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Stress-Induced Phosphorylation of PACT Reduces Its Interaction with TRBP and Leads to PKR Activation

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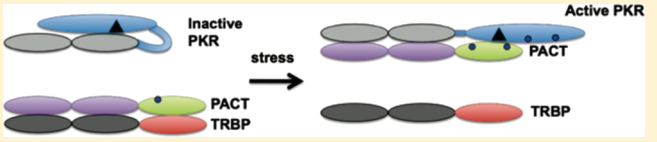
ABSTRACT: PACT is a stress-modulated activator of interferon (IFN)-induced double-stranded (ds) RNA-activated protein kinase (PKR) and is an important regulator of PKR-dependent signaling pathways. Stress-induced phosphorylation of PACT is essential for PACT's association with PKR leading to PKR activation. PKR activation by PACT leads to phosphorylation of translation initiation factor eIF2α, inhibition of protein synthesis, and apoptosis. In addition to positive regulation by PACT, PKR activity in cells is also negatively regulated by TRBP. In this study, we demonstrate for the first time that stress-induced phosphorylation at serine 287 significantly increases PACT's ability to activate PKR by weakening PACT's interaction with TRBP. A non-phosphorylatable alanine substitution mutant at this position causes enhanced interaction of PACT with TRBP and leads to a loss of PKR activation. Furthermore, TRBP overexpression in cells is unable to block apoptosis induced by a phospho-mimetic, constitutively active PACT mutant. These results demonstrate for the first time that stress-induced PACT phosphorylation functions to free PACT from the inhibitory interaction with TRBP and also to enhance its interaction with PKR.



RNA-activated protein kinase (PKR) is an interferon (IFN)-induced serine/threonine kinase that is expressed ubiquitously and plays a central role in mediating IFN's antiviral actions.[1−3] Although IFNs increase PKR's cellular abundance, PKR's kinase activity requires binding to one of its activators leading to autophosphorylation and enzymatic activation.[4,5] Double-stranded (ds) RNA, a replication intermediate for several viruses, was one of the first well-characterized activators of PKR.[6] The best-characterized cellular substrate of PKR is the translation initiation factor, eIF2α, the phosphorylation of which results in an inhibition of protein synthesis.[7,8] 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.[2,9−11] 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.[2,9−11] Thus, PKR is involved in multiple cellular processes such as differentiation, apoptosis, proliferation, and oncogenic transformation.[11]

PKR binds dsRNA via the two dsRNA-binding motifs (dsRBMs),[12−16] and this binding changes the conformation of PKR to expose ATP-binding site,[17,18] which leads to consequent autophosphorylation.[19] The two dsRBMs also mediate dsRNA-independent protein−protein interactions with other proteins that carry similar domains.[20−25] Among these are proteins inhibitory for PKR activity such as TRBP (human immunodeficiency virus (HIV)-1 transactivation-responsive (TAR) RNA-binding protein),[24] Dus2L (dihydrouridine synthase 2-like),[25] and PACT (protein activator).[21,26] PACT's association with PKR activates PKR in the absence of dsRNA.[21,26] 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 tightly 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.[27,28]

Although purified, recombinant PACT can activate PKR by a direct interaction in vitro,[21] PACT-dependent PKR activation in cells occurs in response to a cellular stress signal.[26,29−31] PACT-mediated activation of PKR occurs in response to cellular stresses such as arsenite, peroxide, growth factor withdrawal, thapsigargin, tunicamycin, and actinomycin and leads to phosphorylation of the translation initiation factor eIF2α and cellular apoptosis.[26,29,30] PACT (and its murine homologue RAX) is phosphorylated in response to the stress signals leading to its increased association with PKR causing PKR activation.[26,29,30,32,33] It is known that PACT is phosphorylated on serines 246 and 287 within the third carboxy-terminal copy of dsRBM. Of these, the serine 246 is constitutively phosphorylated in cells and is a prerequisite for the phosphorylation of serine 287 that occurs in response to stress signals.[33]

TRBP also binds PKR via dsRBMs, but unlike PACT, its interaction with PKR leads to an inhibition of PKR. TRBP1 and TRBP2 were originally described for their ability to bind HIV-1 TAR RNA,[34−36] and TRBP2 differs from TRBP1 only by having

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extra 21 amino acids at its amino terminal end.[35] Both the proteins stimulate the expression from HIV-1 long terminal repeat (LTR) in human and murine cells.[36] Similar to PACT, TRBP is a dsRNA binding protein with 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,[37−40] merlin,[41,42] and PACT.[43] TRBP functions to enhance the dicer activity by direct interaction and thus plays a central role in the RNAi pathways.[37,39]

TRBP interacts with PKR to block PKR's kinase activity and thus also eliminates PKR's inhibitory effect on translation,[44] yeast growth,[23,45] HIV expression, and replication.[45,46] In lymphocytes, TRBP inhibits PKR activation during HIV infection and thus plays a major role in suppressing the innate immune antiviral pathways.[23,39,45] Human astrocytes infected with HIV have an enhanced PKR antiviral response due to low endogenous expression of TRBP in these cells.[47] Thus HIV cannot replicate efficiently in this cell type due to a robust PKR activation in the absence of TRBP. Consistent with being a PKR inhibitor, TRBP is an oncogenic protein and its overexpression in NIH3T3 cells makes them tumorigenic in nude mice.[24]

Interestingly, TRBP and PACT are 40% similar at the amino acid level[21] and interact with each other via all three dsRBMs including the third dsRBM that does not bind dsRNA.[43] It is interesting to note that although these two proteins are very homologous, they affect PKR activity in an opposite manner. In virally infected cells, TRBP inhibits PKR by directly binding to it as well as by sequestering PKR's activator dsRNA.[45] However, in uninfected cells TRBP inhibits PKR by direct binding and by forming heterodimers with PACT and thereby keeping PACT from interacting with PKR.[48] Recently, we have shown that cellular stress signals cause PACT to dissociate from TRBP and this leads to PACT-mediated PKR activation.[48] 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 by controlling its accessibility to PACT. In this study, we investigated the possible involvement of stress-induced PACT phosphorylation in mediating the dissociation of TRBP from PACT, thus activating PKR in response to stress signals. Our results for the first time establish that stress-induced phosphorylation of PACT on serine 287 reduces its interaction with TRBP and thus leads to PKR activation. These results explain the functional consequence of serine 287 phosphorylation of PACT and the mechanism of stress-induced PKR activation by PACT.

EXPERIMENTAL PROCEDURES

Reagents, Cells, and Antibodies. HeLa M cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin/streptomycin. pEGFPC1 plasmid (Clontech) was used to detect transfected cells by cotransfection. Anti-FLAG monoclonal M2 antibody and anti-HA (HA-7) monoclonal antibodies were from Sigma. The anti-c-Myc monoclonal antibody (9E10) was from Santa Cruz. The anti-PKR monoclonal antibody (71/10) used for immunoprecipitation of PKR for activity assays was from R and D systems. Anti-PACT antibodies were raised in rabbit by immunizing them with recombinant PACT protein and have been described before (*26*). The phospho-specific anti-PKR antibody (pT451) was from Biosource International. Anti-TRBP antibody was a kind gift from Dr. Anne Gatignol.

Construction of PACT Deletion and Point Mutants. PACT and its deletion mutants M1M2, M2M3, and M3 were subcloned from the BSIIKS+ constructs[28] into the pGBKT7 and pGADT7 yeast vectors (Clontech) into BamH1 and NdeI sites. Point mutations were generated in M3 domain of PACT at S246 and S287 position by substituting these two serines with alanine and aspartic acid by using the appropriate primers for PCR amplification of the corresponding regions from the M3/pGADT7 construct. The primers used were

S246A sense:

5′-GCCATATGGAACCAAATACAGATTACATCCAGC-TGCTTGCTGAAATTGCCAAGG-3′;

S246D sense:

5′-GCCATATGGAACCAAATACAGATTACATCCAGC-TGCTTGATGAAATTGCCAAGG-3′;

S287A antisense:

5′GGGGATCCTTATAAATACTGCAAAGCATTGTGAG-CTGCATCACTTTGTGCATTGCCACAGGAGATACCGG-CGCCATGACAGAC-3′

S287D antisense:

5′GGGGATCCTTATAAATACTGCAAAGCATTGTGAGC-TGCATCACTTTGTGCATTGCCACAGGAGATACCGTCG CCATGACAGAC-3′;

M3 sense:

5′-GCTCTAGACATATGGAACCAAATACAGATTA-CATCC-3′

M3 antisense:

5′-GGGGATCCTTATAAATACTGCAAAGCATTG-3′.

The PCR products were subcloned into pGEMT-easy vector (Promega). Once the sequence of each M3 domain point mutant (S246A, S246D, S287A, S287D, S246AS287A, S246AS287D, S246DS287D and S246DS287A) had been verified, to generate full-length PACT with M3 domain point mutants the M3 domains were subcloned into mammalian expression vector pcDNA3.1$^-$ by three-piece ligation of XbaI -PvuII restriction piece from flag-PACT/BSIIKS$^+$, PvuII-*Bam*HI piece from M3 point mutants/pGBKT7, and XbaI-BamH1 cut pcDNA3.1$^-$. All of the full-length PACT M3 domain point mutants in pcDNA3.1$^-$ have a amino-terminal flag or myc tag. Full-length PACT and its point mutants were then subcloned into yeast expression vectors pGBKT7 and pGADT7 into NdeI-BamH1 sites. To generate myc-tagged full-length PACT, S246AS287A, and S246DS287D mutants the inserts were subcloned into pcDNA3.1$^-$ by using EcoRV and BamH1 restriction sites in pcDNA3.1$^-$ and HincII and *Bam*HI in full-length PACT, S246AS287A, and S246DS287D full-length PACT mutants in pGBKT7. TRBP was subcloned into the pGBKT7 yeast vector from the TRBP/BSIIKS$^+$ construct using the NdeI and BamH1 sites.

In Vitro Protein−Protein Interaction Assay. In vitro translated, ^{35}S-labeled myc-epitope-tagged PACT, its deletion mutants and flag-epitope-tagged TRBP proteins were synthesized using the TNT T7 coupled reticulocyte system from Promega. Five microliters of the in vitro translated ^{35}S-labeled PACT and its deletion mutants were incubated with 5 μL of the in vitro translated ^{35}S-labeled TRBP for 30 min at RT. This protein mixture was incubated 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 4× Laemmli buffer (250 mM Tris-HCl, pH6.8, 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.

For analyzing interactions between the full-length PACT, its point mutants S246AS287A and S246DS287D and TRBP, the same assay was used with anti-HA antibody.

Yeast Two-Hybrid Interaction Assay. The full-length PACT and its point mutants were expressed as a GAL4 DNA-activation domain fusion protein from pGADT7 vector, while TRBP was expressed as a GAL4 DNA-binding domain fusion protein from pGBKT7 vector. Each pGBKT7 and pGADT7 construct was cotransformed into *S. cerevisiae* strain AH109 (clontech) and selected on double dropout SD minimal 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 quadruple dropout histidine, leucine, tryptophan, and adenine-lacking medium, 10 μL of serial dilutions (of OD_{600} = 10, 1.0, 0.1, 0.01) of an overnight liquid culture were spotted for each of the transformants on quadruple dropout SD medium plates that lacked adenine, tryptophan, leucine and histidine and contained 25 mM 3-amino-1,2,4-triazole (3AT). Plates were incubated for three days at 30 °C.

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. 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. Cell extracts were prepared in 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). A 100 μg aliquot of total protein was immunoprecipitated using anti-PKR monoclonal antibody (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 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. 0.116 pmol of each purified full-length PACT mutant protein was added to test its effect on PKR activity. Labeled proteins were analyzed by SDS−PAGE on a 12% gel followed by autoradiography.

Apoptosis Assay. Cells were grown to 50% confluency in six-well plates and cotransfected with 200 ng of the indicated effector (expression constructs for PACT or its mutants) and 400 ng of pmCherry-C1 (Clontech) plasmid using the Effectene (Qiagen) reagent. The cells were observed for mCherry fluorescence (red) 24 h after transfection using an inverted fluorescence microscope. At this point, they were treated with 10 μM sodium arsenite for 4−8 h. The morphology of cells was monitored at regular time intervals. At 4 h after treatment, the cells were stained with PS-Green (Cell Meter Phosphatidylserine Apoptosis assay kit) that stains early apoptotic cells fluorescent green. Cells were observed live without fixing for dual (red and green) fluorescence, which indicates transfected cells that are apoptotic. At least 300 mCherry-positive (red) cells were counted as live or dead based on the staining with PS-Green (green fluorescence). The experiment was repeated three times and the data are represented as averages of these values. The assays were counted in blinded manner to ascertain the validity of observed differences. The percentage of apoptotic cells were calculated using the formula, % apoptosis = (red fluorescent cells with PS-green fluorescence/total red fluorescent cells) × 100. The *P*-values were calculated using statistical analysis software.

Expression and Purification of Recombinant PACT and Its Deletion Mutants. The full-length PACT point mutants were subcloned into pET-15b vector (Novagen) to generate an in-frame fusion of a hexahistidine tag on the amino terminus of these mutants. The expression host BL21 (DE3) was transformed and the recombinant proteins were purified as described before.[21,28]

Expression in Mammalian Cells and Coimmunoprecipitation Assay. HeLa cells were cotransfected in 6-well culture dishes with 250 ng each of (i) flag-TRBP/pcDNA3.1$^-$ alone, (ii) flag-TRBP/pcDNA3.1$^-$ and myc-wtPACT/pcDNA3.1$^-$, (iii) flag-TRBP/pcDNA3.1$^-$ and myc-PACT (S246AS287A)/pcDNA3.1$^-$, or (iv) flag-TRBP/pcDNA3.1$^-$ and myc-PACT (S246DS287D)/pcDNA3.1$^-$ using the effectene (Qiagen) reagent. At 24 h post-transfection, cells were treated with 10 μM hydrogen peroxide or 100 μM sodium arsenite for 30 min in serum-free medium, and cell extracts were prepared in lysis buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 100 U/mL aprotinin, 0.2 mM phenylmethanesulfonyl fluride (PMSF), 20% glycerol, 1% Triton X-100). 100 μg of total protein was used to immunoprecipitate flag-TRBP with anti-flag mAb-agarose in immunoprecipitation buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 U/mL aprotinin, 0.2 mM PMSF, 20% glycerol, 1% Triton X-100). The agarose beads were washed four times with 500 μL of IP buffer each time. The proteins bound to the beads were then analyzed by a Western blot analysis with the anti-myc tag (Santa Cruz Biotechnology) and anti-flag tag monoclonal antibodies (Sigma).

RESULTS

PACT's Interaction with TRBP Is Weakened by Stress Signals. TRBP and PACT interact with each other and also with PKR.[24,43,48] We examined if TRBP−PACT interaction is affected by stress signals using coimmunoprecipitation assays. As seen in Figure 1A, a strong interaction between TRBP and PACT was observed in the absence of stress signal (lane 4). This interaction was significantly weakened when cells were treated with peroxide (lane 5) or sodium arsenite (lane 6) as seen by the fact that significantly less PACT could be coimmunoprecipitated with TRBP from stressed cells. The input lanes (lanes 1−3) demonstrate that an equal amount of PACT was present in the cells, but the PACT association with TRBP was markedly lower after stress treatment. In contrast to the weakened interaction between TRBP and PACT, the interaction of PACT with PKR was enhanced by stress treatment. As seen in Figure 1B, very low amounts of endogenous PKR could be coimmunoprecipitated with PACT in the absence of cellular stress signal (lane 4). Treatment of cells with peroxide (lane 5) as well as with arsenite (lane 6) resulted in efficient coimmunoprecipitation of endogenous PKR with PACT. However, as seen in the TRBP panel, endogenous TRBP immunoprecipitated efficiently with PACT in the absence of a stress signal (lane 4), but this interaction was weakened significantly after

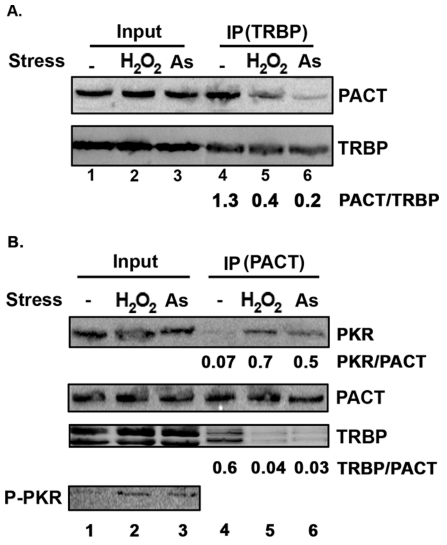

Figure 1. Stress signals reduce the interaction between PACT and TRBP and increase the interaction between PACT and PKR. (A) HeLa cells were transfected with flag-TRBP/pCDNA3.1$^-$. Twenty-four hours after transfection, cells were treated with hydrogen peroxide (10 μM) or sodium arsenite (100 μM) for 30 min. Flag-tagged TRBP protein was immunoprecipitated using anti-flag monoclonal antibody conjugated to agarose. The immunoprecipitates were analyzed by Western blot analysis with anti-PACT polyclonal antibody (PACT panel). The blot was then stripped and reprobed with monoclonal anti-flag M2 antibody to ascertain that an equal amount of TRBP protein is present in each lane (TRBP panel). The ratio of PACT and TRBP band intensities are shown below the respective IP lanes. (B) HeLa cells were transfected with flag-PACT/pCDNA3.1$^-$. Twenty-four hours after transfection, cells were treated with hydrogen peroxide (10 μM) or sodium arsenite (100 μM) for 30 min. Flag-tagged PACT protein was immunoprecipitated using anti-flag monoclonal antibody conjugated to agarose. The immunoprecipitates were analyzed by Western blot analysis with anti-PKR monoclonal antibody. The blot was stripped and reprobed with anti-TRBP antibody and then with monoclonal anti-flag M2 antibody to ascertain that an equal amount of PACT protein is present in each lane. The ratios PKR/PACT and TRBP/PACT band intensities are shown below the respective IP lanes. Total proteins were also analyzed for PKR phosphorylation with a phospho-PKR specific antibody. Input lanes represent 10% of the protein analyzed by immunoprecipitation in A and B.

treatment with peroxide or arsenite (lanes 5 and 6). Since the endogenous TRBP is present as two isoforms and both are detected by anti-TRBP antibody the TRBP panel shows two bands. The input samples (lanes 1−3) show that an equal amount of PKR and TRBP was present in untreated cells and cells treated with stressors. In order to confirm that the stress signals resulted in activation of PKR, we performed a Western blot analysis of total proteins with a phospho-specific anti-PKR antibody that only detects active, phosphorylated form of PKR. As seen in the fourth panel in Figure 1B, this analysis showed the presence of active, phosphorylated PKR (P-PKR) in cells treated with peroxide (lane 2) and arsenite (lane 3) but not in untreated cells. These results demonstrate that the interaction between TRBP and PACT is significantly weakened in response to stress signals and that PKR activation occurs by increased association of PACT with PKR.

PACT's M3 Domain Is Essential for High Affinity Interaction with TRBP. Both TRBP and PACT have three copies of the conserved dsRNA-binding motif.[27,28,43] All three copies in PACT mediate direct protein−protein interaction with the corresponding three motifs in TRBP when tested individually as truncated motifs in Y2H assay.[43] In this assay PACT motif M1 as well as PACT motif M2 each interact with both TRBP motifs M1 and M2. PACT motif M3 interacts with TRBP motif M3 but does not interact with TRBP motifs M1 or M2. In order to test the contribution of PACT motif M3 to interaction with TRBP in a full-length TRBP context and not with isolated motifs, we tested the interaction between TRBP and deletion mutants of PACT. Figure 2A shows representation of the two deletion mutants of PACT used in the coimmunoprecipitation experiment. Co-immunoprecipitation of PACT and its deletion mutants was tested using the in vitro translated ^{35}S-methionine labeled proteins. TRBP was tagged with flag epitope to allow immunoprecipitation with anti-flag monoclonal antibody. PACT and its deletion mutants were mixed with TRBP and allowed to form a complex before immunoprecipitation with flag antibody conjugated to agarose. As seen in Figure 2B (bottom panel), TRBP immunoprecipitated efficiently with the antibody agarose (lane 8), and none of the untagged proteins (PACT, M1M2 mutant, and M2M3 mutant) were pulled down with the antibody in the absence of TRBP (lanes 12−14). PACT (lane 9) and the M2M3 (lane 11) mutant were coimmunoprecipitated efficiently with TRBP, thus indicating a strong interaction. However, the M1M2 deletion mutant of PACT showed significantly weaker interaction with TRBP (lane 10), thus demonstrating that the motif M3 of PACT is essential to mediate a high-affinity interaction with TRBP. The band intensities in the total samples and the immunoprecipitated samples were quantified using the imagequant program, and percentage of the input proteins coimmunoprecipitating with TRBP were calculated. As seen in Figure 2C, both PACT and M2M3 mutant show efficient coimmunoprecipitation and the M1M2 mutant shows markedly less ability to coimmunoprecipitate with TRBP. These results demonstrate that the motif M3 in PACT is required to mediate high-affinity interaction with TRBP.

Serine 287 Phospho-mimetic Mutants of PACT Show Weaker Interaction with TRBP. PACT is constitutively phosphorylated on serine 246 and phosphorylation at this site is essential for stress-induced phosphorylation of PACT on serine 287.[33,49] Since stress signals cause dissociation of PACT from TRBP as shown in Figure 1, we further examined if phosphorylation of PACT at serine 287 causes its reduced interaction with TRBP. We used the yeast two-hybrid (Y2H) system to test the relative strength of interaction between TRBP and various point mutants of PACT. We generated point mutants that are nonphosphorylatable at serines 246 and 287 by substituting these serines by alanines. Similarly, we also generated the phospho-mimic mutants by substituting the serines 246 and 287 by aspartic acids. Thus, we tested four different mutants of PACT for their ability to interact with TRBP: S246A,S287A (AA), S246A, S287D (AD), S246D,S287D (DD), and S246DS287A (DA). Each of these mutants were expressed in yeast as a GAL4 activation domain fusion protein from the yeast expression vector pGADT7 (Clontech). TRBP protein was expressed in yeast as a fusion protein with GAL4 DNA binding domain from yeast expression vector pGBKT7. A positive interaction between TRBP and PACT results in activation of *his* and adenine reporter gene and confers the yeast an ability to grow on growth media lacking histidine and adenine. As shown in Figure 3A, the growth of yeast expressing TRBP and the different PACT mutants was assayed by spotting four different starting cell densities (10 OD units-0.01 OD units). TRBP and wtPACT showed growth at all four concentrations. The point mutant AA showed growth characteristics similar to the wtPACT thereby indicating that PACT protein completely devoid of

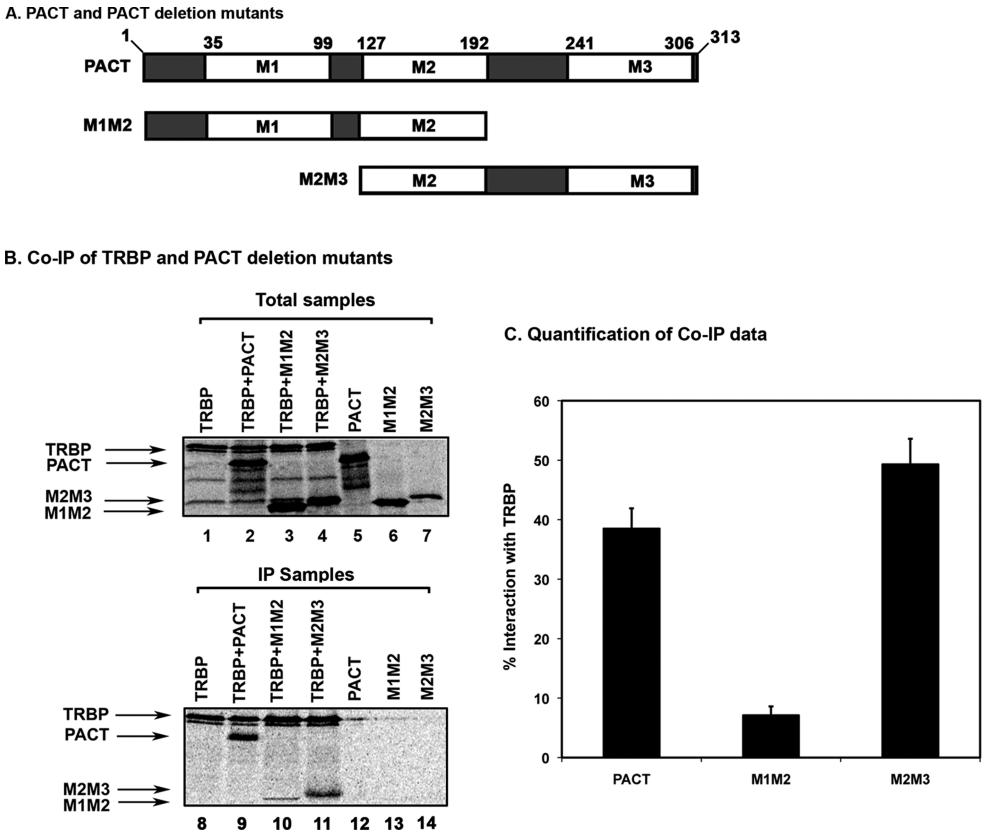

Figure 2. PACT's M3 domain is essential for high-affinity interaction with TRBP. (A) A schematic representation of the PACT and PACT's deletion mutants. The white boxes represent the three conserved motifs M1, M2, and M3 in PACT. The gray boxes represent PACT sequence outside of its conserved motif. The amino acid numbers are indicated on the top of the boxes. (B) In vitro coimmunoprecipitation of TRBP and PACT deletion mutants. The myc-epitope-tagged, [^{35}S]methionine-labeled PACT, its deletion mutants and flag-epitope-tagged, [^{35}S]methionine-labeled TRBP proteins were synthesized using the TNT T7 system. Five microliters of the reticulocyte lysates were used for coimmunoprecipitation assay using anti-flag M2 antibody agarose. Top panel represents the total proteins from the translation mix (20% of input in lanes, 8−14 of immunoprecipitation panel). The positions of the respective bands corresponding to full-length protein sizes are indicated by arrows. TRBP gives two closely spaced bands and two additional smaller bands and PACT also gives four bands due to internal initiation at internal methionine codons. The coimmunoprecipitated wtPACT, its deletion mutants M1M2 and M2M3 bends are also indicated by arrows. Bottom panel represents the coimmunoprecipitated proteins remaining bound to beads after washing. Lane 8: negative control, TRBP alone. Lanes 9−11: proteins coimmunoprecipitated with TRBP. Lanes 12−14: wtPACT, its deletion mutants M1M2 and M2M3 without TRBP. (C) Quantification of the coimmunoprecipitation gel by phosphorimager analysis. The radioactivity present in the bands was measured and the percentage interaction was calculated as 100× (radioactivity present in the coimmunoprecipitated PACT or M1M2 or M2M3 band/the radioactivity present in the PACT or M1M2 or M2M3 band in the total lane). In addition, this value was normalized to the amount of radioactivity present in the flag-TRBP bands to correct for differences in translation/immunoprecipitation steps. The error bars represent the standard deviation calculated from three independent experiments.

phosphorylation at serines 246 and 287 interacts with TRBP as efficiently as the wtPACT protein. The point mutant DA also showed growth characteristics similar to the wtPACT, thus indicating that PACT protein completely devoid of phosphorylation at serine 287 but phosphorylated at serine 246 interacts with TRBP as efficiently as the wtPACT protein. In contrast to this, the DD mutant showed markedly reduced growth abilities as demonstrated by significantly less growth at 0.1 OD unit spot and almost no growth at 0.01 OD unit spot. The AD mutant also showed much reduced growth at 0.1 and 0.01 OD unit spots. These results strongly suggest that phosphorylation of serine 287 results in a reduction in interaction between TRBP and PACT.

The single point mutants S246A, S246D, S287A, and S287D were also tested for their ability to interact with TRBP in comparison to wtPACT. As seen in Figure 3B, the three single mutants S246A, S246D, and S287A showed TRBP interaction that was equal in strength to the wtPACT. In contrast to this, the S287D mutant showed significantly weaker interaction with TRBP, thus confirming that phosphorylation of serine 287 causes weakening of the interaction between TRBP and PACT. This data further strengthens the data from double mutants (Figure 3A) and establishes that weakening of the interaction between TRBP and PACT in response to stress signals occurs primarily as a result of phosphorylation at serine 287.

In order to further confirm the weakening of interaction between TRBP and PACT in response to PACT phosphorylation, we performed coimmunoprecipitation assays with in vitro translated wtPACT and double mutants AA and DD. As seen in Figure 4A, the wtPACT and AA mutants coimmunoprecipitated efficiently with TRBP and mutant DD showed reduced coimmunoprecipitation. We quantified this data and as depicted in Figure 4B, considering the wtPACT interaction with TRBP as 100%, the AA mutant showed about 90% interaction with TRBP. In contrast to this, the DD mutant showed only about 20% interaction with TRBP. These results further strengthen the results obtained with yeast two-hybrid analysis.

Phosphorylation of PACT in Response To Stress Causes Its Dissociation from TRBP in Mammalian Cells. Our previous

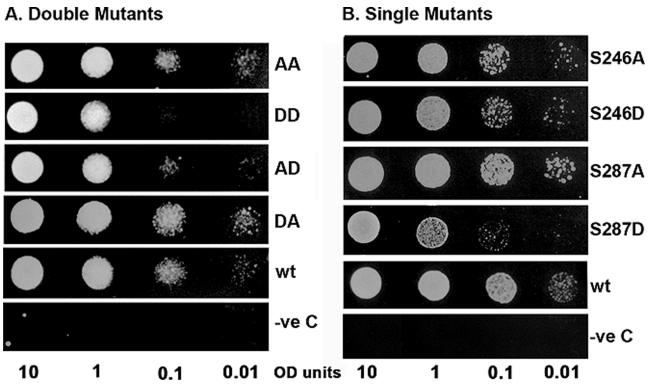

Figure 3. The phospho-mimetic mutation at serine 287 reduces PACT interaction with TRBP. (A) Interaction of TRBP with double point mutants of PACT and (B) with single point mutants of PACT in yeast two-hybrid assay. TRBP in pGBKT7 and wtPACT and its point mutants in pGADT7 were transformed into yeast strain AH109 and selected on double dropout medium lacking tryptophan and leucine. Ten microliters of serial dilutions (of OD_{600} = 10, 1.0, 0.1, 0.01) were spotted for each transformant on quadruple dropout SD medium plate that lacks adenine, tryptophan, leucine, and histidine and have 25 mM 3-amino-1,2,4-triazole (3-AT). Plates were incubated for three days at 30 °C. Transformation of empty vectors pGBKT7 and pGADT7 served as a negative control.

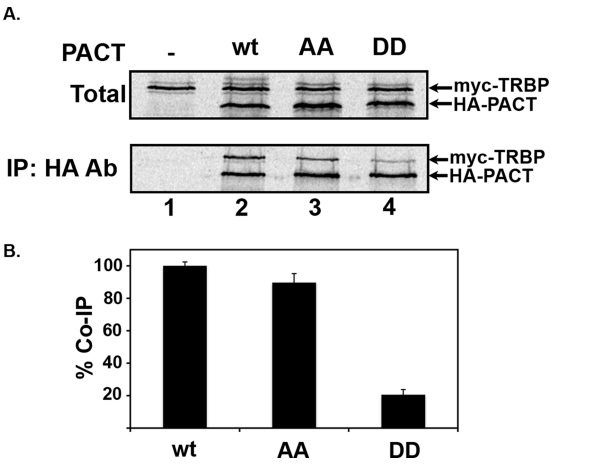

Figure 4. PACT−TRBP interaction is weaker in vitro if the serine 246 and serine 287 of PACT are phosphorylated. (A) In vitro coimmunoprecipitation of TRBP and point mutants of PACT. Hemagglutinin (HA)-epitope-tagged, [^{35}S]methionine-labeled PACT, its point mutants, and the myc-epitope-tagged, [^{35}S]methionine-labeled TRBP proteins were synthesized using the TNT T7 system. Five microliters of the reticulocyte lysates were used for coimmunoprecipitation assay using anti-HA antibody. Top panel represents the total proteins from the translation mix (20% of input in lanes, 1−4 of immunoprecipitation panel). The positions of the respective bands are indicated by arrows. The bottom panel represents the coimmunoprecipitated proteins remaining bound to beads after washing. Lane 1: negative control with only TRBP, Lanes 2−4: TRBP protein coimmunoprecipitated with wtPACT and its point mutants S246AS287A (AA) or S246DS287D (DD). (B) Quantification of the coimmunoprecipitation gel by phosphorimager analysis. The radioactivity present in the bands was measured and the percentage interaction was calculated as 100× (radioactivity present in the coimmunoprecipitated TRBP band/the radioactivity present in the TRBP band in the total lane). In addition, this value was normalized to the amount of radioactivity present in the HA-tag PACT and its point mutant's bands to correct for differences in translation/immunoprecipitation steps. The interaction between wtPACT and TRBP was taken as 100% and other interactions were normalized with respect to that. The error bars represent the standard deviation from three independent experiments.

research established that exposure of mammalian cells to stress signals causes dissociation of PACT from TRBP.[48] To investigate the involvement of PACT phosphorylation in its dissociation from TRBP, we compared the interaction between TRBP and wtPACT to that of the PACT mutants AA and DD using coimmunoprecipitation assays after arsenite treatment of cells. Flag-tagged TRBP and myc-tagged PACT proteins were expressed in HeLa M cells and their association was examined after arsenite treatment. Flag-TRBP was immunoprecipitated with Flag-antibody agarose and the immunoprecipitates were analyzed by a Western blot analysis using anti-myc antibody. As shown in Figure 5A, all the proteins were expressed at comparable levels in cells (input lanes 1−8). The reason for the slightly reduced mobility of DD mutant when expressed in mammalian cells is unclear at present but could be due to some additional modifications that may take place only in mammalian cells since the mobility of the in vitro translated DD mutant is identical to that of wtPACT (Figure 4A). In the absence of any stress signal, wtPACT and AA mutant proteins coimmunoprecipitated very efficiently with TRBP (lanes 10 and 11). However, the PACT DD mutant showed significantly weaker interaction with TRBP even in the absence of cellular stress (lane 12). Exposure of cells to arsenite caused a marked dissociation of wtPACT from TRBP (lane 14) as reflected by significantly lower amount of wtPACT coimmunoprecipitating with TRBP. The AA mutant showed a very marginal weakening of interaction with TRBP since a considerable amount of AA mutant protein coimmunoprecipitated with TRBP after arsenite treatment (lane 15) thereby demonstrating that phosphorylation of PACT at serines 246 and 287 is essential for weakening the TRBP−PACT interaction in response to arsenite. In accordance with this, the DD mutant showed no coimmunoprecipitation with TRBP after arsenite treatment (lane 16). The fact that DD mutant shows markedly weaker interaction with TRBP in the absence of any stress signal and absolutely no interaction with TRBP after arsenite treatment establishes the essential function of serine 287 phosphorylation in dissociation of PACT from TRBP in response to arsenite. This conclusion is further strengthened by the fact that the interaction of AA mutant with TRBP is largely unaffected by stress (lane 11 and 15).

PACT Phosphorylated at Serines 246 and 287 Can Activate PKR More Efficiently. Our previous results established that stress signals cause increased association of PACT with PKR and thus lead to PKR activation.[26] In order to further examine the role of serine 246 and serine 287 phosphorylation in PACT's ability to activate PKR enzymatically, we tested the ability of seven different PACT mutants to activate PKR as compared to the wt PACT protein. Wt PACT and its mutants were expressed as hexahistidine tagged proteins in *Escherichia coli* and were obtained in pure form as recombinant proteins. When tested for their ability to activate PKR, the different mutants demonstrated PKR activation capability to varying degrees. As seen in Figure 6A, there was no PKR activity detected in the absence of any PACT protein (lane 1). The DD mutant exhibited the highest PKR activation (lane 3), which was about 2.5 fold as efficient as wt PACT (lane 2). The double mutant AD activated PKR at about the same efficiency as wt PACT

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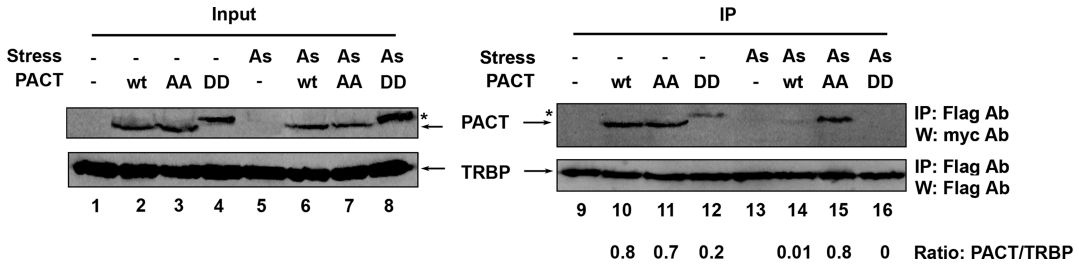

Figure 5. PACT−TRBP interaction is weaker in vivo if the serine 246 and serine 287 of PACT are phosphorylated. HeLa cells were cotransfected in 100 mm culture dishes with 250 ng each of (i) flag-TRBP/pCDNA3.1$^-$ alone, (ii) flag-TRBP/pCDNA3.1$^-$ and myc-PACT/pCDNA3.1$^-$, (iii) flag-TRBP/pCDNA3.1$^-$ and myc-PACT (AA)/ pCDNA3.1$^-$, or (iv) flag-TRBP/pCDNA3.1$^-$ and myc-PACT (DD). Twenty-four hours after transfection, cells were treated with sodium arsenite (100 μM) for 30 min in serum-free medium. Flag-tagged TRBP protein was immunoprecipitated from cell extracts using anti-flag monoclonal antibody conjugated to agarose. The immunoprecipitates were analyzed by Western blot analysis with anti-myc monoclonal antibody. The blot was then stripped and reprobed with monoclonal anti-flag M2 antibody to ascertain that an equal amount of TRBP was pulled down in each lane. The DD mutant shows a slower mobility compared to wtPACT and AA as indicated by an asterisk. The ratio of PACT to TRBP band intensities is shown below the respective IP lanes.

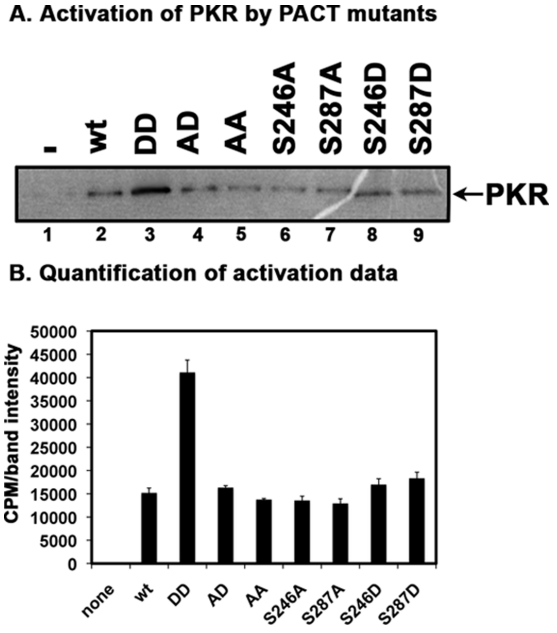

Figure 6. The S246DS287D (DD) mutant of PACT activates PKR better than wtPACT. (A) PKR kinase activity assay. PKR immunoprecipitated from HeLa cell extracts was activated by the addition of purified recombinant wtPACT and its point mutant's proteins. Lane 1: no activator; lane 2: 4 ng of pure wtPACT protein; lanes 3−9: 4 ng of pure PACT point mutants S246DS287D (DD), S246AS287D (AD), S246AS287A (AA), S246A, S287A, S246D and S287D, respectively. Position of autophosphorylated PKR is indicated by an arrow. (B) Quantification of activation data.

thereby indicating that phosphorylation of both serines at 246 and 287 is essential for PACT to activate PKR efficiently. The single mutants S246A, S287A, S246D, and S287D as well as the double mutant AA showed PKR activation very similar to the wt PACT (lanes 5−9). Most significantly, these results correspond well with the in vivo TRBP−PACT interaction results (Figure 5). The DD mutant interacts weakly with TRBP and also conceivably has enhanced interaction with PKR since in vitro this mutant can activate PKR most efficiently and better than wt PACT (lane 2 and lane 3, Figure 6). The single mutants S246D and S287D have PKR activation capabilities similar to wt PACT and not to that of DD mutant thereby indicating that phosphorylation at both 246 and 287 serines is required for efficient activation of PKR.

PACT Double Mutant DD Induces Apoptosis in the Absence of Stress That Is Not Blocked Efficiently by TRBP. Overexpression of wtPACT induces apoptosis in mammalian cells.[26] Since PACT phosphorylated at serines 246 and 287 interacts with TRBP poorly, we tested if it can induce apoptosis in mammalian cells at an efficiency higher than the wt PACT. We also reasoned that apoptosis induced by DD mutant may not be blocked efficiently by an overexpression of TRBP since TRBP may not interact with the DD mutant effectively to block its interaction with PKR. Thus, we tested the ability of DD mutant to induce apoptosis in the presence and absence of a stress signal and also in the presence and absence of TRBP overexpression and compared it to that of wt PACT. As seen in Figure 7A, the vector transfected (EV) cells showed about 4% apoptosis in the absence of stress signal and about 32% apoptosis in response to arsenite treatment. Cotransfection with a TRBP expression construct (EV + TRBP) blocked the arsenite-induced apoptosis significantly since only about 18% cells underwent apoptosis. Transfection of wt PACT expression construct showed apoptosis in 25% cells even in the absence of any stress signals. This percentage further increased to about 55% in response to arsenite treatment thereby confirming our earlier published results and establishing that an overexpression of wt PACT sensitizes cells to apoptosis in response to arsenite.[26] Cotransfection of TRBP expression construct with wt PACT reduced the basal levels of apoptosis as well as apoptosis induced by arsenite. The TRBP overexpressing cells showed only about 10% apoptosis in the absence of stress and about 36% apoptosis in response to arsenite. These results indicate that TRBP can block wt PACT-induced apoptosis both in the absence and presence of a stress signal. An overexpression of the DD mutant of PACT resulted in significantly increased apoptosis in the absence of a stress signal with about 36% cells showing signs of apoptosis. Treatment with arsenite further increased the percentage apoptosis to 53%. Since based on our biochemical analysis the DD mutant would be able to activate PKR more efficiently even in the absence of a stress signal, these results are very significant. In contrast to the cells transfected with wt PACT, where TRBP could block the apoptosis, in the cells transfected with the DD mutant TRBP was not able to block the apoptosis. Cotransfection of TRBP with DD mutant did not alter the percentage apoptosis values since

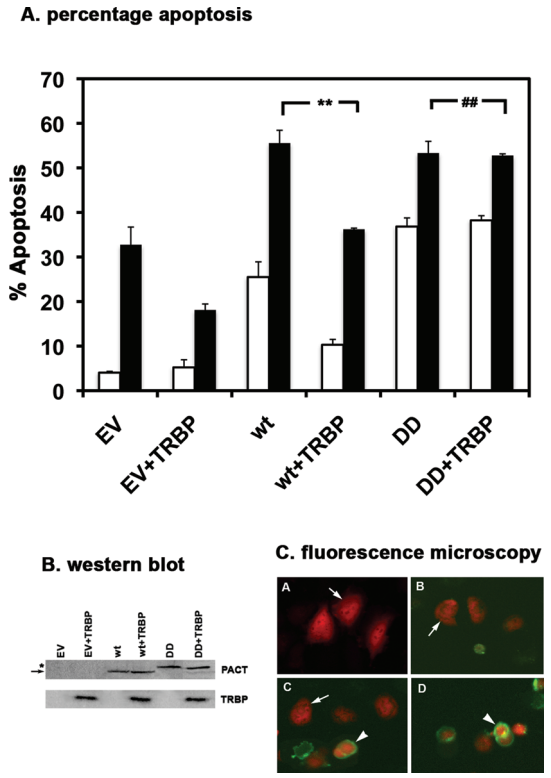

Figure 7. (A) TRBP does not rescue the apoptosis induced by S246-DS287D (DD) mutant efficiently. HeLa cells were cotransfected with (i) pmCherry-C1 + pCDNA3.1$^-$ (EV), (ii) pmCherry-C1 + pCDNA3.1$^-$ (EV) +flag-TRBP/pCDNA3.1$^-$, (iii) pmCherry-C1 + myc-wtPACT/pCDNA3.1$^-$ + pCDNA3.1$^-$, (iv) pmCherry-C1 + myc-wtPACT/pCDNA3.1$^-$ + flag-TRBP/pcDNA3.1$^-$, (v) pmCherry-C1 + myc-PACT S246DS287D (DD)/pcDNA3.1$^-$ + pcDNA3.1$^-$, or (vi) pmCherry-C1 + myc-PACT S246DS287D (DD)/pcDNA3.1$^-$ + flag-TRBP/pcDNA3.1$^-$. Twenty-four hours after transfection, the cells were treated with 10 μM sodium arsenite and at 4 h after treatment, the cells were stained with PS-Green (Cell Meter Phosphatidylserine Apoptosis assay kit) that stains early apoptotic cells fluorescent green. At least 300 mCherry-positive (red) cells were counted as live or apoptotic based on the staining with PS-Green (green fluorescence). The data are represented as averages of three separate experiments. % apoptosis = (red fluorescent cells with intense PS-green fluorescence/total red fluorescent cells) × 100. The ** symbol indicates a P value of 0.017 and the symbol ## indicates a P value of 0.062, with significant values being <0.05. The white bars represent the control treatment and black bars represent the sodium arsenite treatment. (B) Western blot analysis. Samples prepared from HeLa cells transfected in a similar manner as in A were analyzed by Western blot analysis with anti-myc (for PACT) antibody. The same blot was stripped and reprobed with anti-flag (for TRBP) antibody. The positions of PACT and the DD mutant are indicated by an arrow and an asterisk respectively. (C) Morphology of apoptotic cells. A representative fluorescence micrograph of HeLa cells transfected as indicated in A. Only the most relevant samples from the set in Figure 7A are represented. Panel A shows untreated cells, and panels B−D show cells treated with 10 μM sodium arsenite for 4 h. (A) pmCherry-C1 + flag-TRBP/pCDNA3.1$^-$, (B) pmCherry-C1 + flag-TRBP/pCDNA3.1$^-$, (C) pmCherry-C1 + myc-wtPACT/pCDNA3.1$^-$ + flag-TRBP/pcDNA3.1$^-$, and (D) pmCherry-C1 + myc-PACT S246DS287D (DD)/pcDNA3.1$^-$ + flag-TRBP/pcDNA3.1$^-$. White arrows represent transfected cells (red) and the white arrowheads represent transfected cells that are at an early stage in apoptosis (red and green).

about 38% cells underwent apoptosis in the absence of a stress signal and about 52% cells underwent apoptosis in response to arsenite. These results indicate that the DD mutant can induce apoptosis more efficiently than wt PACT in the absence of a stress signal and that TRBP is unable to block the apoptosis induced by the DD mutant. Figure 7B shows a Western blot analysis performed with a parallel set of transfected cells with anti-myc and anti-flag antibodies to ensure that equal amounts of PACT and TRBP proteins were expressed in all transfected samples. Figure 7C shows fluorescence micrographs that depict the morphology of the selected representative samples of transfected cells. Panel A shows the morphology of untreated cells with no PS-Green staining. Panels B−D each show transfected cells that are not apoptotic as indicated by an arrow (red fluorescence) and cells that are apoptotic as indicated by an arrowhead (red and green fluorescence). The apoptotic cells show rounded morphology and annexin-V staining in contrast to flat, spread out morphology and lack of annexin-V staining of nonapoptotic cells. The numbers from such analysis were analyzed for percentage apoptosis and are depicted in Figure 7A.

In order to confirm that the observed induction of apoptosis was due to PKR activation, we performed similar experiments in PKR null mouse embryonic fibroblasts (MEFs). As published by us previously, PKR null MEFs were resistant to PACT-induced apoptosis.[26] Furthermore, the DD mutant of PACT also failed to induce apoptosis in PKR null MEFS (data not shown). These observations strengthen our results presented in Figure 7 by confirming that wtPACT- and DD mutant-induced apoptosis is mediated by activation of PKR.

DISCUSSION

PACT activates PKR in response to stress signals, which leads to phosphorylation of eIF2α and consequent apoptosis.[26,29,31] Thus, PACT is involved in regulating apoptosis in response to arsenite, peroxide, growth factor withdrawal, thapsigargin, ER stressor tunicamycin, and actinomycin.[26,29,31,33] In coimmunoprecipitation assays, PACT associates with PKR efficiently in mammalian cells only in response to a stress signal.[26,49] Recently, it was shown by Peters et al. that the increased affinity of PACT toward PKR in response to stress signals results from PACT phosphorylation at serine 287.[33,49] However, association of PACT with PKR in mammalian cells can be further regulated by TRBP, a protein that complexes with PKR as well as with PACT.[24,43,44] TRBP inhibits PKR activity and interacts both with PKR and PACT via its dsRBM motifs.[43] Since TRBP is an inhibitor of PKR activity, it is possible that stress signals may result in PACT's dissociation from TRBP. The cellular abundance of PACT, TRBP, and PKR remains unchanged in response to stress. Thus, we investigated if PACT's increased association with PKR in response to stress is in part mediated by PACT's decreased interaction with TRBP. Our results clearly indicate that PACT phosphorylation at serine 287 decreases its interaction with TRBP thereby facilitating its binding to PKR. In the in vitro kinase assays, PACT phosphorylated at serines 246 and 287 activates PKR significantly more efficiently than wtPACT or other point mutants. Interestingly, the double mutant AD activated PKR at about the same efficiency as wt PACT (Figure 6) thereby indicating that phosphorylation of both serines at 246 and 287 is essential for PACT to activate PKR efficiently. Since serine 246 phosphorylation did not have any effect on PACT's interaction with TRBP (Figure 3B), the significance of phosphoserine 246 requirement for PKR activation is unclear at present. It remains to be tested if serine 246

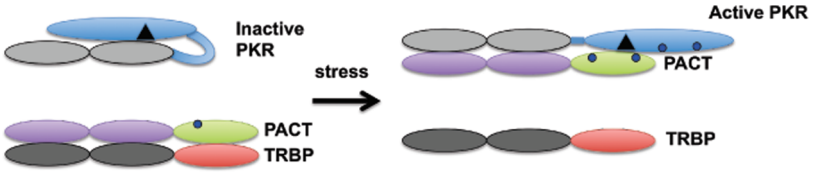

Figure 8. A schematic model for stress-induced changes in PACT's binding partners. PBM: black triangle. The conserved, dsRNA binding motifs: light gray ovals in PKR, purple in PACT, and dark gray in TRBP. PKR's catalytic domain: blue oval. The PACT M3 motif: green oval. The TRBP M3 motif: red oval. The small dark blue circles: phosphoserines 246 and 287 in M3 and phosphothreonines 446 and 451 in PKR. 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. TRBP and PACT interact via all three conserved dsRBMs. Each M1 and M2 domain is capable of interacting with both M1 and M2 in the same as well as the other protein. The M3 domain can only interact with M3 in the same or the other protein and does not interact with M1 or M2 in either protein (our unpublished observations; ref 43). Thus, PACT−PACT, PACT−TRBP, and TRBP−TRBP interactions are possible. PACT is constitutively phosphorylated at serine 246. Stress signal dependent phosphorylation of PACT at serine 287 leads to a dissociation of interaction between TRBP and PACT and 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.[27,28,58] M3 contacts the PACT-binding motif (PBM) in PKR and relieves PKR from the autoinhibitory interaction between PBM and PKR's M2 motif.

phosphorylation is essential for PACT's increased association with PKR or may serve another function. Although Peters et al.[49] have determined the PKR association of a M3 domain mutated at serine 246, the association of full-length PACT with a mutation of serine 246 to alanine or aspartate remains to be determined. In their study, Peters et al reported that the isolated M3 domain of PACT with alanine substitution mutation at serine 246 does not bind to PKR and aspartate substitution mutation at serine 246 binds PKR with same efficiency as wtPACT.[49] The interaction of full-length PACT with PKR may not be affected similarly and will have to be tested in the future. If the results of Peters et al. hold true also for full-length PACT, the decreased activation by the AD mutant may be attributable to its decreased association with PKR.

The ability of the DD mutant of PACT to induce apoptosis in the absence of a stress signal further confirms the biological significance of its ability to activate PKR efficiently. The results shown in Figure 7 are in accordance with a previous report by Peters et al.[33] It is interesting to note that although the DD mutant induces apoptosis in the absence of any stress signal at a significantly higher level than wt PACT, it also responds to stress signals and shows a further increase in percentage apoptosis after arsenite treatment. These results suggest that some additional modifications of PACT (other than phosphorylation at serines 246 and 287) or activation of other pathways that occurs in response to stress signals may contribute to induction of apoptosis. Bennett et al have previously reported that in murine cells PACT is phosphorylated on serine 18 in response to stress signals and that phosphorylation at this residue is required for PKR activation leading to apoptosis.[32] Any significance of phosphorylation of PACT at serine 18 in stress response of human cells is unknown at present. The identity of a kinase that phosphorylates PACT constitutively at serine 246 or at serine 287 in response to stress also remains to be investigated further.

Various proteins from bacteria to humans that belong to the dsRNA-binding protein family are involved in many diverse cellular functions and possess a variable number of dsRBM motifs.[50,51] The dsRBM is an alpha-beta-beta-beta-alpha fold with a well-characterized function to bind dsRNA molecules in a sequence-independent manner.[17,52] dsRBMs can also recognize non-RNA targets (proteins and DNA) and act in combination with other dsRBMs and non-dsRBM to play a regulatory role in a variety of catalytic processes that range from transcriptional regulation to RNA interference.[52,53] Both PACT and TRBP are involved in the RNAi pathways and seem to regulate these processes positively.[37,39,40,54,55] Unlike the RNAi pathways, PACT and TRBP have opposite effects on the PKR activity.[21,44,56] Recently, it was reported that TRBP phosphorylation by mitogen-activated protein kinase (MAPK) Erk in response to growth factors and PMA resulted in a coordinated increase in levels of growth-promoting miRNAs.[57] In this regard, it would be interesting to investigate if stress signal-mediated phosphorylation of PACT may change its function in the RNAi pathways.

There is some variation in steady-state TRBP protein levels in various cell lines. In human astrocytic cell line U251MG that has intrinsically low levels of TRBP, overexpression of PACT can activate PKR in the absence of any stress signals.[48] However, in cell lines with higher TRBP protein levels such as HeLa, HEK293, NIH3T3, and COS7 a stress signal is required for PKR activation by PACT (unpublished observations). Thus, PACT's ability to activate PKR is tightly regulated by TRBP. Our results presented here demonstrate that phosphorylation of PACT at serine 287 in response to stress serves to increase its affinity for PKR while reducing its interaction with TRBP. On the basis of our data, we present a model for stress-induced activation of PKR via modulation of the interaction between PACT and TRBP. As depicted in Figure 8, under normal conditions in the absence of a stress signal PACT−TRBP heterodimers form and PKR is present in its inactive conformation in cells. Under these conditions, PACT is phosphorylated constitutively only on serine 246 as previously shown by Peters et al.[33] and associates with TRBP efficiently (Figure 3B). Stress signals induce PACT phosphorylation at serine 287, which reduces its affinity for TRBP (Figure 3B). The same phosphorylation also increases its association with PKR (refs 33 and 49, our unpublished results) and thus leads to PKR activation. As depicted in Figure 8, PACT and TRBP interact via all three dsRBM copies.[43] Phosphorylation in response to stress occurs on the carboxy-terminal dsRBM copy (M3 domain), which has no dsRNA-binding activity but is essential for PKR activation. The M3 domain is important for PACT's interaction with the PBM motif in the catalytic domain of PKR,[28,58] and the phosphorylation of serines 246 and 287 may facilitate the enhanced PKR interaction in response to stress.

There are numerous proteins that are known to possess dsRBMs, which clearly play a dual function in dsRNA binding as well as protein−protein interactions.[51,52] Many proteins involved in RNA metabolism, innate immunity, and signaling across several different species are known to interact via these motifs.[53] Our results presented here outline for the first time how phosphorylation of specific amino acids within dsRBMs can change the cellular

responses by altering the balance between various protein complexes that involve proteins with dsRBMs.

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ABBREVIATIONS USED

IFN, interferon; ds, double-stranded; PKR, RNA-activated protein kinase; PACT, PKR activator; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; dsRBM, dsRNA-binding motif; HIV, human immunodeficiency virus; TRBP, transactivation-responsive (TAR) RNA-binding protein; LTR, long terminal repeat; 3AT, 3-amino-1,2,4-triazole; wt, wild type; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; PBM, PACT-binding motif

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