The Kinase Insert Domain of Interferon-induced Protein Kinase PKR Is Required for Activity but Not for Interaction with the Pseudosubstrate K3L*

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Interferon-induced protein kinase (PKR) is a member of a family of kinases that regulate translation initiation through phosphorylation of eukaryotic initiation factor 2α. In addition to the conserved catalytic subdomains that are present in all serine/threonine kinases, the eukaryotic initiation factor 2α kinases possess an insert region between catalytic subdomains IV and V that has been termed the kinase insert domain. To investigate the importance of the kinase insert domain of PKR, several deletions and point mutations were introduced within this domain and analyzed for kinase activity both in vitro and in vivo. Here we show that deletion of the kinase insert sequence or mutation of serine 355, which lies within this region, abrogates kinase activity. In addition, the kinase insert domain of PKR and adjacent amino acids (LFIQME) in catalytic subdomain V are not required for binding of the pseudosubstrate inhibitor K3L from vaccinia virus. A portion of the catalytic domain of PKR between amino acids 366 and 415 confers K3L binding in vivo, suggesting a possible role for this region of PKR in substrate interaction.

The double-stranded RNA (dsRNA)°-activated protein kinase, PKR (also called p68, DAI, and P1 kinase) is an interferon-inducible protein that plays a key role in the antiviral and antiproliferative interferon response (for a review see Ref. 1). PKR is a serine/threonine kinase that undergoes autophosphorylation upon binding to dsRNA activators, such as those generated upon viral infection or stem-loop structures in certain RNAs (e.g., Refs. 2 and 3). Upon autophosphorylation, PKR phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2) (2, 4). eIF2 is composed of three subunits (α, β, and γ) and forms a ternary complex (eIF2-Met-tRNA,GTP) that is required for binding of Met-tRNA, to the 40 S ribosomal subunit. Prior to the association of the 60 S ribosomal subunit, GTP hydrolysis results in formation of an inactive eIF2-GDP complex. Upon phosphorylation of eIF2α, the exchange of GDP for GTP is blocked due to inactivation of the guanine nucleotide exchange factor, eIF2B, resulting in the shut off of protein synthesis (5).

PKR plays a critical role in the establishment of the interferon-induced antiviral state (6). Many viruses have evolved strategies to counteract the inhibition of translation mediated by PKR (for review see Ref. 7). For instance, vaccinia virus encodes a protein called K3L, which shares extensive homology with the N terminus of eIF2α (8) and inhibits phosphorylation of eIF2α by PKR in vitro (9) and in vivo (10, 11). Taken together with the finding that K3L binds directly to PKR (9, 11, 12), these data suggest that K3L acts as a pseudosubstrate inhibitor of PKR.

In addition to its antiviral role, PKR plays an important role in the control of cell growth, inasmuch as expression of inactive mutants of PKR (PKRΔ6 (13), PKR-K296R (14), and PKR-M6, M7 (15)) causes the malignant transformation of NIH 3T3 cells, whereas expression of wild-type PKR in yeast causes a slow growth phenotype (16). Also, overexpression of a PKR inhibitor (p58) transforms NIH 3T3 cells (17). PKR mutants cause the malignant transformation of cells possibly through formation of inactive heterodimers with endogenous PKR (18), thus diminishing the levels of eIF2α phosphorylation. In support of this hypothesis, a recent study has shown that expression of the eIF2α subunit, in which the phosphorylation site residue (serine 51) was mutated to alanine, also resulted in malignant transformation of NIH 3T3 cells (19).

Several eIF2α kinases have been cloned: PKR (20, 21), a heme-sensitive kinase (named heme-regulated inhibitor) (22, 23), and a yeast kinase GCN2 (24). Although there is sequence similarity among the catalytic domains of the eIF2α kinases and other serine/threonine kinases, a unique feature that distinguishes eIF2α kinases from other kinases is the large spacer (28 amino acids) between catalytic subdomains IV and V, called the kinase insert domain (21).

In this study we examined the effects of mutations in the kinase insert domain on PKR activity in vitro and in vivo. Also, we tested mutants of PKR for interaction with the pseudosubstrate K3L in vitro and in vivo. Our findings indicate that the kinase insert domain is dispensable for K3L interaction, but it is required for kinase activity, suggesting that the kinase insert domain is not involved in binding of K3L. Mapping studies of K3L binding to PKR implicate a predicted α-helical sequence between amino acids 366 and 415 of the catalytic domain to be sufficient for the binding of K3L to PKR.

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† The abbreviations used are: dsRNA, double-stranded RNA; PKR, double-stranded RNA-dependent protein kinase; eIF, eukaryotic initiation factor; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

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MATERIALS AND METHODS

PKR Mutagenesis and Plasmid Construction—All mutagenesis was carried out by a two-step polymerase chain reaction approach (25). Two polymerase chain reaction products were digested with Msc I and A/FII and ligated into a similarly digested KS-PKR clone, which contained the entire cDNA from pCDNA1neo-p68 (26). PKRAK1 and PKRA330–336 mutants were generated to delete amino acid codons 330–355 and 330–336, respectively. Wild type PKR and mutants were subcloned into the mammalian expression vector pcR/CMV (Invitrogen) at unique HindIII/NotI sites. PKRΔ36 was subcloned into the yeast two-hybrid system vectors pcDP10 and pGAD.GH (gifts from P. Bartel, SUNY Stony Brook, and G. Hannon, Cold Spring Harbor, NY) as described previously (12). An EcoRI/BglII fragment encoding amino acids 366–415 of PKR was subcloned into pGBT9 digested with EcoRI and BamHI. The PKR constructs were also subcloned into the galactose-inducible yeast expression vector pDA1 (27) by digestion of KS-PKR plasmids with HindIII/SmaI and ligation to pDA1 digested with HindIII/PvuII.

For expression of an internal portion of PKR, the pET-HMK vector (a gift from M. Blanar, University of California, San Francisco, CA) was used to provide the initiation codon and 15 additional amino acids (31). The FlagPKR281–415 mutant was generated by digestion of KS-PKR with MscI/A/FII and pET-HMK with EcoRI, followed by blunt ending and ligation. This construct generates a fusion protein containing the "Flag" peptide sequence (31) followed by amino acids 281–415 of PKR. The stop codon is generated from vector sequences. A glutathione peptide sequence (31) followed by amino acids 281–415 of PKR. The fragment encoding amino acids 366–415 of PKR was subcloned into pETDuet-1 digested with EcoRI and BamHI. The PKR constructs were also subcloned into the galactose-inducible yeast expression vector pDA1 (27) by digestion of KS-PKR plasmids with HindIII/SmaI and ligation to pDA1 digested with HindIII/PvuII.

In Vitro Translation and Co-precipitatation—The FlagPKR281–415 mutant was expressed in BL21(DE3) following extensive washing with co-immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40), Laemmli buffer was added, and samples were subjected to SDS-10% PAGE followed by transfer to 0.45-

RESULTS

Mutagenesis of the Kinase Insert Domain of PKR—To understand the function of the kinase insert domain of PKR with respect to catalytic activity and possible substrate interactions, we designed a series of mutants (Fig. 1). The mutations included a large and small deletion (PKRAK1 and PKRA330–336, respectively) and point mutations in three residues (PKR-Y323F, PKR-S337A, and PKR-S355A) in the kinase insert domain, which are conserved in all eIF2α kinases (Fig. 1). The PKRAK1 mutant contains a deletion of amino acids 330–355 that removes the entire kinase insert domain. The PKRA330–336 mutant contains a deletion of a seven-amino acid sequence that is duplicated in the human PKR kinase insert sequence (21), whereas there is only one copy in the murine PKR sequence (21). In addition, we used the dominant negative mutant, PKRAΔ13, which harbors a deletion of amino acids 361–366. This sequence was proposed as a possible substrate interaction site because of the high sequence conservation among the eIF2α kinases (17). Also shown in Fig. 1 is the enzymatically inactive PKR-K296R mutant (14, 26), which harbors a mutation of the conserved lysine in catalytic subdomain II that is involved in the phosphotransfer reaction.

Expression and Characterization of PKR Mutants—To characterize the kinase insert domain mutants, COS-1 cells were transiently transfected with wild type and mutant PKR constructs, and PKR activity was assayed in vitro. Western blot analysis was performed on extracts from transfected cells with a monoclonal antibody that is specific for human PKR (13B8-F9; Ref. 29) at a dilution of 1:1000. The signal was revealed by ECL (Amersham Corp.). In Vitro Kinase Assay—The activity of the PKR proteins expressed in COS-1 cells was tested by immunoprecipitation with monoclonal antibody 71/10 (30), followed by a kinase assay in the presence of purified eIF2 (a generous gift of W. Merrick) and 1 μg/ml poly(rI)poly(C), as described previously (28).

In Vitro Translation and Co-precipitations—PKR plasmids were transcribed in vitro, and mRNAs were translated in rabbit reticulocyte lysate in the presence of [35S]methionine (ICN) as suggested by the manufacturer (Promega). PKR–1–415 and PKR–280 were generated by linearization of pcR/CMV-PKR wt DNA with A/FII and MscI, respectively. Glutathione S-transferase (GST) and the GST-K31 fusion protein were expressed in Escherichia coli and purified using glutathione-Sepharose beads (Pharmacia Biotech, Inc.). Translation products were incubated with GST or GST-K31 proteins (1 μg) for 60 min at 4 °C, and Glutathione-Sepharose beads were added for an additional 30 min. Following extensive washing with co-immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40), Laemmli buffer was added, and samples were subjected to SDS-10% PAGE. 35S-Labeled translation products were detected by autoradiography. The FlagPKR281–415 mutant was expressed in BL21(DE3) bacteria and subjected to co-precipitation with GST and GST-K31 proteins as described above. The precipitated products were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and Western blotted using an anti-Flag monoclonal antibody (M2; Eastman Kodak Co.). Proteins were detected by ECL (Amersham Corp.).

Luciferase Assay—Luciferase assays were performed according to the manufacturer's instructions (Promega) and analyzed using a BIOORBIT bioluminometer. To determine levels of luciferase mRNA, total RNA was prepared from duplicate transfections and harvested by the guanidinium/CsCl method as described (32). RNA was digested with RNase-free DNase I (Boehringer Mannheim) and analyzed using the RNase-One protection assay (as described in Ref. 19), according to the manufacturer's instructions (Promega).

Yeast Transformations—The yeast strain S150–2B (leu3-3,112, his3-11, trpl-1, ura3-52) was transformed with pDA1 constructs using the lithium acetate method (33) and plated on SD-agar plates supplemented with leucine, histidine, and tryptophan. Single colonies were picked and grown overnight in liquid SD medium containing the same amino acids. 20 μl of these cultures were streaked onto parallel SD-agar media containing either glucose or galactose as the source of carbon. Slow growth phenotype was monitored after 3 days. For the two-hybrid assay, the yeast strain Y526 was transformed with various combinations of pGBT9 and pGAD.GH constructs using the lithium acetate method (33) as described (12).
Fig. 1. Schematic diagram of PKR mutants. Wild type PKR is shown at the top. The hatched boxes represent the two N-terminal double-stranded RNA-binding domains, and the black boxes represent the 11 catalytic subdomains present within the C-terminal kinase domain (not to scale). Two previously characterized mutants are shown: PKR-K296R (in which the conserved lysine in subdomain II is mutated to arginine; Ref. 14) and PKRΔ6 (a six-amino acid, LFIQME, deletion in subdomain V; Ref. 13). Deletion and point mutations within the kinase insert region between subdomains IV and V are shown. The sequences of the insert domain from mouse and human PKR are shown, and the three residues mutated (Y323F, S337A, and S355A) are conserved in both sequences. In addition, a small deletion of seven amino acids (PKRΔ330–336) and a larger deletion of 26 amino acids (PKRΔKI) are shown.

The kinase activity of the PKRΔ330–336 mutant was assessed in a similar assay (Fig. 2C). In this experiment, extracts from IFN-induced HeLa cells showed strong autophosphorylation (marked by an open arrow) and eIF2α phosphorylation (marked by a closed arrow; Fig. 2C, lanes 1 and 2). No catalytic activity was obtained in COS cells transfected with the vector alone (lanes 3 and 4). The addition of dsRNA to immunoprecipitates of wild type PKR caused extensive eIF2α phosphorylation (lanes 5 and 6). Only slight eIF2α phosphorylation was detected for the PKRΔ330–336 mutant (lanes 7 and 8). The amount of PKRΔ330–336 protein was 10-fold higher than wild type PKR, yet eIF2α phosphorylation was only 10% of that for wild type PKR (compare lanes 6 and 8), indicating that this mutant retains only approximately 1% of the catalytic activity of wild type PKR. This suggests that this seven-amino acid sequence (amino acids 330–336), although duplicated in human PKR (Fig. 1), is very important for kinase activity.

Effects of PKR Mutants on Yeast Growth—The expression of wild type PKR in yeast causes growth suppression, which results from elevated eIF2α phosphorylation (17, 18, 39). To test for the effect of mutations in PKR on yeast growth, PKR mutants were expressed in yeast under a galactose-inducible promoter. As expected, all yeast transformants grew when plated on glucose-containing media (Fig. 3); the slower growth phenotype of the PKR-S337A transformant reflects a poor streak and is not observed in all experiments. Upon transfer to galactose-containing media, wild type PKR-expressing yeast showed the characteristic slow growth phenotype (17), while yeast transformed with the empty vector or the PKR-K296R mutant displayed normal growth. In accordance with the in vitro results, expression of PKRΔKI and PKR-S355A did not affect yeast growth, consistent with a lack of kinase activity. The PKRΔ330–336 mutant did not cause inhibition of yeast growth, suggesting that the very low level of kinase activity retained by this mutant was not sufficient for growth inhibition. Expression of PKRΔ6 and PKRΔ330–336 did cause growth suppression, indicating that this is not due to diminished amounts of protein, since both mutant proteins were detected by immunoprecipitation followed by Western blotting (data not shown).
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sion of PKR-Y323F and PKR-S337A mutants caused growth suppression similar to that observed for wild type PKR (Fig. 3). The lack of an effect on yeast growth for the inactive PKR mutants is not due to lack of expression, since the proteins were detected by Western blotting (data not shown). The in vivo results, together with the in vitro kinase assay data (Fig. 3B), indicate that deletion of the entire kinase insert domain or deletion of a seven-amino acid motif within this domain (PKRΔ330–336) results in inactivation of PKR. Mutation of serine 355 to alanine also caused a loss of kinase activity, raising the possibility that phosphorylation of this residue might be important for activation of PKR.

**Inactive PKR Mutants Stimulate Translation in Vivo**—To assess the effects of the PKR mutants on translation, the luciferase reporter gene was co-transfected with the PKR constructs into COS-1 cells. Luciferase activities were corrected for the amount of luciferase mRNA as measured by RNase protection (data not shown; less than 30% variability between PKR transfectants). Translation of luciferase mRNA was stimulated (between 2- and 6-fold) by the catalytically inactive PKR-K296R, PKRΔ6, and PKRΔKI mutants (Fig. 4). The PKR-K296R mutant had the largest effect (about 6-fold stimulation), which could be explained by the better expression of this mutant (Fig. 2A, lane 3). The kinase insert deletion mutant and the PKRΔ6 mutant in three different experiments caused a 3-fold stimulation of translation of the reporter mRNA (Fig. 4). The enhanced translation could be explained by dominant negative inhibition, or by sequestration of dsRNA activators from the endogenous kinase, which becomes activated upon transfection with plasmid DNA (28). A study of PKR function in yeast suggests that inactive PKR mutants generated by deletions are dominant alleles, while those containing point mutations are recessive (18). However, in this assay the largest effects were observed for the PKR-K296R and not the deletion mutants (PKRΔ6, PKRΔKI). This could be due to the much higher expression levels of the PKR-K296R protein, compared with PKRΔ6 and PKRΔKI proteins (Fig. 2A). Wild type PKR-expressing cells did not show a significant reduction in luciferase mRNA translation. This was also observed in previous studies using another reporter gene (35). This likely reflects the low level expression of wild type PKR in this expression system (see Fig. 2A).

**Effects of Mutations in the PKR Kinase Domain on Binding of the Pseudosubstrate K3L**—The substrate binding site of PKR has not been characterized, although some regions have been proposed, including the kinase insert domain (40, 41). We attempted to determine whether the kinase insert domain is the binding site of eIF2 by co-immunoprecipitation of in vitro translated PKR products with purified eIF2. However, an interaction between PKR and eIF2 was not detected (data not shown). This is consistent with previous studies in which the binding of eIF2 to PKR was suggested to be much weaker than the binding of PKR to the vaccinia virus K3L protein (11). The K3L protein has extensive homology to the N-terminal portion of eIF2α (8) and is thought to act as a pseudosubstrate inhibitor of PKR, since expression of K3L can inhibit phosphorylation of eIF2α by PKR in vitro (9) and in vivo (10, 11).

Since the K3L protein is a pseudosubstrate inhibitor of PKR, delineation of the K3L binding site may be helpful in identifying the substrate binding site within PKR. In particular, we wished to determine if the kinase insert domain constitutes the K3L binding site on PKR. PKR proteins were synthesized in vitro and incubated in the presence of either recombinant GST or a recombinant GST-K3L fusion protein. Glutathione-Sepharose beads were added to precipitate GST and GST-K3L proteins, and bound 35S-labeled PKR proteins were resolved by SDS-PAGE. Wild type PKR was expressed at lower levels than PKRΔ6 and PKRΔKI proteins (Fig. 5A, compare lane 1 with lanes 4 and 7; full-length product indicated by dot). As reported in earlier studies, a number of 35S-labeled smaller molecular weight products were detected (21, 26). The three PKR products were all co-precipitated with GST-K3L with similar efficiencies but not by GST alone (compare lanes 3, 6, and 9 with lanes 2, 5, and 8). Three to five percent of the 35S-labeled PKR proteins were recovered with the GST-K3L precipitates. The low levels of recovery could be due to partial masking of K3L by the larger GST moiety. Also, endogenous PKR and heme-regulated inhibitor likely compete with the PKR products for K3L binding (9). The results indicate that whereas the kinase insert region is required for kinase activity (Figs. 2 and 3) it is not required for K3L binding. Also, it is noteworthy that the deletion of amino acids 361–366 (PKRΔ6 mutant; Ref. 13), which were proposed to constitute part of the substrate binding site (17), has no effect on the binding of the pseudosubstrate K3L.
To delineate the K3L binding site, C-terminal deletion mutants of PKR were synthesized in vitro and tested for interaction with K3L. The PKR1–415 mutant migrated at its predicted molecular mass (59 kDa, indicated by a dot). However, there were trace amounts of full-length PKR resulting from incomplete digestion of the cDNA (Fig. 5B, lane 1). The PKR1–415 mutant co-precipitated with GST-K3L with efficiency comparable with that of wild type PKR and did not bind the GST control (lanes 1–3). The PKR1–280 migrated according to its expected size (40 kDa, lane 4) and failed to bind K3L (lane 6). These results suggest that there are critical residues between amino acids 281 and 415 of PKR that are involved in K3L binding. However, these results do not rule out the possibility of a conformational change in another portion of the protein, resulting in a loss of K3L binding. To address this issue, we generated an N-terminal deletion mutant of PKR, which contains a Flag motif (encoding 15 amino acids; Ref. 31) at its N terminus fused to a portion of the PKR kinase domain (FlagPKR281–415). The FlagPKR281–415 mutant contains a portion of PKR spanning catalytic subdomains I–VI (amino acids 281–415; Fig. 5D). It was expressed in E. coli and subjected to the K3L binding assay described above (Fig. 5C). The FlagPKR281–415 mutant that encodes a 20-kDa protein was detected using an anti-Flag monoclonal antibody (Fig. 5C, lane 1). This protein was precipitated by K3L (lane 3; 5% of input) but not by the GST control (lane 2). Taken together with the results for the C-terminal deletion mutants (Fig. 5B), these data suggest that the sequence between amino acids 281 and 415 contains critical binding sites for the pseudosubstrate K3L and that the kinase insert domain and LFIQME sequence are dispensable for K3L binding (Fig. 5D).

**Binding of K3L to PKR in Vivo**—To substantiate our results from the in vitro system, the yeast two-hybrid system was used to assay substrate interactions with PKR in vivo (42). In this assay, the proteins of interest are expressed as GAL4 DNA-binding or GAL4 transactivation domain fusion proteins, and their interaction results in the expression of β-galactosidase, which is monitored by blue color formation in media containing 5-bromo-4-chloro-3-indolyl β-D-galactoside. In agreement with previous results (12), we were able to detect binding of the PKR mutants to K3L in this assay (Table I, bottom). As positive controls, we utilized two yeast proteins, SNF1 and SNF4 (42), which gave a strong signal (Table I, top). As negative controls,
TABLE I

| GAL4-TA | GAL4-DB | Colony color$^a$ |
|---------|---------|-----------------|
| SNF4    | SNF1    | +++             |
| SNF4    | PKRΔ6   | -               |
| SNF4    | PKR366-415 | -            |
| SNF4    | K3L     | -               |
| PKRΔ6   | SNF1    | -               |
| K3L     | SNF1    | -               |
| PKRΔ6   | p20     | +               |
| PKRΔ6   | K3L     | ++              |
| p20     | PKRΔ6   | ++              |
| K3L     | PKRΔ6   | +               |
| K3L     | PKR366-415 | +            |

$^a$ Transformants were replica-plated onto selection medium containing 5-bromo-4-chloro-3-indoyl-β-D-galactoside and evaluated for the development of blue color (+++, strong blue; ++, blue; +, light blue; −, white).

K3L. This combination showed a positive signal, suggesting that the K3L binding site identified in vitro (amino acids 281–415; Fig. 5) can be further demarcated to between amino acids 366 and 415 of PKR (Fig. 6, dashed line).

**DISCUSSION**

In this study we report that the kinase insert domain of human PKR is required for kinase activity but does not play a role in binding of PKR to the pseudosubstrate K3L protein from vaccinia virus. We also observed that mutation of a conserved serine residue in the kinase insert domain (serine 355) abolished kinase activity, raising the possibility that phosphorylation of this residue may be critical for activation of PKR. The context of this serine residue is a PKC consensus site (43), but since K3L strongly inhibits the phosphorylation of eIF2α by PKR through direct interaction with PKR (9), and because of the ability of eIF2α to compete out the interaction between PKR and K3L (11), K3L is thought to be a pseudosubstrate inhibitor of PKR. Also, the residues in K3L showing greatest homology to eIF2α are essential for inhibition of substrate phosphorylation by PKR. This suggests that K3L inhibits eIF2α phosphorylation by PKR through binding and blocking at least a portion of the PKR substrate binding site. Therefore, identification of the K3L binding site within PKR may provide important information as to the location of the substrate binding site within PKR. We showed that residues 361–366, which are deleted in the PKRAΔ mutant (13), are not required for K3L binding in vitro or in vivo (Fig. 5A, Table I). These findings demonstrate that the PKRΔ mutant still contains the pseudosubstrate binding domain. In fact, upon alignment of the catalytic domains of PKR and the cell cycle regulatory kinase Cdc2 (44), the LFIQME sequence, which is deleted in the PKRΔ mutant, aligns with the highly homologous sequence LYLIFE, which comprises β-sheet 5 in subdomain V of Cdc2 (Fig. 6). Therefore, the loss of kinase activity in the PKRΔ mutant (13) results from the deletion of a conserved structural motif (β-sheet 5) and not from loss of a substrate binding site.

We have determined that the kinase insert domain is not required for K3L binding (Fig. 5A). We also defined a portion of

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$^a$ T. Dever, personal communication.
PKR between amino acids 281 and 415 that confers K3L binding ability in vitro (Fig. 5C). The binding site was further delineated using the two-hybrid system, in which amino acids 366–415 of PKR were sufficient for interaction with K3L (Table I). In another recent study, it was found that K3L binding is conferred by amino acids 367–551 (45), which is in agreement with our results.

The structural motifs present within the pseudosubstrate binding domain can be predicted from the deduced three-dimensional structures of other serine/threonine kinases. The kinase domain possesses a small N-terminal lobe rich in β-sheets and a C-terminal large lobe that is predominantly α-helical. The cleft between the small and large lobes contains the catalytic site (46, 47). Amino acids 367–415 of PKR, containing the minimal K3L binding site, form an α-helical structure, which links the small and large lobes of the kinase (between subdomains V and VI). This region corresponds to the α-helices present between β-sheets 5 and 6 and is not highly conserved between kinases (46). Studies with protein kinase A and its inhibitor, protein kinase inhibitor (48), have shown that protein kinase inhibitor contacts protein kinase A within the α-helical region that corresponds to the K3L binding site to PKR. Alignment of this sequence of the different eIF2α kinase sequences does not yield a clear sequence motif that is exclusive to this kinase family. Further studies will be necessary to determine the nature of the K3L–PKR interaction.

In summary, we have identified the binding site within PKR of the pseudosubstrate K3L. It is possible that in addition to the K3L binding site (Fig. 6) other portions of PKR, including the kinase insert domain, may interact with the larger eIF2α complex. Further characterization of the substrate binding site of PKR is of importance to the understanding of the role of substrate interactions in growth regulation by PKR.

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