trans-Autophosphorylation by the Isolated Kinase Domain Is Not Sufficient for Dimerization or Activation of the dsRNA-Activated Protein Kinase PKR†

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Received November 11, 2003; Revised Manuscript Received June 3, 2004

ABSTRACT: The double-stranded (ds) RNA-activated protein kinase PKR phosphorylates the α-subunit of the eukaryotic initiation factor 2 (eIF2α) and inhibits translation initiation. PKR contains two dsRNA binding domains in its amino terminus and a kinase domain in its carboxy terminus. dsRNA binding activates PKR from a latent state by inducing dimerization and *trans*-autophosphorylation. Recent studies show that PKR is also activated by caspase cleavage to remove the inhibitory dsRNA binding domains. In this report, we show that the isolated kinase domain of PKR is a constitutively active monomeric kinase that has an activity similar to that of wild-type PKR. We used a solid-phase kinase assay system to show that PKR does not transfer its own phosphate to either PKR or eIF2α but rather uses the γ-phosphate from ATP. In addition, the isolated autophosphorylated kinase domain of PKR phosphorylated intact monomeric PKR in trans in a reaction that did not require dsRNA binding. However, this *trans*-phosphorylation did not occur at the critical Thr446/451 sites and was not sufficient to induce dimerization and/or activation of PKR. The results show that dsRNA binding domains of PKR are not only required for dimerization of PKR but also required for phosphorylation of Thr446/451 sites of PKR. The results imply that even though the isolated kinase domain of PKR phosphorylates intact PKR and eIF2α, it is unable to activate PKR.

The interferon-inducible, double-stranded (ds) RNA-activated protein kinase (PKR) regulates protein synthesis initiation by phosphorylating the α-subunit of eukaryotic initiation factor 2 (eIF2α) at serine residue 51 (*1*). PKR activation and subsequent eIF2α phosphorylation are the primary mechanisms that limit viral replication as part of the interferon antiviral response (*2*). Recently, it has become evident that PKR also plays a critical role in regulation of cell growth (*3*, *4*), the innate immune response (*5*−*8*), dsRNA-dependent transcriptional regulation (*3*, *9*−*12*), induction of cell apoptosis (*12*−*16*), and suppression of cell transformation (*4*, *17*).

PKR is constitutively expressed in most mammalian cells and is induced by type I interferons (α and β). PKR contains two conserved dsRNA binding motifs in its amino terminus and a serine/threonine kinase catalytic domain in its carboxy terminus. PKR is synthesized in a latent form and requires activation that may occur through two independent mechanisms. First, dsRNA binding to PKR induces dimerization with subsequent *trans*-autophosphorylation and activation of the eIF2α kinase activity (*18*−*22*). The structural requirements for the intermolecular interaction between dsRNA and PKR have been extensively studied (*21*−*27*). Mutation of the conserved lysine 64 to glutamic acid (K64E) within the first dsRNA binding motif significantly reduces dsRNA binding to less than 5% (*27*). However, the precise mechanism for dsRNA-induced PKR activation remains in question. PKR autophosphorylation is believed to convert the protein into an active conformation by relieving inhibition from an amino-terminal negative regulatory domain (*22*, *28*, *29*). Although reports support that dimerization is mediated by bridging through a dsRNA molecule (*30*), there is evidence that dimerization is mediated by direct protein−protein interactions between dsRNA binding domains (*19*, *31*). Second, in response to apoptosis, activated caspase-3, caspase-7, or caspase-8 can cleave at Asp251 and activate PKR by removing the inhibitory amino-terminal dsRNA binding domains (*32*). Although the isolated kinase domain, KD (residues 258−551), did not efficiently phosphorylate eIF2α in vivo (*29*, *33*), when this KD was extended 7−30 amino acids (residues 228−251), it efficiently phosphorylated eIF2α and inhibited protein synthesis in vivo (*20*, *32*). In addition, the extended KD (228−251) was autophosphorylated and subsequently *trans*-phosphorylated intact PKR and eIF2α in vitro (*22*, *34*).

Upon activation, multiple sites in PKR become phosphorylated (*35*, *36*). Among these sites, the phosphorylation at Thr446 and Thr451 in the PKR activation loop is required for high level PKR activity (*30*). It was shown that PKR autophosphorylation may occur in trans (*18*). Activated PKR subsequently catalyzes phosphorylation of eIF2α at a single

† Portions of this work were supported by NIH Grants RO1 AI42394 (R.J.K.)and RO1 CA86926 (S.W.).

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10.1021/bi0360105 CCC: $27.50
Published on Web 08/05/2004

serine residue, Ser51 (*37*−*40*). To further elucidate the mechanism of PKR *trans*-phosphorylation and PKR-catalyzed phosphorylation of eIF2α, we established an in vitro solid-phase kinase assay system. First, the kinase domain of PKR and various substrates of PKR were overexpressed in COS-1 cells by transient DNA transfection. The overexpressed proteins were immunoadsorbed to antibodies and then immobilized onto protein A−Sepharose. The immobilized kinase was tested for its ability to utilize different substrates in order to elucidate the requirements for dsRNA binding and *trans*-autophosphorylation in PKR activation. Our results support that dsRNA binding to substrate is not required for intact PKR to be *trans*-phosphorylated and that activated PKR can phosphorylate nonactivated PKR. However, phosphorylation in the absence of dsRNA does not occur at the critical Thr446/451 sites and does not induce PKR dimerization or activation. This phosphorylated intact PKR requires binding to dsRNA for activation and phosphorylation of eIF2α.

MATERIALS AND METHODS

Expression Vectors. The expression plasmid pETFVA$^-$ was previously described (*41*). It contains a transcription unit utilizing the adenovirus major late promoter and simian virus 40 (SV40) enhancer element. In addition, the vectors contain the SV40 origin for replication in COS-1 cells. Where indicated, the bacteriophage T7 epitope tag was placed at the carboxy terminus of the protein. The K296P expression vector pETFVA$^-$-K296P-T7, the dsRNA binding domain mutant K64E expression vector pETFVA$^-$-K64E-T7, the double mutant K64E/K296P expression vector pETFVA$^-$-K64E/K296P-T7, the PKR kinase domain (KD, amino acid residues 228−551) expression vector pETFVA$^-$-KD-T7, and the expression vector for dsRNA binding domain expression vector (BD, amino acid residues 1−243) were described previously (*21*) and are depicted in Figure 1A. The eIF2α expression vectors pETFVA$^-$-eIF2α and pETFVA$^-$-eIF2α-S51A were also described previously (*39*).

DNA Transfection and Solid-Phase Protein Preparation. COS-1 monkey kidney cells were transfected by the DEAE-dextran method (*21*). For each 100 mm plate, 4 μg of plasmid DNA was used in a volume of 4 mL of transfection medium. After 48 h posttransfection, cell extracts were prepared by harvesting the cells in 500 μL of kinase binding buffer [20 mM HEPES, pH 7.5, 300 mM NaCl, 5 mM $Mg(OAc)_2$, 1 mM DTT, and 10% glycerol] containing 0.5% NP-40. The nuclei and cellular debris were removed by centrifugation at 5000 rpm at 4 °C in a microcentrifuge apparatus. The overexpressed KD-T7, K296P-T7, K64E/K296P-T7, eIF2α, and eIF2α51A were incubated with anti-T7 antibody or anti-eIF2α antibody, and the antigen−antibody complexes were adsorbed with protein A−agarose.

Co-immunoprecipitation of the dsRNA Binding Domain (BD-T7) with PKR Mutants. At 48 h after transfection, cells were labeled with [^{35}S]methionine/cysteine protein labeling mix (100 μCi/mL, 1000 Ci/mmol; Amersham Co., Arlington Heights, IL) for 20 min in methionine/cysteine-free minimal essential medium (Life Technologies, Gaithersburg, MD). Cell extracts were prepared by lysis in kinase binding buffer containing 0.5% NP-40 (*21*), and the expressed proteins were immunoprecipitated using anti-T7-tag monoclonal antibody

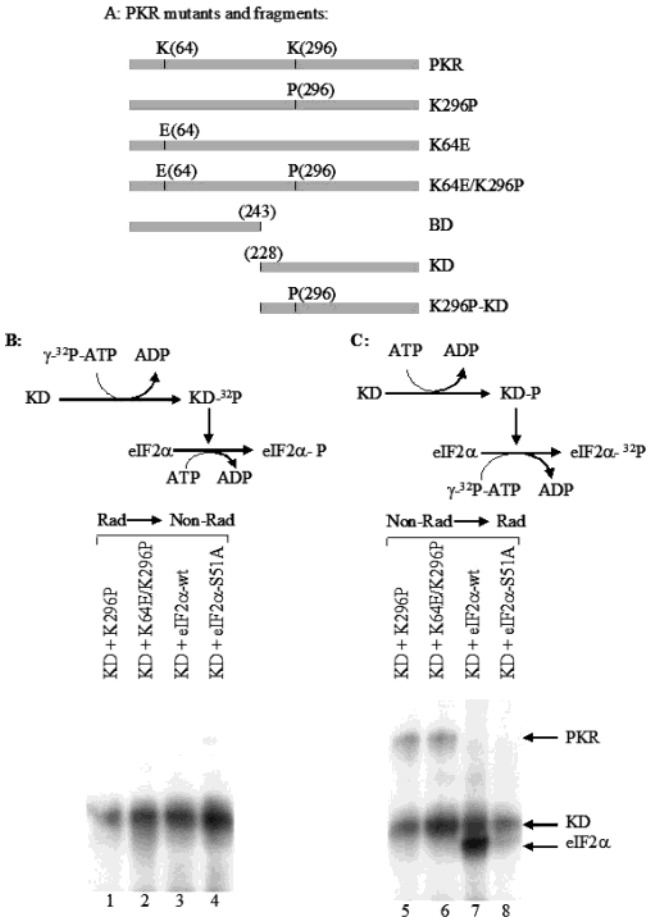

FIGURE 1: PKR utilizes free ATP to phosphorylate substrates. Panel A shows the constructs of PKR mutants and fragments used in these experiments. COS-1 monkey kidney cells were transfected and harvested as described in Materials and Methods. The overexpressed T7-tagged KD (lanes 1−8), K296P (lanes 1 and 5), or K64E/K296P (lanes 2 and 6) and nontagged eIF2α (lanes 3 and 7) or mutant S51A eIF2α (lanes 4 and 8) proteins were incubated with anti-T7 antibody or anti-eIF2α antibody, respectively, and the antigen−antibody complexes were adsorbed onto protein A−agarose. The immunoprecipitates were washed and assayed for in vitro kinase activity. The immunoprecipitated active kinase domain of PKR (KD) was first incubated with (panel B) or without (panel C) [γ-^{32}P]-ATP in the kinase assay buffer at 30 °C for 30 min. The agarose-bound kinase was washed and mixed with immunoprecipitated substrates as indicated. The kinase−substrate mixtures were incubated without (panel B) or with [γ-^{32}P]ATP (panel C) again in kinase assay buffer at 30 °C for 30 min. The samples were washed three times with kinase binding buffer, and protein phosphorylation was analyzed by SDS−PAGE followed by autoradiography.

(Novagen Corp., Madison, WI). Where indicated, the cell extracts were incubated at 30 °C for 30 min in the presence of poly(rI·C) (1 μg/mL; Pharmacia, Piscataway, NJ). The immunoprecipitates were resolved by SDS−PAGE. The gel was then fixed in 30% methanol and 10% acetic acid, prepared for fluorography by treatment with En3Hance (New England Nuclear Corp., Boston, MA), and dried. Autoradiography was performed using Fuji Medical X-ray film (Fuji Medical Systems, Stamford, CT).

In Vitro Kinase Assay. The immunoprecipitates were washed twice with kinase binding buffer (22) and once with a kinase assay buffer [20 mM HEPES, pH 7.5, 50 mM KCl, 1.5 mM DTT, 2 mM $Mg(OAc)_2$, 2 mM $MnCl_2$, and 0.1 mM ATP] and assayed for in vitro kinase activity. The immunoprecipitated kinase domain of PKR (KD) was first

incubated with (Figure 1B) or without (Figure 1C) [γ-^{32}P]-ATP in kinase assay buffer at 30 °C for 30 min. Then the agarose-bound kinase was washed twice with kinase assay buffer and mixed with immunoprecipitated substrates as indicated. The kinase−substrate mixtures were incubated in the presence (Figure 1C) or absence (Figure 1B) of [γ-^{32}P]-ATP in kinase assay buffer. The in vitro kinase assay was performed in 25 μL of kinase assay buffer with 5 μCi of [γ-^{32}P]ATP at 30 °C for 30 min. The samples were analyzed by SDS−PAGE followed by autoradiography with Fuji Medical X-ray film (Fuji Medical Systems).

K64E mutant PKR was immunoprecipitated with anti-PKR(BD) antibody and used to phosphorylate eIF2α. The immunoprecipitated K64E PKR was incubated with cell extracts prepared from COS-1 cells transfected with vector, K296P-KD, or KD in the kinase assay buffer containing [γ-^{32}P]ATP at 30 °C for 30 min. The immunoprecipitates were then washed twice with kinase binding buffer and once with the kinase assay buffer. The phosphorylated K64E PKR immunoprecipitates were incubated with immunoprecipitated eIF2α in the kinase assay buffer supplied with [γ-^{32}P]ATP at 30 °C for 30 min. After three washes with the kinase binding buffer, the samples were subjected to SDS−PAGE and electroblotted to nitrocellulose membrane. The amounts of precipitated eIF2α and K64E PKR were measured by Western blot analysis. The incorporation of ^{32}P into eIF2α and K64E PKR was detected by autoradiography.

In Vivo Kinase Activity Assay. COS-1 cells were transfected with pETFVA$^-$-2α in the presence of pETFVA$^-$ vector, pETFVA$^-$-PKR-T7, and pETFVA$^-$-KD-T7 using the DEAE-dextran method. At 48 h posttransfection, the cells were harvested using NP-40 lysis buffer. The expression and phosphorylation levels of eIF2α were measured by Western blot analysis using antibodies against total eIF2α and phosphorylated eIF2α (Research Genetics, Inc., Huntsville, AL). In parallel, an identical set of transfected COS-1 cells was labeled with [^{35}S]methionine/cysteine (Amersham Co.) in methionine- and cysteine-free Dulbecco's modified essential medium (Life Technologies) for 2 h before being harvested with NP-40 lysis buffer. The expression levels of PKR-T7 and KD-T7 were determined by immunoprecipitation using antibody against the T7 epitope (Novagen Corp.).

Western Blot Analysis. Equal amounts of protein samples were subjected to SDS−PAGE and electroblotted to nitrocellulose membranes. The membranes were probed with antibodies as indicated in the figures and the legends. After extensive washing with Tris-buffered saline with Tween-20 (TBST), the membranes were incubated with HRP-conjugated secondary antibodies. Signals were detected using a SuperSignal chemiluminescent kit according to the manufacturer's procedure (Pierce, Rockford, IL).

RESULTS

Autophosphorylated KD Does Not Transfer Its Phosphoryl Residue to Its Substrate. An in vitro solid-phase kinase assay system was used to study phosphate transfer upon PKR-catalyzed phosphorylation of eIF2α. The T7-tagged isolated kinase domain of PKR (KD, residues 228−551) and its substrates were expressed in COS-1 monkey cells, immunoadsorbed onto antibodies, and immobilized onto protein A−agarose. First, we tested whether the isolated kinase domain (KD, residues 228−551) can transfer its own radioactively labeled phosphoryl residue to its substrate. The immobilized KD was activated by autophosphorylation with [γ-^{32}P]ATP and then used to *trans*-phosphorylate different substrates in the presence of unlabeled ATP (Figure 1B). The results demonstrated that although the activated KD was radioactively labeled, it did not transfer labeled phosphate to intact catalytically inactive K296P mutant PKR (K296P) (*21*, *22*), catalytically inactive and dsRNA-binding-defective double mutant K64E/K296P PKR, eIF2α wild type, or phosphorylation site mutant S51A eIF2α (Figure 1B). For control of this reaction, we analyzed the KD-catalyzed transfer of [γ-^{32}P]ATP to its substrates. The immobilized KD was activated with unlabeled ATP and then used to *trans*-phosphorylate immobilized PKR substrates in the presence of [γ-^{32}P]ATP (Figure 1C). Analysis of ^{32}P incorporation into KD demonstrated some autophosphorylation in the radiolabeled reaction, indicating that KD was not fully phosphorylated during the preincubation with nonradioactive ATP. The PKR substrates, the PKR mutants K296P and K64E/K296P, and wild-type eIF2α were all radioactively labeled under these conditions where S51A mutant eIF2α was not phosphorylated (Figure 1C). These data show that the phosphorylated KD *trans*-phosphorylates PKR and eIF2α using the γ-phosphate of ATP as the donor phosphate and support that activated KD does not transfer its own phosphate to its substrates. In addition, the PKR-mediated phosphorylation detected under these conditions is specific to the S51 residue of eIF2α (Figure 1C, lane 8 vs lane 7).

PKR KD Can Phosphorylate Intact PKR in the Absence of dsRNA Binding. The K64E/K296P double mutant PKR was used to study the requirement for dsRNA binding to intact PKR for the *trans*-phosphorylation reaction catalyzed by the isolated kinase domain KD. Using an in vitro solid-phase phosphorylation assay system, KD was able to *trans*-phosphorylate K296P PKR as well as K64E/K296P PKR (Figure 1C, lane 5 vs lane 6). To confirm that KD phosphorylates K296P PKR and K64E/K296P PKR in living cells, an in vivo phosphorylation assay was performed. PKR KD was cotransfected with PKR K296P or K64E/K296P expression plasmids, and phosphorylation of PKR was measured. The results show that overexpressed K296P PKR and K64E/K296P PKR were both phosphorylated by the expressed PKR KD in vivo (Figure 2A, lanes 2 and 3). In the absence of PKR cotransfection, very little phosphorylated PKR was detectable, as previously shown (*22*). Normalization for the amount of PKR protein (Figure 2B, lanes 5 and 6) demonstrates that PKR KD phosphorylates K64E/K296P PKR at a level similar to that of K296P PKR (Figure 2C). These results support that the dsRNA binding to substrate is not required for intact PKR to be *trans*-phosphorylated.

PKR KD Inefficiently Phosphorylates the Critical Thr446/451 Residues of PKR. Since phosphorylation of the Thr446/Thr451 residues in PKR is critical for its activation, we determined the ability of PKR KD to phosphorylate these residues during *cis*-autophosphorylation and *trans*-phosphorylation using Thr446/Thr451 phosphopeptide-specific antibodies. Upon expression in COS-1 cells, wild-type PKR inhibited its own expression so it was barely detectable by Western blot analysis (Figure 3 bottom, lane 2). However, it was possible to detect this wild-type PKR by reactivity with a Thr446/Thr451 phosphopeptide-specific PKR antibody

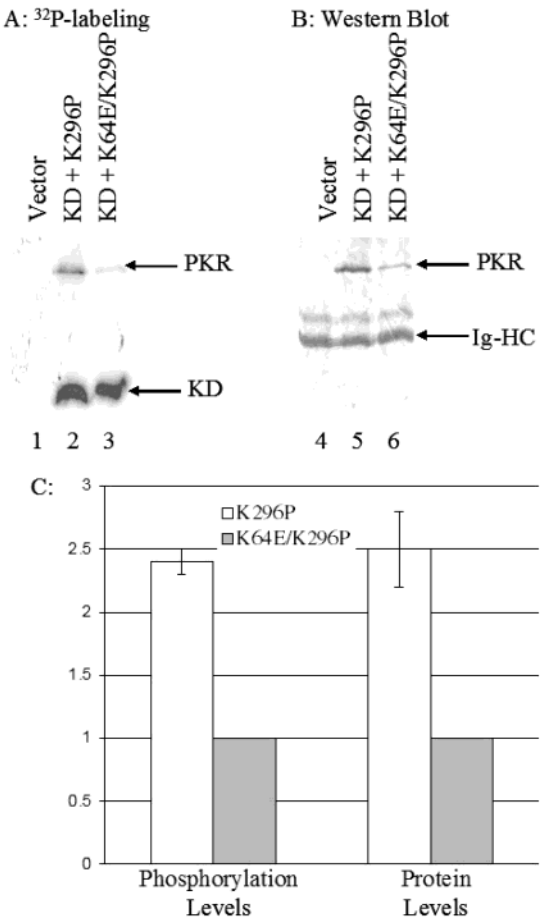

FIGURE 2: Catalytic site and dsRNA binding site PKR mutants are *trans*-phosphorylated by PKR. COS-1 cells were cotransfected with the T7-tagged KD expression vector pETFVA$^-$-KD-T7 in the presence of pETFVA$^-$-K296P-T7 (panels A and B, lanes 2 and 5) or pETFVA$^-$-K64E/K296P-T7 (panels A and B, lanes 3 and 6). Lanes 1 and 4 represent cells that received pETFVA$^-$ vector alone. After 48 h, cells were labeled with [^{32}P]phosphoric acid for 4 h and harvested using NP-40 lysis buffer. The labeled PKR mutant proteins were immunoprecipitated with anti-T7 monoclonal antibody (Novagen Corp.). The immunoprecipitates were subjected to SDS−PAGE and electroblotted onto nitrocellulose membrane. The membrane was autoradiographed to measure the incorporation of [^{32}P]PO_4 into the PKR mutant proteins (panel A). The amount of immunoprecipitated PKR protein on the membrane was detected by Western blot analysis using anti-PKR (BD) polyclonal antibody (panel B). The amount of protein expressed relative to the amount of ^{32}P incorporation is shown in panel C. The data represent the average of two independent experiments.

(Figure 3 top, lane 2). Therefore, wild-type PKR was significantly phosphorylated at the Thr446/Thr451 sites, as well as other sites that resulted in the detection of a doublet (Figure 3, lane 2). In contrast, although the K296P kinase-defective mutant PKR was expressed at a much greater level, it did not react with the Thr446/Thr451 phosphopeptide-specific antibody (Figure 3, lane 3). When KD was coexpressed with K296P mutant PKR, phosphorylation at K296P Thr446/Thr451 was also not detected in K296P PKR, although K296P PKR was expressed at a significant level (Figure 3, lanes 3 and 4). However, PKR KD did autophosphorylate the Thr446/Thr451 at a low level (Figure 3, lanes 4 and 6). The K64E/K296P double mutant PKR is a functionally disabled protein that has 95% reduced dsRNA binding and is unable to form dimers with activated PKR

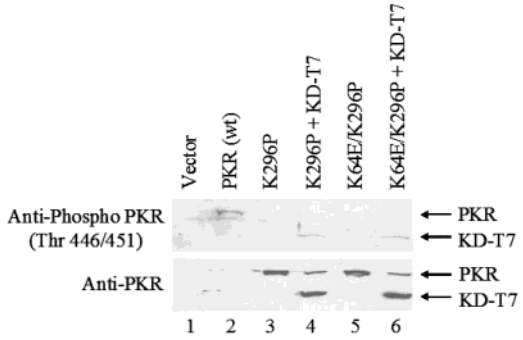

FIGURE 3: The critical Thr446/451 residues of PKR are inefficiently phosphorylated by PKR KD. COS-1 cells were transfected with vector alone, wild-type PKR, K296P PKR, or K64E/K296P PKR in the presence or absence of KD-T7 as indicated. At 48 h posttransfection, the cells were harvested for Western blot analysis using antibody against PKR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The phosphorylation of PKR on residues Thr446/451 was also detected by Western blot analysis using antibodies that specifically react with PKR phorphorylated at Thr446/451 (Cell Signaling Inc., Beverly, MA).

(*22*). Similar analysis with the K64E/K296P mutant PKR demonstrated that it was also not susceptible to phosphorylation at Thr446/Thr451 (Figure 3, lanes 5 and 6). These results show that although PKR KD is a functional kinase able to phosphorylate Ser51 of eIF2α, it does not efficiently phosphorylate the critical Thr446/Thr451 residues of PKR. Although it is also possible that the K296P mutant may disrupt the recognition of the Thr446/Thr451 sites, we believe this is unlikely since these domains are distant from each other within the related PKA protein kinase crystal structure (*42*). The results also support that removal of the dsRNA binding domain may partially expose the Thr446/Thr451 residues so they are phosphorylated inefficiently.

PKR Is More Active than PKR KD in Vivo. Since PKR KD did not significantly phosphorylate the critical Thr446/Thr451 residues of PKR, we measured the relative ability of PKR and PKR KD to phosphorylate eIF2α in vivo. Frequently, eIF2α kinase activity is measured by the ability of an eIF2α kinase to phosphorylate the eIF2α free subunit as opposed to the heterotrimeric eIF2 complex. Previous studies demonstrated that phosphorylation of the free eIF2α subunit directly reflects the phosphorylation status of eIF2α contained in the eIF2 heterotrimer (*40*). Because expression of wild-type PKR and PKR KD inhibits translation, the expression levels of PKR and PKR KD were too low to be detected by Western blot analysis. Therefore, we metabolically labeled the expressed kinases with [^{35}S]methionine/cysteine. As the half-life of PKR is short (our unpublished data), the newly synthesized labeled proteins replace all of the preexisting protein within 2 h and represent the steady-state level of the kinases. Immunoprecipitation with anti-T7 antibody detected approximately 3.7-fold greater levels of KD-T7 than PKR-T7, after correction for the Met and Cys contents (KD, 10, and PKR, 17) (Figure 4A). Expression of either PKR-T7 or KD-T7 reduced the expression of coexpressed eIF2α to similar extents. In addition, the percentages of phosphorylated eIF2α were similar in the presence of either PKR-T7 or KD-T7 (Figure 4B, lanes 2 and 3). The expression and phosphorylation levels of eIF2α, PKR-T7, and KD-T7 were determined using NIH image (Version 1.62, NIH). The kinase activities of PKR-T7 and KD-T7 were

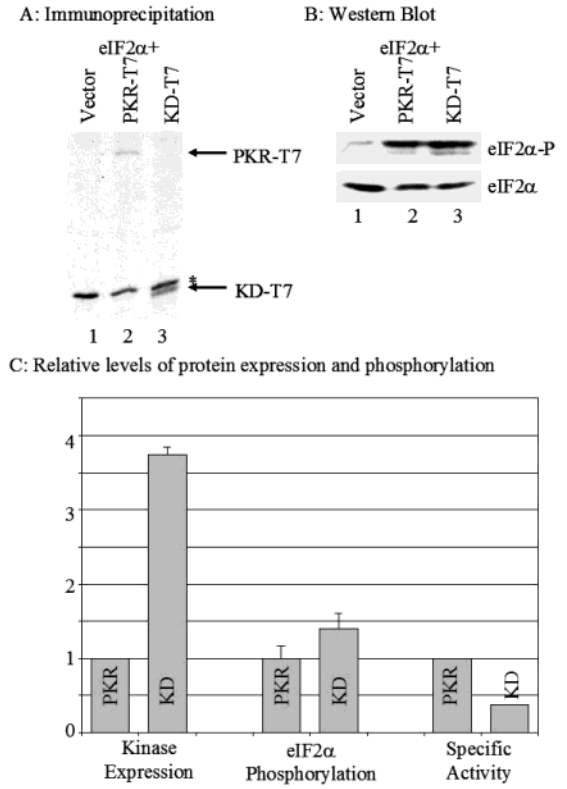

FIGURE 4: PKR KD is less active than wild-type PKR in vivo. COS-1 cells were cotransfected with the eIF2α expression vector (0.5 μg) and the indicated kinase expression vector (1 μg). After 48 h, the transfected COS-1 cells were pulse-labeled for 2 h with [^{35}S]methionine and cysteine. The expressed PKR-T7 and KD-T7 proteins were immunoprecipitated using anti-T7 antibody and resolved by SDS−PAGE. Gels were dried for autoradiography (panel A). A parallel set of transfected cells was harvested with NP-40 lysis buffer at 48 h posttransfection. Total cell extracts from eIF2α transfected COS-1 cells were resolved on 10% SDS−PAGE and electroblotted to nitrocellulose membranes. Western blot analysis was performed using antibody that reacts with total eIF2α and phosphorylated eIF2α (panel B). The relative activities of PKR and KD were calculated by dividing the normalized relative levels of phosphorylated eIF2α by the corresponding expression levels of PKR or KD (panel C). The data represent the average from two sets of measurements. The asterisk indicates a nonspecific band.

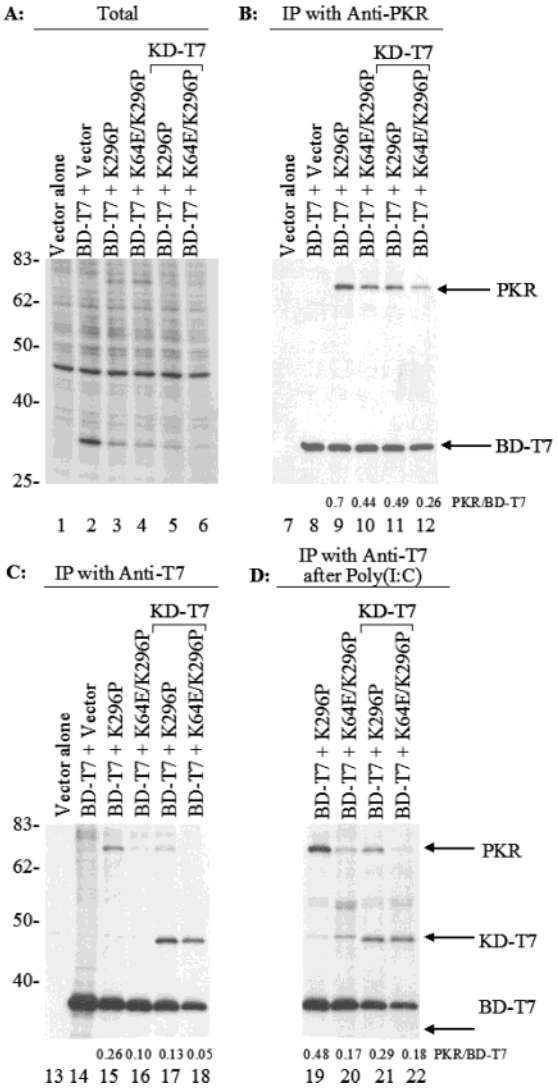

FIGURE 5: Phosphorylation of PKR is not sufficient for dimerization. The T7-epitope-tagged dsRNA binding domain (BD-T7) was cotransfected with untagged intact K296P PKR or double mutant K64E/K296P PKR (contained in the pETFVA$^-$ vector) in the absence or presence of the T7-tagged PKR kinase domain (KD-T7). After 48 h, cells were labeled with [^{35}S]methionine/cysteine in methionine- and cysteine-free minimal essential medium for 20 min. Cell extracts were prepared by lysis in the kinase binding buffer containing 0.5% NP-40 lysis buffer. The expressed proteins were immunoprecipitated with anti-PKR antibody (panel B) or anti-T7 antibody (panel C). The expressed proteins were also immunoprecipitated using anti-T7 monoclonal antibody after treatment with poly(rI•C) (1 μg/mL) at 30 °C for 30 min (panel D). The total cell extracts (panel A) and the immunoprecipitates (panels B−D) were resolved by SDS−PAGE. Gels were fixed and analyzed by fluorography. The heavy chain of immunoglobulin (Ig-HC) was pointed in panel A. The intensities of the bands were quantified by ImageJ (Version 1.31 for Mac OS X, NIH).

calculated by dividing the phosphorylation levels of eIF2α by the corresponding expression levels of kinase. Our data indicate that KD-T7 retains approximately 37% of the activity of wild-type PKR-T7. The superior specific activity of PKR-T7 could be due to the lack of autophosphorylation at the critical Thr446/Thr451 residues in KD-T7. It is also possible that the difference could be due to altered ribosomal association, as we have previously reported that both PKR dsRNA binding domains are required for ribosomal binding (*43*).

trans-Phosphorylation of PKR by PKR KD Is Not Sufficient To Induce Dimerization and/or Activation of PKR. We investigated whether *trans*-phosphorylation of PKR by PKR KD leads to dimerization of intact PKR in the absence of dsRNA binding. We previously described an immunoprecipitation assay to detect PKR dimerization that relied on one of the interacting partners being expressed with a bacteriophage T7 epitope tag at the carboxy terminus (*21*). K296P PKR or K64E/K296P PKR was cotransfected with the T7-tagged dsRNA binding domain comprising amino acid residues 1−243 (BD-T7) in the presence or absence of the T7-tagged PKR kinase domain (KD-T7). Analysis of the total cell extracts indicates that K296P, K64E/K296P, and BD-T7 were expressed in COS-1 cells at a higher level in the absence of KD-T7 (Figure 5A, lanes 3 and 4) compared to the presence of KD-T7 (Figure 5A, lanes 5 and 6). The low level expression of the proteins in the presence of the constitutively active KD is due to the inhibition of protein synthesis as previously described (*22*). Immunoprecipitation with anti-T7 epitope antibody detected the expected polypep-

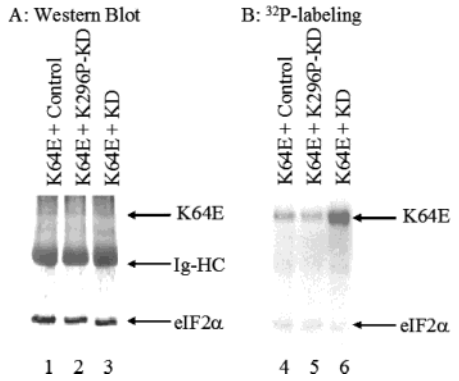

FIGURE 6: PKR phosphorylation is not sufficient for activation. Cell extracts were prepared from pETFVA$^-$-K64E-T7 or pETFVA$^-$-eIF2α transfected cells and incubated with anti-PKR antibody or anti-eIF2α antibody. The antigen−antibody complexes were adsorbed onto protein A−agarose. The precipitated K64E-T7 was incubated with cell extracts prepared from COS-1 cells transfected with pETFVA$^-$ (lanes 1 and 4), pETFVA$^-$-K296P-KD-T7 (lanes 2 and 5), and pETFVA$^-$-KD-T7 (lanes 3 and 6) in the kinase assay buffer containing [γ-^{32}P]ATP. After extensive washing, the immunoprecipitated K64E-T7 was incubated with immunoprecipitated eIF2α in the kinase assay buffer supplied with [γ-^{32}P]ATP. The amount of immunoprecipitated K64E and eIF2α was analyzed by Western blot analysis using anti-PKR and anti-eIF2α antibodies (panel A). The phosphorylation of K64E-T7 and eIF2α was detected by autoradiography (panel B).

tides at 33 kDa (BD-T7) (Figure 5C, lanes 14−18) and 42 kDa (KD-T7) (Figure 5C, lanes 17 and 18) as well as some co-immunoprecipitation of a 69 kDa polypeptide that represented K296P PKR (Figure 5C, lanes 15 and 17). In contrast, K64E/K296P did not coprecipitate with BD-T7 in either the absence or presence of KD-T7 (Figure 5C, lanes 16 and 18). Additionally, in contrast to the demonstration that dsRNA binding induced dimerization between K296P or K64E/K296P and BD-T7 (Figure 5D, lanes 19−22), phosphorylation of K296P did not increase the dimerization between K296P or K64E/K296P and BD-T7 (Figure 5C, lane 17−18 vs lane 15−16). The increased dimerization between K64E/K296P and BD-T7 after poly(rI·C) treatment may result from the residual dsRNA binding activity retained in K64E/K296P as discussed previously (*44*, *45*). Although we cannot rule out that very low affinity or transient dimerization may occur, the results support that *trans*-phosphorylation of PKR by PKR KD is not sufficient to induce significant dimerization in the absence of dsRNA.

To determine whether *trans*-phosphorylation of PKR induces PKR eIF2α kinase activity, we analyzed the ability of dsRNA-binding-defective phosphorylated PKR (K64E) (*44*) to phosphorylate eIF2α. Immobilized K64E PKR was incubated with KD-T7 or catalytically inactive mutant K296P KD in the presence of [γ-^{32}P]ATP. KD-T7 and K296P-KD-T7 were removed by extensive washing, and the remaining K64E PKR was analyzed for its ability to phosphorylate eIF2α in vitro. Western blot analysis demonstrated that similar amounts of K64E PKR and eIF2α were present in each eIF2α kinase reaction (Figure 6A). At a low level, K64E PKR was autophosphorylated and was able to phosphorylate eIF2α (Figure 6B, lanes 4 and 5). Incubation of K64E PKR with KD-T7, but not with control or K296P-KD, increased the autophosphorylation of K64E (Figure 6B, lane 6), although this did not increase the eIF2α kinase activity (Figure 6B, lane 6 vs lane 4−5). Thus, phosphorylation of K64E PKR by KD-T7 was not sufficient to activate the eIF2α kinase activity.

DISCUSSION

PKR exists in a latent form that requires exposure to an activator, such as dsRNA, to undergo dimerization and *trans*-autophosphorylation for activation to phosphorylate eIF2α (*1*). Although the roles of dsRNA binding and dimerization in activation of PKR eIF2α kinase activity have been analyzed (*22*), the role of *trans*-phosphorylation of PKR in the activation process is not known. We previously proposed a model for dsRNA-dependent dimerization and activation of PKR (*22*). Our model, which was supported by NMR spectroscopy (*28*, *46*), suggests that dsRNA binding to PKR induces a conformational change that allows PKR to undergo dimerization and autophosphorylation. In the studies presented here, we further elucidated the role of autophosphorylation in dimerization and activation of PKR. Although there are examples where phosphorylated kinases transfer their own phosphate to substrates (*47*, *48*), and it was previously suggested that PKR utilizes such a mechanism (*48*), our data demonstrate that activated PKR catalyzes substrate phosphorylation by the direct transfer of the γ-phosphate of ATP and not from phosphorylated PKR.

Deletion of the dsRNA binding domain from PKR yields a functional constitutive PKR kinase that has a reduced activity to wild-type PKR in vivo. Our data show that the isolated kinase domain (KD) can *trans*-phosphorylate wild-type PKR and eIF2α in the absence of dsRNA. The findings are consistent with the finding that fully phosphorylated PKR binds poorly to dsRNA, although it is an active eIF2α kinase (*49*, *50*). However, although the phosphorylation of eIF2α was specific for residue Ser51, PKR phosphorylation did not occur on the critical Thr446/451 residues. The PKR KD-mediated phosphorylation did not result from contaminating kinase(s) because (1) the activity was only observed in cells transfected with functional PKR KD and not catalytically inactive mutant PKR KD, (2) the activity was isolated by immunoadsorption of epitope-tagged PKR KD, (3) the immunoadsorbed kinase isolated from metabolically labeled cells was radiolabel pure upon analysis by SDS−PAGE and autoradiography, and (4) PKR KD phosphorylated wild-type eIF2α but did not phosphorylate the phosphorylation site mutant S51A eIF2α. In addition, there are no other kinases that are known to phosphorylate PKR.

Previous studies demonstrated that dsRNA binding induces a conformational change that is required for PKR dimerization (*20*, *28*, *46*). In this study, we tested whether phosphorylation induces a conformational change that leads to dimerization in the absence of dsRNA binding. We demonstrated that dsRNA binding is not required for intact PKR to be *trans*-phosphorylated and that PKR KD can phosphorylate nonactivated PKR (depicted in Figure 7). However, the *trans*-phosphorylation of PKR by PKR KD under these conditions did not induce PKR dimerization or activation. The results support that phosphorylation of Thr446/451 residues is required to induce dimerization and activation. The results support that each phosphorylated PKR molecule requires the binding of an activator, such as dsRNA, to induce kinase activity. This mechanism of activation eliminates the possibility that generation of PKR KD via caspase

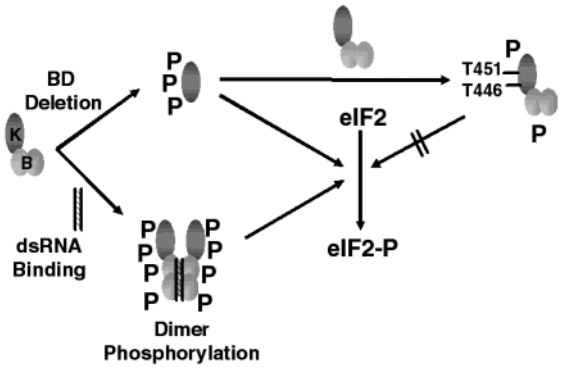

FIGURE 7: Two pathways exist for PKR activation. Two pathways for PKR activation are depicted. In the first pathway, dsRNA induces a conformational change to permit dimerization with subsequent *trans*-autophosphorylation and activation of eIF2α kinase activity. In the second pathway, activated PKR *trans*-phosphorylates PKR. However, this molecule cannot dimerize and/or phosphorylate eIF2α in the absence of dsRNA. K = kinase domain; B = dsRNA binding domains. See Discussion for details.

cleavage would initiate a chain reaction of PKR activation and eIF2α phosphorylation that would irreversibly shut down protein synthesis.

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BI0360105