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Essential Role of PACT-Mediated PKR Activation in Tunicamycin-induced Apoptosis

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Summary

Cellular stresses such as disruption of calcium homeostasis, inhibition of protein glycosylation, and reduction of disulfide bonds result in accumulation of misfolded proteins in the endoplasmic reticulum (ER) and lead to cell death by apoptosis. Tunicamycin, which is an inhibitor of protein glycosylation, induces ER stress and apoptosis. In this study, we examined the involvement of double stranded (ds) RNA-activated protein kinase PKR and its protein activator PACT in tunicamycin-induced apoptosis. We demonstrate for the first time that PACT is phosphorylated in response to tunicamycin and is responsible for PKR activation by direct interaction. Furthermore, PACT-induced PKR activation is essential for tunicamycin-induced apoptosis since PACT as well as PKR null cells are markedly resistant to tunicamycin and show defective eIF2α phosphorylation and **C**/EBP **ho**mologous **p**rotein (CHOP, also known as GADD153) induction especially at low concentrations of tunicamycin. Reconstitution of PKR and PACT expression in the null cells renders them sensitive to tunicamycin, thus demonstrating that PACT-induced PKR activation plays an essential function in induction of apoptosis.

Keywords

interferon; PKR; PACT; apoptosis; eIF2α

Introduction

PKR is an interferon (IFN)-induced serine/threonine kinase that is expressed ubiquitously (1-3). Although IFNs increase PKR's cellular abundance, PKR's kinase activity stays latent until it binds to one of its activators leading to its autophosphorylation and activation (4,5). The well-known activator of PKR is double-stranded (ds) RNA, a replication intermediate for several viruses (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,

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dsRNA, and extracellular stresses (2,3,9,10). 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, have been shown to require PKR activity (2,3,9,10). Thus, PKR is involved in multiple cellular processes such as differentiation, apoptosis, proliferation, and oncogenic transformation (3).

dsRNA binds to PKR via the two dsRNA-binding motifs (dsRBMs) present at the N terminus (11-15), changing the conformation of PKR to expose ATP-binding site (16,17) and consequent autophosphorylation (18). In addition to this, the two dsRBMs can mediate dsRNA-independent protein-protein interactions with other proteins that carry similar domains (19-24). Among these are proteins inhibitory for PKR activity such as TRBP (23), Dus2L (24) and also activator protein PACT (25,26). PACT's association with PKR leads to the activation of PKR in the absence of dsRNA (25,26). PACT contains three identifiable copies of dsRBM, of which motifs 1 and 2 are true dsRBMs and exhibit dsRNA-binding activity. In addition, these two amino-terminal dsRBMs in PACT also bind tightly to the N-terminal dsRBMs of PKR. The third, carboxy-terminal motif 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* (25), PACT-dependent PKR activation in intact cells occurs in the presence of a cellular stress signal (26,29,30). PACT-mediated activation of PKR occurs in response to cellular stresses such as arsenite, peroxide, growth factor withdrawal, thapsigargin, actinomycin and leads to phosphorylation of the translation initiation factor eIF2α and cellular apoptosis (26,29,30). PACT (and its murine homolog RAX) is phosphorylated in response to the stress signals and this leads to its increased association with PKR causing PKR activation (26,29-32). In addition, an overexpression of a truncated form of PACT (PACT305) can also lead to cellular apoptosis in the absence of a stress signal (26,27). The truncated PACT (PACT305) is presumably in an active conformation due to the truncation and thus may not require the stress-induced phosphorylation of the specific serine residues (32) to bring about PKR association and activation.

In this study, we investigated the possible involvement of PACT-mediated activation of PKR in apoptosis induced by ER stressor tunicamycin. Tunicamycin inhibits N-glycosylation of proteins and thus results in an accumulation of misfolded proteins in the ER, a primary cause of ER stress. To counteract the adverse effects of ER stress, cells trigger compensatory responses, which are collectively termed as unfolded protein response (UPR). This includes a generalized suppression of translation (33) while inducing increased expression of molecular chaperones, such as GRP94 (GRP: glucose-regulated protein) and GRP78/Bip, which promote proper folding of proteins (34), and ER-associated degradation (35,36) of misfolded proteins. These three protective responses act transiently to control the accumulation of misfolded proteins within the ER. The inhibition of protein synthesis to cope with ER stress is mainly achieved by phosphorylation of the initiation factor eIF2α (37)˜ The expression of the transcription factor C/EBP homologous protein (CHOP, also

known as GADD153) is induced in response to eIF2α phosphorylation (38,39). Although phosphorylation of eIF2α causes a general block in translation, it paradoxically activates translation of the ATF4 mRNA, which encodes a transcription factor that binds and activates the CHOP promoter. Sustained ER stress leads to apoptosis, with the characteristic fragmentation of nuclei, condensation of chromatin and shrinkage of cells (40). Cells lacking CHOP are significantly protected from ER stress induced apoptosis and thus CHOP is thought to be one of the major inducers of apoptosis in response to ER stress (41,42).

Our results for the first time establish that PACT is phosphorylated in response to tunicamycin treatment causing its increased association with PKR leading to PKR activation. PACT dependent PKR activation in response to tunicamycin is essential for induction of apoptosis since both $PACT^{-/-}$ and $PKR^{-/-}$ cells are resistant to tunicamycin-induced apoptosis. The essential role of PACT-induced PKR activation in this pathway is further underscored by the fact that phosphorylation of eIF2α and induction of CHOP were defective in PACT null cells. Thus, our results presented here uncover a novel functional role of PACT and PKR in the tunicamycin-induced stress response pathway.

Results

PKR null cells are defective in tunicamycin-induced apoptosis

In order to test the involvement of PKR in tunicamycin-induced apoptosis, we compared the apoptotic response in MEFs derived from wild type and PKR null mice. The characteristic sub-diploid peak representing the apoptotic population was compared at 24h, 48h, and 72h after tunicamycin treatment by flow cytometry analysis. The sub-diploid DNA content is a characteristic of apoptotic cells and these cells show a distinct peak before the G1 peak. In the wt MEFs, tunicamycin at 0.1 μg/ml concentration induced apoptosis in 17.8 %, 36.0%, and 38.7% cells at 48h, 72h, and 96h respectively (Fig. 1 A). In contrast to this, the PKR null MEFs showed no apoptosis above the control at any of these time points. Thus, the PKR null cells are markedly resistant to tunicamycin-induced apoptosis. To examine the ability of tunicamycin to induce apoptosis in PKR null MEFs, we further investigated if higher concentrations of tunicamycin could induce apoptosis in the absence of PKR activity. The progression of apoptosis was monitored by DNA fragmentation analysis performed at 48h after tunicamycin treatment. Apoptosis is associated with the fragmentation of chromosomal DNA into multiples of the nucleosomal units, known as DNA laddering. As seen in Fig. 1 B, the wt MEFs show a clear DNA fragmentation ladder at tunicamycin concentrations higher than 0.025 μg/ml (lanes 3-8). In contrast to this, the PKR null cells showed DNA fragmentation only at tunicamycin concentrations higher than 0.25 μg/ml (lanes 6-8). Thus, PKR null cells are markedly resistant to tunicamycin concentrations up to 0.1 μg/ml. Even at the concentrations higher than 0.25μg/ml, the DNA fragmentation in PKR null MEFs was significantly weaker than in wt MEFs. These results establish that PKR null MEFs are significantly resistant to tunicamycin and that PKR plays an important functional role in tunicamycin-induced apoptosis.

PKR is activated in response to tunicamycin

We next examined if PKR activation takes place in response to tunicamycin treatment. As seen in Fig. 2 A, PKR is activated in response to tunicamycin treatment of the MEFs. A western blot analysis with a serine-451 phospho-specific anti-PKR antibody, which specifically detects only the catalytically active PKR, showed PKR activation at 2h after tunicamycin treatment. PKR activation peaked at 4h and showed a gradual decrease after that. We also examined PKR activation in a human neuroblastoma cell line SK-N-SH in response to tunicamycin. As seen in Fig. 2 B, PKR is activated in response to tunicamycin in SK-N-SH cells with the same kinetics as in MEFs. To further ascertain that tunicamycin treatment activates PKR, we also performed PKR activity assays with cell extracts prepared at various time points after the tunicamycin treatment in the absence of any exogenously added PKR activator. As seen in Fig. 2 C, PKR was activated at 2h after tunicamycin treatment in SK-N-SH cells and its activity peaked at 4h after treatment. These results further confirm that PKR is activated by tunicamycin treatment of cells. At later time points of 8h and 12h, PKR activity gradually decreased. PKR activation observed in response to tunicamycin is equally strong as compared to the activation observed with cell extract from untreated sample activated with exogenously added dsRNA or recombinant PACT (positive control lanes). Similar results were obtained in several other cell types including HeLa, and HEK293 cells (data not shown). These results further establish that tunicamycin treatment results in activation of PKR in both murine and human cells. To confirm the apoptotic response of SK-N-SH cells to tunicamycin, the DNA laddering assay was performed. As seen in Fig. 2 D, the SK-N-SH cells showed characteristic DNA laddering in response to tunicamycin at 24h, which further increased at 48h. This demonstrates that the SK-N-SH cells respond to tunicamycin in a manner similar to the MEFs.

PACT associates with PKR and activates its kinase activity in response to tunicamycin

Since PACT activates PKR in response to several stress signals (26,29-32), we examined the involvement of PACT in PKR activation in response to tunicamycin. PACT-induced PKR activation occurs by a direct association of PACT with PKR in response to a stress signal (26,32). Thus, we decided to test association of PACT with PKR in response to tunicamycin. To assay this, we performed a PACT pull-down experiment with pure recombinant hexahistidine tagged PKR bound to Ni-agarose. SK-N-SH cells were treated with tunicamycin and cell extracts prepared at different time points were used to test for binding of the endogenous PACT from cells to recombinant PKR protein immobilized in Ni-agarose beads. As shown in Fig. 3 A, tunicamycin treatment resulted in a time-dependent increase in association of PACT with PKR. In untreated control cells, very little PACT associated with PKR. At 2h and 4h after tunicamycin treatment, PACT association with PKR increased significantly and then declined at 12h. These results establish that PACT associates with PKR in response to tunicamycin treatment leading to its activation. Since it is established that stress-induced PACT phosphorylation triggers association of PACT with PKR (26,32), we next examined if PACT is phosphorylated in response to tunicamycin treatment. We performed *in-vivo* labeling experiment with ^{32}P-orthophosphate during tunicamycin treatment. In order to detect PACT phosphorylation efficiently, we used flag-PACT overexpression in SK-N-SH cells. As represented in Fig. 3 B, tunicamycin treatment showed

significant PACT phosphorylation as compared to the untreated cells. PACT phosphorylation was also detected at a low basal level in untreated cells, and this may be due to the constitutive phosphorylation of PACT at serine 246 in the absence of cellular stress (32). In addition to phosphorylation of PACT, we also observed that the amount of phosphorylated PKR that co-immunoprecipitated with flag-PACT also increased significantly after tunicamycin treatment (^{32}P-PKR panel, Fig. 3 B). These results clearly demonstrate that PACT is phosphorylated in response to tunicamycin and leading to its increased association with PKR causing PKR activation.

PACT null cells are defective in PKR activation in response to tunicamycin

To further evaluate if PACT is essential for PKR activation in response to tunicamycin, we compared the PKR activation in response to tunicamycin in PACT$^{+/+}$ and PACT$^{-/-}$ MEFs. As seen in Fig. 4 A, PKR activation was seen after 2h of tunicamycin treatment in PACT$^{+/+}$ MEFs similar to PKR$^{+/+}$ MEFs and SK-N-SH cells (Fig. 2 A and B). In contrast to this, the serine-451 phospho-specific PKR antibody did not detect any autophosphorylated PKR after tunicamycin treatment in PACT$^{-/-}$ MEFs (Fig. 4 B). Thus, PACT is essential for PKR activation in response to tunicamycin treatment. These results establish that PACT mediates the PKR activation in response to tunicamycin and that PKR activation is defective in the absence of PACT.

PACT null cells are defective in tunicamycin-induced apoptosis

Since PACT acts as the activator of PKR in response to tunicamycin, we next compared tunicamycin-induced apoptosis in PACT wt and null cells using the flow cytometry technique to measure percentage of cells with hypo-diploid DNA content characteristic of apoptotic cells. In the wt MEFs, 0.1μg/ml tunicamycin induced apoptosis in 18.9%, 36.2%, and 39.5% cells at 24h, 48h, and 72h respectively (Fig. 5 A). In contrast to this, the PACT null MEFs showed no apoptosis above the control at any of these time points. Thus, the PACT null cells are markedly resistant to tunicamycin-induced apoptosis. In order to confirm that the hypo-diploid peak represented the apoptotic population, we examined the apoptosis by DNA fragmentation analysis. The progression of apoptosis was monitored at 48h after tunicamycin treatments at different tunicamycin concentrations. As seen in Fig. 5 B, the wt MEFs show a clear DNA fragmentation ladder characteristic only of apoptotic cells at tunicamycin concentrations higher than 0.025 μg/ml (lanes 3-8) and a weak but detectable DNA ladder at 0.01 μg/ml (lane 2). In contrast to this, the PACT null cells showed a relatively weaker DNA fragmentation ladder only at tunicamycin concentrations higher than 0.1 μg/ml (lanes 5-8). At concentrations lower than 0.1 μg/ml, PACT null MEFs showed no DNA fragmentation. Thus, PACT null cells are markedly resistant to tunicamycin concentrations up to 0.1 μg/ml, as compared to wt MEFs, which show equivalent DNA fragmentation even at 10-fold lower (0.01 μg/ml) tunicamycin concentrations (lane 2). These results establish that PACT is essential for tunicamycin-induced apoptosis.

Reconstitution of PKR and PACT expression rescues the defective apoptosis in the corresponding null cells

If the defective apoptotic response of PKR and PACT null cells to tunicamycin is specifically due to lack of these proteins, a reconstitution of PKR or PACT expression is expected to rescue the phenotype. We tested this by transfecting the PKR and PACT expression constructs in PKR and PACT null MEFs respectively. We co-transfected PKR null and PACT null MEFs with FLAG epitope-tagged wtPKR, or PACT expression constructs along with pEGFPC1 (Clontech) that encodes EGFP. As a negative control, we used empty vector pcDNA3.1^{-} co-transfected with pEGFPC1. 24 h after transfection the cells were treated with tunicamycin, and 72 h after the treatment the GFP-positive cells were assessed for hallmark signs of apoptosis such as cell shrinkage, membrane blebbing, and nuclear condensation. Reconstitution of PKR expression in PKR null MEFs caused pronounced chromatin condensation seen as intense DAPI fluorescence after tunicamycin treatment. In contrast, transfection of empty vector showed no nuclear condensation after tunicamycin treatment. To quantify this effect, we counted the percentage of cells showing nuclear condensation within the GFP-positive transfected population. It was seen that 33.5% of FLAG-PKR-transfected PKR null cells and showed nuclear condensation after tunicamycin treatment (Fig. 6 *A*, black bars). In contrast, this percentage was only 6.6% in the pCDNA3.1^{-}-transfected cells (Fig. 6 *A*, white bars). Thus, there was about a 5-fold increase in apoptosis in response to tunicamycin when PKR expression was reconstituted in PKR null MEFs. The wt MEFs showed about 38% apoptosis in response to tunicamycin both with or without PKR reconstitution as expected. Similar results were obtained with PACT null MEFs as seen in Fig. 6 B. It was seen that 35.3% of FLAG-PACT-transfected cells and showed nuclear condensation after tunicamycin treatment (Fig. 6 B, black bars). In contrast, this percentage was only 9.1% in the pCDNA3.1^{-}-transfected cells (Fig. 6 B, white bars). Thus, there was a 3.8-fold increase in apoptosis in response to tunicamycin when PACT expression was reconstituted in PACT null MEFs. The wt MEFs showed about 30% apoptosis in response to tunicamycin both with or without PACT expression construct. Western blot analysis confirmed the expression of the FLAG-tagged PKR and PACT proteins (data not shown). These results establish that the defective apoptosis observed in PKR and PACT null cells indeed is solely due to absence of PKR or PACT respectively.

Both PACT and PKR null cells are defective in CHOP induction in response to tunicamycin

In order to characterize the impaired apoptotic response of PACT and PKR null MEFs, we examined the eIF2α phosphorylation and CHOP induction by western blot analysis. Since the difference in apoptosis was most pronounced at 0.1μg/ml tunicamycin concentration (Fig. 1 and 5), we performed the treatments for the western blot analysis at the same concentration. As shown in Fig. 7 A and B, the PACT$^{+/+}$ and PKR$^{+/+}$ MEFs show rapid phosphorylation of eIF2α and CHOP induction in response to tunicamycin. In contrast to this, eIF2α phosphorylation as well as CHOP induction was absent in PACT$^{-/-}$ MEFS after tunicamycin treatment. These results further demonstrate that the defective apoptosis observed in PACT null cells is due to a lack of eIF2α phosphorylation and consequent CHOP induction in these cells. The lack of CHOP induction and the eIF2α phosphorylation response of PKR$^{-/-}$ MEFs was similar to that of PACT$^{-/-}$ MEFs. As seen in Fig. 7 B,

PKR$^{-/-}$ MEFs did not show any significant eIF2α phosphorylation response, and since eIF2α phosphorylation leads to CHOP induction, the expression of CHOP was barely detectable. These results indicate that PACT-induced PKR activation plays a central role in tunicamycin-induced apoptosis by regulating eIF2α phosphorylation and CHOP induction.

In order to further investigate the response of PKR and PACT null cells to tunicamycin treatment, we studied the eIF2α phosphorylation in response to varying doses of tunicamycin. As represented in Fig. 8, the wt MEFs showed a strong eIF2α phosphorylation response at all concentrations of tunicamycin that were examined. In contrast to this, PKR$^{-/-}$ as well as PACT$^{-/-}$ MEFs showed good eIF2α phosphorylation only at tunicamycin concentrations 0.25 μg/ml and above. At concentrations of tunicamycin 0.1μg/ml and below, there was no observed eIF2α phosphorylation response in both these cell types. These results also explain the lack of apoptotic response of these two cell types as shown in Fig. 1 and 5 at lower concentrations of tunicamycin. The apoptotic defect observed in PKR$^{-/-}$ and PACT$^{-/-}$ cells was also most pronounced below the tunicamycin concentrations of 0.1 μg/ml. Thus, PACT and PKR are essential for eIF2α phosphorylation and apoptosis in response to lower concentrations of tunicamycin. At lower levels of ER stress induced by tunicamycin, PACT-mediated PKR activation seems to be the primary mechanism utilized by the cells to bring about eIF2α phosphorylation. At higher concentrations of tunicamycin an additional, alternate cellular pathway may bring about the eIF2α phosphorylation and consequent apoptosis.

Discussion

The ER is the primary site of synthesis, post-translational modifications, folding, and oligomerization of secreted, membrane bound, and some organelle-resident proteins (43). The ER also functions as an efficient quality-control system, and prevents incompletely folded molecules from moving along secretory pathways. Some forms of cellular stress (known collectively as ER stress), such as depletion of calcium ions from the ER lumen, inhibition of protein glycosylation, reduction of disulfide bonds, expression of mutant proteins, and ischemic insults, lead to the accumulation and aggregation of unfolded proteins in the ER (44,45). Since protein aggregation and misfolding is toxic to cells, several pathophysiological conditions are associated with ER stress, including diabetes, ischemia, and neurodegenerative diseases (46,47). The attenuation of general protein synthesis to cope with ER stress is mainly achieved by phosphorylation of the initiation factor eIF2α on serine-51(37)˜ PKR-like ER resident kinase (PERK) has been shown to be activated in response to misfolded proteins and to phosphorylate eIF2α (43,48,49). Phosphorylation of serine-51 of eIF2α by PERK in response to ER stress is a protective response since it attenuates the new protein synthesis and the cells gain time to manage correct folding or degrade the accumulated misfolded proteins. In accordance with this, homozygous mutations in PERK cause pancreatic beta-cell death and infancy-onset diabetes in human Wollcot-Rallison syndrome and in genetically engineered mice (50-54). Thus, lack of PERK kinase activity and lack of eIF2α phosphorylation makes the cells more vulnerable to apoptosis in response to ER stress (55-58). In contrast to this, PKR activation by ER stressors such as thapsigargin (which causes Ca+ release from the ER) leads to activation of apoptotic pathways (59-62). Although the primary result of PKR activation by ER stress is

also phosphorylation of eIF2α on serine-51, the outcome of this event is opposite of PERK activation and leads to cell death by apoptosis. In accordance with this, inhibition of PKR activation in response to ER stress leads to a reduction in apoptosis (60,63-66).

In this study we investigated the mechanism of PKR activation in response to ER stressor tunicamycin. Our results demonstrate that PACT mediates PKR activation in response to tunicamycin. Using metabolic labeling with ^{32}P-orthophosphate, we established that PACT is phosphorylated after tunicamycin treatment and phosphorylated PACT associates with PKR with higher affinity, thereby leading to its activation. Consequently, PACT$^{-/-}$ cells show no PKR activation in response to tunicamycin. Furthermore, MEFs isolated from PKR$^{-/-}$ and PACT$^{-/-}$ mice are resistant to tunicamycin-induced apoptosis. This defect in null cells specifically results from lack of PKR and PACT activity since a reconstitution of PKR and PACT expression in the null cells rescues the defect in apoptosis. Both eIF2α phosphorylation and the consequent CHOP induction was absent in PACT$^{-/-}$ MEFs in response to tunicamycin. Furthermore, the results of tunicamycin dose response (Fig. 8), clearly indicated that PACT-induced PKR activation seems to be the primary mediator of eIF2α phosphorylation at tunicamycin concentrations 0.1 μg/ml and lower. Our results indicate that the MEFs use two different mechanisms to phosphorylate eIF2α depending on the level of stress they are experiencing. PERK activation, which is known to occur in response to ER stress, may require higher levels of tunicamycin (ER stress). On the contrary, PACT-induced PKR activation occurs at lower concentrations of tunicamycin. A crosstalk between the PERK and PKR pathways has been noted before for eIF2α phosphorylation in response to viral infections (67). Thus, our results for the first time establish PACT phosphorylation in response to tunicamycin as the mechanism of PKR activation during ER stress. In addition, our results also establish that PACT-dependent PKR activation is essential for induction of apoptosis in response to tunicamycin at lower concentrations. However, it is also worth noting that the PACT and PKR null MEFs showed consistently lower amount of apoptosis even at higher tunicamycin concentrations (Figs. 2 and 5).

Recently PKR was identified as one of the mediators of tunicamycin-induced cell death in neuronal cells (63). Interestingly, phosphorylated PKR is present in brain tissues in patients with Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) (68-71). Thus, PKR may not only be involved in apoptosis induced by viral infections, but may also be involved in inducing apoptosis in response to ER stress. Inhibition of PKR activity by overexpression of its trans-dominant mutant or by using a chemical inhibitor confers resistance to apoptosis in response to a variety of stress signals including ER stressors (59,63,72,73). However, the exact mechanism of PKR activation in this pathway had remained elusive thus far. Our results now establish PACT as the activator of PKR in response to ER stressor tunicamycin. Using a siRNA-mediated downregulation of PKR activity in HeLa and HEK 293 cells, it was reported recently that PKR plays a significant role in ER stress mediated apoptosis (73). In the same report it was shown that thapsigargin treatment of HEK293 cells increased the PACT mRNA and protein levels, which was essential for PKR activation. We did not observe any induction of PACT protein levels in response to tunicamycin in MEFs or in SK-N-SH cells. Although the reasons for these differences are unknown at the present, it is possible that they may result from the different cell types or from different inducers of ER stress. On the other hand,

phosphorylation of PACT in response to cellular stress resulting in its increased affinity for PKR and consequent PKR activation has been established (26,29,32). It is known that PACT is phosphorylated on serine 287 in response to stress signals and that constitutive phosphorylation of serine 246 is a prerequisite for the stress-induced phosphorylation (32). Both these residues reside in the PKR activation domain of PACT, which has been mapped to its third carboxy-terminal copy of dsRBM (27,28). Considering that phosphorylation of PACT in response to tunicamycin resulted in an increased association with PKR, it is possible that the phosphorylation observed in response to ER stress occurs at these sites.

There are four eIF2α kinases in mammalian cells, heme-regulated inhibitor (HRI), mammalian homologue of the *Saccharomyces cerevisiae* protein kinase general control nonderepressible-2 (GCN2), PERK, and PKR (74-77). Functional characterization of these kinases has indicated that each of them is involved in phosphorylation of eIF2α in response to a distinct stimulus. HRI is known to regulate eIF2α phosphorylation and protein synthesis in response to heme availability in erythroid cells. GCN2 is involved in regulating translation in response to amino acid deprivation in mammalian cells. In general, it is thought that the ER stress activated kinase PERK phosphorylates the eIF2α in response to ER stressors. Our results establish that PKR also plays a major functional role in tunicamycin-induced eIF2α phosphorylation and induction of apoptosis. We also observed that the apoptotic response of PKR and PACT null cells was identical to their wt counterparts in response to thapsigargin and DTT, which induce ER stress by Ca^{+} mobilization and by inhibition of disulfide bond formation (data not shown). In this regard, it was reported recently that PACT null MEFS were not resistant to thapsigargin-induced apoptosis (78). Thus, it is possible that although seemingly similar, the different inducers of ER stress may utilize separate kinases as effectors to bring about an inhibition of protein synthesis by phosphorylation of eIF2α. The existence of several related eIF2α specific kinases likely provides the cells some degree of redundancy of function. Our work presented here nevertheless establishes that PACT-induced PKR activation plays an important functional role in tunicamycin-induced apoptosis.

Materials and Methods

Reagents, Cells, and Antibodies

Tunicamycin was obtained from Sigma. $PACT^{+/+}$ and isogenic $PACT^{-/-}$ mouse embryonic fibroblasts (MEFs) were a genorous gift from Dr. Ganes Sen (Lerner Research Institute, Cleveland Clinic foundaton) and $PKR^{+/+}$ and isogenic $PKR^{-/-}$ MEFs were a kind gift from Dr. Bryan Williams (Monash Institute of Medical Research, Australia). MEFs, SK-N-SH and HT1080 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/strepto mycin. Following antibodies were used: Anti-FLAG monoclonal M2 (Sigma), anti-PKR(human) monoclonal (71/10-R & D systems), anti-PKR(murine) monoclonal (Transduction Laboratories), anti-phospho-PKR (thr451) (Cell Signaling), anti-phospho-eIF2α (ser51) (Invitrogen), anti-eIF2α, and anti-his tag (Santa Cruz), anti-CHOP (Santa Cruz). PACT polyclonal antibody was has been described (26).

Flow Cytometry analysis to monitor apoptosis

MEFs were treated with 0.1 μg/ml of tunicamycin. 24h, 48h, and 72h after the treatment, the cells were permeabilized using the Vindelov's solution (79), stained with propidium iodide and analyzed by flow cytometry using a Coulter cell sorter.

DNA fragmentation analysis

The MEFs were treated with different concentrations of tunicamycin for 48 h. Low molecular weight fragmented DNA was prepared and analyzed as described (29).

Western lot analysis

The western blot analysis of the total proteins from cell extracts or of the immunoprecipitates was performed as described before (25). For the analysis of eIF2α phosphorylation and CHOP induction from MEFs, the 10 μg of the total protein was analyzed for eIF2α phosphorylation and 50 μg of the total protein was analyzed for CHOP induction.

PKR activity Assay

The SK-N-SH cells were treated with 1μg/ml tunicamycin for indicated time intervals and PKR activity assays were performed as described before (25) without addition of any exogenous activator using anti-PKR monoclonal antibody (R & D systems).

PACT-PKR interactions

SK-N-SH cells grown to 70% confluency in 100-mm plates were treated with 1μg/ml of tunicamycin for indicated times. The cell extracts were prepared in 100 μl of low salt buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 100 units/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 20% glycerol, 0.1% Triton X-100, and phosphatase inhibitor cocktail, Sigma). 500 μg of cell extract was bound to 1 μg of recombinant, hexahistidine tagged PKR protein immobilized on Ni-agarose resin in 100 μl of low salt buffer at 4 °C for 1h. The beads were washed in 500 μl of low salt buffer four times and PACT bound to beads was analyzed by SDS-PAGE followed by western blot analysis using anti-PACT polyclonal antibody. Aliquots of whole cell lysate were examined without immunoprecipitation by Western blot analyses with anti-PACT antibody. To ascertain that equal amounts of pure PKR protein was analyzed in the assays, same blots were stripped and re-probed with hexahistidine tag specific antibody.

In vivo phosphate labeling

For analyzing phosphorylation of PACT, SK-N-SH cells were transfected with flagPACT/pcDNA3.1^{-} construct in 100 mm dishes. 24h after transfection, the transfected cells were kept in phosphate-free DMEM containing 10% serum for 1 h before the tunicamycin treatment. The tunicamycin treatment was done in 3 ml medium in the presence of 500 μCi/ml of ^{32}P-orthophosphoric acid (Perkin Elmer). 4h after the tunicamycin treatment, cell extracts were prepared in 100 μl of low salt buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 100 units/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 20% glycerol, 0.1% Triton X-100, and phosphatase inhibitor cocktail, Sigma). Flag-PACT was immunoprecipitated

from 500 μg total protein extract using the Flag M2 antibody-agarose (Sigma) in low salt buffer at 4°C for 2h. The beads were washed in 500 μl of low salt buffer four times and PACT bound to beads was analyzed by SDS-PAGE followed by phosphorimager analysis. Another sample, similarly immunoprecipitated with Flag M2 antibody-agarose was used for western blot analysis with anti-PKR monoclonal antibody (Transduction Laboratories).

Reconstitution of PKR and PACT expression in MEFs and apoptosis assays

The $PKR^{-/-}$ and $PACT^{-/-}$ cells grown on cover slips were transfected with 600 ng total of the indicated plasmids using the Effectene (Qiagen) reagent. The cells were observed for GFP fluorescence 24 h after transfection using an inverted fluorescence microscope and were treated with 0.1μg/ml tunicamycin. The morphology of cells was monitored at regular time intervals every 12h. At 72 h after treatment, the cells were washed twice with phosphate- buffered saline and fixed in 1:1 acetone/methanol for 1 min, and the cover slips were mounted in Vectashield (Vector Laboratories) mounting medium containing DAPI (4,6-diamidino-2-phenylindole). At least 300 GFP-positive cells were counted as live or dead based on their morphology. The cells showing normal flat morphology were scored as live and the cells showing cell shrinkage, membrane blebbing, rounded morphology, partial detachment from the plate, and nuclear condensation with intense DAPI fluorescence were counted as dead cells. The percentage of apoptotic cells were calculated using the formula, % apoptosis = (fluorescent dead cells/total fluorescent cells) × 100.

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Abbreviations

ER	endoplasmic reticulum
ds	double stranded
IFN	interferon
JNK	c-jun N-terminal kinase
SAPK	stress-activated protein kinase
dsRBMs	dsRNA-binding motifs
CHOP	C/EBP homologous protein
MEFS	mouse embryonic fibroblasts
GRP	glucose-regulated protein
PKR	protein kinase dsRNA-activated
PERK	PKR-like ER resident kinase
eIF2α	α subunit of eukaryotic initiation factor 2 α
GCN-2	general control nonderepressible-2

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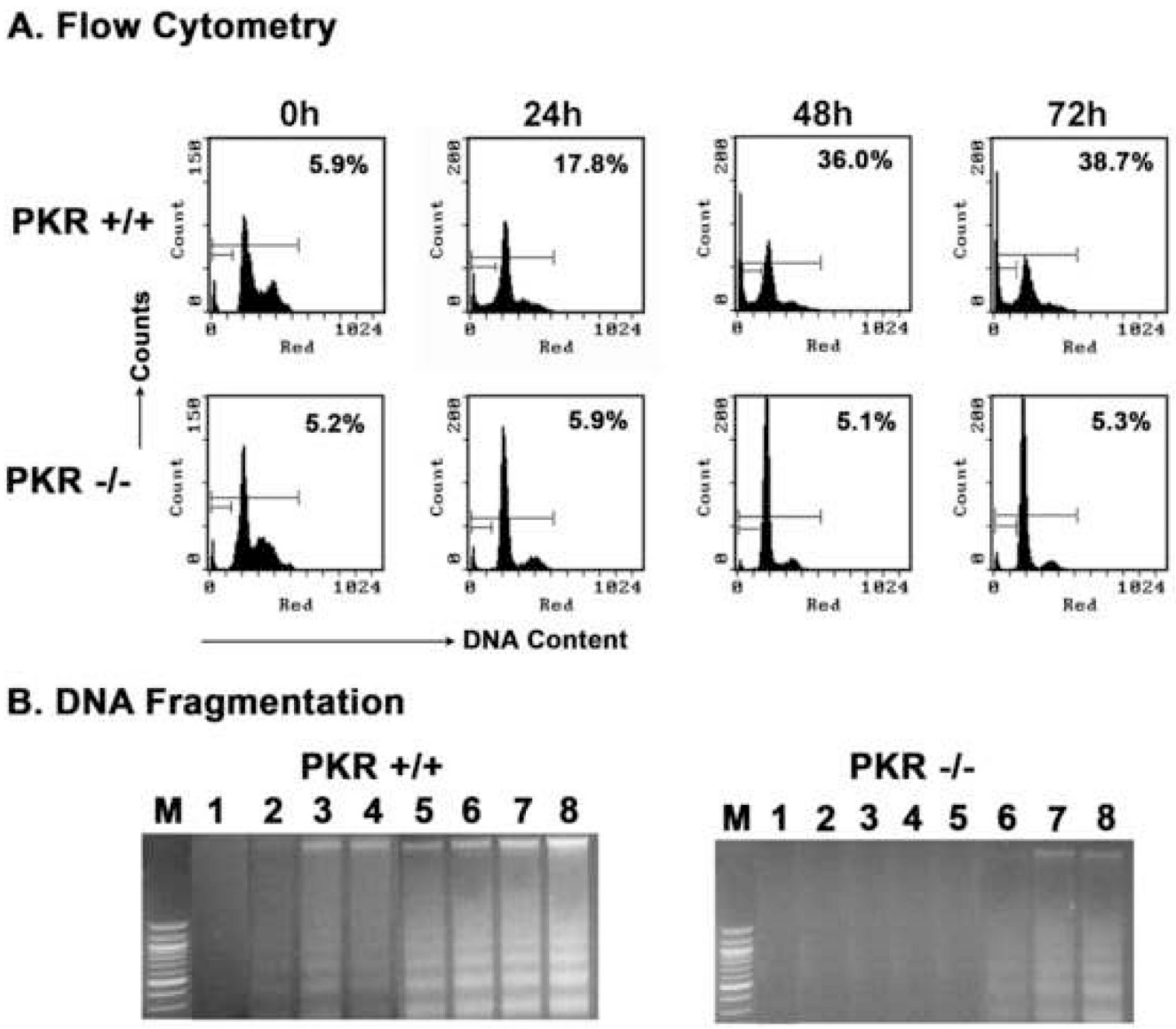

Fig. 1. PKR null MEFs are resistant to tunicamycin-induced apoptosis

A. Flow cytometry analysis of cell-cycle distribution after tunicamycin treatment. $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were treated with 0.1μg/ml tunicamycin. Cells were harvested at 24h, 48h, and 72h after the treatment and subjected to flow cytometry analysis after propidium iodide staining. The sub-G0/G1 cell population represents the apoptotic cells. The sub-G0/G1 percentages are displayed in each panel. These experiments were repeated twice, each time with duplicate samples. The most representative profiles from a single experiment are shown. B. DNA fragmentation analysis in response to tunicamycin. $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were treated with increasing concentrations of tunicamycin. 48 h after tunicamycin treatment, the fragmented DNA was analyzed. The cell type is as indicated on the top of the lanes. Lane 1, untreated cells; Lanes 2-8 cells treated with different concentrations of tunicamycin. Lane 2, 0.01μg/ml; lane 3, 0.025 μg/ml; lane 4, 0.05 μg/ml; lane 5, 0.1 μg/ml;

lane 6, 0.25 μg/ml; lane 7, 0.5 μg/ml, and lane 8, 1 μg/ml. M: 100-bp ladder DNA size markers.

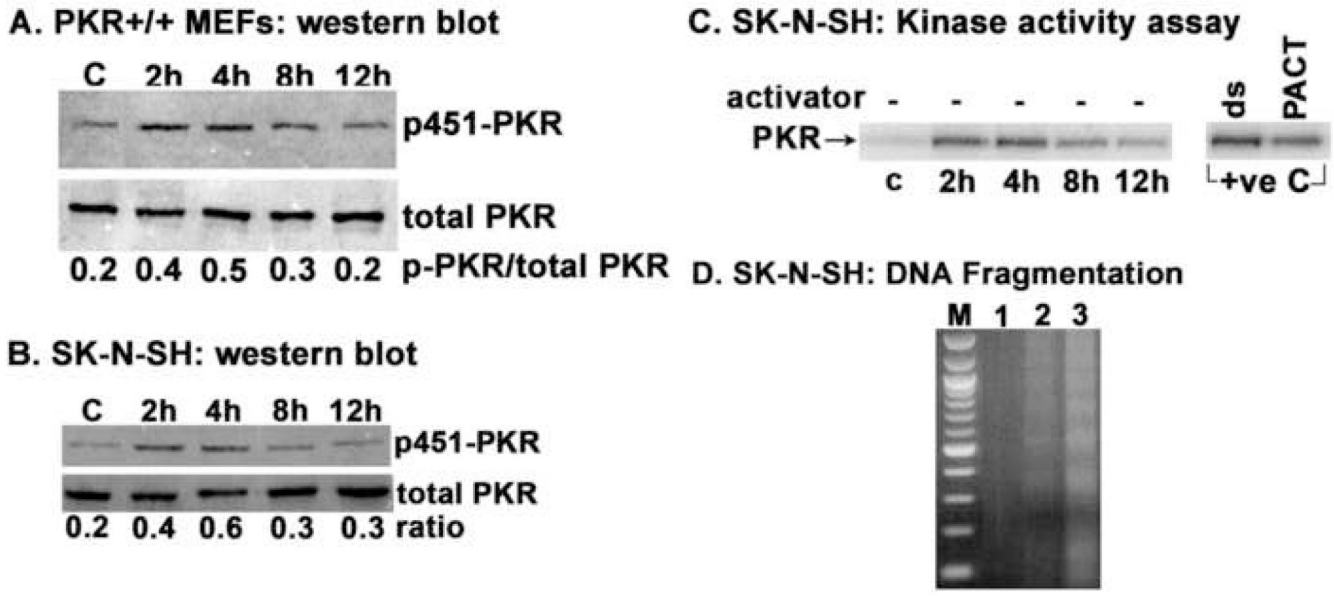

Fig. 2. PKR is activated in both murine and human cells in response to tunicamycin

A. $PKR^{+/+}$ MEFs: Western blot analysis with a phosphoserine-451 specific antibody. The MEFs were treated with 1 μg/ml tunicamycin and cell extracts were prepared at indicated times after the treatments. Phosphorylation of PKR was examined by western blot analysis with a phosphoserine-451 specific antibody. The same blot was stripped and re-probed with an anti-PKR monoclonal antibody to detect total PKR. The ratio of phosphorylated PKR/ total PKR is represented below each lane. B. SK-N-SH: Western blot analysis with a phosphoserine-451 specific antibody. The SK-N-SH cells were treated with 1 μg/ml tunicamycin and cell extracts were prepared at indicated times after the treatments. Phosphorylation of PKR was examined by western blot analysis with a phosphoserine-451 specific antibody. The same blot was stripped and re-probed with an anti-PKR monoclonal antibody to detect total PKR. The ratio of phosphorylated PKR/total PKR is represented below each lane. **C. SK-N-SH: PKR kinase activity assay.** SK-N-SH cells were treated with 1 μg/ml tunicamycin and PKR activity was assayed from the cell extracts prepared at the indicated times. PKR was immunoprecipitated with a monoclonal antibody 71/10 and protein A-sepharose beads. PKR activity assay was performed with PKR attached to the protein A-sepharose beads without any exogenous activator added. Lanes marked +ve C are positive controls to show that activation occurs with extract from untreated cells with 0.1μg/ml polyI-polyC (dsRNA) and 0.2 pmoles of pure recombinant truncated PACT (amino acids 1-305) added to the kinase assay reaction *in vitro*. D. SK-N-SH: DNA fragmentation analysis in response to tunicamycin. SK-N-SH cells were treated with 1 μg/ml tunicamycin. At 24h and 48 h after tunicamycin treatment, the fragmented DNA was analyzed. Lane 1, untreated cells; Lanes 2, 24h treatment; and lane 3, 48h treatment. M: 100-bp ladder DNA size markers.

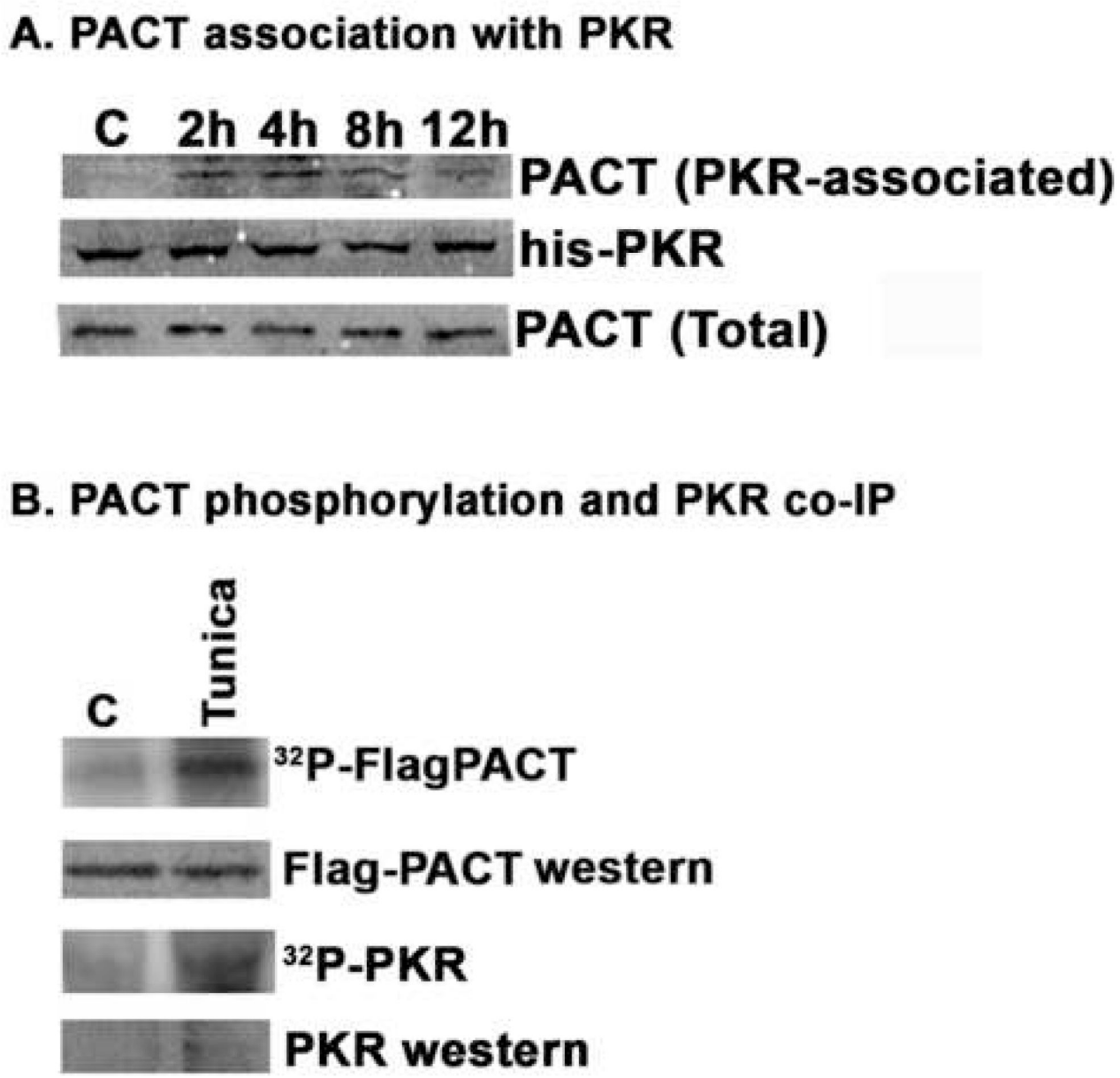

Fig. 3. PACT activates PKR in response to tunicamycin

A. PACT associates with PKR in response to tunicamycin. SK-N-SH cells were treated with 1 μg/ml tunicamycin. The cells extracts were prepared at indicated times and 500 μg of protein from the cell extracts was allowed to interact with 1 μg of purified PKR protein immobilized on Ni-agarose beads. After washing the beads to remove unbound material, the PKR-associated PACT was analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using anti-PACT antibody. The same blot was stripped and re-probed with anti-hexahistidine tag antibody to detect total PKR immobilized on Ni-agarose beads. To analyze total PACT amounts in the extracts, 100 μg of the total protein from extracts was analyzed by western blot analysis with anti-PACT antibody. B. PACT phosphorylation and PKR association in response to tunicamycin. SK-N-SH cells were transfected with flag-

PACT/pCDNA3.1^{-} (full-length/amino acids 1-313) to achieve overexpression of flag-PACT. 24h after transfection, the cells were treated with 1μg/ml tunicamycin for 4h in the presence of 0.5 mCi/ml ^{32}P-orthophosphate in phosphate free medium. Phosphorylation status of PACT was examined by immunoprecipitation with anti-Flag monoclonal antibody followed by phosphorimager analysis (^{32}P-FlagPACT). In the same gel, a weaker ^{32}P-labeled band was observed at a position corresponding to PKR (^{32}P-PKR). Aliquots of cell lysate with 100 μg total protein were also examined by western blot analysis for total flag-PACT with anti-flag antibody (Flag-PACT western). A western blot analysis was performed with anti-PKR monoclonal antibody on 1 g of cell extracts immunoprecipitated with anti-Flag M2 monoclonal antibody (PKR western). Subsequent autoradiographic analysis of the western blot also showed presence of ^{32}P-labeling in the band corresponding to PKR.

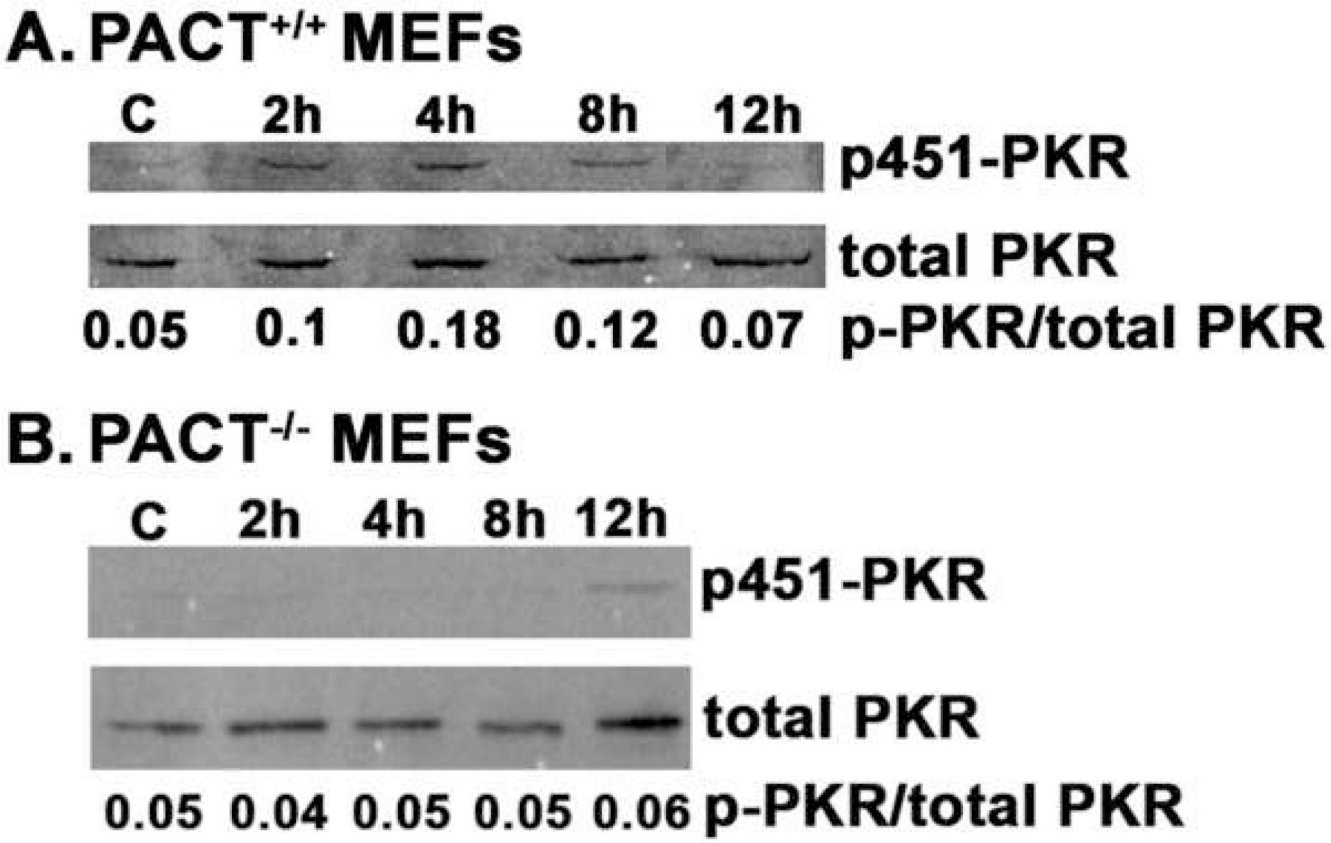

Fig. 4. PKR is not activated in PACT null MEFs

The PACT$^{+/+}$ (A) and PACT$^{-/-}$ (B) MEFs were treated with 1 μg/ml tunicamycin and cell extracts were prepared at indicated times after the treatments. Phosphorylation of PKR was examined by western blot analysis with a phosphoserine-451 specific antibody. The same blot was stripped and re-probed with an anti-PKR monoclonal antibody to detect total PKR. The ratio of phosphorylated PKR/total PKR is represented below each lane.

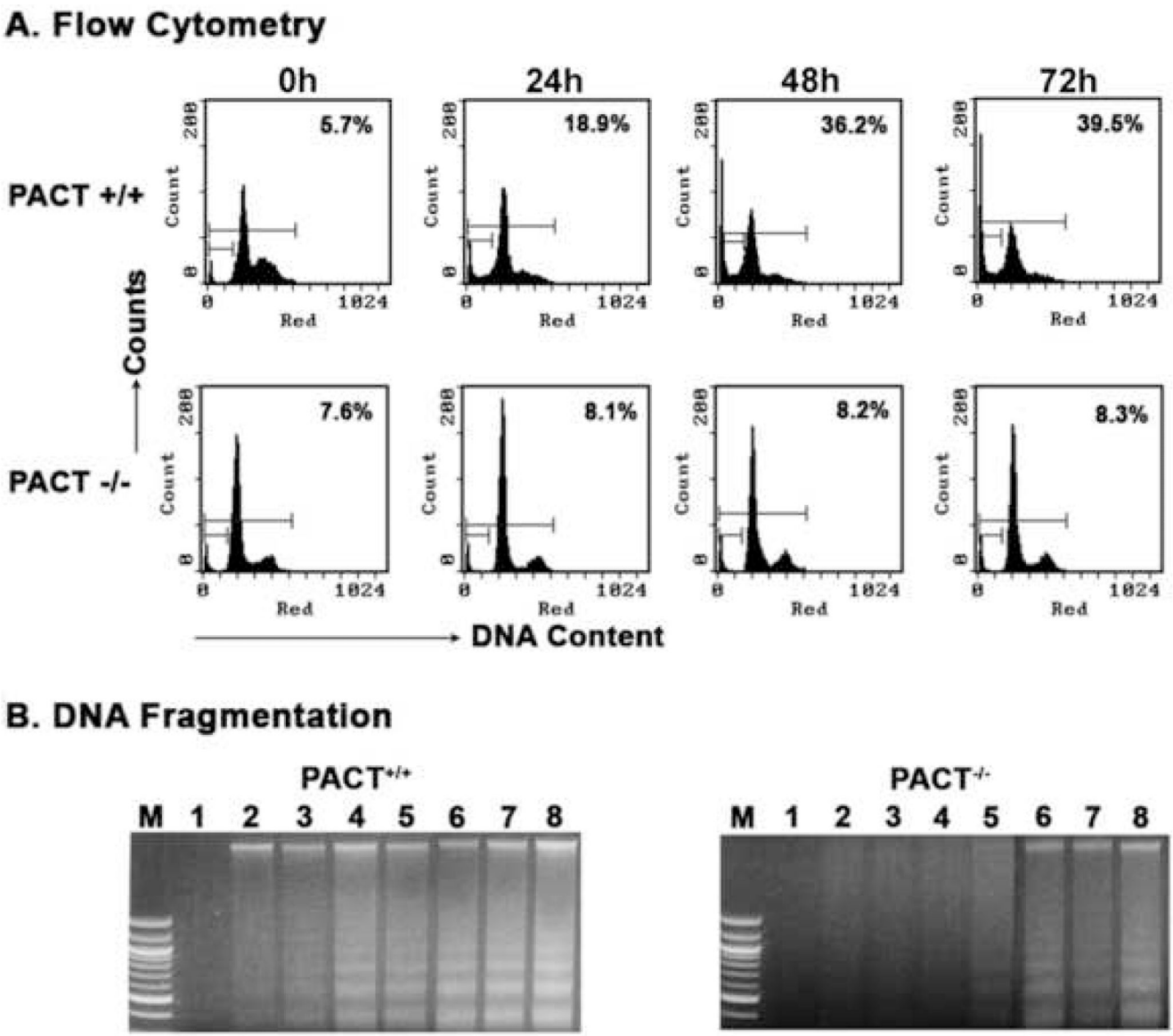

Fig. 5. PACT null MEFs are resistant to tunicamycin-induced apoptosis

A. Flow cytometry analysis of cell-cycle distribution after tunicamycin treatment. PACT$^{+/+}$ and PACT$^{-/-}$ MEFs were treated with 0.1μg/ml tunicamycin. Cells were harvested at 24h, 48h, and 72h after the treatment and subjected to flow cytometry analysis as described. The sub-G0/G1 cell population represents the apoptotic cells. The sub-G0/G1 percentages are displayed in each panel. These experiments were repeated twice, each time with duplicate samples. The most representative profiles from a single experiment are shown. B. DNA fragmentation analysis in response to tunicamycin. PACT$^{+/+}$ and PACT$^{-/-}$ MEFs were treated with increasing concentrations of tunicamycin. 48 h after tunicamycin treatment, the fragmented DNA was analyzed. The cell type is as indicated on the top of the lanes. Lane 1, untreated cells; Lanes 2-8 cells treated with different concentrations of tunicamycin. Lane 2, 0.01μg/ml; lane 3, 0.025 μg/ml; lane 4, 0.05 μg/ml; lane 5, 0.1 μg/ml; lane 6, 0.25 μg/ml; lane 7, 0.5 μg/ml, and lane 8, 1 μg/ml. M: 100-bp ladder DNA size markers.

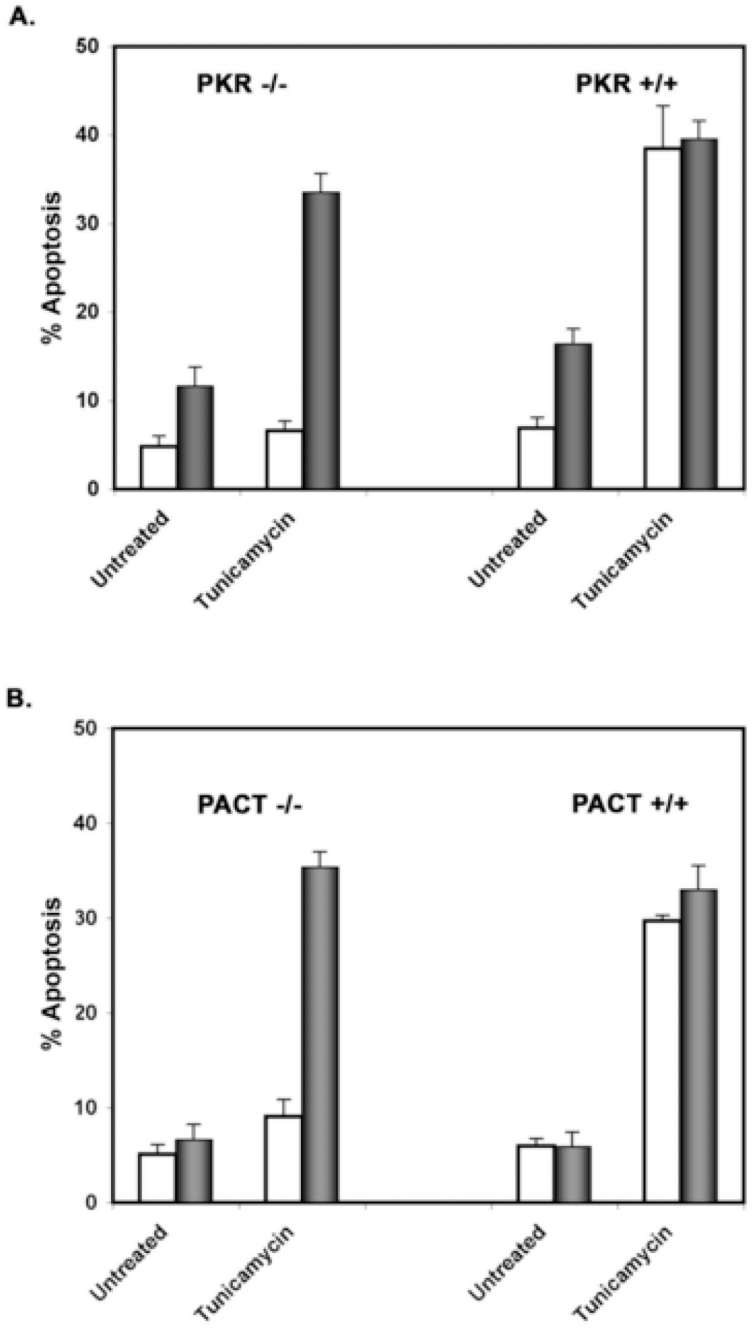

Fig. 6. Reconstitution of PKR and PACT expression restores the apoptotic response in null MEFs

A. Reconstitution of PKR expression. As indicated, $PKR^{-/-}$ or $PKR^{+/+}$ MEFs grown on coverslips were transfected with 500 ng of $pCDNA3.1^{-}$ + 100 ng of pEGFPC1 (white bars) or 500 ng of flag-PKR/$pCDNA3.1^{-}$ + 100 ng of pEGFPC1 (black bars) using Effectene. The cells were observed for EGFP fluorescence 24 h after transfection and were treated with 0.1μg/ml of tunicamycin. The morphology of cells was monitored every 12 h. At 72 h after treatment, the cells were fixed and mounted in Vectashield with DAPI nuclear stain. White bars represent empty vector transfected cells and black bars represent cells with PKR

expression construct transfection. At least 300 fluorescent (EGFP-positive) cells were counted as live or dead based on their morphology and nuclear condensation indicated by intense DAPI fluorescence. % apoptosis = (fluorescent dead cells with intense DAPI fluorescence/total fluorescent cells) × 100. **B. Reconstitution of PACT expression.** As indicated, $PACT^{-/-}$ or $PACT^{+/+}$ MEFs grown on coverslips were transfected with 500 ng of $pCDNA3.1^{-}$ + 100 ng of pEGFPC1 (white bars) or 500 ng of flag-PACT/$pCDNA3.1^{-}$ (encoding full length PACT, amino acids 1-313) + 100 ng of pEGFPC1 (black bars) using Effectene. The cells were subjected to similar treatment and analysis as in A.

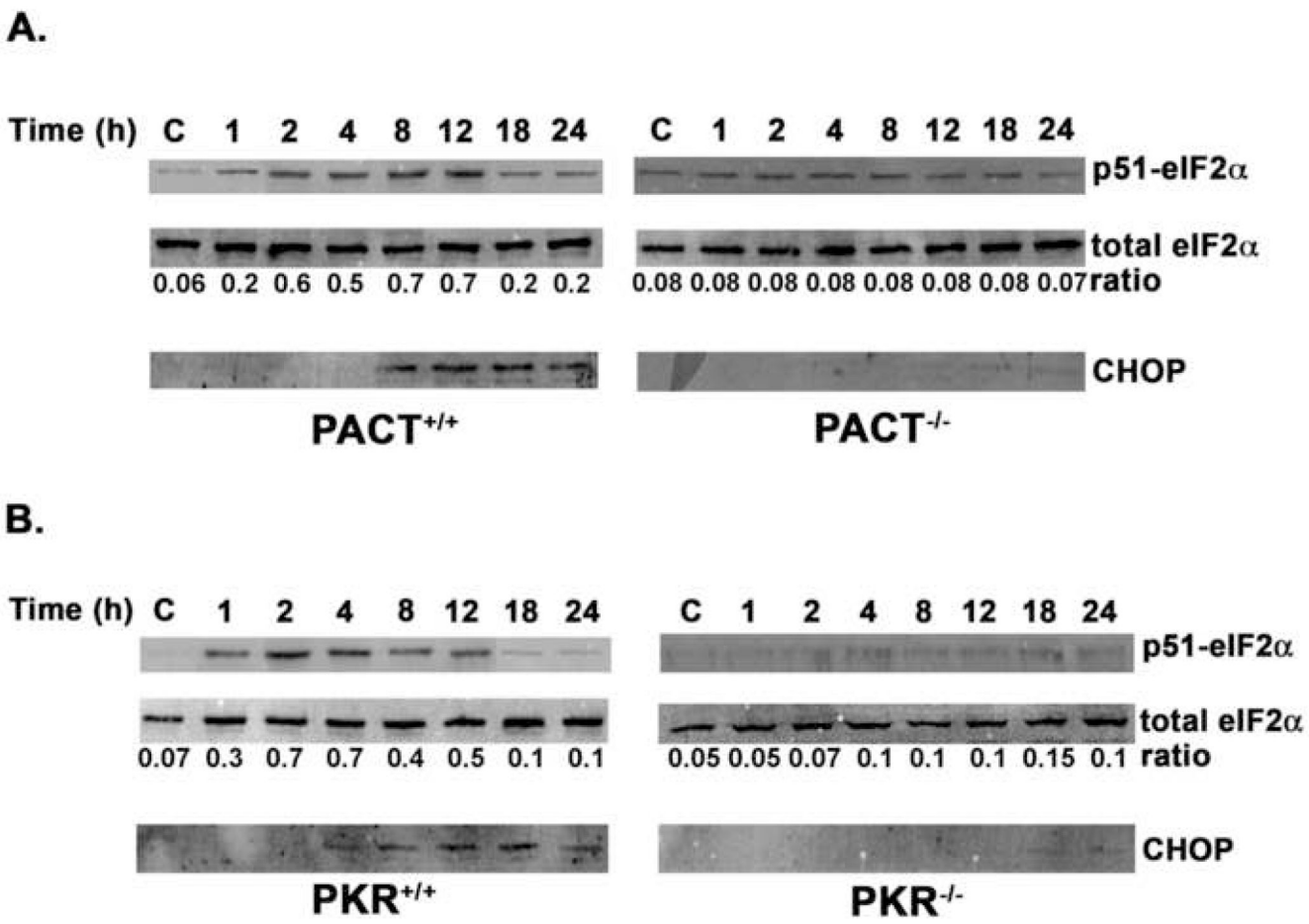

Fig. 7. Functional requirement of PACT and PKR for eIF2α phosphorylation and CHOP expression in response to tunicamycin

A. eIF2α phosphorylation and CHOP induction in PACT null MEFs. $PACT^{+/+}$ and $PACT^{-/-}$ MEFs were treated with 0.1 μg/ml tunicamycin. The cells extracts were prepared at indicated times and analyzed by western blot analysis. The same blot was stripped and re-probed with all antibodies. The numbers at the bottom of the panels represent the ratio of phosphorylated eIF2α to total eIF2α, calculated using the Imagequant software and Storm phosphorimager. The analysis was repeated five times and best representative blots are shown. The fold increases in ratios after tunicamycin treatment compared to the control samples were calculated at each time point and subjected to a statistical analysis with the use of the Student's t test. The observed differences between +/+ and −/− MEFS are considered significant with all probability values being less than 0.05. **B. eIF2α** phosphorylation and CHOP induction in PKR null MEFs. $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were treated with 0.1 μg/ml tunicamycin. The cells extracts prepared at indicated times were analyzed as described in A.

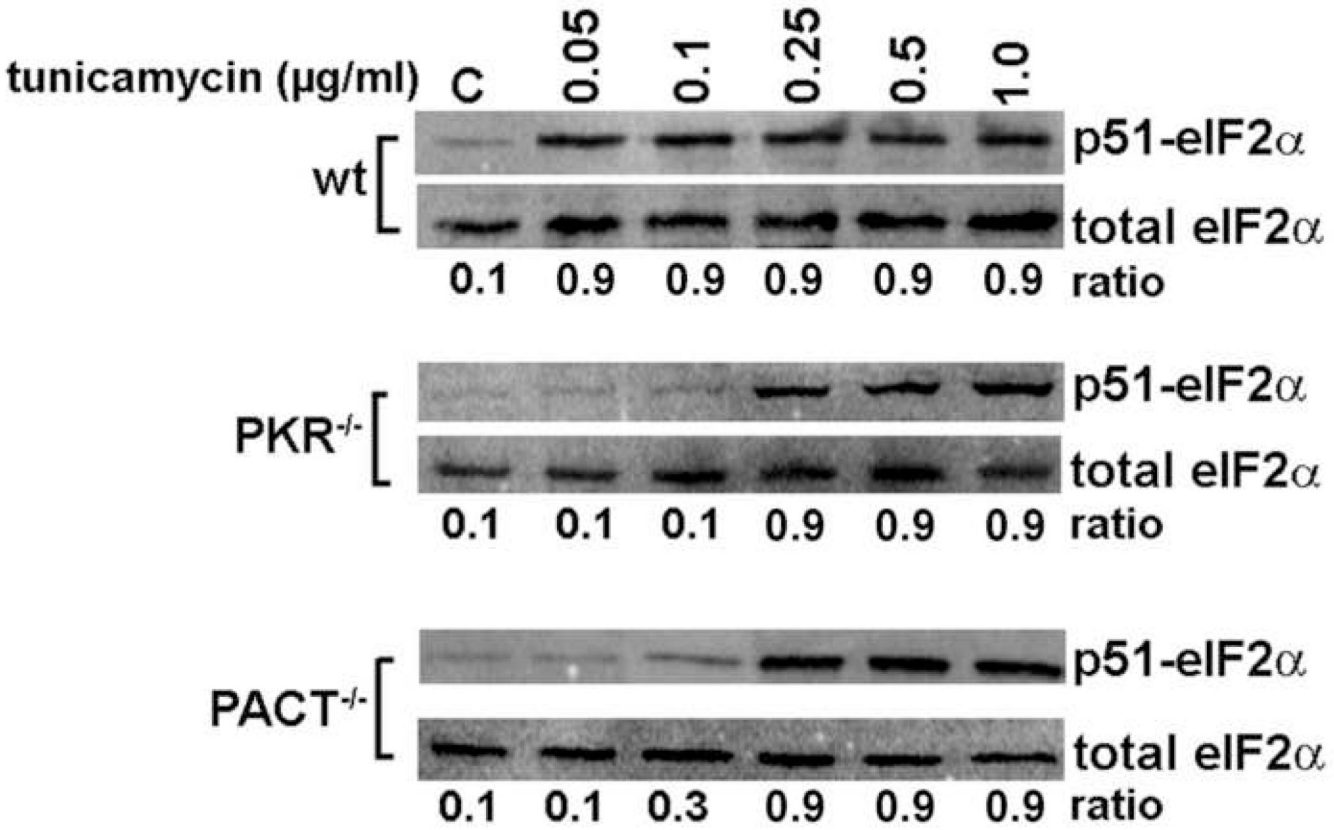

Fig. 8. Tunicamycin dose response

$PKR^{+/+}$, $PACT^{+/+}$, $PKR^{-/-}$, and $PACT^{-/-}$ MEFs were treated with 0.05 μg/ml, 0.1 μg/ml, 0.25 μg/ml, 0.5 μg/ml, and 1.0 μg/ml tunicamycin as indicated on top of the lanes. The cells extracts were prepared at 2 h after the treatments and analyzed by western blot analysis. The same blot was stripped and re-probed with both the antibodies. The numbers at the bottom of the panels represent the ratio of phosphorylated eIF2α to total eIF2α, calculated using the Imagequant software and Storm phosphorimager. Since the results obtained from $PKR^{+/+}$ and $PACT^{+/+}$ MEFS were identical, only the $PKR^{+/+}$ MEF results are represented as wt panel.