Activation of Protein Kinase PKR Requires Dimerization-induced \textit{cis}-Phosphorylation within the Activation Loop\textsuperscript{*}\textsuperscript{[5]}

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\begin{abstract}
Protein kinase R (PKR) functions in a plethora of cellular processes, including viral and cellular stress responses, by phosphorylating the translation initiation factor eIF2\textalpha. The minimum requirements for PKR function are homodimerization of its kinase and RNA-binding domains, and autophosphorylation at the residue Thr-446 in a flexible loop called the activation loop. We investigated the interdependence between dimerization and Thr-446 autophosphorylation using the yeast \textit{Saccharomyces cerevisiae} model system. We showed that an engineered PKR that bypassed the need for Thr-446 autophosphorylation (PKR\textsuperscript{T446\textgamma-P}\textsubscript{bypass} mutant) could function without a key residue (Asp-266 or Tyr-323) that is essential for PKR dimerization, suggesting that dimerization precedes and stimulates autophosphorylation of PKR. We also showed that the PKR\textsuperscript{T446\textgamma-P}\textsubscript{bypass} mutant was able to phosphorylate eIF2\textalpha even without its RNA-binding domains. These two significant findings reveal that PKR dimerization and activation loop autophosphorylation are mutually exclusive yet independent processes. Also, we provide evidence that Thr-446 autophosphorylation during PKR activation occurs in a \textit{cis} mechanism following dimerization.

The kinase domain (KD)\textsuperscript{2} in most eukaryotic protein kinases remains in an inactive precursor state while associated with another domain in the same protein (intradomain) or with another partner protein (intersubunit)\textsuperscript{1}. Inactive-to-active transition of the KD requires binding of a specific modulator to the respective domain or subunit or phosphorylation by an upstream kinase, or both (2–5). The KD then adopts a core catalytic structure composed of a smaller N-terminal lobe (N-lobe) and the larger C-terminal lobe (C-lobe), creating an active site at the interface of these lobes (1). While adopting the core structure, an internal loop called the activation loop is often phosphorylated. The activation loop contains one or two regulatory phospho-acceptor residues that are phosphorylated by another kinase (\textit{trans}-phosphorylation) or by the kinase \textit{per se} (autophosphorylation). For example, the protein kinase PDK1 (phosphoinositide-dependent protein kinase 1) \textit{trans}-phosphorylates the activation loop of PKA (cyclic AMP-dependent protein kinase A) (6), whereas DYRK1 (dual specificity tyrosine-regulated kinase 1) autophosphorylates its own activation loop (7). However, the mechanism of activation loop phosphorylation is still not clear in many kinases.

Regardless of the mechanism of phosphorylation, the phosphorylated activation loop changes its conformation and bridges with both N- and C-lobes of the KD, thus adopting a catalytically competent conformation for substrate phosphorylation. Like a typical KD, the KD of PKR is activated by autophosphorylation in the activation loop (8–10). In PKR, two double-stranded RNA-binding domains (RBDs) at the N terminus serve as docking sites for virally produced dsRNA (11, 12) or PKR-activating protein PACT/RAX (13, 14). Binding of dsRNA or PACT/RAX to these RBDs releases the intramolecular auto-inhibition and brings two RBDs molecules together (15–19). Such effector-induced PKR dimerization promotes autophosphorylation at several residues, especially the residue Thr-446 (8, 20, 21). The phosphorylated KD then phosphorylates the translation initiation factor eIF2\textalpha (9, 22–24) or nuclear factors NF\textalpha1/2 (25) and elicits antiviral defense response, in part, by inhibiting protein synthesis (26), activating NF\kappaB (27), and/or enhancing interferon production (28). X-ray crystal structures have been resolved for the truncated PKR lacking the RBDs in apo form \textit{i.e.} K296R mutant and in a complex with eIF2\textalpha (29). Several mechanistic details of PKR activation have been inferred from these structures although both are inactive under physiological conditions (20). In both structures, the KDs exist as dimers in which protomers associate with each other in a back-to-back orientation so that the catalytic sites face outward. We analyzed these structures and observed that the overall architecture of the apo-KD dimer was similar to the KD dimer complexed with eIF2\textalpha except at the region of the activation loop. This loop in the eIF2\textalpha-bound form adopts an extended conformation with a phosphorylated residue Thr-446 (Fig. 1A), whereas it remains largely

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unstructured in the apo form (Fig. 1A). This structural difference indicates that a Thr-446 phosphorylation-dependent conformational transition must occur within the activation loop during PKR activation. Previously, we have shown that Thr-446 autophosphorylation is directly related to PKR activation and coupled with KD dimerization (20, 30). Such coupled processes lead us to propose three different possible models for Thr-446 autophosphorylation (20).

In the first model, the dimers phosphorylate activation loops on other dimers (trans-interdimer). Second, each protomer in a dimer phosphorylates its own activation loop (cis-intradimer). Third, one protomer in a dimer phosphorylates the activation loop of its partner (trans-intradimer). Several additional questions about the PKR activation mechanism remain unclear: what are the exact mechanisms and roles of Thr-446 autophosphorylation? How does dimerization promote Thr-446 autophosphorylation?

We showed that an engineered PKR that bypassed the need for Thr-446 autophosphorylation could function without a key residue (Asp-266 or Tyr-323) that is essentially required for the PKR dimerization; supporting the conclusion that dimerization precedes and stimulates activation loop autophosphorylation. Then, we showed that the Thr-446 phosphorylation occurs in a mechanism where each protomer within a dimer phosphorylates on its own (cis-intradimer).

Countermeasures

**Mechanism of Activation Loop Phosphorylation of PKR**

**Protein Expression, Purification, and Kinase Assay**—Flag-tagged PKR and its derivatives were introduced into the SS1A strain MY71. Yeast cells were then grown with appropriate nutrient in the presence of 10% galactose until the A$_{600}$ reached 4.0. Cells were harvested, and PKR protein was purified by ANTI-FLAG M2-agarose (Sigma) as described previously (9). The GST-elf2α (1–180) and GST-elf2α-S51A (1–180) fusion constructs were introduced in *Escherichia coli* BL21 (DE3) plus cells (NOVAGEN). A culture of these cells ($A_{600} = 0.5 – 0.8$) was induced with 0.5 mM IPTG (isopropyl-$eta$-thiogalactoside) for 6 h at 37 °C. Cells were collected, and the recombinant GST-elf2α fusion protein was purified by glutathione-agarose resin (Roche), using the standard manufacturer’s protocol. Phosphorylation of elf2α by PKR was performed in a kinase buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 25 mM MgCl$_2$, and 1 mM PMSF (phenylmethylsulfonyl fluoride) with 10 μCi of $[\gamma-3^3P]ATP$. Reactions were quenched after 20 min by addition of 2× SDS gel loading buffer, and the reaction products were separated by SDS-PAGE. The gel was stained with Coomassie blue, and the dried gel was subjected to autoradiography.

**Structure Analysis**—We analyzed protein structure using computer software PyMol (54).

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Condition**—Four yeast *Saccharomyces cerevisiae* strains were used in this study: 1) H2557: *MATa ura3-32 leu2-3 112 gen2Δ*, 2) H17 (elf2B desensitiv); *Matα gcn3-102 leu2-3 leu2-112 ura3-52*, 3) H1894: *MATa ura3-32 leu2-3 leu2-112 trp1-63 gen2Δ*, and 4) MY71 in which chromosomal *SUI2* gene in H2557 was replaced by a *SUI2-S51A* allele. These strains were obtained from Dr. Thomas E. Dever (NICHD, NIH). Synthetic dextrose (SD) minimal medium supplemented with appropriate nutrients was used to maintain the plasmids containing wild type PKR or its derivatives.

**Plasmids**—A wild-type (WT) Flag-(His)$_6$-tagged PKR was used (9). Mutations were introduced in the WT PKR gene by fusion PCR. WT PKR and its site-directed mutants were expressed from a galactose-inducible promoter built into the vector plasmid p1079 (pEMLYex4) or p2444 (pEMLYex4-based vector with a TRP selectable marker). The plasmids used in this report are listed in the supplement Table S1.

**Western Blot Analysis**—Yeast cells were grown in a synthetic dextrose (SD) medium supplemented with appropriate nutrients until the $A_{600}$ reached 0.6 – 0.7. Cells were collected and resuspended in 10% galactose (SGal) medium and allowed to grow for another 4 h at 30 °C. Whole cell extracts were prepared and subjected to Western blot analysis using an appropriate antibody. Four antibodies were used in these studies: 1) a phosphospecific antibody against elf2α phosphorylated on Ser-51 (gift from Dr. Thomas E. Dever, NICHD, NIH), 2) a Thr-446 phosphospecific antibody of PKR (Cell Signaling), 3) polyclonal antibody of total elf2α (gift from Dr. Thomas E. Dever, NICHD, NIH), and 4) polyclonal antibody of the C-terminal end of PKR (Cell Signaling).

**RESULTS**

**Functional Coupling between the Phosphorylated Activation Loop and the Helix-$\alpha$C of PKR**—To gain an insight into how PKR transitions from an inactive-to-active state, we analyzed two x-ray crystal structures of the PKR-KD: 1) one is complexed with elf2α (PDB ID: 2A1A) (29) and 2) the other is in the inactive apo form (K296S mutant, PDB ID: 3U1U). In both structures, each kinase domain is folded into a bi-lobal structure, and two kinase domains orient in parallel to form a back-to-back dimer. We observed that these two KD dimers superimpose well on each other (root mean square deviation = 1.54; supplemental Fig. S1), suggesting that the overall architecture of these proteins is similar. However, a prominent difference was observed in the region of activation loop (residues from Leu-435 to Lys-449) (Fig. 1A). In the apo-form, this loop remains largely unresolved and only three residues Val-Thr-Ser$_{436 - 438}$ have been resolved as a β-strand. In the PKR-KD bound to elf2α, the activation loop adopts an extended conformation with the residue Thr-446 being phosphorylated. These structural differences suggest that, even though the activation loop is unresolved in PDB 3U1U, the inactive-to-active transition likely requires a movement of the activation loop, as observed in numerous other protein kinases (2) so that the substrate, like elf2α, can bind and be phosphorylated.

The structural analyses further revealed that two helix-$\alpha$C residues Lys-304 and Arg-307 co-ordinate with the phosphate moiety of Thr-446 in the activation loop and the spatial positions of the helix-$\alpha$C element remain essentially unchanged in both structures (Fig. 1A). This indicates that the inactive-to-active catalytic transition might not require re-positioning of the helix-$\alpha$C, unlike what has been observed in most active protein kinase domains (3). However, a coupling between the helix-$\alpha$C and the phosphorylated activation loop might be crucial. To determine whether these prominent structural interactions between the helix-$\alpha$C and the phosphorylated activation
loop are likely to be physiologically relevant, we replaced residues Lys-304 with alanine and Arg-307 with alanine or lysine. The alanine substitution was expected to reduce the PKR function, whereas the more conservative lysine substitution might be able to retain the needed hydrogen bond.

We expressed the wild-type (WT) human PKR and its mutants K304A, R307A, and R307K in yeast Saccharomyces cerevisiae from a galactose-inducible promoter. WT PKR in yeast cells expressed poorly from a galactose-inducible promoter when grown on a synthetic dextrose (SD) medium (Fig. 1B). The poor PKR expression did not affect regular cell function and physiological processes, and a normal yeast growth phenotype was observed (Fig. 1B, SD). On a synthetic galactose medium (SGal), PKR expression was induced. This induction interfered with normal cell functions, resulting in a slow-growth phenotype (Slg−) in an eIF2B-dissociative strain (Fig. 1B, SGal, column 1) because PKR excessively phosphorylated eIF2α, leading to partial inhibition of the eIF2B function (20). However, expression of a catalytically inactive PKR-K296R mutant showed no growth defect (column 2). Whole cell extracts from those cells were subjected to Western blot analyses, which showed that both WT and K296R mutant proteins were expressed under inducible conditions (Fig. 1C, PKR). Phosphorylations were observed on the residues PKR-T446 (Fig. 1C, PKR−P lane) and eIF2α-Ser51 (eIF2α−P, lane 1) in cells expressing WT PKR, but not in cells expressing K296R mutant (PKR−P, lane 2 and eIF2α−P, lane 2). These data confirmed that the Slg− phenotype was correlated with eIF2α phosphorylation and that the K296R substitution blocks both PKR and eIF2α phosphorylation, restoring rapid yeast growth (see Refs. 8-10 for other PKR assays in yeast where PKR induction causes a lethal phenotype).

An alanine substitution of the phosphate acceptor residue Thr-446 suppressed the Slg− phenotype (Fig. 1B, SGal, T446A, column 6) and reduced eIF2α phosphorylation (Fig. 1C, T446A, lane 6), supporting our previous observation that residue Thr-446 is a physiologically relevant phosphorylation site. Like the T446A substitution, the alanine substitution of residue Arg-307 (R307A), which was designed to destroy the observed salt-bridge interaction between the helix-αC and the activation loop (Fig. 1A), suppressed the Slg− phenotype (Fig. 1B, SGal, R307A, column 3) and decreased both Thr-446 and eIF2α phosphorylations (Fig. 1C, eIF2α−P, lane 3). These data suggested that a productive interaction between the residue Arg-307 and the phosphorylated Thr-446 was required for PKR function. Unlike PKR-R307A, the PKR-R307K and PKR-K304A mutants retained the Slg− phenotype (Fig. 1B, SGal, columns 4 and 5), autophosphorylated on the residue Thr-446 (Fig. 1C, PKR−P, lanes 4 and 5) and phosphorylated eIF2α (Fig. 1C, eIF2α−P, lanes 4 and 5), indicating that the lysine substitution at position 307 enables formation of a salt-bridge with phosphorylated Thr-446, and that the interaction between residues Lys-304 and...
phosphorylated Thr-446 are not critical for PKR activity. Collectively, these structure-based mutational analyses reveal that a productive interaction between the Thr-446-phosphorylated activation loop and the helix-αC is important for the PKR catalytic function.

**Mechanism of Activation Loop Phosphorylation of PKR**

**Thr-446 Phosphorylation Occurs in a cis Mechanism following Dimerization**—To determine the mechanisms of Thr-446 autophosphorylation during the KD dimerization, we used a LIM-Ldb dimerization system (31). In this system, only the kinase domain of PKR was expressed in yeast cells as a monomer or forced to form a homodimer. The KD (residues 258 to 551) was fused to the *Xenopus* protein LIM domain (residues 1 to 58) and to LIM-domain binding protein Ldb (residues 290 to 350) creating LIM-PKRKD and Ldb-PKRKD fusion proteins, respectively. Yeast cells expressing either LIM-PKRKD (WT or D414A) and Ldb-PKRKD fusion protein were grown in the SGal medium and whole cell extracts were subjected to Western blot analysis using either a Thr-446-phosphospecific (T446P) or Ser-51 phosphospecific (eIF2α-P) antibody followed by a polyclonal antibody of PKR or eIF2α. C, models represent mono- or dimeric form of LIM- and Ldb-PKRKD fusion proteins. The bi-lobal PKR-KD is shown in gray whereas the activation loops in purple solid lines with a phosphorylated residue (red circle). The inactive form of the kinase domain is colored orange.

**FIGURE 2. Thr-446 phosphorylation occurs in a cis mechanism following dimerization. A**, heterologous dimerization domains activate the PKR KD. The LIM-PKRKD fusion gene in the plasmid pC901 with a URA3 selectable marker and the Ldb-PKRKD fusion gene in the plasmid pC903 with a TRP selectable marker were introduced into WT yeast strain H2557 as well as S51A strain. The sign (+) means the presence, whereas the sign (−) means the absence of pC901/pC903 plasmids but contains the respective vector plasmid. The indicated D414A mutation was introduced in to the LIM-PKRKD fusion construct. Transformants were tested for growth on SD and SGal media. The PKR was inactive when the yeast grew on SGal medium, but active if the yeast did not grow. **B**, analysis of Thr-446 autophosphorylation and eIF2α phosphorylation. Yeast strains expressing indicated LIM-PKRKD (WT or D414A) and Ldb-PKRKD fusion protein were grown in the SGal medium and whole cell extracts were subjected to Western blot analysis using either a Thr-446-phosphospecific (T446P) or Ser-51 phosphospecific (eIF2α-P) antibody followed by a polyclonal antibody of PKR or eIF2α. C, models represent mono- or dimeric form of LIM- and Ldb-PKRKD fusion proteins. The bi-lobal PKR-KD is shown in gray whereas the activation loops in purple solid lines with a phosphorylated residue (red circle). The inactive form of the kinase domain is colored orange.
were expressed together in a same cell (Fig. 2B, lane 4 and in the right panel, lane 2), suggesting that dimerization resulting from LIM-Ldb interaction (Fig. 2C) resulted in Thr-446 phosphorylation.

We propose three models that might explain coupling of PKR dimerization and Thr-446 phosphorylation: (I) trans-intradimer, (II) cis-intradimer, or (III) trans-interdimer. To test the first two models, we introduced a kinase-inactivating mutation in the LIM-PKRKD (D414A mutation in the conserved HRD motif, data not shown) and expressed it alone or co-expressed with the Ldb-PKRKD. Expression of LIM-PKRKD-D414A alone did not affect yeast cell growth on SD and SGal media (Fig. 2A, yeast growth, SD, and SGal, row 5), whereas co-expression with the Ldb-PKRKD fusion protein exhibited an Sg⁰ phenotype on SGal medium (Fig. 2A, rows 6), but not in the isogenic elf2α-SS1A strain (phosphorylation site Ser-51 mutated to an Ala, Fig. 2A, right panel), suggesting that the reduced growth was due to elf2α phosphorylation. These data suggested that the LIM-PKRKD-D414A derivative retained the ability to activate the partner domain, even though it lacked the kinase activity. The residue Thr-446 in LIM-PKRKD-D414A fusion protein (Fig. 2B, Western blots, right panel, LIM-PKRKD-D414A, lane 3) remained almost un-phosphorylated, ruling out the possibility of a trans-intradimer model of Thr-446 autophosphorylation. Phosphorylation of the residue Thr-446 in Ldb-PKRKD protein was observed when co-expressed with the LIM-PKRKD-D414A partner (Fig. 2B, Western blots, right panel, Ldb-PKRKD-D414A, compare lanes 1 and 3). Consistent with the Thr-446 autophosphorylation, elf2α was phosphorylated in yeast cells expressing LIM-PKRKD together with Ldb-PKRKD or Ldb-PKRKD-D414A (Fig. 2B, Western blots, right panel, elf2α-P, lanes 2 and 3). These results suggest that phosphorylation on residue Thr-446 in Ldb-PKRKD protein occurs in a cis mechanism following dimerization (see Fig. 2C).

Thr-446 Phosphorylation Does Not Occur in a trans Mechanism—To determine whether or not Thr-446 phosphorylation occurs in a trans-interdimer mechanism, a homodimeric GST-PKRKD fusion protein (a functional homodimer, see Ref. 31) was mixed with a kinase-dead PKR-K296R protein in a reaction buffer (Fig. 3A, Anti-PKR, 1×). Then, the phosphorylation status of the activation loops of both proteins was examined by Western blot analysis using a Thr-446 phosphospecific antibody of PKR (Fig. 3A, Anti-T446-P). The same experiment was repeated with 5-fold higher protein concentration to examine any positive effect on Thr-446 phosphorylation (Fig. 3A, Anti-PKR, 5×). The residue Thr-446 was phosphorylated in the GST-PKRKD fusion protein in the presence or absence of PKR-K296R protein (Fig. 3A, GST-PKRKD-P, lanes 1 and 3; and also in 5×, lanes 4 and 6); however, the residue Thr-446 was unphosphorylated in the PKR-K296R protein (lanes 2, and also in 5× lane 5) even when it was mixed with GST-PKRKD fusion protein (lane 3 and in 5× lane 6); arguing against the trans mechanism of Thr-446 phosphorylation.

To confirm these results under a more physiological cellular environment, we co-expressed the GST-PKRKD fusion protein and WT PKR or PKR-K296R mutant in yeast. Whole cell extracts were subjected to Western blot analysis using a Thr-446 phosphospecific antibody of PKR followed by an antibody against PKR. The residue Thr-446 in WT PKR (Fig. 3B, Anti-T446-P, lane 1) or GST-PKRKD fusion protein (lanes 1 and 2) was phosphorylated. However, the residue Thr-446 in the PKR-K296R protein (Fig. 3B, lane 2) was not phosphorylated. Expressions of both of these proteins (Fig. 3B, Anti-PKR) were nearly equal, indicating that the trans phosphorylation of the Thr-446 residue is not a likely mechanism.

A PKR<sup>trans</sup> Chimera Bypasses the Requirement for Activation Loop Phosphorylation—PKR activation requires both dimerization and Thr-446 autophosphorylation. To further understand whether these processes are dependent, independent, and interdependent, we engineered the PKR gene so that it could bypass the requirement for activation loop phosphorylation. Initially, two PKR mutants (PKR-T446D and PKR-T446E) were created with the expectation that aspartate/glutamate could function as a phospho-mimetic substitution. As shown in Fig. 4A, yeast cells expressing the PKR-T446D mutant (column 4), like PKR-K296R (column 2), and PKR-T446A mutants (column 3), grew well on SGal medium. Western blot analysis showed that phosphorylation of elf2α was substantially reduced in those cells (Fig. 4B, elf2α-P, lanes 2, 3, and 4), although mutant PKR proteins expressed comparatively better than WT (Fig. 4B, Western blots, compare lanes 1–4, PKR). These results indicated that the ability of PKR-T446D or PKR-T446E (data not shown) mutant to phosphorylate elf2α is substantially lower than WT PKR. Collectively, we conclude that phospho-mimetic aspartate or glutamate does not bypass the requirement for activation loop phosphorylation.
Mechanism of Activation Loop Phosphorylation of PKR

Then we attempted to replace the activation loop by a loop that would mimic the phosphorylation state. Thus, we performed a global search of kinase domain sequences and structures and identified kinases Phk1 (phosphorylase kinase 1) (32) and Chk1 (checkpoint kinase 1) (33) in which the activation loop residues (Fig. 5), we substituted each residue in PKR (PKRPHK1 chimera) by alanine and expressed these chimeras in yeast. Yeast cells expressing PKRPHK1 chimera, but not PKR (PKRCHK1 chimera) grew slowly like WT PKR on SGal medium (Fig. 4A, compare columns 1, 5, and 6). Western blot analysis showed that the slow-growth correlated with eIF2α phosphorylation (Fig. 4B, Western blots, eIF2α−P, lanes 5 and 6). These results indicated that activation loop residues of Phk1, but not of Chk1, functionally substitute the corresponding residues of PKR.

We replaced the activation loop residues of PKR by the respective residues from Phk1 or Chk1, generating PKRPHK1 and PKRCHK1 chimeras, and expressed these chimeras in yeast. Western blot analysis showed that the slow-growth correlated with eIF2α phosphorylation (Fig. 4B, Western blots, eIF2α−P, lanes 5 and 6). These results indicated that activation loop residues of Phk1, but not of Chk1, functionally substitute the corresponding residues of PKR.

FIGURE 4. Functional substitution of activation loop residues of PKR by residues from the activation loop of phosphorylase kinase 1 (Phk1). A, in vivo analysis of PKR mutants by growth in yeast. The yeast strain H17 (eIF2β desensitized) or MY71 (eIF2α−S51A) harboring indicated WT PKR and its derivatives were serially diluted and spotted on SD and SGal media. B, in vivo analysis of eIF2α phosphorylation by PKR mutants. Whole cell extracts were prepared from yeast cells indicated in A and subjected to Western blot analysis using phosphospecific antibodies against Ser-51 (eIF2α−P). The membrane was stripped and re-probed with a polyclonal antibody of eIF2α and PKR. C, in vitro analysis of eIF2α phosphorylation by PKR mutants. Purified PKR protein (WT, K296R, or PKRPHK1 chimera) was mixed with the recombinant eIF2α and [γ−32P]ATP in a reaction buffer for 10 min. The reaction products were then separated using SDS-PAGE. The gel was stained, dried, and subjected to autoradiography to monitor the incorporation of 32P in eIF2α proteins. D, catalytic function of PKRPHK1 chimera requires an active interaction between the activation loop and the helix-αC. Left panels, eIF2β desensitization strain H17 expressing indicated PKR mutants were tested for growth on SD and SGal media. Right panels, yeast cells were grown in the presence of galactose (10%) and harvested after 2 h. Whole cell extracts were then prepared and subjected to Western blot analyses using antibodies of phosphorylated eIF2α (eIF2α−P), eIF2α, and PKR.
PKRphk1 R307A (column 4) grew rapidly like the PKR-R307A mutant (column 3) on SGal medium. Western blot analyses showed that rapid growth correlated with reduced eIF2α phosphorylation (Fig. 4D, Western blots, compare lanes 1–4) though expression of these PKR derivatives was better than WT (Fig. 4D, Western blot, PKR, lanes 1–4). These results suggest that, in the PKRphk1 chimera, the positively charged residue Arg-307 in helix-αC and the phospho-mimetic glutamate in the activation loop (Glu-182 corresponding to Thr-446) play important roles in the PKR catalytic function. The residue Phk1-E182 aligns with the phosphorylated Thr-446 of PKR (Fig. 5); thus it appears that the residue Glu-182 in the PKRphk1 activation loop maintains the salt bridge interaction with the helix-αC.

Dimerization Precedes and Stimulates Activation Loop Phosphorylation—Though PKR has been reported to exist as a monomer (17, 34), numerous studies have challenged whether PKR functions as a monomeric protein. These studies include: 1) PKR forms a dimer at higher concentration (34) and 2) PKR exists as a monomer-dimer equilibrium that is enhanced by autophosphorylation (35). Furthermore, the crystal structure reveals that the PKR-KD forms a dimer by a back-to-back interaction between two N-lobes where several intra- and inter-molecular salt-bridges and hydrogen bonds stabilize the interaction. The prominent interactions include a salt-bridge between residues Arg-262 and Asp-266 and a hydrogen bond network between residues Asp-289, Tyr-293, and Tyr-323 (Fig. 6A).
have shown previously that mutations of residues Arg-262 and Asp-266 reduce the ability of PKR both to autophosphorylate Thr-446 and to phosphorylate eIF2α, whereas reciprocal exchange of these two residues restores both functions (30). We conclude that the KD dimerization is coupled with Thr-446 phosphorylation in the activation loop. But, it is still unclear how these processes are inter-related.

We used the PKRphk1 chimera to understand the importance of dimerization on Thr-446 autophosphorylation. We hypothesized that PKRphk1 should be non-functional or partially functional if the RBD-mediated KD dimerization was required for Thr-446 autophosphorylation. To test this, residues Asp-266 and Tyr-323 in the PKRphk1 chimera were individually substituted to Arg and Ala, generating PKRphk1D266R and PKRphk1Y323A mutants, respectively. We then expressed these PKR derivatives in yeast, tested for growth on SGal medium, and examined eIF2α phosphorylation. The results were then compared with cells expressing WT PKR and PKR mutant K296R, D266R, or Y323A (Fig. 6B).

As expected, expression of WT PKR exhibited a lethal phenotype (Fig. 6B, SGal, column 1). The lethality was suppressed in cells expressing PKR mutants K296R, D266R or Y323A (Fig. 6B, SGal, columns 2, 3, and 4). Whole cell extracts from these cells were subjected to Western blot analyses using antibodies against phosphorylated eIF2α (eIF2α—P) and eIF2α. Western blot showed that eIF2α was phosphorylated in cells expressing WT PKR (Fig. 7B, Western blots, eIF2α—P, column 1), but not in cells expressing K296R mutant (Fig. 6B, Western blots, eIF2α—P, column 2). Phosphorylation of eIF2α was substantially impaired in cells expressing PKR-D266R and PKR-Y323A mutants (Fig. 6B, Western blot, eIF2α—P, columns 3 and 4). These results together with our previous observation that the reduced eIF2α phosphorylation was correlated with the reduced Thr-446 phosphorylation (20) suggest that KD dimerization is required for the catalytic activation of PKR.

Like WT PKR, expression of PKRphk1 exhibited a lethal phenotype on SGal and resulted in excessive phosphorylation of eIF2α (Fig. 6B, Western blot, eIF2α—P, column 5). Expression of PKRphk1D266R or PKRphk1Y323A derivative increased the yeast growth (Fig. 6B, SGal, columns 6 and 7) when compared with PKRphk1 (column 5). The increased growth was correlated with a decreased eIF2α phosphorylation (Fig. 6B, Western blots, eIF2α—P, compare columns 5, 6, and 7), but increased eIF2α phosphorylation when compared with PKR-D266R or PKR-Y323A derivative (compare columns 3, 4, 6, and 7). These increased eIF2α phosphorylation indicated the PKRphk1D266R or PKRphk1Y323A became activated without key dimer interface residues. Collectively, these data suggest that the RBD-mediated dimerization was partially important for PKR activation, even though activation loop autophosphorylation was bypassed. Furthermore, these data suggest that PKR dimerization precedes and stimulates the activation loop phosphorylation.

The Kinase Domain of PKRphk1 Functions without RNA Binding Domains—Next, to test the hypothesis that the kinase domain of PKRphk1 should be functional without its N-terminal RNA binding domains, we expressed only the kinase domain of the PKRphk1 in yeast, tested cell growth on SGal medium and examined eIF2α phosphorylation in those cells. Expression of LIM-PKRphk1-KD fusion (Fig. 7, eIF2α—WT, lane 4), like PKRphk1 (lane 2), substantially reduced yeast growth on SGal medium. However, no growth defect was observed in an isoegenic S51A strain (Fig. 7, eIF2α—S51A), suggesting that growth reduction was due to eIF2α phosphorylation. As expected, expression of the PKR-KD only like a vector control (lane 1) showed no growth defect in both WT and S51A yeast strains (Fig. 7, lanes 1 and 4, see also Fig. 2A, column 2). As expected, Western blot analysis showed that PKRphk1 phosphorylated eIF2α (Fig. 7B, lane 2) and no phosphorylation of eIF2α was observed in yeast expressing PKR-KD (lane 1). Interestingly, we observed that the PKRphk1KD phosphorylated eIF2α (Fig. 7B, lane 3), suggesting that PKRphk1KD bypassed the requirement of absolute requirement for KD dimerization for its catalytic activity.

To test further that the PKRphk1KD actively phosphorylated eIF2α, yeast cell expressing PKR-KD or PKRphk1KD was transformed with a GCN4 LacZ reporter plasmid p180 (36). Transformants were grown till A600 reached ~0.6. Then, cells were harvested, resuspended on a medium containing 10% galactose,
and grown for another 6 h. Whole cell extracts were prepared, and β-gal activity was monitored (Fig. 7C). The LacZ expression was not induced in cells expressing PKR-KD, but was significantly induced in PKRphk1KD cell (5-fold), confirming that p53 induction was not induced in cells expressing PKR-KD, but was significant because the LIM and Ldb domains form a heterodimer protein. Consistently, we showed that the residue Thr-446 of LIM-PKRKD-D414A remained un-phosphorylated. Supporting this hypothesis, it was shown that the residue Thr-255, and Thr-258. Alanine mutation of these residues has no or minimum effect on the catalytic function of PKR (38), suggesting that those sites are not physiologically relevant phosphorylation sites. Therefore, we conclude that the in vitro PKR autophosphorylation reaction may not explain adequately the mechanism of Thr-446 phosphorylation.

In our earlier report (20), we ruled out that the cis-intradimer Thr-446 autophosphorylation is not a possible mechanism because the in vitro PKR autophosphorylation reaction shows second order kinetics with respect to PKR concentration (35, 37). The autophosphorylation reaction occurs at multiple residues other than Thr-446, including residues Ser-340, Ser-343, Ser-344, Ser-351, Ser-354, Ser-355, Ser-357, Thr-359, Ser-242, Thr-255, and Thr-258. Alanine mutation of these residues has no or minimum effect on the catalytic function of PKR (38), suggesting that those sites are not physiologically relevant phosphorylation sites. Therefore, we conclude that the in vitro PKR autophosphorylation reaction may not explain adequately the mechanism of Thr-446 phosphorylation.

Dar et al. reported that the PKR kinase domains form a back-to-back dimer (29). If this geometry needs to be maintained in an active PKR protein, the mechanism of trans-intradimer Thr-446 autophosphorylation is not a likely mechanism because a protomer in a dimer would not access the active site of the partner. Supporting this hypothesis, it was shown that the residue Thr-446 of LIM-PKRKD-D414A remained un-phosphorylated in an Ldb-LIM-PKRKD heterodimer (Fig. 2). However, the heterodimer was able to activate the catalytic function of its partner Ldb-PKRKD fusion protein while inducing Thr-446 phosphorylation (Fig. 2), fitting perfectly with the possibility

![Mechanism of Activation Loop Phosphorylation of PKR](image-url)
Mechanism of Activation Loop Phosphorylation of PKR

that Thr-446 phosphorylation occurs in a cis mechanism following dimerization.

Although the structure of the PKR holoenzyme is still lacking, it has been shown that the isolated RBDs forms a dumbbell shaped monomer in solution (39). It has also been shown that the RDB2 interacts with the C-lobe of PKR-KD (40). Together with these results, we propose that RDB2 remains bound to the inactive kinase domain until an effector molecule (dsRNA, PACT, or RAX) binds and stimulates its release (Fig. 8). The allosteric interaction between the RBDs and the dsRNA, PACT, or RAX favors a conformation that triggers Thr-446 autophosphorylation in a cis mechanism. Then, PKR autophosphorylates in trans at multiple residues as reported earlier (38) and becomes fully active. The active PKR induces antiviral defense responses and functions in regulatory pathways of a plethora of biological processes, including myogenesis (41), cell cycle progression (42), and inflammation (43).

PKR belongs to a family of eIF2α kinases that also contain GCN2, PERK, and HRI (44). Each of these kinases carries distinct regulatory domains that sense a specific cellular stress. HRI contains heme-binding domains that sense the intracellular heme deprivation (45), PERK contains a luminal domain that senses the accumulation of unfolded protein in the endoplasmic reticulum (46), and GCN2 contains a HisRS-like (histidyl-tRNA synthetase) domain that senses the metabolite stress (47). Upon sensing stress through the sensor domain, each one activates its kinase domain and phosphorylates eIF2α to down-regulate protein synthesis. Each of these kinases functions as a dimer, and is regulated by autophosphorylation at a conserved activation-loop mechanism (48). The allosteric interaction between the RBDs and the dsRNA, PACT, or RAX is mediated by a novel transitional intermediate domain that interacts with PIF and the C-terminal residues of PKR (49). These pseudokinases positively or negatively regulate the activity of the partner domain (50). For example, a pseudokinase domain functions of the partner domain (50). These pseudokinases positively or negatively regulate the activity of the partner domain (50). These pseudokinases positively or negatively regulate the activation mechanism. Then, PKR autophosphorylates in trans at multiple residues as reported earlier (38) and becomes fully active. The active PKR induces antiviral defense responses and functions in regulatory pathways of a plethora of biological processes, including myogenesis (41), cell cycle progression (42), and inflammation (43).

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