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Increased Interaction Between PACT Molecules in Response to Stress Signals Is Required for PKR Activation

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ABSTRACT
PKR (protein kinase, RNA activated) is an interferon (IFN)-induced serine-threonine protein kinase and is one of the key mediators in IFN's cellular actions. Although double-stranded (ds) RNA is the most relevant PKR activator during viral infections, PACT acts as a stress-modulated activator of PKR and is an important regulator of PKR dependent signaling pathways in the absence of viral infections. Stress-induced phosphorylation of PACT is essential for PACT's association with PKR leading to PKR activation. PKR activation by PACT leads to phosphorylation of translation initiation factor eIF2α, inhibition of protein synthesis, and apoptosis. In the present study, we have investigated the functional significance of PACT–PACT interaction in mediating PKR activation in response to cellular stress. Our results suggest that enhanced interaction between PACT molecules when PACT is phosphorylated in response to stress signals on serines 246 and 287 is essential for efficient PKR activation. Using a point mutant of PACT that is deficient in PACT–PACT interaction, we demonstrate that PACT–PACT interaction is essential for efficient PKR activation. J. Cell. Biochem. 113: 2754–2764, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: INTERFERON; PKR; PACT; KINASE; dsRNA; dsRBM

PKR is an interferon (IFN)-induced serine/threonine kinase that is expressed ubiquitously and plays a central role in mediating IFN's antiviral actions [Garcia et al., 2006a]. Although IFNs increase PKR's cellular abundance, PKR's kinase activity requires binding to one of its activators leading to autophosphorylation and enzymatic activation [Meurs et al., 1990]. The double-stranded (ds) RNA, a replication intermediate for several viruses, was one of the first well-characterized activators of PKR [Hovanessian and Galabru, 1987; Meurs et al., 1990]. The best-characterized cellular substrate of PKR is the translation initiation factor, eIF2α, the phosphorylation of which results in an inhibition of protein synthesis [Samuel, 1993]. Although PKR's antiviral activities are the most studied, PKR is also implicated in the signal transduction pathways activated by cytokines, growth factors, dsRNA, and extracellular stresses [Williams, 1995]. Optimal activation of p38, c-Jun N-terminal kinase (JNK), stress-activated protein kinases (SAPKs), and the downstream transcription factors induced by these kinases such as NF-κB, IRF-1, p53, STAT1, ATF, STAT3, and AP-1 require PKR activity [Williams, 2001]. Thus, PKR is involved in multiple cellular processes such as differentiation, apoptosis, proliferation, and oncogenic transformation [Garcia et al., 2006b].

PKR binds dsRNA via the two dsRNA-binding motifs (dsRBMs) [Green and Mathews, 1992; McCormack et al., 1992; Patel and Sen, 1992], which changes the conformation of PKR to expose the ATP-binding site [Nanduri et al., 2000] and leads to consequent autophosphorylation [Cole, 2007]. The two dsRBMs also mediate dsRNA-independent protein–protein interactions with other proteins that carry similar domains [Patel et al., 1995; Chang and Ramos, 2005]. Among these are proteins inhibitory for PKR activity such as TRBP (human immunodeficiency virus (HIV)-1 transactivation-responsive (TAR) RNA-binding protein) [Benkirane et al., 1997], Dus2L (dihydrouridine synthase 2-like) [Mittelstadt et al., 2008] and also PKR activator protein PACT (protein activator) [Patel and Sen, 1998a]. PACT's association with PKR activates PKR in the absence of dsRNA [Patel and Sen, 1998a; Patel et al., 2000]. PACT contains three copies of dsRBM, of which the two amino-terminal motifs 1 and 2 are true dsRBMs and exhibit dsRNA-binding activity. In addition, these two dsRBMs in PACT also bind to the amino-terminal dsRBMs of PKR. The third, carboxy-terminal motif 3 shows significant homology to a consensus dsRBM but is not a functional dsRBM since it does not bind dsRNA. However, this third motif is essential for PKR activation and binds to a specific region in the kinase domain of PKR with low affinity [Peters et al., 2001; Huang et al., 2002].

Although purified, recombinant PACT can activate PKR by direct interaction in vitro [Patel and Sen, 1998a], PACT-dependent PKR activation in cells occurs in response to a cellular stress signal [Ito

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et al., 1999; Patel et al., 2000; Bennett et al., 2006; Singh et al., 2009]. PACT-mediated activation of PKR occurs in response to cellular stresses such as arsenite, peroxide, growth factor withdrawal, thapsigargin, tunicamycin, actinomycin and leads to phosphorylation of the translation initiation factor eIF2α and cellular apoptosis [Ito et al., 1999; Patel et al., 2000; Bennett et al., 2006]. PACT (and its murine homolog RAX) is phosphorylated in response to the stress signals leading to its increased association with PKR causing PKR activation [Ito et al., 1999; Patel et al., 2000; Bennett et al., 2006].

Similar to PACT, TRBP is also a dsRNA-binding protein but unlike PACT, it inhibits PKR. TRBP also has three copies of dsRBMs and the two amino terminal copies are capable of binding dsRNA. The third carboxy terminal copy does not bind dsRNA but mediates protein–protein interactions with several proteins including dicer [Daniels et al., 2009], merlin [Lee et al., 2004], and PACT [Laraki et al., 2008]. TRBP interacts with PKR to block PKR's kinase activity and thus eliminates PKR's inhibitory effect on translation [Park et al., 1994], yeast growth, [Cosentino et al., 1995; Daher et al., 2001], HIV expression, and replication [Daher et al., 2001]. In lymphocytes, TRBP inhibits PKR activation during HIV infection and thus plays a major role in suppressing the innate immune antiviral pathways [Cosentino et al., 1995; Daher et al., 2001]. Consistent with being a PKR inhibitor, TRBP is an oncogenic protein and its overexpression in NIH3T3 cells makes them tumorigenic in nude mice [Benkirane et al., 1997].

Interestingly, TRBP and PACT are 40% similar at the amino acid level [Patel and Sen, 1998a] and interact with each other via all three dsRBMs including the third dsRBM that does not bind dsRNA [Laraki et al., 2008]. It is interesting to note that although these two proteins are very homologous, they affect PKR activity in opposite manner. In virally infected cells TRBP inhibits PKR by directly binding to it as well as by sequestering PKR's activator dsRNA [Daher et al., 2001]. However, in uninfected cells TRBP inhibits PKR by direct binding [Cosentino et al., 1995] and by forming heterodimers with PACT and thereby keeping PACT from interacting with PKR [Daher et al., 2009]. Recently we have shown that cellular stress signals cause PACT to dissociate from TRBP and this leads to PACT-mediated PKR activation [Daher et al., 2009]. TRBP-PACT heterodimers are present in unstressed cells and PACT dissociates from TRBP in response to oxidative stress and serum starvation. Thus TRBP regulates the activation of PKR in response to stress signals by controlling its accessibility to PACT.

Phosphorylation of two serine residues S246 and S287 in PKR activation domain (M3) is required for PACT's ability to activate PKR in response to stress signals. Constitutive phosphorylation of serine 246 is a pre-requisite for stress-induced phosphorylation of serine 287 [Peters et al., 2006]. Our recent results have demonstrated that stress-induced phosphorylation at serine 287 has a dual role in PACT mediated PKR activation in response to stress. Phosphorylation of serine 287 causes dissociation of PACT-TRBP complex and at the same time increases PACT's affinity for PKR, thereby leading to PKR activation [Singh et al., 2011]. Since PACT interacts with itself via the conserved dsRBMs, it is possible that phosphorylation of serine 287 affects PACT–PACT interaction in addition to affecting PACT–TRBP and PACT–PKR interactions. Thus, in the present study, we used a yeast two-hybrid screen to map the interaction domains within PACT that mediate PACT–PACT interactions. Next, we tested the effect of phosphorylation at serine 246 and 287 on PACT–PACT interaction by creating the phosphorylation defective alanine substituted and phospho-mimetic aspartic acid substituted PACT mutants. In order to address a longstanding question whether PACT monomer can activate PKR, we created a point mutant of PACT (L99E) that is defective in mediating PACT–PACT interaction, but interacts efficiently with PKR. Using this mutant we demonstrate for the first time here that PACT–PACT interaction is essential for PKR activation. Our results presented here establish that phosphorylation of PACT on S246 and S287 leads to an enhanced PACT–PACT interaction, which is essential for efficient PKR activation.

MATERIALS AND METHODS

CELL CULTURE AND PLASMIDS

HeLa cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin–streptomycin, and L-glutamine. The M1, M2, and M3 deletion constructs of PACT were made by using appropriate primers for PCR amplification of the corresponding regions from the PACT/BSIIKS$^+$ construct described previously [Huang et al., 2002]. PACT and its deletion mutants M1, M2, and M3 were sub-cloned into the pGBKT7 and pGADT7 yeast vector into *Nde*I-*Bam*H1 sites. Point mutations were generated in M3 domain of PACT at S246 and S287 position by substituting these two serines with alanines and aspartic acids by using the appropriate primers for PCR amplification of the corresponding regions from the M3/pGADT7 construct used as a template [Singh et al., 2011]. The PCR products were sub-cloned into pGEMT-easy vector (Promega). Once the sequence of each M3 domain point mutant [S246A, S246D, S287A, S287D, S246AS287A (AA), S246AS287 (AD), S246DS287D (DD), and S246DS287A (DA)] had been verified, each of these was sub-cloned into pGBKT7 and pGADT7 yeast vector into *Nde*I-*Bam*H1 sites. Full-length PACT and its point mutants were sub-cloned into yeast expression vectors pGBKT7 and pGADT7 into *Nde*I-*Bam*HI sites. The bigger piece (194–313) of M3 (M3*) was made by using appropriate primers for PCR amplification of the corresponding regions from the PACT/BSIIKS+ construct. The primers used for M3 (195–313) were:

195 M3 sense: 5′-GCTCTAGACATATGGAAAATATTTCTCCAGAGAACCAC-3′, and 313 M3 Antisense: 5′-GGGGATCCTTACTTTCTTTCTGCTATTATC-3′. Point mutations were generated in M3 domain (195–313) of PACT at S246 and S287 position by substituting these two serines with alanine and aspartic acid by using the primers 195 M3 sense: 5′-GCTCTAGACATATGGAAAATATTTCTCCAGAGAACCAC-3′, and full-length M3 Antisense: 5′-GCGGATCCTTACTTTCTTTCTGCTATTATCTTTAAATACTGCAAAGCATTGTGAGC-3′ for PCR amplification of the corresponding regions from the PACT S246AS287A (AA)/pcDNA3.1$^-$ and PACT S246DS287D (DD)/pcDNA3.1$^-$ constructs used as a template [Singh et al., 2011]. The PCR products were sub-cloned into pGEMT-easy vector (Promega). Once the sequence of each M3 domain point mutant (S246AS287A, S246DS287D) had been verified each of these was sub-cloned into pGBKT7 and pGADT7 yeast vector into *Nde*I-*Bam*H1 sites. L99E mutant was created in PACT/BSIIKS+ using the

Gene Editor (Promega) site directed mutagenesis kit and after sequence verification was sub-cloned from L99E/BSIIKS$^+$ construct into the pGBKT7 and pGADT7 vectors at *Nde*I-*Bam*HI sites.

EXPRESSION AND PURIFICATION OF RECOMBINANT WT PACT AND L99E MUTANT

The L99E mutant was sub-cloned into pET-15b vector (Novagen) to generate an in-frame fusion of a hexahistidine tag on the amino terminus of M3 domain. The wt PACT and L99E mutant were expressed and purified as described before [Patel and Sen, 1998a].

YEAST TWO-HYBRID INTERACTION ASSAY

M1, M2, M3 domains and wt PACT were expressed as a GAL4 DNA-binding domain fusion protein and GAL4 DNA-activation domain fusion protein from yeast two-hybrid vectors pGBKT7 and pGADT7. Each pGBKT7 and pGADT7 construct was co-transformed into *Saccharomyces cerevisiae* strain AH109 (clontech) and selected on SD minimal double dropout medium lacking tryptophan and leucine. Transformation of empty vectors pGBKT7 and pGADT7 served as a negative control. In order to check for the transformants' ability to grow on histidine-lacking medium, 10 μl of serial dilutions (OD_{600} = 10, 1.0, 0.1, 0.01) were spotted for each transformant on triple dropout SD medium lacking tryptophan, leucine, and histidine. Plates were incubated for 3 days at 30°C. The interactions between the wt M3* (195–313) and its mutants were tested in a similar manner. The interaction of wt PACT and its point mutants (S246A, S246D, S287A, S287D, S246AS287A, S246AS287D, S246DS287D, S246DS287A, and L99E) was also tested using the above strategies, except that these were plated on quadruple dropout SD medium that lacks tryptophan, leucine, adenine, and histidine and have 10 mM 3-Amino-1,2,4-triazole (3-AT). For liquid growth curves, the transformed yeast strains were grown to an OD_{600} of about 1.5 in synthetic medium lacking leucine, tryptophan. The cultures were harvested and washed with synthetic medium lacking leucine, tryptophan, histidine and containing 10 mM 3-AT. The cultures were then diluted to an OD_{600} of about 0.4 in synthetic medium lacking leucine, tryptophan, histidine and containing 10 mM 3-AT. The cell growth was monitored at various time points by measuring the OD_{600}.

β-GALACTOSIDASE FILTER ASSAY

AH109 yeast reporter strain containing the LacZ-Gal4-inducible gene were co-transformed with the indicated plasmids and plated on selective medium lacking tryptophan, and leucine. Double transformants were streaked on selective medium, replica-lifted on nitrocellulose filters and tested for β-galactosidase activity [Mittelstadt et al., 2008].

CO-IMMUNOPRECIPITATION ASSAY

In vitro translated, ^{35}S-labeled flag-epitope-tagged wt PACT, its point mutant L99E and wt PKR proteins were synthesized using the TNT T7 coupled reticulocyte system (Promega). 5 μl of the in vitro translated ^{35}S-labeled PACT and L99E proteins were incubated with 5 μl of the in vitro translated ^{35}S-labeled PKR for 30 min at RT. This protein mixture was immunoprecipitated with 20 μl of anti-flag M2 antibody agarose (Sigma) in 200 μl of immunoprecipitation buffer (30 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 0.4% Igepal) at RT for 30 min on a rotating wheel. The beads were washed in 500 μl of immunoprecipitation buffer four times and the washed beads were boiled in 1× Laemmli buffer (250 mM Tris–HCl, pH 6.8, 8% SDS, 10% β-mercaptoethanol, 40% glycerol, 0.1% bromophenol blue) for 2 min, and eluted proteins were analyzed by SDS–PAGE on a 12% gel followed by phosphorimager analysis.

PKR KINASE ACTIVITY ASSAY

PKR activity assays were performed using an anti-PKR monoclonal antibody (R&D system; 71/10). HeLa M cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. HeLa cells were treated with INF-β for 24 h. The cells were harvested when they were at 70% confluency. Cells were washed in ice-cold PBS and collected by centrifugation at 600*g* for 5 min. They were lysed by addition of an equal volume of lysis buffer (20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 400 mM NaCl, 2 mM DTT, 1% Triton X-100, 100 U/ml aprotinin, 0.2 mM PMSF, 20% glycerol). The lysates were centrifuged at 10,000*g* for 5 min and the supernatants were assayed for PKR activity. A 100 μg aliquot of total protein was immunoprecipitated using anti-PKR monoclonal antibody (Ribogene; 71/10) in high salt buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 100 U/ml aprotinin, 0.2 mM PMSF, 20% glycerol) at 4°C for 30 min on a rotating wheel. Then 20 μl of Protein A-agarose beads were added and incubation was carried out for a further 1 h. The Protein A-agarose beads were washed four times in 500 μl of high-salt buffer and twice in activity buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM PMSF, 5% glycerol). The PKR assay was performed with PKR still attached to the beads in activity buffer containing 250 ng purified rabbit eIF2 (a generous gift from William Merrick, Case Western Reserve University), 0.1 mM ATP and 1 μCi of [γ ^{32}P] ATP at 30°C for 10 min. The standard activator of the enzyme was 0.116 pmol of pure PACT protein, 0.1 μg/ml poly(I).poly© and 50 μg/ml of heparin. Purified L99E PACT mutant in amounts varying from 4 pg to 400 ng were added to test its effect on PKR activity. Labeled proteins were analyzed by SDS–PAGE on a 12% gel followed by autoradiography.

RESULTS

PACT–PACT INTERACTION OCCURS VIA M1, M2, AND M3 DOMAINS

The effect of stress-induced PACT phosphorylation on PACT–PACT interactions has never been studied. When we performed a yeast two-hybrid screen, using PACT as bait in order to identify PACT-interacting proteins that may regulate its activity and subsequent activation of PKR, PACT itself was identified as one of the interacting proteins (unpublished observations). Thus, in order to define the effects of stress-induced phosphorylations of PACT on its interaction with itself, we first wanted to define the interaction domains within PACT. PACT has three copies of dsRBM, a conserved protein motif involved in dsRNA binding as well as in protein–protein interactions. In order to investigate the contribution of each dsRBM motif towards PACT–PACT interaction, we tested the interaction between each of the individual domains (M1, M2, M3) of PACT. We generated three deletion constructs of PACT, as shown

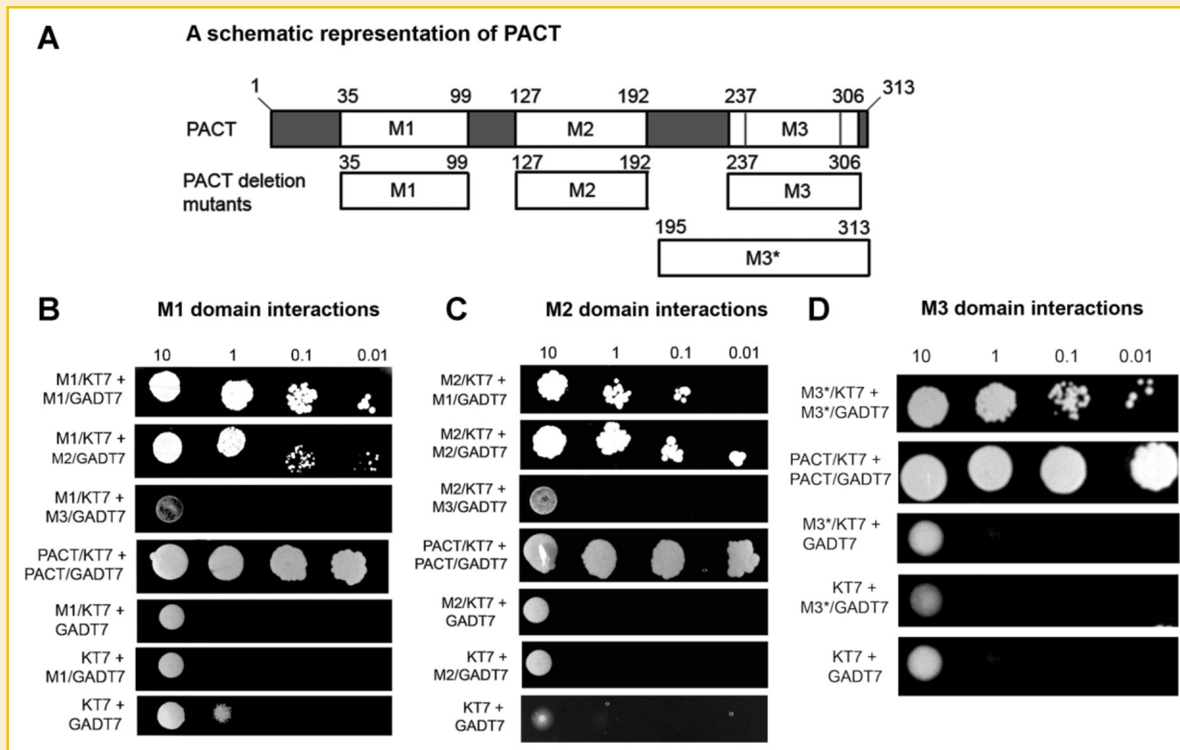

Fig. 1. PACT interacts with itself through M1, M2, and M3 domains. A: PACT domains and deletion mutants. The white boxes represent three conserved motifs M1, M2, and M3 in PACT. The gray box represents PACT sequence outside of the conserved motifs. The amino acid numbers are indicated on the top of the boxes. The various deletion mutants M1, M2, M3, and M3* are as indicated. The amino acid positions spanned by the mutants are indicated above the boxes. The two vertical lines within M3 motif indicate positions of serine 246 and 287, which are phosphorylated. B: M1 domain interactions (C) M2 domain interactions, and (D) M3 domain interactions in yeast two-hybrid assay. PACT domains M1, M2, M3, M3*, and wt PACT in pGBKT7 and in pGADT7 were transformed into yeast strain AH109 as indicated and the colonies were selected on double dropout medium lacking tryptophan and leucine. Ten microliter of serial dilutions ($OD_{600} = 10$, 1.0, 0.1, 0.01) were spotted for each transformant on triple dropout SD medium plates lacking tryptophan, leucine, and histidine. Plates were incubated for three days at 30°C. Transformation of empty vectors pGBKT7 and pGADT7 served as negative controls.

in Figure 1A. M1 domain was used as bait, being expressed as the GAL4-DNA-binding domain fusion protein from the corresponding pGBKT7 construct. Its interaction with M1, M2, and M3 domains was investigated by co-transformation of these constructs in yeast strain AH109 where these three domains were expressed as GAL4 activation domain fusion protein from the yeast expression vector pGADT7 (Clontech). Plates containing supplemental media lacking leucine, tryptophan, and histidine provide the strongest selection for the yeast two-hybrid system where growth itself indicates a positive interaction between the candidate proteins. Four different starting cell densities (10–0.01 OD units) were spotted on the plates. As seen in Figure 1B, M1 domain showed positive interaction with M1 and M2 domain. In contrast to this, M1 showed no interaction with M3. The positive control (PACT/pGBKT7 with PACT/pGADT7) showed growth as expected. The three negative controls M1/pGBKT7 with pGADT7, pGBKT7 with M1/pGADT7 and pGBKT7 with pGADT7 showed no growth. Using a strategy similar to the one above, the interaction of M2 domain with M1, M2, and M3 domains was investigated. Similar to M1 domain, M2 domain showed positive interaction with M1 and M2 domains but not with M3 (Fig. 1C). The positive control PACT/pGBKT7 with PACT/pGADT7 showed growth as expected. The negative controls M2/pGBKT7 with pGADT7, pGBKT7 with M2/pGADT7 and pGBKT7 with pGADT7 showed no growth. These results established that both M1 and M2 domains interact with each other.

The yeast two-hybrid approach that worked well for domains M1 and M2 did not work for M3 domain (residues 237–306) since this domain gave false positive results and showed activation of both reporters (β-galactosidase and his) in the absence of any activation domain fusion protein. In order to test if M3 domain contributes to interaction between two PACT molecules, we used a bigger piece of M3 domain (Fig. 1A, M3*) to avoid getting false positive results. Using this bigger M3 domain (M3*-residues 195–313), we tested for interaction in yeast two-hybrid assay. M3* domain showed a positive interaction with itself (Fig. 1D). As expected the negative control M3*/pGBKT7 with pGADT7, pGBKT7 with M3*/pGADT7 and pGBKT7 with pGADT7 showed no growth. These results established that M3 domain within PACT also mediates PACT–PACT interactions. Western blotting confirmed that each domain of PACT (M1, M2, and M3) as well as full-length PACT is expressed as a GAL4 DNA-binding and GAL4-Activation domain fusion in yeast cells (data not shown).

In order to further confirm the positive interaction between M1, M2, and M3 domains we also tested activation of another reporter, β-galactosidase. Expression of β-galactosidase in yeast cells can be analyzed by performing an enzyme activity assay that results in

formation of a blue color and occurs only if the protein encoded by pGADT7 and pGBKT7 constructs interact. As seen in Figure 2A, both M1 and M2 domains each showed positive interactions with both M1 and M2 domains as indicated by the blue color. Both M1 and M2 showed no interaction with M3 as indicated by the white color. As seen in Figure 2B, M3* domain (195–313) showed a blue color with itself and thus confirmed that M3*–M3* interaction. The negative controls M1/pGADT7 and M2/pGADT7 with pGBKT7 showed white color indicating no interaction. Similarly, opposite combinations also showed white color. As seen in Figure 2B, the Negative control for M3* domain M3*/pGBKT7 with pGADT7 also showed white color. These results confirm that M1 and M2 each can interact with both M1 and M2 and that M3* domain only interacts with M3* but not with M1 or M2.

PHOSPHO-MIMETIC MUTATION OF SERINES 246 AND 287 ENHANCES THE M3–M3 INTERACTION

Previous work from our laboratory has indicated that M3 domain of PACT is essential to activate PKR. The data presented in Figure 1D demonstrates that M3 domain interacts with itself. It is known that the phosphorylation of two serine residues S246 and S287 in M3 domain is required for PKR activation. Thus, we planned to investigate if phosphorylation at these sites changes M3 domain's ability to interact with itself.

We tested the effect of specific point mutations at positions serine 246 and 287. We substituted these two serines with phosphorylation defective alanine (AA) and phospho-mimetic aspartic acid (DD). We tested these M3 domain point mutants for their ability to interact in a yeast two-hybrid assay. Compared to wt M3, the phosphomimetic DD mutant showed a stronger interaction as it showed enhanced growth on all four concentrations (Fig. 3). The point mutant AA showed growth characteristics similar to the wt M3 indicating that when M3 is not phosphorylated on S246 and S287 it has no adverse effect on M3–M3 interaction (Fig. 3). As expected, none of the negative controls showed interaction. These results strongly suggest that phosphorylation at S246 and S287 position results in enhanced interaction between the M3 domains. Western blotting confirmed that wt M3 and its point mutants AA and DD were expressed as a GAL4 DNA-binding and GAL4-Activation domain fusion proteins in yeast cells (data not shown).

PHOSPHO-MIMETIC MUTATION OF SERINES 246 AND 287 ALSO ENHANCES THE PACT–PACT INTERACTION

After establishing that phosphorylation at S246 and S287 results in a better interaction between the M3 domains, we next wanted to test the effect of phosphorylation at these sites on PACT–PACT

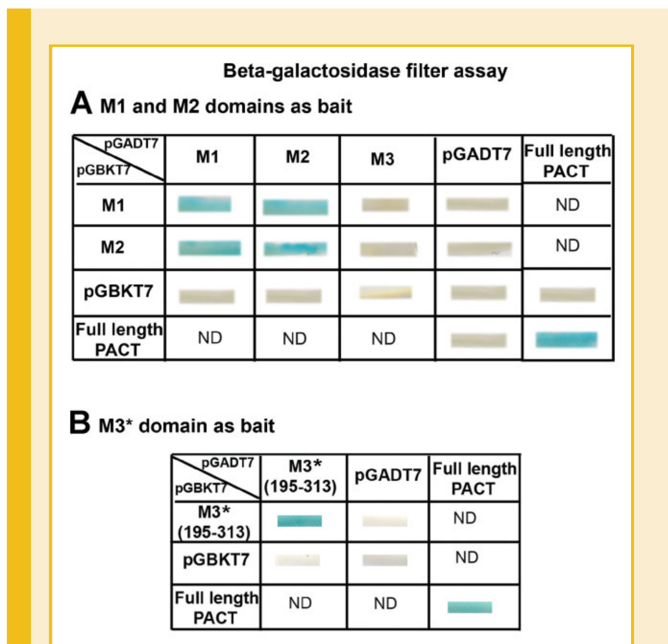

Fig. 2. β-galactosidase filter assay. A: M1 and M2 domains as bait and (B) M3 domain as bait. The indicated plasmids were transformed into yeast strain AH109 and the colonies were streaked on double dropout medium lacking leucine, and tryptophan. After 4 days, the growth was lifted on nitrocellulose membranes and β-galactosidase activity assay was performed after lysis of yeast cells on the membrane. Blue color indicates a positive interaction and white color indicates no interaction. ND, not done. M3 = 237–305, M3* = 195–313.

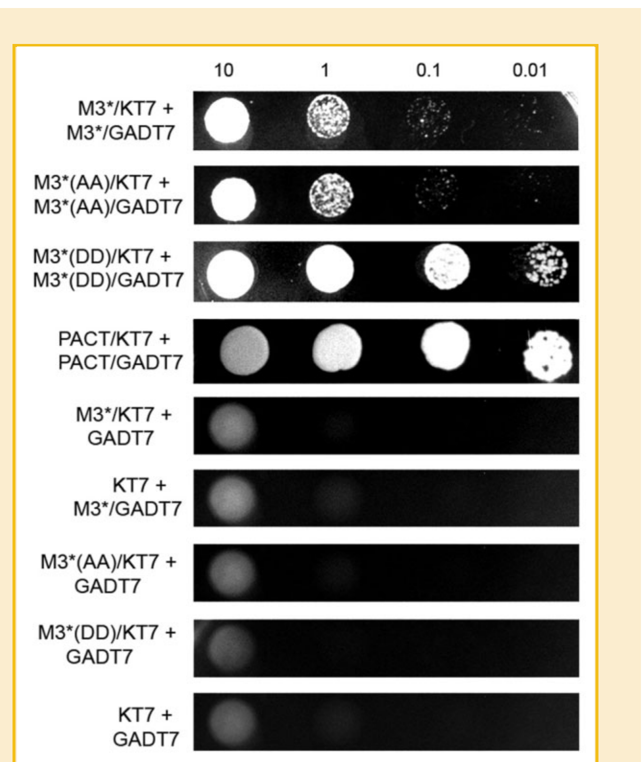

Fig. 3. A phospho-mimetic mutation S246DS287D (DD) within M3* exhibits stronger interaction with itself than the wild type or a phosphorylation-defective S246AS287A (AA) mutation. M3* and its point mutants S246AS287A (AA) and S246DS287D (DD) in pGBKT7 and in pGADT7 were transformed into yeast strain AH109 and selected on double dropout medium lacking tryptophan and leucine. Ten microliter of serial dilutions (OD_{600} = 10, 1.0, 0.1, 0.01) were spotted for each transformant on triple dropout SD medium plates lacking tryptophan, leucine and histidine. Plates were incubated for 3 days at 30°C. Transformation of pGBKT7 and M3*/pGADT7, M3* (AA)/pGBKT7 and pGADT7, M3* (DD)/pGBKT7 and pGADT7 and pGBKT7 and pGADT7 served as negative controls.

interactions. We created the M3 domain point mutations AA and DD in full-length PACT and examined the effects on PACT–PACT interaction in a yeast-two hybrid assay. Since wt PACT interacts very strongly with itself on triple drop-out media (Figs. 1 and 3), we increased the stringency of the assay by including 10 mM 3-AT in the plates in order to assess if DD mutation will cause enhanced interaction of full-length PACT protein molecules. As seen in Figure 4, DD point mutant showed a stronger PACT–PACT interaction compared to wt PACT as it showed growth on all four starting cell densities (10–0.01 OD units). PACT point mutant AA showed significantly less growth when compared to wt PACT indicating that prevention of phosphorylation at serines 246 and 287 affect PACT–PACT interaction negatively. As expected, none of the negative controls showed any growth. These results strongly suggest that phosphorylation at S246 and S287 results in an enhanced PACT–PACT interaction. These results strongly support and extend our wt M3 domain results. Western blotting confirmed the expression of the wt and mutant proteins as a GAL4 DNA-binding and GAL4-Activation domain fusion proteins in a yeast cells (data not shown). In order to further confirm the differences in growth patterns between the AA and DD mutants, we tested the growth of the yeast transformants in liquid synthetic medium. The results obtained on solid medium were reproduced in liquid medium as seen in Figure 4B. The DD mutant showed significantly accelerated growth rate compared to wt PACT and the AA mutant exhibited significantly reduced growth rate compared to wt PACT. These results further confirm that DD mutation increases the strength of PACT–PACT interaction and that AA mutation reduces it significantly. Thus, phosphorylation at serines 246 and 287 increases PACT–PACT interaction and a complete absence of phosphorylation at these sites reduces PACT–PACT interaction. Presumably, the wt PACT may be constitutively phosphorylated on serine 246 in yeast as in mammalian cells.

PACT–PACT INTERACTION IS NECESSARY FOR PKR ACTIVATION

The observed enhanced PACT–PACT interaction of the DD point mutant (Fig. 4) indicates that stress-induced PACT phosphorylation leads to increased PACT–PACT interaction. We have previously shown that stress-induced PACT phosphorylation weakens the PACT–TRBP interaction [Singh et al., 2011]. Taken together, these results raise a question whether PACT–PACT interaction may in fact be essential for PKR activation since DD point mutant of PACT associates better with PKR. [Peters et al., 2006; Peters et al., 2009]. In order to test this, we created a point mutant that interacts efficiently with PKR but does not interact with itself and thus PACT–PACT interaction would be absent. We have previously shown that L75E mutant of PKR is defective in dimerization [Patel and Sen, 1998b]. Since, the dsRBMs of PKR and PACT are very homologous to each other, we decided to mutate the corresponding leucine residue to a glutamic acid (L99E) in PACT. Next we investigated whether L99E mutant is defective in PACT–PACT interactions similar to L75E mutant of PKR. In order to test this, we cloned the L99E mutant in the

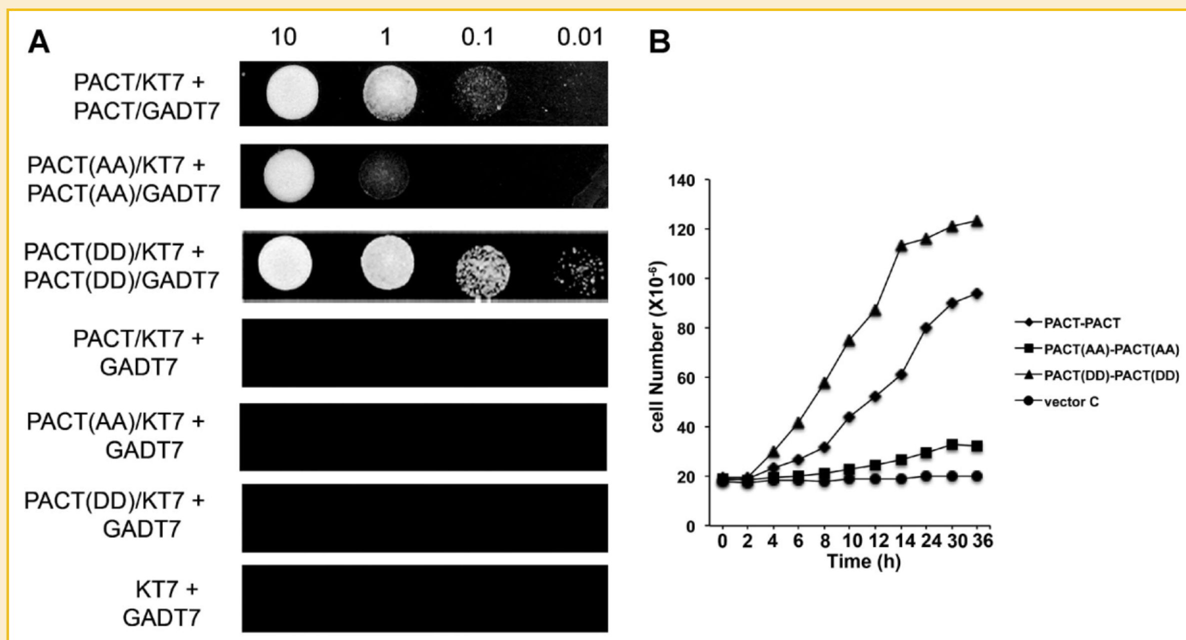

Fig. 4. A phospho-mimetic mutation S246DS287D (DD) in PACT exhibits stronger interaction with itself than the wild type or a phosphorylation-defective S246AS287A (AA) mutation. A: PACT and its point mutants S246AS287A (AA) and S246DS287D (DD) in pGBKT7 and in pGADT7 were transformed into yeast strain AH109 and selected on double dropout medium lacking tryptophan and leucine. Ten microliter of serial dilutions (OD_{600} = 10, 1.0, 0.1, 0.01) were spotted for each transformant on triple dropout SD medium plates lacking tryptophan, leucine, and histidine and containing 10 mM 3-Amino-1,2,4-triazole (3-AT). Plates were incubated for three days at 30°C. Transformation of PACT/pGBKT7 and pGADT7, PACT (AA)/pGBKT7 and pGADT7, PACT (DD)/pGBKT7 and pGADT7 and pGBKT7 and pGADT7 served as negative controls. B: Growth rate analysis. The growth rate of the transformed yeast strains was analyzed in synthetic medium lacking leucine, tryptophan, histidine, and containing 10 mM 3-amino-1,2,4-triazole. At various time points, cell growth was monitored by measuring OD_{600}. The lines represent growth curves of four strains tested as indicated in the marker legend.

yeast expression vectors and examined its ability to interact with itself in a yeast-two hybrid assay. As seen in Figure 5A, L99E point mutant did not show any growth on the plate where four different starting cell densities (10–0.01 OD units) were spotted. As expected, both of the positive controls wt PACT and DD PACT showed growth on the plates while none of the negative controls showed growth. These results indicate that L99E point mutant does not interact with itself. This result also indicates that although all three domains M1, M2, and M3 interact as isolated domains, in a full-length PACT protein, the PACT–PACT interaction may be mainly mediated via M1–M1 interaction. In a similar experiment, we next tested whether L99E point mutant interacts with K296R mutant of PKR. It is not possible to test the interaction of wild-type PKR with L99E since expression of wild-type PKR inhibits the growth of yeast cells.

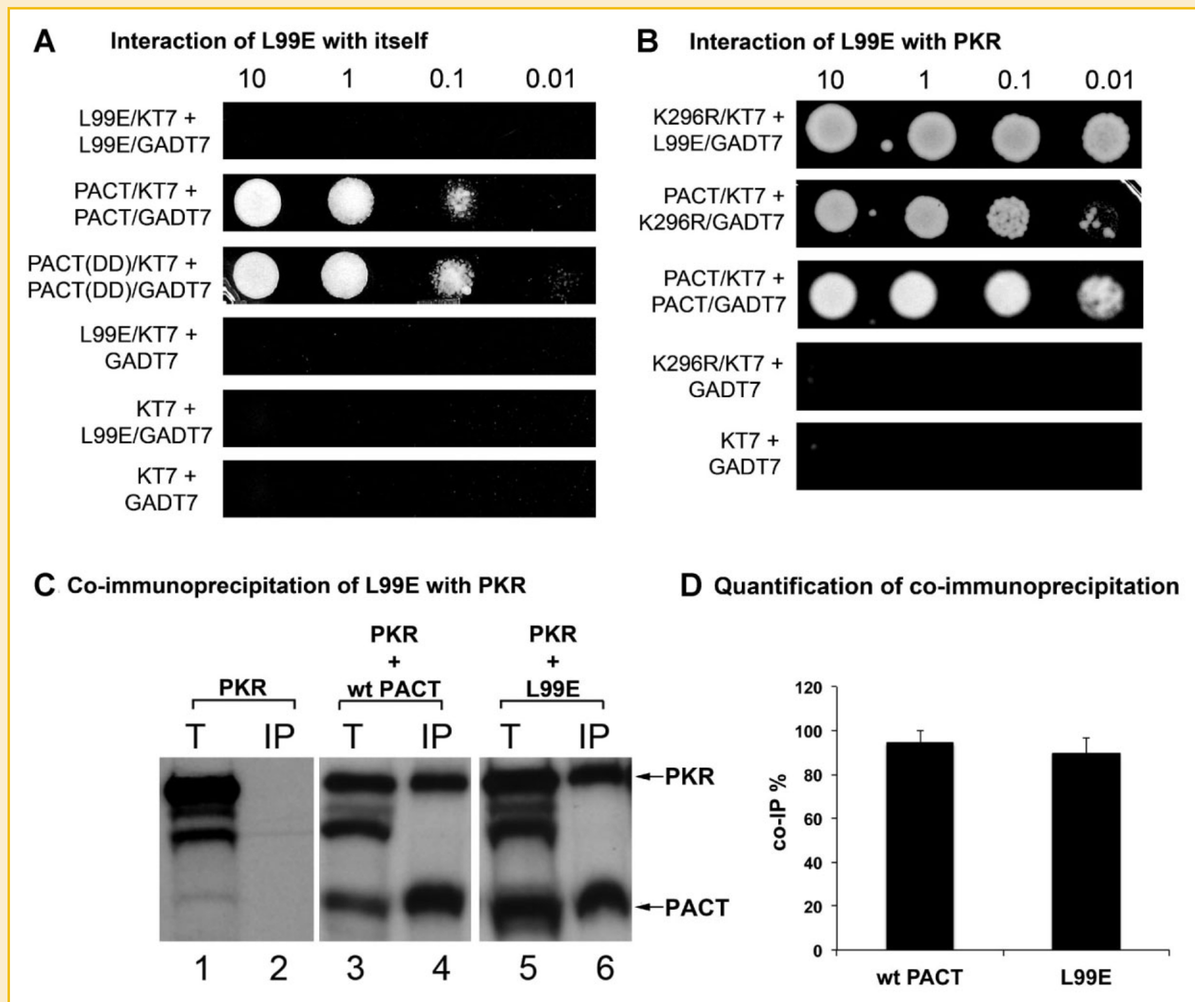

Fig. 5. L99E point mutant of PACT does not interact with itself but interacts with PKR. A: L99E mutant does not interact with itself. Wt PACT and its point mutant L99E in pGBKT7 and in pGADT7 were transformed into yeast strain AH109 and selected on double dropout medium lacking tryptophan and leucine. Ten microliter of serial dilutions ($OD_{600} = 10, 1.0, 0.1, 0.01$) were spotted for each transformant on quadruple dropout SD medium plates lacking tryptophan, leucine, adenine, and histidine and containing 10 mM 3-Amino-1,2,4-triazole (3-AT). Plates were incubated for three days at 30°C. Transformation of L99E/pGBKT7 and pGADT7, pGBKT7 and L99E/pGADT7 and pGBKT7 and pGADT7 served as negative controls. Transformation of PACT (DD)/pGBKT7 and PACT (DD)/pGADT7 served as a positive control. B: L99E point mutant of PACT interacts with PKR. K296R and L99E point mutant of PACT in pGBKT7 and in pGADT7 were transformed into yeast strain AH109 and selected on double dropout medium lacking tryptophan and leucine. Ten microliter of serial dilutions ($OD_{600} = 10, 1.0, 0.1, 0.01$) were spotted for each transformant on triple dropout SD medium plates lacking tryptophan, leucine and histidine. Plates were incubated for 3 days at 30°C. Transformation of K296R/pGBKT7 and pGADT7 and pGBKT7 and pGADT7 served as negative controls. Transformation of PACT/pGBKT7 and PACT/pGADT7 served as a positive control. C: Co-immunoprecipitation of wt PACT and L99E with PKR. The flag epitope-tagged wt PACT, L99E and untagged PKR proteins were synthesized labeled with [^{35}S] methionine using the TNT T7 coupled reticulocyte system. Five microliters of the reticulocyte lysates were used for co-immunoprecipitation assay using anti-flag M2 antibody agarose. T lanes (lanes 1, 3, and 5) represent the total proteins from the translation mix (20% of immunoprecipitation (IP) lanes 2, 4, and 6). Positions of the PKR and PACT bands are as indicated by arrows. Lane 2 represents the negative control where only PKR was added to anti-flag M2 antibody agarose. D: Quantification of co-immunoprecipitation by phosphorimager. The radioactivity present in bands was measured and the percentage co-immunoprecipitation was calculated as 100 × (radioactivity present in co-immunoprecipitated PKR/the radioactivity present in the PKR band in the total lane). In addition, this value was normalized to the amount of radioactivity present in the flag-PACT or Flag-L99E bands to correct for differences in the translation/immunoprecipitation steps. The error bars represent the standard deviation calculated from three different independent experiments.

However, it has been established before that the kinase dead K296R mutant of PKR can be used successfully to define PKR's dimerization as well as its interactions with other proteins [Cosentino et al., 1995; Patel and Sen, 1998b; Coolidge and Patton, 2000]. Our results demonstrated that L99E point mutant of PACT interacts with K296R mutant of PKR (Fig. 5B). In order to further confirm the interaction between L99E mutant and PKR, we performed co-immunoprecipitation assay. In vitro translated ^{35}S-labelled proteins were immunoprecipitated as indicated in Figure 5C. PKR co-immunoprecipitated efficiently with the wt PACT (lane 4) and its point mutant L99E (lane 6). In order to compare the strength of interaction between wt PACT and PKR to that between L99E mutant and PKR, we quantified the results of the co-immunoprecipitation assay. As seen in Figure 5D, both wt PACT and L99E co-immunoprecipitated equally well with PKR. These results further strengthen the results obtained with yeast two-hybrid analysis and establish that L99E mutant interacts efficiently with PKR.

After establishing that L99E mutant is defective in PACT–PACT interaction but it does interact with PKR, we examined if it can activate PKR efficiently. In order to test the ability of L99E point mutant to activate/inhibit PKR in a biochemical assay, we expressed L99E and wt PACT as a hexahistidine-tagged proteins in *Escherichia coli* and purified them using affinity chromatography on Ni-agarose beads. The pure recombinant proteins were used to activate PKR in an in vitro kinase activity assay that measures eIF2α phosphorylation in addition to PKR autophosphorylation. As seen in Figure 6, there was no PKR activity detected in the absence of any activator. Addition of increasing amounts of L99E protein as an activator (lane 2–7) resulted in very poor PKR activation as compared to activation obtained with wt PACT (lane 8), dsRNA (lane 9), or heparin (lane 10). These results establish that L99E point mutant does not activate PKR.

Taken in view of our previous results that stress-induced phosphorylation of PACT at serine 287 weakens its association with PKR inhibitor TRBP [Singh et al., 2011], these results further establish that stress-induced phosphorylation of PACT leads to a change in its binding partners in cells and thereby causes efficient PKR activation. In particular, serine 246 and 287 phosphorylation increases the interaction between PACT molecules and this increased interaction seems to bring about efficient PKR activation. The inability of PACT–PACT interaction defective L99E mutant to activate PKR further demonstrates that PACT–PACT interaction is essential for PKR activation especially since L99E interacts with PKR with similar efficiency as wt PACT.

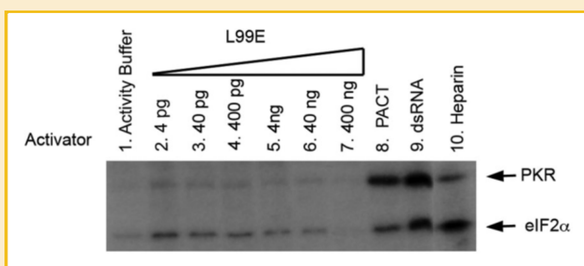

Fig. 6. L99E point mutant of PACT activates PKR very inefficiently. PKR immunoprecipitated from HeLa cell extracts using monoclonal anti-PKR antibody and protein A-agarose was activated by the addition of purified recombinant wt PACT, dsRNA and heparin. The effects of addition of increasing amounts of recombinant L99E protein was assayed for its ability to activate PKR. Lane 1: no activator added, lanes 2–7: increasing amounts of pure recombinant L99E point mutant of PACT, lane 8: 0.116 pmol of pure recombinant wt PACT, lane 9: 0.1 μg/ml poly(I).poly(C), and lane 10: 50 μg/ml of heparin added as PKR activators.

DISCUSSION

A variety of stress signals lead to phosphorylation of PACT and its increased association with PKR, which causes PKR activation [Ito et al., 1999; Patel et al., 2000; Bennett et al., 2006]. Thus, PACT plays a central role in mediating apoptosis-inducing pathways in response to stress. The mouse embryonic fibroblasts (MEFs) isolated from PACT null mice show significantly decreased apoptosis in response to ER stressor tunicamycin [Singh et al., 2009]. Accordingly, PACT overexpressing cells exhibit enhanced sensitivity to a variety of stresses [Ito et al., 1999; Patel et al., 2000; Bennett et al., 2006]. The mechanism of stress-induced, PACT-mediated PKR activation is not completely understood. It is known however, that PACT phosphorylation in response to stress signals is required for activation of PKR. Recently, Peters et al. [2006] established that PACT is constitutively phosphorylated on serine 246 and gets phosphorylated on serine 287 in response to stress. Furthermore, phosphorylation of PACT on these two serines is required for its increased association with PKR and PKR activation in response to cellular stress [Peters et al., 2009]. PACT and PKR belong to the family of dsRNA-binding proteins (dsRBPs) that are known to interact with each other in mammalian cells to form various complexes [Chang and Ramos, 2005]. In the context of PKR activation the interactions between PKR, TRBP (PKR inhibitor), and PACT are the most relevant. Thus, it is interesting to examine if stress signals cause any changes in PACT–PKR, PACT–PACT, and PACT–TRBP interactions. The effect of stress-induced PACT phosphorylation on PACT–PKR and PACT–TRBP interactions is known and our recent results have established that serine 287 phosphorylation decreases the interaction between PACT–TRBP and increases the interaction between PACT–PKR [Singh et al., 2011].

In this report, we further investigated if the stress-induced phosphorylation of PACT also may cause a change in its ability to interact with itself. Our results presented here demonstrate that a phospho-mimetic mutant of PACT with an aspartic acid substitution at both serines 246 and 287 shows enhanced interaction with itself as compared to wild-type PACT. This indicates that PACT–PACT interaction may promote PACT–PKR interaction and consequently its activation. Thus, we further investigated if PACT–PACT interaction may be essential for PACT's ability to activate PKR by generating a point mutation within the first dsRBM (M1 motif) that is known to destroy the protein–protein interactions. A similar mutation in PKR's first dsRBM (L75E) destroys PKR–PKR interaction and consequently renders PKR inactive [Patel and Sen, 1998b]. Our results indicate that the L99E mutation in PACT eliminates PACT–PACT interaction but PACT's interaction with PKR is unaltered. Thus, although all three domains M1, M2, and M3 show capability to interact when present as isolated domains, within a full-length PACT

protein context, PACT–PACT interaction seems to be regulated mainly by the M1 motif. However, this mutant is unable to activate PKR thereby demonstrating that PACT–PACT interaction is essential for PKR activation. Although we have not investigated the nature of PACT multimers in detail, preliminary results indicate that PACT may form dimers (unpublished observations). It can thus be concluded that stress-induced PACT phosphorylation on serine 287 causes enhanced PACT–PACT interaction which is essential for efficient activation PKR in response to stress. It has been established that the AA mutant of PACT (phosphorylation-defective alanine substitution mutant at 246 and 287) is unable to activate PKR. Since the AA mutation also affects the PACT–PACT interaction, and since PACT–PACT interaction is essential for PACT's ability to activate PKR, it is possible that the inability of the AA mutant to activate PKR is partly due to the lack of PACT–PACT interaction. These results are very significant for understanding the stress-induced signaling pathways leading to apoptosis since they add an additional layer of complexity brought about by phosphorylation-mediated changes in the relative affinity of various binding partners within the dsRBM family. This is a novel aspect that has not been explored to date. In this regard, a recent report indicated that mitogenic signals cause phosphorylation of TRBP and this changes the expression profile of micro RNAs (miRNAs) in cells [Paroo et al., 2009]. Although protein–protein interactions were not explored in this study, it is possible that the observed changes in miRNA expression result from a phosphorylation mediated change in TRBP's interactions with other proteins involved in the miRNA pathway.

The potential use of type I IFNs as antitumor agents has been significantly limited due to tumor resistance mediated by the survival pathways that counteract the apoptotic pathways [Caraglia et al., 2009; Erdmann et al., 2011]. In order to improve IFN's therapeutic potential, various strategies are being tested to antagonize the survival pathways induced by IFN treatments [Caraglia et al., 2004; Vitale et al., 2012]. Targeted PKR activation has been shown to lead to selective apoptosis in a chronic myelogenous leukemia (CML) cell culture model [Li et al., 2011]. It can certainly be tested in future if selective activation of PKR by PACT pathway can be utilized to induce apoptosis in cancer cells. In this context it may be possible to design peptides based on PACT's M3 domain that may induce PKR activation and lead to cancer cell apoptosis. Thus, the studies presented here may have potential clinical applications in cancer therapeutics.

Several biochemical, biophysical, and structural studies have established the need for PKR dimerization in activating its kinase function when dsRNA is the activating agent [Lemaire et al., 2008; Heinicke et al., 2009]. PKR activation occurs when two PKR molecules bind to a single dsRNA molecule [Robertson and Mathews, 1996; Zhang et al., 2001]. PKR's second dsRBM motif interacts with the catalytic domain to keep PKR in a closed conformation that precludes ATP binding [Nanduri et al., 2000; Vattem et al., 2001]. In the prevalent autoinhibition model for PKR activation, binding to dsRNA induces a conformational change that leads to the release of the dsRBM from the catalytic domain, thus relieving the inhibition of the latent enzyme and allowing for ATP-binding. Structural and biophysical data favors a model whereby dsRNA principally functions to induce dimerization of PKR via the kinase domain [Taylor et al., 2005]. Although PKR exists in a equilibrium between monomeric and dimeric states in the absence of its interaction with dsRNA, binding to dsRNA shifts this equilibrium towards the dimeric form and also induces a conformational change necessary to relieve the autoinhibition [Lemaire et al., 2005]. In contrast to this, activated PKR that is phosphorylated on several serines and threonines has been shown to exist in monomeric as well as dimeric forms and both forms are competent in kinase function and active in phosphorylating eIF2α [Anderson et al., 2010]. Thus, it is possible that two molecules of PKR are brought together in close proximity by virtue of their interaction with PACT dimer or multimer. Thus, PACT may serve the same function that a dsRNA molecule of sufficient length serves, which is to allow for binding of two PKR molecules and allow for trans-autophosphorylation.

The dsRBM is widely distributed in eukaryotic proteins, as well as in proteins from bacteria and viruses [St Johnston et al., 1992; Chang and Ramos, 2005]. Many proteins that belong to the dsRBM family of RNA-binding proteins interact with other members of the same family and the interactions mediated by the individual domains show specificity such that they mediate interactions with some but not all members. The exact amino acids that determine and maintain the specificity in such interactions to mediate precise biological functions have not been characterized as yet. The biochemical studies of four Arabidopsis Dicer-like proteins (DCL1-4), and HYL1 and four of its homologs (DRB2, DRB4, DRB5, and OsDRB1), each containing one or two dsRBMs have indicated that each HYL1/DRB family protein interacts with only one specific partner among the four Dicer-like proteins [Hiraguri et al., 2005]. Our results presented here also establish that each dsRBM dictates its binding partners as the M3 motif in PACT does not interact with M1 or M2 but does interact with M3. *Xenopus laevis* (Xlrbpa) RNA-binding protein A has been shown to form multimers only via its third dsRBM, although similar to PACT, it contains three copies of dsRBM [Hitti et al., 2004]. Members of the family of adenosine deaminases acting on RNA (ADARs) can catalyze the hydrolytic deamination of adenosine to inosine and thereby change the sequence of specific mRNAs with highly double-stranded structures. The ADARs all contain one or more copies of the dsRBM and in case of rat ADAR, dsRBM2 but not dsRBM1 is necessary and sufficient for dimerization and activity of the enzyme [Poulsen et al., 2006].

Based on some of the previous reports about stress-induced PACT phosphorylation and our results presented here we have proposed a model for PKR activation by PACT (Fig. 7). According to this model the intra-molecular inhibitory interaction between the PBM within the catalytic domain and dsRBM2 maintains PKR in an inactive conformation [Li et al., 2006]. In response to stress signals PACT gets phosphorylated on S287 residue, and PACT phosphorylated in serines 246 and 287 forms stable PACT–PACT interactions via its M3 domain. PACT phosphorylated at S246 and S287 residues also binds to PKR more efficiently and brings two PKR molecules into close proximity thereby facilitating PKR dimerization and trans-autophosphorylation leading to PKR's catalytic activation. Thus, our results for the first time establish that stress-induced phosphorylation of PACT on S287 enhances PACT–PACT interaction, which is essential for PKR activation. In the absence of stress signals, PACT interacts with TRBP (a PKR inhibitory protein). Phosphorylation at serine 287

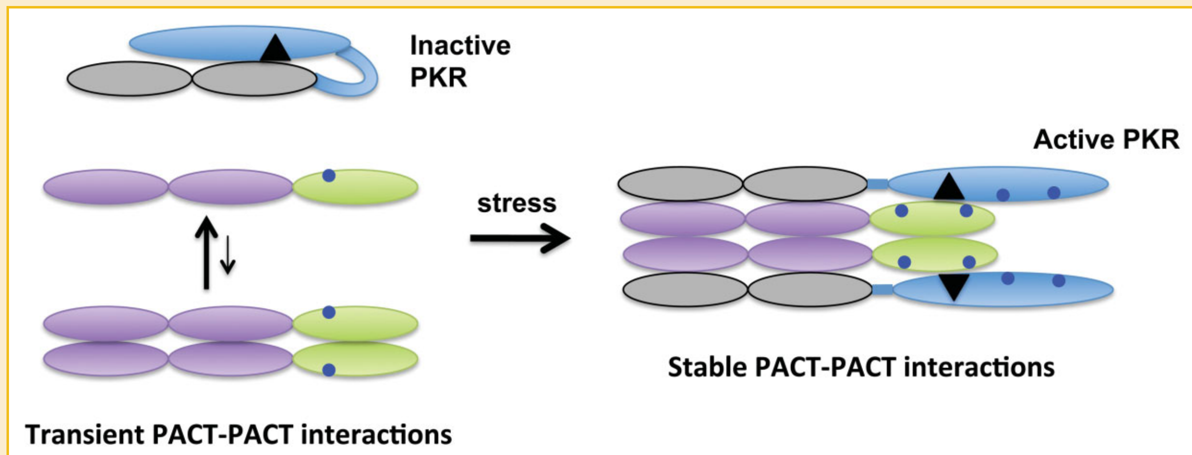

Fig. 7. A schematic model for stress-induced changes in PACT–PACT and PACT–PKR interactions. Gray ovals in PKR and purple ovals in PACT: the conserved, dsRNA-binding motifs M1 and M2, green oval: the M3 domain of PACT, blue oval: catalytic domains in PKR, black triangle: PACT-binding motif (PBM) within PKR's catalytic domain, and small blue circles: phosphoserines 246 and 287 in PACT's M3 domain and phosphothreonines 446 and 451 in PKR's catalytic domain. In the absence of stress signals, PKR exists in an inactive conformation in which PKR's M2 motif interacts with PACT-binding motif (PBM) in the catalytic domain and PACT does not interact with PKR efficiently. Under these conditions, PACT is constitutively phosphorylated at serine 246, and mainly exists as a monomer. Although PACT's M1 and M2 domains are capable of interacting with both M1 and M2 (but not M3) this interaction is transient in the absence of stress-induced phosphorylation of serine 287. Stress-dependent phosphorylation of PACT at serine 287 leads to a stronger PACT--PACT interactions and also promotes the PACT–PKR interaction. Although PACT interacts with PKR mainly via M1 and M2, which mediate high affinity interaction, the M3 is indispensable for PKR activation. M3 contacts the PACT-binding motif (PBM) in PKR and relieves PKR from the auto-inhibitory interaction between PBM and PKR's M2 motif.

leads to weakening of this interaction, thereby releasing PACT from TRBP and allowing for its efficient dimerization leading to PKR activation [Singh et al., 2011]. Thus, phosphorylation at a single serine residue may be able to achieve weakening of PACT's interaction with TRBP and strengthening of its interaction with itself as well as PKR.

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