PACT, a protein activator of the interferon-induced protein kinase, PKR

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PKR, a latent protein kinase, mediates the antiviral actions of interferon. It is also involved in cellular signal transduction, apoptosis, growth regulation and differentiation. Although in virus-infected cells, viral double-stranded (ds) RNA can serve as a PKR activator, cellular activators have remained obscure. Here, we report the cloning of PACT, a cellular protein activator of PKR. PACT heterodimerized with PKR and activated it in vitro in the absence of dsRNA. In mammalian cells, overexpression of PACT caused PKR activation and, in yeast, co-expression of PACT enhanced the anti-growth effect of PKR. Thus, PACT has the hallmarks of a direct activator of PKR.

Keywords: double-stranded RNA/heterodimer/interferon/ PKR/protein kinase

Introduction

The interferon (IFN)-induced, double-stranded (ds) RNA-activated protein kinase (PKR), is a key mediator of the antiviral and antiproliferative effects of IFN (Hovanesian, 1989; Sen and Ransohoﬀ, 1993; Williams, 1995; Clemens and Elia, 1997). PKR is present at low constitutive levels in cells and its expression can be induced by treatment with IFN. The most well known activator of PKR is dsRNA, but other polyanionic agents such as heparin have also been shown to activate it in vitro (Galabru and Hovanesian, 1987; Hovanesian and Galabru, 1987). The only known physiological substrate of PKR activity is the α subunit of the eukaryotic initiation factor eIF-2 (Lengyel, 1993; Samuel, 1993). Phosphorylation of eIF-2α on Ser51 by PKR leads to inhibition of protein synthesis (Colthurst et al., 1987; Hershey, 1991). eIF-2α phosphorylation leads to its increased affinity for eIF-2B, thus sequestering eIF-2B in an inactive complex with phosphorylated eIF-2 and GDP (Clemens et al., 1994; De Haro et al., 1996). As a result, eIF-2B is not available to catalyze nucleotide exchange on non-phosphorylated eIF-2. Because eIF-2B is present in cells at a lower molar concentration than eIF-2, phosphorylation of a small fraction of cellular eIF-2α can lead to a severe block in protein synthesis.

Upon viral infection of IFN-treated cells, PKR is activated by viral dsRNA which leads to an inhibition of viral and cellular protein synthesis (Samuel et al., 1984; Rice et al., 1985). Thus, PKR plays a major role in the antiviral activity of IFN. The importance of antiviral effects of PKR is manifested by the fact that many viruses employ a variety of mechanisms to counteract the actions of PKR (Sonenberg, 1990; Katze, 1992). Although the majority of research on PKR has focused on its participation in the inhibitory action of IFN on viral infection, there is now substantial evidence for PKR’s role in uninfected cells as well (Proud, 1995). PKR has been implicated in several diverse cellular functions such as growth regulation, apoptosis, differentiation and signaling pathways (Proud, 1995; Williams, 1997). A potential role for PKR in cell growth regulation has been suggested by the fact that overexpression of human PKR inhibits cell proliferation in yeast (Chong et al., 1992), insect (Barber et al., 1992) and mammalian cells (Koromilas et al., 1992). On the other hand, expression of catalytically inactive mutants of PKR in NIH 3T3 cells results in tumorigenicity in nude mice (Koromilas et al., 1992; Meurs et al., 1993). This result has been ascribed to a transdominant inhibitory effect of mutant enzyme on the endogenous wild-type PKR, resulting in derepression of growth. It is relevant to the proposed role of PKR in growth regulation that oncogenic Ras protein has been reported to induce an inhibitor of PKR activation (Mundschau and Faller, 1992, 1994). Surprisingly, mice devoid of functional PKR develop normally and are free of any tumors (Yang et al., 1995), suggesting that a cellular system, absent in NIH 3T3 cells, may be able to replace PKR functionally in the PKR null mice. Several studies have shown the involvement of PKR in cellular apoptosis (Yeung et al., 1996; Kibler et al., 1997). Overexpression of PKR in HeLa cells leads to apoptosis (Lee and Esteban, 1994), and recent studies with mouse embryonic fibroblasts derived from the PKR null mice have also shown its involvement in dsRNA- and lipopolysaccharide (LPS)-induced apoptosis (Der et al., 1997).

PKR has also been implicated in the onset of differentiation. Activation of PKR has been shown to be an important regulatory signal in controlling growth arrest of mouse 3T3-F442A fibroblasts and subsequent differentiation to adipocytes (Judware and Petryshyn, 1991, 1992; Petryshyn et al., 1997). However, the identity of an activator of PKR under differentiation permissive conditions has remained unknown. A more recent observation also correlates the levels of active PKR with the degree of cellular differentiation. It was observed that in the myogenic line L8, transforming growth factor-β (TGF-β) and EGTA reduced both the PKR activity and the level of muscle-specific proteins (Salzberg et al., 1995).

PKR participates in transcriptional signaling pathways used by dsRNA and IFN-γ: in PKR null cells, dsRNA fails to activate NFκB and IRF-1, and the IFN-γ responses of these cells are also impaired (Maran et al., 1994; Yang et al., 1995; Kumar et al., 1997; Wong et al., 1997). Treatment of cells with IFN-γ has been shown to result
in phosphorylation and activation of PKR (Kumar et al., 1997). However, no cellular activator of PKR in response to IFN-γ in the absence of dsRNA has been identified thus far. In addition, PKR has also been implicated in platelet-derived growth factor (PDGF) (Mundschau and Faller, 1995) and interleukin-3 (IL-3) (Ito et al., 1994) signaling.

The dsRNA-binding domain (DRBD) of PKR lies near its N-terminus (Katze et al., 1991; Feng et al., 1992; Green and Mathews, 1992; McCormack et al., 1992; Patel and Sen, 1992) and it contains two dsRNA-binding motifs conserved in a large family of dsRNA-binding proteins (St. Johnston et al., 1992). Deletion or specific substitution mutations in this region result in the loss of the dsRNA binding and activation (McCormack et al., 1994; Patel et al., 1994, 1996; Green et al., 1995; McMillan et al., 1995). These mutants can, however, be activated in vitro by heparin (Patel et al., 1994, 1996) and may be functionally active in yeast (Millan et al., 1995; Romano et al., 1995) and mammalian cells (Lee et al., 1994), suggesting the existence of additional cellular activators of PKR. The N-terminal domain of PKR also mediates its dsRNA-independent dimerization in vitro and in vivo (Cosentino et al., 1995; Patel et al., 1995; Romano et al., 1995; Wu and Kaufman, 1997). Similar dimerization domains are present in other dsRNA-binding proteins, and these proteins can heterodimerize with PKR through these domains (Cosentino et al., 1995; Benkirane et al., 1997). Since dsRNA is thought to be present only in virally infected cells, it is unclear what activates PKR in the absence of virus infection, and potential protein activators of PKR are likely to exist in cells. Here we report the identification of a new protein, PACT, which interacts with PKR through dimerization domains similar to PKR’s own and can activate PKR in vitro and in vivo in the absence of dsRNA.

Results

Cloning of PACT, a new dsRNA-binding protein that interacts with PKR

PACT was cloned by virtue of its interaction with PKR. K296R, an enzymatically inactive mutant of PKR (Katze et al., 1991), was used as the bait in a yeast two-hybrid screen of a human cDNA library, and a novel 1.8 kb PKR-interacting cDNA containing an open reading frame encoding a protein of 313 residues was isolated (Figure 1A). PACT was represented in the database only as several expressed sequence tags, and its sequence contains three motifs (Figure 1A, underlined residues) similar to the dimerization motifs present in PKR and other dsRNA-binding proteins. A comparison of the first two motifs of PACT with the first motif of PKR and two motifs of TAR RNA-binding protein (TRBP) (St. Johnston et al., 1992), another human protein of this family, revealed strong sequence conservation (Figure 1B). Primer extension analysis of cellular mRNA confirmed that the clone represented a full-length cDNA (data not shown), and Northern analysis showed a corresponding mRNA of 2.0 kb. PACT mRNA was expressed at varying levels in all of the cell lines tested (Figure 1C). Since PKR is an IFN-inducible protein, it was of interest to investigate if either IFN or dsRNA treatment had any effect on PACT mRNA levels. To test this, we have used the human glioblastoma cell line, GRE, in which the IFN genes have been deleted. This makes it suitable for studying the dsRNA-mediated transcriptional induction because these cells do not induce IFN production upon treatment with dsRNA. The transcriptional induction of PACT was assessed by an RNase protection assay. As represented in Figure 1C, PACT mRNA levels were the same in untreated cells as in cells treated with IFN-β, -γ or dsRNA. As expected, the 561 mRNA was induced both by IFN-β and dsRNA treatments, and the 561 mRNA was induced by treatment with IFN-γ (Leonard and Sen, 1997).

PACT interacts with PKR through its dsRNA-binding motifs

The interaction of PACT with PKR was confirmed by co-immunoprecipitation of the in vitro translated products. 35S-Labeled, flag epitope-tagged PACT protein was synthesized by in vitro translation (Figure 2A, lane 1). This protein could be immunoprecipitated using anti-flag monoclonal antibody (mAb)–agarose (Figure 2A, lane 2), but not by agarose alone (lane 3). The specificity of this immunoprecipitation is shown by the fact that PKR protein, similarly synthesized, was not immunoprecipitated either with the anti-flag mAb-agarose or with agarose alone (lanes 5 and 6). Since flag-PACT could be immunoprecipitated specifically, we then assayed for PKR’s interaction with PACT by co-immunoprecipitation and mapped the PACT-interacting domain within PKR. PKR could be co-immunoprecipitated with flag epitope-tagged PACT (Figure 2B, lane 5). A similar interaction was also observed between DRBD, the dimerization domain of PKR, and PACT (lane 6). A170, the C-terminal half of PKR (residues 171–551), did not co-immunoprecipitate with PACT (lane 7), thereby indicating that PACT interacts with PKR through its DRBD. The specificity of these co-immunoprecipitations was confirmed by the fact that another dsRNA-binding protein, the 69 kDa 2′−5′ oligoadenylate synthetase (Marie et al., 1997), did not co-immunoprecipitate with PKR. We further checked if when a fixed amount of PACT protein was mixed with increasing amounts of PKR protein, the same stoichiometric ratio was maintained in the co-immunoprecipitations. Since we were using flag-PACT to pull down untagged PKR, we varied the amount of PKR protein, the same stoichiometric ratio was maintained with PKR protein could be immunoprecipitated using anti-flag monoclonal antibody (mAb)–agarose (Figure 2A, lane 2), but not by agarose alone (lane 3). The specificity of this immunoprecipitation is shown by the fact that PKR protein, similarly synthesized, was not immunoprecipitated either with the anti-flag mAb-agarose or with agarose alone (lanes 5 and 6). Since flag-PACT could be immunoprecipitated specifically, we then assayed for PKR’s interaction with PACT by co-immunoprecipitation and mapped the PACT-interacting domain within PKR. PKR could be co-immunoprecipitated with flag epitope-tagged PACT (Figure 2B, lane 5). A similar interaction was also observed between DRBD, the dimerization domain of PKR, and PACT (lane 6). A170, the C-terminal half of PKR (residues 171–551), did not co-immunoprecipitate with PACT (lane 7), thereby indicating that PACT interacts with PKR through its DRBD. The specificity of these co-immunoprecipitations was confirmed by the fact that another dsRNA-binding protein, the 69 kDa 2′−5′ oligoadenylate synthetase (Marie et al., 1997), did not co-immunoprecipitate with PKR. We further checked if when a fixed amount of PACT protein was mixed with increasing amounts of PKR protein, the same stoichiometric ratio was maintained in the co-immunoprecipitations. Since we were using flag-PACT to pull down untagged PKR, we varied the amount of PKR in the mixture while keeping the amount of flag-PACT constant. As seen in Figure 2C (lanes 6–10), a fixed amount of PACT was able to co-immunoprecipitate increasing quantities of PKR from the mixtures containing increasing amounts of PKR. About 30% of the total PKR protein could be co-immunoprecipitated from the mixture at all ratios. The amount of PKR co-immunoprecipitated with PACT followed the same ratio at which these two proteins were mixed. The ratios at which these proteins were mixed (lanes 1–5) were 1, 2, 5, 10 and 30, and the ratios of the immunoprecipitated PKR (lanes 6–10) were 1, 1.8, 4.25, 9.4 and 21.3.

PACT binds dsRNA

Since the same motifs of PKR that mediate interaction with PACT also mediate dsRNA binding by PKR, we examined the ability of PACT to bind dsRNA (Figure 3). This was assayed by a poly(I)−poly(C)−agarose binding assay previously described for assaying the binding of PKR to dsRNA (Patel and Sen, 1992). The 35S-labeled...
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Fig. 1. (A) Protein sequence of PACT. The deduced amino acid sequence of PACT cDNA is represented. The conserved dsRNA-binding motifs are underlined. (B) Alignment of the three human protein sequences that contain the dsRNA-binding domain. Each of the three proteins has the full-length domain and C-terminal short domains. The shaded residues indicate amino acids that are identical in at least two motifs. The consensus sequence derived from St. Johnston et al. (1992) is shown below. Upper case letters indicate residues that occur in at least three motifs, and lower case letters indicate residues shared by two motifs or conservative substitutions. The sequences are as follows: PACT-1, human TAR-binding protein 10–74; PKR-1, human dsRNA-activated kinase 10–75; PACT-2, human dsRNA-activated kinase 10–75; PACT-2, PACT 127–192; TRBP-2, human TAR-binding protein 139–204. The similarities between PACT, TRBP and PKR were identified initially in searches of the GenBank and EMBL databases. The multiple motifs in these sequences subsequently were aligned using the MegAlign v3.05 (DNASTAR Inc.) program. The following nucleic acid sequences were used to obtain protein sequences: human TRBP, M60801; human PKR, M35663. (C) Northern blot analysis. A multiple human cancer cell line blot (Clontech) was hybridized to random primer-labeled PACT cDNA insert. Each lane contains ~2 μg of poly(A) RNA isolated from the following cell lines; lane 1, melanoma G361; lane 2, lung carcinoma A549; lane 3, colorectal adenocarcinoma SW 480; lane 4, Burkitt's lymphoma Raji; lane 5, T lymphoblastic leukemia CEM; lane 6, chronic myelogenous leukemia K-562; lane 7, HeLa S3; and lane 8, promyelocytic leukemia HL-60. (D) Ribonuclease protection assay. Total RNA isolated from untreated GRE cells, or GRE cells treated for 6 h with IFN-β (500 U/ml), IFN-γ (500 U/ml) or 100 μg/ml of poly(I)·poly(C) was analyzed by RNase protection assay. Thirty μg of total RNA was hybridized with 32P-labeled PACT (296 bases), IRF-1 (220 bases), 561 (195 bases) and γ-actin (140 bases) antisense RNA probes. Following RNase digestion, the protected RNA probes (PACT, 196 bases; IRF-1, 175 bases; 561, 142 bases; and γ-actin, 135 bases) were resolved in a 6% polyacrylamide, 8 M urea gel and visualized by autoradiography. The positions of the undigested probes are indicated on the left and the positions of the protected fragments are indicated on the right. The RNA samples are as indicated on the top of the lanes.

in vitro synthesized PACT and PKR proteins were assayed for binding to poly(I)·poly(C)–agarose at 50, 300 and 500 mM salt. As represented in lanes 2–4, PKR bound strongly, showing 54, 50 and 26% binding at 50, 300 and 500 mM salt concentrations, respectively. PACT showed similar binding characteristics, with 40, 31 and 6% binding at these salt concentrations (lanes 6–8). To ascertain the specificity of dsRNA binding by PACT, various nucleic acids were added in excess during the binding step as competitors (lanes 9–12). The binding was not affected by single-stranded RNA and DNA or DNA–RNA hybrid but was competed out by dsRNA. Thus, PACT was identified as a new dsRNA-binding protein that could heterodimerize directly with PKR.

PACT activates PKR in a dsRNA-independent manner

To determine if PACT could affect the activity of PKR, polyhistidine-tagged PACT was expressed in Escherichia coli and purified under denaturing conditions in the presence of 6 M urea, to prevent RNA binding to PACT. The validity of this procedure was confirmed by adding 32P-labeled dsRNA to the bacterial lysate before purification. Under native conditions, 90% of the added dsRNA co-purified with PACT, but no detectable radioactivity was associated with PACT that had been purified in the presence of 6 M urea. From the specific activity of the labeled dsRNA that was added to the bacterial lysate, it was calculated that 10⁻¹³ g of dsRNA may have been
associated with PACT after purification under denaturing conditions. This amount of dsRNA is far less (~10^4-fold less) than that required for activation of PKR. Denatured PACT, thus purified, was renatured by stepwise dialysis against decreasing concentrations of urea. The purified renatured PACT was homogeneous as confirmed by silver staining after SDS–PAGE analysis (data not shown). Purified PACT, devoid of any associated RNA, was incubated with immunopurified PKR, eIF-2 and [γ-32P]-ATP. In the absence of activators, neither PKR nor eIF-2 was phosphorylated (Figure 4A, lane 1), whereas the addition of two known activators, dsRNA and heparin, caused phosphorylation of both proteins (lanes 8 and 9). PACT could also activate PKR, causing the phosphorylation of both PKR and eIF-2. PKR activation by PACT was dose-dependent and biphasic: the maximum activation was observed between 400 pg and 4 ng of PACT, which was similar to that achieved by 1 μg/ml of dsRNA (lane 8). These results clearly demonstrated that PACT could activate PKR directly. To confirm further that PACT activates PKR without the involvement of any dsRNA, we assayed the ability of heat-inactivated and micrococcal nuclease-treated PACT to activate PKR (Figure 4B, lanes 3 and 4). Heat inactivation is expected to result in loss of activity due to protein denaturation, but the dsRNA-dependent activation will be unaffected by this treatment. As expected, the heat inactivation resulted in a loss of PACT’s ability to activate PKR (lane 3), while micrococcal nuclease treatment did not (lane 4). To rule out the possibility of any dsRNA remaining protected because it is bound by PACT, we assayed the effect of artificially added dsRNA during the micrococcal nuclease treatment. However, since PACT treated in such a way would activate PKR in the absence of dsRNA, we needed to inactivate PACT by heat after the nuclease treatment. Any protected dsDNA during this treatment could now be assayed for its ability to activate PKR. As seen in lane 5, the artificially added dsRNA was not protected during the nuclease treatment. As seen in lane 6, dsRNA’s ability to activate PKR is not destroyed by heat inactivation. When an activating amount of PACT was incubated in the kinase assay buffer with purified K296R, an inactive mutant of PKR, no phosphorylation of K296R protein was detected (data not shown). This result rules out the possibility of a bacterial kinase co-purifying with PACT and phosphorylating PKR.

We previously have identified two mutants of PKR, K150A and A158D, which can neither bind to nor be activated by dsRNA (Patel et al., 1996). These mutants retained their ability to interact with PACT in a co-immunoprecipitation assay (Figure 5A), thereby enabling us to test if they could be activated by PACT. As shown in Figure 5B, these mutants could be activated by PACT, confirming that activation of PKR by PACT can be achieved in the absence of dsRNA binding. Although these mutants are unable to bind dsRNA, it is conceivable that their interaction with PACT may complement this defect, thereby enabling them to bind dsRNA that may be associated with either protein. Since our preparation

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**Fig. 2.** (A) In vitro translation and immunoprecipitation of flag-tagged PACT. In vitro translated, 35S-labeled wild-type PKR and flag-PACT were synthesized using the TNT T7 coupled rabbit reticulocyte lysate system from Promega. Three μl of the reticulocyte lysate proteins were immunoprecipitated using anti-flag mAb–agarose (IBI). Total lanes represent proteins from 1 μl of the lysate, and the anti-flag mAb and agarsanes represent the proteins immunoprecipitated from 3 μl of the lysates. The positions of the molecular weight markers are indicated on the left. The positions of the two proteins are as indicated on the right. (B) PACT interacts with PKR through its DRBD. In vitro translated, 35S-labeled proteins were synthesized using the TNT T7 coupled rabbit reticulocyte lysate system from Promega. Flag-tagged PACT, wild-type PKR, Δ170, 2′−5′ oligoadenylate synthetase and DRBD were translated independently. Three μl of the reticulocyte lysate containing the flag-PACT were mixed with 3 μl of the lysates containing either wild-type PKR, Δ170, 2′−5′ oligoadenylate synthetase or DRBD. Flag-tagged PACT was immunoprecipitated from the reticulocyte lysate using the anti-flag mAb–agarose (IBI), and the proteins co-immunoprecipitating with it were analyzed by SDS–PAGE analysis followed by fluorography. Lanes 1–4 show all proteins in the mixture before immunoprecipitation, and lanes 5–8 represent immunoprecipitated proteins. Lanes 1 and 5, wild-type PKR and flag-PACT; lanes 2 and 6, flag-PACT and DRBD; lanes 3 and 7, flag-PACT and Δ170; lanes 4 and 8, flag-PACT and 2′−5′ oligoadenylate synthetase. The ‘total’ lanes contain 1 μl of the reticulocyte lysates each and the ‘CO-IP’ lanes contain 3 μl of the lysates each. The positions of the molecular weight markers are indicated on the right. The positions of different proteins are as indicated on the left. (C) Dose-dependent association between PKR and PACT. In vitro translated, 35S-labeled wild-type PKR and flag-PACT proteins were mixed in different proportions. Two μl of the reticulocyte lysate containing the flag-PACT was mixed with 0.1–3 μl of the lysate containing wild-type PKR. Flag-tagged PACT was immunoprecipitated from the reticulocyte lysate using the anti-flag mAb–agarose (IBI), and the wild-type PKR co-immunoprecipitating with it was analyzed by SDS–PAGE analysis followed by fluorography. The relevant bands were quantitated by phosphorimager analysis. Lane 1, 3 μl of flag-PACT + 0.1 μl of wild-type PKR; lane 2, 2 μl of flag-PACT + 0.2 μl of wild-type PKR; lane 3, 3 μl of flag-PACT + 0.5 μl of wild-type PKR; lane 4, 3 μl of flag-PACT + 1 μl of wild-type PKR; lane 5, 3 μl of flag-PACT + 3 μl of wild-type PKR. The positions of PKR and flag-PACT are as indicated on the right.
of purified PACT was devoid of any dsRNA, we needed to ascertain that no dsRNA was associated with the in vitro translated mutant PKR molecules. For this purpose, we added labeled dsRNA to the reticulocyte lysate containing the in vitro translated PKR mutant proteins and analyzed its association with these proteins after the immunoprecipitation. No significant counts were found to be associated with immunoprecipitates. Since neither of the protein partners (PACT and PKR mutants) in the kinase reaction mix had any dsRNA bound to them, it can be concluded that PACT activates K150A and A158D mutants by direct interaction.

**Overexpression of PACT in mammalian cells leads to activation of PKR, enhanced phosphorylation of eIF-2α and inhibition of translation**

To determine if PACT could activate PKR in vivo, an expression construct of flag-tagged PACT was transfected into human HT1080 cells and co-immunoprecipitation experiments performed. The endogenous cellular PKR could be co-immunoprecipitated with transfected PACT protein (Figure 6A). PKR precipitated from PACT-expressing cells was more active, as judged by autophosphorylation and eIF-2α phosphorylation (Figure 6B), than PKR from the vector control. As expected, PKR from the K296R-transfected cells was even less active than the vector control, since K296R inhibits PKR activity. Western blotting confirmed that all immunoprecipitates contained similar amounts of PKR.

The above results suggested that PACT could activate PKR in vivo. This was confirmed by examining the level of phosphorylation of PKR and eIF-2 in vivo in the transfected cells. The cells transfected with expression constructs of PACT and K296R were labeled with $^{35}$S-labeled PKR and eIF-2α were assayed by immunoprecipitation with monoclonal antibodies (Figure 7A). The assay was done as described in (A). The additions to the assay mixture were as follows: lane 1, no activator; lane 2, 4 ng of native PACT; lane 3, 4 ng of heat-inactivated (90°C for 15 min) PACT; lane 4, 4 ng of PACT treated with micrococcal nuclease; lane 5, 4 ng of PACT with 1 ng of poly(I)·poly(C), treated with micrococcal nuclease and then heat inactivated; and lane 6, 1 ng of dsRNA heated at 90°C for 15 min.

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enhanced translation of the reporter, and co-transfection of a PKR activator would be expected to result in inhibition of its translation. As shown previously by us and others (Patel et al., 1996; Wu and Kaufman, 1996), co-transfection with expression constructs of K296R and DRBD caused a marked increase in luciferase activity. In contrast, co-expression of PACT decreased the level of luciferase expression, indicating an enhanced activation of PKR (Figure 7B), further demonstrating that PACT expression leads to activation of PKR in mammalian cells.

**Co-expression of PKR and PACT gives an anti-growth phenotype in Saccharomyces cerevisiae**

Since expression of PKR in yeast causes pronounced inhibition of growth (Chong et al., 1992; Romano et al., 1995), we determined whether PACT could enhance this effect by activating PKR co-expressed in yeast. The yeast strain that we have used for this analysis is H17 (Bushman et al., 1993), which carries a mutation in the α subunit (GCN3) of the yeast eIF-2B. This mutation reduces the deleterious effect of eIF-2α phosphorylation on growth. It has been shown that phosphorylation of eIF-2 regulates translation by reducing eIF-2B function, and that GCN3 interacts directly with the α subunit of eIF-2 and mediates the increased binding of phosphorylated eIF-2 to eIF-2B, thereby preventing recycling of eIF-2 for the next round of translation initiation. Expression of wild-type PKR in H17 does not cause as pronounced an effect as in wild-type yeast strains (Dever et al., 1993), making it possible to assay for enhancement of the slow growth phenotype by PACT. PACT, K296R or DRBD were expressed alone or together with PKR in the H17 strain using a galactose-inducible promoter. In glucose-containing medium, as expected, all transfectants grew equally well, but, in the presence of galactose, wild-type PKR, but not PACT, caused a mild slow growth (data not shown). Upon co-expression of PKR and PACT, the slow growth phenotype was enhanced (compare 2 with 1 in Figure 8A, plate B). Wild-type PKR was needed for the action of PACT, and the K296R mutant could not substitute for it (compare 3 with 1). As reported previously, unlike PACT, DRBD inhibited the anti-growth effect of PKR (compare 4 with 1). A Western blot analysis confirmed that comparable levels of PKR, PACT and DRBD were expressed in the different transfected yeasts (Figure 8B). The growth rates of the different transfected yeast strains in liquid cultures were also determined (Figure 8C). It was seen that co-expression of PACT and PKR resulted in a significantly slower growth rate compared with cells expressing PKR alone. As seen with growth on solid medium, co-expression...
of DRBD with PKR resulted in reversal of the slow growth phenotype. Expression of PACT alone or K296R alone did not show any slow growth rate phenotype. These experiments demonstrated that PACT could activate PKR in yeast and enhance its anti-growth effects. We reasoned that the K150A and A158D mutants of PKR may not have a slow growth phenotype in yeast because they do not bind dsRNA. When assayed for their effect on yeast growth rate, however, it was seen that they were as inhibitory as wild-type PKR (Figure 8D). Similar results have been reported by other workers for the mutant K150P (Romano et al., 1995). Like wild-type PKR, our mutants showed an enhancement of their slow growth phenotype when PACT was co-expressed (Figure 8D), thereby confirming our in vitro activation results. A Western blot analysis was done to confirm that all proteins were expressed at comparable levels (Figure 8E).

**Discussion**

PACT is a new member of the family of dsRNA-binding proteins to which PKR belongs. These proteins are characterized by structural motifs (St. Johnston et al., 1992) which mediate their homo- (Patel et al., 1995; Romano et al., 1995; Ortega et al., 1996; Wu and Kaufman, 1997) and heterodimerization (Cosentino et al., 1995) and binding to dsRNA. PACT contains three such motifs without any other remarkable structural features. PACT clearly binds PKR and activates it by a dsRNA-independent mechanism. This was shown by the purification scheme, which precluded the co-purification of associated dsRNA. Moreover, treatment of the purified PACT with micrococcal nuclease, which degrades dsRNA, had no effect on its ability to activate PKR (Figure 4B). Genetic evidence for a dsRNA-independent activation of PKR by PACT was provided by the activation of two PKR dsRNA-binding mutants. The conclusion that PACT activates PKR directly, and not by recruiting dsRNA, is supported further by the observation that other dsRNA-binding proteins, such as the DRBD of PKR and TRBP that can also heterodimerize with PKR, do not activate PKR but rather inhibit the activation of PKR (Cosentino et al., 1995; Patel et al., 1995; Benkirane et al., 1997; Wu and Kaufman, 1997). dsRNA is known to activate PKR by promoting a conformational change of the protein (Carpick et al., 1997). Our data suggest that PACT, by binding to the same region of PKR as dsRNA, may induce a similar conformational change. The observed diminution of PKR activation by PACT at higher concentrations could be due to promotion of PACT homodimerization or due to two different PACT molecules binding to the two dimerization motifs of PKR under those conditions. Since PACT has more than one DRBD, it is also possible that PACT may bind to a PKR dimer, thus allowing one PKR molecule to trans-phosphorylate its partner. At high PACT concentrations, this is less likely to occur since, under these conditions, only one PKR molecule may be bound to each PACT molecule. Further studies such as determination of the exact subunit composition are likely to throw some light on the exact mechanism of activation.

The demonstration of PKR activation by PACT in mammalian cells in vivo relied on transient transfection and, thus, underestimates the extent of the effect because not all cells were transfected. However, repeated attempts to isolate permanent overexpressors of PACT in several mammalian cell lines failed (data not shown), presumably because of its anti-cellular effects. The small, but consistent, enhanced phosphorylation of eIF-2α observed in the PACT-transfected cells is significant physiologically because phosphorylation of only 30% of the cellular pool of eIF-2α has been shown to cause a severe inhibition of protein synthesis (Hershey, 1989). Several other proteins which belong to the same family of dsRNA-binding proteins have been cloned. Two of the mammalian proteins in this family, human TRBP (Park et al., 1994) and mouse Prbp (Lee et al., 1996), have been shown to be involved in translational regulation. The TRBP gene was cloned from a HeLa cell expression library based on its ability to bind to the HIV-1 TAR RNA in vitro (Gatignol et al., 1991) and then was cloned again based on its ability to bind the HIV-1 Rev-responsive element (Park et al., 1994). The function of TRBP in non-infected cells is unknown, but it has been suggested to regulate translation by functioning as an inhibitor of PKR in a dsRNA-independent manner. Overexpression of TRBP in mouse cells leads to a transformed phenotype, which is consistent with its
Fig. 8. (A) PACT enhances the slow growth phenotype of PKR in yeast. Growth of transformed yeast strains containing (1) GAL1-wild-type PKR/pRS315 + pYES2, (2) GAL1-wild-type PKR/pRS315 + PACT/pYES2, (3) GAL1-K296R/pRS315 + PACT/pYES2 and (4) GAL1-wild-type PKR/pRS315 + DRBD/pYES2. Cells were grown for 2 days at 30°C on synthetic medium lacking uracil and leucine with 2% glucose (plate A) or 10% galactose and 1% raffinose (plate B) as sole carbon source. (B) Western blot analysis. A 100 μg aliquot of the total protein extracts from transformed yeast strains was analyzed by Western blot analysis with anti-PKR and anti-flag monoclonal antibodies. Lane 1, GAL1-wild-type PKR/pRS315 + pYES2; lane 2, GAL1-wild-type PKR/pRS315 + PACT/pYES2; lane 3, GAL1-K296R/pRS315 + PACT/pYES2; and lane 4, GAL1-wild-type PKR/pRS315 + DRBD/pYES2. (C) Growth rate analysis. The growth rate of the transformed yeast strains was analyzed in synthetic medium containing 10% galactose and 1% raffinose. At various time points, cell growth was monitored by measuring OD$_{600}$. ▲, GAL1-K296R/pRS315 + pYES2; ○, GAL1-PACT/pRS315; ●, GAL1-wild-type PKR/pRS315 + pYES2; ▼, GAL1-wild-type PKR/pRS315 + PACT/pYES2; ▲, GAL1-wild-type PKR/pRS315 + DRBD/pYES2; and ▼, GAL1-K296R/pRS315 + PACT/pYES2. (D) PACT enhances the slow growth phenotype of PKR's dsRNA-unresponsive mutants. The growth rate of the transformed yeast strains was analyzed in synthetic medium containing 10% galactose and 1% raffinose. At various time points, cell growth was monitored by measuring the OD$_{600}$. ▲, GAL1-K296R/pRS315 + pYES2; ○, GAL1-K150A/pRS315 + pYES2; ▼, GAL1-A158D/pRS315 + pYES2; ▼, GAL1-A158D/pRS315 + PACT/pYES2; ○, GAL1-A158D/pRS315 + PACT/pYES2; ▲, GAL1-K150A/pRS315 + PACT/pYES2; and ▼, GAL1-A158D/pRS315 + PACT/pYES2. (E) Western blot analysis. A 100 μg aliquot of the total protein extracts from transformed yeast strains was analyzed by Western blot analysis with anti-PKR and anti-flag monoclonal antibodies. Lane 1, GAL1-K296R/pRS315 + PACT/pYES2; lane 2, GAL1-K150A/pRS315 + pYES2; lane 3, GAL1-K150A/pRS315 + PACT/pYES2; lane 4, GAL1-A158D/pRS315 + PACT/pYES2; and lane 5, GAL1-A158D/pRS315 + PACT/pYES2.

inhibitory role towards PKR activity (Benkirane et al., 1997). Prbp, a mouse homolog of TRBP, was cloned from mouse testis based on its ability to bind the 3'-untranslated region of mouse protamine-1 mRNA. Prbp has been proposed to be a general translational inhibitor and was found to repress translation of protamine-1 mRNA in a non-specific manner. Thus, TRBP and PACT, in spite of sharing a high degree of homology, have different effects on PKR activity. Only PACT has the ability to activate PKR by direct protein-protein interaction. It should be noted that several mutants of PKR, that cannot bind to or be activated by dsRNA in vitro, still maintain their anti-
growth effects in yeast (Romano et al., 1995). Two examples are shown in Figure 8D. Many of these mutants have been shown to be activated in vitro by heparin, and it is conceivable that similar polyanionic compounds activate them in yeast. It is also possible that yeast contains a functional homolog of PACT that can activate PKR. The anti-growth effect of PACT required the presence of PKR. This effect was specific because other dsRNA-binding proteins such as DRBD had the opposite effect of neutralizing PKR’s anti-growth effect. Thus, PACT’s PKR. This effect was specific because other dsRNA-binding proteins were synthesized using the TNT T7 coupled reticulocyte system from Promega. The PKR and DRBD were translated alone or co-translated with flag-tagged PACT. Five μl of the in vitro translated 35S-labeled proteins were incubated with 20 μl of anti-flag mAb–agarose (IBI) in 200 μl of IP buffer (20 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 100 μM aprotinin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 20% glycerol, 1% Triton X-100) at 4°C for 30 min on a rotating wheel. The beads were washed in 500 μl of IP buffer four times and the washed beads were then boiled in 2× Laemmli buffer (150 mM Tris–HCl pH 6.8, 5% SDS, 5% β-mercaptoethanol, 20% glycerol) for 2 min and eluted proteins were analyzed by SDS-PAGE on a 12% gel. Fluorography was performed at –80°C with intensifying screens.

dsRNA binding assay

The in vitro translated, 35S-labeled PACT protein was synthesized using the TNT T7 coupled reticulocyte lysate system from Promega. The dsRNA-binding activity was measured by poly(I)poly(C)–agarose binding assay. The translation products (4 μl) diluted with 25 μl of binding buffer (20 mM Tris–HCl, pH 7.5, 0.3 M NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF, 0.5% NP-40, 10% glycerol) were mixed with 25 μl of poly(I)poly(C)–agarose (Pharmacia) beads and incubated at 30°C for 30 min with intermittent shaking. The beads were then washed with 500 μl of binding buffer four times. The proteins bound to beads after washing were analyzed by SDS-PAGE followed by fluorography.

Expression and purification of PACT from E.coli

The PACT protein coding region was subcloned into pET15b (Novagen) to generate PACT/pET15b. This results in the in-frame fusion of PACT sequence to the histidine tag. The expression host BL21(DE3) was transformed with PACT/pET15b. The bacteria were grown overnight in Luria broth and harvested at 5000 g for 10 min at 25°C. The cell pellet was suspended in 3-fold the initial culture volume of fresh LB, and isopropyl-β-D-thiogalactoside (IPTG) was added at a final concentration of 1 mM. The culture was incubated at 37°C with vigorous shaking for 2–3 h, at which point the cells were harvested. The cell pellet from 500 ml of culture typically was suspended in 10 ml of binding buffer (5 mM imidazole, 200 mM NaCl, 20 mM Tris–HCl pH 7.9, 0.1% NP-40) and sonicated at a high setting for five 30 s pulses on ice. The homogenate was centrifuged at 12 000 g for 30 min. The pellet was resuspended in 25 ml of the same buffer and pelleted again at 12 000 g for 30 min. The pellet was then resuspended in 10 ml of denaturing buffer (6 M urea, 5 mM imidazole, 200 mM NaCl, 20 mM Tris–HCl pH 7.9, 0.1% NP-40) and sonicated at a high setting for five 30 s pulses on ice. The homogenate was centrifuged at 12 000 g for 30 min. The supernatant was mixed with 2 ml of Ni-charged his-bind resin (Novagen). The mixture was incubated at 4°C for 30 min on a slow rotating shaker. After binding, the resin was washed four times with 50 ml each of denaturing buffer and six times with 50 ml each of wash buffer (6 M urea, 60 mM imidazole, 200 mM NaCl, 20 mM Tris–HCl pH 7.9, 0.1% NP-40). The washed resin was packed into a column and his-PACT was eluted with 25 ml of strip buffer (6 M urea, 100 mM EDTA, 200 mM NaCl, 20 mM Tris–HCl pH 7.5). The eluted protein was dialyzed against 2 l of buffer at 4°C in four steps of decreasing urea concentrations (4, 2, 1 and 0.5 M, and finally no urea). The refolded protein was then concentrated to 400 μg/ml using Centriprep concentrators.

Kinase assays

HeLa M cells were maintained in DMEM with 10% FCS. The cells were harvested when they were 70% confluent in ice-cold phosphate-buffered saline (PBS) and packed by centrifugation at 600 g for 5 min. They were lysed by addition of an equal volume of lysis buffer (20 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 50 mM KCl, 400 mM NaCl, 2 mM DTT, 1% Triton X-100, 100 μM aprotinin, 0.2 mM PMSF, 20% glycerol). The lysates were centrifuged at 10 000 g for 5 min and the supernatants were assayed for PKR activity. A 100 μg
 aliquot of total protein was immunoprecipitated using PKR monoclonal antibody (Ribogene) in the high salt buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 U/ml aprotinin, 0.02 mM PMSE, 20% glycerol, 1% Triton X-100) at 4°C for 30 min on a rotating wheel. Then 10 μl of protein A-Sepharose slurry was added and incubation was carried out for a further 1 h. The protein A-Sepharose beads were washed in 500 μl of high salt buffer four times and twice in activity buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM MgCl2, 2 mM MnCl2, 100 U/ml aprotinin, 0.1 mM PMSE, 5% glycerol). PKR assay was performed with PKR still attached to the beads in activity buffer containing 500 ng of purified elf-2, 0.1 mM ATP and 10 μCi of [γ-32P]ATP at 30°C for 10 min. One μg/ml of poly(I)·poly(C) or 10 U/ml of heparin was used as the standard activator for the enzyme. Purified PACT in amounts varying from 4 pg to 400 ng was added to test its effect on PKR activity. Micrococcal nuclease (Boehringer) treatment was done on 50 μg of purified PACT as suggested by the manufacturer. The treated PACT protein was then re-purified on the Ni-agarose column and was used in the kinase assays. Heat inactivation of PACT was done at 90°C for 15 min. Labeled proteins were analyzed by SDS-PAGE on a 12% gel. Autoradiography was performed at –80°C with intensifying screens.

Expression in mammalian cells and co-immunoprecipitation assay

HT 1080 cells were transfected in 100 mm culture dishes with 5 μg of pCB6 vector and flag-PACT/pCB6 DNA using the lipofectamine (Gibco-BRL) reagent. At 24 h post-transfection, cells were harvested and cell extracts were prepared; 100 μg of total cell extracts were used to immunoprecipitate flag-PACT with anti-flag mAb-agarose as described under In vitro interaction assay. The immunoprecipitates were then analyzed by a Western blot analysis with the anti-PKR and anti-flag polyclonal antibodies (Santa Cruz Biotech. Inc.). For the kinase assays from transfected cells, HT 1080 cells were transfected in 100 mm culture dishes with 5 μg of pCB6 vector, flag-PACT/pCB6 or K296R/pCB6 DNA. At 24 h post-transfection, cells were harvested and cell extracts were prepared; 100 μg of total cell extracts were used to immunoprecipitate PKR with monoclonal antibody. The immunoprecipitates were assayed for kinase activity as described under Kinase assays. No activator of PKR was added to these kinase assays.

In vivo phosphate labeling

HT 1080 cells were transfected in 100 mm culture dishes with 5 μg of pCB6 vector, flag-PACT/pCB6 or K296R/pCB6 DNA. At 24 h post-transfection, cells were metabolically labeled with 100 μCi/ml of [32P]orthophosphate (DuPont NEN, 8500 Ci/mmol) for 3 h in phosphate-free medium. Cell extracts were prepared as described under Kinase assay and were subjected to immunoprecipitation with monoclonal antibodies to PKR and elf-2α and analyzed by SDS-PAGE followed by autoradiography. Western blot analysis was performed on these extracts to ascertain that equal amounts of PKR and elf-2α were analyzed by phosphate labeling.

Translation inhibition assay

The effect of co-transfection of pCB6 vector, flag-PACT/pCB6, K296R/pCB6 and DRBD/pCB6 on the reporter pGL2-Control (Promega) in HT 1080 cells was measured as described before (Patel et al., 1996). HT 1080 cells were transfected in 6-well plates in triplicate with 200 ng of each of the two (expression construct and the reporter plasmid pGL2-Control from Promega) plasmid DNAs by the lipofectamine procedure. At 24 h after transfection, the cells were treated with 100 U/ml of IFN-β. Cells were harvested 48 h after transfection and assayed for luciferase activity after normalizing for the transfection efficiency by measuring the total protein.

Expression in yeast and growth analysis

Wild-type PKR and K296R coding regions were subcloned into the pYES2 vector (Invitrogen). The fragments which contained the Gal1 promoter–wild-type PKR or Gal1 promoter–K296R were then subcloned into pRS315 vector (Sikorski and Hieter, 1989). This gave us the galactose-inducible expression of wild-type PKR or K296R in a vector with a Gal1 promoter. The PACT and DBD coding regions were subcloned into the pYES2 vector to obtain their expression in yeast in a galactose-inducible manner. Expression plasmids were introduced into the yeast strain H17 (Matα, gen3-102, his3-52, leu2-3, 112) using the lithium acetate procedure (Chong et al., 1992). Transformed yeast strains were selected on synthetic medium containing 2% glucose and lacking the appropriate amino acids (either ura-, or ura- and leu-). The colonies were then streaked on synthetic medium plates lacking the appropriate amino acids and containing either 2% glucose or 1% raffinose and 10% galactose. The growth of yeast was monitored every day. For liquid-grown yeast strains, the transformed yeast strains were grown to an OD600 of <1.5 in synthetic medium containing 2% glucose lacking uracil and leucine. The cultures were then harvested and washed with synthetic medium containing 10% galactose and 1% raffinose. The cultures were then diluted to an OD600 of 0.4 in synthetic medium lacking uracil and leucine and containing 10% galactose and 1% raffinose. At various time points, cell growth was monitored by measuring the OD600.

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References


PACT: a protein activator of PKR


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