impact of Jak1 P1084A in IFN-α and IFN-γ signaling**

The studies described above suggested that a Pro-to-Ala mutant, either Tyk2 or Jak1, can be inducibly phosphorylated on the activation loop through the initiating action of the facing WT partner and that this reaction is productive for Stat activation. This model predicts that the WT enzyme may become rate-limiting when the mutant Jak is highly expressed. This possibility was tested by comparing the IFN-α response of cells expressing Jak1 (WT or P1084A) at near-endogenous level (referred as low) or at levels 15–20-fold higher (referred as hi). Jak1 WTlow and P1084Alow cells responded similarly to IFN-α (Fig. 7A, lanes 11–20). In contrast, Jak1hi cells responded differently, with phospho-Stat1 being more abundant in WThi than in P1084Ahi cells (lanes 1–10). This suggested that, in the presence of excess Jak1 P1084A mutant, endogenous Tyk2 activity can become limiting.

Jak1 is involved in signaling through the IFN-γ receptor complex, where it is juxtaposed to Jak2. Thus, using the clones described above, we analyzed the rescuing capacity of Jak1 P1084A by measuring IFN-γ–induced Stat1 activation. As shown in Fig. 7B, IFN-γ signaling was comparable in Jak1 low WT and P1084Alow cells (lanes 1–8). Cells reconstituted with the kinase-dead Jak1 K904E mutant, and parental 2C4 cells were monitored.

**FIGURE 6.** Functional analyses of the engineered Jak1 P1084A mutant. (A) In vitro kinase (IKV) activity of Jak1 WT, Jak1 P1084A, and the ATP-deficient Jak1 K908E. U4C cell–derived clones reconstituted with the indicated tagged Jak1 form were used. Jak1 was immunoprecipitated (IP) with anti–VSV-G from either nonstimulated cells or IFN-α–stimulated cells and subjected to in vitro kinase reaction. Phosphorylated proteins were revealed by blotting the reaction product with 4G10, anti–phospho-Jak1–Tyr1022–1023, and anti-Jak1. (B) IFN-α–induced Jak/Stat activation in U4C-derived clones expressing Jak1 WT, Jak1 P1084A, or Jak1 K908E. Cells were treated with increasing doses of IFN-α for 15 min. Phosphorylation levels of the indicated Stat were analyzed by Western blot with phospho-tyrosine specific Abs. Total Jak1 and Stat1 levels are shown. (C) In vitro trans phosphorylation of Tyk2 P1104A and Tyk2 K930R by Jak1 WT. U4C cells expressing tagged Jak1 WT were engineered to stably coexpress either tagged Tyk2 WT or Tyk2 P1104A. Tagged proteins were coimmunopurified on beads coated with anti–VSV-G and subjected to in vitro kinase (IKV) reaction. Phosphorylated proteins were revealed by blotting the reaction products with 4G10 (top panel) and, after stripping, with anti–VSV-G (bottom panel). (D) In vitro trans phosphorylation of Jak1 P1084A by Tyk2 WT or Tyk2 P1104A. U4C cells expressing tagged Jak1 P1084A were engineered to stably coexpress either tagged Tyk2 WT or Tyk2 P1104A. Tagged proteins were coimmunopurified on beads coated with anti–VSV-G and subjected to in vitro kinase reaction. Phosphorylated proteins were revealed by blotting the reaction products with 4G10 (top panel) and, after stripping, with anti–VSV-G (bottom panel).
as controls (Fig. 7B, lanes 9–15). Interestingly, Jak1WT hi and P1084A hi cells exhibited a comparable robust IFN-γ signaling (Fig. 7C, lanes 6–15). Thus, when overexpressed, Jak1 P1084A is unable to support high signaling from the type I IFN receptor but can do it from the IFN-γ receptor. Overall, these results indicate that the specific role of a Jak, and thus the impact of a variant, can differ in different cytokine receptors.

Discussion

The exact mechanism that controls basal-state and ligand-induced activation of Jak proteins in complex with cytokine receptors remains ill defined. Over the years, this issue has been addressed by measuring the signaling capacity of kinase-dead Jaks mutated in the conserved lysine of the ATP-binding pocket of the JH1 domain. For type I IFN, analysis of Jak1 K907E-expressing cells and Tyk2 K930R cells led to propose a hierarchical asymmetric activation model, with Jak1 playing the initiator role (30, 36). Studies of IFN-γ signaling were performed on cells overexpressing Jak1 or Jak2 lysine mutants and concluded on a hierarchical activation as well, with Jak2 playing the initiator role (36). The question was recently re-examined for IFN-γ with the same mutants expressed at near-endogenous levels, and the data pointed, instead, to Jak1 as the initiator (37). These studies emphasized the impact of Jak expression level and indicated a certain degree of catalytic hierarchy between two juxtaposed Jaks. Interestingly, the Jak1 and Tyk2 lysine mutants were found to have defective interactions with their cognate receptors in the IFN-γ and IFN-α context, respectively (30, 37), suggesting that the alteration of the ATP-binding pocket disturbs the conformational integrity of the enzyme (see below).

In the present work, we describe three mutants (Tyk2 I684S, Tyk2 P1104A, and Jak1 P1084A) that are catalytically impaired and yet indistinguishable from the WT proteins. Furthermore, Jak1 P1084A rescues IFN-γ signaling as well. Contrary to the lysine mutants, the Pro-to-Ala mutants are good activation loop phosphorylation substrates, both in cells and in vitro. These data suggest that, at least for these cytokine receptors, one catalytically competent enzyme is sufficient to trigger signaling provided that the juxtaposed partner, although inactive, acts as a proper scaffold and/or phospho-acceptor substrate. This unidirectional activation mode is nonhierarchical and somehow reminiscent of the allosteric mechanism of kinase activation proposed for epidermal growth factor receptor family members (38). Moreover, it is expected to be productive only in cytokine receptors bringing together two different Jaks. Consistent with this, one study showed that a catalytically inactive Jak2 mutant (Jak2 I1065A, Fig. 1C) rescued signaling to IFN-γ but not to erythropoietin, a cytokine that homodimerizes the EpoR/Jak2 complex (39).

The difference in signaling competence between the natural variants and the conventional lysine mutants, all catalytically impaired, is remarkable. As mentioned above, mutants of the lysine in the ATP-binding pocket behave as poor scaffolds and substrates and thus may be constrained in an inhibited conformation, less prone to release the activation loop. As in other protein kinases, ATP binding may prime for critical conformational changes (40, 41). Indeed, structural studies showed that the isolated JH1

FIGURE 7. Stat1 activation by IFN-α or IFN-γ in cells expressing high or endogenous-like levels of Jak1 P1084A. (A) IFN-α–induced Stat1 activation in U4C cells reconstituted with either Jak1 WT or Jak1 P1084A expressed at high (hi) or endogenous-like level (low). Cells were treated with IFN-α for 15 min. Stat1/2 phosphorylation was analyzed by Western blot. Total Jak1 and Stat1 levels are shown. (B) IFN-γ–induced Stat1 activation in U4C cells reconstituted with endogenous-like level of Jak1 WT, Jak1 P1084A, or the ATP-binding–deficient Jak1 K908E and in the parental 2C4 cells. Cells were treated with IFN-γ as indicated for 15 min. Stat1 phosphorylation was analyzed with phospho-tyrosine–specific Abs. Total Jak1 and Stat1 levels are shown. (C) IFN-γ–induced Stat1 activation in parental 2C4 cells and U4C cells expressing high Jak1 WT or Jak1 P1084A levels. Cells were treated with IFN-γ for 15 min. Stat1 phosphorylation was analyzed with phospho-tyrosine–specific Abs. Total Jak1 and Stat1 levels are shown. Note that the phospho-Stat1 membrane was exposed for shorter time than in (B). 2C4 cells are present in both panels as internal reference.
domains of the four Jaks complexed with ATP-competitive inhibitors adopt an active conformation with an exposed loop (42–44). With regard to intact Jaks, specific ATP competitive inhibitors that block catalytic function also promote activation loop phosphorylation in cells (45). Moreover, some JH2 mutants of Tyk2 and Jak3 have been described that are catalytically dead but constitutively phosphorylated on the activation loop. Importantly, this phosphorylation was abrogated when the ATP-binding lysine of JH1 was mutated (46, 47). Overall, these data support the concept that scaffolding function, ATP binding- and conformational flexibility of a Jak protein can be more critical than its phospho-transfer activity for signaling through heteromeric receptors.

Although detailed mechanistic understanding of the catalytic defect exhibited by the two Tyk2 variants will require advanced structural knowledge, the positions of each residue in the protein is informative. The isoleucine residue (Tyk2 Ile<sub>684</sub>) is located in the regulatory JH2 domain, which has negative and positive effects on JH1 (33, 46). As shown in Fig. 8A, the corresponding isoleucine in Jak2 JH2 is located in the N lobe, at the end of the loop connecting the β4 and β5 strands, and is a few residues away from Val<sub>617</sub> (48). Interestingly, it was shown that the activating V617F mutation rigidifies the JH2 αC helix through π-stacking interactions with two phenylalanines (Phe<sub>594</sub>, Phe<sub>595</sub>) confirming previous mutational studies of Jak2 V617F by Constantinescu’s group (49). Yet, the mode by which the rigidified αC helix brings about JH1 activation in Jak2 V617F remains unclear. In this study, we show that the V617F and I684S single substitutions in Tyk2 have opposite consequences on catalytic activity and thus may impact in opposite manner on the JH2 αC helix. Indeed, in Jak2 JH2, the isoleucine side chain does not point toward the αC helix, as is the case for the valine, but faces more toward the inner hydrophobic core of the N lobe (Fig. 8A). Thus, substitution of the isoleucine with the small polar serine may disturb the N lobe and compromise productive positioning of the αC helix. Overall, Tyk2 I684S appears to be impaired only in the positive regulatory function of JH2 toward JH1. This contrasts with the more severe loss of function of two point mutants in the C lobe of JH2 (Tyk2 R856G and Tyk2 E782K). These mutants are not only kinase-dead, but also highly phosphorylated on the activation loop and signaling incompetent (6, 46).

Pro1104 is located at the edge of a unique insert (13–15 residues) in the JH1 domain. This insert is conserved in the Jak family with the exception of Drosophila Hopscotch, is not found in the JH2 domain nor in other tyrosine kinases, and has been called αH helix, FG helix, Jak-specific insertion, or Lip (43). The insert folds into a short α helix (αH) that is adjacent to the αG helix of the C lobe and close to the catalytic cleft (Fig. 8B). Mutational studies in Jak2 indicated the key role of the αH helix in autophosphorylation (39). Indeed, Jak2 point mutants in conserved residues of αH showed reduced, abrogated, or enhanced phosphorylation in cells. In these studies, the proline corresponding to Tyk2 Pro<sub>1104</sub> was not analyzed nor was in vitro kinase activity of the Jak2 mutants measured. As discussed above, both Pro-to-Ala Tyk2 and Jak1 mutants that we studied in this paper rescued signaling, indicating no impairment of their respective scaffolding role. In cells, both mutants were indubitably phosphorylated in response to ligand, but failed to auto-transphosphorylate on the activation loop and to phosphorylate an exogenous substrate in vitro.

Several observations suggest that Pro<sub>1104</sub> and the adjacent αH helix are important in substrate recognition. The catalytic defect of Tyk2 P1104A is dominant in that it could not be rescued by the V678F substitution, which per se causes enzyme hyperactivation. When stimulated by IFN-α in cells and tested in vitro, both Pro-to-Ala mutants exhibited a very weak catalytic activity on tyrosines outside of the activation loop (Figs. 4A, 6A), which may indicate altered substrate recognition. As shown in Fig. 8B, Pro<sub>1104</sub> appears to pack in a ring-stacking interaction with a tryptophan residue (Trp<sub>1067</sub>), which is conserved in tyrosine kinases. Interestingly, in place of this tryptophan, Ser/Thr kinases have a tyrosine residue, which, in cAMP-dependent protein kinase studies, was shown to be critical for substrate binding and orienting catalytic residues (50). Moreover, adjacent to the αH helix is the exposed Gly-Gln-Met motif (Figs. 1C, 8B), which is part of a binding surface for the negative regulator suppressor of cytokine signaling 3. Through this

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**FIGURE 8.** (A) Location of Jak2 Ile<sub>623</sub> and Val<sub>617</sub> residues corresponding to Tyk2 Ile<sub>684</sub> and Val<sub>617</sub>. The side chains of Jak2 Ile<sub>623</sub> and Val<sub>617</sub>, corresponding to Tyk2 Ile<sub>684</sub> and Val<sub>617</sub>, respectively, are highlighted in blue sphere representation on the crystal structure of the active form of the JH2 domain (PDB entry 4FVQ) (48). The N lobe of the kinase domain is in beige and the C lobe in cyan. Note that a unique large insert is present only in Tyk2 between the β2 and β3 strands (arrow). (B) Location of Pro<sub>1104</sub> in the JH1 domain of Tyk2. The side chain of Pro<sub>1104</sub> is highlighted in yellow sphere representation on the crystal structure of Tyk2 JH1 domain complexed with the Jak inhibitor CP-690550 (PDB entry 3LXN) (42). The N lobe of the kinase domain is in orange, the C lobe in light blue, with the αH helix in darker blue. Facing the proline, a conserved tryptophan residue (Trp<sub>1067</sub>) is shown in yellow stick representation (see text). The side chain of the glutamine residue of the Gly-Gln-Met motif is depicted (see text) (51). Note that the activation loop, phosphorylated on Tyr<sub>1104</sub>, is partly disordered.
interaction, suppressor of cytokine signaling 3 was reported to disturb phosphate transfer to the tyrosine substrate without affecting ATP hydrolysis (51).

Tyk2 is an essential element of the tonic signal induced by homeostatic IFN-β and is implicated in signaling networks initiated by a large number of immunoregulatory cytokines (9, 52). As first proposed by Shaw et al. (6) and later supported by human disease-genetic association studies, notably MS, naturally occurring mutations in the Tyk2 gene may underlie altered susceptibility to autoimmune disorders (Table I). The two rare disease-associated Tyk2 polymorphisms that we studied in this paper have a clear damaging effect, as they give rise to hypomorphic proteins devoid of kinase activity. Yet, the kinase-independent scaffolding function of these proteins appears sufficient for Stat activation by type I IFN, IL-6, and IL-10 in fibroblastic and/or B cell lines. Also, our comparative analysis of IFN-α and IFN-γ signaling in cells expressing different levels of Jak1 P0184A illustrates that a catalytic loss-of-function Jak can hamper signaling in a cytokine-specific manner.

Studies in mice have shown that the requirement for Tyk2 in signaling by type I IFN and other immune cytokines can be cell-context dependent (2, 3, 53). Moreover, it is increasingly clear that the functional impact of many polymorphisms, notably in immune response imbalance, can differ according to cell or tissue types and their particular biological state. A modest reduction of IFN-β signaling has been observed in ex vivo–expanded T cells from heterozygote Tyk2 P1104A carriers (16). As this is not the case in heterozygote B cell lines and reconstituted fibroblasts, it suggests that this Tyk2 variant may have a differential impact depending on cell type. Given the variety of Tyk2-activating cytokines, their in-context genotyping and sequencing; and all of the members of the Cytokine Signaling Unit for discussion. We also thank Vincenzo Di Bartolo, Mark M. Neubauer, B. McElvany, W. F. Hickey, E. P. Blankenhorn, and C. Teuscher. A single nucleotide polymorphism in Tyk2 controls susceptibility to experimental allergic encephalomyelitis. J. Immunol. 182: 7776–7783.


FUNCTIONAL IMPACT OF TWO DISEASE-ASSOCIATED Tyk2 VARIANTS


