The PKR protein kinase is among the best-studied effectors of the host interferon (IFN)-induced antiviral and antiproliferative response system. In response to stress signals, including virus infection, the normally latent PKR becomes activated through autophosphorylation and dimerization and phosphorylates the eIF2α translation initiation factor subunit, leading to an inhibition of mRNA translation initiation. While numerous virus-encoded or modulated proteins that bind and inhibit PKR during virus infection have been studied, little is known about the cellular proteins that counteract PKR activity in uninfected cells. Overexpression of PKR in yeast also leads to an inhibition of eIF2α-dependent protein synthesis, resulting in severe growth suppression. Screening of a human cDNA library for clones capable of counteracting infection of PKR dimers.

Eukaryotic cells generally down-regulate protein synthesis in response to stress conditions presumably to protect against the harmful effects of toxic agents, to conserve resources that are needed to survive under adverse conditions, or to activate apoptosis (1). A major control mechanism for this cellular stress response involves protein phosphorylation of the α subunit of the translation initiation factor 2 (eIF2α) on serine 51 (reviewed in Ref. 2). When bound to GTP, eIF2 promotes the assembly of the translation initiation complex between Met-tRNA, and the 40 S ribosomal subunit, a process that results in GTP hydrolysis and an eIF2-GDP complex. Phosphorylation of eIF2α subverts the recycling step required for the formation of an active eIF2-GTP complex, thereby reducing the rate of mRNA translation initiation and, ultimately, an inhibition of global cellular protein synthesis. At least four structurally related serine/threonine protein kinases, each responding to specific stress stimuli, phosphorylate eIF2α (reviewed in Ref. 3): the yeast GCN2 kinase, activated by amino acid starvation; the reticulocyte-specific HRI kinase, activated by heme depletion; the endoplasmic reticulum-associated PERK/PEK kinase, activated by stresses that impair protein folding in the endoplasmic reticulum; and the interferon (IFN)-inducible PKR serine/threonine kinase, activated primarily by virus infection.

The PKR protein kinase is one of the few well characterized IFN-induced gene products that directly mediate the antiviral effects of IFNs (reviewed in Ref. 4). PKR is ubiquitously expressed but is normally inactive, presumably because the ATP-binding site or the catalytic domain of PKR is masked by intramolecular interactions (5, 6). Upon binding to dsRNA, or to RNA with secondary structures similar to viral replicative intermediates, PKR is autophosphorylated on multiple serine and threonine residues, which may induce a conformational change that leads to the disclosure of the ATP-binding site and the catalytic domain. This is followed by PKR dimerization, which is thought to promote the intermolecular autophosphorylation of PKR molecules, resulting in maximal activation of the enzyme (7–11). Binding to dsRNA may also serve to recruit PKR molecules to the ribosomes for localized action, where phosphorylation of eIF2α by PKR leads to a block in global protein synthesis, ultimately limiting virus replication within the infected cell (11, 12). The important role of PKR in host innate immunity is underscored by the numerous strategies employed by different viruses to antagonize PKR (4). Furthermore, mice devoid of functional PKR display increased susceptibility to infection by some viruses (13–18).

1 The abbreviations used are: IFN, interferon; dsRNA, double-stranded RNA; PP, protein phosphatase; SD, synthetic defined; Ura, uracil; GST, glutathione S-transferase.
PP1C Inhibition of PKR

Activation of PKR can also lead to apoptosis (4). The translational inhibition and pro-apoptotic properties of PKR have led to the suggestion that PKR may be a tumor suppressor. Indeed, overexpression of PKR is growth-suppressive in insect, yeast, and mammalian cells (19–21), whereas overexpression of dominant-negative forms of PKR, or cellular or viral inhibitors of PKR, leads to malignant transformation of NIH 3T3 cells (22–26). Although the exact mechanisms are still not clear, PKR may function through its ability to regulate transcription factors NFκB (27, 28), STAT1 (29, 30), and the tumor suppressor p53 (31, 32). Accordingly, PKR activity should be tightly modulated in the cell. While viral studies have revealed different strategies for PKR countermeasures, including the inhibition of dsRNA-mediated activation of PKR, the interference with PKR dimerization process, and the degradation of PKR protein (4), the regulation of PKR by post-translational protein modifications in uninfected cells is poorly understood. Of particular interest to this study is the modulation of PKR by reversible protein phosphorylation, which is widely used for rapid signal desensitization of enzymes and is commonly used in many signal transduction pathways.

Previous studies have suggested that a type 1-protein phosphatase (PP1) may be responsible for the inactivation of PKR (33), whereas a type 2 PP (PP2) is thought to regulate HRI activity (34). However, the exact protein phosphatases involved have not been determined. To gain insights into the cellular mechanisms of PKR regulation, we undertook experiments to identify negative regulators of PKR. To this end, we used a yeast-based functional assay for PKR to screen a human cDNA expression library for clones capable of repressing PKR activity. This screen yielded the catalytic subunit of type 1 protein phosphatase (PP1α or PP1C). Using various in vitro and in vivo assays, we verified the ability of PP1C to inhibit PKR and further demonstrated a specific and direct interaction between the two proteins. Co-expression of PP1C interfered with PKR dimerization, whereas a catalytically inactive mutant PP1C did not. Our results suggest a potential mechanism for tight control of PKR activity and in turn points to a role for PP1C in translational control.

Experimental Procedures

Yeast Strains and Culture Conditions—For the yeast-based PKR functional assay, we used Saccharomyces cerevisiae RTY1−1 (MATa, ura3-52, leu2-3, leu12-112, gcn2, trep-1, 353, LEU::GAL-CYC1-PRKα (provided by Drs. A. G. Hinnebusch and P. R. Romano). For the yeast two-hybrid assay, S. cerevisiae H7C (MATa, ura3-52, his3-200, lys2-801, ade2-101, 112, trep-1, 301, leu2-3, gal4-542 gal80-538, LYS2::Gal1-HIS3, URA3::Gal1-7-mers)-YCV1iacZ (CLONTECH) was used. Basic methods for the growth and manipulation of yeast were carried out as described by Romano et al. (9) and the CLONTECH manual. Reagents for preparation of media were purchased from Bio-101, and media were prepared according to the manufacturer’s specifications.

Yeast Transformation and Library Screening—A human cDNA expression library in a YES-R (a gift from Dr. S. J. Elledge) was used to infect Escherichia coli BHN132, and plasmid DNA was prepared as described by Elledge et al. (35). Purified library plasmid DNA (500 μg) was transformed into E. coli BL21(DE3) (Novagen) and induced with 1 mM isopropyl β-D-galactoside. Cells were grown overnight at 30 °C in LB supplemented with antibiotics, 10 mM MgSO₄, and 0.2% galactose. Cultures were pelleted by centrifugation and washed three times with lysis buffer containing 0.25% Triton X-100. The bead pellet was boiled in equal volume of 1X Laemmli sample buffer for 5 min. Immunoprecipitated complexes were resolved by SDS-PAGE (12%). Proteins were transferred onto nitrocellulose membrane and immunoblotted with either anti-PKR (provided by Dr. B. Schneider) or anti-PKR polyclonal primary antibody (19), followed by donkey anti-rabbit secondary antibody (Jackson ImmunoResearch). Proteins were visualized by ECL (Amer sham Biosciences) and autoradiography of the immunoblots.

Functional Assays for PKR Activity—For PKR in vitro kinase assays, native PKR protein, affinity-purified from IFN-treated human 293 cells, was used (39). GST-PP1C and GST-Tat (obtained from Dr. M. Mathews) proteins were purified as described by Miki et al. (40). SDS-PAGE with bovine serum albumin as a standard was used to determine the concentration of all purified proteins. In vitro kinase reactions were performed essentially as described (10), except that PKR (0.3 μg) was incubated with increasing amounts (0.1, 0.2, and 0.4 μg) of purified rabbit PP1C (from Upstate Biotechnology) in a 30 μl phosphate reaction buffer (Upstate Biotechnology) at 30 °C for 30 min. When indi...
cated, rabbit PP1C, the amino acid sequence of which is identical to human PP1C, was inactivated by the addition of Inhibitor-2 (Upstate Biotechnology) according to the manufacturer’s instructions. PKR was immunoprecipitated from the reaction mixture using a PKR-specific monoclonal antibody (7/10; Ribogene) and subjected to kinase reaction containing [γ-32P]ATP (10 μCi) and histone H1 (10 μg) in the presence or absence of poly(I·C) (3 μg/ml). The reaction was terminated by the addition of 30–μl 2× sample buffer (100 mm Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.2% bromphenol blue). One half of each sample (30 μl) was subjected to SDS-PAGE (14%), followed by autoradiography. To determine PKR activity in vivo, eIF2α phosphorylation within RY1–1 yeast cells harboring various expression plasmids was determined by isoelectric gel focusing and immunoblot analysis as described by Gale et al. (40). A rabbit polyclonal antiserum specific to yeast eIF2α (a generous gift from Dr. T. E. Dever) was used to detect eIF2α by immunoblot analysis. Construction of pYX233-PP1C was achieved by subcloning a 1-kb EcoRI-SalI DNA fragment from GAL4A/PPI1, into pYX233 (Novagen) linearized with EcoRI and Xhol. Dr. P. B. Romano provided plasmid p1470, which expresses PKR K296R. The relative levels of protein phosphorylation were determined by quantifying the immunobLOTS using a Molecular Dynamics Phosphor-Imager and ImageQuant software (version 5.1).

RESULTS

Identification of PP1C as an Inhibitor of PKR—To identify novel cellular inhibitors of PKR, we adopted a genetic screening strategy using S. cerevisiae. Overexpression of human PKR protein in yeast cells is lethal because constitutive hyperphosphorylation of eIF2α by PKR leads to severe inhibition of mRNA translation (20). We reasoned that co-expression of mammalian genes that negatively regulate PKR would suppress the PKR-induced lethality. We used a gen2Δ yeast strain (RY1–1), which carries two copies of the human PKR allele under the control of a galactose-inducible promoter (9). We chose this strain because PKR protein expression is suppressed when the cells are grown in glucose-containing medium, thus allowing the cells to grow normally. Upon transfer to galactose-containing medium, which induces the GAL promoter and hence PKR expression, these yeast cells cease to grow. We assumed that reversion to normal growth because of mutations would be minimal because the high expression levels of mutations would block cell division, a prerequisite for the generation of mutants. To this end, a galactose-inducible human cDNA expression library was introduced into RY1–1, and transformants that overcame the PKR-mediated growth-inhibitory effect were selected. Plasmids containing cDNAs were extracted from these transformants and retransformed into fresh RY1–1 to verify the reversal of growth arrest phenotype. One of the cDNA clones that reproducibly restored RY1–1 viability on galactose-containing medium encoded the catalytic subunit of type 1 protein phosphatase, PPI1 (41). As shown in Fig. 1A, RY1–1 cells grew normally on raffinose medium, on which PKR expression was suppressed (left panel). However, RY1–1 cells were unable to grow when PKR expression was induced on galactose medium (right panel, lane 1). Coexpression of PPI1 partially rescued the growth defect of RY1–1 (right panel, lane 2).

Because relatively little is known about the cellular mechanisms controlling PKR activity, we chose to investigate the mode of action of PPI1. PPI1 could conceivably restore RY1–1 growth on galactose-containing medium through pathways that are independent of PKR. To confirm that the effect of PP1C was at least in part specific to PKR, we took note of previous observations that the catalytically inactive PKR K296R is repressive to wild-type PKR in yeast (9). We thus reasoned that co-expression of PKR K296R in RY1–1 might reverse the inhibitory effect of PPI1 on PKR by sequestering PPI1, in nonfunctional PKR K296R–PP1C complexes. Alternatively, PKR K296R might dimerize with PKR, and because these dimers would be functional in yeast, they could titrate out the PPI1.

As predicted, the PKR-mediated toxicity was partially restored when PKR K296R was coexpressed with PPI1 (Fig. 1B, right panel, lane 3). PKR proteins in these samples were expressed to comparable levels as shown by immunoblot analysis using a PKR-specific antibody (Fig. 1B, left panel). The more intense PKR band in lane 3 presumably represents the co-migrating wild-type PKR and PKR K296R. These results support our hypothesis that the PPI1 rescues RY1–1 from cell growth retardation, at least in part, via the PKR pathway.

PP1C Inhibits PKR Auto-phosphorylation—To examine whether PPI1 directly dephosphorylates PKR, we took advantage of the fact that PKR is hyperphosphorylated when expressed in yeast (9). Treatment of protein extracts with excess λ protein phosphatase converted the slower-migrating PKR protein band to a faster-migrating band (lane 2), indicating that the difference of migration was due to differential phosphorylation of PKR (Fig. 2A). Importantly, co-expression of PPI1 with PKR resulted in a similar hypophosphorylated form of PKR (lane 9). Moreover, as a control, expression of the catalytically inactive PKR K296R also produced a faster-migrating band, indicating that PPI1 dephosphorylates PKR (lane 4). Further support for this was obtained by the experimental results shown in Fig. 2B. Purified PKR proteins were first labeled by autophosphorylation in the presence of [γ-32P]ATP and poly(I·C) and then purified and used as a substrate for PPI1 in a phosphatase assay as described under “Experimental Procedures.” Indeed, PPI1 could dephosphorylate PKR in vitro in a dose-dependent manner (Fig. 2B, top panel). Western blotting showed that the PKR phosphorylation...
PP1C Inhibits PKR Substrate Phosphorylation—To confirm the functional significance of the PKR inhibitory effect of PP1C, we used isoelectric focusing to measure the phosphorylation level of eIF2α, the physiological substrate of PKR, in RY1–1 cells (9). In this assay, PKR-phosphorylated eIF2α can be distinguished from the non-phosphorylated form by its gel mobility pattern. As previously reported, eIF2α was not phosphorylated in the parental gcn2Δ yeast strain lacking PKR (Fig. 3A, no PKR). In contrast, induced expression of PKR led to hyperphosphorylation of eIF2α (Fig. 3A, PKR). When PP1C was coexpressed, a reduction of eIF2α hyperphosphorylation was observed (PKR + PP1C), which is comparable to that caused by coexpression of a PKR dominant-negative mutant (PKRΔ7). Although the majority of eIF2α remained in the hyperphosphorylated state, this is in agreement with previous results that relatively small changes (15–20%) in the overall level of eIF2α phosphorylation can have dramatic effects on cell growth (9, 40). Taken together, these results support the notion that PP1C is capable of antagonizing PKR function in vitro.

We next used purified components in an in vitro kinase assay (43) to determine whether PP1C could directly inhibit the ability of PKR to phosphorylate H1 histones. We chose to use H1 histones in this assay because PP1C is capable of dephosphorylating H1 histones in a dsRNA-dependent manner when the preincubation step did not include PP1C. Preincubation with PP1C, however,
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Fig. 4. PP1C interacts with PKR in vitro and in vivo. A, GST pulldown analysis. Crude lysates (500 μg) from HeLa cells were incubated with GST or the indicated GST fusion protein (0.2 or 0.5 μg) immobilized on glutathione-agarose beads as described under “Experimental Procedures.” The beads were washed, and bound proteins were resolved by SDS-PAGE (12.5% acrylamide). After transfer to nitrocellulose, the blots were probed with a PKR-specific monoclonal antibody (α-PKR; left panel A) or a PP1C-specific polyclonal antibody purchased from Upstate Biotechnology (α-PP1C; right panel). The PKR and PP1C proteins are indicated by arrows. B, summary of two-hybrid analysis. The Hf7c reporter strain was transformed with the indicated plasmids. The interaction between the two-hybrid proteins was scored by the induction of HIS3 expression (growth on SD agar plates lacking histidine). + indicates growth on SD medium-His (indicative of interaction) and − denotes no growth. C, co-immunoprecipitation of endogenous PKR and PP1C. Immunoprecipitation was performed using either PKR-specific monoclonal antibody or normal mouse serum (NMS), and the blots were probed with a PP1C-specific polyclonal antibody obtained from Dr. K. Schlender (α-PP1C; top panel) or a PKR-specific polyclonal antibody (α-PKR; bottom panel).

ever, significantly inhibited the ability of PKR to phosphorylate histones (lanes 3 and 4). As a further control in this experiment, PKR pre-incubated with inactive PP1C (inhibited by the PKI inhibitor I-2) retained its ability to phosphorylate histones (lane 5), indicating that the activity of PP1C is required to inhibit PKR in vitro. These results are consistent with the notion that PP1C directly inactivates PKR function.

PP1C Forms a Physical Complex with PKR—An emerging theme in cell signaling is the ability of protein kinases to form stable complexes with their corresponding phosphatases to ensure rapid and transient signal transduction mediated through reversible protein phosphorylation. Furthermore, the results shown thus far suggested that PP1C likely interacts with PKR. To test this possibility, we used a GST fusion protein-mediated co-sedimentation and immunoblotting assay. GST-PP1C-containing agarose beads were incubated with human HeLa cell extracts. Bound proteins were pulled down by centrifugation, washed, and subjected to SDS-PAGE and immunoblotting analyses using an anti-PKR antibody. As predicted, we found that endogenous PKR from HeLa cell extracts co-sedimented with recombinant GST-PP1C, but not with the GST control (Fig. 4A, left panel). The PKR-PP1C interaction was verified by reciprocal experiments, which showed that GST-PKR was able to pull down endogenous PP1C from HeLa cell lysates, although to a lesser extent (right panel). It is not clear why GST-PP1C is more effective than GST-PKR in pulling down the interacting partner. A possible explanation for this is that the fused GST tag is partially masking a region or affecting the protein conformation that is required for PKR interaction with PP1C.

We next used the yeast two-hybrid system to confirm the interaction between PP1C and PKR. Because wild-type PKR is toxic to S. cerevisiae, we used the catalytically inactive protein, PKR K296R. As shown in Fig. 4B, yeast strain Hf7c co-transformed with GAL4BD-PKR K296R and the GAL4BD expression vector, or a fusion control to GAL4BD (GAL4BD-P53), was unable to activate the HIS3 reporter genes and was therefore unable to grow in the absence of histidine (His). In addition, yeast cells co-transformed with GAL4BD PP1C and the GAL4BD expression vector, or a control fusion to GAL4BD (GAL4BD SV40 T ag), were unable to trans-activate the reporter construct. However, when the PP1C and PKR hybrid proteins were coexpressed in Hf7c, transactivation of HIS3 occurred, allowing the cells to grow in the absence of His. A similar effect was observed using the positive control proteins P53 and SV40 T Ag (45). The specificity of PP1C interaction was further demonstrated by the observation that the p53 protein did not bind PKR in this system, nor did we detect an interaction between PP1C and eIF2α, which is consistent with published observations (44, 46). It is interesting to note that the catalytic subunit of PP2A (PP2A C), the other major protein phosphatase, interacted with PKR very weakly in light of recent findings that PKR interacts with the regulatory subunit of PP2A (47).

To examine whether PKR and PP1C interact in living cells, we immunoprecipitated endogenous PKR from protein lysates prepared from Huh7 cells with an antibody to PKR. The precipitates were then analyzed by Western blotting using a PP1C-specific antibody that also reacts with an unknown 100-kDa protein (Fig. 4C; lysate). A significantly large portion of endogenous PP1C, but not the 100-kDa protein, could be detected in PKR immunoprecipitates (Fig. 4C; α-PKR). Furthermore, we did not detect PP1C in control immunoprecipitates obtained using normal mouse serum. Taken together, these results strongly support the notion that PP1C specifically interacts with PKR in intact cells.

PP1C Binds to the Regulatory Domain of PKR via a PP1C-Binding Consensus Motif—We next performed GST pulldown assays to identify the region of PKR that interacts with PP1C. Lysates from HeLa cells were incubated with recombinant proteins consisting of GST fused to deletion or point mutants of PKR. As shown in Fig. 5A, PP1C bound to the N-terminal regulatory domain, but not to the C-terminal catalytic domain of PKR (top panel, lanes 1 and 2). Consistent with this result, the catalytic activity of PKR was not required for the interaction with PP1C, because the catalytically attenuated PKR K296R retained its ability to bind PP1C (lane 3). The amount of the various GST fusion proteins that were co-immunoprecipitated in these experiments was revealed by Western blot analysis using an antibody against GST (bottom panel; indicated by asterisks).

To identify the PKR domain participating in interacting with PP1C in an in vivo environment, we used the two-hybrid sys-
tem. We found that PP1C bound to the N-terminal 242 residues of PKR (Fig. 5B), consistent with the *in vitro* results above. The undetectable interaction between PP1C and PKR 244–551 cannot be explained by the lack of protein expression or incorrect protein folding because the latter interacted effectively with the vaccinia virus K3L protein positive control, consistent with published results (37). Because PKR binds dsRNA, it is possible that the interaction between PP1C and PKR is tethered via an RNA bridge. To test this possibility, we used a truncated mutant PKR (amino acids 1–220) containing a mutation at lysine 64 (K64E), which abrogates its ability to bind dsRNA (48). The results show that this mutant retained its ability to bind efficiently to PP1C, supporting our contention that PP1C binds PKR via a direct protein-protein contact mechanism. However, we cannot rule out completely the presence of residual dsRNA mediating the interaction between PKR 1–220 (K64E) and PP1C in this assay.

Many PP1C-interacting proteins share a short PP1C-binding consensus motif, defined as (R/K)(V/I/L)X(F/W/Y) (49). Sequence analysis revealed that PKR has two potential motifs that are analogous to the PP1C-binding consensus sequence: the first is located at position 164–167 and the second at position 297–300 of PKR. Because removal of the C-terminal part of PKR, which includes the second PP1C-binding consensus motif, did not abrogate PP1C binding (Fig. 5B), we reasoned that the first PP1C-binding consensus motif might be important for PP1C binding. To validate this, we mutated the Tyr residue to Ala in the motif and tested the ability of the mutant (PKR Y167A) to bind PP1C. As predicted, the mutant PKR was unable to interact with PP1C in the two-hybrid assay (Fig. 5B).

**FIG. 5.** **PP1C** binds to the regulatory domain of PKR via a PP1C-binding motif. **A**, GST pulldown assay. GST pulldown assays were performed as described in the legend of Fig. 2A. HeLa cell lysates were incubated with GST or the indicated GST fusion protein immobilized on glutathione-agarose beads. The beads were washed, and bound proteins were resolved by SDS-PAGE (12.5% acrylamide). After transfer to nitrocellulose, the blots were probed with anti-PP1C (α-PP1C top panel) or anti-GST (α-GST bottom panel) antibody. The PP1C protein is indicated by arrow; GST fusion proteins are indicated by an asterisk. **B**, yeast two-hybrid assay. A schematic representation of domain structures of wild-type (WT) PKR and PKR mutant constructs used is shown. DSRM1 and DSRM2 denote the positions of the dsRNA-binding motifs 1 and 2, respectively. The protein kinase catalytic domain begins at residue 265 and contains the conserved kinase homology subdomains labeled I–XI. Positions of terminal amino acids and point mutations are indicated. The yeast two-hybrid results are shown on the right where growth on SD medium-His is indicative of interaction.
As a control, PKR Y167A retained the ability to bind K3L, indicating that the mutant protein was properly expressed and translocated to the nucleus.

**PKR Binds to a Conserved C-terminal Non-catalytic Region of PP1C Isoforms**—We also examined whether PKR could bind to PP1o2 (or PP1C2), an isoform that differs from PP1C by an N-terminal 11-amino acid insert (50). GST or GST fusions containing PP1C or PP1C2 immobilized on agarose beads were incubated with HeLa cell extracts, and the bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting using an antibody against PKR or GST. As shown in Fig. 6A (top panel), PKR interacted with PP1C2 (lane 4). GST and GST-PP1C were used as a negative and positive control, respectively (lane 2 and 3). Because PP1C1 and PP1C2 share a common C-terminal non-catalytic region, we suspected that this region might be sufficient to mediate PKR interaction. To test this, we used a GST fusion containing the C-terminal 161 residues of PP1C. As predicted, PKR was still capable of interacting with this truncated PP1C protein (lane 5). Importantly, all GST fusions were expressed efficiently (bottom panel). Thus the PKR-interactive region appears to be localized within the C-terminal non-catalytic region conserved among PP1C isoforms.

The Catalytic Domain of PP1C Is Required for Inhibition of PKR Dimerization—To obtain corroborative evidence that PP1C binds to the N-terminal region of PKR, which is critical for enzyme dimerization, we turned to the λ repressor fusion dimerization assay (51). In this system, full-length inactive PKR is expressed in *E. coli* as a fusion to the N-terminal domain of λ cI repressor (λN), which contains the DNA-binding domain but lacks the dimerization domain of cI. Dimerization of PKR reconstitutes the DNA-binding activity of λN fusion, leading to repression of the λ P~R~ promoter that can be scored by the resistance of the *E. coli* cells to lysis by the λ phage (10, 36). As summarized in Fig. 6B, we found that co-expressing full-length GST-PP1C or GST-PP1C2, but not GST or GST-Tat, blocked dimerization by the λN-PKR K296R fusion. Interestingly, a GST fusion containing the C-terminal 161 residues of PP1C, despite retaining the ability to bind PKR (Fig. 6A), did not block PKR dimerization in this assay. Taken together, these results suggest that PP1C does not inhibit PKR by merely binding to the kinase and that the catalytic domain of PP1C is required to disrupt the dimerization process of PKR.

**DISCUSSION**

Compared with the widely studied mechanisms for the phosphorylation and activation of PKR, the mechanisms underlying the inactivation of the enzyme are largely uncharacterized. Although viral studies have led to the identification of many virus-encoded or -directed factors that bind PKR or inhibit its activity (4), little progress has made in understanding the mechanisms that normally control PKR activity during cellular homeostasis. Identification of protein phosphatases that reverse the activating phosphorylation of PKR will provide a better understanding of its regulation and function. Previous two-hybrid screens for PKR-interacting proteins have yielded only activators or substrates of PKR (47, 52–54). We thus chose to use a functional screen to identify novel cellular PKR antagonists. Expression of PKR in yeast inhibits growth by phosphorylating eIF2α, which leads to the disruption of the cellular translational apparatus (20). This PKR-mediated toxicity can be partially reversed by the co-expression of viral protein inhibitors (39, 41, 45). Using this functional assay, we screened a human cDNA expression library for clones capable of counteracting the growth-suppressive effect of PKR, which led to the identification of PP1C (Fig. 1).

Both PP1 and PP2 are capable of dephosphorylating eIF2α kinases (33, 34). However, the exact protein phosphatase for PKR has not been identified, nor is the mechanism of action known. In this report, we present evidence that PP1C is a bona fide antagonist of PKR. Co-expression of PP1C leads to reduced phosphorylation of PKR and its physiological substrate, eIF2α (Figs. 2 and 3). The interaction of PP1C with PKR appears to be specific and functional, because the dephosphorylation of PKR *in vitro* by purified PP1C was concentration-dependent and could be blocked by a PP1 inhibitor. Furthermore, an *in vitro* binding assay, the two-hybrid system, and co-immunoprecipitation experiments collectively demonstrated a specific and direct interaction between PP1C and PKR (Figs. 4 and 5). The finding that PKR phosphorylation may be transiently modulated by PP1C is consistent with the proposed mechanism of action of viral RNA inhibitors of PKR (55). The RNA binding affinity of PKR is regulated by its phosphorylation states; autophosphorylated PKR molecules display low affinity for RNA and high eIF2α kinase activity. Thus, viral RNA inhibitors would not be able to effectively inhibit autophosphorylated PKR unless the kinase is dephosphorylated by a cellular phosphatase(s). It remains to be seen whether viruses have evolved a mechanism to activate PP1C, or recruit a PP1C-like phosphatase to dephosphorylate PKR, resulting in PKR forms that are susceptible to viral RNA-mediated inhibition.

To begin to delineate the mechanisms of PP1C action, we...
performed deletion analysis and found that PP1\textsubscript{C} bound to the N-terminal regulatory region of PKR independently of the dsRNA-binding capability of the kinase (Fig. 5\textbf{B}). Importantly, we found that the N terminus of PKR contains a PP1\textsubscript{C}-binding motif, which is present in most PP1\textsubscript{C}-interacting proteins, and which was required for the interaction of PKR with PP1\textsubscript{C}. PKR is phosphorylated at multiple serine and threonine residues, including those in the N-terminal regulatory domain (56). While we do not know whether PP1\textsubscript{C} directly binds to those sites, there is increasing precedence for both kinases and phosphatases interacting with sites other than the phosphorylation sites in their substrates. We mapped the PKR-interacting region to the C-terminal non-catalytic region, which is conserved between PP1\textsubscript{C} and PP1\textsubscript{C2} isoforms. Consistent with this observation, we found that both PP1\textsubscript{C} and PP1\textsubscript{C2} were capable of disrupting PKR dimer formation in the \(\lambda\) repressor fusion assay (Fig. 6). Interestingly, a truncated PP1\textsubscript{C} lacking the catalytic domain, but retaining its ability to interact with PKR, did not prevent PKR dimerization. Based on these results, we propose that PP1\textsubscript{C} and PKR interact directly through their respective non-catalytic regions. However, the catalytic domain of PP1\textsubscript{C} is required to dephosphorylate PKR, resulting in monomeric PKR forms due to their higher affinities for RNA (55). Alternatively, PP1\textsubscript{C}-mediated dephosphorylation of PKR may produce a protein conformation that is unable to dimerize independently of RNA binding. These complex interactions should be more apparent when a three-dimensional structure of the PKR-PP1\textsubscript{C} complex is solved.

PP1\textsubscript{C} modulates an enormous variety of cellular functions and normally exists as a heterodimer consisting of a core catalytic subunit and one of a number of different regulatory subunits. It has been suggested that the substrate specificity of PP1\textsubscript{C} is dictated by the interaction of PP1\textsubscript{C} with different regulatory subunits, which may target the catalytic subunit to specific subcellular locations (57). Regulation of PP1\textsubscript{C} in response to extracellular and intracellular signals occurs mostly through changes in the levels, conformation, or phosphorylation status of targeting subunits. Most of these bind to a small hydrophobic groove on the surface of PP1\textsubscript{C} through a short conserved binding motif-the (R/K)(V/I/L)X(F/W/Y) motif, which is often preceded by further basic residues, although several putative targeting subunits do not possess an (R/K)(V/I/L)X(F/W/Y) motif but nevertheless interact with the same region of PP1\textsubscript{C}. In this regard, the herpes simplex virus type 1 (HSV-1)-encoded 34.5 protein contains such a motif, which interacts with PP1\textsubscript{C} to redirect the phosphatase to dephosphorylate eIF2\(\alpha\) (44, 46). Selective dephosphorylation of eIF2\(\alpha\) may be a clever strategy used by HSV-1 to circumvent the PKR-induced shut-off of protein synthesis while maintaining PKR activity for other biological functions that are essential to the viral life cycle. Here, we demonstrated that PKR also contains the (R/K)(V/I/L)X(F/W/Y) motif, which is required for its binding to PP1\textsubscript{C}. However, we cannot exclude the possibility that a cellular regulatory subunit mediates PP1\textsubscript{C} specificity toward PKR. One candidate is the glycogen-targeting subunit of PP1, termed PP1\textsubscript{GL} (10). PP1\textsubscript{GL}, which is expressed in heart and skeletal muscle, plays a pivotal role in rat skeletal muscle cell myogenesis via its regulation of PP1\textsubscript{C} activity (58). PKR also plays an important regulatory role in murine myogenic processes (59, 60), prompting the speculation of a possible localized role for PKR in skeletal muscle via its association with PP1\textsubscript{C} and PP1\textsubscript{GL}. Finally, PP1\textsubscript{C}-catalyzed dephosphorylation of PKR may be implicated in insulin signaling; both PP1\textsubscript{C} activity (61) and PP1\textsubscript{GL} phosphorylation (62) are stimulated by insulin. Interestingly, insulin induces a decrease in eIF2\(\alpha\) phosphorylation (62) are stimulated by insulin. Interestingly, insulin induces a decrease in eIF2\(\alpha\) phosphorylation in chondrocytes (63), although it is not known whether this decrease is an insulin-mediated increase in PP1\textsubscript{C} activity toward PKR and/or eIF2\(\alpha\). Our findings suggest an updated model for PKR regulation within and outside the context of virus infection (Fig. 7). This model should provide the basis for future studies to examine whether a regulatory subunit is involved in PKR interaction with and/or inhibition of PKR under specific conditions. Such studies may begin to ascribe the consequences of the PP1\textsubscript{C} dephosphorylation of PKR to specific biological effects.

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**Fig. 7. Model for PKR regulation by PP1.** A, antiviral and antiproliferative effects of PKR resulting from eIF2\(\alpha\) phosphorylation. B, neutralization of PKR-mediated effects by direct dephosphorylation and monomerization of PKR by PP1\textsubscript{C}, during normal cell physiology or by PP1\textsubscript{C}-mediated eIF2\(\alpha\) dephosphorylation during HSV-1 infection. It is not clear why HSV-1 does not target PKR directly, but it appears that the virus also encodes two additional gene products, US11 and US12, to deliberately activate PKR while encoding a separate function that selectively prevents the phosphorylation of eIF2\(\alpha\). Presumably, this represents a mechanism by which the virus maintains other biological functions of PKR, such as cell differentiation or apoptosis, that are important during different stages of the viral life cycle. See “Discussion” for details.
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