The P58 Cellular Inhibitor Complexes with the Interferon-induced, Double-stranded RNA-dependent Protein Kinase, PKR, to Regulate Its Autophosphorylation and Activity*

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The 58-kDa protein, referred to as P58, is a cellular inhibitor of the interferon-induced, double-stranded RNA-activated protein kinase, PKR. The P58 protein inhibits both the autophosphorylation of PKR and the phosphorylation of the PKR natural substrate, the α subunit of eukaryotic translation initiation factor eIF-2. Sequence analysis revealed that P58 is a member of the tetratricopeptide family of proteins. Utilizing experimental approaches, which included coprecipitation and coseduction of native and recombinant wild-type and mutant proteins, we found that P58 can efficiently complex with the PKR protein kinase. Attempts to map the P58 interactive sites revealed a correlation between the ability of P58 to inhibit PKR in vitro and bind to PKR. The DnaJ sequences, present at the carboxyl terminus of P58, were dispensable for binding in vitro, whereas sequences containing the eIF-2 α similarity region were essential for efficient complex formation. Furthermore, not all tetratricopeptide motifs were necessary for PKR-P58 interactions. Initial experiments to map the binding domains present in PKR showed that P58 complexed with PKR molecules that lacked the first RNA binding domain but did not bind to a PKR mutant containing only the amino terminus. These data, taken together, demonstrate that P58 inhibits PKR through a direct interaction, which is likely independent of the binding of double-stranded RNA to the protein kinase.

PKR1 (for protein kinase RNA-dependent) is a 68-kDa serine-threonine kinase that is induced in cells upon interferon treatment (1–4). In the presence of double-stranded RNA (or other polyanions), PKR becomes autophosphorylated and phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF-2) (5, 6). Phosphorylation of eIF-2 by PKR prevents recycling of eIF-2GDP to eIF-2GTP, and as a result, protein synthesis initiation is globally blocked within the cell (7, 8). PKR activation and phosphorylation of eIF-2 therefore can represent a major effector of the interferon antiviral response. Interestingly, PKR, due to its translationally repressive functions (9, 10), also may be involved in the control of cell growth. In this regard, introduction of catalytically inactive and select regulatory domain mutants of PKR into NIH 3T3 cells results in their malignant transformation (11–13). In certain cases this may be due to dominant negative inhibition of the endogenous murine PKR by the mutants, leading to deregulated protein synthesis (11, 13).

Both RNA and DNA viruses produce RNA intermediates that can activate PKR. As a result, a number of these viruses encode or activate proteins or RNAs that specifically inhibit the action of PKR (14–17). If this down-regulation of PKR failed to occur, viral protein synthesis would become severely compromised. We have shown previously that PKR activation is inhibited in influenza virus infected cells by a cellular protein (18–22). The PKR inhibitor, termed P58 based on its molecular weight of 58 kDa, was purified to homogeneity from Madin-Darby bovine kidney cells, and subsequently the gene was molecularly cloned from Madin-Darby bovine kidney cells, and subsequently the gene was molecularly cloned using reverse genetics (19, 20). Recombinant P58 is capable of inhibiting PKR activation and eIF-2 phosphorylation (20). Sequence analysis indicated that P58 is a member of the tetratricopeptide (TPR) family of proteins, which is characterized by internal 34 amino acid repeats. Moreover, it was found that P58 possessed sequence similarity to the DnaJ heat shock family of proteins and more limited similarity to the eIF-2 α subunit.

We found that overexpression of P58 in NIH 3T3 cells caused their malignant transformation, demonstrating the oncogenic potential of the PKR inhibitor (23). The molecular mechanisms underlying P58-induced down-regulation of PKR and transformation of murine cells remain unknown. Earlier work from our laboratory demonstrated that P58 did not retain ATPase, phosphatase, or protease activity (20, 21). Furthermore, P58 did not sequester dsRNA activator nor did the inhibitor degrade dsRNAs.2 The current study was initiated to determine whether, like the adenovirus VAI RNA inhibitor (24) and the vaccinia virus K3L PKR inhibitor (25), P58 functioned through a direct interaction with PKR. Utilizing recombinant wild-type and mutant proteins, we now show that P58 and PKR form a complex in vitro. Moreover, P58 variants, which fail to inhibit PKR in vitro, do not interact with PKR. Finally we demonstrate that P58 can interact with a PKR variant lacking RNA binding domain 1 but not to a mutant containing both dsRNA binding domains.

MATERIALS AND METHODS

Recombinant PKR and P58 Constructs—Fig. 1A depicts the recombinant P58 and PKR proteins utilized in this study. Fig. 1A shows a schematic diagram of wild-type and mutant P58 (20). The positions of

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1 The abbreviations used are: PKR, double-stranded RNA-dependent protein kinase; eIF, eukaryotic translation initiation factor; TPR, tetratricopeptide; GST, glutathione S-transferase; RBD, RNA binding domain; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ds, double-stranded.

2 T. G. Lee and M. G. Katze, unpublished results.
ThenineTPRdomainsandregionsofIF-2

...the panel is depicted the P58 gene with the nine TPR motifs. The text, this mutant was cloned and purified as a histidine fusion protein. Terminal 97 amino acids and RNA binding domain 1. As described in the...under "Material and Methods." Fig. 1A shows the wild-type PKR and mutant PKR proteins. Wild-type PKR contains the amino-terminal RNA binding domains 1 and 2 (RBD-I and -II) in the regulatory amino terminus and the 11 catalytic domains found at the carboxyl terminus. Also depicted in panel C is a mutant PKR, which lacks the first amino-terminal 97 amino acids and RNA binding domain 1. As described in the text, this mutant was cloned and purified as a histidine fusion protein.

The nine TPR domains and regions of elf-2-a and DnaJ similarities are highlighted. The histidine-tagged wild-type and mutant are depicted below and are described under "Material and Methods." Panel B, the GST-P58 fusion protein (and GST tag alone as control) expressed from vector pGEX2T-P58 are shown. Panel C, the wild-type PKR is depicted, which contains the two dRNA binding domains (RBD-I and -II) in the regulatory amino terminus and the 11 catalytic domains found at the carboxyl terminus. Also depicted in panel C is a mutant PKR, which lacks the first amino-terminal 97 amino acids and RNA binding domain 1. As described in the text, this mutant was cloned and purified as a histidine fusion protein.

Antisera—The mouse monoclonal antibody (mAb) 2F8 recognizes an epitope contained within the amino terminus of PKR (23). The 3D7 monoclonal antibody recognizes an epitope contained within the histidine-thrombin tag present at the amino terminus of all recombinant P58 proteins. Normal mouse serum and an irrelevant IgG1 monoclonal antibody derived from mineral oil plasma cells (28) were used as controls in selected immunoprecipitation reactions. The monoclonal and polyclonal antibody prepared against PKR have been extensively described elsewhere (13, 29–31).

Purification and Activation of Native and Recombinant Human PKR—To prepare purified native PKR, Daudi cells were propagated at 37°C in RPMI medium containing 10% fetal bovine serum, 500 units/ml of penicillin G, 0.1 mg/ml streptomycin sulfate, 1% l-glutamine, 1 mM sodium pyruvate, and 1% nonessential amino acids. Prior to harvesting, cells were treated with human interferon at 500 units/ml for 24 h. PKR was purified by immunoaffinity chromatography utilizing PKR monoclonal antibody linked to CNBr-activated Sepharose as described earlier (5). A recombinant PKR lacking amino acids 1–97 was cloned as a histidine fusion protein and purified on a nickel column under native conditions (32). For activation, an aliquot of purified PKR was diluted in KCl buffer (20 mM Tris, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 25 mM KCl) including heparin and 10 μCi of [32P]ATP and incubated for 15 min at 30°C (33).

Expression and Purification of Recombinant P58—Two systems for expression of P58 were employed. The first involved the fusion of wild-type bovine P58 to a GST tag. P58 was cloned into the plasmid pGEX2T (20). For induction of the GST-P58 fusion protein, overnight cultures were diluted 1:10 and grown for 1 h at 37°C. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.1 mM. Cells were grown at 37°C for an additional hour prior to harvesting by centrifugation. Induced cells were washed once in ice-cold PBS and resuspended in PBS containing 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. Samples were centrifuged for 5 s and centrifuged at 14,000 rpm for 5 min at 4°C (34). Recombinant bovine P58 fused to a histidine-thrombin tag served as the second P58 expression system (HIS-P58). Wild-type bovine P58 and various mutants of P58 were cloned into pet15b (Novagen) as described previously (20). Expression of the recombinant proteins was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.2 mM followed by incubation at 30°C for 2 h. The fusion proteins were then purified by chromatography over a nickel column for the histidine fusion proteins or a glutathione-agarose column for the GST proteins (20).
The following series of experiments was performed. Initially, purity
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2-mercaptoethanol) and boiling. The samples were then analyzed by

RESULTS AND DISCUSSION

To determine whether the cellular P58 PKR inhibitor func-
tioned by directly complexes with the PKR protein kinase, the
following series of experiments was performed. Initially, puri-
fied, in vitro activated, [32P]-labeled native P58 was mixed with
increasing amounts of crude lysates prepared from E. coli over-
expressing either the recombinant fusion protein, GST-P58, or,
as a control, GST alone. After incubation at 30 °C in the pres-
ence of PBS, 1% Triton X-100, GST-P58 and GST control pro-
tiens in the lysates were selected on glutathione-agarose beads
as described under “Materials and Methods,” and subjected to
gel electrophoresis. Coexpressed proteins were then visualized
by autoradiography or by Coomassie Brilliant Blue staining of
the nitrocellulose filter obtained after blotting the
SDS-polyacrylamide gel. Staining of the filter demonstrated
that the predominant bound proteins, as expected, were either
GST-P58 or GST (Fig. 2A). Most significantly, autoradiography
of the identical nitrocellulose filter revealed that radiolabeled
PKR was coexpressed on glutathione-agarose beads containing
GST-P58 but not GST, despite the large molar excess of the
latter. The binding of PKR to GST-P58 increased with increasing
concentrations of lysates (Fig. 2B, lanes 2–4). Phosphor-
Imager quantitation revealed that up to 9–15-fold more PKR
was selected on beads containing GST-P58 compared with
beads containing GST alone. Furthermore, no PKR binding to
the glutathione-agarose beads alone was observed (Fig. 2B,
lane 1). Approximately 13% of the input radiolabeled PKR
was estimated to bind to GST-P58 at the highest levels of extracts
tested.

To provide additional evidence for this PKR-P58 association,
a reciprocal type experiment was performed with [35S]methio-
one-labeled GST-P58 and unlabeled heparin-activated native
PKR. Following the addition of PKR to a preparation of purified
radiolabeled GST-P58 (or radiolabeled GST alone as control)
and incubation in the presence of PBS, 1% Triton X-100, the
mixture was immunoprecipitated using a polyclonal antiserum
specific for PKR. There was a molar excess of both GST-P58
and GST added to the binding reaction: at the highest concen-
trations (lanes 3 and 6) 12 pmol of GST-P58 and 40 pmol of GST
were mixed with 2 pmol of PKR as described in detail in the
figure legend. Increasing amounts of [35S]methionine-labeled
GST-P58 (approximately 10% of input) were coprecipitated
with PKR, whereas no detectable radiolabeled GST was copre-
icipitated with PKR (Fig. 3, compare lanes 1–3 with lanes 4–6).

We also determined that the radiolabeled GST-P58 itself was
not recognized by the PKR polyclonal antibody (data not
shown). Taken together with the previous experiments, these
results show that PKR interacts with P58 and that this inter-
action is specific and not dependent on the GST tag.

The next group of experiments attempted to map the sites on
the P58 protein, which were required for PKR binding. We
analyzed wild-type and variant histidine fusion P58 recombin-
ant proteins that had been previously analyzed for their abil-
ity to inhibit PKR autophosphorylation activity in vitro (20).

Purified P58 histidine fusion proteins were incubated with
[32P]-labeled native PKR at approximately a 15–20-fold molar
excess followed by immunoprecipitation with either P58
specific monoclonal antibody or an irrelevant monoclonal antibody
prepared against IgG1. Coprecipitation of radiolabeled PKR
with the P58-specific antibody would indicate that a specific
interaction between PKR and P58 occurred. Four mutant P58
recombinant proteins were tested in these studies (Fig. 1). (i)
SER241 contains a serine to alanine mutation at amino acid
241 within an ELStripeptide in the P58 protein. A similar ELS
triptpeptide, containing serine 51, the residue phosphorylated
by PKR, is found within the eF-2 α subunit. Although this se-
sequence similarity suggested an important role of this serine
for P58 function, the variant nonetheless retained full PKR inhib-
itory activity (20). (ii) Mutant 8-1 lacked amino acids 167–504
and was nonfunctional in our in vitro PKR autophosphoryla-
tion assay (20). (iii) Mutant 8-2 lacked amino acids 278–504
but contained the region of similarity with the eIF-2 α subunit
and retained kinase inhibitory function (20). (iv) Finally, mu-
tant 9-1 lacked the amino-terminal 167 amino acids and also
Fig. 3. [35S]Methionine-labeled GST-P58 complexes with PKR. Increasing amounts of either [35S]methionine-labeled purified GST-P58 (lanes 1–3, representing 1.0, 2.0, and 12.0 pmol, respectively) or GST (lanes 4–6, representing 4.0, 8.0, and 40.0 pmol, respectively) were mixed with unlabeled, heparin-activated PKR (2 pmol), and immunoprecipitated with polyclonal PKR antiserum derived from baculovirus expressed PKR. An aliquot of radiolabeled purified GST-P58 or GST is shown in lanes 7 and 8, respectively. The migration of GST-P58 and GST are shown by arrows on the right. Phosphorimager quantitation revealed that approximately 10% of input radiolabeled GST-P58 bound to PKR.

retained PKR inhibitory function (20). We first verified that the specific P58 monoclonal antibody immunoprecipitated each of these recombinant proteins. [35S]Methionine-labeled extracts were prepared from E. coli overexpressing each of these four mutants (Fig. 4A). The P58 specific monoclonal antibody, 2F8, efficiently immunoprecipitated SER241, 8-1, and 8-2 (Fig. 4A) but could not immunoprecipitate 9-1, which lacked the amino terminus (data not shown). Mutant 9-1 was efficiently immunoprecipitated, however, with monoclonal antibody 3D7, which we previously determined recognized the histidine-thrombin tag present at the very amino terminus of the fusion protein (Fig. 4A). Previously the functional activity of these variants were assayed by the inhibition of PKR autophosphorylation (20). Before performing the binding experiments, we tested the ability of the P58 variants to inhibit PKR activity as measured by the phosphorylation of exogenously added histones (Fig. 4B).

Both P58, 9-1, and SER241 inhibited PKR function and reduced histone phosphorylation (40–50%), while 8-1 failed to significantly inhibit PKR activity (<10%) compared with the control. Variant 9-1 also inhibited PKR-mediated histone phosphorylation (data not shown).

The binding or coprecipitation experiments were then executed using 3D7 mAb for 9-1, and 2F8 mAb for the other three constructs (and the irrelevant IgG1 antiserum as a negative control). As an additional negative control, we tested the binding of PKR to material, which bound to and eluted from a nickel column exposed to extracts from E. coli, which expressed the histidine fusion vector alone (Fig. 4C, CON). For binding experiments using the histidine fusion proteins, we determined that, although binding did occur in the presence of PBS/Triton, the specificity of binding was greatly enhanced utilizing a binding buffer containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.5% SDS. The binding of mutants 8-1, 8-2, and 9-1 was compared with SER241, which we earlier demonstrated bound to PKR with equal efficiency to the wild-type PKR (data not shown). Radiolabeled PKR bound to both 8-2 and 9-1 but failed to specifically interact with mutant 8-1 (Fig. 4C). We estimate that approximately 7% of input PKR bound to SER241 and 8-2, whereas 4% bound to mutant 9-1. PKR failed to react with control extracts eluted from the nickel column and, furthermore, was not coprecipitated with the irrelevant monoclonal antibody. In addition, we determined that radiolabeled PKR alone failed to react with the P58-specific mAb (data not shown). It is unclear whether the slightly reduced binding to 9-1 relative to the other mutants is due to the difference in monoclonal antibody used or a bona fide difference in the affinity between 9-1 and PKR. It is clear, however, that the binding of the P58 variants correlated well with their ability to inhibit PKR in vitro since SER241, 8-1, and 9-1 are functional while 8-1 is unable to inhibit PKR in vitro.

The final series of experiments were intended to gain additional knowledge of the molecular mechanisms by which P58 inhibits PKR. We were particularly interested in determining whether P58 bound to the dsRNA binding sites on PKR. Other PKR inhibitors that bind PKR, such as the adenovirus VA1 RNA (24), function by competitively inhibiting dsRNA binding to the kinase. We first examined whether P58 bound a PKR variant, which lacked the RBD-1 and which therefore failed to bind detectable levels of dsRNA. Construction and characterization of this histidine fusion PKR mutant has been extensively described elsewhere (13, 32). Despite the absence of RBD-1, the PKR mutant can be efficiently autophosphorylated and radiolabeled in vitro in the presence of the polyanion heparin (32). Recombinant wild-type P58 (also expressed as a histidine fusion protein; 40 pmol) was incubated with two concentrations of 32P-labeled RBD-1 minus PKR (20 pmol, Fig. 5A, lanes 2 and 5; 40 pmol, Fig. 5A, lanes 3 and 6) followed by immunoprecipitation either by the P58-specific monoclonal antibody, 2F8, or, as a control, normal mouse serum. The RBD-1 minus PKR was only coprecipitated with P58 when the specific antibody was utilized (Fig. 5A, lanes 2 and 3). Furthermore, coprecipitation of PKR depended on the presence of the recombinant P58, demonstrating that there was no cross-reactivity between PKR and the P58-specific antibody. Details of quantitation are provided in the legend to Fig. 5.

The last experiment was designed to directly test whether P58 bound a PKR mutant containing only the amino half and therefore both dsRNA binding domains but no catalytic domains (33). Since such a PKR mutant was catalytically inactive, it was necessary to develop another binding assay and prepare in vitro translated wild-type and mutant PKR radiolabeled with [35S]methionine. Both full-length PKR (as control) and PKR containing amino acids 1–242 (Fig. 5B, lanes 1 and 2, respectively) were prepared as described in detail previously (33). Both the in vitro translated full-length and mutant PKR proteins were previously found to efficiently bind dsRNA (33). The in vitro translated proteins were then mixed with crude lysates prepared from E. coli overexpressing either the recombinant fusion protein, GST-P58, or, as a control, GST alone. After incubation, GST-P58 and GST control proteins were selected on glutathione beads and subjected to gel electrophoresis. Coselected proteins were visualized by autoradiography (Fig. 5B, lanes 3–10). Radiolabeled full-length PKR was co-selected on agarose beads containing GST-P58 (lanes 5 and 6) but not GST (lanes 3 and 4). In contrast, the in vitro translated PKR mutant, containing amino acids 1–242, was not co-selected with GST-P58 and thus did not bind PKR (lanes 9 and 10). These data, taken together, suggest that PKR binding site(s) for P58 and dsRNA are likely distinct and that P58 inhibits PKR by mechanisms independent of dsRNA-mediated activation.

The current work was undertaken to determine the molecular mechanisms underlying P58 action. Utilizing two independent recombinant GST and histidine P58 fusion proteins, glutathione-agarose coselection, and a variety of both monoclonal and polyclonal antibodies, we determined that P58 forms a
complex with PKR in vitro. The interaction between P58 and PKR is likely direct and does not involve other cellular proteins, since complexes were obtained utilizing highly purified proteins. Furthermore, this is likely a high affinity interaction since complex formation occurred in crude extracts and in the presence of 0.5% SDS and sodium deoxycholate. Unlike the adenovirus-encoded PKR inhibitor, VAI RNA, P58 does not function by binding to the dsRNA binding sites on PKR and can thus bind to PKR variants, which lack the crucial first RNA binding domain but not to a mutant containing only the RNA binding domains. It is therefore likely that P58 binds to regions within the catalytic domains of PKR. P58 is a member of the TPR family of proteins, characterized by the presence of internal 34-amino acid repeat motifs, which are thought to form helix turn structures, each with a knob and hole, acting as helix-associating domains. TPR proteins are reported to have helix-turn-helix-associating domains. TPR proteins are reported to have helix-turn-helix-associating domains. TPR proteins are reported to have helix-turn-helix-associating domains.

**Fig. 4.** Binding of wild-type and mutant histidine fusion P58 proteins to 32P-radio labeled PKR and analysis of P58 function. Panel A, recombinant proteins were labeled in the presence of [35S]methionine as described under "Materials and Methods." Mutants SER241, 8-1, and 8-2 were immunoprecipitated by 2F8 mAb, while 9-1 was immunoprecipitated by 3D7 (lanes A). As control, all extracts were immunoprecipitated with an irrelevant IgG1 mAb (lanes B). A profile of total cellular proteins derived from extracts containing SER241, 8-1, 8-2, and 9-1 proteins is shown on the right of panel A. Panel B, approximately equimolar amounts of the P58 wild-type and variants were tested for their ability to inhibit PKR-mediated histone phosphorylation as described under "Materials and Methods." As a negative control, we tested the PKR inhibitory activity of material which bound to and eluted from a nickel column exposed to extracts from E. coli, which expressed the histidine fusion vector alone (CON). The histone bands were subjected to phosphorimager analysis for quantitation. Relative to the control, PKR wild-type inhibited 51%, SER241 inhibited 46%, 8-2 inhibited 41%, and 8-1 inhibited approximately 82% (lanes B). Relative to the control, PKR wild-type inhibited 51%, SER241 inhibited 46%, 8-2 inhibited 41%, and 8-1 inhibited approximately 82% (lanes B). As an additional control (CON) coprecipitations with 2F8 (A) or IgG1 (B) mAbs were performed on a mixture containing radiolabeled PKR and elute from a nickel column to which was added extracts from E. coli expressing the vector alone. Phosphorimager quantitation revealed that approximately 7% of input radio labeled PKR bound to SER241 and 8-2 and approximately 4% to 9-1. Quantitation showed that compared with the control, 8-fold more PKR bound to SER241, 5-fold more PKR bound to 8-2, 4-fold more PKR bound to 9-1, and 0.25-fold more PKR bound to the negative 8-1 mutant.

**Fig. 5.** P58 complexes with a PKR mutant lacking RBD-1 but not a PKR mutant containing only the amino terminus. Panel A, approximately 20 pmol (lanes 2 and 5) or 40 pmol (lanes 3 and 6) of PKR lacking RBD-1 was radiolabeled in vitro in the presence of heparin and incubated with purified histidine-tagged P58 (40 pmol). The mixture was then immunoprecipitated with either PSB-specific 2F8 mAb (lanes 1-3) or normal mouse serum (NMS) (lanes 4-6). As an additional control, precipitations were performed with 20 pmol of radiolabeled PKR in the absence of P58 (lanes 1 and 4). The migration of delta RBD-1 PKR is shown on the right of the panel. Phosphorimager quantitation revealed that 8% of radiolabeled input PKR bound when 20 pmol was tested and 20% input radiolabeled PKR bound when 40 pmol was tested. Quantitation also revealed that 12-18-fold more PKR bound when the specific 2F8 antibody was utilized to coprecipitate PKR and P58 compared with normal mouse serum. Panel B, left side of panel, aliquots of 35S-labeled protein, GST-P58 (20 and 50 pmol), PKR (20 pmol), GST (50 pmol), GST-P58 containing the amino terminus, GST-P58 containing the amino terminus but lacking the RBD (10 pmol), GST-P58 containing the amino terminus but lacking the RBD and the first 3 motifs (5 pmol), GST-P58 containing the amino terminus but lacking the RBD and the first 3 motifs but containing the central 34 amino acids (1 pmol), GST-P58 containing the amino terminus but lacking the RBD and the first 3 motifs but containing the central 34 amino acids but lacking the RNA binding domain (0.5 pmol), or GST (20 pmol) were analyzed by SDS-polyacrylamide gel electrophoresis. In vitro translated full-length PKR proteins were prepared from in vitro transcribed RNA as described previously (33). Right side of panel, increasing amounts of GST-P58 (20 and 50 pmol, lanes 3 and 6 and lanes 9 and 10) or GST (20 and 50 pmol, lanes 3 and 4 and lanes 7 and 8) containing lysates were mixed with the in vitro translated full-length PKR (lanes 3-6) or mutant PKR (lanes 7-10) proteins. Following selection on glutathioneagarose beads, samples were washed, resuspended in SDS disruption buffer, and electrophoresed by SDS-polyacrylamide gel electrophoresis (lanes 3-10).
currently unknown, it remains possible that P58, based on its homology to the PKR substrate, interacts with a similar site on PKR. However it should be stressed that P58 blocks the auto-phosphorylation of PKR as well as eIF-2α phosphorylation, indicating that P58 may need to interact with PKR at multiple sites to regulate enzyme function.

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REFERENCES
1. Meurs, E., Chong, K., Galabru, J., Thomas, N., Kerr, I., Williams, B. R. G. & Hovanessian, A. G. (1990) Cell 62, 379–390
2. Samuel, C. E. (1993) J. Biol. Chem. 268, 7603–7606
3. Sen, G. C. & Lengyel, P. (1992) J. Biol. Chem. 267, 5017–5020
4. Hovanessian, A. G. (1989) J. Interferon Res. 9, 641–647
5. Galabru, J. & Hovanessian, A. (1987) J. Biol. Chem. 262, 15538–15544
6. Hovanessian, A. G. & Galabru, J. (1987) Eur. J. Biochem. 167, 467–473
7. Hershey, J. W. B. (1991) Annu. Rev. Biochem. 60, 717–755
8. Rhoads, R. E. (1993) J. Biol. Chem. 268, 3017–3020
9. Barber, G. N., Wambach, M., Wong, M.-L., Dever, T. E., Hinnebusch, A. G. & Katze, M. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4621–4625
10. Thomis, D. C. & Samuel, C. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10837–10841
11. Koromilas, A. E., Roy, S., Barber, G. N., Katze, M. G. & Sonneberg, N. (1992) Science 257, 1685–1689
12. Meurs, E., Galabru, J., Barber, G. N., Katze, M. G. & Hovanessian, A. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 232–236
13. Barber, G. N., Wambach, M., Thompson, S., Jagus, R. & Katze, M. G. (1995) Mol. Cell. Biol. 15, 3138–3146
14. Katze, M. G. (1993) Semin. Virol. 4, 259–268
15. Mathews, M. B. (1993) Semin. Virol. 4, 382–386
16. Samuel, C. E. (1991) Virology 183, 1–11
17. Katze, M. G. (1995) Trends Microbiol. 3, 75–78
18. Katze, M. G., Tomita, J., Black, T., Krug, R. M., Safer, B. & Hovanessian, A. G. (1988) J. Virol. 62, 3710–3717
19. Lee, T.-G., Tomita, J., Hovanessian, A. G. & Katze, M. G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6208–6212
20. Lee, T. G., Tang, N., Thompson, S., Miller, J. & Katze, M. G. (1994) Mol. Cell. Biol. 14, 2331–2342
21. Lee, T. G., Tomita, J., Hovanessian, A. G. & Katze, M. G. (1992) J. Biol. Chem. 267, 14238–14243
22. Garfinkel, M. S. & Katze, M. G. (1994) Sci. Am. Sci. & Med. 1, 64–73
23. Barber, G. N., Thompson, S., Lee, T. G., Strom, T., Jagus, R., Darveau, A. & Katze, M. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4278–4282
24. Katze, M. G., DeCerotto, D., Safer, B., Galabru, J. & Hovanessian, A. G. (1987) EMBO J. 6, 689–697
25. Carrell, K., Elroy-Stein, O., Moss, R. & Jagus, R. (1993) J. Biol. Chem. 268, 12373–12382
26. Green, S. R. & Mathews, M. B. (1992) Genes & Dev. 6, 2478–2490
27. St. Johnston, D., Brown, N. R., Gall, J. G. & Jantsch, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10979–10983
28. Yosida, T. H., Imai, H. T. & Potter, M. (1968) J. Natl. Cancer Inst. 41, 1083–1097
29. Barber, G. N., Tomita, J., Hovanessian, A. G., Meurs, E. & Katze, M. G. (1991) Biochemistry 30, 10356–10361
30. Barber, G. N., Tomita, J., Garfinkel, M. S., Hovanessian, A. G., Meurs, E. & Katze, M. G. (1992) Virology 191, 670–679
31. Laurent, A. G., Krust, B., Galabru, J. & Svab, J. & Hovanessian, A. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4341–4345
32. Barber, G. N., Jagus, R., Meurs, E., Hovanessian, A. G. & Katze, M. G. (1995) J. Biol. Chem. 270, 17423–17428
33. Katze, M. G., Wambach, M., Wong, M.-L., Garfinkel, M. S., Meurs, E., Chong, K. L., Williams, B. R. G., Hovanessian, A. G. & Barber, G. N. (1991) Mol. Cell. Biol. 11, 5497–5505
34. Smith, D. B. & Johnson, K. S. (1988) Gene (Amst.) 67, 31–40
35. Blackwood, E. M., Luscher, B. & Eisenman, R. N. (1990) Genes & Dev. 6, 71–80
36. Goedl, M. & Yanagida, M. (1991) Trends Biochem. Sci. 16, 173–177
37. Sikorski, R. S., Michaud, W. A., Wootton, J. C., Boguski, M. S., Connelly, C. & Hieter, P. (1993) Cold Spring Harbor Symp. Quant. Biol. 58, 663–673
38. Sikorski, R. S., Michaud, W. A. & Hieter, P. (1993) Mol. Cell. Biol. 13, 1212–1221