The Catalytic Activity of the Eukaryotic Initiation Factor-2α Kinase PKR Is Required to Negatively Regulate Stat1 and Stat3 via Activation of the T-cell Protein-tyrosine Phosphatase

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Tyrosine phosphorylation of the transcription factors Stat1 and Stat3 is required for them to dimerize, translocate to the nucleus, and induce gene transcription. Nuclear Stat1 and Stat3 are dephosphorylated and deactivated by the T-cell protein-tyrosine phosphatase (TC-PTP), which facilitates the return of both proteins to the cytoplasm. The protein kinase PKR plays an important role in transcellular control through the modulation of eukaryotic initiation factor-2α phosphorylation. Previous data have implicated PKR in cell signaling via regulation of Stat1 and Stat3, but the molecular mechanisms underlying these events have remained elusive. Using PKR−/− mouse embryonic fibroblasts and a conditionally active form of human PKR, we demonstrate herein that tyrosine (but not serine) phosphorylation of either Stat1 or Stat3 is impaired in cells with activated kinase. This reduction in Stat1 and Stat3 tyrosine phosphorylation by active PKR proceeds through TC-PTP, which is a substrate of the eukaryotic initiation factor-2α kinase both in vitro and in vivo. TC-PTP phosphorylation alone is insufficient to increase its in vivo proteinase activity unless accompanied by the inhibition of protein synthesis as a result of PKR activation. These data reveal a novel function of PKR as a negative regulator of Stat1 and Stat3 with important implications in cell signaling.

The Stat§ family of proteins plays a role in many cellular processes, including development, cell growth and proliferation, and apoptotic cell death (1, 2). These transcription factors remain latent in the cytoplasm until activated by extracellular signaling proteins, primarily cytokines and growth factors (1, 2). Stat1 plays an important role in the cellular response to interferon (IFN) and viral infection and in the regulation of proliferation and apoptosis (1). Stat1 knock-out mice are extremely susceptible to infection with viruses and other pathogens, demonstrating the essential role of this transcription factor in innate immunity (3, 4). At the molecular level, IFN treatment leads to Stat1 activation by phosphorylation at Tyr701 mediated by activated Jaks, a family of tyrosine kinases that are associated with the cytoplasmic portions of IFN receptors (1). Tyrosine phosphorylation is essential for Stat1 dimerization, nuclear translocation, DNA binding, and gene transcription (1). Phosphorylation at Ser727 in the C-terminal domain of Stat1 enhances its transactivation capacity (5). Stat3, another member of the Stat family, was discovered as a result of its response to interleukin-6 (IL-6) in hepatocytes (6). Stat3 is a major signal transducer downstream of gp130-like receptors, and its activation induces many responses, including proliferation of B-lymphocytes, activation of terminal differentiation and growth arrest in monocytes, and maintenance of the pluripotency of embryonic stem cells (1, 6). As with Stat1, Stat3 is tyrosine-phosphorylated at a site close to the C terminus and serine-phosphorylated within the transactivation domain (1, 7). Stat3 knock-out mice exhibit early embryonic lethality, and loss of Stat3 is even lethal in embryonic stem cells; therefore, Stat3 is also required for embryogenesis (6). Although both Stat1 and Stat3 are hyperphosphorylated in numerous cancers (8), Stat3 is categorized as a proto-oncogene, whereas Stat1 is generally believed to act as a tumor suppressor (9).

The cell has developed numerous mechanisms to regulate both the duration and magnitude of Stat activation so that it can formulate appropriate responses to cytokine stimulation. Phosphorylation of Stats is inhibited by proteins known as SOCS (suppressors of cytokine signaling), which either interfere with activation of Jaks or compete with Stats for binding to cytokine receptors (10, 11). Stat activation is also limited by dephosphorylation of upstream signaling components (i.e. receptors and Jaks) by the SH2 domain-containing phosphatases (2, 10). In the nucleus, the transcriptional activities of Stats are regulated by Pias (protein inhibitors of activated Stats), which prevent DNA binding (2), or by specific phosphatases that remove phosphate groups from the tyrosine residues of active Stat molecules (2, 10). In particular, the nuclear T-cell protein-tyrosine phosphatase (TC-PTP) (12) decreases both the cytokine-induced phosphorylation and transcriptional activities of Stat1 and Stat3, thus inhibiting the signaling through each pathway (13, 14).

PKR is ubiquitously expressed serine/threonine protein kinase that...
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is induced by IFN-α/β and activated by double-stranded RNA, cytokines, growth factors, and cellular stress (15). The most extensively characterized role of PKR is its ability to inhibit the initiation of translation through phosphorylation of the eukaryotic initiation factor-2 α-subunit (eIF2α) at Ser51 (15, 16). Through its capacity to regulate protein synthesis, PKR has been considered as a major mediator of the antiviral and anti-proliferative effects of IFN (17). We previously reported that PKR physically interacts with Stat1, an interaction that is diminished in cells treated with IFN or double-stranded RNA (18, 19). We showed that PKR impairs both the nuclear function of Stat1 and the induction of Stat1-dependent gene transcription in response to IFNs (18). Despite the clear inhibitory role of PKR in Stat1 function (18, 19), the precise molecular events underlying this regulation have remained elusive. Herein, using mouse embryonic fibroblasts (MEFs) from a catalytic knock-out of PKR (20) and a conditionally active form of PKR (21, 22), we demonstrate that PKR negatively regulates Stat1 and Stat3. We show that this regulation occurs through activation of TC-PTP, which in turn specifically decreases the tyrosine phosphorylation of Stat3. We show that this regulation occurs through activation of TC-PTP, which in turn specifically decreases the tyrosine phosphorylation of Stat3. We show that this regulation occurs through activation of TC-PTP, which in turn specifically decreases the tyrosine phosphorylation of Stat3. We show that this regulation occurs through activation of TC-PTP, which in turn specifically decreases the tyrosine phosphorylation of Stat3. We show that this regulation occurs through activation of TC-PTP, which in turn specifically decreases the tyrosine phosphorylation of Stat3. We show that this regulation occurs through activation of TC-PTP, which in turn specifically decreases the tyrosine phosphorylation of Stat3. We show that this regulation occurs through activation of TC-PTP, which in turn specifically decreases the tyrosine phosphorylation of Stat3. We show that this regulation occurs through activation of TC-PTP, which in turn specifically decreases the tyrosine phosphorylation of Stat3.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—HT1080 cells expressing GyrB-wild-type (wt) PKR or GyrB-PKR(K296H) were established as described (22). Isogenic MEFs from PKR+/− and catalytic PKR−/− mice (20) were generated as described (23). Isogenic PERK+/− and PERK−/− MEFs were generated as described (24). HT1080 cells and MEFs were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Invitrogen) and 100 units/ml penicillin/streptomycin (Wisent Inc.). eIF2α S/S (homozygous wild-type mouse bearing two Ser51 wild-type alleles) and A/A (homozygous knock-in mouse bearing an S51A mutation) MEFs were generated as described (27). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% non-heat-inactivated calf serum (Invitrogen), 1× amino acids (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and 100 units/ml penicillin/streptomycin. phiNX retroviral packaging cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin/streptomycin, 1 mM hygromycin (Roche Applied Science). For cytokine treatment, cells were serum-starved overnight and then incubated with 100 IU/ml human IFN-γ (BIOSOURCE), 10 ng/ml human IL-6 (BIOSOURCE), 120 IU/ml recombinant murine IFN-γ (Cedarlane Laboratories Ltd.), or 10 ng/ml recombinant murine IL-6 (BIOSOURCE). GyrB-wtPKR and GyrB-PKR(K296H) were activated with 100 ng/ml coumermycin.

**Viral Infection**—PKR+/− and PKR−/− MEFs (4 × 105) were seeded in 6-cm plates and infected with vesicular stomatitis virus (VSV; Indiana strain) as described (19).

**Small Hairpin RNA (shRNA) and Retroviral Infection**—The shRNA construct for TC-PTP or SHP-2 was made by cloning the TC-PTP sequence (5’-GATCCCCCGAGGTTGATACGCTGGTCTTCAAGAGAGGCGTCTGATATCCAATCTTGTGGAAA-3’) or the SHP-2 sequence (5’-GATCCCCGTACAATCGGAGACCTACTACCTTCAAGAGAGTGAAGTCCATCCAGGGTACCTTTTGGAAA-3’) into the pSUPER.retro vector (OligoEngine) according to the manufacturer’s instructions. The shRNA constructs were transfected in phiNX retroviral packaging cells, which were selected with puromycin (2.5 μg/ml; Sigma) and hygromycin (200 μg/ml). Then, the resultant retrovirus were used to infect HT1080 cells expressing GyrB-wtPKR in the presence of Polybrene (6 μg/ml; Sigma) as described (19). Targeted cells were selected with 2.5 μg/ml puromycin for 2 weeks, and polyclonal populations were pooled, expanded, and characterized.

**Purification of Glutathione S-Transferase (GST) Fusion Proteins and PKR Kinase Assays**—GST-wtPKR, GST-PKR(K296R), GST-wtTC-PTP, and GST-TC-PTP(D182A) were purified from bacteria according to the instructions provided with glutathione-Sepharose (Amersham Biosciences). GST-PKR kinase assays with GST-TC-PTP as substrate were performed as described (19).

**Phosphatase Assay**—GST-TC-PTP bound to glutathione-Sepharose beads was washed three times with 5 volumes of 1× phosphate assay buffer (25 mM Tris-HCl (pH 7.4) and 1.6 mM dithiothreitol). After centrifugation at 900 × g for 1 min, the supernatant was removed, and 200 μl of phosphatase assay buffer containing 1.0 mM p-nitrophenyl phosphate (Sigma) was added to each sample, followed by incubation at 37°C. Reactions were quenched with 20 μl of 2M NaOH at 0, 15, 30, and 60 min, and color production was determined spectrophotometrically at 405 nm.

**Immunostaining**—Cell fixation and immunostaining were performed as described (26). The cells were stained with anti-Stat1α monoclonal antibody (mAb; 2 μg/ml; C-11, Santa Cruz Biotechnology, Inc.) or anti-human TC-PTP mAb (1–2 μg/ml; 3E2). Alexa Fluor 488-conjugated anti-mouse IgG (20 μg/ml; Molecular Probes) was applied as the secondary antibody. To visualize the nucleus, cells were counterstained with 0.1 μg/ml 4’,6-diamidino-2-phenylindole (Sigma).

**Luciferase Assay**—Stat1- and Stat3-dependent gene transactivation was measured using the Dual Luciferase reporter system (Promega Corp.) following the manufacturer’s instructions. The Stat1 reporter construct (pGL-2XIFP53-GAS) was obtained from BD Biosciences, and the Stat3 construct (6X APRE (acute-phase response element)) was a gift from T. Decker (University of Vienna). Firefly luciferase levels were normalized to Renilla luciferase levels (pRL-TK; Promega Corp.), which was used as an internal control. GyrB-wtPKR-expressing HT1080 cells were transfected with 1.0 μg of pRC/CMV-eIF2α/S51A (27) or empty vector, and the Stat1 luciferase activity in each condition was measured as described above.

**Northern Blot Analysis**—PKR+/− and PKR−/− MEFs were washed twice with 1× phosphate-buffered saline and lysed with TRIZol reagent (Invitrogen). RNA was prepared according to the manufacturer’s recommendations. Total RNA (15 μg) was electrophoretically resolved on denaturing agarose gels, transferred to nylon membranes, and hybridized to [α-32P]dCTP-labeled random-primed cdNA probe (5 × 106 cpm/ml) consisting of a 750-bp fragment of human interferon regulatory factor-1 (IRF-1) or the entire rat gliceraldehyde-3-phosphate dehydrogenase cdNA at 65°C for 16 h. Radioactive bands were visualized by autoradiography.

**Two-dimensional Gel Electrophoresis**—This was performed as described (22).

**Immunoprecipitation and Immunoblotting**—Protein extraction and immunoprecipitation of Stat proteins were performed as described (18, 19). Immunoblotting was performed as described (28). For immunoprecipitation and/or immunoblotting, the following antibodies were used: anti-Stat1α mAb (C-111), rabbit anti-Stat1α polyclonal antibody (pAb; M-23), anti-phospho-Tyr705 Stat1 mAb (A-2), rabbit anti-Stat3 pAb (C-20), anti-phospho-Tyr705 Stat3 mAb (B-7), rabbit anti-SHP-2 pAb (C-18), anti-c-Myc mAb (9E10), rabbit anti-IRF-1 pAb (C-20), and anti-actin mAb (Santa Cruz Biotechnology, Inc.). Rabbit anti-
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**FIGURE 1. Stat1 and Stat3 phosphorylation and transcriptional activities in PKR−/− and PKR+/+ MEFs.** A and B, PKR+/+ and PKR−/− MEFs were treated with 120 IU/ml murine IFN-γ (A) or 10 ng/ml murine IL-6 (B). Protein extracts (500 μg) were immunoprecipitated (IP) with anti-Stat1α mAb (C-111), followed by immunoblotting with anti-phospho-Tyr705 (pY705) Stat1 MAb (panel a) or anti-Stat3 antibody (panel b). Total RNA (15 μg) was probed with [α-32P]dCTP-labeled IRF-1 fragment (panel c) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; panel d). The normalized ratio of IRF-1 protein and RNA to actin protein and glyceraldehyde-3-phosphate dehydrogenase RNA levels, respectively, for each lane is indicated. D and E, PKR+/+ and PKR−/− MEFs were transiently transfected with a firefly luciferase reporter gene under the control of a promoter containing two IFN-γ-activated sequence elements (D) or six acute-phase response elements (E). Transfected cells were left untreated or were treated with 100 IU/ml IFN-γ (D) or 100 ng/ml recombinant human IL-6 (E) overnight prior to assessment of luciferase activity. For all transfections, a second vector containing a protein extracts (500 μg) were immunoprecipitated (IP) with anti-Stat1 antibody using whole protein extracts (supplemental Fig. 1). Immunoblotting with phospho-specific antibodies showed that Tyr701 phosphorylation of Stat1 in MEFs with a targeted deletion of the catalytic domain of PKR (20). PKR+/+ and PKR−/− MEFs were treated with recombinant mouse IFN-γ, and protein extracts were immunoprecipitated with the anti-Stat1α mAb (C-111) (Fig. 1A). Immunoblotting with phospho-specific antibodies showed that Tyr701 phosphorylation of Stat1 was stronger and persisted longer in PKR−/− cells compared with PKR+/+ cells (Fig. 1A, panel a, ratio a/c). Contrary to tyrosine phosphorylation, Ser727 phosphorylation of Stat1 did not significantly differ between PKR+/+ and PKR−/− MEFs treated with IFN-γ (Fig. 1A, panel b, ratio b/c). The higher induction of Stat1 Tyr701 phosphorylation in PKR−/− cells than in PKR+/+ cells by IFN-γ was further verified by immunoblotting with a second phospho-specific antibody using whole protein extracts (supplemental Fig. 1A). We also noticed that Stat1 Tyr701 phosphorylation was more highly induced in PKR−/− MEFs than in PKR+/+ MEFs after VSV infection (supplemental Fig. 1B). Induction of Stat1 Tyr701 phosphorylation was not observed in IFN-γ-treated PERK−/− MEFs compared with isogenic PERK+/+ MEFs (supplemental Fig. 2), indicating a specific inhibitory effect of PKR on

Increased Phosphorylation of Stat1 and Stat3 in Mouse Cells Deficient in PKR—We previously observed an inhibitory role of PKR on Stat1 transcriptional activity (18). We followed up this observation by examining the tyrosine phosphorylation of Stat1 in MEFs with a targeted deletion of the catalytic domain of PKR (20). PKR+/+ and PKR−/− MEFs were treated with recombinant mouse IFN-γ, and protein extracts were immunoprecipitated with the anti-Stat1α mAb (C-111) (Fig. 1A). Increased phosphorylation of Stat1 and Stat3 were observed in PKR−/− MEFs compared with PKR+/+ MEFs by IFN-γ (Fig. 1A, panel a, ratio a/c). Contrary to tyrosine phosphorylation, Ser727 phosphorylation of Stat1 did not significantly differ between PKR+/+ and PKR−/− MEFs treated with IFN-γ (Fig. 1A, panel b, ratio b/c). The higher induction of Stat1 Tyr701 phosphorylation in PKR−/− cells than in PKR+/+ cells by IFN-γ was further verified by immunoblotting with a second phospho-specific antibody using whole protein extracts (supplemental Fig. 1A). We also noticed that Stat1 Tyr701 phosphorylation was more highly induced in PKR−/− MEFs than in PKR+/+ MEFs after VSV infection (supplemental Fig. 1B). Induction of Stat1 Tyr701 phosphorylation was not observed in IFN-γ-treated PERK−/− MEFs compared with isogenic PERK+/+ MEFs (supplemental Fig. 2), indicating a specific inhibitory effect of PKR on
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Stat1 activation among the other eIF2α kinases. We further examined Stat3 tyrosine phosphorylation in PKR+/+ and PKR−/− MEFs (Fig. 1B). Similar to Stat1, Tyr705 phosphorylation of Stat3 in response to IL-6 was higher in PKR−/− cells compared with PKR+/+ cells (Fig. 1B, panel a). Collectively, these data indicate that the catalytic activity of PKR exhibits a negative effect on Stat1 and Stat3 tyrosine phosphorylation.

Because tyrosine phosphorylation is essential for Stat protein function (1), we examined Stat1 transcriptional activity in an in vivo setting. We evaluated the expression of IRF-1 protein and mRNA levels upon IFN-γ treatment (Fig. 1C, panel a). IRF-1 protein levels were induced more quickly and to a higher degree in PKR−/− cells as opposed to PKR+/+ cells following IFN-γ treatment. Examination of IRF-1 mRNA levels by Northern blot analysis revealed that IRF-1 transcripts were higher in PKR−/− cells after 6 and 12 h of IFN-γ treatment (Fig. 1C, panel c). The transcriptional activity of Stat1 in PKR+/+ and PKR−/− cells was further examined using cells transiently transfected with a luciferase reporter gene under the control of a Stat1-dependent promoter containing two tandem IFN-γ-activated sequence elements (19).

We observed a 2.2-fold increase in the transcriptional activity of Stat1 in PKR−/− cells compared with their wild-type counterparts upon IFN-γ stimulation (Fig. 1D). We also performed a luciferase assay to evaluate Stat3 transcriptional activity using a luciferase reporter construct containing six consecutive repeats of the acute-phase response element. Regulation of Stat3 activity was analogous to that of Stat1 activity in the two cell lines. Stat3-dependent transcriptional activity was increased by 2.6-fold in PKR−/− cells relative to the wild-type cells upon treatment with IL-6 (Fig. 1E). This confirms our previous observation that PKR negatively affects Stat1 transcriptional activity (18, 19) and further indicates that Stat3 is subject to the same regulation.

TyrosinePhosphorylationofStat1andStat3IsDecreasedbyConditionallyActivePKR—We previously reported that catalytic PKR+/+ MEFs express a truncated PKR protein that still contains the N-terminal double-stranded RNA-binding domain of the kinase (29). As such, increases in Stat1 and Stat3 tyrosine phosphorylation in these cells may not have been due to the lack of catalytic activity, but due to a gain of function of the truncated PKR protein. To better address the role of catalytically active PKR in regulating Stat1 and Stat3, we employed an inducible PKR system in which the kinase domain of PKR is expressed as a fusion protein with the first 220 amino acids of the Escherichia coli GyrB protein (21). Chemical cross-linking of the GyrB domain with the drug coumermycin leads to GyrB-PKR dimerization, autophosphorylation, and consequently eIF2α phosphorylation at Ser51 (21). We established a human fibrosarcoma cell line (HT1080) stably expressing either GyrB-wtPKR or the catalytically inactive GyrB-PKR(K296H) mutant (22). In these cells, treatment with coumermycin results in GyrB-wtPKR activation and phosphorylation of endogenous eIF2α as opposed to
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Figure 3. PKR induces Stat1 and Stat3 tyrosine dephosphorylation via TC-PTP. (A) Effects of sodium vanadate on Stat1 phosphorylation in cells with activated PKR. HT1080 cells expressing either GyrB-wtPKR or GyrB-PKR(K296H) were treated with 100 ng/ml coumermycin for 6 h. Cells were then treated with 100 IU/ml IFN-γ in the absence (lanes 1–3 and 7–10) or presence (lanes 4–6 and 10–12) of 0.2 mM Na3VO4. Extracts (500 μg of protein) were immunoprecipitated (IP) with anti-Stat1 mAb (C-111), and Western blots were probed with anti-phospho-Tyr701 Stat1 (pY701) mAb (panel a) or anti-Stat1 mAb (C-111; panel b). The ratio of phosphorylated Stat1 to total Stat1 is shown for each lane. The differences in Stat1 phosphorylation levels between cells expressing GyrB-wtPKR or GyrB-PKR(K296H) caused by sodium vanadate treatment are also shown. (B) Total protein extracts (50 μg) from EFM7 TC-PTP+/− (lane 1) and EFM4 TC-PTP−/− (lane 2) MEFs and from HT1080 cells expressing GyrB-wtPKR and infected with viruses containing either the control shRNA (lane 3) or TC-PTP shRNA-125 (lane 4) were subjected to SDS-PAGE and immunoblotting with anti-TC-PTP antibody (panel a). TC-PTP protein levels were normalized to actin levels by immunoblotting (panel b). The ratio of TC-PTP to actin levels for each lane is shown. (C and D) GyrB-wtPKR-expressing HT1080 cells containing either TC-PTP shRNA-125 (lanes 1–4 and 9–12; and D, lanes 1–5 and 11–15) or the control shRNA (C, lanes 5–8 and 13–16; and D, lanes 6–10 and 16–20) were left untreated (C, lanes 1–8; and D, lanes 1–10) or were treated with 100 ng/ml coumermycin for 6 h (C, lanes 9–16; and D, lanes 11–20) and then treated with 100 IU/ml IFN-γ (C) or 10 ng/ml IL-6 (D). Extracts (500 μg of protein) were immunoprecipitated with either anti-Stat1 mAb (C-111; C) or anti-Stat3 antibody (D), and Western blots were probed with anti-phospho-Tyr701 Stat1 mAb (C, panel a), anti-Stat1 mAb (C, panel b), anti-phospho-Tyr705 (pY705) Stat3 mAb (D, panel a), or anti-Stat3 antibody (D, panel b). The ratio of phosphorylated Stat1 or Stat3 to total protein is shown for each lane.
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GyrB-PKR(K296H), which is unable to induce eIF2α phosphorylation (22). We employed HT1080 cells expressing either GyrB-wtPKR or GyrB-PKR(K296H) to examine the role of PKR activation in Stat1 and Stat3 phosphorylation (Fig. 2). Each cell type was pretreated with coumermycin for 6 h to reach the optimal GyrB-PKR activation (22), followed by stimulation with either IFN-γ(Fig. 2A) or IL-6 (Fig. 2B). In cells containing GyrB-wtPKR, Stat1 tyrosine phosphorylation was reduced by >50% after 30 min of IFN-γ treatment compared with both the parental cells and the GyrB-PKR(K296H)-expressing cells (Fig. 2A, panel a, ratio a/b). GyrB-PKR(K296H) does not function as a dominant-negative mutant to suppress the activity of endogenous PKR present in the parental cell line (22), which explains why no variation is observed in the tyrosine phosphorylation levels of the GyrB-PKR(K296H)-expressing and parental HT1080 cells. As in the PKR−/− cells (Fig. 1A), very minimal changes in serine phosphorylation were detected upon GyrB-wtPKR activation (Fig. 2A, panel c, ratio c/d). Similar to Stat1, Stat3 phosphorylation was reduced by ~40% in coumermycin-treated cells expressing GyrB-wtPKR compared with those expressing GyrB-PKR(K296H) after IL-6 stimulation (Fig. 2B, panel a, ratio a/b). In fact, tyrosine phosphorylation of Stat3 was both stronger and of longer duration in GyrB-PKR(K296H) cells than in GyrB-wtPKR cells (compare lanes 6–10 with lanes 1–5). It is noteworthy that the inhibition of Stat1 Tyr701 phosphorylation by activated GyrB-wtPKR in response to IFN-γ was verified by immunoblotting of whole protein extracts with a second phospho-specific antibody (supplemental Fig. 1C). We also characterized the effect of coumermycin treatment and GyrB-wtPKR activation on the phosphorylation of endogenous PKR by two-dimensional gel analysis (supplemental Fig. 3). The altered banding pattern observed for GyrB-wtPKR upon coumermycin treatment was not observed for endogenous PKR under the same treatment. This confirms that coumermycin treatment specifically activates GyrB-wtPKR.

Because Stat1 tyrosine phosphorylation in IFN-γ-treated cells is mediated by the activity of Jak1 (2), we were interested in examining whether Jak1 activation is also affected by GyrB-PKR. To this end, coumermycin-treated HT1080 cells expressing either GyrB-wtPKR or GyrB-PKR(K296H) were treated with IFN-γ for short periods of time, followed by immunoprecipitation of Jak1 and immunoblotting with anti-phosphotyrosine antibody (Fig. 2C). When phosphorylated Jak1 levels were normalized to total Jak1 levels from the same immunoprecipitated extracts, we observed that a higher amount of Jak1 was tyrosine-phosphorylated in GyrB-PKR(K296H)-expressing cells 30 min after IFN-γ treatment, indicating that GyrB-wtPKR activation interferes with Jak1 activation.

Reduction of Stat1 Tyrosine Phosphorylation by PKR Is Mediated through a Tyrosine Phosphatase—Previous work has provided evidence that tyrosine phosphorylation of Stat1 precedes serine phosphorylation and that serine phosphorylation takes place preferentially in a pool of tyrosine-phosphorylated Stat1 (30). Because serine phosphorylation of Stat1 was not affected by PKR activation, we hypothesized that the kinase induces the activity of a tyrosine phosphatase specific for Stat1 and/or Stat3. To gain better evidence for the role of a tyrosine phosphatase, we examined the effect of the tyrosine phosphatase inhibitor sodium vanadate (Na3VO4) on Stat1 phosphorylation in HT1080 cells expressing GyrB-wtPKR or GyrB-PKR(K296H) (Fig. 3A). We observed that the presence of Na3VO4 increased Stat1 Tyr701 phosphorylation levels in both GyrB-wtPKR and GyrB-PKR(K296H) cells after stimulation with IFN-γ; however, this rescue was more pronounced in GyrB-wtPKR cells. Specifically, after 30 min of IFN-γ treatment, GyrB-wtPKR-expressing cells showed a 2.6-fold increase in Stat1 phosphorylation in the presence of Na3VO4, whereas GyrB-PKR(K296H)-expressing cells showed a 2.1-fold increase. At 60 min of IFN-γ treatment, GyrB-wtPKR-expressing cells treated with Na3VO4 showed a 12.8-fold increase in Stat1 phosphorylation compared with a 4.4-fold increase shown by GyrB-PKR(K296H)-expressing cells. These results indicate not only that a tyrosine phosphatase regulates Stat1 dephosphorylation in cells with activated PKR, but that cells with catalytically active PKR are more sensitive to treatment with a phosphatase inhibitor.

Decreased Stat1 and Stat3 Tyrosine Phosphorylation by PKR Requires TC-PTP—The two tyrosine phosphatases for whichStat1 is a substrate are SHP-2 and TC-PTP (2). SHP-2 is a dual specificity phosphatase that targets both serine- and tyrosine-phosphorylated Stat1 (31), whereas TC-PTP targets specifically tyrosine-phosphorylated Stat1 and Stat3 (14). From these two candidates, TC-PTP was more likely than SHP-2 to be involved because serine phosphorylation of Stat1 was not affected by activated PKR. To confirm this hypothesis, we attempted to knock down TC-PTP in HT1080 cells using the shRNA approach. Of three different shRNA vectors targeting TC-PTP, only one (shRNA-125) was able to down-regulate the endogenous levels of TC-PTP in GyrB-wtPKR-expressing cells by ~75% relative to cells expressing a control shRNA (Fig. 3B, compare lanes 3 and 4). TC-PTP−/− and TC-PTP+/− cells were used as controls (Fig. 3B, lanes 1 and 2). We then examined Stat1 tyrosine phosphorylation upon IFN-γ treatment in GyrB-wtPKR-expressing cells containing shRNA-125 compared with GyrB-wtPKR-expressing cells containing the control shRNA. We found that, in cells containing the control shRNA (Fig. 3C, lanes 5–8 and lanes 13–16), Stat1 Tyr701 phosphorylation by IFN-γ was significantly decreased upon activation of GyrB-wtPKR by coumermycin (lanes 14–16) compared with cells without coumermycin treatment (lanes 6–8). However, the same decrease in Stat1 Tyr701 phosphorylation was not observed in cells with decreased TC-PTP levels, indicating a reduced sensitivity to activation of GyrB-wtPKR (compare lanes 10–12 with lanes 2–4). The difference in Stat1 Tyr701 phosphorylation between cells with decreased TC-PTP levels and those expressing the control shRNA was much more significant in coumermycin-treated cells (compare lanes 10–12 with lanes 14–16) than in untreated cells (compare lanes 2–4 with lanes 6–8), which had an ~2-fold difference after 30 min of IFN-γ treatment.

We similarly evaluated Stat3 Tyr705 phosphorylation in cells containing shRNA-125 to determine whether reduced TC-PTP activity also affects Stat3 phosphorylation. Activation of GyrB-wtPKR by coumermycin decreased the phosphorylation of Stat3 at Tyr705 upon IL-6 stimulation in control shRNA cells as opposed to cells without GyrB-wtPKR activation (Fig. 3D, compare lanes 17–20 with lanes 7–10). However, Stat3 Tyr705 phosphorylation in response to IL-6 was significantly rescued in cells containing shRNA-125 with activated GyrB-wtPKR (lanes 12–15) compared with cells containing shRNA-125 cells in the absence of GyrB-wtPKR activation (lanes 2–5). These findings demonstrate that the reduction of both Stat1 Tyr701 and Stat3 Tyr705 phosphorylation by activated PKR requires TC-PTP.

Catalytically Active PKR Decreases the Transcriptional Function and Nuclear Localization of Stat1—Because of our previous observation that the tyrosine phosphorylation and transcriptional activities of Stat1 and Stat3 are decreased in the presence of catalytically active PKR, we assessed the effects of GyrB-wtPKR on Stat1- and Stat3-dependent gene transcription. GyrB-wtPKR cells containing either the control shRNA or TC-PTP shRNA-125 were transiently transfected with the luciferase reporter constructs as described for Fig. 1. We observed that Stat1-dependent gene transcription was induced in both cell types upon IFN-γ treatment in the absence of GyrB-wtPKR activation (Fig. 4A). However,
Stat1-dependent gene transactivation by IFN-γ/H9253 was diminished by 50% in control shRNA cells with activated GyrB-wtPKR upon coumermycin treatment compared with cells with knocked down TC-PTP (shRNA-125 cells) (Fig. 4A). Similar to the effect observed with IFN-γ/H9253 in the Stat1 system, Stat3-dependent transcription was induced to the same degree in both cell types upon IL-6 treatment (Fig. 4B). Treatment of control shRNA cells with coumermycin caused a 67% decrease in Stat3 transcriptional activity compared with only a 13% decrease observed in TC-PTP shRNA cells. These data show that the effects of GyrB-wtPKR on Stat1 Tyr701 and Stat3 Tyr705 phosphorylation through TC-PTP profoundly affect the transcriptional function of both proteins.

Tyrosine phosphorylation of Stat1 is a prerequisite for its nuclear translocation (32). We therefore sought to characterize the cellular localization of Stat1 in response to PKR activation and treatment with IFN-γ using fluorescence microscopy. GyrB-wtPKR-expressing HT1080 cells containing either the control shRNA or TC-PTP shRNA-125 were treated with a combination of coumermycin and IFN-γ/H9253 (Fig. 4A). Similar to the effect observed with IFN-γ in the Stat1 system, Stat3-dependent transcription was induced to the same degree in both cell types upon IL-6 treatment (Fig. 4B). Treatment of control shRNA cells with coumermycin caused a 67% decrease in Stat3 transcriptional activity compared with only a 13% decrease observed in TC-PTP shRNA cells. These data show that the effects of GyrB-wtPKR on Stat1 Tyr701 and Stat3 Tyr705 phosphorylation through TC-PTP profoundly affect the transcriptional function of both proteins.

Untreated cells, Stat1 exhibited both nuclear and cytoplasmic localization, which did not significantly change in cells treated with coumermycin to activate GyrB-wtPKR. However, treatment with IFN-γ resulted in the nuclear localization of Stat1 in both control shRNA and shRNA-125 GyrB-wtPKR-expressing cells. Interestingly, the nuclear localization of Stat1 by IFN-γ was diminished in coumermycin-treated control shRNA cells, but not in coumermycin-treated shRNA-125 cells, indicating that activation of PKR diminishes the nuclear localization of Stat1 in a TC-PTP-dependent manner.

**TC-PTP Is Phosphorylated by PKR**—To better understand the functional interaction between PKR and TC-PTP, we first examined whether TC-PTP is phosphorylated by PKR in vitro. Recombinant GST-PKR and GST-TC-PTP proteins were incubated in the presence of [γ-32P]ATP, and the incorporation of labeled phosphate in each protein was measured by autoradiography. Incubation of GST-wtPKR with GST-wtTC-PTP resulted in the phosphorylation of both proteins (Fig. 5A, lane 2), which was abolished when the catalytically inactive GST-PKR(K296R) mutant was used (lane 3). GST-wtPKR also phosphorylated the inactive GST-TC-PTP(D182A) mutant (lane 5) to a higher degree than GST-wtTC-PTP.

**FIGURE 4. Regulation of Stat1 nuclear function by PKR.** A and B, control of Stat1- and Stat3-dependent gene expression by PKR and TC-PTP. HT1080 cells expressing GyrB-wtPKR and targeted with either the control shRNA or TC-PTP shRNA-125 were transfected with a firefly luciferase reporter gene under the control of a promoter containing two IFN-γ activated sequence elements (A) or six acute-phase response elements (B). Transfected cells were left untreated or were treated overnight with coumermycin (Cou) and/or 100 IU/ml IFN-γ (A) or 100 ng/ml recombinant human IL-6 (B) prior to the assessment of luciferase activity. For all transfections, a second vector containing a renilla luciferase reporter gene was used as an internal control. The results shown are the means ± S.D. of three experiments performed in triplicate. C, cellular localization of Stat1. HT1080 cells expressing GyrB-wtPKR and either the control shRNA or TC-PTP shRNA-125 were treated with 100 ng/ml coumermycin alone, 100 IU/ml IFN-γ alone, or a combination of 100 ng/ml coumermycin and 100 IU/ml IFN-γ for 1 h. Cells were fixed and immunostained for Stat1 (green). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).
PKR Regulates Stat1 and Stat3 Dephosphorylation

We next investigated whether TC-PTP is phosphorylated by GyrB-wtPKR in vivo. Cell labeling with \(^{32}P\)orthophosphate and detection of TC-PTP phosphorylation by immunoprecipitation from coumermycin-treated GyrB-wtPKR expressing cells were inconclusive due to the high background phosphorylation of proteins that co-immunoprecipitated with TC-PTP (data not shown). To bypass this limitation, we assessed the phosphorylation of TC-PTP by two-dimensional gel electrophoresis (Fig. 5B). When we examined endogenous TC-PTP, we found that a fraction of TC-PTP (Fig. 5B, indicated by arrows) was shifted to more acidic pH in cells treated with coumermycin (panel b) compared with untreated cells (panel a), which is characteristic of phosphorylated proteins. Similar results were obtained in cells containing transfected Myc-tagged TC-PTP (data not shown). Because PKR is an IFN-γ-inducible protein, we examined the effect of IFN-γ stimulation on TC-PTP phosphorylation and observed that endogenous TC-PTP underwent an acidic shift in PKR+/+ MEFs (data not shown). Taken together, these findings show that activation of PKR leads to TC-PTP phosphorylation in vivo.

**DISCUSSION**

This study substantiates our previous findings of a negative role of PKR in Stat1 activation (18, 19) and reveals a novel mechanistic role of the kinase in the Jak-Stat pathway via activation of TC-PTP. Data obtained both from a conditionally inducible PKR system and from cells lacking catalytically active PKR show that Stat1 tyrosine (but not serine) phosphorylation was decreased in the presence of active PKR (Figs. 1A and 3A). The same regulation was seen for Stat3 tyrosine phosphorylation upon stimulation with IL-6 (Figs. 1B and 3B). This regulation carried through to the transactivation activities of both Stat1 (Figs. 1D and 5A) and Stat3 (Figs. 1E and 5B). In addition, the mRNA and protein expression of the Stat1 target IRF-1 was up-regulated in PKR+/−/− cells in response to IFN-γ treatment (Fig. 1C). The introduction of shRNA targeting TC-PTP into this same system ablated the effect of PKR activation on Stat1 and Stat3 tyrosine phosphorylation (Fig. 3, C and D), indicating that catalytically active PKR may not prevent the phosphorylation of the Stat proteins, but rather facilitate their dephosphorylation. Activation of PKR also diminished the phosphorylation of Jak1 in response to IFN-γ (Fig. 2C), consistent with a role of TC-PTP in the dephosphorylation of Jak1 (33).
PKR Regulates Stat1 and Stat3 Dephosphorylation

FIGURE 6. Role of eIF2α in regulating Stat1 phosphorylation and transcriptional activity. A, eIF2α S/S and A/A cells were treated with recombinant mouse IFN-γ (100 IU/ml) for the indicated times. Extracts were immunoprecipitated (IP) with anti-Stat1α mAb (C-111), followed by immunoblotting with anti-phospho-Tyr701 (pY701) Stat1 mAb (panel a) or anti-Stat1α pAb (M-23, panel b). The ratio of phosphorylated Stat1 to total Stat1 is shown for each lane. B, GyrB-wtPKR-expressing HT1080 cells were transiently transfected with the pRC/CMV-eIF2α(S51A) construct or empty vector as a control, followed by transfection with a firefly luciferase reporter construct containing two tandem IFN-γ-activated sequence elements. Cells were left untreated or were treated with 100 ng/ml coumermycin (Cou) or 100 IU/ml IFN-γ as indicated, and the luciferase activity was assessed. In each case, a Renilla luciferase construct was cotransfected as an internal control.

Phosphorylation in cells with activated PKR (data not shown), indicating that TC-PTP is a specific effector of PKR.

Disruption of TC-PTP also rescued the nuclear localization of Stat1 in the presence of activated PKR (Fig. 4C). Non-phosphorylated Stat1 constantly shuttles between the nucleus and the cytoplasm, regardless of cytokine stimulation (32, 34). Cytokine signals trigger not the nuclear import of phosphorylated Stat1 proteins, but rather their nuclear retention, as Stat1 must be dephosphorylated prior to its export by CRM-1-dependent and -independent mechanisms (32, 34). Our data show that activation of PKR affects the dephosphorylation of Stat1, and these changes in the phosphorylation status and nuclear localization of Stat1 are reflected by changes in its transcriptional activity. GyrB-wtPKR cells with reduced TC-PTP protein levels were resistant to the decrease in Stat1 transcriptional activity seen in control cells upon activation of the kinase by coumermycin treatment (Fig. 4A), and the same was true for Stat3 (Fig. 4B). Although the 45-kDa variant of TC-PTP is localized primarily in the nucleus, it can translocate to the cytoplasm in response to various signals, including epidermal growth factor, insulin, and hyperosmotic and oxidative stresses (35–37). We observed no change in the cellular localization of TC-PTP upon PKR activation (data not shown), indicating that PKR regulates another characteristic of this phosphatase. Most protein-tyrosine phosphatases, including TC-PTP, contain a non-catalytic segment involved in subcellular localization and regulation of enzyme activity (38). Amino acids 288–352 at the C-terminal end of TC-PTP may function as an autoinhibitory domain regulating enzyme activity (33), and it is possible that the site of phosphorylation lies within this domain or affects a conformational change in TC-PTP that alters its activity. However, in vitro phosphatase assays performed with recombinant TC-PTP phosphorylated by recombinant PKR indicated no change in phosphatase activity (supplemental Fig. 4), suggesting that phosphorylation may be necessary but not sufficient for TC-PTP activation in vivo. Similarly, phosphorylation of TC-PTP at Ser304, a site within the autoregulatory loop, by cyclin-dependent kinases does not significantly alter its phosphatase activity (39). Introduction of phosphorylation-deficient mutants of TC-PTP may therefore be insufficient to rescue Stat1 phosphorylation in the presence of activated PKR.

When we examined the effect of translation inhibition on TC-PTP protein levels, we discovered that TC-PTP is a very stable protein, showing no decrease in stability upon PKR activation or treatment with cycloheximide (data not shown). Further investigation into the role of eIF2α in regulating Stat phosphorylation revealed that eIF2α phosphorylation at Ser51 caused a decrease in Stat1 phosphorylation and that inhibition of eIF2α phosphorylation ablated the observed decrease in a manner similar to PKR−/− cells (Fig. 6A). The decrease in phosphorylation we observed in this case mimics the effect of activated PKR, suggesting that PKR may inhibit the translation of a cofactor that typically limits the interaction between TC-PTP and its Stat substrates (Fig. 7). Given the important roles of PKR and Stat1 in protecting host cells against viral infection, the inactivation of Stat1 by PKR appears to be a paradoxical finding. One plausible explanation is that PKR controls the duration and strength of cytokine signaling through the induction of TC-PTP activity. Another possibility is, however, that activation of PKR in response to viral infection can also be employed by specific viruses to usurp the normal host defense particularly under conditions in which
the eIF2α phosphorylation pathway is blocked. Thus, efficient viral replication may be controlled by PKR at different levels. For example, previous reports showed a high susceptibility of PKR−/− mice to VSV replication and VSV-mediated death after intranasal inoculation (23, 40, 41). This susceptibility was explained by an impaired capacity of PKR−/− mice to contain viral replication at the translational level (40). Together with our findings, these results indicate that combinations of different signaling pathways in infected cells can determine the outcome of viral replication in response to PKR activation in a manner that is dependent on the cell type and/or viral type. Our data may also serve to explain the lack of any significant phenotype in mice with a targeted disruption of the catalytic activity of PKR (20). These PKR−/− mice do not exhibit growth abnormalities or increased tumorigenesis (20) and, in contrast to Stat1 knock-out mice (3, 4), do not display significant susceptibility to most viral infections (20). In the case of Stat3, previous data provided evidence for a positive role of PKR in Stat3 activation in platelet-derived growth factor-treated cells (42). However, this regulation was described in MEFs from a PKR−/−/H11002 mouse lacking the N-terminal domain of the kinase (43). Given that these mice still express a truncated catalytically active form of PKR that lacks the N-terminal double-stranded RNA-binding domain (29), it remains unclear whether activation of Stat3 in these MEFs is a side effect of the truncated protein or a specific effect of platelet-derived growth factor signaling. Stat3 functions as an oncogene, and its phosphorylation is induced in many types of human tumors (8). Inhibition of Stat3 tyrosine phosphorylation by PKR may reveal a novel mechanism utilized by the activated kinase to inhibit the proliferation of cells and to induce the destruction of tumor cells by apoptosis (44).

FIGURE 7. Model for the induction of Stat1 and Stat3 dephosphorylation by PKR. Activation of PKR leads to TC-PTP phosphorylation, which is necessary but not sufficient to mediate the dephosphorylation of Stat1 or Stat3. The phosphorylation of eIF2α and the subsequent inhibition of protein synthesis by activated PKR facilitate the dephosphorylation of Stat1 and Stat3 by TC-PTP possibly by blocking the expression of a protein that negatively regulates the tyrosine phosphatase.

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