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Regulatable Expression of the Interferon-Induced Double-Stranded RNA Dependent Protein Kinase PKR Induces Apoptosis and Fas Receptor Expression

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PKR is an interferon-induced dsRNA-dependent protein kinase involved in the antiviral response as well as in cell growth and differentiation. Studies using a transdominant negative mutant of PKR also have implicated the kinase in tumor suppression and apoptosis. However, functional studies of PKR have been hampered by the lack of a suitable expression system. In this study, we used a tetracycline-regulated inducible system in NIH3T3 cells to investigate the involvement of PKR in programmed cell death (apoptosis). We show that expression of wild-type PKR causes apoptosis and correlates with increased mRNA levels for the Fas receptor, a member of the tumor necrosis family of proteins. Expression of an inactive form of PKR (K296R) or the vector alone did not induce apoptosis or elevate Fas mRNA levels. Our results clearly demonstrate that expression of an active form of PKR triggers apoptosis, possibly through upregulation of the Fas receptor.
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INTRODUCTION

Programmed cell death (apoptosis) plays a central role in development and homeostasis in metazoans. Apoptosis is involved in pathological processes, in which it participates in defense mechanisms to remove damaged cells such as tumorigenic and virus-infected cells. Apoptosis can be triggered by intracellular and extracellular stimuli. Conditions that lead to the onset of programmed cell death include intracellular conflicting signals of proliferation and differentiation. Additionally, the withdrawal of survival factors or the exposure to stresses also may lead to apoptosis. Mediators of the latter effects have been studied extensively. These include members of the TNF receptor superfamily of proteins, which includes the TNFR1, TNFR2, CD40, and the CD95/FAS receptors. A number of immediate downstream effectors such as FADD, FLICE, TRADD, and RIP have been identified. The recruitment of these proteins to their respective receptors is thought to activate the caspase protease cascade that ultimately leads to cell death (Wallach, 1997). The link between the mediators and upstream effectors of apoptosis during virus and interferon-mediated cell death is still unclear (Nagata, 1997).

PKR is an interferon-induced, serine/threonine protein kinase (Samuel, 1991). Its activity is dependent on double-stranded RNA (dsRNA) and manifests two distinct kinase activities: one for autophosphorylation (regulated by dsRNA) and the other for phosphorylation of eIF-2α. The phosphorylation of eIF-2α blocks the exchange of GDP for GTP and consequently the inhibition of formation of the eIF-2.GTP.Met.tRNA heterotrimeric complex. This results in inhibition of translation (for a review, see Hershey, 1991). PKR plays an important role during viral infection, in cell growth control and differentiation (Petryshyn *et al.,* 1984; Chong *et al.,* 1992; Samuel, 1993; Donzé *et al.,* 1995). PKR also is believed to be a tumor suppressor because the expression of dominant negative mutants of PKR in mouse NIH3T3 fibroblasts causes malignant transformation (Koromilas *et al.,* 1992; Lengyel, 1993; Meurs *et al.,* 1993; Sonenberg, 1993). Moreover, expression of wt PKR in yeast increases phosphorylation of eIF-2α and results in the inhibition of cell growth (Chong *et al.,* 1992; Dever *et al.,* 1993). PKR is also of central importance in the antiviral defense by interferon (Yang *et al.,* 1995).

Several reports suggest that PKR is involved in programmed cell death after viral infection and in tumor necrosis factor-induced apoptosis. Expression of a *trans*-dominant negative mutant of PKR or antisense for PKR blocks apoptosis mediated by influenza and vaccinia viruses, dsRNA, or TNF-α (Lee and Esteban, 1994; Takizawa *et al.,* 1995; Takizawa *et al.,* 1996; Yeung *et al.,* 1996; Anderson, 1997; Der *et al.,* 1997; Kibler *et al.,* 1997; Srivasta *et al.,* 1998). Although these studies suggest a role for PKR in programmed cell

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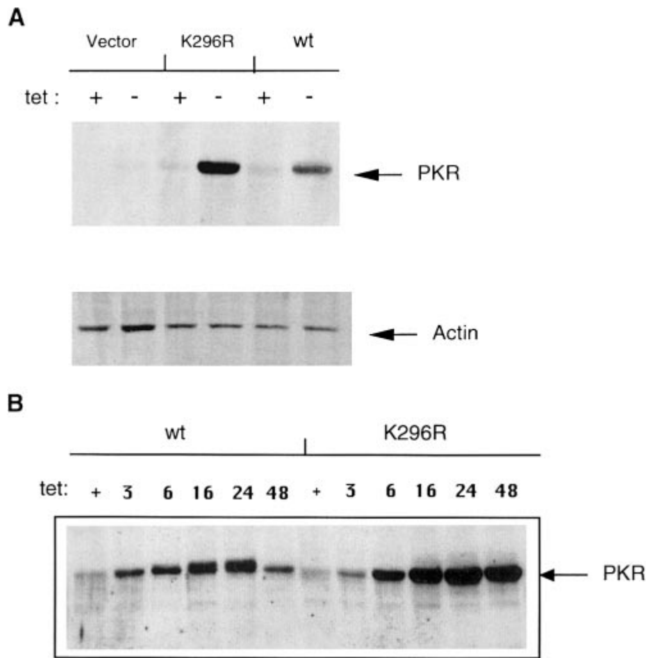

FIG. 1. Inducible human PKR wt and mutant (K296R) in NIH3T3 cell lines were generated using the tetracycline inducible system. Immunoblots are shown of lysates from NIH3T3 cells in which either PKR wt, K296R, or vector alone were induced for 24 h (A). NIH3T3 cell lines were grown in the absence of tetracycline (tet) and extracts were obtained at different times as indicated in the figure (B). Lysates (10 μg) were subjected to SDS–7.5% PAGE, and PKR was detected using a monoclonal antibody against human PKR (Laurent *et al.,* 1985). Actin was used to normalize for loading variability.

death, such a role has not been shown directly by overexpressing wt PKR. Consequently, the mechanism by which PKR induces apoptosis has been difficult to address.

Functional studies of PKR have been hampered by the toxicity of the protein in tissue culture cells (Koromilas *et al.,* 1992). To overcome this problem, we generated PKR-inducible cell lines, using a tetracycline-regulated transactivator system. This system should facilitate the study of the growth-inhibiting properties of PKR and its function in apoptosis and determine its effect on endogenous target genes. Here, we show that the expression of PKR wt induces programmed cell death in NIH3T3. Moreover, we show that PKR induces expression of Fas receptor mRNA.

RESULTS

Expression of PKR using the tetracycline inducible system in NIH3T3

Expression of the tetracycline-repressible transactivator (tTA) allows strict regulation of a promoter containing *tet* operator sequences (Gossen and Bujard, 1992). Constructs coding for wt or a dominant negative mutant (K296R) of PKR under the control of tetracycline regulatable promoter were transfected into parental S2–6 cells, which stably express the transactivator (tTA) (Shockett *et al.,* 1995). As a control, cells were transfected with the vector alone. Two stable cell lines for wt PKR (wt 2 and 5), one for the mutant (K296R) and one for the vector, were chosen for further studies. No protein expression was detected by immunoblot analysis for the transfected PKR plasmid in the presence of tetracycline (lanes indicated by +, Fig. 1A). Tetracycline withdrawal induced PKR protein expression (either wt or mutant). Control cells showed no PKR expression in the presence or absence of tetracycline (Fig. 1A). As observed in earlier studies (Barber *et al.,* 1993), PKR protein expression levels were lower in cell lines expressing PKR wt as compared with mutant PKR, reflecting the autoinhibitory translational effect of PKR wt. After tetracycline removal, the expression of the wild-type kinase was detectable within 3 h and reached maximum expression at 24 h. The induction kinetic of PKR K296R was delayed compared with wt (Fig. 1B). Phosphorylation of PKR wt was assessed by the slower migration of the phosphorylated form on a SDS–7.5% polyacrylamide gel. Interestingly, 3 h after tetracycline removal, PKR migrated as a single band. At 6 h, an additional faint upper band appeared, which most likely represents the phosphorylated form (Ito *et al.,* 1994; Koromilas *et al.,* 1995). At 16 and 24 h after tetracycline removal, PKR migrated as a clear doublet. At 48 h, PKR is visible only as a single lower band, suggesting that it is dephosphorylated and probably inactivated. The level of PKR wt decreased dramatically at 48 h, probably due to translational arrest triggered by PKR wt. This effect requires kinase activity since the reduction was not seen for PKR K296R (compare Fig. 1B lane 48 h for PKR wt and K296R). The activity of the different PKR kinase variants was examined by an autophosphorylation assay (Fig. 2). As expected, kinase activity was detected only in cell lines expressing the wild-type protein. Taken together,

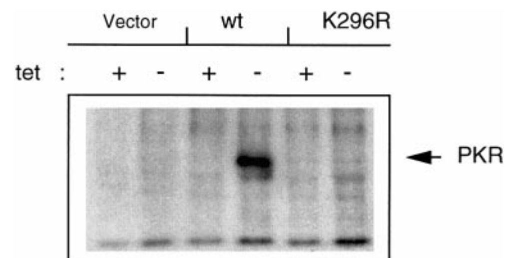

FIG. 2. *In vitro* kinase assay. Cell extracts from NIH3T3 cells expressing PKR wt, K296R, or the vector were performed in the presence of dsRNA as described under Materials and Methods. PKR proteins were induced (−tet) for 24 h.

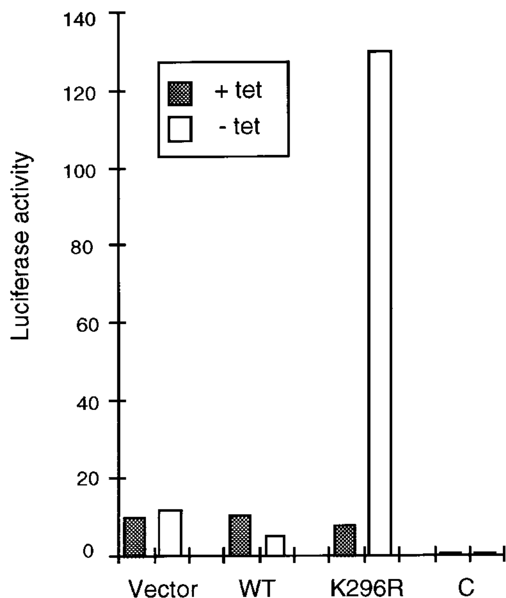

FIG. 3. Expression of luciferase in NIH3T3 cells expressing PKR wt, K296R PKR, or the vector alone. Cells were transfected with pcDNA3-luc. Luciferase activity was measured 48 h after transfection in a luminometer using the luciferase assay system (Promega). The experiments were carried out twice with <20% difference. V, NIH3T3 cells expressing the vector; C, NIH3T3 cells expressing PKR mutant (K296R) that were mock transfected (no DNA). PKR proteins were induced by tetracycline removal for the 24 h before harvesting.

these results demonstrate that a functional PKR protein can be induced in NIH3T3 cells.

To ascertain that PKR function was abrogated in cells expressing the *trans*-dominant negative mutant of PKR (K296R), we studied the expression of a luciferase reporter plasmid as reported previously (Kaufman *et al.,* 1989; Donzé *et al.,* 1995). Transient transfections of some plasmids result in the activation of PKR and specific translational inhibition of the transfected plasmids (Kaufman *et al.,* 1989). Expression of a PKR *trans*-dominant negative mutant reverses this translational inhibition (Barber *et al.,* 1993). We analyzed the expression of the luciferase gene when transfected in either PKR wt or PKR K296R-expressing cells. Luciferase expression in NIH3T3 cell expressing PKR K296R was enhanced as expected (Fig. 3) (Kaufman *et al.,* 1989; Donzé *et al.,* 1995). In cells expressing PKR wt, reporter gene expression was reduced as compared with control cells (Fig. 3). Thus we generated an inducible-system expressing either a functional kinase or a *trans*-dominant negative mutant of PKR.

PKR suppresses growth of NIH3T3 cell lines

Next, we investigated the effect of PKR overexpression on growth of NIH3T3 cells. Induction of PKR wt dramatically inhibited cell growth (Fig. 4). When PKR wt began to be expressed, cell growth decreased and stopped completely after <3 days. Reduction in cell number was detected as early as 16 h after tetracycline removal. No effect on the growth of cells containing the mutant or the vector alone was observed (Fig. 4, data not shown). Based on the results of Fig. 1, it is evident that the increase in functional PKR wt protein causes reduction in cell growth. It is noteworthy, however, that in the absence of tetracycline when both PKR mutant and/or the transactivator (tTA) are induced, cell growth is affected slightly, probably due to the effect of the tTA protein (Fig. 4) (Shockett *et al.,* 1995).

PKR wt induces programmed cell death in NIH3T3 cell lines

After PKR induction upon tetracycline removal, the PKR wt cells showed changes in morphology and loss of adherence to the tissue culture dish within 24 h of PKR wt expression (data not shown). To determine whether apoptosis occurred, flow cytometry was used (FACS) (Fig. 5). NIH3T3 cells displayed DNA content from 2 to 4 N, representing cells in Go/G1, S, and G2/M phase. Twenty-four hours after removal of tetracycline, the number of cells in G1 phase began to decrease with a concomitant appearance of a peak of hypodiploid cells characteristic of apoptosis (Fig. 5A, region h). Forty-eight hours after tetracycline removal, >40% of the cells contained hypodiploid DNA. In the absence of PKR induction, the FACS analysis was identical to control cells with a large peak of cells at Go/G1 (+ tet, Fig. 5A). NIH3T3 cells expressing the PKR mutant (K296R) or the vector alone did not contain cells in the hypodiploid fraction either in the presence or the absence of tetracycline (Fig. 5B). Electrophoretic analysis of DNA from dying cells revealed the fragmentation of chromatin into nucleosomal-size fragments (ladder) characteristic of apoptosis (Fig. 6, lane 4). These results indicate that induction of an active form of PKR is sufficient to induce apoptosis in NIH3T3 cells.

PKR wt induces expression of the Fas receptor

To start investigating the mechanism by which PKR induces apoptosis in NIH3T3 cells, we assayed for one potential target, the Fas antigen (CD95, APO-1) (for a review, see Nagata, 1997). Increased expression of the Fas antigen upon virus infection or double-stranded RNA addition has been reported recently (Takizawa *et al.,* 1995, 1996). Because PKR is activated either by dsRNA or by virus infection, it is conceivable that Fas is a target of PKR (Der *et al.,* 1997). Total RNA from NIH3T3 in which PKR wt has been induced for

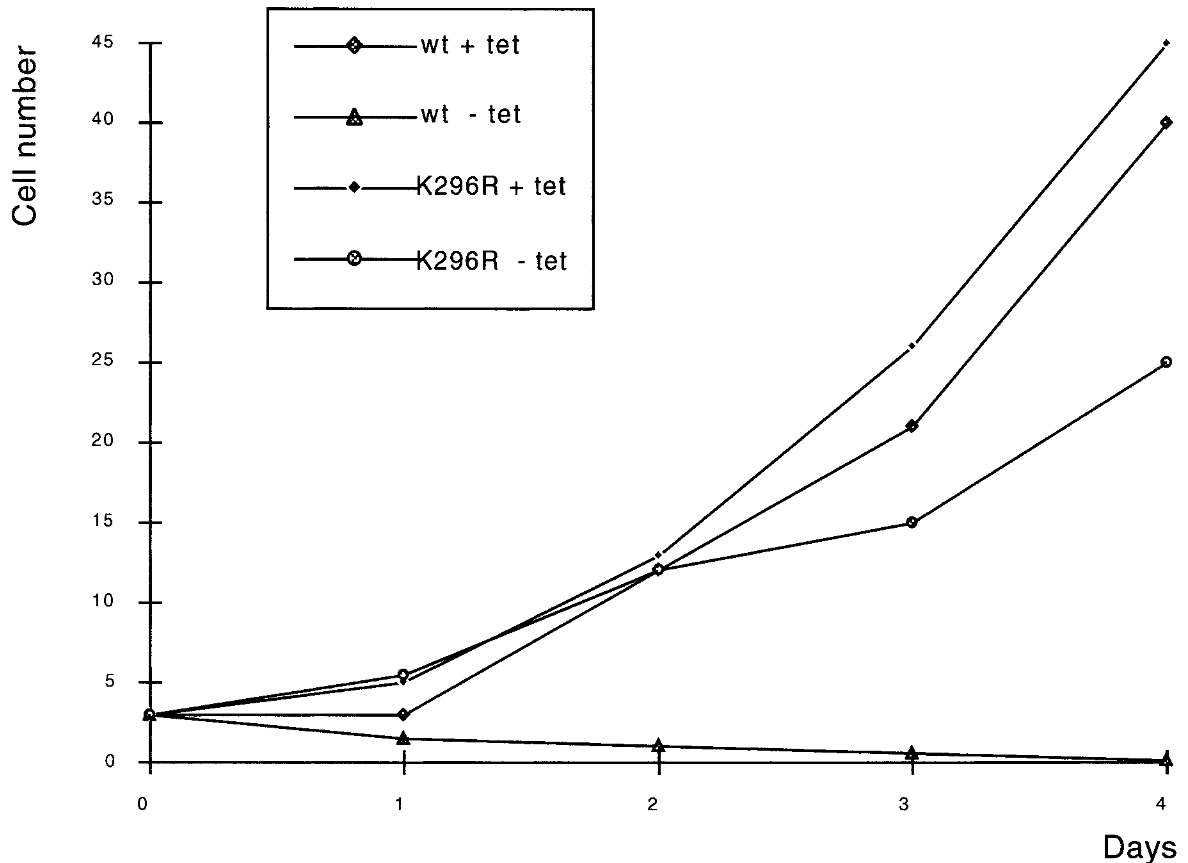

FIG. 4. Growth curve of NIH3T3 cells expressing PKR wt or PKR K296R. NIH3T3 cells expressing wt or mutant (K296R) PKR were grown in the presence or absence of tetracycline (±tet). Cells were seeded at 3×10^4 cells/plate in the presence of tetracycline. Three hours after plating, cultures were extensively washed with TBS to remove residual drug, and live cells were counted in duplicate plates at daily intervals. The number of cells/plate is plotted as log ($\times 10^4$).

24 h was subjected to RT–PCR with primers from the murine Fas gene. As shown in Fig. 7, Fas mRNA is expressed in response to PKR wt at 24 h; a 400-nt band is visible as expected. The PCR product was characterized further by restriction digest analysis to confirm the identity of the Fas sequence (data not shown). In contrast, when PKR K296R is expressed, no Fas mRNA synthesis was observed. This indicates that PKR induces Fas mRNA expression.

DISCUSSION

Functional studies of PKR have been hampered by the lack of a suitable expression system. Due to the toxicity of the protein, the establishment of stable cell lines was not possible. To solve this problem, we generated tetracycline-inducible cell lines expressing PKR. Previous reports suggested a potential role for PKR in the process of cell death. However, they were based on indirect studies involving virus-based expression system, mutants, or knock-out mice of PKR (Lee and Esteban, 1994; Takizawa *et al.,* 1995, 1996; Yeung *et al.,* 1996; Anderson, 1997; Der *et al.,* 1997; Kibler *et al.,* 1997; Srivasta *et al.,* 1998). Here we demonstrate that expression of an active form of PKR is sufficient to induce apoptosis in NIH3T3 cells. Also the occurrence of cell death is shown to correlate with the induction of PKR wt.

Numerous studies have shown that PKR is activated during viral infection (Samuel, 1991; Takizawa *et al.,* 1996). In many instances, viral infection ultimately leads to apoptosis by a mechanism that is still unknown (Takizawa *et al.,* 1993, Uehara *et al.,* 1993). Epstein–Barr (EB) virus (Uehara *et al.,* 1993) and influenza (Takizawa *et al.,* 1993, 1996), which cause apoptosis also lead to increased expression of Fas antigen. This observation indicates that Fas could play an important role in the virus-induced apoptosis (Takizawa *et al.,* 1995). Fas belongs to the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor family (Steller, 1995). It triggers apoptosis when bound to Fas ligand (Nagata, 1997). We thus considered Fas as a putative target of PKR-induced

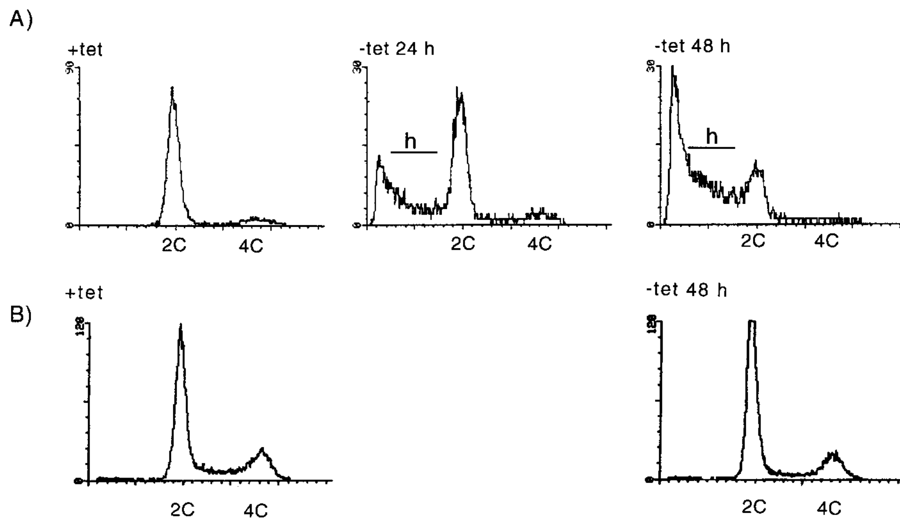

FIG. 5. Flow cytometric analysis of NIH3T3 cells expressing wt (A) or K296R PKR (B). NIH3T3 cells were grown in the presence or absence of tetracycline and analyzed by flow cytometry as described under Materials and Methods. 2C and 4C represent DNA content of cells. Hypodiploid DNA is indicated with h.

apoptosis. We show that PKR expression leads to Fas mRNA upregulation. This result further suggests that Fas may be a downstream effector of PKR during viral infection. It is conceivable that Fas oligomerization with FasL participates in transmitting the death signal during PKR-induced apoptosis.

It is not known how PKR induces Fas mRNA expression. However, the Fas gene contains one NF-kB consensus sequence in its promoter (Kimura *et al.,* 1997). PKR is known to participate in the activation of NF-kB by dsRNA (Maran *et al.,* 1994). Therefore, it is possible that PKR induces Fas mRNA expression at the transcriptional level by activation of NF-kB. The mechanism of activation is still unclear but might involve the inhibitor I-kB. One study reported that PKR could phosphorylate I-kB (Kumar *et al.,* 1994). We attempted to detect I-kB phosphorylation in the NIH3T3 cells at different times after PKR wt expression but failed (Donzé and Sonenberg, unpublished data). This observation is consistent with the lack of I-kB phosphorylation using a vaccinia virus recombinant expressing PKR (Lee *et al.,* 1997). The mechanism of NF-kB activation by PKR is still unclear but perhaps involves translational control of I-kB by the PKR/eIF-2 pathway. Further studies using cells expressing a nonphosphorylatable mutant of eIF-2a will help to solve this issue.

How is PKR activated in the absence of virus infection? Previous studies suggested that PKR is activated by its own mRNA (Barber *et al.,* 1993, Thomis and Samuel, 1995). In our system, one could follow the phosphorylation of PKR by visualizing the appearance of a doublet on a SDS–PAGE. The doublet is not present at 6 h but appears at 16 h (Fig. 1B). The appearance of phosphorylated PKR may result from the increase of its own mRNA levels after induction.

In conclusion, using a tetracycline-based inducible system, we show that expression of the interferon-induced, dsRNA-dependent protein kinase PKR is sufficient to trigger apoptosis in NIH3T3. We also observed that the Fas receptor mRNA is upregulated during this process. This system should facilitate a detailed study of the mechanism by which PKR acts as a tumor-suppressor and as a pre-apoptotic agent. Particularly, it would be important to identify the targets, that are activated during these processes.

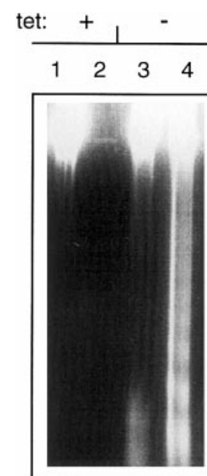

FIG. 6. DNA fragmentation induced by PKR wt. DNA was isolated from adherent (1) or floating (2) NIH3T3 cells expressing PKR wt in the presence of tetracycline or from adherent (3) or floating cells (4) in the absence of tetracycline. DNA was fractionated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

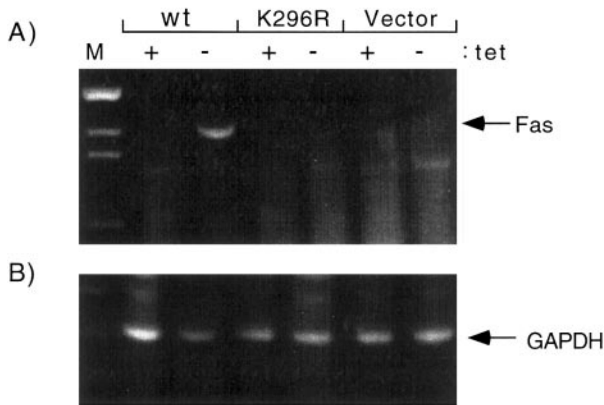

FIG. 7. Fas mRNA is induced in NIH3T3 cells expressing PKR wt. NIH3T3 cells expressing PKR wt, PKR mutant (K296R), or the vector were induced for 24 h. Fas mRNA levels were detected by reverse transcription–PCR (35 cycles) and analysed using 2% agarose gel electrophoresis. To normalize for RNA levels in the RT–PCR reaction, GAPDH mRNA was used for reverse transcription–PCR (30 cycles).

After submission of this paper for publication, a report by Balachandran *et al.* (1998) appeared in which the authors show that induction of PKR in 3T3-L1 cells causes apoptosis but only in the presence of dsRNA. In our system, apoptosis occurred in the absence of dsRNA. The reason for this difference is not immediately clear.

MATERIALS AND METHODS

Establishment of PKR-inducible cell lines

wt or mutant (K296R) (Meurs *et al.,* 1990) PKR cDNA was subcloned into the tetracycline-regulatable expression vector, pUHD 10–3 (Gossen and Bujard, 1992) to generate p10–3 PKR wt and PKR K296R constructs. A NIH3T3-derived cell line, S2–6 expressing the transactivator, tTA (Shockett *et al.,* 1995), was cotransfected with p10–3 (control) or with p10–3 PKR clones and selected for resistance to G418 (800 μg/ml). Cell lines were kept in the presence of 1 μg tetracycline/ml until induction. Cell lines were screened for PKR expression by immunoblotting. Seven clones were chosen for PKR wt expression, three for PKR K296R, and two for the control vector. All wt clones began to die 24 h upon tetracycline withdrawal, and two clones demonstrating tightly regulated induction were selected for this study.

DNA fragmentation analysis

Equal number of cells were seeded into 100-mm dishes, and tetracycline was withdrawn after cells had attached to the dish. At the times indicated in the figure legends, cells were harvested and lysed in 0.5 ml buffer A [10 mM Tris–HCl (pH 7.5), 400 mM NaCl, 1% SDS, 1 mM EDTA, and 0.2 mg/ml proteinase K]. For DNA isolation, lysates were incubated at 50°C for 2 h, treated twice with phenol/chloroform/isoamyl alcohol, followed by chloroform/isoamylalcohol extraction and precipitation with one volume of isopropanol. DNA was resuspended in Tris–EDTA buffer [10 mM Tris–HCl (pH 8.0) and 1 mM EDTA] and treated with RNase A for 15 min at 37°C.

Flow cytometry analysis

Attached and floating cells were pooled after withdrawal of tetracycline. Cells were gently suspended in 0.5 ml hypotonic fluorochrome solution (propidium iodide, 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100). DNA content was measured using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA) (Nicoletti *et al.,* 1991).

In vitro kinase assay

PKR wt and mutant were immunoprecipitated from cell extracts with an antiserum against human PKR (a kind gift of Dr. M. Matthews). An *in vitro* kinase reaction was performed as described previously (Koromilas *et al.,* 1992). The resulting products were separated on a SDS–7.5% PAGE.

Protein analysis

Cells (5×10^6) were washed twice with cold phosphate-buffered saline [140 mM NaCl, 15 mM KH_2PO_4 (pH 7.2), and 2.7 mM KCl] and incubated on ice with 500 ml of lysis buffer [10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 50 mM KCl, 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 0.2 mM phenylmethylsulfonylfluoride (PMSF), and 0.2 mg/ml aprotinin]. The lysate was centrifuged at 10,000 *g* for 5 min, and aliquots containing equal amounts of protein (determined by Bradford) were electrophoresed on an SDS–7.5% polyacrylamide gel (Sambrook *et al.,* 1989). Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) in 25 mM Tris–HCl (pH 7.5), 190 mM glycine, and 20% (v/v) methanol for 1 h at 100 V. Filters were incubated with 5% (w/v) non-fat dried skimmed milk powder in PBS and 0.2% Tween 20 for 30 min at room temperature and then with PBS plus 0.2% Tween 20 and 2% BSA containing a mouse monoclonal antibody to PKR (Laurent *et al.,* 1985) or rabbit antibody against mouse actin (ICN Biomedicals) for 1 h. After washings with PBS and 0.2% Tween 20, the filter was incubated for 1 h with HRP goat antibody against mouse immunoglobulin G (Amsersham). After washings the membrane was dried and exposed to an X-ray film.

Reverse transcription–PCR

Total RNA (1 μg) was reverse-transcribed using poly(dT) primer with MuLV reverse transcriptase (GIBCO)

as described by the manufacturer. PCR was performed on 2-μl aliquots from each cDNA reaction, using primer sets for detecting Fas (5′-CATCTCC-GAGAGTTTAAAG-CTGAGG, 5′-GTTTCCTGCAGTTTGTATTGCT-GGTTGC) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′-CCATGGAGAAGGCTGGGG, 5′-CAAAGTTGTCATGG-ATGACC) (Der *et al.,* 1997).

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