Inactivation of the PKR Protein Kinase and Stimulation of mRNA Translation by the Cellular Co-Chaperone P58^{IPK} Does Not Require J Domain Function†

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ABSTRACT: P58^{IPK} was discovered as an inhibitor of the interferon-induced, protein kinase, PKR. Upon virus infection, PKR can, as part of the host defense system, inhibit mRNA translation by phosphorylating the α subunit of protein synthesis eukaryotic initiation factor 2 (eIF-2α). We previously found that influenza virus recruits the cellular P58^{IPK} co-chaperone to inhibit PKR activity and thus facilitate viral protein synthesis. P58^{IPK} contains nine tetratricopeptide repeat (TPR) motifs in addition to the highly conserved J domain found in all DnaJ chaperone family members. To define the role of molecular chaperones in regulating cell growth in addition to PKR regulation, we performed a detailed analysis of the P58^{IPK} J domain. Using growth rescue assays, we found that the P58^{IPK} J domain substituted for the J domains of other DnaJ proteins, including DnaJ in *Escherichia coli* and Ydj1 in *Saccharomyces cerevisiae*. This is the first time a cellular J domain from a mammalian DnaJ family member was shown to be functional in both prokaryotic DnaJ and eukaryotic Ydj1 constructs. Furthermore, point mutations within the conserved HPD residue cluster of the P58^{IPK} J domain disrupted P58^{IPK} J function including stimulation of ATPase activity of Hsp70. However, the P58^{IPK} HPD mutants still inhibited PKR activity and thus supported cell growth in a yeast rescue assay. Overexpression of the HPD mutants of P58^{IPK}, similar to their wild-type counterpart, also stimulated mRNA translation in a mammalian cell system. Taken together, our data necessitate a model of P58^{IPK} inhibition of PKR kinase activity and stimulation of mRNA translation, which does not require classical J domain function found in the DnaJ molecular chaperone family.

The interferon-induced serine-threonine protein kinase PKR[1] (protein kinase RNA-dependent) plays a pivotal role in the host defense system against virus infection (*1*). Upon activation by double-stranded RNA (dsRNA), PKR becomes autophosphorylated and phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF-2α). Phosphorylation of eIF2α inhibits initiation of mRNA translation and blocks global protein synthesis, including the synthesis of viral proteins (*2*). To antagonize the anti-viral function of PKR, viruses have developed mechanisms to suppress PKR activity. For example, the vaccinia-virus protein K3L, which has sequence similarity to eIF-2α, interacts with PKR as a pseudosubstrate (*3*, *4*). Binding of K3L inhibits PKR activity and releases the PKR-mediated translational shutoff. In addition, adenovirus encodes virus-associated I (VAI) RNA, which competes with dsRNA activators for binding to PKR. Sequestering PKR from dsRNA activation therefore down-regulates PKR activity (*5*, *6*). Influenza virus inactivates PKR by recruiting a cellular inhibitor, P58^{IPK} (*7*). Upon viral infection, P58^{IPK} is released from its own inhibitor, Hsp40 (Hdj1) and forms an inactive complex with PKR (*8*). Binding of P58^{IPK} inhibits both kinase activity and dimerization of PKR and thereby blocks the autophosphorylation of PKR and phosphorylation of eIF2α (*9*, *10*). In addition to recruiting P58^{IPK}, influenza virus also inactivates PKR through its nonstructural protein NS1, by both direct interaction of NS1 with PKR, and by sequestering dsRNA activator from PKR (*11*, *12*).

P58^{IPK} belongs to the family of tetratricopeptide repeat (TPR) containing proteins. It consists of nine tandemly arranged TPR motifs, which are known to mediate protein−protein interaction (Figure 1A). In addition, it contains a C-terminal J domain, which identifies P58^{IPK} as a member of the DnaJ molecular chaperone family (*13*). DnaJ molecular chaperones, together with Hsp70s, another family of molecular chaperones, function in a variety of cellular process, including protein translation, translocation, and folding (*14*−*16*). In addition, DnaJ proteins and Hsp70s are involved in Hsp90-mediated signal transduction pathways, regulating the biological activity of steroid hormone receptors and protein kinases including Src, Raf, Mek, casein kinase II, and eEF-2

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[1] Abbreviations: PKR, protein kinase RNA-dependent; dsRNA, double-stranded RNA; Hsp, heat shock proteins; eIF-2α, the α subunit of eukaryotic translational initiation factor 2; TPR, tetratricopeptide repeat; PCR, polymerase chain reaction; SEAP, secreted embryonic alkaline phosphatase; GST, glutathione *S*-transferase; ER, endoplasmic reticulum; EF1α, human elonglation factor-1α; CMV, cytomegalovirus; WT, wild-type.

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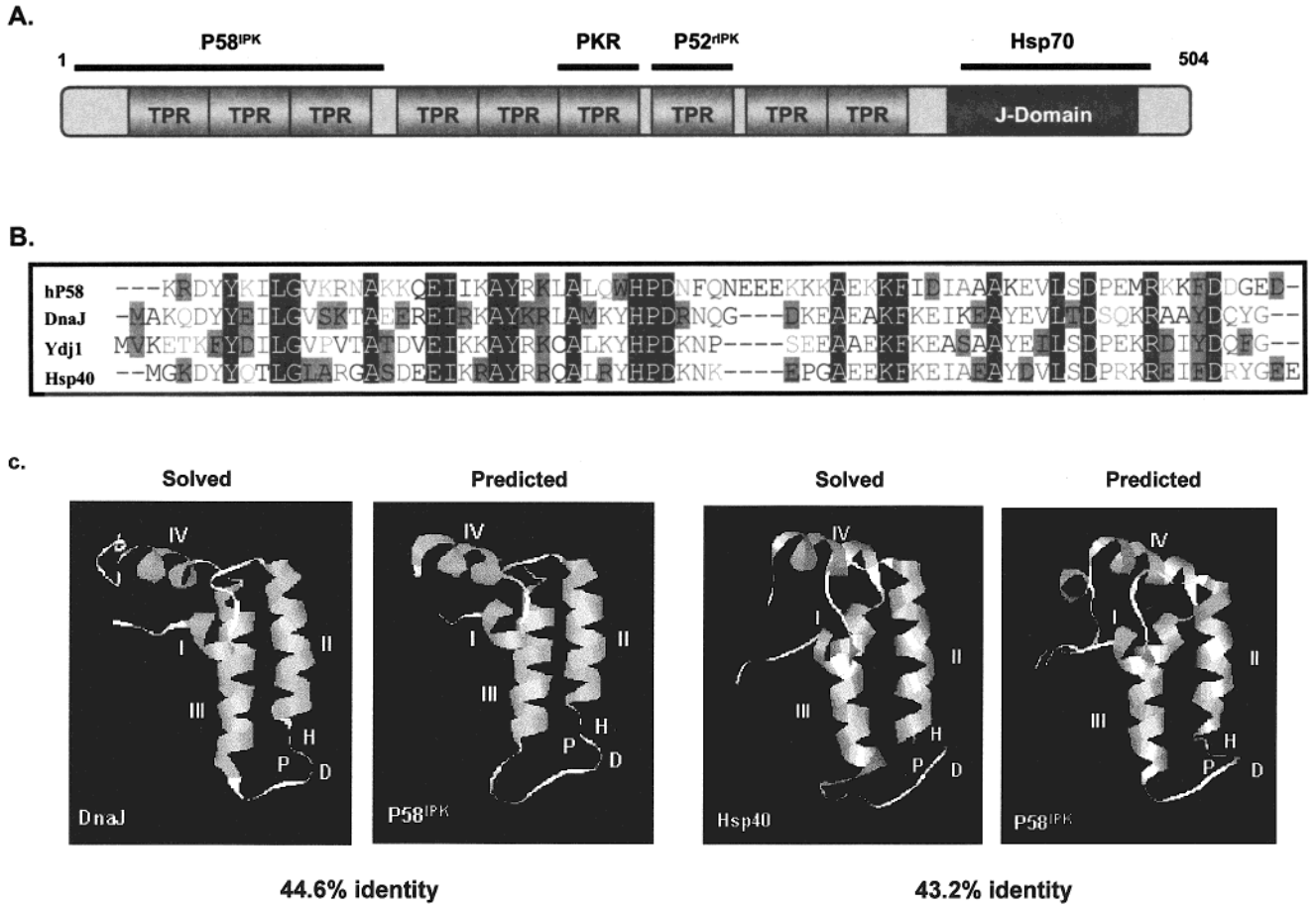

FIGURE 1: J domain sequence alignment and structure modeling. (A) Schematic representation of the domain structures of P58IPK, including nine tetratricopeptide repeat (TPR) motifs and the C-terminal J domain. Domains involved in P58IPK dimerization or interaction with PKR, P52rIPK, or Hsp70 are indicated by solid lines. The J domain is a region of approximately 70 amino acids and is highly conserved among all DnaJ chaperone family members. (B) J domain sequences of human P58IPK (amino acids 393−465), DnaJ (amino acids 1−70), Ydj1 (amino acids 1−70) and human Hsp40 (amino acids 1−70) were aligned using AlignX (InforMax, Inc., Bethesda, MD). Identical amino acids are highlighted, including the conserved HPD residues. (C) The three-dimensional structure of the P58IPK J domain was predicted using the SWISS-MODEL computer program (*62*) based on the available NMR structures of DnaJ and Hsp40 (*63*−*66*). The predicted model was visualized using the RasMol Molecular Visualization Program (*67*). The sequence identities between P58IPK and DnaJ/Hsp40 are indicated. The HPD loops between helices II and helices III are also marked.

kinase (*17*). Interaction between DnaJ proteins and Hsp70s is believed to be important for their cooperative function. DnaJ stimulates the weak intrinsic ATPase activity of Hsp70 and thereby regulates substrate-binding affinity of Hsp70, which is dependent on its nucleotide binding form (*18*−*20*). The J domain of DnaJ, a signature structure of this protein family, is the interacting domain with Hsp70. The highly conserved HPD sequence, which is located in the loop between the second and third helices of the J domain, is absolutely required for interaction with Hsp70 (*18*, *19*, *21*, *22*). Mutations of the HPD sequence in several DnaJ chaperones, including *Escherichia coli* DnaJ, yeast Ydj1, Sis1, and Sec63, mammalian Hsp40 (hdj1), hsj1 and $hTid1_{L/S}$, as well as the large T antigen of simian virus 40 (SV40), have been shown to disrupt the J domain functions of these co-chaperones (*22*−*28*).

Molecular chaperones are also involved in eIF-2α kinase-mediated translational regulation. Maturation and regulation of several eIF2α kinase family members, including PKR, PERK (PKR-like ER kinase), Gcn2 (an eIF2α kinase related to amino acid biosynthesis), and HRI (a heme-regulated eIF-2α kinase), requires the participation of molecular chaperones such as Hsp70, Hsp90, and co-chaperone CDC37 (*29*−*36*). Both Hsp70 and Hsp40 (hdj1) interact with P58IPK (*37*). We recently identified another cellular regulator of P58IPK, called P52rIPK, which shares extensive homology with a segment of Hsp90 (*38*). Because multiple chaperones or chaperone-like molecules interact with P58IPK, and since P58IPK itself contains a J domain, we conducted the present study to further delineate the molecular mechanisms of P58IPK and PKR regulation. We succeeded in demonstrating that the J domain of P58IPK functioned as a classical J domain of the DnaJ family, able to replace the J domain of prokaryotic DnaJ and eukaryotic Ydj1 to support cell growth in a yeast growth rescue assay. Unexpectedly, the function of the highly conserved P58IPK J domain was not required to inhibit PKR kinase activity, nor was it required for the resultant P58IPK stimulation of mRNA translation.

EXPERIMENTAL PROCEDURES

Bacterial and Yeast Strains. E. coli WKG190 (*araD139* Δ*ara714 dnaJ::Tn 10*−*46* Δ*cbpA::kan*), kindly provided by William L. Kelley, University of Geneva (*39*), was grown on LB-ampicilin plates containing 0.01% arabinose at 30, 35, or 37 °C. *Saccharomyces cerevisiae ACY-17b* (MATα, *ade2-1 leu2-3 112 his3-11, 15 trp1-1 ura3-1 can1-100 ydj1-*

2::HIS3 LEU2::ydj1-151), a generous gift from Avrom J. Caplan, Mount Sinai Medical Center (*40*, *41*), and *RY1-1* [MATa *ura3-52 leu2-3 leu2-112 gcn2*Δ *trp1*-Δ*63 (GAL-CYC1-PKR LEU2)$_2$ at leu2*] (*42*), were grown on media containing either 2% glucose or 10% galactose at various temperatures.

Construction of Plasmids. All P58^{IPK} constructs used in this study were derived from the human P58^{IPK} cDNA which is different from the bovine P58^{IPK} used in the previous studies. To construct P58-pYX233, the *Eco*RI−*Bam*HI fragment of pBD-P58^{IPK} (*43*) was cloned into the same sites of the vector pYX233 (Novagen Co., Madison, WI), which contains the selectable marker *TRP1*. Point mutations in the HPD loop of P58^{IPK} (H-Q or HPD-AAA) were then introduced into P58-pYX233 using the QuickChange Site-Directed Mutagenesis Kit (Stratagene Co., La Jolla, CA). The *Eco*RI−*Xho*I fragment of wild-type or mutant P58-pYX233 was then cloned into the same sites of the pYes2 vector (Invitrogen Co., Carlsbad, CA), which contains the selectable marker *URA3*. P58^{IPK} expression in pYES2 is under the control of a galactose-inducible promoter. To express wild-type and mutant P58^{IPK} under the control of the EF1α promoter in mammalian cells, the *Eco*RI (blunt)−*Bam*HI fragment from wild-type or mutant P58-pYX233 constructs was cloned into the *Asp* 718 (blunt) and *Bam*HI sites of pEF4/HISA (Invitrogen Co., Carlsbad, CA).

To make P58/DnaJ chimeras, an *Eco*RI−*Kpn*I PCR fragment, containing the J domain of P58 (either wild-type, mutant H-Q, or mutant HPD-AAA), was cloned into the same sites of pWKG90 to replace the original J domain of DnaJ (*39*). The *Eco*RI−*Kpn*I PCR fragment of the J domain of P58 (either wild-type, H-Q, or HPD-AAA), together with a *Kpn*I−*Bam*HI PCR fragment containing the Ydj1 N-terminal J domain deletion, was triply ligated into the *Eco*RI and *Bam*HI sites of pYX233 to construct wild-type and mutant P58/Ydj1 fusion proteins. All PCR products were sequenced to ensure that no extra mutations were introduced during amplification.

GST-P58 fusion constructs were prepared by cloning the *Eco*RI (blunt)−*Xho*I fragment of wild-type or mutant P58-pYX233 into the *Sma*I and *Xho*I sites of pGEX4T-3 (Amersham Pharmacia Biotech Inc., Piscataway, NJ). GST-P58 fusion proteins (wild-type or HPD-AAA mutant) were then purified from *E. coli* and used in the ATPase assay described below.

Transfection Procedures and Secreted Embryonic Alkaline Phosphatase (SEAP) Assays. COS-1 cells, grown in six-well plates, were cotransfected with 2 μg of SEAP DNA and 2 μg of individual PKR inhibitor construct DNA per well using SuperFect Transfection Reagent (Qiagen Inc., Valencia, CA). Forty hours after transfection, culture medium was replaced by pre-warmed fresh medium. Following a 2 h incubation, culture medium was collected from each well, heated to 65 °C for 5 min, and assayed for SEAP activity as previously described (*44*).

P58^{IPK} Protein Purification and ATPase Assay. E. coli cells transformed with GST-P58 constructs (wild-type or HPD-AAA mutant), or the GST cloning vector alone, were treated with 0.6 mM IPTG for 4 h at 37 °C. Cell lysates were then prepared by sonication in PBS and incubated with glutathione agarose beads for 1 h at 4 °C. The beads were then washed with 1× PBS containing 1 M NaCl and 1% Triton X-100 and subsequently eluted in buffer containing 200 mM Tris hydrochloride (pH 8.0) and 50 mM reduced glutathione. The eluted proteins were dialyzed against ATPase assay buffer (50 mM Tris hydrochloride [pH 7.5], 2 mM $MgCl_2$, 10 mM KCl, 17 mM β-mercaptoethanol). The ATPase assay was performed at 30 °C for 15 min in 40 μL of ATPase assay buffer (see above) containing 10 μM [α-^{32}P]ATP, 0.2 μM Hsp70 (gift from Dr. Richard I. Morimoto, Northwestern University), and 1 μM GST, Hsp40 (Stressgen Co., Victoria, BC, Canada), or GST-P58 (WT and mutant). The reaction mixtures were preequilibrated at 30 °C for 10 min before adding substrate ATP to initiate the reaction. Aliquots were collected at each time point and applied to polyethyleneimine thin-layer chromatography plates and developed as previously described (*37*, *45*). PhosphorImager analysis (Molecular Dynamics, Inc.) was used to measure the fraction of ATP hydrolyzed to ADP. Slopes were determined by linear regression analysis and the ATPase activity (mole of ATP hydrolyzed per minute per mole of Hsp70) was calculated and presented.

Immunoblot Analysis. Yeast cells were disrupted using a bead-beater at 4 °C. Cells were lysed in buffer containing 50 mM Tris hydrochloride (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 1% Igepal CA-630, 10% glycerol, and EDTA-free complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cell lysates with equal number of proteins were subjected to SDS−PAGE and immunoblot analysis using monoclonal antibodies against P58^{IPK} (9F10 and 2F8) as described previously (*46*).

RESULTS

P58^{IPK} J Domain Functionally Substitutes for the J Domains of DnaJ and Ydj1. P58^{IPK} contains a C-terminal J domain that is a signature structure of the molecular chaperone DnaJ family members (Figure 1A). It shares high sequence identity with DnaJ family members including *E. coli* DnaJ (44.6%), yeast Ydj1 (44%), and human Hsp40 (43.2%) (Figure 1B). Computer modeling, based on their sequence similarity and available NMR structures of DnaJ and Hsp40, suggests that the J domain structure of P58^{IPK} is highly conserved (Figure 1C). To dissect the function of the P58^{IPK} J domain, we first constructed a fusion protein in which the J domain of human P58^{IPK} replaced the J domain of DnaJ. The fusion protein P58^{IPK}/DnaJ was then transformed into *E. coli WKG190*, in which the two *E. coli* DnaJ homologues of DnaJ and CbpA are deleted. The fresh transformants were then spotted onto plates containing 0.01% arabinose to induce the expression of chimeras under the control of the pBAD promoter. As shown in Figure 2, the P58^{IPK}/DnaJ fusion protein could complement for bacterial growth at both 35 and 37 °C, similar to the positive control which carried a wild-type DnaJ protein. Negative control experiments showed that transformation with the pBAD22ΔHB2 vector alone did not support cell growth. These results suggest that the P58^{IPK} J domain can functionally substitute for the DnaJ J domain to support *E. coli* cell growth at the nonpermissive temperatures.

Similar experiments were performed in the budding yeast *S. cerevisiae* to determine whether the P58^{IPK} J domain could functionally substitute for the J domain of a eukaryotic DnaJ homologue, the yeast Ydj1 protein. The J domain of P58^{IPK}

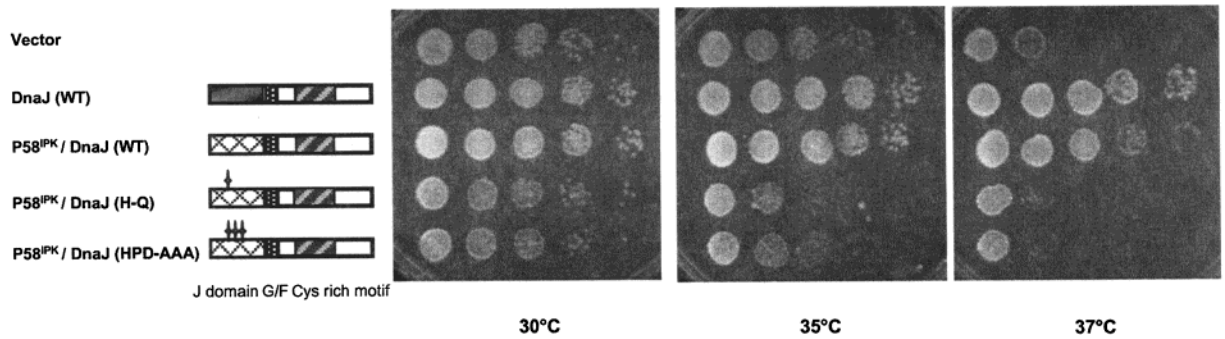

FIGURE 2: J domain of P58IPK can substitute for the J domain of DnaJ to support *E. coli* growth. Chimeric DnaJ constructs containing the J domain from P58IPK (WT, H-Q, and HPD-AAA mutants) are depicted on the left. Mutations within the J domain are indicated by one (H-Q) or three stars (HPD-AAA). G/F refers to the Glycine/Phenylalanine-rich motif and Cys refers to the Cysteine-rich zinc-finger motif of the DnaJ J domain. Chimeras, as well as the vector control (pBAD22ΔHB2), were transformed into *E. coli* strain *WKG190*, in which DnaJ and CbpA genes are deleted. Fresh transformed cells were counted and an equal number of cells were spotted in serial 10-fold dilutions onto LB-ampicillin plates containing 0.01% arabinose to induce chimera expression under the control of the pBAD promoter. Cells were grown overnight at the indicated temperatures before being photographed.

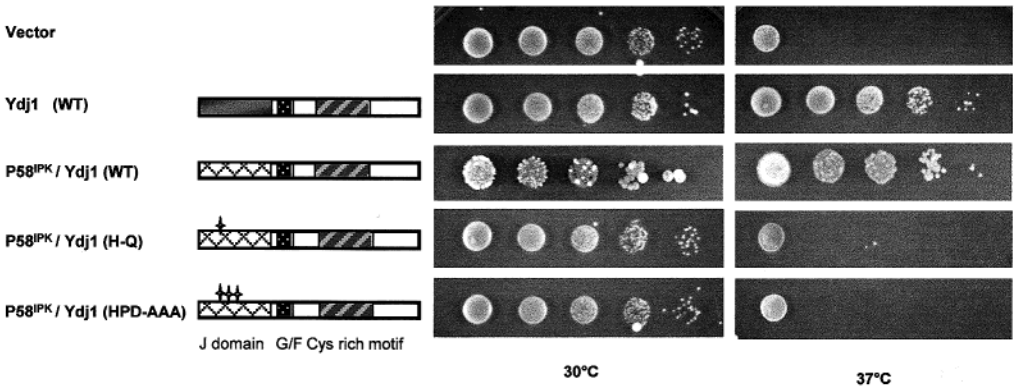

FIGURE 3: J domain of P58IPK can replace the J domain of Ydj1 to suppress the temperature sensitive phenotype of *ydj1−151*. Chimeric constructs containing the P58IPK J domain and Ydj1 backbone (WT and mutants) are depicted on the left. Mutations within the J domain are indicated by one (H-Q) or three stars (HPD-AAA), respectively. G/F refers to the Glycine/Phenylalanine-rich motif and Cys refers to the Cysteine-rich zinc finger motif of the Ydj1 J domain. Chimeras constructed in plasmid pYX233 were transformed into yeast *ACY-17b*, which carries the temperature sensitive *ydj1−151* mutation. Fresh transformed yeast cells were counted and an equal number of cells were spotted in serial 10-fold dilutions onto plates containing 10% galactose to induce expression of P58IPK/Ydj1 chimeric proteins. Cells were grown at both 30 and 37 °C for 4 days before being photographed.

was fused to Ydj1 to replace the N-terminal native J domain of Ydj1. Then the fusion construct, P58IPK/Ydj1 was transformed into *S. cerevisiae* strain *ACY-17b*, which carries a temperature sensitive allele of *ydj1-151*. As seen in Figure 3, the fusion protein P58IPK/Ydj1 could support yeast growth at 37 °C to the same extent as the wild-type Ydj1 protein, while the vector alone did not support cell growth at this temperature. On the basis of these data, from both prokaryotic and eukaryotic systems, we suggest that the P58IPK J domain functions as a classical J domain of the DnaJ protein family and thus probably retains other J domain functions, including dictating interactions with its Hsp70 partner.

HPD Point Mutations Abolish P58IPK J Domain Function. The P58IPK J domain model structure is similar to that of DnaJ and Hsp40 (Figure 1C). The HPD loop is conserved in the predicted J domain structure of P58IPK between helices II and III (Figure 1C). To further validate P58IPK J domain function, two point mutations, H-Q and HPD-AAA, were introduced into the HPD residues of the P58IPK J domain. Analogous mutations in a variety of DnaJ family proteins, such as DnaJ in *E. coli*, Ydj1, Sis1, Sec63, and Mdj1 in yeast, Hsp40, hsj1, and hTid-$1_{L/S}$ in human, as well as the large T antigen in simian virus 40, have been reported to disrupt their J domain functions (*22*−*28*). Fusion constructs containing these two point mutations were transformed into bacteria and yeast to test for cell growth rescue at nonpermissive temperatures. As revealed in Figures 2 and 3, the introduction of point mutations in the HPD residue cluster, either H-Q or HPD-AAA, resulted in loss of function for the P58IPK/DnaJ and P58IPK/Ydj1 chimeras. Neither was able to support cell growth compared to their wild-type counterparts. Importantly, the mutant P58IPK/DnaJ and P58IPK/Ydj1 proteins were produced at a level comparable to their wild-type counterparts (data not shown).

DnaJ molecular chaperones were shown to stimulate Hsp70-mediated ATP hydrolysis in the in vitro biochemical assays. For example, DnaJ was shown to stimulate the steady-state ATPase activity of DnaK, an *E. coli* Hsp70 member, by ~3-fold (*47*). Similar stimulation was observed in mammalian system as Hsp40 was reported to stimulate the ATPase activity of Hsc70, the constitutively expressed Hsp70 in the mammalian cytosol, by 3−7-fold depending on the particular preparations of the proteins and assay conditions (*47*, *48*). Mutations in the HPD loop of DnaJ or Ydj1 were shown to abolish the stimulation of ATPase activities of their Hsp70 partners (*22*, *27*). Previously we showed that P58IPK was able to stimulate the ATPase activity of Hsp70, similar to other DnaJ family members (*37*). We

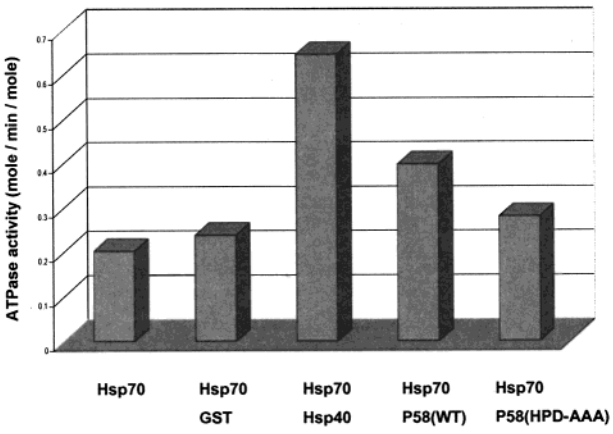

FIGURE 4: Stimulation of the ATPase activity of Hsp70 is impaired in the HPD mutant of P58^{IPK}. Hsp70 (0.2 μM) was incubated with substrate [α-^{32}P]ATP (10 μM) for 15 min at 30 °C in a 40 μL reaction mixture containing 1 μM GST, Hsp40, P58$^{IPK(WT)}$, or P58^{IPK}(HPD-to-AAA) as indicated. Additional experimental details are described in Experimental Procedures. ATPase activity is expressed as moles of ATP hydrolyzed per minute per mole of Hsp70.

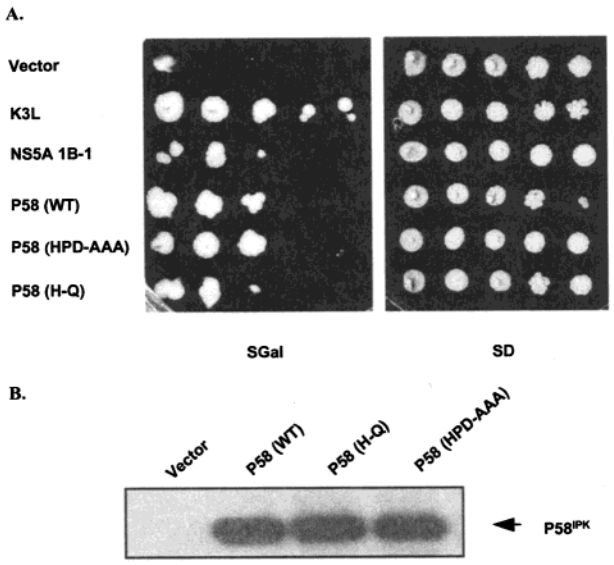

FIGURE 5: J domain function of P58^{IPK} is not required to inhibit PKR kinase activity in a yeast growth-suppression assay. (A) An equal number of yeast cells (strain *RY1-1*), carrying control plasmids or P58^{IPK} constructs (WT and mutants) as indicated, were spotted in serial 10-fold dilutions onto plates containing 10% galactose (SGal) or 2% glucose (SD). Growth on SGal plates induced expression of PKR as well as PKR inhibitors as indicated. The plates were incubated at 30 °C for 9 days before being photographed. (B) Yeast cells carrying vector (pYX233) or P58^{IPK} constructs (WT and mutants) were lysed as described in Experimental Procedures. Cell lysates, containing equal amounts of total protein, were subjected to SDS−PAGE and immunoblotting using anti-P58^{IPK} antibody to test P58^{IPK} protein expression in yeast.

are interested in investigating whether the HPD mutation of P58^{IPK} would affect its ability to stimulate the ATPase activity of Hsp70. Wild-type and mutant (HPD-AAA) P58^{IPK} proteins were purified and utilized in the steady-state ATPase assays. Using the mammalian Hsp70, the heat inducible Hsp70 member, we observed a 3.2-fold increase of ATP hydrolysis stimulated by Hsp40 (Figure 4). In agreement with our previous observation, the wild-type P58^{IPK} was shown to stimulate the ATPase activity of Hsp70 (~2-fold), although not as efficient as the positive control Hsp40 (Figure 4). However, we observed that the stimulation of the Hsp70 mediated ATP hydrolysis was impaired by introduction of the mutation in the HPD loop of P58^{IPK} (Figure 4), similar to the analogous HPD mutants of DnaJ or Ydj1 (*22*, *27*). Therefore, both the cell growth data from in vivo and the ATPase assay data from in vitro, suggest that the mutations within the HPD loop of P58^{IPK} abolish the J domain function of P58^{IPK}.

P58^{IPK} HPD Mutations Do Not Alter Suppression of PKR-Mediated Yeast Growth Arrest. We were interested in knowing whether these loss of J domain function mutants of P58^{IPK} would affect P58^{IPK} function such as PKR inhibition. An in vivo P58^{IPK} functional assay in *S. cerevisiae*, which measured PKR inhibition, was therefore attempted as previously described (*10*, *38*). In the yeast strain *RY1-1* (Δ*gcn2*), PKR was overexpressed under the control of the galactose-inducible promoter. Yeast cells containing this high level of PKR displayed a growth arrest phenotype since PKR overexpression resulted in eIF2α hyperphosphorylation and a subsequent suppression of yeast protein synthesis and cell growth. This growth arrest is known to be reversed by coexpression of PKR inhibitors inside the yeast cell. Several PKR inhibitors, including a hyperactive mutant of vaccinia virus protein K3L, hepatitis C virus protein NS5A, P58^{IPK}, and a dominant-negative PKR mutant (PKRΔ295−300), were all found to suppress growth arrest (*10*, *38*, *49*). Wild-type P58^{IPK}, when it was overexpressed in the yeast strain *RY1-1* under the control of the galactose-inducible promoter in the presence of galactose, could partially rescue PKR-mediated cell growth arrest at a similar level to other PKR inhibitors, including the hepatitis C virus NS5A protein (Figure 5A). Rather unexpectedly, we found that the two P58^{IPK} HPD mutants, although compromising the J domain function, still reversed PKR-mediated cell growth arrest, at least in part (Figure 5A). Both wild-type and mutant P58^{IPK} were expressed in yeast cells as detected by immunoblot using anti-P58^{IPK} antibody (Figure 5B). These data strongly suggest that the J domain function of P58^{IPK} is not required for P58^{IPK} inhibition of PKR kinase activity, at least under the assay conditions we used.

P58^{IPK} HPD Mutants Stimulate mRNA Translation in Mammalian Cells. Having established that P58^{IPK} J domain function was not required for the inhibition of PKR activity in our yeast growth-rescue assay, we were interested in determining whether this J domain function was required for P58^{IPK} stimulation of protein synthetic rates in mammalian cells. Previously, using transient transfection analysis in the COS-1 cell line, we reported that P58^{IPK} stimulated mRNA translation of secreted embryonic alkaline phosphatase (SEAP) reporter gene by inhibiting PKR kinase activity (*44*), similar to other PKR inhibitors including adenovirus virus-associated I (VAI) RNA and reovirus σ3 protein (*50*−*54*). This kind of assay measures quantitatively mRNA translational stimulation as a result of PKR inhibition. We therefore applied this SEAP assay on the two P58^{IPK} HPD mutants. As shown in Figure 6, expression of adenovirus VAI RNA, which inhibits PKR very efficiently, stimulated SEAP activity by approximately 2-fold, compared with the vector alone control. The expression of wild-type P58^{IPK} also resulted in higher level of SEAP activity although not as high as VAI RNA. This stimulation pattern is consistent with what we observed previously (*44*). We

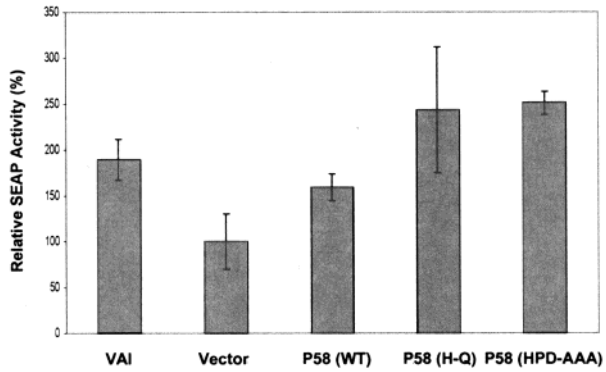

FIGURE 6: HPD mutations do not affect P58IPK stimulation of SEAP mRNA translation in mammalian cells. COS1 cells were cotransfected with SEAP expression plasmid (2 μg) and each inhibitor construct (2 μg) as indicated. SEAP activity was analyzed 40 h after transfection as described in Experimental Procedures. SEAP activity is presented relative to the basal SEAP activity measured in cells transfected with the pEF4/HISA vector (set at 100%). The results shown represent the mean activity from three independent experiments.

noticed that the absolute stimulation level is not as dramatic as we observed previously, this is possibly due to less VAI and P58IPK DNA being transfected (2 μg) compared with what we did previously (6 μg) (*44*). In addition, our human P58IPK constructs used in this study were under the control of the EF1α promoter instead of the stronger CMV promoter in our previous bovine P58IPK constructs. However, since SEAP mRNA levels were similar in cells transfected with the different constructs (data not shown), the relative increase in SEAP activity likely reflects PKR inhibition and resultant stimulation of protein synthesis. Most relevant, expression of either the H-Q or the HPD-AAA mutants resulted in equivalent SEAP activity, and even somewhat enhanced enzymatic activities compared to the wild-type P58IPK. These data extend and complement our yeast data and strongly suggest that P58IPK J domain function is not required for PKR inhibition and resultant stimulation of mRNA translation.

DISCUSSION

In this study, we analyzed the requirement for P58IPK J domain function in the regulation of PKR kinase activity and its resultant effects on mRNA translation. We found that the P58IPK J domain functioned as a classical DnaJ chaperone family J domain and could functionally substitute for both the prokaryotic DnaJ and eukaryotic Ydj1 J domains. Introduction of point mutations in the conserved HPD loop resulted in loss of the J domain function and disruption of the stimulation of ATPase activity of Hsp70. However, the lack of J domain function did not affect P58IPK inhibition of PKR activity and regulation of mRNA translation inside the cell.

To our knowledge, this is the first time that a J domain from a human DnaJ molecular chaperone has been shown to functionally replace the J domain of a molecular chaperone from single-cell organisms, including both bacteria and yeast. Previously, it was reported that the J domains from two yeast cytosolic DnaJ chaperones, Ydj1 and Sis1, were functionally interchangeable (*55*), similar to the case of the J domains of the two yeast endoplasmic reticulum (ER) DnaJ proteins, Sec63 and Scj1 (*56*). However, J domain swapping between

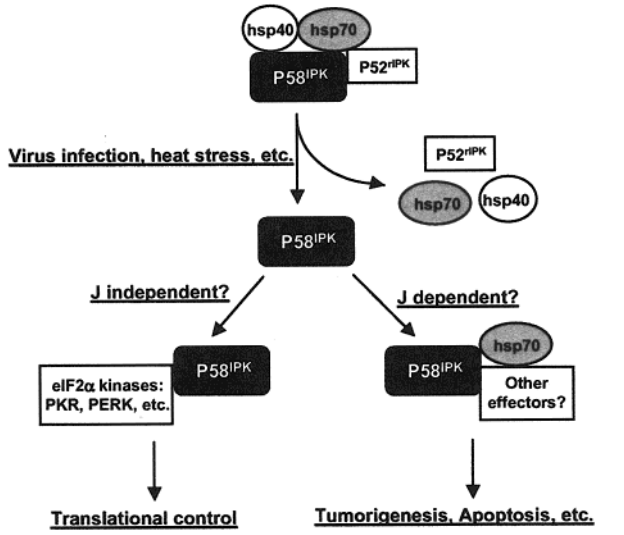

FIGURE 7: Model for the role of molecular chaperones in P58IPK-mediated cellular pathways. Under normal physiological conditions, P58IPK is present in a complex with Hsp70, Hsp40, and/or P52rIPK and retained in an inactive conformation. Upon virus infection or other stress signals, the chaperone complex is disrupted and P58IPK is released from the inhibitory complex. The free P58IPK is thus available to regulate eIF2α kinases, including PKR. This regulation of PKR by P58IPK does not require the J domain. In addition, molecular chaperones, including Hsp70, may participate with P58IPK to regulate the function of cellular effectors other than PKR. It remains to be determined whether such regulation depends on J domain function. See Discussion for additional details.

mammalian and bacterial DnaJ family members has not been reported except for studies on J domains from viral proteins (the T antigens of SV40 and murine polyomavirus). These viral protein domains functionally replace the J domain of DnaJ (*39*). Our data presented here thus argue forcefully in favor of the notion that the J domains in the DnaJ molecular chaperone family are functionally conserved during evolution, from bacteria to humans. Finally, it is noteworthy that studies in yeast suggest J domain interchangeability might be restricted by the cellular compartments of these DnaJ family members, as the J domain of the cytosolic Sis1 was unable to substitute for the J domain of ER-localized Sec63, whereas the J domain of ER-localized DnaJ protein Scj1 is able to substitute for the Sec63 J domain (*56*). This is consistent with our previous observation that P58IPK is localized predominantly in the cytosol (*57*), and thus, its J domain could replace the J domains of the cytosolic DnaJ proteins such as DnaJ and Ydj1.

We must ask why P58IPK contains a conserved J domain and what is the role of P58IPK chaperone activity, given that the J domain is not required for PKR inhibition. Previously, we reported that P58IPK was retained in an inactive form by Hsp40 in uninfected cells (*8*). When cells were infected by influenza virus, or treated by heat shock, Hsp40 was released from P58IPK and the liberated P58IPK inhibited PKR kinase activity. Considering that both P58IPK and Hsp40 interact with Hsp70 biochemically, and P58IPK is complexed with Hsp70 in an Hsp40-dependent manner (*37*), we tend to favor a new working model in which a molecular chaperone complex, minimally including Hsp70, Hsp40, and possibly P52rIPK, plays a critical role in keeping P58IPK in a latent conformation under nonstressed conditions (Figure 7). This is not without precedence, as molecular chaperone complexes, including Hsp90, Hsp70, and Hsp40, reportedly regulate the activities

of various substrates, including the glucocorticoid receptor, estrogen receptor, and src kinase, as well as other eIF2α kinases PERK, Gcn2 and HRI (*17*, *29*−*36*, *58*). Upon virus infection or other stress responses, P58^{IPK} is released to inhibit PKR or perhaps other effectors. This revised working model is supported by our yeast and mammalian data. For example, overexpression of wild-type P58^{IPK} in our transient SEAP assays would be expected to titrate out the molecular chaperone inhibitors including Hsp70. The additional non-chaperoned P58^{IPK} thus actively inhibited PKR and stimulated the rate of SEAP synthesis. The P58^{IPK} HPD mutants, presumably losing interactions with the Hsp70 inhibitor, would be expected to suppress PKR function even when expressed at low levels. Indeed, our SEAP data indicated that the P58^{IPK} HPD mutants inhibited PKR and stimulated SEAP synthetic rate at a slightly higher level than wild-type P58^{IPK}. It would be interesting to see whether the P58^{IPK} HPD mutants inhibit PKR even if they were not overexpressed. Such an analysis would require constructing P58^{IPK} HPD mutant transgenic cells in the absence of wild-type P58^{IPK}.

The data presented in this report also suggest that P58^{IPK} has cellular functions that are independent of its ability to inhibit PKR. These other functions may require the J domain activity. Previously, we reported that P58^{IPK} can suppress programmed cell death in a PKR-independent manner, and that expression of a P58^{IPK} mutant that lacks the ability to interact with or inhibit PKR can still malignantly transform cells (*59*). Very recently it was reported that P58^{IPK} was associated with TIRAP, an adapter molecule of Toll-like receptor family (TLR4) and possibly involved in activation of NF-κB and other Toll signaling pathways (*60*). In addition, it is relevant to mention that the J domain of the SV40 T antigen is required for inactivation of the retinoblastoma tumor suppressor protein (pRb) and for cellular transformation (*61*). We cannot exclude the possibility that the co-chaperone activity of P58^{IPK} might be involved in similar cellular processes (Figure 7). An additional intriguing possibility is that P58^{IPK} may regulate the activity of additional eIF2α kinases, such as the ER-localized protein kinase, PERK. Indeed, we recently found that P58^{IPK} was induced during the ER stress response and interacted with PERK (Wei Yan, Marcus Korth, and Michael Katze, unpublished data). Previously it was reported that PERK kinase activity was regulated by the ER Hsp70 chaperone (BiP) (*32*). Therefore, it is possible that a major function of P58^{IPK} is to regulate PERK kinase activity under ER stress conditions, which might require the participation of molecular chaperones, including Hsp70, Hsp40, and/or Hsp90.

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