Phosphorylation of Specific Serine Residues in the PKR Activation Domain of PACT Is Essential for Its Ability to Mediate Apoptosis*

Received for publication, August 11, 2006, and in revised form, September 18, 2006. Published, JBC Papers in Press, September 18, 2006, DOI 10.1074/jbc.M607714200

Gregory A. Peters‡, Shoudong Li‡§, and Gaines C. Sen‡§

From the Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195 and the Graduate Program in Molecular Virology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Activation of the latent protein kinase, PKR, by extracellular stresses and triggering of resultant cellular apoptosis are mediated by the protein, PACT, which itself gets phosphorylated in stressed cells. We have analyzed the underlying biochemical mechanism by carrying out alanine-scanning mutagenesis of the PKR activation domain of PACT. Among the indispensable residues identified were two serine residues, whose phosphorylation was essential for the cellular actions of PKR. Two-dimensional gel analysis, Western analysis using phosphoamino acid-specific antiserum, and in vivo $^{32}$P labeling of PACT demonstrated that constitutive phosphorylation of one of the two residues, Ser$^{246}$, was required for stress-induced phosphorylation of the other, Ser$^{287}$. Substitution of either of them by threonine or aspartic acid, but not alanine, was tolerated. Substitution of both residues with the phosphoserine mimetic, aspartic acid, produced a mutant PACT whose cellular abundance is elevated by interferon (IFN) treatment of cells (1–3). PKR enzymatic activity is latent, and it needs to be activated by autophosphorylation. The classical activator of PKR is double-stranded (ds) RNA, a replicative intermediate for many viruses. The most studied cellular target of PKR is the translation initiation factor, eIF2α, whose phosphorylation results in an inhibition of protein synthesis. Although its antiviral activities are the most well known, PKR has been implicated in the transcriptional signal transduction pathways activated by specific cytokines, growth factors, dsRNA, and extracellular stresses (4). Optimal activation of p38 and c-Jun N-terminal kinase (JNK), proteins known as stress-activated protein kinases (SAPKs), as well as transcription factors such as NF-κB, IRF-1, p53, STAT1, ATF, STAT3, and AP-1, have each been shown to require PKR (5–11). PKR is also involved in a broad array of cellular processes such as cellular differentiation, apoptosis, cell growth, and oncogenic transformation (3).

dsRNA binding to PKR takes place at two dsRNA-binding motifs (dsRBM) present at the N terminus of the protein (12, 13), changing the conformation of PKR to an active state in which it can bind ATP (14, 15) and autophosphorylate (16). The same domain of PKR can also mediate dsRNA-independent protein-protein interactions with proteins containing similar domains (17). One such protein is PACT, whose binding to PKR leads to the activation of PKR in the absence of dsRNA (18, 19). Thus, PACT is a protein activator of PKR. PACT contains three identifiable domains of which domains 1 and 2 bind tightly to the N-terminal dimerization domain (DD) of PKR. On the other hand, domain 3, which, in vitro, can activate PKR by itself, binds, with low affinity, to a specific region in the kinase domain of PKR (20). We have recently identified the PACT binding motif (PBM) in this region of PKR (21). Moreover, we demonstrated intramolecular interaction between PBM and dsRBM2 of PKR. This interaction, as mediated by specific residues of the two partners, keeps PKR locked in the inactive conformation; its activation requires disruption of the interaction as a result of the binding of dsRNA to dsRBM2 or PACT domain 3 to PBM. As anticipated, point mutations of the residues of PKR, that mediate the intramolecular interaction, caused constitutive activation of the protein. In addition to its ability to activate PKR, PACT has other important physiological functions as well. This was most strikingly demonstrated by the developmental defects of Pact$^{-/-}$ mice that are not present in Krr$^{-/-}$ mice (22).

In cells, PACT-mediated activation of PKR leads to phosphorylation of the translation initiation factor eIF2 and cellular apoptosis. In order for PACT to activate PKR in vivo, it requires in addition to its domain 3, either of the other two domains (20). The function of the latter domain(s) is to either anchor the protein strongly to PKR or mediate PACT dimerization, so that the activation process can occur efficiently. In vivo, PKR is not activated by PACT, unless the cells are stressed. When cells are...
exposed to a variety of stresses such as, withdrawal of growth factors or treatment with a low dose of actinomycin D, arsenite, thapsigargin, or peroxide (20, 24, 25), PACT or its murine homolog RAX, is phosphorylated and associates with PKR with increased affinity (24, 25). In this study, we investigated further the mechanism of PACT-mediated activation of PKR in vivo. For this purpose, we have used an experimental system of expressing a variety of PACT mutants in human HT1080 cells, which express little endogenous PACT. They undergo apoptosis, when functional exogenous PACT is expressed and extra-cellular stress is applied to them. We identified two serine residues in PACT domain 3, whose phosphorylation was essential for the cellular actions of PACT. Our results indicate that constitutive phosphorylation of one of these residues was required for stress-induced phosphorylation of the other, leading to strong association of PACT with PKR and its activation.

**EXPERIMENTAL PROCEDURES**

**Reagents, Cells, and Antibodies**—Actinomycin D was obtained from Sigma. HT1080 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and penicillin/streptomycin. Transfections were done with Lipofectamine 2000 reagent (Invitrogen). Anti-FLAG monoclonal M2 antibody was from Sigma, anti-PKR monoclonal antibody was from Ribogene, anti-phospho-PKR (Thr451), anti-eIF2α, and anti-phospho-eIF2α were from Cell Signaling, anti-histidine antibody was from Santa Cruz Biotechnology, and anti-phosphothreonine antibody was from Biodesign International. PACT anti-domain 3 peptide antibody (770) (20, 26) was custom produced by Bio-Synthesis, Inc.

**Construction of PACT Mutants**—The generation of PACT mammalian expression constructs was described previously (20). Overlap extension PCR was used to construct all PACT mutants (27). PACTΔ1 was used as a template to construct each mutant used for mammalian transfection, with the exception of S18A, S18D, S246A, and S287A, which used full-length PACT as the template. PCR fragments containing the desired PACT mutant were ligated into restriction enzyme-digested pcDNA3. A FLAG epitope tag was added at the N-terminal coding end of all PACT constructs.

**Apoptosis Assays**—TUNEL: HT1080 cells growing on glass coverslips in 6-well dishes were cotransfected with pcDNA3-FLAG PACT or pcDNA3-FLAG PACT mutant. At 6 h after transfection, the cells were stressed by treatment with 40 μM of Lipofectamine 2000 and 50 ng/ml actinomycin D. Cells were fixed in 4% methanol-free formaldehyde 24 h after transfection. TdT-mediated dUTP nick-end labeling (TUNEL) assay using the Dead End Fluorometric TUNEL System (Promega) was performed using the manufacturer’s protocol. After washing, cells were stained with primary anti-FLAG antibody and secondary anti-mouse IgG Texas Red conjugate (Molecular Probes) as described (20). The cells were mounted on glass slides in Vectashield with DAPI (4’-6’-diamino-2-phenylindole) (Vector Laboratories), and examined under a fluorescence microscope. Quantitative Cell Survival Assay: HT1080 cells were cotransfected in 100-mm culture dishes with pcDNA3 vector or pcDNA3-PACT, and pEGFP-C1 in a 8:1 ratio using the Lipofectamine 2000 reagent (Invitrogen). At 6 h after transfection, the cells were stressed and allowed to undergo cell killing. At 48 h after transfection, the medium containing dead cells was removed, and the plate washed with phosphate-buffered saline. The population of cells remaining was collected, and 20,000 cells were monitored for GFP expression using a flow cytometer. The nonspecific background was 4–7% from the loss of stressed GFP-expressing cells transfected with vector, and has been subtracted from all values presented in the figures and tables. Each value presented is an average of three independent experiments.

**Western Blotting for Phosphorylated and Unphosphorylated PACT, PKR, and eIF2α**—HT1080 cells were transfected with FLAG-PACT or its mutants using the Lipofectamine 2000 reagent. At 23 h after transfection, cells were stressed for 1 h where indicated. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, 20% glycerol) on ice. The cell extract was used in Western blot analysis with anti-FLAG (M2) monoclonal antibody as described before (20). For the detection of phosphothreonine residues, the extract was used to immunoprecipitate FLAG-PACT or its mutant with anti-FLAG (M2) agarose. The agarose beads were washed six times with RIPA buffer, and the immunoprecipitates were analyzed by Western blotting with anti-FLAG or with a mixture of three monoclonal anti-phosphothreonine antibodies as described by the manufacturer (Biodesign International). For the detection of phosphorylated PKR or phosphorylated eIF2α, at 47 h after transfection with PACT or mutant, cells were stressed for 1 h with 50 ng/ml actinomycin D when indicated. Cells were then lysed in RIPA buffer and extracts Western-blotted with anti-phospho-PKR, anti-PKR, anti-phospho-eIF2α, anti-eIF2α, or anti-FLAG (M2) antibody.

**Two-dimensional Gel Analysis**—HT1080 cells were transfected with 5 μg of expression vector containing FLAG-PACT or its mutant using Fugene 6 reagent (Roche Applied Science). At 6 h post-transfection, the cells were washed with phosphate-buffered saline once and incubated with fresh complete medium for an additional 18 h. The cells were lysed with 1 ml of cell lysis buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 200 mM NaCl, 1% Triton 100, 1 mM EDTA, 100 units of aprotinin per ml, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM Na3VO4, 10 mM β-glycerophosphate), and PACT-immunoprecipitated using anti-FLAG M2 affinity gel. FLAG-PACT was eluted from the gel by adding 125 μl of rehydration buffer (8 M urea, 4% CHAPS, 10 mM dithiothreitol, 0.5% IPG buffer 3/10). IPG strips (7 cm, pH 3–10; Amersham Biosciences) were rehydrated with rehydration buffer containing FLAG-PACT for 12 h before running isoelectric focusing (IEF). The IEF electrophoresis was performed with an IEPgphor™ Isoelectric Focusing System (Amersham Biosciences) according to the instruction manual. The focusing time was 40,000 vhr. After focusing, the strips were laid on top of SDS-polyacrylamide gels for second-dimension separation. FLAG-PACT was examined by Western blotting using an anti-FLAG M2 monoclonal antibody. For alkaline phosphatase (calf intestine) (CIP, Invitrogen) treatment, after washing four times with cell lysis buffer, the immunoprecipitate was washed once with 1× phosphatase...
buffer, divided into two 1.5 ml Eppendorf tubes, and incubated in 100 μl of phosphatase buffer with or without 10 units of CIP at 37 °C for 1 h. After the CIP treatment, the immunoprecipitate was washed twice with cell lysis buffer and subjected to IEF electrophoresis as indicated above.

_In Vivo Phosphate Labeling_—HT1080 cells were transfected in 100-mm culture dishes with 5 μg of FLAG-tagged PACT or PACT mutant DNA. At 24 h after transfection, cells were passaged at a 1:10 dilution into fresh growth medium. At 48 h after transfection, media was replaced with complete medium containing 800 μg/ml G418, and selection pressure applied for a minimum of 3 weeks. A pool of PACT-expressing cells was metabolically labeled with 1000 Ci/ml of [32P]orthophosphate (PerkinElmer Life Sciences) for 2.5 h in phosphate-free medium. Cells were stressed for the last 30 min where indicated. Cells were washed with phosphate-buffered saline, flash-frozen on liquid nitrogen, and cell extracts prepared using modified RIPA buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na4P2O7, 2 mM Na3VO4, 0.1% SDS, 1% Triton-X 100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM protease inhibitor mixture (Sigma)). For the detection of phosphorylated PACT, the extract was used to immunoprecipitate FLAG-PACT or its mutant with anti-FLAG (M2) agarose. The agarose beads were washed six times with RIPA buffer, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

_Coimmunoprecipitation Assay_—HT1080 cells were transfected in 100-mm culture dishes with 10 μg of total DNA (5 μg of PKR (K296R) DNA and 5 μg of FLAG-PACT or mutant DNA) using the Lipofectamine 2000 reagent (Invitrogen). At 23 h after transfection, cells were stressed. At 24 h after transfection, cells were lysed in immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% Triton-X 100, 1 mM dithiothreitol, 100 units/ml aprotinin, 2 mM MgCl2, 20% glycerol). The cell extract was used to immunoprecipitate FLAG-PACT with anti-FLAG (M2) agarose as described (20), washing the beads six times with IP buffer. The immunoprecipitates were analyzed by Western blotting with anti-PKR polyclonal (Santa Cruz Biotechnology) and anti-FLAG monoclonal antibodies (Sigma) (26).

**RESULTS**

_Essential Residues in PACT Domain 3_—Our previous study (20) has established that domain 3 of PACT is essential for activating PKR (Fig. 1A). For exerting its action in vivo, this domain needs to be attached to another domain of PACT or a heterologous domain that interacts with PKR strongly. We wanted to identify the residues within domain 3 that are required for PACT activity. For this purpose, progressive deletions of ten residues were introduced from the N terminus or the C terminus of domain 3 (Fig. 1B). PACT proteins carrying these mutations were expressed in cells that were stressed by treatment with a low dose of actinomycin D, and apoptosis was measured by TUNEL assays. PACT expression and TUNEL positivity of each cell was determined by double immunofluorescence assay as described before (20, 26). Deletion of ten residues from the C terminus of domain 3 (mutant C1) was tolerated, but removal of another ten residues

**FIGURE 1. Apoptotic activities of PACT deletion mutants.** _A_, map of human wild-type PACT protein. Wild-type PACT has three domains: the PKR interaction domains 1 and 2, and the PKR activation domain 3. Small numbers indicate amino acid residue number. _B_, induction of apoptosis by N-terminal or C-terminal PACT domain 3 deletion mutants. Maps of wild-type PACT domain 3 and its N-terminal (N1–N6) and C-terminal (C1–C6) deletion constructs with amino acid numbers are shown. Each mutant was tested for apoptotic activity in stressed cells. Each domain 3 mutant was tested in the context of PACTΔ1, containing the strong PKR binding domain 2 and PKR activation domain 3, which are necessary for PKR activation in vivo. Immunoﬂuorescence assays were performed to detect PAC- expressing cells, which were scored for apoptosis using TUNEL assay. _C_, representative results of the assays from cells transfected with vector, N1, C1, C2, or wild-type PACT. For each transfection, panels show independent photographs of the same cells, with different filters, to indicate nuclei (DAPI), cells undergoing apoptosis (TUNEL), and PACT-expressing cells (IF). The TUNEL results are representative of three independent experiments. _D_, PACT protein levels in transfected cells.
PACT Action Regulated by Serine Phosphorylation

To identify the essential residues within the above region, we carried out alanine scanning mutagenesis using a quantitative cell survival assay. In this assay, a GFP expression vector was co-transfected with PACT or its mutants so that the transfected cells could be identified by GFP expression. After stressing by a very low dose of actinomycin D treatment, the wild-type PACT-expressing cells started undergoing apoptosis and massive cell death was obvious as dead cells floated off the monolayers. For quantifying the survival index of transfected cells expressing GFP and different PACT proteins, cells still attached to the plate were removed from each plate, and the same number of cells analyzed by FACS for GFP expression. In this quantitative assay, almost no GFP expressing stressed cells survived when wild-type PACT was co-expressed (Fig. 2A). The point mutants carrying substitution of a single residue with alanine had different properties: most of them were as active as the wild-type protein, whereas ten mutants lost their activity almost completely. The inactive mutants were Q243A, S246A, D260A, D262A, S265A, Q271A, S279A, S287A, C291A. In addition, a few mutants had intermediate activities; these residues were located very close to the essential residues. The ten inactive mutant proteins were expressed at the same level as the wild type or an active mutant (244) protein (Fig. 2B). We confirmed that the active PACT mutant protein-expressing cells were undergoing apoptosis by performing TUNEL assays with selected mutants. Cells expressing the active L244A mutant protein were TUNEL-positive, whereas those expressing the S246A or the S287A proteins were TUNEL-negative (Fig. 2C).

Because we are interested in the mechanism of PACT activation by its stress-induced phosphorylation, we focused our attention to those residues in domain 3 that can potentially be phosphorylated and whose replacement by Ala produced inactive PACT. This notion was supported by the properties of two other mutants, S246T and S287T (Table 1). Although Ala was not tolerated in these sites, Thr was tolerated, partially for 246

FIGURE 2. Alanine-scanning mutagenesis: requirement of specific residues in domain 3 for cell killing. A, quantitative cell survival assay of PACT or PACT mutant-expressing cells. Amino acid residues between 240 and 295 in PACT domain 3 were independently mutated to alanine, and each mutant was quantitatively assayed for stress-induced cell killing. Each mutant contained domain 3 and the strong PKR binding domain 2, which are necessary for PKR activation in vivo. Numbers and letters indicate amino acid number and residue, respectively. Residues 249, 266, 274, and 294 are alanine in wild-type PACT. The data shown are an average of three independent experiments; any deviation was less than 10%. B, PACT protein levels in cells transfected with selected mutants. Cells expressing the active L244A mutant protein were TUNEL-positive, whereas those expressing the S246A or the S287A proteins were TUNEL-negative (Fig. 2C).

FIGURE 3. Inability of PACT mutants S246A and S287A to activate PKR in stressed cells. Western blotting of phosphorylated PKR, PKR, and PACT was done as described under “Experimental Procedures.” Full-length PACT or full-length point mutants were tested. Lanes 1 and 2, pcDNA3 vector; lanes 3 and 4, wild-type PACT; lanes 5 and 6, S246A; lanes 7 and 8, S246A. Only in even-numbered lanes, the cells were stressed.

Stress-activated Phosphorylation of PACT and Its Mutants—To investigate further the role of Ser246 and Ser287 in PACT function, we wondered whether these two residues are the targets of phosphorylation by cellular protein kinases. This notion was supported by the properties of two other mutants, S246T and S287T (Table 1). Although Ala was not tolerated in these sites, Thr was tolerated, partially for 246...
and completely for 287, suggesting that Thr, another phosphorylatable residue, can substitute for Ser at these sites. Further support came from the observation that substitution of either Ser residue with the phosphoserine mimic Asp was fully tolerated (Table 1).

Evidence for phosphorylation of PACT on these two Ser residues was provided by biochemical analyses of wild-type PACT and mutant proteins purified from cell extracts. Two dimensional gel analyses followed by Western blotting showed that bulk of the wild-type protein (Spot 1, Fig. 4A) had a higher isoelectric point than that of the minor spot (Spot 2). Applying cellular stress caused the appearance of a new spot (Spot 3) with the lowest isoelectric point, suggesting that Spot 2 and Spot 3 could be a partially and the fully phosphorylated forms of PACT respectively. As expected, treatment with protein phosphatase caused Spots 2 and 3 to collapse into Spot 1 (Fig. 4B). The S246A mutant protein did not produce any phosphorylated species either before or after stress (Fig. 4C), whereas the S287A mutant protein contained Spot 2, but not Spot 3 even after stress (Fig. 4D). These results indicate that Ser246 is a target of phosphorylation even in unstressed cells whereas Ser287 gets phosphorylated after the cells are stressed. Moreover, it appears that Ser287 cannot be phosphorylated without Ser246 phosphorylation. Neither the constitutive nor the stress-induced phosphorylation of PACT was mediated by PKR, because both phosphorylations were observed in Pkr-/- MEF cells (Fig. 4E).

Phosphorylation of residues 246 and 287 was tested by a different approach in the experiment shown in Fig. 5; we used the S246T and the S287T mutants and a phosphothreonine-specific antibody for this purpose. Because these two mutants were functionally active in our cell survival assay (Table 1), we anticipated that the introduced threonine residues would be phosphorylated in vivo. As expected, the wild type protein was not phosphorylated in Thr residues even after stressing the cells (Fig. 5, lanes 6 and 7). The S287T mutant was Thr-phosphorylated only after stressing (Fig. 5, lanes 4 and 5) whereas the S246T mutant was constitutively Thr-phosphorylated before and after stressing (Fig. 5, lanes 2 and 3). We ensured that the transfected PACT proteins were expressed to similar levels (lower panel, Fig. 5).

Finally, stress-induced PACT phosphorylation was confirmed by its in vivo labeling with 32P. Cell lines expressing wild type or mutant PACT were generated; wild-type and the S287A mutant proteins were expressed at similar levels whereas the S246A mutant protein was expressed more highly, probably

### Table 1

**Survival of stressed cells expressing PACT mutants**

A quantitative cell survival assay was used to measure the survival of PACT or PACT mutant-transfected HT1080 cells by cotransfecting with a GFP expression vector (8:1) so that the transfected cells could be identified by GFP expression. After stressing, PACT/mutant-expressing cells that underwent apoptosis floated off the monolayers. Cells still attached to the plate were removed from each plate, and the same number of cells analyzed by FACS for GFP expression as a measure of cell survival. Each PACT mutant contained the strong PKR binding domain 2 and PKR activation domain 3, which are necessary for PKR activation in vivo.

| Mutations | % Cell survival |
|-----------|----------------|
| Wild type | 2              |
| Vector    | 100            |
| S246A     | 100            |
| S246T     | 42             |
| S246D     | 2              |
| S287A     | 88             |
| S287T     | 1              |
| S287D     | 2              |
| S18A      | 2              |

**Figure 4. Detection of in vivo phosphorylation of PACT and its domain 3 mutants by two-dimensional gel analysis.** In the first dimension of electrophoresis the pH gradient was basic to acidic, left to right. The second dimension of SDS-electrophoresis was from the top to bottom. Western analysis was done with anti-FLAG antibody. Only the relevant portions of the gels are shown. In each two-dimensional gel analysis, the number of spots observed for each treatment is indicated. Each PACT mutant contained in addition to domain 3, the strong PKR binding domain 2. A, protein from unstressed and stressed wild-type PACT-expressing cells. B, CIP-treated protein from unstressed and stressed wild-type PACT-expressing cells. C, protein from unstressed and stressed S246A-expressing cells. D, protein from unstressed and stressed S287A-expressing cells. E, protein from unstressed and stressed Pkr-/- MEF cells expressing wild-type PACT.

**Figure 5. Phosphorylation of PACT mutants as detected by anti-phosphothreonine antibody.** PACT proteins were immunoprecipitated with anti-FLAG (M2) agarose from extracts of stressed or unstressed cells. PACT protein levels were analyzed by Western blotting with anti-FLAG antibody. Proteins were tested in the context of PACTΔ1. Lane 1, pcDNA3 vector; lanes 2 and 3, S246T; lanes 4 and 5, S287T; lanes 6 and 7, wild-type PACT. In the odd-numbered lanes, cells were stressed.
because the latter mutant protein was constitutively inert, and cells could tolerate its higher expression (bottom panel, Fig. 6). There was no detectable labeling of wild-type PACT or its mutants in cells that had not been stressed (top panel, Fig. 6), indicating that we could detect only stress-inducible phosphorylation under our labeling conditions. When stress was applied, strong labeling of PACT was observed only in cells expressing the wild-type protein, not the mutants (middle panel, Fig. 6). Thus, both Ser\textsuperscript{246} and Ser\textsuperscript{287} were required for stress-inducible phosphorylation of PACT.

From the above observations, we concluded that phosphorylation of both Ser\textsuperscript{246} and Ser\textsuperscript{287} of PACT, one constitutive and the other stress-inducible, is necessary for its ability to activate PKR \textit{in vivo}. Moreover, constitutive phosphorylation of Ser\textsuperscript{246} was a prerequisite for inducible phosphorylation of Ser\textsuperscript{287}.

Properties of Asp Substitution Mutants—To further solidify the above conclusions, we generated several PACT mutants, in which Ser\textsuperscript{246} and Ser\textsuperscript{287} were replaced with Ala (AA) or the phosphoserine mimetic Asp (DD). As expected, the AA mutant was completely inactive, whereas the DD mutant was as active as wild-type PACT in stressed cells (Table 2). The AD mutant was also inactive, indicating the absolute need for phosphorylation of the 246 residue. Finally, the DD mutant, unlike the wild-type protein, was highly active even in unstressed cells causing death of 86% of cells expressing it. These results were confirmed by TUNEL assay as well (Fig. 7 and data not shown).

To probe the cause of the observed apoptosis in cells expressing the DD mutant, we examined the extent of PKR activation by measuring the levels of phosphorylation of eIF2\textalpha, a substrate of PKR. PKR activation \textit{in vivo}, as measured by eIF2\textalpha phosphorylation, was equally strong, before and after stress, in cells expressing the DD mutant (lanes 2 and 7, Fig. 8A). There was little eIF2\textalpha phosphorylation in cells expressing the AA mutant even after the application of stress (lanes 4 and 5, Fig. 8A), and the wild-type protein caused eIF2\textalpha phosphorylation only after the cells were stressed (lanes 2 and 3, Fig. 8A). To seek the reason behind the above observations, we wondered whether the enhanced eIF2\textalpha phosphorylation was caused by a stronger association between PACT and PKR. Indeed, more PKR coimmunoprecipitated with wild-type PACT after the cells were stressed (lanes 3 and 4, Fig. 8B). In contrast, when the AA mutant of PACT was expressed, only a small amount of PKR was coimmunoprecipitated even after stress (lanes 5 and 6, Fig. 8B). Strikingly, the DD mutant of PACT coimmunoprecipitated PKR more efficiently, even without stress (lanes 7 and 8, Fig. 8B). These results demonstrated that phosphorylated wild-type PACT bound PKR more strongly \textit{in vivo}, as did the DD mutant.

DISCUSSION

We have used a combination of genetic and biochemical experiments to explore the mechanism by which various extracellular stresses use PACT to activate PKR and cause apoptosis (20, 24, 25). We used a human cell line that did not express detectable PACT and consequently was a good recipient for testing various mutant PACT proteins \textit{in vivo} expressed by transfection. Because the cell survival assay was convenient and quantitative, it was used for screening mutants of domain 3.
This assay was in perfect concordance with the apoptosis assay (Fig. 2). Out of the fifty-six residues needed for activity (Fig. 1), replacement of any of ten specific residues with alanine almost completely destroyed the activity. Further structural studies will be needed for understanding why alanine substitution of these ten residues disrupted the function of domain 3. It is conceivable that the mutated Asp and Gln residues form salt bridges to stabilize its structure and Cys279 may be involved in a disulfide linkage. In contrast, the four serine residues may be structurally required or be the targets of phosphorylation. We explored the latter possibility because of the information in the literature that PACT gets phosphorylated in stressed cells. Our results indicate that Ser246 and Ser287 are the targets of phosphorylation.

While our work was in progress, Bennett et al. (28) reported that the S18A mutant of RAX (murine homolog of PACT) was inactive in their assay system of PKR activation in vivo. Moreover, the mutant protein could interact with PKR as effectively as the wild-type protein and functioned as a dominant negative over, the mutant protein could interact with PKR as effectively as the wild-type protein and functioned as a dominant negative. Most importantly, because the mutant protein isolated from stressed or unstressed cells was unphosphorylated. The two-dimensional gel analysis of wild-type and mutant PACT proteins present in stressed and unstressed cells revealed that Ser246 was partially phosphorylated even in unstressed cells, suggesting that a constitutively active kinase phosphorylates Ser246. In contrast, Ser287 was phosphorylated only after stress, suggesting that it is the target of a stress-activated protein kinase pathway. Phosphorylation of the S246T and the S287T mutants in threonine residues (Fig. 5) unequivocally proved that these two residues are indeed the targets of constitutive and induced phosphorylation respectively. In vivo radiolabeling of the stress-activated phosphoserine residue further confirmed the above conclusion (Fig. 6). The properties of the mutants uncovered a surprising aspect of phosphorylation of these two serine residues: not only was it sequential, but the order was also fixed. It appears that Ser287 phosphorylation required prior phosphorylation of Ser246, because the S246A mutant produced only unphosphorylated protein even after stress, whereas the S287A mutant produced a monophosphorylated species, before and after stress (Fig. 4). However, the S246A/S287D mutant was as ineffective in cell killing as the S246A/287D mutant (Table 2) indicating that the phosphoserine at 246 was needed for another purpose in addition to allowing the phosphorylation of Ser287. Substitution of the two other essential Ser residues, Ser265 and Ser279 (Fig. 2A), with Asp did not produce constitutively active mutants (data not shown), indicating that their role is structural and they are not targets of phosphorylation.

Until the structure of PACT, or at least its domain 3, is solved, we can only speculate about the reasons for the observed need of most of the ten essential residues in domain 3, as identified by alanine-scanning mutagenesis. However, the results presented here provide strong evidence for the roles of Ser246 and Ser287 as targets of necessary phosphorylation. Our observation that residues in domain 3, but not elsewhere, regulate PACT activation, is consistent with our earlier observation that domains 1 and 2 of PACT could be functionally substituted by the corresponding dimerization domains of PKR (20). The fusion protein could induce apoptosis only in stressed cells, indicating that the stress-sensing residues were located in domain 3. Our data support a two-step phosphorylation model of PACT, one constitutive and the other stress-activated. Future biochemical analyses will be needed for identifying the respective protein kinases that...
mediate the phosphorylation of Ser\textsuperscript{246} and Ser\textsuperscript{287}. Our results with various mutants, especially the DD mutant, strongly indicate that the phosphorylation of these two serine residues is both necessary and sufficient for PACT activation. Results in Fig. 8B presented a biochemical basis for understanding why phosphorylated PACT could activate PKR \textit{in vivo}: it associated with PKR more efficiently. This observation is in line with that made by others using wild-type PACT (25). In contrast, the S18A inactive mutant of RAX, associated with PKR as strongly as the wild-type protein (28) indicating that it does not regulate PKR activation, a prediction supported by our experiments. However, it remains possible that different kinds of stresses can activate PACT through phosphorylation of different residues, a paradigm established for the activation of transcription factors such as NF\textsubscript{κ}B, IRF-3, or p53.

Acknowledgments—We thank Paul Fox and Prabha Sampath for anti-phosphothreonine antibodies, Judy Drazba for advice on fluorescence microscopy, Xinna Li and Mark Kader for help with PACT mutant construction, and Cathy Shemo and Dolly Klingman at the Lerner Research Institute Flow Cytometry core facility for experimental assistance.

REFERENCES
1. Clemens, M. J., and Elia, A. (1997) \textit{J. Interferon Cytokine Res.} 17, 503–524
2. Sen, G. C. (2001) \textit{Annu. Rev. Microbiol.} 55, 255–281
3. Williams, B. R. (1999) \textit{Oncogene} 18, 6112–6120
4. Williams, B. R. (2001) \textit{Science’s STKE} http://stke.sciencemag.org/cgi/content/full/sigtrans;2001/89/re2
5. Chu, W. M., Ostertag, D., Li, Z. W., Chang, L., Chen, Y., Hu, Y., Williams, B., Perrault, J., and Karin, M. (1999) \textit{Immunity} 11, 721–731
6. Cuddihy, A. R., Wong, A. H., Tam, N. W., Li, S., and Koromilas, A. E. (1999) \textit{Oncogene} 18, 2690–2702
7. Iordanov, M. S., Paranjape, J. M., Zhou, A., Wong, J., Williams, B. R., Meurs, E. F., Silverman, R. H., and Magun, B. E. (2000) \textit{Mol. Cell. Biol.} 20, 617–627
8. Kumar, A., Haque, J., Lacoste, J., Hiscott, J., and Williams, B. R. (1994) \textit{Proc. Natl. Acad. Sci. U. S. A.} 91, 6288–6292
9. Kumar, A., Yang, Y. L., Flati, V., Der, S., Kadereit, S., Deh, A., Haque, J., Reis, L., Weismann, C., and Williams, B. R. (1997) \textit{EMBO J.} 16, 406–416
10. Wong, A. H., Tam, N. W., Yang, Y. L., Cuddihy, A. R., Li, S., Kirchhoff, S., Hauser, H., Decker, T., and Koromilas, A. E. (1997) \textit{EMBO J.} 16, 1291–1304
11. Zamanian-Daryoush, M., Mogensen, T. H., DiDonato, J. A., and Williams, B. R. (2000) \textit{Mol. Cell. Biol.} 20, 1278–1290
12. Green, S. R., and Mathews, M. B. (1992) \textit{Genes Dev.} 6, 2478–2490
13. Patel, R. C., and Sen, G. C. (1992) \textit{J. Biol. Chem.} 267, 7671–7676
14. Bischoff, J. R., and Samuel, C. E. (1985) \textit{J. Biol. Chem.} 260, 8237–8239
15. Carpick, B. W., Graziano, V., Schneider, D., Maitra, R. K., Lee, X., and Williams, B. R. G. (1997) \textit{EMBO J.} 16, 9123–9136
16. Romano, P. R., Garcia-Barrio, M. T., Zhang, X., Wang, Q., Taylor, D. R., Zhang, F., Herring, C., Mathews, M. B., Qin, J., and Hinnebusch, A. G. (1998) \textit{Mol. Cell. Biol.} 18, 2282–2297
17. Patel, R. C., Stanton, P., McMullan, N. M., Williams, B. R., and Sen, G. C. (1995) \textit{Proc. Natl. Acad. Sci. U. S. A.} 92, 8283–8287
18. D’Acquisto, F., and Ghosh, S. (2001) \textit{Science’s STKE} http://stke.sciencemag.org/cgi/content/full/sigtrans;2001/89/re1
19. Patel, R. C., and Sen, G. C. (1998) \textit{EMBO J.} 17, 4379–4390
20. Peters, G. A., Hartmann, R., Qin, J., and Sen, G. C. (2001) \textit{Mol. Cell. Biol.} 21, 1908–1920
21. Li, S., Peters, G. A., Ding, K., Zhang, X., Qin, J., and Sen, G. C. (2006) \textit{Proc. Natl. Acad. Sci. U. S. A.} 103, 10005–10010
22. Rowe, T. M., Rizzii, M., Hirose, K., Peters, G. A., and Sen, G. C. (2006) \textit{Proc. Natl. Acad. Sci. U. S. A.} 103, 5823–5828
23. Tibbles, L. A., and Woodgett, J. R. (1999) \textit{Cell. Mol. Life Sci.} 55, 1230–1254
24. Ito, T., Yang, M., and May, W. S. (1999) \textit{J. Biol. Chem.} 274, 15427–15432
25. Patel, C. V., Handy, L., Goldsmith, T., and Patel, R. C. (2000) \textit{J. Biol. Chem.} 275, 37993–37998
26. Peters, G. A., Khoo, D., Mohr, I., and Sen, G. C. (2002) \textit{J. Virol.} 76, 11054–11064
27. Pogulis, R. J., Vallejo, A. N., and Pease, L. R. (1996) in \textit{In Vitro Mutagenesis Protocols} (Trower, M. K., ed) vol. 57, pp. 167–176, Humana Press, Inc., Totowa, NJ
28. Bennett, R. L., Blalock W. L., and May W. S. (2004) \textit{J. Biol. Chem.} 279, 42687–42693