P52^{rIPK} Regulates the Molecular Cochaperone P58^{IPK} To Mediate Control of the RNA-Dependent Protein Kinase in Response to Cytoplasmic Stress†

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ABSTRACT: The 52 kDa protein referred to as P52^{rIPK} was first identified as a regulator of P58^{IPK}, a cellular inhibitor of the RNA-dependent protein kinase (PKR). P52^{rIPK} and P58^{IPK} each possess structural domains implicated in stress signaling, including the charged domain of P52^{rIPK} and the tetratricopeptide repeat (TPR) and DnaJ domains of P58^{IPK}. The P52^{rIPK} charged domain exhibits homology to the charged domains of Hsp90, including the Hsp90 geldanamycin-binding domain. Here we present an in-depth analysis of P52^{rIPK} function and expression, which first revealed that the 114 amino acid charged domain was necessary and sufficient for interaction with P58^{IPK}. This domain bound specifically to P58^{IPK} TPR domain 7, the domain adjacent to the TPR motif required for P58^{IPK} interaction with PKR, thus providing a mechanism for P52^{rIPK} inhibition of P58^{IPK} function. Both the charged domain of P52^{rIPK} and the TPR 7 domain of P58^{IPK} were required for P52^{rIPK} to mediate downstream control of PKR activity, eIF2α phosphorylation, and cell growth. Furthermore, we found that P52^{rIPK} and P58^{IPK} formed a stable intracellular complex during the acute response to cytoplasmic stress induced by a variety of stimuli. We propose a model in which the P52^{rIPK} charged domain functions as a TPR-specific signaling motif to directly regulate P58^{IPK} within a larger cytoplasmic stress signaling cascade culminating in the control of PKR activity and cellular mRNA translation.

Stress-induced translational control pathways provide the cell with a rapid mechanism by which to alter gene expression in response to a wide spectrum of signals, including heat and chemical stressors, osmotic shock, ultraviolet radiation, nutrient deprivation, and virus infection (*1*−*3*). A key feature of these pathways is the reversible phosphorylation of translation factors, which enforces translational control programs by modifying translation factor function (*4*). The major points of translational control reside within the processes of translation initiation. In this often rate-limiting step, the initiator Met-tRNA$_i$ is delivered to the 40S ribosome preinitiation complex by eukaryotic initiation factor 2 (eIF2)[1] (*5*). In mammalian cells, eIF2 function is regulated through phosphorylation of its α subunit (eIF2α) by the actions of a family of eIF2α protein kinases that includes HRI, GCN2, PERK/PEK, and PKR (*6*, *7*). Phosphorylation of eIF2α blocks a critical guanine nucleotide exchange reaction, leaving eIF2 in an inactive GDP-bound form that is unavailable for initiation of mRNA translation (*8*).

The eIF2α protein kinases possess common structural features within their catalytic domains that direct substrate specificity for eIF2α (*7*). However, these kinases exhibit significant sequence divergence within their regulatory domains, which likely contributes to the differing array of signals to which each kinase responds. In erythroid cells, HRI regulates protein synthesis in response to hemin levels and is activated by heme deprivation (*9*). In *Saccharomyces cerevisiae*, GCN2 is activated in response to nutrient deprivation that leads to amino acid (aa) limitation. By phosphorylating eIF2α, GCN2 stimulates the translation of *GCN4* mRNA to induce aa biosynthesis (*10*). GCN2 homologues have also been identified in mammalian and insect tissues (*8*, *11*−*13*); however, the extent to which the metazoan GCN2 homologues regulate aa biosynthesis, and their role in translational signaling, remains to be determined. The PERK/PEK eIF2α kinase is localized to the endoplasmic reticulum (ER) membrane and is activated in response to ER stress (*14*). PERK/PEK participates in the unfolded protein response by phosphorylating eIF2α to limit local mRNA translation (*15*), which is thought to relieve ER stress

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[1] Abbreviations: aa, amino acid; dsRNA, double-stranded RNA; eIF2, eukaryotic initiation factor 2; ER, endoplasmic reticulum; IEF, isoelectric focusing; IFN, interferon; PKR, protein kinase RNA dependent; TPR, tetratricopeptide repeat.

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by reducing the de novo synthesis of proteins destined for the ER.

PKR is a cytoplasmic protein present in most mammalian tissues and is activated upon binding to double-stranded RNA (dsRNA) or by interacting with specific protein activators (*16*, *17*). PKR levels increase approximately 3−10-fold in response to interferons (IFNs), whereupon the kinase plays a prominent role in mediating the antiviral and antiproliferative actions of these cytokines (*18*). PKR also mediates the response to dsRNA signals that stimulate apoptosis and that activate NFκB and IRF-1 (*19*). During virus infection, activation of PKR by dsRNA leads to a suppression of mRNA translation that serves to limit virus replication (*20*). Many viruses have therefore evolved mechanisms to inhibit PKR (*18*), including influenza virus, which activates P58IPK, a cellular PKR inhibitor (*21*−*23*).

P58IPK is a member of the tetratricopeptide repeat (TPR) domain protein family and encodes nine tandemly arranged TPR domains. Like other TPR proteins, the TPR domains of P58IPK are important for directing protein interactions, which in the case of P58IPK includes self-association. P58IPK binds to PKR through TPR domain 6 (*24*) and inhibits PKR by disrupting the process of kinase dimerization that is required for catalytic function (*22*). In addition, P58IPK possesses a C-terminal DnaJ domain that formally classifies it as a member of the stress-response protein superfamily (*21*, *25*, *26*). We have also shown that P58IPK undergoes regulatory interactions with heat shock protein (Hsp) 40 that result in stimulation of Hsp70 function during the heat shock response (*23*, *27*). These results define P58IPK as a protein cochaperone that inhibits PKR, possibly by directing Hsp-dependent protein refolding or kinase denaturation. The inhibition of PKR by P58IPK can stimulate cell growth by disrupting PKR-dependent control of mRNA translation and by blocking PKR-dependent apoptosis (*25*, *28*, *29*).

P58IPK is also regulated through interaction with P52rIPK, a protein with limited homology to the charged domains of Hsp90 (*30*). We found that P52rIPK binds to P58IPK in vivo and in vitro and that this interaction blocks the PKR regulatory function of P58IPK. Moreover, when these components are coexpressed in yeast, P52rIPK removes the block to PKR function imposed by P58IPK, resulting in restoration of eIF2α phosphorylation (*30*). These studies indicate that P52rIPK functions as an upstream regulator of PKR, eIF2, and mRNA translation through its ability to interact with and inhibit P58IPK, though the mechanisms of this regulation have not been defined. In the present report, we describe the domains of P52rIPK and P58IPK that are responsible for the interaction of these proteins and present evidence that P52rIPK functions as an upstream signal transducer to regulate P58IPK in response to cytoplasmic stress.

EXPERIMENTAL PROCEDURES

Plasmids and Recombinant DNA Construction. The GAL4 DNA-binding domain (BD) or activation domain (AD) fusion constructs pBD-P58IPKwt, pBD-P58IPK ΔTPR6, pBD-P58IPK 8−1, pBD-P58IPK 8−2, pBD-P58IPK 9−1, pAD-PKR K296R, and pAD-P52rIPKwt were described previously (*24*, *30*). Plasmids pBD-P58IPK ΔTPR5 and pBD-P58IPK ΔTPR7 were constructed by recovering the respective internal *Bst*XI/*Bam*HI fragments from pCDNA1neo-P58IPK ΔTPR5 and ΔTPR7, respectively (*29*), and cloning them into *Bst*XI/*Bam*HI-digested pBD-P58IPKwt. The pBD-P58IPK constructs 8−4, 10−1, and TPR7, encode P58IPK aa 1−302, 154−267, and 268−302, respectively, and were cloned from PCR products into the *Eco*RI/*Bam*HI sites of pGBT9 to yield in-frame fusion proteins with the GAL4 DNA-binding domain. The pAD-P52rIPK deletion constructs were cloned from PCR products derived from pAD-P52rIPKwt using restriction site-linked oligonucleotide primer pairs. PCR products were first subcloned into pCR2.1 (Invitrogen). Insert DNA encoding P52rIPK aa 1−243, 237−492, 1−203, 1−84, or 86−203 was released from pCR2.1 by *Eco*RI/*Bam*HI digestion, and the gel-purified fragments were cloned into the corresponding sites of pGAD424 (Clontech) to yield pAD-P52rIPK deletion constructs fused in-frame to the GAL4 activation domain. DNA encoding P52rIPK aa 201−492 was released from pCR2.1 by *Bam*HI cleavage and subsequently cloned into *Bam*HI-digested pAD-P52rIPK 1−84 to yield pAD-P52rIPK Δ85−200. Plasmids pGex2T-P58IPK and pBSK-P52 encode full-length P58IPK and P52rIPK, respectively (*30*). Inducible expression of P58IPK and P52rIPK in yeast cells was achieved by utilizing the galactose-inducible 2 μm yeast expression vectors pEMBLyex4 (pYex4, encodes uracil selection) (*31*) and pYX233 (pYX, encodes tryptophan selection) (Novagen), respectively. Construction of pYex4-P58IPKwt and pYX-P52rIPKwt was described previously (*30*). Plasmids pYX-P52rIPK 86−200 and pYX-P52rIPK Δ86−200 were constructed by cloning the *Eco*RI/*Bam*HI fragments from the respective pAD constructs into the *Eco*RI/*Bam*HI sites of pYX233. Plasmid pYex4-P58IPK ΔTPR7 was derived by cloning the *Sma*I/*Xba*I fragment from pCDNA1neo- P58IPK ΔTPR7 into *Eco*RV/*Xba*I-digested pYex4. All cloning products were sequenced by the dye termination method with an Applied Biosystems automated DNA sequencer to ensure that no mutations were introduced during PCR amplification or cloning.

Cell Culture. HeLa, 293, NIH3T3, Cos1 monkey kidney cells, and Madin Darby bovine kidney cells (MDBK) were obtained from ATCC. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics, and 200 μM L-glutamine. For stress induction experiments, cultured monolayers (at approximately 60% confluency) were rinsed twice with sterile PBS and cultured for the indicated time in media containing 150 μM sodium arsenite (Sigma), 6 mM tunicamycin, or 0.2 mM thapsigargin. For heat shock experiments, media were removed from HeLa cell monolayers and replaced with media prewarmed to 42 °C, after which cells were incubated for a total of 30 min at 42 °C prior to harvesting. For serum deprivation experiments, HeLa cell monolayers were rinsed twice with sterile PBS and incubated for 4 h in media lacking FBS. Control cultures were similarly rinsed with PBS but were incubated for 4 h in media containing 10% FBS.

Protein Analysis. Yeast extracts were prepared from 20 mL liquid cultures (*24*), and mammalian cell protein extracts were prepared in lysis buffer as described previously (*30*). Total protein concentration in cell extracts was determined using the Bio-Rad protein assay. Immunoblot analyses of proteins separated by SDS−PAGE, or single-dimension isoelectric focusing, were conducted as described (*30*). PKR or P58IPK expression was detected using monoclonal antibody

(Mab) 71/10 (*32*) or 9F10, respectively (*28*). P52^{rIPK} expression was detected using anti-P52^{rIPK} polyclonal rabbit serum (*30*) or the 5D12 Mab described below. BD- and AD-fused proteins were detected using anti-GAL4 BD and anti-GAL4 AD Mab, respectively (Clontech).

For production of 5D12 Mab specific to human P52^{rIPK}, mice were immunized with recombinant GST-P52^{rIPK} as described (*28*). Immune serum was screened for reactivity to GST-P52^{rIPK} or GST alone, and mice with specific reactivity to P52^{rIPK} were selected and euthanized, and their splenocytes were prepared for hybridoma production using standard procedures (*33*). Hybridoma culture supernatants were screened by immunoblot analysis for the presence of antibodies specific for recombinant P52^{rIPK}. Cells from positive cultures were cloned by limiting dilution, and the resulting culture supernatants were rescreened for P52^{rIPK}-specific antibodies (IgG_1 subtype).

For immunoprecipitation reactions, 1×10^5 cpm of [^{35}S]-methionine-labeled in vitro translation products, or unlabeled cell extract (400 or 600 μg), was mixed with the indicated amount of Mab or polyclonal antiserum for 1 h at 4 °C. After the addition of protein G−agarose beads, mixtures were incubated for an additional 1 h at 4 °C. Immunocomplexes were recovered by centrifugation at 12000*g* and washed four times in lysis buffer (*30*) containing an additional 200 mM NaCl (for a final NaCl concentration of 350 mM). After the final wash, bead-bound immunocomplexes were released by the addition of 50 μL of SDS sample buffer, incubated in a 100 °C bath, and placed on ice prior to loading on gels. Eluted proteins were resolved by 10% or 12.5% SDS−PAGE, and in some experiments, the resolved proteins were blotted to nitrocellulose membranes and subjected to immunoblot analysis.

In Vitro Translation Reactions and GST Pull-Down Assay. Full-length and truncated P52^{rIPK} constructs were transcribed from the T7 promoter of pBSK-P52^{rIPK} and translated in vitro in the presence of [^{35}S]methionine using the TNT reaction system (Promega). To generate P52^{rIPK} aa 1−384 or 1−239, we digested pBSK-P52^{IPK} with *Mun*I and *Bgl*II restriction enzymes, respectively, and gel purified the linear plasmids prior to the TNT reaction. Radiolabel incorporation of in vitro translated products was quantified by scintillation counting of trichloroacetic acid-precipitable material. Expression of GST and GST-P58^{IPK} in *Escherichia coli* and GST pull-down assays were carried out as previously described (*24*). GST pull-down assays using glutathione−Sepharose-bound GST or GST-P58^{IPK}, and 1×10^5 cpm of radiolabeled P52^{rIPK} translation products, were conducted and analyzed exactly as described (*30*).

Yeast Methods. The yeast two-hybrid assay was used to identify in vivo protein−protein interactions as described previously (*24*, *30*). Plasmids encoding GAL4 AD or BD fusion proteins were introduced into *S. cerevisiae* Hf7c [*MATa ura3-52 hi3-200 lys2-801 ade2-101 trp1-901 leu2-3, 112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3(GAL4 17-mers)$_3$-CYC-lacZ*; Clontech]. Transformants were plated onto synthetic defined (SD) medium supplemented with histidine (His$^+$), but lacking leucine and tryptophan, to select for the retention of the pAD and pBD plasmids. Colonies were subsequently streaked onto SD medium lacking His, Trp, and Leu (His$^-$) and grown at 30 °C for 3 days to deplete endogenous histidine stores. Colonies were then replica printed onto His$^+$ and His$^-$ medium and incubated at 30 °C for 2−4 days. Colonies were scored positive for a two-hybrid protein interaction if they exhibited growth on both His$^-$ and His$^+$ medium. Liquid β-Gal assays, using the fluorogenic substrate 4-methylumbelliferyl β-D-galactoside (MUG; Sigma), were conducted on yeast two-hybrid strains as previously reported (*24*).

To assess protein function in vivo, pYex4-P58^{IPK} and pYX-P52^{rIPK} wild-type and mutant constructs were introduced into *S. cerevisiae* RY1-1 [*MATa ura3-52 leu2-3 leu2-112 gcn2*Δ *trp1-*Δ*63 LEU2::(GAL-CYC1-PKR)$_2$*] (*34*). This strain lacks the endogneous yeast eIF2α kinase, GCN2, but contains two copies of human *PKR* integrated into the *LEU2* locus under control of the galactose-inducible *GAL*/*CYC1* hybrid promoter. In the presence of galactose, PKR is expressed and phosphorylates eIF2α, resulting in suppression of cell growth. However, the coexpression of PKR regulatory proteins results in modulation of PKR function, which can be scored by the amount of galactose-specific growth and by analysis of eIF2α phosphorylation (*30*, *34*). RY1-1 transformants were plated onto SD medium lacking uracil and tryptophan and incubated at 30 °C for 2−4 days. Resultant colonies were plated onto synthetic defined galactose (SGal) medium (containing 2% galactose as the sole carbon source), incubated for 5−9 days at 30 °C, and scored for growth as described previously (*30*).

Isoelectric Focusing Analysis. Isoelectric focusing (IEF) analysis to determine the phosphorylation state of yeast eIF2α was carried out essentially as described (*35*). Approximately 20 μg of total protein from each extract was separated by single-dimension IEF and blotted to nitrocellulose membranes. Blots were probed with rabbit polyclonal antiserum specific to yeast eIF2α.

RESULTS

The Charged Domain of P52^{rIPK} Is Necessary and Sufficient for Interaction with P58^{IPK}. The charged domain of P52^{rIPK} exhibits homology to the charged domains of Hsp90, which include an N-terminal geldanamycin-binding domain and a region located at the C terminus of the protein (*30*, *36*, *37*). Both Hsp90 and P52^{rIPK} bind to TPR proteins, and in the case of Hsp90, the charged domains appear to be TPR domain acceptor sites that mediate protein−protein interactions (*38*, *39*). In previous work, we demonstrated that P52^{rIPK} binds P58^{IPK} and inhibits the PKR regulatory properties of P58^{IPK} in vitro and in vivo (*30*). To understand the mechanism by which P52^{rIPK} regulates P58^{IPK} and PKR function, we conducted detailed structure−function analyses of the P52^{rIPK}−P58^{IPK} interaction. We hypothesized that the P52^{rIPK} charged domain might directly bind to P58^{IPK}, possibly in a TPR domain-dependent fashion.

In initial experiments, we measured the relative binding of in vitro translated wild-type P52^{rIPK}, and progressive C-terminal P52^{rIPK} truncation products, to GST-P58^{IPK} in a cell-free GST pull-down assay. As shown in Figure 1A, full-length P52^{rIPK}, and P52^{rIPK} aa 1−384 in vitro translation products, bound efficiently in a dose-dependent manner to immobilized GST-P58^{IPK} but not to immobilized GST alone. P52^{rIPK} aa 1−239 translation products retained the ability to specifically bind to GST-P58^{IPK}, albeit at a reduced level (Figure 1A, lanes 9−12). These results indicate that the P58^{IPK}-binding activity resides within the N-terminal region

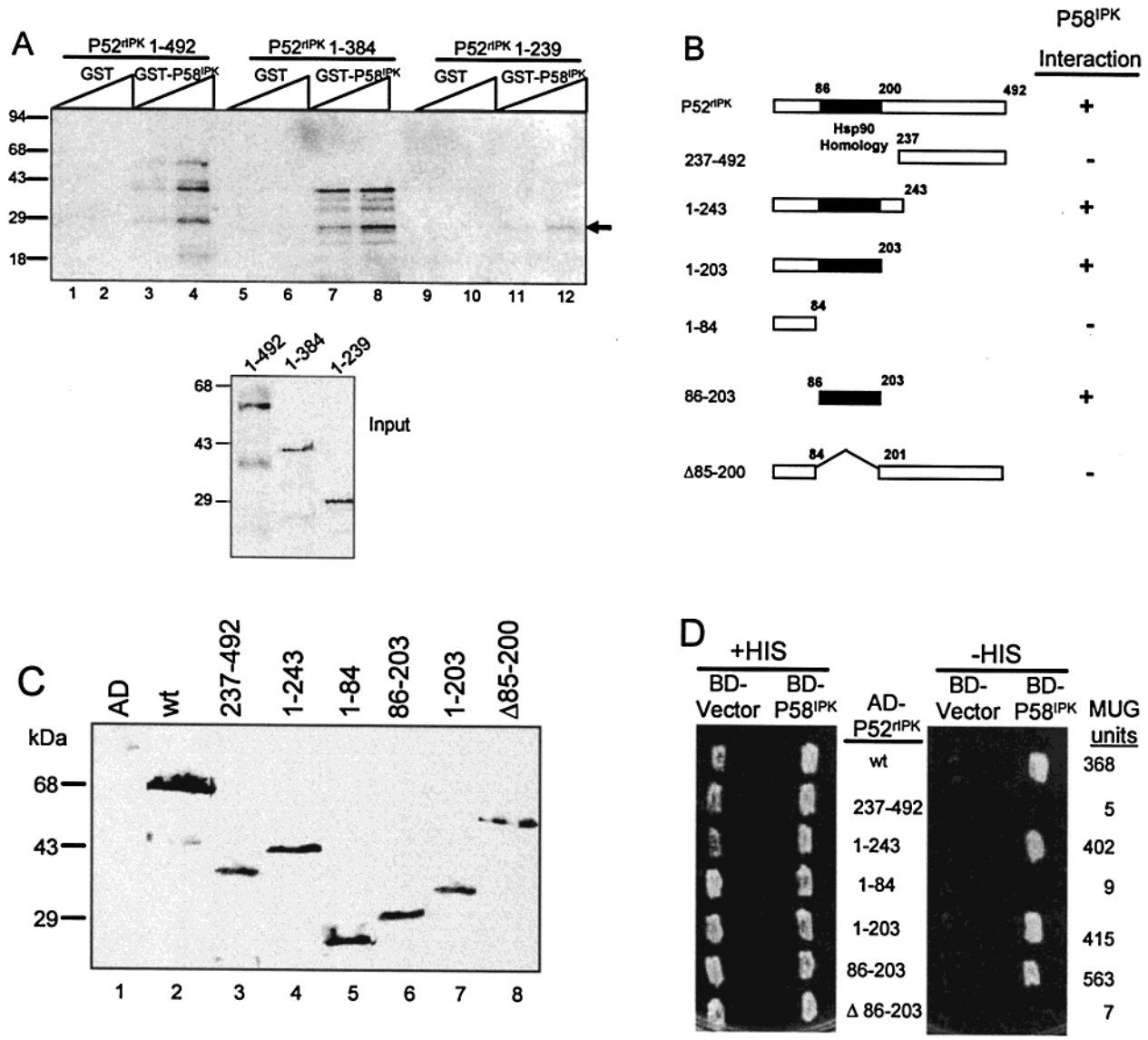

FIGURE 1: Mapping the P58IPK interactive domain of P52rIPK. (A) GST pull-down assay. Equal molar amounts of GST or GST-P58IPK were immobilized on glutathione−Sepharose beads, and increasing amounts of bead-bound complexes were mixed with 1×10^5 cpm of ^{35}S-labeled in vitro translation products corresponding to full-length P52rIPK (aa 1−492; lanes 1−4), P52rIPK aa 1−384 (lanes 5−8), or P52rIPK aa 1−239 (lanes 9−12). After the beads were washed, protein complexes were eluted and then separated by SDS−PAGE. An autoradiogram of the dried gel is shown. The arrow points to the 1−239 translation product. The positions of protein standards are indicated in kDa on the left. The lower panel shows the amount of input P52rIPk translation products used in the binding assays. (B) Schematic representation of wild-type P52rIPK (top) and P52rIPK deletion mutants used in mapping the P58IPK interactive domain by yeast two-hybrid analysis. Each construct was expressed in *S. cerevisiae* Hf7c as a GAL4 activation domain fusion and is identified by the encoded aa residues shown on the left. The dark region denotes the 114 aa charged domain of P52rIPK that exhibits homology to Hsp90 (*30*). This region is deleted from the Δ85−200 construct. The results from P58IPK interaction studies are summarized on the right. (C) Expression of AD-P52rIPK fusion proteins in yeast. Extracts from yeast coexpressing BD-P58IPK and the indicated AD constructs were subjected to immunoblot analysis using an antibody specific to the GAL4 activation domain. Lane 1 shows an extract from yeast coexpressing the AD vector. Lanes 2−8 show extracts from yeast coexpressing AD fusion proteins corresponding to P52rIPK aa 1−492 (wild type, lane 2), 237−492 (lane 3), 1−243 (lane 4), 1−84 (lane 5), 86−203 (lane 6), 1−203 (lane 7), and Δ85−200 (lane 8). The positions of protein standards are shown on the left. (D) Yeast two-hybrid assay. Hf7c yeast cells coexpressing the indicated AD fusion proteins with the GAL4 DNA-binding domain alone (BD vector), or BD-P58IPK, were replica printed onto medium in the presence (+HIS, left panel) or absence (−HIS, right panel) of histidine and incubated at 30 °C for 4 days. In this assay, growth on both His^+ and His^- medium is indicative of a two-hybrid protein interaction (*24*). To confirm the growth phenotype, we conducted liquid β-Gal assays of the encoded β-Gal reporter using the MUG substrate. Results are shown to the right of each panel and are the average of three separate experiments. Numbers shown were rounded to the nearest whole value.

of P52rIPK and that aa 1−239, which include the charged domain, are sufficient to mediate interaction with P58IPK in vitro. Variations in the ability of the different P52rIPK deletion constructs to bind to P58IPK could be due to conformation changes resulting from protein truncation.

To verify the in vitro binding data, and to test the role of the 114 aa charged domain in mediating interaction with P58IPK, we constructed a series of P52rIPK deletion mutants for use in a yeast two-hybrid in vivo interaction assay (Figure 1B). Each construct was coexpressed as a GAL4 activation domain (AD) fusion protein in *S. cerevisiae* strains expressing the GAL4 DNA-binding domain (BD) or a BD-P58IPK fusion protein. Each AD-P52rIPK construct was efficiently expressed (Figure 1C) and was scored for its ability to

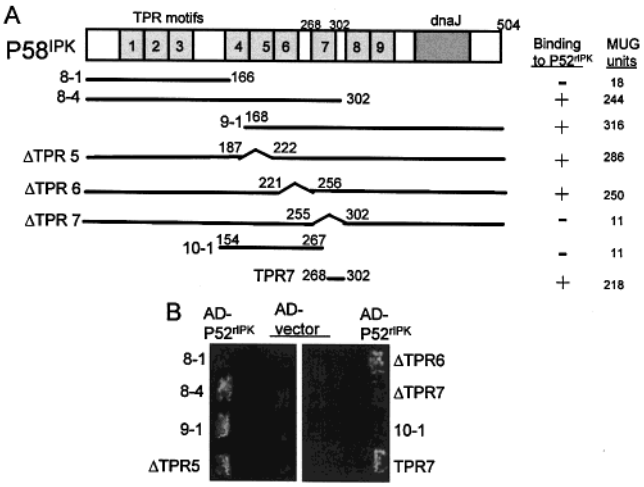

FIGURE 2: Mapping the P52^{rIPK} interacting domain of P58^{IPK}. (A) Structural representation of P58^{IPK} and TPR domain deletion constructs. TPR domains are indicated by number in gray-shaded squares, and aa positions defining TPR domain 7 are indicated. Amino acid positions are shown to denote full-length P58^{IPK}, the terminal aa positions within the P58^{IPK} 8−1, 8−4, 9−1, 10−1, and TPR7 truncation mutants, and the positions of deleted TPR domain 5 (ΔTPR5), 6 (ΔTPR6), and 7 (ΔTPR7). The results from protein interaction studies with P52^{rIPK} are summarized on the right, together with β-Gal assay results (in MUG units). Values shown are the average of two independent experiments and are rounded to the nearest whole number. (B) Analysis of BD-P58^{IPK} and TPR deletion mutants in the yeast two-hybrid assay. Hf7c yeast cells coexpressing the indicated BD-P58^{IPK} fusion proteins and AD-P52^{rIPK} or the GAL4 activation domain alone (AD vector) were streaked onto His$^-$ medium, and plates were incubated for 4 days at 30 °C. Growth is indicative of a two-hybrid protein interaction. All AD and BD fusion proteins were expressed in each strain, and all strains grew efficiently on control His$^+$ medium (data not shown).

specifically bind to BD-P58^{IPK} by its ability to promote the growth of each yeast strain on His$^-$ medium. Whereas all stains grew equally well on His$^+$ medium (Figure 1D, left panel), exclusion of the P52^{rIPK} charged domain resulted in abrogation of P58^{IPK} interaction and a lack of growth on His$^-$ medium (Figure 1D, right panel). In particular, P52^{rIPK} aa 86−203 were sufficient to mediate interaction with P58^{IPK}, since yeast cells expressing this construct maintained robust growth on His$^-$ medium. Conversely, the P52^{rIPK} Δ85−200 construct, which lacks the charged domain, failed to confer growth on selective medium, even though this construct was efficiently expressed (see Figure 1C,D). Analysis of the encoded β-Gal activity (MUG units in Figure 1D) within each strain confirmed the growth assay data and indicated that P52^{rIPK} aa 86−203 bound to P58^{IPK} at a relative strength approximating or slightly surpassing that of the wild-type protein. Taken together, these studies demonstrate that the charged domain of P52^{rIPK} is both necessary and sufficient for interaction with P58^{IPK}. The localization of P58^{IPK}-binding activity to within the charged domain of P52^{rIPK} is of interest, since the charged acidic domains in the N- and C-terminal regions of Hsp90 also interact with TPR proteins (*38*−*40*).

P58^{IPK} TPR Domain 7 Is Necessary and Sufficient for Interaction with P52^{rIPK}. TPR domains are modular protein interaction motifs that are present in a diverse family of proteins that includes many cell-signaling effectors (*41*). As shown in Figure 2, P58^{IPK} contains nine tandemly arranged TPR motifs (*21*). We previously demonstrated that TPR domain 6 directs the interaction between P58^{IPK} and PKR (*24*). To determine if the interaction with P52^{rIPK} was similarly TPR domain dependent, we conducted a series of protein interaction studies using the yeast two-hybrid system to score for protein interaction in vivo. GAL4 DNA-binding domain fusion proteins of wild-type P58^{IPK}, or various P58^{IPK} deletion constructs, were coexpressed with the GAL4 activation domain or the AD-P52^{rIPK} wild-type fusion protein, and each strain was scored for growth on His$^-$ medium. The relative strength of the two-hybrid protein interactions was scored by β-Gal assay (MUG units). In initial experiments, we tested a series of P58^{IPK} N- and C-terminal deletion constructs for their ability to bind P52^{rIPK}. Expression of BD fusion proteins P58^{IPK} 8−4 or 9−1, which respectively encode P58^{IPK} aa 1−302 or 168−504, was sufficient for interaction with AD-P52^{rIPK}, as determined by growth of the corresponding strains on His$^-$ medium (Figure 2). In contrast, P58^{IPK} aa 1−166, encoded by the BD-P58^{IPK} 8−1 protein, failed to mediate interaction with AD-P52^{rIPK}. From these initial studies, we preliminarily mapped the P52^{rIPK} interactive domain of P58^{IPK} to within aa 168−302, which extends from midway within TPR domain 4 through TPR domain 7 (Figure 2A).

To further define the P52^{rIPK} interactive domain of P58^{IPK}, we tested the ability of P58^{IPK} TPR domain deletion mutants, lacking either TPR domains 5, 6, or 7 (encoded in the BD-P58^{IPK} ΔTPR 5, ΔTPR6, and ΔTPR 7 fusion constructs, respectively), to interact with P52^{rIPK}. Each construct was coexpressed in yeast with either the GAL4 activation domain or AD-P52^{rIPK}. Deletion of TPR domain 6 did not affect P58^{IPK} binding to P52^{rIPK}. Indeed, yeast cells coexpressing AD-P52^{rIPK} with wild-type P58^{IPK}, ΔTPR 5, or ΔTPR 6 BD fusion proteins exhibited robust growth on His$^-$ medium, and the encoded β-Gal activity approximated that of the wild-type P58^{IPK} protein. In contrast, the 10−1 construct (encoding TPR domains 4−6 and the flanking 12 aa) failed to interact with P52^{rIPK}. Similarly, deletion of TPR domain 7 ablated the ability of P58^{IPK} to bind P52^{rIPK} in this assay (Figure 2). The 34 -aa TPR domain 7 was sufficient to mediate an interaction with P52^{rIPK} that was equivalent in strength (MUG units) to the wild-type protein. We confirmed that all BD and AD constructs were efficiently expressed in the corresponding yeast cells (data not shown). The ΔTPR 5 and ΔTPR 7 constructs retained their ability to bind PKR in parallel yeast two-hybrid studies (data not shown), indicating that deletion of each TPR domain had little impact on overall protein conformation. These results demonstrate that P58^{IPK} TPR domain 7 is both necessary and sufficient for interaction with P52^{rIPK} in vivo.

P58^{IPK} TPR Domain 7 Is Required for P52^{rIPK}-Dependent Regulation of PKR. Our results demonstrate that P58^{IPK} binds to PKR and P52^{rIPK} at independent, but adjacent, sites within TPR domains 6 and 7, respectively. We previously showed that P58^{IPK} inhibition of PKR requires TPR domain 6 (*24*) and that this likely occurs through formation of a TPR 6-mediated P58^{IPK}-PKR inhibitory complex in vivo (*22*, *25*). We sought to determine if P52^{rIPK} control of P58^{IPK} was similarly a TPR-dependent event, possibly mediated by the P52^{rIPK} interaction with P58^{IPK} TPR domain 7. For these experiments, we utilized a yeast genetic system to assess the role of P52^{rIPK} in the P58^{IPK}-dependent regulation of PKR and eIF2α phosphorylation (*34*). This experimental system is based upon the observation that PKR is growth suppressive

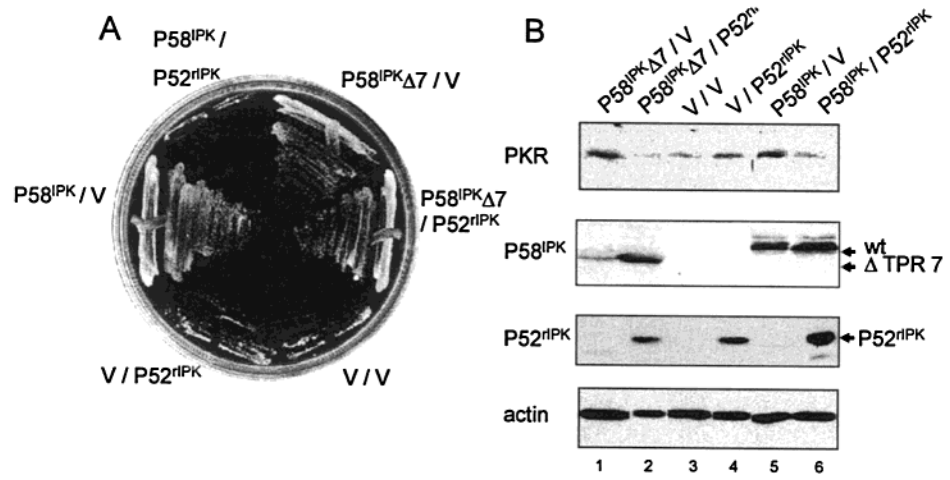

FIGURE 3: P52rIPK mediates control of P58IPK and PKR through P58IPK TPR domain 7. (A) Yeast growth assay for the analysis of PKR regulation in vivo. RY1-1 yeast cells were streaked onto selective SGal medium to induce coexpression of PKR and the indicated P58IPK and P52rIPK alleles. The plate shown was incubated for 6 days at 30 °C. In this assay, yeast growth is indicative of PKR inhibition. Growth suppression indicates that PKR is active. All strains exhibited efficient growth on SD medium (not shown). V denotes the empty expression vector in place of P58IPK or P52rIPK. (B) Protein expression in RY1-1 yeast cells. Extracts from the yeast cells shown in panel A were subjected to immunoblot analysis with antibodies specific to human PKR (top panel), P58IPK (second panel from top), P52rIPK (third panel from top), or actin (lower panel). Results were reproducible over three experiments and are shown from a single blot that was first probed with antiserum to P52rIPK, stripped, and then sequentially reprobed with each individual antibody. Lanes show protein expression in extracts prepared from yeast cells coexpressing PKR with P58IPK Δ7 (lane 1), P58IPK Δ7 and P52rIPK (lane 2), vectors only (V/V, lane 3), P52rIPK (lane 4), P58IPK (lane 5), or P58IPK and P52rIPK (lane 6). Arrowheads at right indicate the positions of wild-type P58IPK, P58IPK Δ7, and P52rIPK.

when expressed in yeast, due to high levels of PKR-mediated eIF2α phosphorylation (*35*). We employed the gcn2Δ yeast strain RY1-1, which lacks the endogenous yeast eIF2α kinase but harbors two integrated copies of human PKR placed under the transcriptional control of a galactose-inducible promoter (*34*). This strain grows well when streaked onto noninducing dextrose medium but exhibits a slow growth phenotype when streaked onto galactose medium, due to induced expression and activity of PKR.

We previously demonstrated that, when expressed in RY1-1, P58IPK represses PKR-mediated eIF2α phosphorylation and relieves PKR-mediated growth suppression (*22*, *30*). However, the introduction of P52rIPK into RY1-1 expressing both PKR and P58IPK results in restoration of PKR activity and eIF2α phosphorylation, concomitant with growth suppression due to inhibition of P58IPK function (*30*). The effects of this reconstituted PKR regulatory pathway on yeast growth are shown in Figure 3A. RY1-1 harboring vectors alone (V/V), or in combination with P52rIPK (V/P52rIPK), failed to grow when streaked onto galactose medium. Expression of P58IPK was sufficient to reverse PKR-mediated growth suppression (Figure 3A, P58IPK/V), confirming that P58IPK can repress PKR function in yeast (*22*, *30*). In comparison, the introduction of P52rIPK into this strain restored the slow growth phenotype (Figure 3A, compare P58IPK/V and P58IPK/P52rIPK strains), again demonstrating that P52rIPK can inhibit P58IPK function to restore PKR activity in vivo. RY1-1 expressing P58IPK that lacked TPR domain 7 (P58IPKΔ7/V) exhibited growth on galactose medium, indicating that deletion of TPR domain 7 does not hinder the PKR regulatory properties of P58IPK. P52rIPK coexpression had no effect on the growth of this strain on galactose medium (compare P58IPK Δ7/V with P58IPK Δ7/P52rIPK, Figure 3A). Examination of protein expression in these cells revealed that each expression construct, and PKR, was efficiently expressed when the cells were grown on galactose medium (Figure 3B). It is important to note that P52rIPK itself is not toxic when expressed in yeast (*30*), and this observation was confirmed using RY1-1 coexpressing P58IPK Δ7 with P52rIPK. Our results therefore demonstrate that TPR domain 7 of P58IPK is required for control of P58IPK function by P52rIPK. When taken together with our protein interaction data, these studies indicate that P52rIPK mediates control of P58IPK, and thereby regulation of PKR, by binding to P58IPK TPR domain 7. In this manner, P52rIPK releases the block to PKR activity imposed by P58IPK, resulting in inhibition of growth on galactose medium (Figure 3A).

The Charged Domain of P52rIPK Is Necessary and Sufficient for Control of P58IPK Function in Vivo. Our protein interaction data demonstrated that the charged domain of P52rIPK, encoding aa 86−200, was both necessary and sufficient to mediate interaction with P58IPK in vivo (see Figure 1). We therefore sought to determine if this region of P52rIPK was similarly required for protein function. When coexpressed with P58IPK in RY1-1, a P52rIPK mutant lacking the entire 114 aa charged domain (P52rIPK Δ85−200) failed to inhibit P58IPK function, as demonstrated by growth on galactose medium (Figure 4, P58IPK/P52rIPK Δ85−200). This is in stark contrast to the ability of wild-type P52rIPK to inhibit P58IPK function and restore the PKR growth-suppressive phenotype (Figure 4, compare P58IPK/P52rIPK to P58IPK/P52rIPK Δ85−200). As a control for potential construct toxicity, we confirmed that P52rIPK Δ85−200 had no effect on P58IPK Δ7-dependent growth rescue (P58IPK Δ7/P52rIPK Δ85−200). Similarly, RY1-1 expressing either P58IPK or P58IPK Δ7 exhibited growth on galactose, indicative of P58IPK repression of PKR (Figure 4). Coexpression of P52rIPK aa 86−200 was sufficient to block the PKR inhibitory function of wild-type P58IPK but not of the P58IPK ΔTPR7 mutant (Figure 4, lower panel). We again confirmed that all constructs were efficiently expressed (data not shown). Thus, P52rIPK aa 86−200 are both necessary and sufficient to

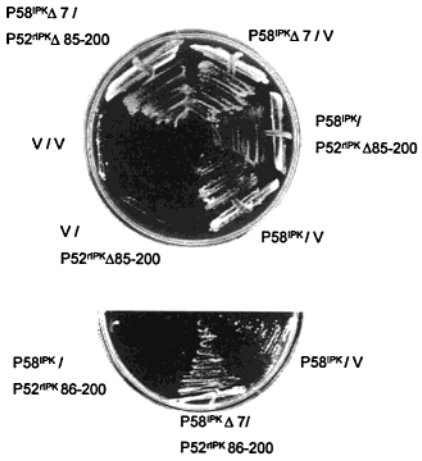

FIGURE 4: The charged domain of P52^{rIPK} is necessary and sufficient for the control of P58^{IPK} and regulation of PKR function in vivo. RY1-1 yeast cells were streaked onto selective SGal medium to induce coexpression of PKR with the indicated P58^{IPK} and P52^{rIPK} alleles. The panels shown are of plates that were incubated at 30 °C for 7 days. V denotes the empty expression vector in place of P58^{IPK} or P52^{rIPK}. Growth is indicative of PKR inhibition by P58^{IPK}. All strains exhibited efficient growth on SD medium (data not shown).

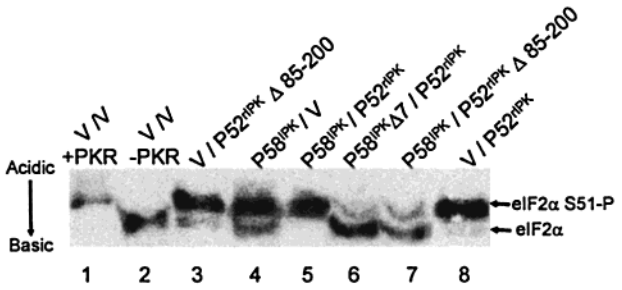

FIGURE 5: eIF2α phosphorylation analysis. Yeast extracts were separated by single-dimension IEF and blotted to nitrocellulose, and eIF2α was detected by immunoblot analysis. This procedure allows for the discrimination and detection of the serine 51 phosphorylated eIF2α isoform (phosphorylated by PKR) from the less acidic, basely phosphorylated eIF2α isoform (*35*). Lanes 1 and 2 correspond to extracts from RY1-1 control cells harboring the expression plasmids alone and grown in SGal or SD medium to induce (+PKR) or suppress PKR expression (−PKR). Lanes 3−8 correspond to extracts prepared from RY1-1 cells grown in SGal medium and coexpressing PKR with P52^{rIPK} Δ85−200 (lane 3), P58^{IPK} (lane 4), P58^{IPK} and P52^{rIPK} (lane 5), P58^{IPK} Δ7 and P52^{rIPK} (lane 6), P58^{IPK} and P52^{rIPK} Δ85−200 (lane 7), or P52^{rIPK} (lane 8). Arrows at the right denote the positions of basely phosphorylated eIF2α and the serine 51 phosphorylated eIF2α isoforms.

suppress the PKR regulatory function of P58^{IPK}, demonstrating that the charged domain of P52^{rIPK} is required for inhibition of P58^{IPK} in vivo.

P58^{IPK} TPR Domain 7 and the Charged Domain of P52^{rIPK} Are Required To Control eIF2α Phosphorylation by PKR. As upstream regulators of PKR function, P52^{rIPK} and P58^{IPK} are proposed to modulate the phosphorylation of PKR substrates, including eIF2α. To determine how the P58^{IPK}−P52^{rIPK} interaction may influence eIF2α phosphorylation, we examined the levels of endogenous eIF2α in yeast cells coexpressing PKR and various forms of P58^{IPK} and P52^{rIPK}. We used a sensitive IEF procedure to separate the PKR phosphorylated isoform of eIF2α (phosphorylated on serine 51) from the non-PKR phosphorylated eIF2α isoform in strains grown in liquid SGal media. Induction of PKR expression resulted in high levels of eIF2α phosphorylation (Figure 5, compare lanes 1 and 2) and was not significantly affected by expression of P52^{rIPK} (lane 8) or P52^{rIPK} Δ85−200 (lane 3). In contrast, expression of P58^{IPK} resulted in an accumulation of the hypophosphorylated eIF2α species. Although the majority of eIF2α remained phosphorylated in the presence of P58^{IPK}, we have observed that relatively small changes in the overall level of eIF2α phosphorylation can have a significant effect on cell growth (*30*, *42*). The coexpression of wild-type P52^{rIPK} blocked the P58^{IPK}-mediated reduction of eIF2α phosphorylation (Figure 5, compare lanes 4 and 5), whereas coexpression of P52^{rIPK} lacking the charged domain had no effect on P58^{IPK} function (lane 7). This result confirms that the 114 aa charged domain is responsible for the downstream regulation of P58^{IPK} and PKR. When compared to cells expressing wild-type P58^{IPK}, cells expressing P58^{IPK} Δ7 exhibited a marked increase in the accumulation of hypophosphorylated eIF2α. Moreover, this reduction in eIF2α phosphorylation was maintained even in the presence of P52^{rIPK} (Figure 5, lane 6). In general, we found that cells expressing P58^{IPK} Δ7 exhibited significantly shorter doubling times when grown in the presence of galactose (data not shown). These results suggest that TPR domain 7 confers negative regulation to P58^{IPK} and that deletion of this domain results in hyperactive protein function. When taken together with the protein interaction and cell growth data, our analysis of eIF2α phosphorylation demonstrates that the 114 aa charged domain of P52^{rIPK} regulates P58^{IPK} through an interaction with P58^{IPK} TPR domain 7 and that this interaction modulates PKR-mediated eIF2α phosphorylation.

Regulation of the P52^{rIPK}−P58^{IPK} Complex in HeLa Cells. Our results indicate that P52^{rIPK} and P58^{IPK} mediate a regulatory pathway that responds to stress-induced signals. This notion is supported by our previous work that defined P58^{IPK} as a stress-activated cochaperone and regulator of PKR (*23*, *27*). We therefore sought to determine if the formation of a P52^{rIPK}−P58^{IPK} complex was regulated in response to cellular stress. We began by analyzing this interaction in HeLa cells that were deprived of serum for a period of 4 h in order to induce an acute stress response. Cell extracts prepared from serum-starved and control cultures were then subjected to immunoprecipitation analysis to assess endogenous complex formation between P58^{IPK} and P52^{rIPK}. To detect complex formation, anti-P52^{rIPK} immunoprecipitation products were recovered and subjected to immunoblot analysis with a Mab specific to P58^{IPK}. As shown in Figure 6A, we failed to detect significant complex formation between P58^{IPK} and P52^{rIPK} within control HeLa cells cultured in complete medium. However, removal of serum from the growth media induced the association of P58^{IPK} with P52^{rIPK}, and P58^{IPK} was recovered within anti-P52^{rIPK} immunoprecipitation reactions (Figure 6A, compare lanes 2 and 3). No detectable changes in the level of P52^{rIPK} protein were observed between control and serum-starved cultures (data not shown). Importantly, P58^{IPK} was not present in control immunoprecipitation products recovered from serum-starved cells using preimmune rabbit control serum (Figure 6A, lane 1). These results demonstrate that P58^{IPK} and P52^{rIPK} form a stable complex in cells stressed by serum deprivation.

We next examined P52^{rIPK}−P58^{IPK} complex formation in response to heat shock, or treatment with sodium arsenite,

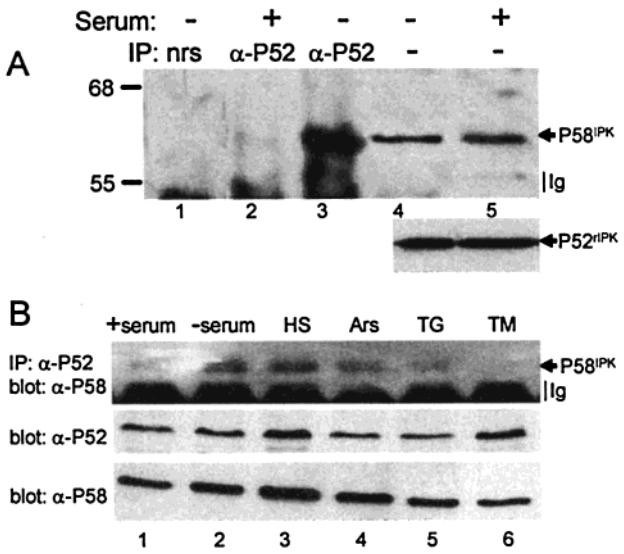

FIGURE 6: Stress-regulated complex formation of P52rIPK and P58IPK in HeLa cells. (A) HeLa cells were cultured for 4 h in the presence (lanes 2 and 5) or absence of serum (lanes 1, 3, and 4). Extracts were prepared, and 600 μg of protein was subjected to immunoprecipitation with normal (preimmune) rabbit serum (nrs, lane 1) or anti-P52rIPK rabbit serum (lanes 2 and 3). After washing, the immunoprecipitation products were resolved by electrophoresis in a 10% acrylamide gel and subjected to immunoblot analysis with a P58IPK-specific Mab. To adequately separate the immunoglobulin heavy chain (dark smear) from the P58IPK band, the electrophoresis was conducted such that the 55 kDa standard was resolved to very near the bottom of the gel. The positions of the 68 and 55 kDa protein standards are shown on the left. Lanes 4 and 5 show P58IPK and P52rIPK (arrowheads, upper and lower panels, respectively) in 50 μg of the input extract that was used in the immunoprecipitation reactions. (B) Cells were untreated (lane 1), serum starved for 4 h (lane 2), heat shocked (lane 3), or cultured with sodium arsenite for 2 h (lane 4), thapsigargin for 4 h (lane 5), or tunicamycin for 4 h (lane 6), and harvested, and 400 μg of each extract was subjected to immunoprecipitation with α-P52rIPK serum as described above. P58IPK was identified in the immunocomplexes by Western blot analysis (upper panel), and arrows point to the positions of the recovered P58IPK or immunoglobulin (Ig) heavy chain. The lower panels show P52rIPK and P58IPK protein levels in 50 μg of the input extract from each immunoprecipitation reaction.

thapsigargin, or tunicamycin. As shown in Figure 6B, serum withdrawal, heat shock, and treatment with sodium arsenite all resulted in the induction of a stable P52rIPK−P58IPK complex. In contrast, we were unable to detect significant levels of the P52rIPK−P58IPK complex in cells treated with thapsigargin or tunicamycin. Serum withdrawal, heat shock, and sodium arsenite treatment each trigger cytoplasmic stress-response programs, whereas thapsigargin and tunicamycin are specific inducers of ER stress (*1*, *43*). The induction of the P52rIPK−P58IPK interaction only in response to inducers of cytoplasmic stress suggests that P52rIPK regulation of P58IPK is specific to cytoplasmic stress-response pathways.

DISCUSSION

The P52rIPK Charged Domain and Regulation of P58IPK. It has become increasingly clear that TPR proteins participate in a diverse range of signaling processes, including protein kinase regulation and stress-signaling cascades (*44*−*46*). Here we have shown that P52rIPK binds to P58IPK and inhibits its function in vivo in a TPR domain 7-dependent manner. TPR domain 7 is separated from TPR domain 6, which mediates binding to PKR, by only 12 aa. The close proximity of binding sites for P52rIPK and PKR raises the possibility that the binding of these proteins to P58IPK is mutually exclusive. Thus, P52rIPK may inhibit the PKR regulatory function of P58IPK by acting as a noncompetitive inhibitor, binding P58IPK and excluding the PKR−P58IPK interaction.

We have also shown that the 114 aa charged domain of P52rIPK is necessary and sufficient for interaction with P58IPK and that this region is required for the regulation of P58IPK in vivo. This region of P52rIPK is similar to the charged domains of Hsp90, which act as protein interaction motifs. In particular, there is evidence that the C-terminal charged domain of Hsp90 binds to the TPR proteins Hop, FKBP52, FKBP51, Cyp40, and PP5 (*47*, *48*). When we used computer-assisted homology search programs to look for sequence similarity between overlapping 5−10 aa regions of the P52rIPK and Hsp90 charged domains, we found that both proteins contained the 6 aa motif KRIKEL (corresponding to aa 101−106 of P52rIPK). This motif, or a conserved variant, is also present within the TPR-binding regions of other cellular and viral proteins, including c-Myb (*49*), human immunodeficiency virus Vpu and Gag (*50*), and human and murine PKR (*24*, *25*). The presence of this motif within a diverse group of cellular and viral TPR-binding proteins suggests that it may participate in binding to the TPR domain. Indeed, mutations that localize very near and around this motif within the C terminus of Hsp90 preclude its ability to interact with PP5, FKBP52, and Hop (*38*).

A Role for P52rIPK Regulation of P58IPK in Stress-Induced Signaling and Cell Growth Control. Serum withdrawal, heat shock, and sodium arsenite treatment all induce the activation and regulation of PKR (*1*). Each of these regimens also induced the formation of a recoverable P52rIPK−P58IPK complex. Importantly, these treatments all induce cytoplasmic stress-response programs, either through serum-responsive signaling cascades and the direct accumulation of heat shock proteins or, in the case of sodium arsenite, by inducing the inactivation of protein sulfhydryl groups (*1*). In contrast, thapsigargin or tunicamycin treatment, which indirectly activates PERK by inducing ER stress through the depletion of ER calcium stores or inhibition of N-linked glycosylation, respectively (*15*), did not induce discernible P52rIPK−P58IPK complex formation. These results indicate that P52rIPK likely mediates control of P58IPK function and PKR activity in response to cytoplasmic stress signaling. Although we cannot formally exclude a role for P52rIPK in the response to ER stress, our results imply that P52rIPK regulates P58IPK in stress-response pathways that are functionally distinct from those that impact the activity of PERK. This is particularly intriguing since P58IPK expression is induced during ER stress (*51*), and the lack of association with P52rIPK may correlate with a need for increased P58IPK function. In this regard, we have recently found that P58IPK interacts with PERK in ER-stressed cells, suggesting that P58IPK may also function as a regulator of PERK activity (unpublished data).

It is notable that PACT, a protein activator of PKR, is also signaled to bind and activate the kinase in response to a diverse range of stress signals, including serum deprivation (*52*). In response to cellular stress, PACT stimulates the PKR-dependent phosphorylation of eIF2α, which correlates with PACT phosphorylation. This suggests that PACT is signaled to specifically activate PKR during the cellular stress response. The observation that PACT and P52rIPK bind PKR

and P58^{IPK}, respectively, in response to serum withdrawal suggests that these proteins participate in a common pathway. Thus, the binding of P52^{rIPK} to P58^{IPK}, and the inhibition of P58^{IPK} function, may potentiate PACT-mediated activation of PKR in response to stress-induced signals. This may provide the cell a rapid mechanism by which to downmodulate gene expression during the stress response.

The cellular response to stress is clearly dependent upon the nature and duration of the stress-induced signals. We previously demonstrated that when cells are subjected to heat shock, the association between P58^{IPK} and Hsp40 is disrupted during the recovery phase (*23*). However, the role of P52^{rIPK} in this process was not addressed. In the present study, we demonstrated the formation of a P52^{rPK}−P58^{IPK} complex in heat-shocked cells. It is possible that Hsp40 and P52^{rIPK} respond to different signals to mediate regulation of P58^{IPK} at different times and under different circumstances. It will therefore be important to elucidate the signaling mechanisms that lead to activation of P52^{rIPK}. In this regard, preliminary studies from our laboratory suggest that P52^{rIPK} is phosphorylated during the stress response induced by serum withdrawal (unpublished data).

Finally, through its ability to inhibit P58^{IPK}, P52^{rIPK} may participate in cell growth regulation by acting as an upstream modulator of both PKR-dependent and independent events. Expression of trans-dominant negative PKR mutants induces oncogenic transformation of murine fibroblast cell lines, presumably by disrupting PKR-dependent processes of translational control and apoptotic signaling (*53*−*55*). Similarly, the overexpression of cellular and viral inhibitors of PKR, including P58^{IPK}, induces a transformed phenotype in NIH3T3 cells (*28*, *56*, *57*). We also demonstrated that P58^{IPK} possesses oncogenic properties that are independent of PKR, since the overexpression of P58^{IPK} ΔTPR6, which fails to interact with or inhibit PKR, also induces a transformed phenotype (*29*). Taken together, these studies indicate that regulation of PKR and P58^{IPK} function is critical for maintaining control of cell growth and proliferation. Thus, P52^{rIPK} may itself possess antiproliferative properties by modulating the oncogenic potential of P58^{IPK}.

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REFERENCES

1. Brostrom, C. O., and Brostrom, M. A. (1998) Regulation of translational initiation during cellular responses to stress, *Prog. Nucleic Acid Res. Mol. Biol. 58*, 79−125.
2. Sheikh, M. S., and Fornance, A. J., Jr. (1999) Regulation of translation initiation following stress, *Oncogene 18*, 6121−6128.
3. Gale, M., Jr., Tan, S.-L., and Katze, M. G. (2000) Translational control of viral gene expression in eukaryotes, *Microbiol. Mol. Biol. Rev. 64*, 239−280.
4. Frederickson, R. M., and Sonenberg, N. (1992) Signal transduction and regulation of translation initiation, *Semin. Cell Biol. 3*, 107−115.
5. Merrick, W. C., and Hershey, J. W. B. (1996) The pathway and mechanism of eukaryotic protein synthesis, in *Translational control* (Hershey, J., Mathews, M., and Sonenberg, N., Eds.) pp 31−70, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
6. Clemens, M. J. (1996) Protein kinases that phosphorylate eIF-2 and eIF-2B, and their role in eukaryotic cell translational control, in *Translational control* (Hershey, J., Mathews, M., and Sonenberg, N., Eds.) pp 139−172, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7. Silverman, R. H., and Williams, B. R. G. (1999) Translational control perks up, *Nature 397*, 208−211.
8. Hinnebusch, A. G. (1994) The eIF-2α kinases: regulators of protein synthesis in starvation and stress, *Semin. Cell Biol. 5*, 417−426.
9. Chen, J.-J. (2000) Heme-regulated eIF2α kinase, in *Translational control of gene expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.) pp 529−546, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Hinnebusch, A. G. (1996) Translational control of GCN4: gene-specific regulation by phosphorylation of eIF2, in *Translational control* (Hershey, J., Mathews, M., and Sonenberg, N., Eds.) pp 199−244, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
11. Sood, R., Porter, A. C., Olsen, D., Cavener, D. R., and Wek, R. C. (2000) A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2α, *Genetics 154*, 787−801.
12. Olsen, D. S., Jordan, B., Chen, D., Wek, R. C., and Cavener, D. R. (1998) Isolation of the gene encoding the *Drosophila melanogaster* homologue of the *Saccharomyces cerevisiae GCN2* eIF-2α kinase, *Genetics 149*, 1495−1509.
13. Berlanga, J. J., Santoyo, J., and de Haro, C. (1998) Characterization of a mammalian homologue of the GCN2 eukaryotic initiation factor 2α kinase, *Eur. J. Biochem. 265*, 754−762.
14. Ron, D., and Harding, H. P. (2000) Perk and translational control by stress in the endoplasmic reticulum, in *Translational control of gene expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.) pp 547−560, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
15. Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000) Perk is essential for translational regulation and cell survival during the unfolded protein response, *Mol. Cell 5*, 897−904.
16. Williams, B. R. G. (1997) Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation, *Biochem. Soc. Trans. 25*.
17. Clemens, M. J., and Elia, A. (1997) The double-stranded RNA-dependent protein kinase PKR: structure and function, *J. Interferon Cytokine Res. 17*, 503−524.
18. Gale, M., Jr., and Katze, M. G. (1998) Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase, *Pharmacol. Ther. 78*, 29−46.
19. Kumar, A., Yang, Y.-L., Flati, V., Der, S., Kadereit, S., Deb, A., Haque, J., Reis, L., Weissmann, C., and Williams, B. R. G. (1997) Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: role of IRF-1 and NF-κB, *EMBO J. 16*, 406−416.
20. Kaufman, R. J. (2000) Double-stranded RNA-activated protein kinase PKR, in *Translational control of gene expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.) pp 503−528, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Lee, T. G., Tang, N., Thompson, S., Miller, J., and Katze, M. G. (1994) The 58,000-dalton cellular inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (PKR) is a member of the tetratricopeptide repeat family of proteins, *Mol. Cell. Biol. 14*, 2331−2342.
22. Tan, S.-L., Gale, M. J., Jr., and Katze, M. G. (1998) Double-stranded RNA-independent dimerization of interferon-induced protein kinase PKR and inhibition of dimerization by the cellular P58^{IPK} inhibitor, *Mol. Cell. Biol. 18*, 2431−2443.
23. Melville, M. W., Tan, S.-L., Wambach, M., Song, J., Morimoto, R. I., and Katze, M. G. (1999) The cellular inhibitor of the PKR protein kinase, P58^{IPK}, is an influenza virus-activated co-chaperone that modulates heat shock protein 70 activity, *J. Biol. Chem. 274*, 3797−3803.
24. Gale, M., Jr., Tan, S.-L., Wambach, M., and Katze, M. G. (1996) Interaction of the interferon-induced PKR protein kinase with inhibitory proteins P58^{IPK} and vaccinia virus K3L is mediated by

unique domains: implications for kinase regulation, *Mol. Cell. Biol. 16*, 4172−4181.
25. Tang, N. M., Ho, C. Y., and Katze, M. G. (1996) The 58-kDa cellular inhibitor of the double stranded RNA-dependent protein kinase requires the tetratricopeptide repeat 6 and DnaJ motifs to stimulate protein synthesis *in vivo*, *J. Biol. Chem. 271*, 28660−28666.
26. Yan, W., Gale, M. J., Jr., Tan, S.-L., and Katze, M. G. (2002) Inactivation of the PKR protein kinase and stimulation of mRNA translation by the cellular co-chaperone P58IPK does not require J-domain function, *Biochemistry 41*, 4938−4945.
27. Melville, M. W., Hansen, W. J., Freeman, B. C., Welch, W. J., and Katze, M. G. (1997) The molecular chaperone hsp40 regulates the activity of P58IPK, the cellular inhibitor of PKR, *Proc. Natl. Acad. Sci. U.S.A. 94*, 97−102.
28. Barber, G. N., Thompson, S., Lee, T. G., Strom, T., Jagus, R., Darveau, A., and Katze, M. G. (1994) The 58-kilodalton inhibitor of the interferon-induced double-stranded RNA-activated protein kinase is a tetratricopeptide repeat protein with oncogenic properties, *Proc. Natl. Acad. Sci. U.S.A. 91*, 4278−4282.
29. Tang, N. M., Korth, M. J., Gale, M., Jr., Wambach, M., Der, S. D., Bandyopadhyay, S. K., Williams, B. R. G., and Katze, M. G. (1999) Inhibition of double-stranded RNA- and tumor necrosis factor alpha-mediated apoptosis by tetratricopeptide repeat protein and cochaperone, P58IPK, *Mol. Cell. Biol. 19*, 4757−4765.
30. Gale, M., Jr., Blakely, C. M., Hopkins, D. A., Melville, M. W., Wambach, M., Romano, P. R., and Katze, M. G. (1998) Regulation of interferon-induced protein kinase PKR: modulation of P58IPK inhibitory function by a novel protein, P52rIPK, *Mol. Cell. Biol. 18*, 859−871.
31. Cesareni, G., and Murray, J. A. H. (1987) Plasmid vectors carrying the replication origin of filamentous single-stranded phages, in *Genetic engineering: principles and methods* (Setlow, J., Ed.) 1st ed., pp 135−154, Plenum Publishing Corp., New York.
32. Laurent, A. G., Krust, B., Galabru, J., Svab, J., and Hovanessian, A. G. (1985) Monoclonal antibodies to interferon induced 68,-000 Mr protein and their use for the detection of double-stranded RNA dependent protein kinase in human cells, *Proc. Natl. Acad. Sci. U.S.A. 82*, 4341−4345.
33. Harlow, E., and Lane, D. (1988) *Antibodies: a laboratory manual*, pp 310−310, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. Romano, P. R., Green, S. R., Barber, G. N., Mathews, M. B., and Hinnebusch, A. G. (1995) Structural requirements for double-stranded RNA binding, dimerization, and activation of the human eIF-2α kinase DAI, in *Saccharomyces cerevisiae*, *Mol. Cell. Biol. 15*, 365−378.
35. 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) Mammalian eukaryotic initiation factor elF2α kinases functionally substitute for GCN2 protein kinase in the GCN4 translational control mechanism of yeast, *Proc. Natl. Acad. Sci. U.S.A. 90*, 4616−4620.
36. Hickey, E., Brandon, S. E., Smale, G., Lloyd, D., and Weber, L. A. (1989) Sequence and regulation of a gene encoding a human 89 kilodalton heat shock protein, *Mol. Cell. Biol. 9*, 2615−2626.
37. Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent, *Cell 89*, 239−250.
38. Ramsey, A. J., Russell, L. C., Whitt, S. R., and Chinkers, M. (2000) Overlapping sites of tetratricopeptide repeat protein binding and chaperone activity in heat shock protein 90, *J. Biol. Chem. 275*, 17857−17862.
39. Russell, L. C., Whitt, S. R., Chen, M. S., and Chinkers, M. (1999) Identification of conserved residues required for the binding of tetratricopeptide repeat domain to heat shock protein 90, *J. Biol. Chem. 274*, 20060−20063.
40. Chen, S., Sullivan, W. P., Toft, D. O., and Smith, D. F. (2000) Differential interactions of p23 and the TPR-containing proteins Hop, Cyp40, FKBP52, and FKBP51 with Hsp90 mutants, *Cell Stress Chaperones 3*, 118−129.
41. Blatch, G. L., and Lässle, M. (1999) The tetratricopeptide repeat: a structural motif mediating protein−protein interactions, *BioEssays 21*, 932−939.
42. Gale, M., Jr., Korth, M. J., Tang, N. M., Tan, S.-L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R., and Katze, M. G. (1997) Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein, *Virology 230*, 217−227.
43. McLauchlan, J. (2000) Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes, *J. Viral Hepatitis 7*, 2−14.
44. Sikorski, R. S., Michaud, W. A., Wootton, J. C., Boguski, M. S., Connelly, C., and Hieter, P. (1991) TPR proteins as essential components of the yeast cell cycle, *Cold Spring Harbor Symp. Quant. Biol. 56*, 663−673.
45. Stepanova, L., Leng, X., and Harper, J. W. (1997) Analysis of mammalian Cdc37, a protein kinase targeting subunit of heat shock protein 90, *Methods Enzymol. 283*, 220−229.
46. Prodromou, C., Siligardi, G., O'Brien, R., Woolfson, D. N., Regan, L., Panaretou, B., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1999) Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones, *EMBO J. 18*, 754−762.
47. Carrello, A., Ingley, E., Minchin, R. F., Tsai, S., and Ratajczak, T. (1999) The common tetratricopeptide repeat acceptor site for steroid receptor-associated immunophilins and hop is located in the dimerization domain of Hsp90, *J. Biol. Chem. 274*, 2682−2689.
48. Young, J. C., Oberman, W. M. J., and Hartl, F. U. (1998) Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90, *J. Biol. Chem. 273*, 18007−18010.
49. Leverson, J. D., and Ness, S. A. (1998) Point mutations in v-Myb disrupt a cyclophilin-catalyzed negative regulatory mechanism, *Mol. Cell 1*, 203−211.
50. Callahan, M. A., Handley, M. A., Lee, Y. H., Talbot, K. J., Harper, J. W., and Panganiban, A. T. (1998) Functional interaction of human immunodeficiency virus type 1 Vpu and Gag with a novel member of the tetratricopeptide repeat protein family, *J. Virol. 72*, 5189−5197.
51. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R. J. (2001) Translational control is required for the unfolded protein response and in vivo glucose homeostasis, *Mol. Cell 7*, 1165−1176.
52. Patel, C. V., Handy, I., Goldsmith, T., and Patel, R. C. (2000) PACT, a stress-modulated cellular activator of interferon-induced double-stranded RNA-activated protein kinase, PKR, *J. Biol. Chem. 275*, 37993−37998.
53. Koromilas, A. E., Roy, S., Barber, G. N., Katze, M. G., and Sonenberg, N. (1992) Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase, *Science 257*, 1685−1689.
54. Barber, G. N., Wambach, M., Thompson, S., Jagus, R., and Katze, M. G. (1995) Mutants of the RNA-dependent protein kinase (PKR) lacking double-stranded RNA binding domain I can act as transdominant inhibitors and induce malignant transformation, *Mol. Cell. Biol. 15*, 3138−3146.
55. Balachandran, S., Kim, C. N., Yeh, W.-C., Mak, T. W., and Barber, G. N. (1998) Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling, *EMBO J. 17*, 6888−6902.
56. Benkirane, M., Neuveut, C., Chun, R. F., Smith, S. M., Samuel, C. E., Gatignol, A., and Jeang, K.-T. (1997) Oncogenic potential of TAR RNA binding protein TRBP and its regulatory interaction with RNA-dependent protein kinase PKR, *EMBO J. 16*, 611−624.
57. Gale, M., Jr., Kwieciszewski, B., Dossett, M., Nakao, H., and Katze, M. G. (1999) Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase, *J. Virol. 73*, 6506−6516.

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