Conserved Intermolecular Salt Bridge Required for Activation of Protein Kinases PKR, GCN2, and PERK*

Received for publication, August 17, 2006, and in revised form, December 14, 2006 Published, JBC Papers in Press, January 3, 2007, DOI 10.1074/jbc.M607897200

Madhusudan Dey†, Chune Cao‡, Frank Sicheri§¶, and Thomas E. Dever††1

From the †Laboratory of Gene Regulation and Development, NICHD, National Institutes of Health, Bethesda, Maryland 20892, the ‡Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada, and the §Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario MSS 1A8, Canada

The protein kinases PKR, GCN2, and PERK phosphorylate translation initiation factor eIF2α to regulate general and gene-specific protein synthesis under various cellular stress conditions. Recent x-ray crystallographic structures of PKR and GCN2 revealed distinct dimeric configurations of the kinase domains. Whereas PKR kinase domains dimerized in a back-to-back and parallel orientation, the GCN2 kinase domains displayed an antiparallel orientation. The dimerization interfaces on PKR and GCN2 were localized to overlapping surfaces on the N-terminal lobes of the kinase domains but utilized different intermolecular contacts. A key feature of the PKR dimerization interface is a salt bridge interaction between Arg262 from one protomer and Asp266 from the second protomer. Interestingly, these two residues are conserved in all eIF2α kinases, although in the GCN2 structure, the two residues are too remote to interact. To test the importance of this potential salt bridge interaction in PKR, GCN2, and PERK, the residues constituting the salt bridge were mutated either independently or together to residues with the opposite charge. Single mutations of the Asp (or Glu) and Arg residues blocked kinase function both in yeast cells and in vitro. However, for all three kinases, the double mutation designed to restore the salt bridge interaction with opposite polarity resulted in a functional kinase. Thus, the salt bridge interaction and dimer interface observed in the PKR structure is critical for the activity of all three eIF2α kinases. These results are consistent with the notion that the PKR structure represents the active state of the eIF2α kinase domain, whereas the GCN2 structure may represent an inactive state of the kinase.

Protein synthesis in mammalian cells is regulated by four protein kinases (PKR, PERK, GCN2, and HRI) that specifically phosphorylate the α subunit of the translation initiation factor eIF2 in response to various environmental stress conditions (reviewed in Refs. 1 and 2–5). The double-stranded RNA-activated protein kinase PKR is a component of the cellular antiviral defense mechanism, whereas the endoplasmic reticulum (ER)2-resident kinase PERK senses misfolded proteins in the ER and transduces this signal to attenuate protein synthesis. The heme-regulated kinase HRI is activated under conditions of heme deprivation and coordinates globin chain synthesis with heme availability in reticulocytes. Finally, uncharged tRNAs that accumulate in cells under amino acid starvation conditions bind to and activate GCN2. Unique regulatory domains enable each of these kinases to respond to different stress conditions, whereas their conserved kinase domains specifically phosphorylate eIF2α on Ser511 (see Refs. 1–5). Phosphorylation of eIF2α on Ser51 converts the factor from a substrate to a competitive inhibitor of its guanine nucleotide exchange factor eIF2B. The resulting impaired nucleotide exchange blocks protein synthesis by preventing the recycling of inactive eIF2α-GDP to functional eIF2α-GTP that binds the initiator methionyl-tRNA to the 40 S ribosomal subunit in the first step of translation initiation (reviewed in Ref. 3).

Interestingly, phosphorylation of eIF2α in the yeast *Saccharomyces cerevisiae* regulates both general and gene-specific mRNA translation. Activation of the sole eIF2α kinase GCN2 is required for growth under amino acid starvation conditions as imposed by the histidine analog 3-aminotriazole (3-AT). By regulating translation reinitiation at upstream open reading frames in the *GCN4* mRNA, phosphorylation of eIF2α in yeast by GCN2 paradoxically stimulates expression of the transcription factor GCN4 (reviewed in Ref. 6). The GCN4 then stimulates expression of amino acid biosynthetic enzyme genes under its control, resulting in a 3-AT-resistant phenotype. Thus, growth on medium containing 3-AT is a simple test for eIF2α kinase activity in yeast. In contrast, high level phosphorylation of eIF2α by mutationally activated forms of GCN2 impairs general translation initiation in yeast and results in a slow growth phenotype (3, 7). Whereas yeast cells lacking GCN2 are unable to grow on medium containing 3-AT, low level expression of heterologous PKR or PERK kinases confers a 3-AT-resistant phenotype (8, 9). In addition, high level expression of PKR or PERK inhibits yeast cell growth by impairing general translation (8, 10, 11). Biochemical analyses demonstrated that PKR and PERK phosphorylate yeast eIF2α on Ser511 both in vivo and in vitro (8, 11). Moreover, replacement of Ser511 in eIF2α by nonphosphorylatable Ala (eIF2α-S51A) suppresses the growth phenotypes associated with expression of PKR and

---

1 To whom correspondence should be addressed: National Institutes of Health, Bldg. 6A, Rm. B1A-03, Bethesda, MD 20892-2427. Tel.: 301-496-4519; Fax: 301-496-8576; E-mail: tdever@nih.gov.

2 The abbreviations used are: ER, endoplasmic reticulum; KD, kinase domain; WCE, whole cell extract; 3-AT, 3-aminotriazole; GST, glutathione S-transferase; WT, wild type.
PERK in yeast (8, 11). Finally, mutations that inactivate PKR kinase function in vitro abolish the ability of PKR to regulate GCN4 and general translation in yeast cells (8), indicating that yeast is an excellent system to study structure-function properties of the eIF2α kinases.

A common feature in the activation of all four kinases eIF2α kinases is dimerization. Whereas GCN2 and the heme-regulated form of HRI are constitutive dimers (3, 5, 12, 13), activation of both PKR and PERK is coupled with dimerization of the regulatory and kinase domains of these proteins (1, 2, 14, 15). In addition to its kinase domain (KD), GCN2 contains a tRNA-binding domain that resembles HisRS (histidyl-tRNA synthetase) and a C-terminal domain. The isolated, KD, HisRS, and C-terminal domain of GCN2 were found to dimerize in vivo, and these dimer contacts are thought to maintain the protein in a dimeric state (13). Binding of double-stranded RNA molecules promotes dimerization of the double-stranded RNA-binding motifs at the N terminus of PKR, which is thought to promote subsequent dimerization of the kinase domains (15–18). Finally, in the absence of ER stress, it is proposed that ER chaperones prevent dimerization of the N-terminal IRE1-like domains of PERK that reside in the ER lumen. Under stress conditions, unfolded proteins are thought to titrate the ER chaperones and to directly bind to the IRE1-like domains of PERK, leading to dimerization of the N-terminal domains and the KDs of the protein (14, 19, 20). Consistent with the idea that KD dimerization is critical for kinase activity, fusion of constitutive (GST) or regulated (GyrB and Fv2E) dimerization domains to the PKR or PERK KD was sufficient to promote kinase activation (16, 21, 22).

The recent crystal structure of the PKR KD in complex with eIF2α has provided additional insights into KD dimerization (23). In the crystal structure, the catalytic domain of PKR consisted of the typical smaller N-terminal lobe and larger C-terminal lobe connected by a hinge region. A symmetric dimeric configuration of the PKR KD was observed in the crystal, and each KD was stoichiometrically phosphorylated on Thr446 in the activation segment, indicating that the structure represents an active form of the kinase (23). Further supporting this notion, each KD in the crystal was bound to its substrate eIF2α. PKR dimerization was mediated by back-to-back positioning of the N-terminal lobes of the KD and involved residues that were preferentially conserved among the eIF2α family of kinases (23). Significant among these conserved residues were Arg262 and Asp266, which form intermolecular salt bridges between the two PKR protomers in the dimer. Individual mutations of Arg262 to Asp or Asp266 to Arg blocked PKR toxicity in yeast and impaired PKR autophosphorylation and eIF2α substrate phosphorylation in yeast cells (15). Moreover, when both the R262D and D266R mutations were introduced into the same PKR molecule, which was predicted to reestablish the salt bridge interaction with opposite polarity, PKR function was restored (15). Thus, the back-to-back dimeric configuration of PKR observed in the crystal structure is critical for PKR activity.

Additional structural insights into the eIF2α kinases were provided by recent crystal structures of the wild type yeast GCN2 KD and derivatives bearing activating mutations (24). Whereas the KD in the PKR structure was phosphorylated on a positive regulatory site (Thr446) within the activation segment, the KD of the GCN2 structures was not. Thus, the GCN2 structure may represent an inactive form of the KD, whereas the PKR structure represents an active conformation. Consistent with this notion, the activation segment and N-lobe catalytic elements of PKR adopt productive conformations, whereas those of GCN2 are nonproductive. Interestingly, the GCN2 KD also adopted a symmetrical but antiparallel dimer configuration involving primarily the N-lobes of two KDs (24). This configuration contrasts with the symmetrical but parallel dimer configuration of the PKR KDs (Fig. 1, B and C). In addition, the GCN2 KD dimer interface was located on a similar face of the N-terminal lobe as compared with the PKR dimer. Specifically, the PKR and GCN2 dimerization surfaces display 80% overlap of contacting residues with the two dimer orientations differing roughly by a 180° rotation normal to the plane of the contact surfaces. Thus, whereas the side chains of Arg262 and Asp266 are located 2.4–2.7 Å apart in the PKR dimer, the corresponding residues, Arg594 and Asp598, are too far apart (13.14 Å) to form a salt bridge interaction in the GCN2 dimer (Fig. 1, B and C). To gain further molecular insights into the dimeric configuration of the eIF2α kinases, we mutated the predicted salt bridge-interacting residues in the GCN2 and PERK KDs. Whereas single mutations to oppositely charged residues eliminated kinase function, reciprocal exchanges restored kinase activity. Thus, the salt bridge interaction and dimer interface observed in the PKR structure is conserved among PKR, GCN2, and PERK and is critical for eIF2α kinase activation.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—Yeast strain H17 (MATα gcn3-102 leu2-3 ura3-112) was described previously (25). The wild-type SUI2 allele in strain H2557 (MATα ura3-52 leu2-3 ura3-112 gcn2Δ) was replaced with SUI2-SS1A to generate strain J223 (15). Strain H1894 (MATα ura3-52 leu2-3 ura3-112 trpl-Δ 63 gcn2Δ) was described previously (26).

**Plasmids**—Plasmid pC1685 expressing FLAG- and His6-tagged PKR under the control of a hybrid GAL-CYC1 promoter and related plasmids expressing mutant forms of PKR were described previously (15). A SacI-PstI fragment encoding FLAG- and His6-tagged *Caenorhabditis elegans* PERK (residues 26–1077) was obtained by PCR using plasmid pS51 (11) as a template. The PCR product was inserted into the yeast expression vector pEMBLyex4 (27), generating plasmid pC2706. Plasmids pC2707, pC2708, pC2709, and pC2710 expressing PERK-R598E, PERK-D603R, the double mutant PERK-R598E,E603R, and PERK-K633R, respectively, were generated by using fusion PCR to introduce the appropriate mutations in pC2706. Plasmid p722 is a low copy number *URA3* vector that expresses GCN2 from its native promoter (7). Site-directed mutagenesis of p722 was used to generate the plasmids pC2745, pC2746, pC2747, and pC2748 expressing GCN2-K628R, GCN2-R594D, GCN2-D598R, and GCN2-R594D,D598R, respectively. Plasmid pC2436 expressing FLAG- and His6-tagged GCN2 under the control of the hybrid GAL-CYC1 promoter in pEMBLyex4 was derived from pH103 (28). The related plasmid pH109 was used to express GCN2-K628R (28). The GCN2 single mutations R594D and
**elf2α Kinase Activation Requires Trans-dimer Salt Bridge**

D598R and the double mutation R594D,D598R were introduced into pC2436, generating plasmids pC2437, pC2438, and pC2439, respectively.

**Immunoblot Analysis of Kinase Expression in Yeast**—Transformants of strain H17 expressing PKR under the control of the GAL-CYC1 hybrid promoter were grown as described previously (15). Transformants of strains H2557 and J223 expressing PERK under the control of the GAL-CYC1 hybrid promoter were grown in SC-Ura medium (synthetic minimal medium with all amino acids) overnight at 30 °C to saturation, diluted in fresh medium to A600 ≈ 0.1, and grown at 30 °C until A600 ≈ 0.6. Cells were harvested and transferred to SGal-Ura medium (SC-Ura except 10% galactose) for 2 h at 30 °C to induce PERK expression. Transformants of strain H1894 expressing GCN2 from its native promoter were grown in SC-Ura medium overnight at 30 °C to saturation, diluted in fresh medium to A600 ≈ 0.1, grown at 30 °C to A600 ≈ 0.6, and then treated with 3-AT (30 mM) for 1 h.

Cells were harvested, and whole cell extracts (WCEs) were prepared as described previously (29). The WCEs (5 or 35 μg) were resolved by SDS-PAGE and subjected to immunoblot analysis using rabbit phospho-specific antibodies directed against phosphorylated Ser51 of elf2α (BioSource International), rabbit polycional antiserum that recognizes yeast elf2α irrespective of Ser51 phosphorylation, rabbit polycional anti-FLAG antibodies (Sigma), mouse monocional penta-His antibodies (Qiagen), or rabbit antiserum directed against the N terminus of yeast GCN2 (29).

**In Vitro Immunokinase Assay**—FLAG- and His6-tagged PKR and PERK were overexpressed in yeast strain J223, and FLAG- and His6-tagged GCN2 was overexpressed in yeast strain H1894. Yeast transformants were grown to saturation in 5 ml of SC-Ura medium at 30 °C; the cells were harvested, transferred to 50 ml of SGR medium (SC medium containing 10% galactose and 2% raffinose), and incubated at 30 °C for 48 h. The cells were then harvested, washed with ice-cold water, and resuspended in FLAG binding buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 50 mM β-glycerophosphate, 125 μM Na3VO4, and complete protease inhibitor mixture (Roche Applied Sciences)). Cells were broken by high speed mixing with glass beads on a vortex. WCEs were mixed with 100 μl of anti-FLAG-M2 affinity gel (50% slurry; Sigma) preequilibrated with FLAG binding buffer, and incubated with gentle rocking at 4 °C for 1 h. Immune complexes were collected by centrifugation and washed three times with FLAG binding buffer and once with 1× kinase buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 25 mM MgCl2, and 1 μM phenylmethylsulfonyl fluoride). Bound proteins were eluted with 25 μl of FLAG-peptide (500 μg/ml), and individual aliquots were used for immunoblot analysis and for protein kinase assays with recombinant yeast elf2α as described previously (15).

**Mammalian Cell Experiments**—Plasmids PERK.WT.9E10, pCDNA.amp and PERK.K618A.9E10.pCDNA.amp that express...
Kinase reactions were resolved on SDS-PAGE, stained with Coomassie Blue (D), and exposure time was required to detect PKR (top). WCEs prepared from cells expressing no PKR (middle) and phosphorylated PKR (middle) were subjected to SDS-PAGE followed by immunoblot analysis using phosphospecific antibodies directed against phosphorylated Ser51 of eIF2α. The membrane was then stripped and probed using polyclonal antisera against yeast eIF2α (bottom) or monoclonal anti-human PKR antibodies (top). In vitro protein kinase assay with immunopurified PKR. WCEs prepared from cells expressing no PKR (Vector), wild type PKR, or the indicated PKR mutants were incubated with anti-FLAG M2-agarose. Immunoprecipitated proteins were eluted with FLAG-peptide and mixed with recombinant GST-eIF2α (bottom) or anti-Myc antibodies (to detect PERK).

**RESULTS AND DISCUSSION**

**Salt Bridge Interaction of Arg**<sup>262</sup> **and Asp**<sup>266</sup> **at the Dimer Interface of PKR Is Critical for eIF2α Phosphorylation**—To initiate our studies, we added a FLAG and His<sub>6</sub> tag to the N terminus of full-length human PKR and expressed the kinase in yeast cells. Whereas high level expression of PKR from a yeast virus promoter (30) was a kind gift of David Ron (Skirball Institute, New York University School of Medicine). Using fusion PCR, mutations were introduced into the plasmid PERK-WT.9E10.pCDNA.amp to generate derivatives expressing PERK-K584D, PERK-D588R, or PERK-R584D, D588R. HEK 293 EbnAT cells (1.6 × 10<sup>6</sup> cells/well in a 12-well plate) were transfected using Lipofectamine 2000 reagent (4 μl; Invitrogen) with derivatives of the vector pcDNA1 (1.6 μg) designed to express wild type mouse PERK, PERK-K618A, PERK-R584D, PERK-D588R, or PERK-R584D, D588R. Thirty hours after transfection, cells were harvested and lysed, and aliquots of WCEs were subjected to SDS-PAGE and immunoblot analysis using phosphospecific antibodies against phosphorylated Ser<sup>51</sup> of eIF2α, monoclonal antibodies against eIF2α, or anti-Myc antibodies (to detect PERK).

**Structural Models and Figures**—Structural models were generated using the coordinates of the PKR-eIF2α complex (Protein Data Bank code 2A1A) and GCN2 (Protein Data Bank code 1ZYC) using PyMOL software (31).

**FIGURE 2.** Intermolecular salt bridge interaction required for PKR and PERK kinase activity. A, reciprocal exchange of PKR residues Arg<sup>262</sup> and Asp<sup>266</sup> restores PKR toxicity in yeast. Plasmids expressing FLAG (FL)- and His-tagged wild type or the indicated PKR mutants under the control of a galactose-inducible promoter were introduced into yeast strains H17 (eIF2α) and J223 (eIF2α-S51A). Transformants were grown to saturation in SD (glucose) medium, and 5 μl of serial dilutions (of A<sub>660</sub> = 1.0, 0.1, and 0.01) were spotted on minimal SGal (galactose) medium and incubated 3 days at 30 °C. B, immunoblot analysis of eIF2α phosphorylation in yeast expressing PKR. WCEs were prepared from yeast transformants described in A or from a vector control transformant not expressing PKR and then subjected to SDS-PAGE followed by immunoblot analysis using phosphospecific antibodies directed against phosphorylated Ser<sup>51</sup> of eIF2α (middle). The membrane was then stripped and probed using polyclonal antisera against yeast eIF2α (bottom) or monoclonal anti-human PKR antibodies (top). C, in vitro protein kinase assay with immunopurified PKR. WCEs prepared from cells expressing no PKR (Vector), wild type PKR, or the indicated PKR mutants were incubated with anti-FLAG M2-agarose. Immunoprecipitated proteins were eluted with FLAG-peptide and mixed with recombinant GST-eIF2α (residues 1–180) and [γ<sup>32</sup>P]ATP. Kinase reactions were resolved on SDS-PAGE, stained with Coomassie Blue (bottom), and subjected to autoradiography to visualize phosphorylated eIF2α (eIF2α→P; middle) and phosphorylated PKR (PKR→P; top). A longer exposure time was required to detect PKR→P than to detect phosphorylated eIF2α. D, reciprocal exchange of PERK residues Arg<sup>599</sup> and Glu<sup>603</sup> restores PERK toxicity in yeast. Plasmids expressing FLAG (FL)- and His-tagged wild type or the indicated PERK mutants under the control of a galactose-inducible promoter were introduced into yeast strains H2557 (eIF2α) and J223 (eIF2α-S51A). Transformants were grown to saturation in SD (glucose) medium, and 5 μl of serial dilutions (of A<sub>660</sub> = 1.0, 0.1, and 0.01) were spotted on minimal SGal (galactose) medium and incubated 3 days at 30 °C. E, immunoblot analysis of eIF2α phosphorylation in yeast expressing PERK. WCEs were prepared from yeast transformants described in D or from a vector control transformant not expressing PERK and then subjected to SDS-PAGE followed by immunoblot analysis using phosphospecific antibodies directed against phosphorylated Ser<sup>51</sup> of eIF2α (middle). The membrane was then stripped and probed using polyclonal antisera against yeast eIF2α (bottom) or anti-FLAG antibodies to detect FLAG (FL)-tagged PERK (top). F, in vitro protein kinase assay with immunopurified PERK. WCEs prepared from cells expressing no PERK (Vector), wild type PERK, or the indicated PERK mutants were incubated with anti-FLAG M2-agarose. Immunoprecipitated proteins were eluted with FLAG-peptide and mixed with recombinant GST-eIF2α (residues 1–180) and [γ<sup>32</sup>P]ATP. Kinase reactions were resolved on SDS-PAGE, stained with Coomassie Blue (bottom), and subjected to autoradiography to visualize phosphorylated eIF2α (eIF2α→P; top).
and various mutant forms of PKR were expressed at similar levels. Analysis using anti-PKR antibodies revealed that the wild type and the kinase-dead mutant (Fig. 2A, rows 3 and 4). When the two single mutations (R262D and D266R) were combined in one protein (PKR-R262D,D266R), PKR toxicity in yeast was restored (Fig. 2A, row 5). Thus, the salt bridge interaction between residues 262 and 266, with either normal or opposite polarity, is required for the growth-inhibitory effects of PKR in yeast.

The suppression of PKR toxicity in the eIF2α-S51A strain indicates that the growth-inhibitory affects of wild type PKR and the PKR-R262D,D266R mutant were due to phosphorylation of eIF2α. To directly examine eIF2α phosphorylation in the yeast cells, whole cell extracts were subjected to immunoblot analysis using anti-phospho-Ser51-specific antibodies. Subsequently, the blot was stripped and probed with anti-yeast eIF2α antiserum that detects eIF2α independently of Ser51 phosphorylation. As shown in Fig. 2B, no Ser51 phosphorylation was detected in cells not expressing PKR (vector control; lane 1) or in cells expressing kinase-dead PKR-K296R (lane 3) or the salt bridge mutants PKR-R262D or PKR-D266R (lanes 4 and 5). In contrast, phosphorylated eIF2α was readily detected in cells expressing wild type PKR or the PKR-R262D,D266R double mutant (Fig. 2B, lanes 2 and 6). Additional immunoblot analysis using anti-PKR antibodies revealed that the wild type and various mutant forms of PKR were expressed at similar levels (Fig. 2B, top). Moreover, both wild type PKR and PKR-R262D,D266R migrated with a slower mobility on SDS-PAGE than inactive PKR-K296R or the single salt bridge mutants. This latter observation is consistent with the notion that the mutations disrupting the intermolecular salt bridge interaction impair PKR autophosphorylation.

To directly test the impact of the mutations on PKR activity, we partially purified the PKR proteins from yeast using anti-FLAG M2 resin and performed immunokinase assays using recombinant GST-eIF2α (residues 1–180) as a substrate. Whereas wild type PKR readily autophosphorylated and phosphorylated eIF2α (Fig. 2C, lane 2), PKR-K296R as well as the PKR-R262D and PKR-D266R salt bridge mutants were defective for both autophosphorylation and eIF2α phosphorylation (Fig. 2C, lanes 3–5). Consistent with the in vivo results, the R262D,D266R double mutation restored PKR autophosphorylation and eIF2α phosphorylation activity in vitro (Fig. 2C, lane 6). Thus, we conclude that the salt bridge interactions between residue 262 in one PKR protomer and residue 266 in the other protomer at the PKR dimer interface are critical for kinase activation.

Reciprocal Exchange of Residues Arg599 and Glu603 Restores PERK Function in Vivo and in Vitro—Like PKR, high level expression of PERK in yeast lacking GCN2 results in phosphorylation of eIF2α and inhibition of cell growth (11). Consistent with these previous results, we found that high level expression of FLAG- and His6-tagged C. elegans PERK (residues 26–1077) from a galactose-inducible promoter impaired the growth of the gcna2Δ strain H2557 (Fig. 2D, left, row 2) and resulted in significant phosphorylation of eIF2α on Ser51 (Fig. 2E, lane 2). This growth inhibition was attributed to PERK kinase activity and to eIF2α phosphorylation, since it was relieved both by a K633R mutation in the essential and conserved subdomain II residue of the kinase domain (Fig. 2D, left, row 3) and in the eIF2α-S51A strain J223 (Fig. 2D, right, row 2). Residues Arg599 and Glu603 in PERK correspond to the salt bridge-interacting residues Arg262 and Asp266 in PKR (Fig. 1A). To test the importance of the putative salt bridge in PERK, we substituted individually the residues Arg599 and Glu603 by the oppositely charged residues Glu and Arg, respectively. The single mutations R598E and E603R eliminated PERK toxicity and Ser51 phosphorylation in yeast (Figs. 2, D and E, rows 4 and 5) but did not affect kinase expression (Fig. 2E, top). In contrast, and consistent with the idea that PERK residues 599 and 603 form an essential trans-dimer salt bridge interaction, the double mutation R599E,E603R restored PERK toxicity (Fig. 2D, row 6) and Ser51 phosphorylation (Fig. 2E, lane 6) in vivo. Like PKR, functional forms of PERK migrate with a slower mobility on SDS-PAGE than inactive mutants. Consistent with the notion that the double mutation restored PERK function, wild type PERK and PERK-R599E,E603R migrated more slowly on the gels than the three PERK single mutants (Fig. 2E, top).

To examine the impact of the salt bridge mutations on PERK kinase activity in vitro, wild type PERK and the various mutants were overexpressed in yeast and immunoprecipitated using anti-FLAG M2-agarose resin. The immune complexes were then incubated with recombinant GST-eIF2α and [γ-33P]ATP under in vitro kinase reaction conditions, and the products were resolved by SDS-PAGE. Wild type PERK readily phosphorylated eIF2α (Fig. 2F, lane 2); however, the subdomain II PERK-K663R mutant and the putative salt bridge mutants PERK-R599E and PERK-E603R failed to phosphorylate eIF2α in vitro (Fig. 2F, lanes 3–5). The PERK-R599E,E603R double mutant regained eIF2α kinase activity (Fig. 2F, lane 6), albeit not up to the level observed with wild type PERK. Immunoblot analysis of the PERK immune complexes revealed greater amounts of the three inactive mutant forms of PERK than of the wild type and double mutant (Fig. 2F, middle), indicating that the lack of kinase activity associated with the single mutations was not due to poor kinase expression. Interestingly, wild type PERK and the active PERK-R599E,E603R double mutant migrated with a slower mobility on SDS-PAGE than the three inactive mutants (Fig. 2F, middle). Since changes in PERK mobility on SDS-PAGE have been attributed to autophosphorylation of the kinase (1, 30), the faster mobility of the PERK-R599E and E603R single mutants versus the PERK-R599E,E603R double mutant indicates that the salt bridge interaction is required for PERK kinase activation and autophosphorylation.

It is noteworthy that Arg599 in C. elegans PERK corresponds to Arg587 in human PERK and Arg262 in mouse PERK. Mutation of Arg587 to Gln in human PERK was found in a patient with Wolcott-Rallison syndrome, a rare form of diabetes (32). To assess the impact in mammalian cells of disrupting the putative salt bridge interaction in PERK, the single mutations R584D and D598R were introduced into a mouse PERK expression vector. Expression of wild type mouse PERK in HEK 293 Ebna T...
eIF2α Kinase Activation Requires Trans-dimer Salt Bridge

FIGURE 3. Intermolecular salt bridge interaction required for PERK kinase activity in mammalian cells. HEK 293 EbnTa cells were transfected with the empty vector pcDNA1 or derivatives designed to express Myc-tagged wild type mouse PERK or the indicated mutants. Thirty hours following transfection, cells were harvested and lysed, and 3-µg aliquots of WCEs were subjected to SDS-PAGE and immunoblot analysis using phosphospecific antibodies directed against phosphorylated Ser51 of eIF2α (middle). The membrane was then stripped and probed using monoclonal antibodies against eIF2α (bottom). On a separate gel, 3-µg aliquots of the WCEs were subjected to 4–12% SDS-PAGE and immunoblot analysis using monoclonal anti-Myc antibodies to detect PERK (top). Results are representative of at least four independent experiments.

cells significantly elevated (2–5-fold) the levels of phosphorylated eIF2α compared with cells transfected with an empty vector (Fig. 3, middle, lane 2 versus lane 1). In contrast, cells expressing inactive PERK-K618A or either single salt bridge mutant, PERK-R584D or PERK-D588R, displayed low levels of eIF2α phosphorylation, similar to what was observed in cells transfected with the empty vector (Fig. 3, middle, lanes 3–5). Thus, disruption of the putative salt bridge interaction blocked PERK activity in mammalian cells. Importantly, expression of the double mutant PERK-R584D,D588R, predicted to restore the salt bridge interaction, resulted in elevated levels of eIF2α phosphorylation (2–4-fold), similar to what was observed in cells expressing wild type PERK (Fig. 3, middle, lane 6 versus lane 2). Examination of PERK expression using the Myc epitope tag confirmed that the various forms of mouse PERK were expressed to similar levels in the transfected cells (Fig. 3, top). In addition, WT PERK and the double mutant PERK-R584D,D588R migrated with a slower mobility on SDS-PAGE than the inactive PERK mutants, consistent with activation and autophosphorylation of WT PERK and the PERK double mutant (Fig. 3, top). Since the R584D mutation inactivated mouse PERK, we propose that the corresponding R587Q mutation in PERK from the patients with Wolcott-Rallison syndrome inactivates the kinase by blocking formation of a trans-dimer salt bridge. This notion is consistent with the inability of the human PERK-R587Q mutant to phosphorylate eIF2α either in vitro or in yeast cells (33). We conclude that the intermolecular salt bridge identified in PKR is also critical for the function of PERK following its oligomerization in response to ER stress. Moreover, the apparent Arg-Glu salt bridge in C. elegans PERK differs from the observed Arg-Asp salt bridge in PKR, suggesting at least some plasticity in the dimer interface of the eIF2α kinases.

Reciprocal Exchange of GCN2 Residues Arg594 and Asp598 Restores GCN4 Translational Control—To test the importance of the putative salt bridge-interacting residues in yeast GCN2, we substituted Arg594 and Asp598 with the oppositely charged residues Asp and Arg, respectively. Whereas yeast lacking GCN2 grow well on synthetic minimal medium, the gcn2Δ strains fail to grow on medium containing 3-AT, because they cannot derepress GCN4 expression (6) (see also Fig. 4A, row 1). Like the K628R mutation in kinase subdomain II, the R594D and D598R mutations in GCN2 impaired growth on 3-AT medium (Fig. 4A, rows 3–5). However, when the salt bridge mutations were combined in GCN2-R594D,D598R, growth on 3-AT medium was restored (Fig. 4A, row 6). Consistent with these growth phenotypes, eIF2α phosphorylation on Ser51 was readily detected in extracts of cells treated with 3-AT and expressing either wild type GCN2 or the GCN2-R594D,D598R double mutant (Fig. 4B, lanes 2 and 6). In contrast, no eIF2α phosphorylation was detected in extracts of yeast expressing the kinase-dead GCN2-K628R mutant or either of the single salt bridge mutants (Fig. 4B, lanes 3–5). Since the various GCN2 mutants were expressed at levels similar to wild type GCN2 (Fig. 4B, top), the differences in eIF2α phosphorylation can be attributed to changes in kinase activity rather than kinase expression. Thus, like PKR and PERK, reversing the polarity of the putative salt bridge-interacting residues in GCN2 yielded a phenotype equivalent to the wild type kinase. These results strongly indicate that a salt bridge interaction forms between Arg594 and Asp598 during GCN2 activation.

To test the importance of the salt bridge for GCN2 function in vitro, FLAG- and His6-tagged forms of wild type and the various mutant versions of GCN2 were expressed in yeast under the control of a galactose-inducible promoter. The GCN2 proteins were immunoprecipitated from crude cell extracts using anti-FLAG M2 resin and then tested in immunokinase assays using recombinant eIF2α and [γ-32P]ATP. Wild type GCN2 readily autophosphorylated and phosphorylated eIF2α (Fig. 4C, lane 2), whereas the subdomain II GCN2-K628R mutant and the salt bridge GCN2-R594D and GCN2-D598R single mutants failed to autophosphorylate or phosphorylate eIF2α in vitro (Fig. 4C, lanes 3–5). The GCN2-R594D,D598R double mutant showed significant eIF2α phosphorylation activity (Fig. 4C, lane 6), consistent with the in vivo results. Surprisingly, we did not detect restoration of kinase autophosphorylation by the GCN2-R594D,D598R double mutation (Fig. 4C, lane 6). However, since eIF2α phosphorylation is a more sensitive assay than kinase autophosphorylation, the apparent lack of autophosphorylation may indicate that reversal of the salt bridge polarity does not fully restore GCN2 function. Taking the in vivo and in vitro studies together, we conclude that salt bridge interaction detected in the PKR crystal structure is conserved in GCN2 and is critical for kinase activity.

From the crystal structures of PKR and GCN2, two different dimeric configurations for the eIF2α kinases were identified. In this report, we presented genetic and biochemical data indicating that the dimer interface observed in the PKR crystal structure is functionally important for activation of PKR, PERK, and GCN2. Although mutation of the predicted salt bridge-interacting residues in the fourth eIF2α kinase HRI impaired the ability of HRI to complement the function of GCN2 in yeast, the double mutation designed to restore the salt bridge interaction with opposite polarity failed to restore HRI function (data not
eIF2α Kinase Activation Requires Trans-dimer Salt Bridge

A reciprocal exchange of GCN2 residues Arg594 and Asp598 restores GCN4 translational control and eIF2α phosphorylation. A, reciprocal exchange of GCN2 residues Arg594 and Asp598 restores GCN4 translational control in yeast. Plasmids expressing wild type GCN2, the indicated GCN2 mutants, or no GCN2 (vector) were introduced into yeast strain H1894 expressing wild type eIF2α. C, in vitro protein kinase assay prepared, and 5-μg aliquots were subjected to SDS-PAGE followed by immunoblot analysis using phosphospecific antibodies directed against phosphorylated Ser51 of eIF2α (middle). The membrane was then stripped and probed using polyclonal antiserum against yeast eIF2α (bottom). C, in vitro protein kinase assay prepared from cells expressing no GCN2 (vector) or the indicated wild type or mutant forms of FLAG-tagged GCN2, GST-eIF2α (residues 1–180) and [γ-32P]ATP. Kinase reactions were resolved on SDS-PAGE, stained with Coomassie Blue (bottom), and subjected to autoradiography to visualize phosphorylated eIF2α (eIF2α-P) and phosphorylated GCN2 (GCN2-P) (top).

Additional mutational studies on PKR and GCN2 provide further support for the importance of the dimer interface detected in the PKR crystal structure. The side chains of the salt bridge-interacting residues project from helix α0, and the aliphatic portion of Arg262 in PKR is oriented in part by van der Waals interactions with Trp327 (15). Our previous mutational studies on PKR revealed that only the aromatic residues Tyr and Phe could functionally substitute for Trp327 (15). Similarly, we found that substitution of Val for Trp662 in GCN2, which corresponds to Trp327 in PKR and is predicted to alter the orientation of Arg599, eliminated GCN4 translational control. Thus, a specific orientation of the salt bridge-interacting residues may be required for proper dimer formation and kinase activation.

Based on our studies, we propose that the PKR crystal structure provides a view of the active state of the eIF2α kinase domain. This view is supported by the fact that the PKR molecules in the crystal structure are stoichiometrically phosphorylated on Thr446 in the activation loop and bound to the substrate eIF2α in a manner such that Ser51 can access the kinase active site (23). Accordingly, we propose that the altered architecture of the GCN2 crystal structure represents an inactive state. Consistent with this notion, the GCN2 kinase domains were not phosphorylated, and the activation loop was disordered in the structure (24). Since GCN2 is a constitutive dimer, it is plausible to propose that binding of uncharged tRNA to the HisRS domain allosterically induces a conformational change in which the GCN2 kinase domains rotate 180° normal to the dimerization surface and thus transpose from their antiparallel orientation to a parallel orientation as observed in the PKR structure. Future mutational studies on the PKR kinase domain as well as a structure of the inactive form of PKR should provide prepared, and 5-μg aliquots were subjected to SDS-PAGE followed by immunoblot analysis using phosphospecific antibodies directed against phosphorylated Ser51 of eIF2α (middle). The membrane was then stripped and probed using polyclonal antiserum against yeast eIF2α (bottom). C, in vitro protein kinase assay prepared from cells expressing no GCN2 (vector) or the indicated wild type or mutant forms of FLAG-tagged GCN2, GST-eIF2α (residues 1–180) and [γ-32P]ATP. Kinase reactions were resolved on SDS-PAGE, stained with Coomassie Blue (bottom), and subjected to autoradiography to visualize phosphorylated eIF2α (eIF2α-P) and phosphorylated GCN2 (GCN2-P) (top).

3 M. Dey, unpublished observation.
additional insights into the role of dimerization for eIF2α kinase domain activation.

Acknowledgments—We thank Alan Hinnebusch, Elaine Chiu, Dante Neculai, Stefan Rothenburg, and members of the Dever, Sicheri, and Hinnebusch laboratories for valuable discussions, and we thank David Ron and Heather Harding for the pCDNA-PERK expression vectors and for advice on mammalian cell experiments.

REFERENCES

1. Ron, D., and Harding, H. P. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 547–560, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Kaufman, R. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 503–527, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. Hinnebusch, A. G. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 185–243, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
4. Dever, T. E. (2002) Cell 108, 545–556
5. Chen, J.-J. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 529–546, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
6. Hinnebusch, A. G. (2005) Annu. Rev. Microbiol. 59, 407–450
7. Ramirez, M., Wek, R. C., Vazquez de Aldana, C. R., Jackson, B. M., Freeman, B., and Hinnebusch, A. G. (1992) Mol. Cell. Biol. 12, 5801–5815
8. Dever, T. E., Chen, J. J., Barber, G. N., Cigan, A. M., Feng, L., Donahue, T. F., London, I. M., Katze, M. G., and Hinnebusch, A. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4615–4620
9. Shi, Y., Vattem, K. M., Sood, R., An, J., Liang, J., Stramm, L., and Wek, R. C. (1998) Mol. Cell. Biol. 18, 7499–7509
10. Chong, K. L., Feng, L., Schappert, K., Meurs, E., Donahue, T. F., Friesen, J. D., Hovanessian, A. G., and Williams, B. R. G. (1992) EMBO J. 11, 1553–1562
11. Sood, R., Porter, A. C., Ma, K., Quilliam, L. A., and Wek, R. C. (2000) Biochem. J. 346, 281–293
12. Yang, J. M., London, I. M., and Chen, J. J. (1992) J. Biol. Chem. 267, 20519–20524
13. Qiu, H., Garcia-Barrio, M. T., and Hinnebusch, A. G. (1998) Mol. Cell. Biol. 18, 2697–2711
14. Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000) Nat. Cell. Biol. 2, 326–332
15. Dey, M., Cao, C., Dar, A. C., Tamura, T., Ozato, K., Schier, F., and Dever, T. E. (2005) Cell 122, 901–913
16. Ung, T. L., Cao, C., Lu, J., Ozato, K., and Dever, T. E. (2001) EMBO J. 20, 3728–3737
17. Wu, S., and Kaufman, R. J. (1997) J. Biol. Chem. 272, 1291–1296
18. Zhang, F., Romano, P., Nagamura-Inoue, T., Tian, B., Dever, T. E., Mathews, M. B., Ozato, K., and Hinnebusch, A. G. (2001) J. Biol. Chem. 276, 24946–24958
19. Ma, K., Vattem, K. M., and Wek, R. C. (2002) J. Biol. Chem. 277, 18728–18735
20. Credle, J. J., Finer-Moore, J. S., Papa, F. R., Stroud, R. M., and Walter, P. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 18773–18784
21. Dar, A. C., and Sicheri, F. (2002) Mol. Cell 10, 295–305
22. Lu, P. D., Jousses, C., Marciniak, S. J., Zhang, Y., Novoa, I., Scheuner, D., Kaufman, R. J., Ron, D., and Harding, H. P. (2004) EMBO J. 23, 169–179
23. Dar, A. C., Dever, T. E., and Sicheri, F. (2005) Cell 122, 887–900
24. Padyana, A. K., Qiu, H., Roll-Mecak, A., Hinnebusch, A. G., and Burley, S. K. (2005) J. Biol. Chem. 280, 29289–29299
25. Harashima, S., Hannig, E. M., and Hinnebusch, A. G. (1987) Genetics 117, 409–419
26. Kawagishi-Kobayashi, M., Silverman, J. B., Ung, T. L., and Dever, T. E. (1997) Mol. Cell. Biol. 17, 4146–4158
27. Cesareni, G., and Murray, I. A. H. (1987) in Genetic Engineering: Principals and Methods (Setlow, J. K., and Hollaender, A., eds) pp. 135–154, Plenum Press, New York, NY
28. Dong, J., Qiu, H., Garcia-Barrio, M., Anderson, J., and Hinnebusch, A. G. (2000) Mol. Cell 6, 269–279
29. Romano, P. R., Garcia-Barrio, M. T., Zhang, X., Wang, Q., Taylor, D. R., Zhang, F., Herring, C., Mathews, M. B., Qin, J., and Hinnebusch, A. G. (1998) Mol. Cell. Biol. 18, 2282–2297
30. Harding, H. P., Zhang, Y., and Ron, D. (1999) Nature 397, 271–274
31. DeLano, W. L. (2004) The PyMol Users Manual, DeLano Scientific, San Carlos, CA
32. Delepine, M., Nicolinolo, M., Barrett, T., Golamouly, M., Lathrop, G. M., and Julier, C. (2000) Nat. Genet. 25, 406–409
33. Senee, V., Vattem, K. M., Delepine, M., Rainbow, L. A., Haton, C., Lecoz, A., Shaw, N. J., Robert, J. I., Rooman, R., Dietloff-Zito, C., Michaud, J. L., Bin-Abbas, B., Taha, D., Zabel, B., Franceschini, P., Topaloglu, A. K., Lathrop, G. M., Barrett, T. G., Nicolinolo, M., Wek, R. C., and Julier, C. (2004) Diabetes 53, 1876–1883