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The Role of PACT in Mediating Gene Induction, PKR Activation, and Apoptosis in Response to Diverse Stimuli

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PACT, the protein activator of the double-stranded (ds)RNA-activated protein kinase (PKR) has been shown to strongly interact with and activate PKR in cultured cells and *in vitro*. To further analyze the functions of PACT we have recently generated PACT knockout (KO) mice and described several developmental defects that are absent in PKR KO mice. Importantly, PACT has been previously suggested to be involved in different signaling pathways that include endoplasmic reticulum stress, serum deprivation, growth factor withdrawal, viral infection, and cytokine responses. In this study, we have analyzed the contribution of PACT to these pathways using cells derived from wildtype (WT) and PACT KO mice. Notably, we have been unable to detect any significant differences in the responses to stress stimuli comparing WT and PACT KO cells, although we have been able to validate the specific interaction between PACT and PKR. Taken together, our results reinforce the importance of genetic loss of function analysis to infer protein function.

Introduction

The cDNA encoding PACT was originally isolated in our laboratory using a yeast two-hybrid screen from a human cDNA library using the K296R mutant of human double-stranded (ds)RNA protein kinase (PKR) as bait (Patel and Sen 1998). Subsequent experiments indicated that PACT could interact with and activate PKR in the absence of dsRNA and in response to stress stimuli (Patel and Sen 1998; Peters and others 2001). Hence it was named *PACT*, for protein activator of PKR (Patel and Sen 1998). Using a similar strategy, another group identified with yeast two-hybrid the cDNA for mouse PACT (termed *RAX*) from a mouse cDNA library using the mouse equivalent of human K296R (mouse K271R) mutant PKR as bait (Ito and others 1999). Since its discovery, PACT has been implicated in the activation of PKR under a variety of stress and inflammatory stimuli. These include proinflammatory cytokines such as tumor necrosis factor (TNF) and interferon (IFN) γ, endoplasmic reticulum (ER) stress, arsenite, peroxide, ceramide, growth factor withdrawal, viral infection, and ethanol (Patel and others 2000; Ruvolo and others 2001; Bennett and others 2004, 2006; Chen and others 2006). Although the majority of information about PACT relates to its interaction with PKR, some PKR-independent functions have also been proposed for PACT. Notably, PACT has also been reported to interact with the RNase III Dicer and participate in the mammalian RNA interference (RNAi) pathway (Lee and others 2006; Kok and others 2007). Interestingly, other reports have indicated that PACT can enhance expression of cotransfected reporter genes, which could be related to its effects on the aforementioned pathways (Li and Sen 2003; Yang and others 2003). Others have reported that PACT can enhance IFN induction by Newcastle disease virus and colocalize with viral replication complexes (Iwamura and others 2001).

PACT is a 313–amino acid protein that contains three conserved dsRNA binding motifs (dsRBM) (Patel and Sen 1998). dsRBMs 1 and 2 but not 3 can bind to dsRNA but domain 3 is required for the activation of PKR by PACT (Peters and others 2001; Huang and others 2002). PACT is 60% similar to another mammalian dsRNA binding protein, TAR RNA binding protein (TRBP), and 69% similar to the *Xenopus leavis* dsRNA binding protein (Xlrbpa) (Ito and others 1999; Gupta and others 2003;). Despite the amino acid sequence similarity between these proteins, TRBP is an inhibitor of PKR activation in contrast to PACT. Interestingly, swapping dsRBM 3 of PACT and TRBP can revert their effects on PKR,

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indicating that the effect of these proteins on PKR activation is dictated by their dsRBM 3 (Gupta and others 2003).

To address the physiological role of PACT we have recently generated PACT knockout (KO) mice. In contrast to PKR KO mice, which develop normally (Yang and others 1995; Abraham and others 1999), PACT KO mice have developmental defects, leading to small size, defective ear development, and reduced fertility (Rowe and others 2006) (data not shown). In this study, we used cells derived from PACT KO mice to address the contribution of PACT in the activation of PKR in response to stress and inflammatory stimuli. Surprisingly, the absence of PACT had no effects on several of the pathways in which experiments in cell cultures and biochemical analyses suggested it played a role.

Materials and Methods

Cells, viruses, and reagents

Mouse embryo fibroblasts (MEFs) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Wildtype (WT) and PACT KO primary MEF lines were generated from littermate embryos resulting from matings of PACT heterozygous mice that had been backcrossed to the C57Bl/6 background for at least 10 generations and genotyped by Southern blot, as previously described (Rowe and others 2006). Viral stocks for VSV Indiana strain and EMCV were prepared in VERO and L929 cells, respectively, as described (Marques and others 2005). Sendai virus Cantell strain was purchased from Charles Rivers laboratories (Wilmington, MA, USA). Antibodies against interferon regulatory factor (IRF)-1, signal transducer and activator of transcription (STAT-1), total eukaryotic translation initiation factor (eIF)2α, and PKR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-Actin was obtained from Sigma (St. Louis, MO, USA), PARP and Phospho-eIF2α were from Cell Signaling (Beverly, MA, USA), mouse IRF-3 was from Zymed laboratories (South San Francisco, CA, USA), and GAPDH was from Chemicon International (Temecula, CA, USA). Antibodies against p54 were described in other studies (Terenzi and others 2005). Rabbit polyclonal antibodies against PACT were raised against full-length recombinant human PACT by the Hybridoma Core facility at the Cleveland Clinic Foundation. Thapsigargin, polyriboinosinic-ribocytidylic acid (I:C) and LPS from *Escherichia coli* 0111:B4 were purchased from Sigma (St. Louis, MO, USA). FuGENE reagent was purchased from Roche Diagnostics (Indianapolis, IN, USA).

Bone marrow–derived macrophages preparation and treatment

PACT KO mice have been previously described (Rowe and others 2006). Bone marrow–derived macrophages (BMDM) were derived from bone marrow by *in vitro* differentiation for 6 days in 10% FCS RPMI containing 20% L-cell conditioned medium and 50 μM β-mercaptoethanol. For the initial 24 h, cells were differentiated on tissue-culture–treated culture dishes to facilitate cell adhesion at a density of 10^6 cells/mL. For the final 5 days of culture, cells were grown on bacteriological plates at a density of 2×10^5 cells/mL. For stimulation, cells were plated at 5×10^4 cells/well in 200 μL/well of 10% FCS RPMI containing 10% L-cell conditioned medium and 50 mM β-mercaptoethanol in a 96-well plate. Supernatants were harvested 24 h after stimulation and stored at –20°C before enzyme-linked immunosorbent assay (ELISA).

Poly(I:C) transfections

Poly(I:C) was transfected using FuGENE reagent (Roche Applied Science, Mannheim, Germany) in all experiments according to protocols provided by the manufacturer. Briefly, 2 μg of poly(I:C) per 3 μL of FuGENE were incubated in 100 μL of serum-free DMEM for 15 to 30 min before being added to the supernatant of cells containing 10% FBS.

Western blot

Western blots were performed as described elsewhere (Marques and others 2005). Briefly, cells were lysed in 50 mM Tris buffer, pH 7.4, containing 150 mM of NaCl, 50 mM of NaF, 10 mM of β-glycerophosphate, 1% Triton X-100, 0.1 mM of EDTA, and 10% glycerol and protease/phosphatase inhibitors. The samples were kept on ice for 10 min and were vortexed and centrifuged for 15 min at 16,000 ×g; the supernatant was collected in a new tube and protein concentrations were determined using the Protein assay kit from Bio-Rad (Hercules, CA, USA). Total protein, 30 μg, was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon™-PSQ membranes (Millipore Corp., Belford, MA, USA), and probed with the indicated antibodies.

Immunoprecipitation

For immunoprecipitations, cells were lysed in Tris/NaCl lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100). Total protein, 1 mg, was incubated with a polyclonal antibody against PACT for 16 h at 4°C. The immunocomplex was precipitated using protein-G sepharose beads (Amersham Biosciences, Piscataway, NJ, USA). The beads were washed six times with Tris-buffered saline (50 mM Tris HCl, pH 7.4, 150 mM NaCl) and the immunoprecipitate was separated on SDS-PAGE before proceeding to the Western blot with the appropriate antibodies.

Caspases 3/7 activity

Cells were grown in black wall 96-well plates with a transparent bottom and treated as indicated. All treatments were performed in biological triplicates, and the results shown represent the average of the three independent wells. Caspase activity was measured directly from the 96-well plate using the Apo-ONE™ Homogeneous Caspase-3/7 assay according to protocols provided by the manufacturer (Promega, Madison, WI, USA).

Cell viability determination

To determine the viability of adherent macrophages, supernatant was removed and cells were fixed with 100 μL/

well of 10% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ, USA) in PBS. Following incubation for 10 min at room temperature and washing with distilled water, cells were stained with 30 µL/well of 1% crystal violet solution (Sigma). Following incubation for 15 min at room temperature and extensive washing to remove excess dye, plates were left to dry before reading the absorbance at 490 nm with a Wallac Victor^2 plate reader (Turku, Finland). Media controls at 24 h were considered to be 100% viable. Absorbencies of media controls at 24 h were 100–110% of those obtained at 0 h.

Statistical analysis

Statistical analysis was performed using Prism version 4.0 Software (Graphpad Software Inc., San Diego, CA, USA) or Microsoft Excel 2003 (Microsoft, Redmond, WA, USA).

Results

PACT associates with PKR

Based on previous data from our laboratory and others implicating PACT in stress-induced pathways (Patel and others 2000; Ruvolo and others 2001; Bennett and others 2004, 2006; Chen and others 2006) and PKR activation (Patel and Sen 1998; Peters and others 2002), the potential interaction of PACT and PKR was investigated in resting cells. The results showed that PKR co-immunoprecipitated with PACT in WT MEFs (Fig. 1). The specificity of the interaction was confirmed by the absence of PKR in immunoprecipitates from PACT KO MEFs using the anti-PACT polyclonal antibody or from WT cells using the preimmune serum (Fig. 1 and data not shown).

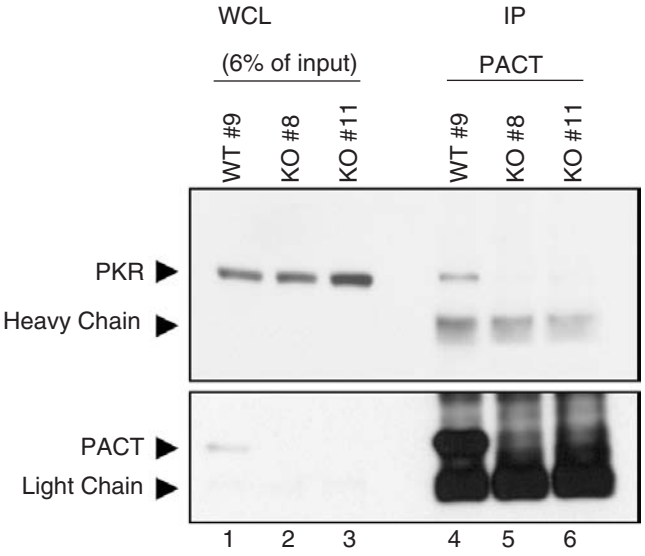

FIG. 1. PACT and PKR can associate in a complex, Total protein extracts from WT and PACT KO MEFs were subjected to immunoprecipitation (IP) using a polyclonal antibody against PACT and analyzed by Western blotting with the indicated antibodies. WCL indicates whole cell lysate.

PACT is not involved in the activation of PKR nor IRF-3-mediated gene induction in response to dsRNA

The dsRBM motifs present in PACT suggest a role in innate recognition of dsRNA. Thus different responses to dsRNA in WT and PACT KO MEFs were compared. Activation of PKR leads to phosphorylation of the eIF2α [reviewed in Williams (2001)]. Upon transfection of poly(I:C), a synthetic analog of dsRNA, we observed similar eIF2α phosphorylation in both WT and PACT KO MEFs (Fig. 2A). This is in accord with the established role of PKR in responding to intracellular dsRNA and the fact that PACT has been described as the protein activator of PKR in the absence of dsRNA (Patel and Sen 1998; Peters and others 2001).

PKR KO mice have defective induction of IRF-1 and consequently IRF-1–mediated gene induction (Kumar and others 1997). Thus we analyzed IRF-1 induction in MEFs treated with poly(I:C) and observed normal or even increased induction in PACT KO cells compared to WT (Fig. 2B).

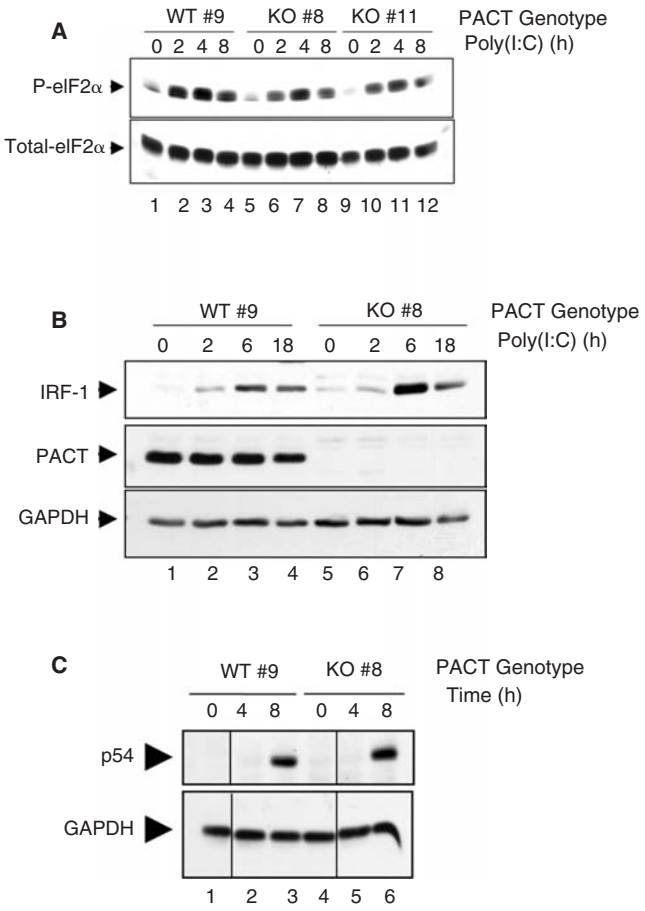

FIG. 2. PACT is not required for the activation of dsRNA-signaling pathways. (**A–C**) WT and PACT KO MEFs were transfected with 8 µg/mL of poly(I:C) for the indicated times, and total protein extracts were prepared and analyzed by Western blot with the indicated antibodies.

dsRNA is also recognized by the RNA helicases retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (Mda-5) that trigger a transcriptional response by activating the transcription factors IRF-3 and nuclear factor κB (NF-κB) [reviewed by Kawai and Akira (2006)]. *ISG54*, an IRF-3 target gene that encodes p54, is highly induced in response to dsRNA treatment and provides a good measure of IRF-3 activation (Terenzi and others 2005). Accordingly, the induction of *ISG54* was analyzed in response to poly(I:C) treatment but no differences were observed in p54 accumulation between WT and PACT KO MEFs (Fig. 2C), suggesting that IRF-3 induction in response to dsRNA is not impaired in PACT KO MEFs. Studies of induction of NF-κB–induced genes including IL-6, IL-12, and TNFα in WT and PACT KO BMDM showed similar induction of these genes in both cell types, suggesting that NF-κB activation is not defective when PACT is absent in these cells (data not shown).

PACT is not required for apoptosis in response to dsRNA, LPS, and serum withdrawal

PACT has also been implicated in response to different stress stimuli, many of which result in cell death (Patel and others 2000; Bennett and others 2006). Thus, we investigated the role of PACT in apoptosis induced by dsRNA, LPS, serum deprivation, and growth factor withdrawal. WT and PACT KO MEFs showed the same levels of apoptosis induced by dsRNA as indicated by caspase 3 activation (Fig. 3A). WT and PACT KO MEFs also showed the same levels of apoptosis induced by serum deprivation as indicated by poly-ADP ribose polymerase (PARP) cleavage (Fig. 3B). Accordingly, serum deprivation–induced PKR activation to similar levels in WT and PACT KO MEFs (Fig. 3B), as indicated by a band shift due to phosphorylation (Marques and others 2005). To confirm these results in a more physiologically relevant system, the effects of poly(I:C), LPS, or withdrawal of macrophage-colony stimulating factor (MCSF) in BMDM from WT and PACT-deficient mice were measured. Cell viability was decreased by poly(I:C) and more dramatically in response to MCSF withdrawal, but not in response to LPS (Fig. 3C). Importantly, there were no significant differences in cell viability between WT and PACT KO BMDM (Fig. 3C).

Role of PACT in ER stress

ER stress triggers a coordinated response involving diverse signaling pathways that includes translation inhibition, which allows cells to recover from the initial insult (Jiang and others 2004). PACT has been implicated in translation inhibition induced by thapsigargin, a classical inducer of ER stress (Ito and others 1999). Interestingly, phosphorylation of eIF2α induced by ER stress is dependent on the kinases, PKR-like ER kinase (PERK), and general control nonderepressible-2 (GCN2), but not PKR (Jiang and others 2004; Silva and others 2007). Consistent with this, similar levels of eIF2α phosphorylation were observed in response to thapsigargin treatment in WT, PKR KO, and PACT KO MEFs (Fig. 4 and data not shown).

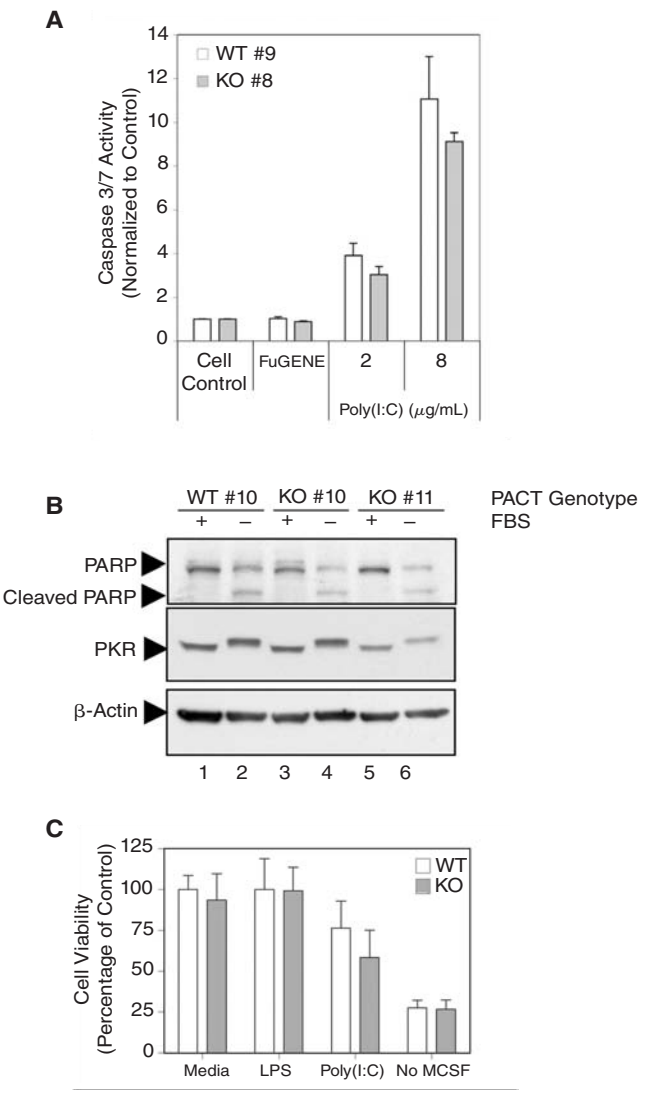

FIG. 3. PACT deficiency does not affect apoptosis in response to dsRNA or serum deprivation. (**A**) WT and PACT KO MEFs were transfected with either 2 or 8 μg/mL of poly(I:C), and caspase 3/7 activity was determined after 18 h. Data are presented as mean ± SD. The *p*-values ranged between 0.14 and 0.42 when caspase 3/7 activity in wildtype and PACT KO cells was compared by *t*-test for each stimulation condition. (**B**) WT and PACT KO MEFs were subjected to serum deprivation for 18 h, and total protein extracts were prepared and analyzed by Western blot. (**C**) BMDM from WT and PACT KO mice were treated with 100 μg/mL of poly(I:C), 100 ng/mL of LPS, or deprived of MCSF for 24 h and cell viability was determined. Data are presented as mean ± SD. The *p*-values ranged between 0.0683 and 0.330 when viability of wildtype and PACT KO cells was compared by *t*-test for each stimulation condition.

PACT is not required for expression of IRF-1 induced by TNF and IFN-γ

Induction of IRF-1 by TNF and IFN-γ with consequent induction of cell death has also been suggested to be

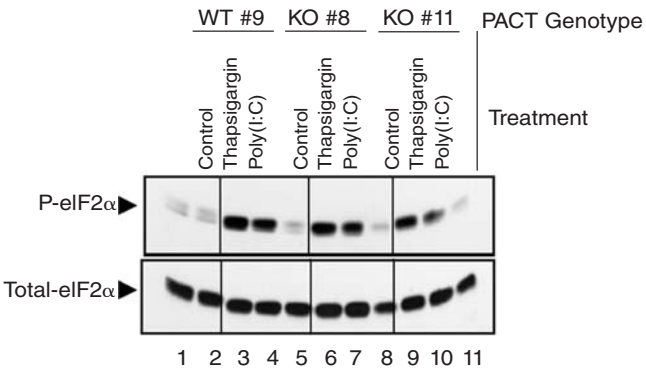

FIG. 4. PACT deficiency does not affect responses to ER stress. WT and PACT KO MEFs were transfected with 8 μg/mL of poly(I:C) for 6 h or treated with 500 nM of thapsigargin for 1 h, and total protein extracts were prepared and analyzed by Western blot.

dependent on PACT (Bennett and others 2006). However, TNF and IFN-γ, alone or in combination, induced similar levels of IRF-1 in WT and PACT KO MEFs (Fig. 5). In addition, no differences in cell viability were observed between WT and PACT KO BMDM following TNF and IFN-γ treatment (data not shown). As NFκB is required for IRF-1 induction by TNFα and IFN-γ (Ohmori and others 1997), this also implies that NF-κB activation is not impaired in PACT KO MEFs.

PACT is not involved in antiviral defense against different RNA viruses: EMCV, VSV, and Sendai virus

PACT was suggested to also play a role in antiviral defense against VSV and to enhance IFN induction in response to Newcastle disease virus (Iwamura and others 2001; Bennett and others 2006). Thus, we analyzed the accumulation of p54 and STAT-1 induced by Sendai virus infection and observed no differences between WT and PACT KO cells (Fig. 6A). To investigate a more general role of PACT in antiviral defense, we infected WT and PACT KO MEFs with VSV and EMCV and compared to virus yield obtained from these cells. Notably, we observed no significant differences in EMCV or VSV yields obtained from WT and PACT KO cells (Fig. 6B).

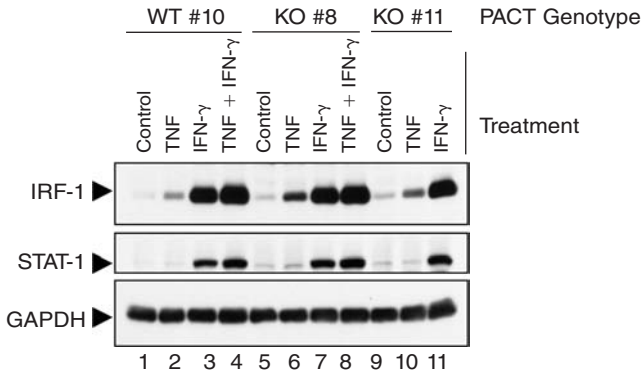

FIG. 5. PACT is not required for expression of IRF-1 induced by TNF and IFN-γ. WT and PACT KO MEFs were treated with 100 ng/mL of TNF or 1000 U/mL of IFN-γ for 6 h, total protein extracts were prepared and analyzed by Western blot.

Discussion

Our results indicate that PACT has no apparent role in mediating PKR activation in response to different stress stimuli in MEFs. In contrast, previously published results from our group and others have indicated that PACT has an important role in mediating these responses in other cell types (Patel and others 2000; Peters and others 2001; Ruvolo and others 2001; Bennett and others 2004; Bennett and others 2006; Chen and others 2006). The basis for these discrepancies remains unclear. However, it is tempting to speculate that differences in the systems utilized may account for contradictory conclusions. The majority of published work has relied on overexpression studies that might not reflect the physiological function of PACT. Overexpression of full-length or truncated PACT may result in gain of function phenotypes and explain the discrepancy with previous results (Patel and Sen 1998; Peters and others 2001). It is more difficult to account for the results of more recent work using RNAi-mediated knockdown to study the effect of loss of PACT in different pathways (Bennett and others 2006). The results in this study were very different from our observations using PACT KO cells. One possible explanation is that RNAi did not completely ablate PACT expression in the system described in the study of Bennett and others (2006), with the potential for low levels of residual PACT to confound results. There are also still many nonspecific effects associated with the use of RNAi (Marques and Williams 2005; Pei and Tuschl 2006) that are not completely understood and may have altered the signaling pathways being studied. Finally, PACT has also been implicated in the RNAi pathway (Lee and others 2006; Kok and others 2007). While we have not yet seen any differences in the RNAi-mediated knockdown of reporter genes or endogenous genes in PACT KO cells (data not shown) this may also have implications for studies involving RNAi-mediated knockdown of PACT.

The PACT KO mice used to generate cells used in this study carried a disruption of domain 3 of the *Pact* gene, and it remains possible that a truncated protein containing dsRBMs 1 and 2 could be produced in these cells. However, a truncated form of PACT has not been detected (Rowe and others 2006) and so should not be modulating signaling in cells used in this study or contributing to the discrepancies observed between our current results and data from other experimental systems.

Taken together, our results indicate that the major physiological role of PACT is different from the predicted functions from previous work. Nevertheless, as PACT expression message level varies between tissues (Fasciano and others 2007), it is possible that PACT has cell-type or tissue-specific functions or different functions at different times during development that depend on high endogenous levels of PACT and/or the

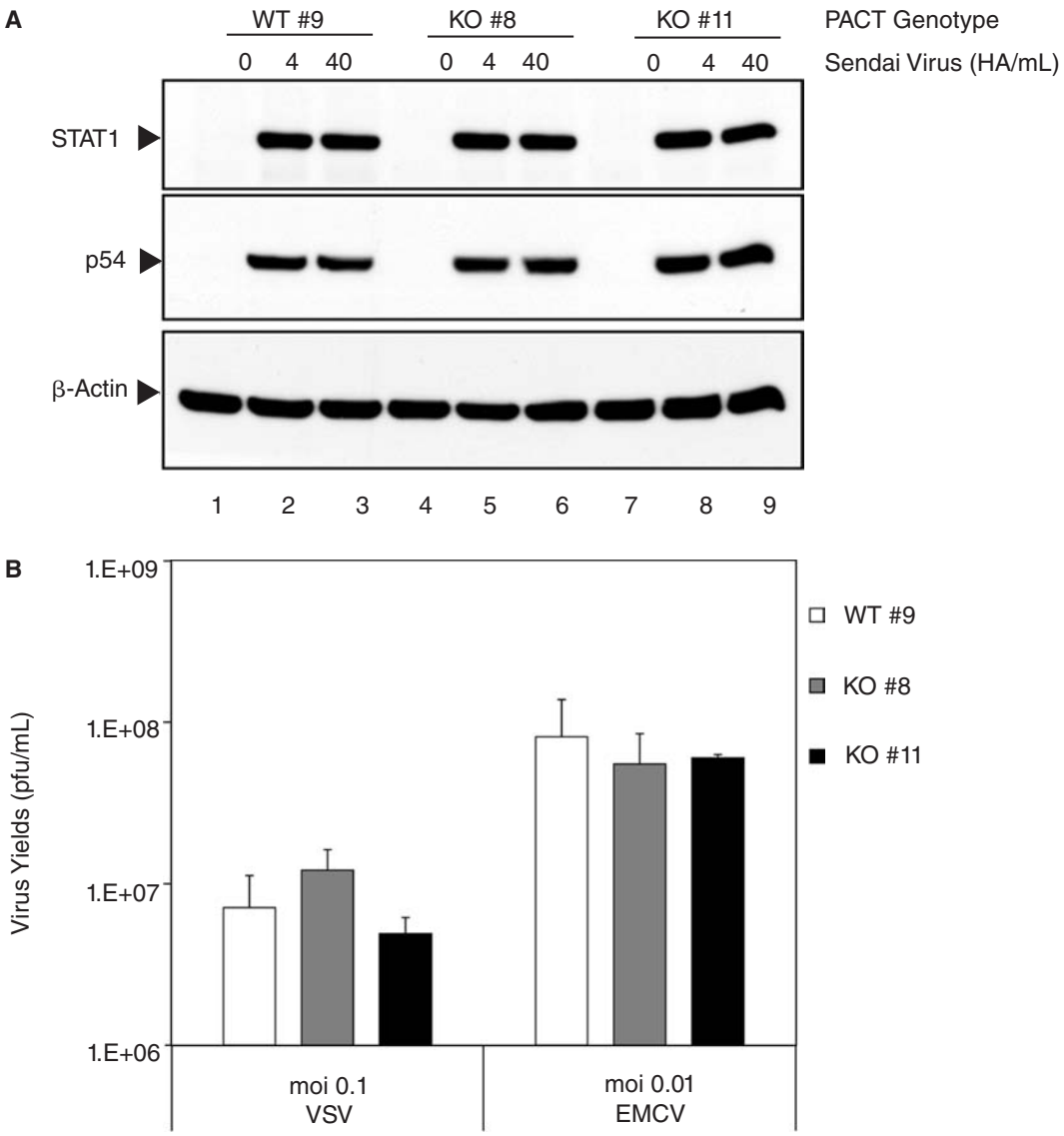

FIG. 6. PACT is not required for antiviral defense against VSV, EMCV, and Sendai virus. (**A**) WT and PACT KO MEFs were infected with Sendai virus at the indicated multiplicity of infection (moi) for 16 h, and total protein extracts were prepared and analyzed by Western blot. (**B**) WT and PACT KO MEFs were infected at the indicated moi with EMCV or VSV for 24 h and virus yields were determined in CV1 cells. Data are presented as mean ± SD. The *p*-values ranged between 0.10 and 0.41 when virus yield was compared between wildtype and either PACT KO #8 or PACT KO #11 cells by *t*-test for the infection conditions shown.

expression of protein partners. A requirement for high levels of PACT expression in specific tissues or at particular times of development may account for the developmental defects observed in PACT KO mice in spite of the lack of signaling defects we observed in PACT KO MEFs and macrophages. Alternatively, it is possible that TRBP can compensate for the absence of PACT under some circumstances, which would mask the phenotype of PACT KO mice. However, it is important to point out that PACT and TRBP have opposing roles to activating PKR (Gupta and others 2003). It is clear that PACT can interact and regulate PKR activation in different *in vitro* and cell culture studies. However, we have not been able to identify conditions where the absence of PACT has an effect on PKR activation. We are currently characterizing PACT KO mice and investigating pathways where the phenotype dictates the PACT that may be required.

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Contribution: J.T.M and C.L.W performed experiments. J.T.M., C.L.W., and G.A.P. analyzed results. J.T.M and C.L.W. drafted the manuscript. G.C.S. and B.R.G.W designed the research. G.A.P., G.C.S., and B.R.G.W. reviewed manuscript drafts.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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