Mutations in the Double-stranded RNA-activated Protein Kinase Insert Region That Uncouple Catalysis from eIF2α Binding*

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The interferon-induced, double-stranded RNA (dsRNA)-activated protein kinase, PKR, inhibits protein synthesis via phosphorylation of the alpha subunit of the translation initiation factor eIF2. A kinase insert region N-terminal of PKR kinase subdomain V, which is conserved among eIF2α kinases, has been proposed to determine substrate specificity of these kinases. To investigate the function of this kinase insert region, selective PKR mutants were generated, and kinase activities and eIF2α affinities were analyzed in vitro. The in vivo function was investigated by growth inhibitory assays in yeast and translational assays in COS cells. Among the 13 mutations, 5 lost kinase activity and 3 exhibited less than 30% of wild-type eIF2 activity but also is important for substrate interaction. It was determined that the hydrophobicity of the conserved sequence of PKR is required for kinase activity but is not crucial for eIF2α binding. The amino acid residue Glu-367 in the conserved motif was shown to be directly involved in substrate binding but was not important for kinase activity. These results suggest that the activation of PKR is not a prerequisite for its binding to the substrate and that the conserved motif in subdomain V contributes to the interaction of PKR and eIF2α.

Protein synthesis is divided into three phases: initiation, elongation, and termination. The reactions in each phase are promoted by soluble protein factors that transiently interact with the ribosome, mRNA, and aminoacyl-tRNA (1). The phosphorylation of the α subunit of translation initiation factor 2 (eIF2α) is a well characterized translational control mechanism. During the first step of translation initiation, a ternary complex eIF2-GTP-Met-tRNA is formed and subsequently binds to the 40 S ribosomal subunit of the ribosomes. After one round of initiation, eIF2 is released as an eIF2-GDP complex. To re-form the ternary complex, the GDP bound to eIF2 must be replaced with GTP, and this reaction is catalyzed by the guanine nucleotide exchange factor eIF2B. Phosphorylation of eIF2α sequesters the function of eIF2B, thus blocking the recycling, and results in the inhibition of protein synthesis (1).

Three distinct eIF2α kinases have been characterized in eukaryotic cells, including the hemi-regulated inhibitor (HR1, or HCR), the double-stranded RNA activated protein kinase (PKR), and the yeast kinase GCN2 (general control nondepressible). All three kinases phosphorylate eIF2α at Ser-51 in response to cellular stress conditions (2, 3). HR1 mediates protein inhibition in heme-deficient reticulocyte lysate and is also activated by heat shock, sulfhydryl reagents, and heavy metal ions (4). PKR is up-regulated by interferon and becomes activated upon viral infections that produce dsRNA or single-stranded RNA with double-stranded secondary structure (5). GCN2 is activated by uncharged tRNA under amino acid starvation conditions. Phosphorylation of eIF2α by GCN2 mediates gene-specific translational control of GCN4, a transcriptional activator of amino acid biosynthetic genes in yeast. GCN2 is required for increased translation of GCN4 mRNA in amino acid-starved cells (3). Sequence analysis shows that PKR, HRI, and GCN2 share sequence and structural features distinct from other eukaryotic protein kinases. Most notable is the presence of an insert sequence located between kinase subdomains IV and V, consisting of 24 amino acid residues for human PKR, 11 amino acid residues for murine PKR, 128 amino acid residues for HRI, and 112 amino acid residues for GCN2 (Fig. 1A) (6). Although subdomain V is less well conserved among eukaryotic protein kinases (6), the eIF2α kinases share significant homology in this domain, suggesting that this motif might contain a putative substrate-specific recognition domain (7, 8).

PKR is a serine-threonine kinase that mediates the antiviral and antiproliferative effects of interferon through phosphorylation of eIF2α (5). Discovered as an enzyme responsible for the inhibition of translation in response to dsRNA in reticulocytes (9), PKR has two distinct kinase activities: autophosphorylation, requiring the presence of dsRNA, divalent cations (Mn2+ and Mg2+), and ATP (10, 11), and phosphorylation of exogenous substrates. This second activity is independent of dsRNA but dependent on ATP and divalent cations (4). As phosphorylation of eIF2α by PKR provides a mechanism for cell defense against virus infection, many viruses have evolved specific mechanisms to inactivate PKR, including degradation of PKR (12), sequestering of dsRNA activator (13), binding to PKR to inhibit its activation (14), or activating a cellular PKR inhibitor (15). DNA transfection of certain plasmids can also activate PKR and result in poor translation of plasmid-derived mRNAs. Translation can be restored by coexpression of PKR inhibitors of a mutant form of eIF2α where serine residue 51 has been replaced by alanine (16). Because of the homology between

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human and yeast eIF2α. PKR is also able to phosphorylate yeast eIF2α at Ser-51 and inhibit yeast growth (7).

Human PKR is a 551-amino acid protein consisting of an N-terminal regulatory domain and a C-terminal catalytic domain (17). The regulatory domain contains two dsRNA binding motifs that are homologous to those in other dsRNA-binding proteins (18). Motif I (amino acids 55–75) is essential for dsRNA binding, whereas motif II (amino acids 145–166) plays a less important role (19–21). The catalytic domain contains 11 conserved kinase subdomains characterizing PKR as a serine/threonine kinase (17). The invariant lysine (Lys-296) in subdomain II is essential for the phosphate transfer reaction. Mutation of this lysine to arginine or proline results in a catalytically inactive protein (7, 22). As mentioned above, PKR has a 24-amino acid kinase insert between subdomains IV and V and the highly conserved eIF2α kinase motif LFIQMFECFD in subdomain V. A full-length PKR containing a deletion of six amino acids (LFIQME) at this motif was unable to undergo autophosphorylation or to phosphorylate eIF2α (23), underlining the importance of this region to PKR activity.

To delineate the individual contribution of conserved individual amino acids in and around the kinase insert region of PKR to eIF2α binding and kinase activity, we generated 13 PKR mutants in this region, analyzed their in vitro auto- and substrate phosphorylation activities and eIF2α affinity, and examined the function of selected mutants in yeast and in mammalian cells. We found that the hydrophobicity of the conserved motif in subdomain V is essential for PKR kinase activity but is not necessarily required for the interaction with eIF2α. We also identified a specific amino acid (Glu-367) that is involved in eIF2α binding but not in PKR auto- and substrate phosphorylation. We conclude that the conserved motif in subdomain V is required for PKR kinase activity and also contributes to its interaction with eIF2α.

### EXPERIMENTAL PROCEDURES

**PKR Mutagenesis and Plasmid Constructions**—PKR mutants were generated as described (24) using the Transformer site-directed mutagenesis kit (CLONTECH). PKR cDNA was cloned into the LEU2 gene (20). The bacterial expression vector pQE-eIF2α was constructed by insertion of PKR/eIF2α expression vectors were constructed by insertion of PKR/eIF2α

**Expression of the Human PKR in Saccharomyces cerevisiae**—Expression plasmids were introduced into the haploid yeast strain, W303-1a (MATa, can1-100, his3-11, 15, leu2-3, 112, trpl-1, ura3-1, ade2-1) using the lithium acetate procedure (25). Transformed yeast cells were grown on a minimal medium lacking tryptophan, amino acids (DBGA), and lacking uracil or leucine. 10 ng/ml ampicillin. Followed by growth on the agar plates was monitored.

**PKR Expression in Mammalian Cells**—PKR mutants were subcloned into the mammalian expression vector pRcCMV. COS-1 cells were transiently transfected with 400 ng of pRc-PKR and 2 μg of reporter plasmid pGL2-Control (Promega) by the lipofectamine procedure (Life Technologies Inc.). Cells were harvested 48 h after transfection and assayed for luciferase activity after normalizing for transfection efficiency by measuring the total protein (27).

**Immunopurification of PKR from COS-1 Cells**—COS-1 cells transiently transfected with PKR were harvested 48 h after transfection, and the cells lysed in buffer I (400 mM NaCl, 50 mM KCl, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 100 μg/ml aprotinin, 20% (v/v) glycerol, 1% (v/v) Triton X-100). PKR was immunoprecipitated from lysate containing 2 mg of total protein using anti-PKR monoclonal antibody (28) and detected by Western blot with anti-PKR polyclonal antibody.

**RESULTS**

**Site-directed Mutation of PKR**—The eIF2α kinases (HRI, GCN2, and PKR) are distinguished in the family of the serine/threonine protein kinases by possessing a kinase insert between subdomains IV and V. Sequence analysis shows that the amino acid residues spanning the insert regions are highly conserved, including two amino acids in subdomain IV (Tyr-232 and Trp-237 in PKR) and a nine-amino acid motif in subdomain V (LFIQMFECFD) (Fig. 1A). It has been proposed that these amino acids may contribute to substrate recognition (7, 8). To investigate the function of these residues, we designed and constructed 13 mutated forms of PKR, including a deletion of the conserved motif (M12), a deletion of the insert region together with part of the kinase insert (M13), and single point mutations of different conserved residues (Fig. 1B). Site-directed mutagenesis was performed in pBluescript, confirmed by sequencing, and the mutated cDNAs were subcloned into expression vectors for in vitro and in vivo functional analysis.

**Expression and Kinase Analysis of PKR Mutants in Vitro**—
Wild-type and PKR mutant cDNAs were expressed in E. coli as GST fusion proteins in the bacterial expression vector pGEX (Fig. 2). The GST fusion proteins were purified by glutathione-Sepharose affinity chromatography, and their expression was monitored by SDS-PAGE with Coomassie Blue staining (data not shown). All mutants were well expressed except M5, which may be intrinsically unstable. The GST fusion proteins showed different mobilities on SDS-PAGE, which were likely due to different phosphorylation levels since recombinant PKR can be partially phosphorylated in bacteria (29).

To assess the autophosphorylation and substrate phosphorylation activities of PKR mutants, kinase assays were performed using the purified GST-PKR proteins. The levels of GST-PKR proteins used in the kinase assays were determined by Western blot using anti-PKR polyclonal antibody (Fig. 2, upper panel). PKR autophosphorylation (Fig. 2, middle panel) and phosphorylation of eIF2α (Fig. 2, lower panel) were detected by autoradiography. As expected, wild-type PKR showed a high level of autophosphorylation and eIF2α phosphorylation, but no kinase activity was detected for the K296R mutant (Fig. 2, middle panel). The two deletion mutants (M12, M13) showed no auto- and substrate phosphorylation activities, whereas the single point mutants showed varying levels of auto- and substrate phosphorylation activities. By comparing the intensities of the phosphorylated protein bands with the PKR concentration, the specific kinase activities for each PKR mutant could be calculated (Fig. 2C). For most of the mutants, autophosphorylation and substrate phosphorylation activities were well correlated. An exception to this pattern is mutant M3, which shows autophosphorylation activity but no eIF2α phosphorylation. The single point mutants M3 (L362Q), M4 (I364N), and M8 (F363S), which lost all or most of their kinase activity, contain substitutions of hydrophobic to hydrophilic amino acids within the conserved sequence of subdomain V.

Analysis of eIF2α Binding—To study the interaction of PKR with eIF2α, the bacterially expressed GST-PKR proteins were reacted with histidine-tagged eIF2α. Glutathione-Sepharose beads were used to immobilize the GST-PKR/eIF2α complex, and PKR and eIF2α were detected in Western blots probed with monoclonal antibodies against PKR and eIF2α, respectively (Fig. 2B). The interaction between PKR and eIF2α was specific because GST alone was not able to bind histidine-tagged eIF2α (Fig. 2B, lower panel), and wild-type GST-PKR was not able to bind an unrelated histidine-tagged protein His-WT1 (data not shown). All the PKR mutants were able to bind to eIF2α but with different affinities (Fig. 2B). The relative affinity of different mutants for eIF2α was calculated by counting the ratios of how much eIF2α bound to certain amounts of PKR (Fig. 2D). Interestingly, there was no correlation between the affinity of the PKR mutants for eIF2α and

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**Fig. 1. Mutagenesis of human PKR.** A, amino acid sequence alignment of PKR with other protein kinases. The eIF2α kinases (human PKR, murine PKR, yeast GCN2, and rabbit HRI) have a kinase insert between subdomains IV and V. The sequences of kinase subdomain IV and V are shown. The sizes of the inserts are indicated by the number in parentheses. The conserved amino acid residues around the insert among the eIF2α kinases are shadowed. According to the crystal structure of the catalytic subunit of PKA, subdomain IV forms a β strand and an α-helix (see “Discussion”). B, human PKR mutants. The amino acid sites for the point mutants (M1-M11) and the deletion mutants (M12, M13) used in this study are indicated.
their kinase activities (Fig. 2, C and D). For example, M4 has no kinase activity yet has almost twice the affinity of wild-type PKR for eIF2α. In contrast, mutant M11 has only 20% of wild-type affinity for eIF2α but retains 90% of wild-type kinase activity. Furthermore, M11 can phosphorylate eIF2α very well, suggesting that the high affinity of PKR for eIF2α might not be a prerequisite for PKR function.

Effects of PKR Mutants on Yeast Cell Growth—To analyze the activities of the PKR mutants in vivo, we expressed PKR mutants in yeast cells (7, 24, 30). There is 80% homology between yeast and human eIF2α when conservative substitutions are considered (31), and yeast eIF2α can be phosphorylated by human PKR, leading to growth inhibition (7). Accordingly, the different PKR mutants were subcloned into the vector pEMBLyex4 and transformed into S. cerevisiae strain W303a. PKR protein expression was controlled via the GAL10-
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**FIG. 3. Expression of PKR in yeast.** The PKR expression vector pEMBL-yex-PKR was transformed into the yeast strain w303–1a. Yeast growth was monitored with or without galactose induction. A, yeast growth in glucose medium. B, yeast growth in galactose medium. Colonies 1–13 are yeast transformed with PKR mutants M1–M12, whereas 14, 15, and 16 are yeast transformed with wild-type PKR, K296R mutant, and the vector pEMBL-yex4, respectively.

Cyc1 hybrid promoter, whereby growth on galactose as the sole carbon source activates expression. In glucose medium, yeast transformed with M1, M2, M6, M7, or wild-type PKR grew very slowly (Fig. 3A). This was likely caused by a low basal level expression of PKR. When PKR expression was induced in galactose medium (Fig. 3B), the growth of yeast transformed with M1, M2, M4, M6, M7, M9, M10, M11, and wild-type PKR was completely inhibited. Significantly, all of these mutants showed kinase activities *in vitro* except M4 (Fig. 2A). Because M4 has a high affinity for eIF2α *in vitro* (Fig. 2), we speculated that the yeast slow growth phenotype might be caused by the direct binding of M4 to eIF2α. To prove this hypothesis, we co-expressed M4 with sui2M, a yeast eIF2α allele that contains a mutation (Ser-51 → Ala) that completely abolishes phosphorylation by PKR. We have previously shown that sui2M is able to rescue the slow growth phenotype when co-expressed with wild-type PKR (7). We found that sui2M also reverted the slow-growth phenotype caused by M4 (Fig. 4B). This suggests that M4 inhibits yeast growth by direct interaction with eIF2α independent of auto- or substrate phosphorylation.

**Expression of PKR Mutants in Mammalian Cells—Transient transfection of COS-1 cells with expression plasmids encoding a reporter gene such as DHFR has been shown to result in inefficient translation due to PKR activation and eIF2α phosphorylation (16).** A variety of agents can stimulate the synthesis of the reporter protein, including vaccinia virus gene products E3L and K6L, the catalytically inactive PKR mutant K296R, and the dsRNA binding-deficient mutant K64E (32, 33). The luciferase reporter plasmid pGL2-control was also successfully used in this system (27). Therefore, we used the COS-1 expression system to analyze the function of some of our PKR mutants in mammalian cells. Three PKR mutants (M3, M4, M11) together with wild-type PKR and the K296R mutant were subcloned into the mammalian expression vector pRc/CMV and transiently transfected into COS-1 cells together with the luciferase reporter vector. Transfection of wild-type PKR almost completely inhibited luciferase translation, whereas the K296R mutant had a dominant-negative effect, resulting in enhanced luciferase activity (Fig. 5A). Mutant M3, which has very little autophosphorylation activity and no eIF2α phosphorylation activity in *vitro*, was not able to inhibit translation in COS cells, consistent with its function in yeast. However, mutant M4, which inhibited yeast growth, also enhanced luciferase activity, suggesting that it is inactive in COS cells. As with wild-type PKR, mutant M11 inhibited luciferase gene translation, consistent with its function in yeast. As shown previously, although M11 has a low eIF2α affinity for eIF2α, it can phosphorylate eIF2α almost as well as wild-type PKR *in vitro* (Fig. 2). In *vivo* experiments demonstrate that M11 is fully functional in both yeast and COS cells.

PKR has been shown to be autoregulatory in transfected COS cells (16, 34). Therefore, we analyzed the expression of PKR and its mutants in transfected COS cells. PKR was immunoprecipitated from transfected cell lysate by anti-PKR monoclonal antibody and detected by Western blot with polyclonal antibody. Consistent with their kinase activities *in vitro*, wild-type PKR and mutant M11 were expressed at low levels in the transfected cells (Fig. 5B, lanes 3 and 7), whereas K296R mutant and M3 were expressed very well (Fig. 5B, lanes 4 and 5). M4 was expressed better than wild-type PKR (Fig. 5B, lane 6), whereas in another experiment, its expression was close to that of the K296R mutant (data not shown). Thus, the lack of inhibitory function of M4 in the COS cell assay is due to the loss of kinase activity rather than deficient protein expression.

**DISCUSSION**

The anti-viral effect of PKR is an important mechanism for host defense against viral infection and is mediated through the phosphorylation of eIF2α and inhibition of viral protein synthesis. In addition, PKR has been shown to have an anti-proliferative function and to regulate cell growth (7, 23, 35), which is at least in part linked to eIF2α phosphorylation. In this study, we have examined the effects of mutations of the conserved amino acids around the PKR kinase insert region *in vitro* and *in vivo*. By using bacterially expressed PKR and eIF2α, we were able to study their direct interaction *in vitro*. We found that amino acids in the conserved sequence LFQMEFC (amino acids 362–370) in subdomain V are important for PKR kinase activity, as the single point mutations L362Q (M3), I364N (M4), and F363S (M8) resulted in a loss of kinase activity rather than deficient protein phosphorylation. This suggests that this region is important for PKR kinase activity because all mutants bound eIF2α, including the two deletion mutants, although some mutants bound eIF2α at a relatively low level compared with wild-type PKR. For example, M11 (E367R), which has kinase activity and is fully functional *in vivo*, showed only about 20% of normal (wild-type) eIF2α binding activity.

Because of the homologous nature of protein kinase domains, they all fold into topologically similar three-dimensional core structures and carry out phosphotransfer by a common mechanism (6). The crystal structures of more than ten eukaryotic protein kinases have been reported, and their kinase domains...
human PKR wild-type and mutant expression in COS-1 cells. shows the endogenous monkey PKR of COS-1 cells. Lane 1 and detected by Western blotting with anti-PKR polyclonal antibody. Precipitated PKR proteins were separated by 10% SDS-PAGE immunoprecipitation of PKR protein. COS-1 cell lysates containing 2 

expression and inhibiting yeast growth. The reversal of this effect by the eIF2\(\alpha\) mutant su2M could then be explained by competition with endogenous eIF2\(\alpha\) for binding to M4, freeing eIF2\(\alpha\) to release the suppression of GCN4 expression. The mechanism of action of M4 in COS-1 cells is likely independent of eIF2\(\alpha\) binding and may involve the formation of inactive heterodimers with endogenous PKR.

Finally, our data show that phosphorylation of PKR is not required for binding to its substrate, eIF2\(\alpha\). The catalytically inactive mutant K296R has no kinase activity but is able to bind eIF2\(\alpha\). Moreover, mutant M4 can bind eIF2\(\alpha\) more readily than wild-type PKR. This increased affinity of M4 for eIF2\(\alpha\) may result from a conformational change due to the replacement of the hydrophobic isoleucine residue with a hydrophilic asparagine. In any case, high affinity of the PKR mutants for eIF2\(\alpha\) may be involved in substrate anchoring through an ion pair may be involved in substrate anchoring through an ion pair.

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