Tyrosine Phosphorylation of Protein Kinase D2 Mediates Ligand-inducible Elimination of the Type 1 Interferon Receptor*

Received for publication, May 22, 2011, and in revised form, July 22, 2011 Published, JBC Papers in Press, August 24, 2011, DOI 10.1074/jbc.M111.263608

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Type 1 interferons (including IFNα/β) activate their cell surface receptor to induce the intracellular signal transduction pathways that play an important role in host defenses against infectious agents and tumors. The extent of cellular responses to IFNα is limited by several important mechanisms including the ligand-stimulated and specific serine phosphorylation-dependent degradation of the IFNAR1 chain of Type 1 IFN receptor. Previous studies revealed that acceleration of IFNAR1 degradation upon IFN stimulation requires activities of tyrosine kinase TYK2 and serine/threonine protein kinase D2 (PKD2), whose recruitment to IFNAR1 is also induced by the ligand. Here we report that activation of PKD2 by IFNα (but not its recruitment to the receptor) depends on TYK2 catalytic activity. PKD2 undergoes IFNα-inducible tyrosine phosphorylation on specific phospho-acceptor site (Tyr-438) within the plekstrin homology domain. Activated TYK2 is capable of facilitating this phosphorylation in vitro. Tyrosine phosphorylation of PKD2 is required for IFNα-stimulated activation of this kinase as well as for efficient serine phosphorylation and degradation of IFNAR1 and ensuing restriction of the extent of cellular responses to IFNα.

Tyrosine phosphorylation of PKD2 is required for de novo tyrosine phosphorylation on specific Ser residues within a defined phospho-acceptor site (Tyr-438) within the plekstrin homology domain. Activated TYK2 is capable of facilitating this phosphorylation in vitro. Tyrosine phosphorylation of PKD2 is required for IFNα-stimulated activation of this kinase as well as for efficient serine phosphorylation and degradation of IFNAR1 and ensuing restriction of the extent of cellular responses to IFNα.

Tyrosine Phosphorylation of Protein Kinase D2 Mediates Ligand-inducible Elimination of the Type 1 Interferon Receptor*

Type 1 interferons (IFNs) play a critical role in modulating the immune responses against many pathogens and directly mounting the anti-viral defenses (for review, see Refs. 1–4). A family of these cytokines (including IFNβ and diverse species of IFNα) elicit the responses through the cognate Type 1 IFN receptor on the cell surface (5–7). Interaction of IFNAR1 and IFNAR2c chains of this receptor with the ligands triggers the signal transduction pathway that involves activation of the Janus kinases, JAK (TYK2 and JAK1) and subsequent tyrosine phosphorylation and ensuing activation of signal transducers and activators of transcription (STAT1 and STAT2). Transcriptionally active STAT1/2 further associate with IRF3, translocate to the nucleus and interact with IFN-stimulated response elements (ISRE) to induce de novo expression of the IFN-stimulated genes. Protein products of these mediated immunomodulatory and anti-viral responses as well as inhibit proliferation and survival of cells exposed to Type 1 IFN (for review, see Refs. 1–4).

To alleviate these detrimental effects of Type 1 IFN, cells evolved to develop the mechanisms that limit the magnitude and duration of their responses to these cytokines. For example, some of the IFN-stimulated genes encode the proteins that may interfere with the recruitment of JAK to IFNAR chains (8). Additional modes of negative regulation that is commonly shared between most of cytokines-induced JAK-STAT pathways include inhibition of JAK activity/stimulation of JAK degradation by SOCS proteins, inhibition of tyrosine phosphorylation by phosphatases, and inhibition of STAT-induced transcription by PIAS (for review, see Refs. 6, 9). In addition to these modes of negative regulation, which occur in cells that have already executed the IFN-induced programs of signal transduction and transcriptional activation, a rapid elimination of Type 1 IFN receptors from the cell surface serves as a rapid and important mechanism that limits cell sensitivity to continuous exposure to the ligands.

Elimination of the entire receptor is driven by ubiquitination and subsequent endocytosis and lysosomal degradation of the IFNAR1 chain (6, 10). This ubiquitination is facilitated by the SCFβTrcp E3 ubiquitin ligase that is recruited to IFNAR1 upon its phosphorylation on specific Ser residues within a defined degron (534–DSGNYS) (11, 12). Stimulation of this phosphorylation in cells exposed to IFNα/β appears to play a key role in subsequent recruitment of βTrcp and stimulation of IFNAR1 ubiquitination and degradation (11, 13) in a manner that requires catalytic activity of TYK2 (14, 15).

Our previous studies also revealed the role of protein kinase D2 (PKD2) in ligand-stimulated IFNAR1 phosphorylation, ubiquitination, and degradation (16). Whereas an increase in both recruitment of PKD2 to IFNAR1 and in catalytic activity of PKD2 were observed in cells treated with IFNα/β, the mechanisms that govern the ligand-inducible JAK and PKD2-stimulated phosphorylation of IFNAR1 degron remains largely to be understood. Here we report that kinase function of TYK2 is dispensable for basal activity of PKD2 or for induction of its recruitment to IFNAR1. Instead, TYK2 activity plays an important role in stimulation of kinase activity of PKD2 by IFNα through phosphorylation of specific tyrosine residue, Tyr-438. The latter mechanism is important for IFNAR1 degradation and for tempering the IFNα-induced signaling and anti-viral defenses.

* This work was supported, in whole or in part, by National Institutes of Health Grants C929900 and CA142425 (to S. Y. F.). All authors involved in this study have no conflict of interest to declare except Dr. Darren P. Baker, who is a Biogen Idec employee and owns a company stock.

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‡ The abbreviations used are: IFNAR1, IFNα/β receptor chain 1; ISRE, IFN-stimulated response element; TYK, tyrosine kinase; PKD2, protein kinase D2.
**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—Vectors for mammalian expression of FLAG-IFNAR1 and bacterial expression of GST-IFNAR1 (12), and HA-tagged TYK2 (a gift from J. Krolewski) (17), as well as the 5×ISRE-luciferase reporter (a gift from C. Horvath) (18) have been described elsewhere. Vectors for mammalian expression of human GST-tagged PKD2 (19) were kindly provided by V. Malhotra. Silent mutations, as well as replacement of Tyr-438 with tyrosine were generated by site-directed mutagenesis. All resulting mutants were verified by dideoxy sequencing. Lentiviral shRNAs against PKD2 constructed in the backbone of the pLKO.1-puro vector were purchased from Sigma (MISSION shRNA, SHGLY-NM_016457). Control shRNA vector targeted against GFP (20) was a gift from J. W. Harper. Recombinant human IFNo2 (Roferon) was purchased from Roche Applied Science. Cycloheximide and other chemicals were purchased from Sigma.

**Cell Culture, Treatment, and Viral Infection**—Human HeLa, 293T and 2fTGH cells were obtained from ATCC. 2fTGH-isogenic 11.1-TYK2-null cells and their derivatives reconstituted with catalytically inactive TYK2 (KR-2 cells) or wild-type TYK2 (WT-5 cells) were a generous gift of S. Pellegrini. All cell lines were maintained in DMEM supplemented with 10% (v/v) FBS (HyClone) and various selection antibiotics where indicated.

**Antibodies and Immunotechniques**—Monoclonal antibodies against human IFNAR1 that were used for immunoprecipitation (EA12) or immunoblotting (GB8) were described in detail elsewhere (22). Commercially available antibodies against Tyr(P)-463 in PKD1/PKCζ (Abcam; ab59415), anti-pan-Tyr(P) (4G10), phospho-STAT1, STAT1 (Cell Signaling), FLAG, GST, and β-actin (Sigma), HA (12CA; Roche Applied Science), Ser(P)-710 of PKD2 (Bethyl Laboratories), PKD1/2, TYK2, and the intracellular domain of hIFNAR1 (Santa Cruz) were purchased. Antibody against IFNAR1 phosphorylated on Ser-535 (Ser(P)-535) (11) was described previously. Secondary antibodies conjugated to HRP were purchased from Chemicon and LI-COR. Immunoprecipitations, immunoblotting, and assessment of the kinetics of IFNAR1 degradation by cycloheximide chase (i.e. measurement of protein levels at different times after blocking translation with cycloheximide at 20 μg/ml) were carried out as described previously (11, 12, 23–27). Briefly, for co-immunoprecipitation, cells harvested from a 10-cm plate (1–5 × 10⁶) were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, PMSF (50 μg/ml), and protease inhibitor mixtures (Sigma) on ice. Lysates were subjected to immunoprecipitation by the respective antibody for 1 h followed by a 2-h incubation with protein G-coupled Sepharose beads at 4 °C under rotary agitation. After washing by the above lysis buffer three times, immune complexes were resolved by SDS-PAGE and then were subjected to further analyses. Immunoblot detection and quantification were carried out using the LI-COR Odyssey infrared imaging system.

**In Vitro Kinase Assays**—In vitro phosphorylation of PKD2 (purified from HeLa cells) by HA-tagged TYK2 immunopurified from 293T cells (untreated or treated with IFNo) or by recombinant Src (purchased from Cell Signaling) was carried out in a total volume of 20 μl in 50 mM MOPS (pH 7.4), 10 mM MgCl₂, 5 mM MnCl₂, 2 mM DTT, and 0.2 mM ATP at 30 °C for 20 min. The samples were resolved by SDS-PAGE and analyzed by immunoblotting with an anti-phosphotyrosine antibody (4G10).

Alternatively, in vitro tyrosine phosphorylation of purified from 293T cells GST-PKD2 (WT or Y438F, “YF”) by the recombinant truncated GST-TYK2 1683–1187 kinase (molecular mass 68.8 kDa; PV4790, Invitrogen) was carried out in kinase buffer (50 mM MOPS (pH 7.4), 10 mM MgCl₂, 5 mM MnCl₂, and 2 mM DTT) at 30 °C with ATP (0.3 mM). The samples were resolved by SDS-PAGE and analyzed by immunoblotting with an anti-phosphotyrosine antibody (4G10) followed by immunoblotting using an anti-GST antibody.

**Immunocytochemistry**—2fTGH or 11.1 cells were plated on coverslips for 24 h and then treated with IFNo (2,000 units/ml) for 5 min. Cells were fixed with 3.7% paraformaldehyde at once and permeabilized with 0.3% Triton X-100. The cells were incubated with anti-Flag and anti-GST antibodies and scanned as described elsewhere (22). Cells were embedded in Prolong Gold Antifade mounting medium (Invitrogen). The confocal images were acquired on a Leica Inverted DM14000 microscope equipped with a 100× HCX PL APO 1.46 NA oil objective, a Yokogawa CSU-10 spinning disk confocal system, and an ImagEM 16-bit cooled EMCCD camera (Hamamatsu). Laser excitation was provided by a 488-nm (Spectra Physics) and a 561-nm laser (Cobolt Jive) controlled through LMM5 (Spectral Applied Research).

**Viral Infection**—The anti-viral effect of Type 1 IFN was determined in the derivatives of 2fTGH cells that harbor diverse PKD2 status. These cells were pretreated with IFNo (5 units/ml) for 1 h prior to infection with vesicular stomatitis virus (Indiana serotype, a gift from R. Hart; propagated in HeLa cells) at a multiplicity of infection of 0.1 for 1 h. After removing the virus inoculums, cells were then fed with fresh medium and incubated for 20 h. Culture supernatant was harvested, and the viral titer was determined in HeLa cells overlaid with methylcellulose as described elsewhere (28) and plaque-forming units (pfu/ml) calculated.

**RESULTS**

IFNo/β-stimulated and TYK2-dependent phosphorylation on Ser-535 was shown to play a key role in ligand-inducible ubiquitination and degradation of IFNAR1 (11, 12, 15). Furthermore, our recent studies revealed a key role for PKD2 in this signaling (16). Although PKD2 was expressed in either
human fibrosarcoma 2fTGH cells or their isogenic TYK2-null 11.1 derivatives, the activation of PKD2 by IFNα was not observed in 11.1 cells (16). Previous work by Pellegrini group established that these cells fail to retain IFNAR1 on cell surface due to constitutive endocytosis of IFNAR1 (29). Reconstitution of these cells with wild-type TYK2 restored the sensitivity of resulting cells (WT-5) to Type 1 IFN. When isogenic cell line (KR-2) was engineered using catalytically incompetent TYK2, it exhibited an attenuated JAK-STAT signaling (15, 30) and could only moderately respond to IFN treatment (Fig. 1A). This result suggests that, similar to an increase in WT-5 cells (Fig. 1C). This result suggests that IFNα-stimulated recruitment of PKD2 to IFNAR1 does not depend on catalytic activity of TYK2.

We next sought to determine whether TYK2 activity is involved in regulating the IFNα-induced increase in PKD2 kinase activity. To this end, we expressed GST-tagged PKD2 in either WT-5 or KR-2 cells, treated these cells with IFNα for various times, purified PKD2 using affinity beads, and incubated this kinase with bacterially produced GST-IFNAR1 protein as a substrate and ATP in vitro. The resulting kinase activity toward phosphorylation of Ser-535 on GST-IFNAR1 was measured by immunoblotting using phospho-specific antibody as the mode for detection. Basal activity of GST-PKD2 (assessed by its ability to phosphorylate on Ser-535 in vitro) was detected in untreated WT-5 cells (Fig. 2A). Remarkably, whereas treatment of cells with IFNα noticeably increased the kinase activity of GST-PKD2 toward Ser-535 phosphorylation in WT-5 cells, this induction was not seen in KR-2 cells (Fig. 2A). Importantly, the status of TYK2 had no bearing on the basal activity of expressed PKD2. These data suggest that TYK2 stimulated by IFNα contributes to catalytic activation of PKD2 in response to IFNα.

We next investigated whether PKD2 and TYK2 interact in cells. Immunoprecipitation reactions carried out using anti-TYK2 antibody revealed the presence of PKD2 in a complex with TYK2 in the untreated cells. Treatment of cells with IFNα rapidly increased this interaction within 2–5 min followed by subsequent decrease in this interaction at later time points (Fig. 2B). We further sought to determine localization of endogenous PKD2 and TYK2 proteins using immunocytochemistry. We first characterized suitability of antibodies for these analyses. Signals detected using an antibody against TYK2 were readily seen in 2fTGH cells but not in TYK2-negative 11.1 cells (Fig. 2C). The pattern of staining in 2fTGH cells was similar to that reported by Lukashova et al. in monocytoid MonoMac-1 cells (32). In addition, immunoochemical detection of PKD2 was much more efficient in 2fTGH cells that received control shRNA compared with those that were transduced with shRNA against PKD2 (Fig. 2D). We further used these antibodies against TYK2 and PKD2 for co-localization studies in 2fTGH cells. These analyses showed that PKD2 and TYK2 co-localize in untreated cells and that this co-localization is noticeably increased in cells treated with IFNα (Fig. 2E). In all, these data suggest that PKD2 is capable of interacting with TYK2 and that this interaction can be transiently stimulated by the ligand.

Two nonexclusive mechanisms have been proposed for activation of a better studied PKD2-related kinase PKD1 by numerous stimuli. One is a phorbol ester-induced phosphorylation of the activation loop of PKD on Ser-744/Ser-748 (33, 34) attributed to function of protein kinases C (PKC) (33, 35). Another is a tyrosine phosphorylation of Tyr-463 that relieves an autoinhibitory effect of the plekstrin homology domain; this phosphorylation could be stimulated by Src (36). Intriguingly, previous reports demonstrated that PKD2 can undergo tyrosine phosphorylation in cells (37). We next assessed phosphorylation status of endogenous PKD2 in WT-5 and KR-2 cells. Phosphorylation of Ser-710 (analog of Ser-748 within PKD1) was indeed stimulated upon treating either WT-5 or KR-2 cells with IFNα (Fig. 3A). This result suggests that, similar to an increase in PKD2-IFNAR1 binding (Fig. 1), phosphorylation of Ser-710 can be stimulated by IFNα independently of TYK2 kinase activity.
stimulus and that an additional signal is required. Given that
TYK2 is a tyrosine kinase we next assessed tyrosine phospho-
rylation of PKD2. Indeed a ligand-inducible phosphorylation
on Tyr residues observed on immunoprecipitated endogenous
PKD2 in WT-5 (Fig. 3A) or HeLa (Fig. 3B) but not in KR-2 cells
(Fig. 3A). The kinetics of this reaction in TYK2-competent cells
revealed a peak at 2–5 min (Fig. 3, A and B) that resembled the
kinetics of the PKD2-TYK2 interaction shown in Fig. 2B. In all,
these results are consistent with a hypothesis that IFNα-in-
duced catalytic activity of TYK2 directly contributes to Tyr
phosphorylation of PKD2.

To test this possibility, we turned to kinase assays in vitro. In
these experiments, the HA-tagged TYK2 was expressed in and
immunopurified from 293T cells, which were treated or not
with IFNα prior to harvesting. After that, HA-TYK2 was incu-
bated with PKD2 (purified from serum-starved and untreated
HeLa cells) in the presence of ATP. The reaction was analyzed
by immunoblotting using an anti-phosphotyrosine antibody. In
this assay, incubation of PKD2 with ATP in the absence of
added tyrosine kinase did not produce any phosphotyrosine
signals consistent with the fact that PKD2 is a Ser/Thr protein
kinase (Fig. 3C, lane 1). Conversely, the tyrosine phosphoryla-

FIGURE 2. IFNα stimulates kinase activity of PKD2 and its interaction with TYK2. A, activity of GST-PKD2 expressed in cells harboring wild-type or
kinase-dead TYK2, and left untreated or treated with IFNα, was analyzed by in vitro Ser-535 phosphorylation of GST-IFNAR1 as assessed by immunoblotting
using a Ser(P)-535-specific antibody. Levels of substrate and kinase were also analyzed by immunoblotting. B, interaction between endogenous TYK2 and
PKD2 in HeLa cells treated as indicated was analyzed by co-immunoprecipitation (IP) and immunoblotting. WCL, whole cell lysate. IgG, isotype antibody
control. C, localization of endogenous TYK2 in 2fTGH cells or isogenic 11.1 cells was detected using primary anti-TYK2 antibody (1o Ab, where indicated) and
secondary antibodies conjugated with Alexa Fluor 488. Nuclei were counterstained using DAPI. D, localization of endogenous PKD2 in 2fTGH cells transduced
with the indicated shRNA was detected using primary anti-PDK2 antibody (1o Ab, where indicated) and secondary antibodies conjugated with Alexa Fluor 594.
Nuclei were counterstained using DAPI. E, co-localization of TYK2 and PKD2 in 2fTGH cells treated with IFNα (2000 units/ml for 5 min) or not was determined
by confocal microscopy analysis using indicated antibodies.
Tyr phosphorylation of PKD2 regulates IFNAR1 signaling

A phosphorylation and levels of endogenous PKD2 immunopurified (IP) from WT-5 or KR-2 cells treated with IFNα as indicated were analyzed by the indicated antibodies. B, tyrosine phosphorylation and levels of endogenous PKD2 immunopurified from HeLa cells treated with IFNα as indicated were analyzed by immunoblotting. C, HA-tagged TYK2 was expressed in and immunopurified from 293T cells that were IFNα-treated (lanes 1–3 and 5) or not (lane 4). TYK2 proteins were then incubated with PKD2 purified from serum-starved and untreated 2TGH cells (except in lane 2) in the presence of ATP (except in lane 2). The reaction was analyzed by immunoblotting using an anti-phospho-tyrosine antibody. The recombinant Src protein was used as positive control.

Stimulation of PKD2 was easily detected when recombinant Src protein was used as positive control (Fig. 3C, lane 6). In the presence of activated HA-TYK2, a noticeable tyrosine phosphorylation signal on PKD2 was also observed in an ATP-dependent manner (compare lanes 2 and 5). A lesser extent of in Tyr phosphorylation of PKD2 was seen when HA-TYK2 was purified from untreated cells (Fig. 3C, lanes 2 and 5), indicating that ligand-stimulated TYK2 is capable of directly phosphorylating PKD2 on Tyr residues.

Soluble tyrosine kinases including Abl and Src were shown to phosphorylate a related PKD1 on Tyr-463 (36). An increase in this phosphorylation in response to various PKD inducers could be detected using a phospho-specific antibody (38). A homologous tyrosine residue, Tyr-438, was found on PKD2 (Fig. 4A); this residue was proposed to play a role in modulating its activity (39). We next aimed to determine the role of this site in IFNα-induced PKD2 activation and IFNAR1 phosphorylation. We generated the GST-PKD2<sup>V438F</sup> mutant and expressed it in and purified from 293T cells to use in an in vitro kinase assay as a substrate. Compared with analogous wild-type GST-PKD2, the mutant lacking Tyr-438 revealed a lesser extent of overall tyrosine phosphorylation upon its incubation with recombinant truncated GST-TYK2<sup>933–1187</sup> kinase (Fig. 4B, lanes 3 and 4). Given that the autophosphorylation activity of TYK2 was not inhibited by the presence of GST-PKD2<sup>V438F</sup> mutant (Fig. 4B, lane 4), this result indicate a possibility that Tyr-438 may represent one of the phospho-acceptor sites used by TYK2 for PKD2 phosphorylation. Consistent with this possibility, overall tyrosine phosphorylation of GST-PKD2<sup>V438F</sup> expressed in 293T cells treated with IFNα was noticeably less pronounced than that observed in GST-PKD2<sup>WT</sup> protein (Fig. 4C) despite the fact that Tyr-438 mutation did not affect the recruitment of PKD to IFNAR1 (Fig. 4D). Importantly, phosphorylation of these two proteins on Ser-710 was comparable (Fig. 4C), indicating that mutation of Tyr-438 does not affect either basal or IFNα-induced phosphorylation of the PKD2 activation loop. Together, these results suggest that ligand phosphorylation of PKD2 on Tyr-438 and that activated TYK2 is directly capable of this phosphorylation.

Given the similarities between key Tyr residue-encompassing sequences within the plekstrin homology domains of PKD1 and PKD2 (Fig. 4A), we further used an antibody developed against Tyr-463 within PKD1 for PKD2 analysis. This antibody was used efficiently for analyses of PKD1 phosphorylation in cells treated with various stimuli (38). Immunoblotting analysis of GST-PKD2 species expressed in 293T cells and purified by affinity beads revealed a robust IFNα-inducible signal detected on wild-type PKD2 but not on Y438F mutant (Fig. 4E). This result suggests that anti-Tyr(P)-463 antibody can be used for the specific detection of Tyr-438 phosphorylation within PKD2. Subsequent analysis of endogenous proteins also revealed that there is an increase in recognition of PKD2 by this antibody when PKD2 is immunopurified from IFNα-treated cells (Fig. 4F). These results strongly suggest that IFNα stimulates phosphorylation of Tyr-438 within PKD2.

We next compared kinase activities of GST-tagged PKD2<sup>WT</sup> and PKD2<sup>V438F</sup> proteins expressed in HeLa cells using an in vitro assay that enables detection of the phosphorylation of IFNAR1 on Ser-535. The basal Ser-535-phosphorylating activity of GST-PKD2<sup>V438F</sup> mutant was similar to that of wild-type enzyme. However, activation of this mutant kinase in response to IFNα treatment was visibly impaired (Fig. 5A). This result suggests that phosphorylation of Tyr-438 might be required for stimulation of PKD2 activity by IFNα.

To test this possibility further, we generated GST-PKD2 expression constructs (depicted as GST-PKD2<sup>WT</sup>) that contained silent mutations, making them insensitive to shPKD2 that we previously used for kinase knockdown (16). For subsequent experiments we chose to use human 2TGH cells because, similar to HeLa, the role of PKD2 in modulating IFNAR1 phosphorylation and degradation was firmly established; yet, unlike HeLa cells, 2TGH cells do not harbor human papillomavirus genes and exhibit a robust anti-viral effects in response to IFNα (16). 2TGH cells were stably transduced with control shRNA or shPKD2 and with either empty vector or GST-PKD2<sup>WT</sup> constructs (wild type or Tyr-438 mutant). Consistent with our previous report (16), knockdown of endogenous PKD2 impeded IFNα-induced phosphorylation of IFNAR1 on Ser-535 (Fig. 5B, lane 2 versus lane 4). Whereas reexpression of wild-type PKD2 partially restored this phosphorylation, such an effect was not observed when PKD2 was mutated at Tyr-438 (Fig. 5B, lane 6 versus lane 8). These results collectively suggest that ligand-induced phosphorylation of this tyrosine residue within PKD2 is required for IFNα-stimulated PKD2 activation and ensuing PKD2-mediated phosphorylation of the IFNAR1 degron.

We next sought to investigate the role of these mechanisms in regulating IFNAR1 stability and signaling. Cycloheximide chase was used to determine the rate of IFNAR1 degradation in cells treated with IFNα. Under these conditions, proteolytic turnover was noticeably delayed in cells that received shRNA against PKD2 (Fig. 6A). These results are in line with our previous report and are consistent with the model wherein PKD2 plays a key role in regulation of the ligand-inducible IFNAR1 degradation (16). Subsequent expression of shPKD2-insensitive wild-type GST-PKD2<sup>WT</sup> in these cells noticeably reverted the
delay in IFNAR1 turnover. Remarkably, mutation of Tyr-438 rendered this construct incapable of restoring the rates of IFNAR1 degradation (Fig. 6A). These results suggest that tyrosine phosphorylation of PKD2 on Tyr-438 is important for rapid degradation of IFNAR1 in the presence of IFNα/H9251.

Our previous studies demonstrated that PKD2 plays a major role in negative regulation of magnitude and duration of IFNα/H9251 signaling via mediating the ligand-stimulated IFNAR1 degradation (16). The role of PKD2 tyrosine phosphorylation in limiting the extent of cellular responses to IFNα/H9251 was next assessed using transactivation assay based on expression of the ISRE-driven luciferase in 2fTGH cells. A pulse IFNα/H9251 treatment of 2fTGH cells that received irrelevant control shRNA led to a noticeable activation of ISRE-dependent transcription (Fig. 6B). Consistent with previously reported results (16), knockdown of PKD2 significantly augmented the IFNα-stimulated luciferase activity. Importantly, expression of shRNA-insensitive wild-type GST-PKD2* (but not of the Tyr-438 mutant) tempered the effects of PKD2 knockdown (Fig. 6B).

We further used these functional PKD2 knock-in approaches in 2fTGH cells to assess the antiviral effects of the pulse treatment with a low dose (5 units/ml) of IFNα/H9251. Pretreatment of cells with this dose did not significantly decrease the titer of vesicular stomatitis virus upon subsequently infection with this virus unless PKD2 was knocked down (Fig. 6C). The knockdown phenotype was rescued by expression of shRNA-insensitive wild-type PKD2 but not of Tyr-438 mutant. A similar trend was observed when the dose of IFNα was increased to 50 units/ml (Fig. 6C). The extent of the observed differences is probably underestimated due to an incomplete knockdown of endogenous PKD2 (Fig. 5). Nevertheless, these results collectively suggest that Tyr-438 phosphorylation of PKD2 play an important role in restricting the effects of IFNα signaling and in limiting its ability to mount an efficient anti-viral state.

DISCUSSION

Continuous responses of cells to Type 1 IFN are disrupted by proteolytic elimination of IFNAR1 (10). These events are facilitated by PKD2-dependent phosphorylation of IFNAR1 (16) followed by the recruitment of SCF/Trcp E3 ubiquitin ligase and ensuing IFNAR1 ubiquitination, endocytosis, and postinternalization sorting into the lysosomes (11–13). Activation of PKD2 by Type 1 IFN was shown to play an important role in these processes (16); in addition, the role of TYK2 catalytic activity in ligand-stimulated IFNAR1 degradation was revealed by Pellegri group (15). Although we previously proposed that PKD2 may function downstream of TYK2, the detailed mechanisms by which this signaling occurs remained poorly characterized. Here we describe studies that reveal that TYK2 interacts with PKD2 and that this interaction is noticeably stimulated by IFNα/H9251 (Fig. 2). Furthermore, activated TYK2 is capable of directly phosphorylating PKD2 on tyrosine residues. A specific Tyr-438 within the plekstrin homology domain of PKD2 has been characterized as an important amino acid residue that can serve as a

FIGURE 4. TYK2 is capable of phosphorylating PKD2 on Tyr-438. A, alignment of fragments of primary sequences of the plekstrin homology domains within human PDK1 (Prkd2) and PKD2 (Prkd2); Tyr-463 and Tyr438 are underlined. B, in vitro tyrosine phosphorylation of purified from cells GST-PKD2 (wild-type or Y438F mutant, YF) by recombinant truncated GST-TYK2833–1187 kinase analyzed by immunoblotting (IB). C, phosphorylation and levels of GST-PKD2 (wild type or Y438F-mutant) expressed in 293T cells determined by immunoprecipitation (IP) followed by immunoblotting using the indicated antibodies. D, interaction of endogenous IFNAR1 with exogenous GST-tagged PKD2 (wild type or Y438F mutant) expressed in 293T cells determined by immunoprecipitation (IP) followed by immunoblotting using the indicated antibodies. E, specific Tyr-438 phosphorylation of GST-tagged PKD2 species (wild type or Y438F mutant) purified from 293T cells treated or not with IFNα for 5 min analyzed by immunoblotting using the indicated antibodies. F, specific Tyr-438 phosphorylation of endogenous PKD2 immunopurified from HeLa cells treated with IFNα for the indicated times analyzed by immunoblotting using the indicated antibodies.
phospho-acceptor site for IFNα-induced and TYK2-facilitated phosphorylation (Fig. 4). The integrity of this Tyr-438 site plays a key role in the ligand-inducible activation of PKD2 and ensuing phosphorylation of IFNAR1 degron (Fig. 5). Furthermore, Tyr-438 appears to be important for PKD2-dependent regulation of the IFNAR1 stability and the restrictions imposed on the extent of IFNAR1 degradation following decreased sensitivity of cells to subsequent effects of IFNα.

Based on these data, we propose a model wherein the interaction of Type 1 IFN with the cognate receptor chains and ensuing activation of catalytic activity of JAKs may lead to a direct phosphorylation of recruited PKD2 on Tyr-438 by TYK2 (and, most likely, by JAK1 as well). This phosphorylation mediates catalytic activation of PKD2 and results in the phosphorylation of IFNAR1 degron leading to IFNAR1 degradation followed by decreased sensitivity of cells to subsequent effects of IFNα.

Given the reports suggesting that phosphorylation of analogous Tyr-463 in the pleckstrin homology domain of PKD1 leads to activation of this related kinase in response to oxidative stress via the Src-Abl pathway (40), it is plausible that analogous Tyr-438 phosphorylation of PKD2 by JAK would achieve a similar outcome. Indeed, TYK2 catalytic activity and PKD2 phosphorylation on Tyr-438 appears to be dispensable for basal activity of PKD2 yet play an important role in its activation by IFNα (Figs. 2A and 5A). However, the exact mechanisms of activation of PKD2 by IFNα and of PKD1 by H2O2 might be rather distinct. It was proposed that phosphorylation of Tyr-463 releases the pleckstrin homology domain, thus exposing the activation loop of PKD1 for subsequent phosphorylation by PKC (40). A later report indeed suggested that hydrogen peroxide-induced PKD1 activation includes sequential Tyr-463 phosphorylation by c-Abl followed by the loop phosphorylation on Ser-738/Ser-742 by PKC8 (41). Although IFNα treatment also increases phosphorylation of PKD2 on its activation loop (Ser-710), this phosphorylation is dependent neither on TYK2 activity (Fig. 3A) nor on Tyr-438 phosphorylation (Fig. 4C). This distinction is likely to reflect the intrinsic biochemical and biologic differences between PKD2 and PKD1. The latter kinase is poorly recruited to IFNAR1 (16) (Fig. 1B) and exhibits a strong preference for specific phospho-acceptor motif (L1/I/V-X-R/K-XX-s/t) (42–44), that is absent in IFNAR1 degron. Seemingly for that reason PKD1 does not efficiently phosphorylate IFNAR1 on Ser-535 (16). In addition, soluble c-Abl kinase activated by H2O2 might act differently from the receptor-associated JAK; accordingly, treatment of cells with hydrogen peroxide does not efficiently stimulate IFNAR1 phosphorylation in cells.3

The mechanisms by which the recruitment of PKD2 to IFNAR1 and to TYK2 is stimulated by IFNα remain to be understood. Neither this increase in recruitment (Fig. 1C) nor in IFNα-stimulated phosphorylation of PKD2 on Ser-710 (Fig. 3A) required TYK2 activity. Although participation of a remaining JAK member (JAK1) cannot be ruled out, the dichotomy between requirements for Tyr phosphorylation and recruitment/Ser-710 phosphorylation of PKD2 strongly suggests that a separate signal transduction pathway must regulate these latter events. Given that a great wealth of published studies implicated various PKC species in PKD1 loop activation (33, 45–49), it is plausible that this mechanism could be also engaged by the Type 1 IFN pathway. Indeed, extensive work by many groups has demonstrated that IFNα/β are capable of activating diverse PKC species and that this activation plays an important role in mediating the signal transduction induced by these cytokines (50–55). Future studies will identify specific members of PKC superfamily that may contribute to PKD2 loop phosphorylation in a manner that does not depend on TYK2 kinase function. Additional studies are also warranted to delineate the mechanisms that govern the recruitment of PKD2 into the vicinity of IFNAR1 as well as to determine the role of these mechanisms in regulation of IFNAR1 stability and signaling. As IFNα/β are often used as anti-cancer or anti-viral drugs

3 H. Zheng and S. Y. Fuchs, unpublished data.
Tyr Phosphorylation of PKD2 Regulates IFNAR1 Signaling

FIGURE 6. Role of PKD2 Tyr-438 site in regulating IFNAR1 stability and the extent of cellular responses to IFNα. A, 2fTGH cells with stable knockdown of PKD2 were transfected with FLAG-IFNAR1 and GST-PKD2* (wild type or Y438F mutant). Cells were treated with IFNα for the indicated times. Immunoblot (IB) analysis of levels of FLAG-IFNAR1 and transfected GST-PKD2 using indicated antibodies is shown. B, relative activity of ISRE-driven firefly luciferase activity was normalized to Renilla luciferase activity in 2fTGH cells with stable knockdown of PKD2 that were transfected with empty vector or GST-PKD2* (wild type or Y438F mutant). Cells were treated with IFNα (5 units/ml for 1 h, black bars) as indicated and then incubated without this cytokine for 24 h until harvesting and assessment of the activity of ISRE-luciferase normalized per Renilla luciferase activity. Data from three independent experiments (each in triplicate) are shown in arbitrary units as mean ± S.E. Asterisks denote p < 0.01 between groups depicted by filled and open symbols.

(56–58), targeting the mechanisms that desensitize cells to these cytokine is of potential clinical importance.

Acknowledgments—We thank Drs. J.W. Harper (Harvard University, Boston, MA), R. Harvy (University of Pennsylvania, Philadelphia, PA), C. M. Horvath (Northwestern University, Evanston, IL), J. Krolewski (University of California, Irvine, CA), V. Malhotra (Center for Genomic Regulation, Barcelona, Spain), and S. Pellegrini (Pasteur Institute, Paris, France) for the reagents. We thank Dr. Ling-Li Zhang (Penn Vet Imaging Facility, University of Pennsylvania, Philadelphia, PA) for help with confocal microscopy.

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