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Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling

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The dsRNA-dependent protein kinase (PKR) is considered to play a key role in interferon-mediated host defense against viral infection and conceivably malignant transformation. To investigate further the mechanisms of PKR-induced growth inhibition, we have developed tetracycline-inducible murine cell lines that express wild-type PKR or a catalytically inactive PKR variant, PKRΔ6. Following induction, the growth of the wild-type PKR-expressing cells was similar to that of cells transfected with vector alone, while cells expressing PKRΔ6 became malignantly transformed. Significantly, treatment with dsRNA caused the wild-type PKR-overexpressing cells to undergo programed cell death while, conversely, cells expressing PKRΔ6 were completely resistant. Our studies demonstrated that activation of PKR induces the expression of members of the tumor necrosis factor receptor (TNFR) family, including Fas (CD95/Apo-1) and pro-apopotic Bax. In contrast, transcripts representing Fas, TNFR-1, FADD (Fas-associated death domain), FLICE, Bad and Bax were ablated in cells expressing PKRΔ6. The involvement of the death receptors in PKR-induced apoptosis was underscored by demonstrating that murine fibroblasts lacking FADD were almost completely resistant to dsRNA-mediated cell death. Thus, PKR, a key cellular target for viral repression, is a receptor/inducer for the induction of pro-apoptotic genes by dsRNA and probably functions in interferon-mediated host defense to trigger cell death in response to virus infection and perhaps tumorigenesis.

Keywords: apoptosis/dsRNA-dependent protein kinase/FADD/tumorigenesis/viral infection

Introduction

The interferons (IFNs) are a family of related cytokines that can influence the regulation of cellular differentiation and viral replication through the induction of >30 responsive genes (reviewed by Sen and Ransohoff, 1994). The double-stranded (ds)RNA-dependent, serine/threonine protein kinase (PKR) is a key IFN-inducible gene that exists as a 68 000 and 65 000 Da protein in human and murine cells, respectively (Meurs *et al*., 1990; Icely *et al*., 1991). Following interaction with dsRNA, PKR autophosphorylates and, in turn, is able to catalyze phosphorylation of substrate targets, the most well characterized being the α-subunit of eukaryotic protein synthesis initiation factor 2 (eIF2α). This causes a dramatic inhibition of protein synthesis since phosphorylated eIF2α sequesters a key component of the translation machinery, eIF2B, required for the initiation of protein synthesis in the cell (Levin and London, 1978; Panniers and Henshaw, 1983). In addition to eIF2α, however, it is almost certain that other, as yet undefined substrates for PKR exist. For example, PKR has been reported to function as a second messenger in a number of signal transduction pathways, including those involving platelet-derived growth factor (PDGF) and nuclear transcription factor (NF)-κB (Kumar *et al*., 1994; Mundschau and Faller, 1995; Yang *et al*., 1995).

Several lines of evidence suggest that PKR plays an important role in the anti-viral response mediated by IFN. Following infection, PKR is known to be activated by many viruses, including influenza virus, adenovirus and human immunodeficiency virus type 1, (HIV-1) (Samuel, 1991; Katze, 1993; Clemens and Elia, 1997). Since phosphorylation of PKR caused by viral RNAs can affect dramatically the regulation of viral replication, numerous viruses appear to have developed strategies to suppress this kinase (Mathews, 1993). One such example is vaccinia virus, which encodes at least two proteins, E3L and K3L, to ensure the suppression of PKR (Beattie *et al*., 1991; Chang *et al*., 1992; Davies *et al*., 1992, 1993; Carroll *et al*., 1993). In addition to these viruses, adenovirus, hepatitis C virus, Epstein–Barr virus, influenza virus and HIV-1 have all reportedly devised mechanisms to inhibit PKR, underscoring the importance of the kinase in host defense mechanisms (Roy *et al*., 1990; Mathews and Shenk, 1991; Miller and Samuel, 1992; Seliger *et al*., 1992; Clemens *et al*., 1994; Langland *et al*., 1994; Lee *et al*., 1994; McMillan *et al*., 1996; Brand *et al*., 1997; Gale *et al*., 1997).

A number of studies have also shown that overexpression of wild-type PKR in mammalian and insect cells as well as yeast, results in the inhibition of cellular growth (Barber *et al*., 1992, 1993; Chong *et al*., 1992; Thomis and Samuel, 1992). At least in yeast, the mechanism of growth inhibition was demonstrated to be at the level of translation through inhibition of the yeast eIF2α homolog, SUI2 (Dever *et al*., 1992, 1993). Although the mechanism(s) of growth suppression remains to be clarified in higher order eukaryotic cells, the toxic phenotype is also presumed to involve the repression of translation through inhibition of the eIF2α pathway. Interestingly, the expression of catalytically inactive, dominant-negative PKR molecules causes the transformation of immortalized cells

(Koromilas *et al*., 1992; Meurs *et al*., 1993). Possibly, such PKR variants could function by inhibiting eIF2α phosphorylation to disrupt this important checkpoint in protein synthesis regulation and stimulate translation in the cell (Barber *et al.*, 1995; Donze *et al.*, 1995). These observations have led to the hypothesis that the growth-suppressive properties of PKR may be utilized in the anti-proliferative effects mediated by IFN (Lengyel, 1993).

Importantly, PKR has been proposed to play a role in the induction of programed cell death. For example, dsRNA alone has been reported to induce apoptosis in HeLa cells, although the exact mechanism remains undefined (Kibler *et al*., 1997). HeLa cells infected with recombinant vaccinia viruses expressing wild-type PKR, but not a catalytically inactive PKR variant, were also reported to undergo apoptosis (Lee and Esteban, 1993, 1994). Related studies have revealed that HeLa cells infected with vaccinia viruses lacking the putative PKR inhibitor E3L are more sensitive to host-mediated apoptosis compared with control viruses that encoded the inhibitor (Kibler *et al*., 1997). Furthermore, correlative evidence has been obtained using murine fibroblasts that lack PKR activity (Yang *et al*., 1995; Srivastava *et al*., 1998). Following treatment with actinomycin D, PKR-deficient cells were more resistant to dsRNA-mediated cell death than control cells (Der *et al.*, 1997).

To clarify further the mechanisms of PKR's growth-suppressive phenotype, we have established murine cell lines that inducibly express heterologous wild-type PKR or a dominant-negative PKR variant. By adopting this strategy, we have been able to monitor PKR's role in the regulation of cell growth, including possible mediation of apoptosis. Following induction, cells overexpressing wild-type PKR became extremely sensitive to apoptosis when treated with dsRNA. Interestingly, the mechanism did not appear to significantly involve translational suppression since the inhibition of protein synthesis in PKR-expressing cells did not give a similar apoptotic phenotype. In fact, treatment of PKR-expressing cells with dsRNA induced the expression of death receptors involved in mediating cell suicide, including Fas (CD95/Apo-1). Significantly, cells expressing dominant-negative PKR exhibited down-regulation of effector molecules involved in forming death-induced signaling complexes (DISC) such as Fas, TNFR-1, FADD (Fas-associated protein with death domain) and FLICE (caspase 8). These cells became transformed and were completely resistant to apoptosis induced by dsRNA or triggered by tumor necrosis factor-α (TNF-α) or Fas ligation. The mechanistic importance of the death receptors was emphasized by demonstrating that murine fibroblasts lacking FADD were significantly resistant to dsRNA-induced apoptosis. Our data confirm that in addition to being able to inhibit the eIF2α pathway, PKR functions as a receptor/inducer for dsRNA signaling and can trigger apoptosis.

Results

Inducible expression of functional PKR in 3T3 L1 cells

Functional, *in vivo* analysis of PKR has so far been hindered by an inability to obtain cell lines that overexpress this kinase. Therefore, to analyze the mechanism of growth suppression by PKR, we established novel cell lines that inducibly express wild-type or catalytically inactive dominant-negative PKR proteins. In our studies, we used a tetracycline- [or doxycycline (Dox)-] repressible transcriptional activator (tTA) that recognizes Tet operator sequences placed upstream of the PKR gene. Removal of Dox from the culture medium activates transcription in a dose-dependent manner (Gossen *et al*., 1995). Two independent cell lines expressing functional PKR or a non-functional PKR variant (PKRΔ6), isolated under non-induced conditions were characterized for this study. As an additional control, we used cell lines transfected with non-recombinant empty vector (pTRE) alone. To detect recombinant PKR expression, an anti-PKR monoclonal antibody that recognizes the human and not endogenous murine PKR (Laurent *et al*., 1985) was utilized. In the presence of Dox, only small amounts of heterologous PKR were detectable by immunoblot analysis with the anti-human PKR-specific monoclonal antibody, indicating that the tTA was largely suppressed (Figure 1A). Following removal of Dox from the medium, expression of both wild-type PKR and PKRΔ6 was highly induced within 1 day, peaking at ~1–2 days (Figure 1A, lanes 1–4 and 6–9). The level of heterologous PKR expression was comparable with that seen in non-IFN-treated HeLa cells (lanes 10 and 11). Analysis of the cell extracts with polyclonal antibody to PKR, which recognizes both the human and murine kinase, revealed that recombinant PKR was being induced ~6-fold more than the endogenous PKR (Barber *et al*., 1992; Figure 1B).

To confirm that the heterologous PKR was functional in the wild-type expressing cells, the recombinant kinase was immunoprecipitated using the anti-human PKR monoclonal antibody. Immunopurified PKR was then tested for activity using an *in vitro* kinase assay (Barber *et al*., 1995). Essentially, increasing amounts of dsRNA were added to immunoprecipitated PKR in the presence of a radiolabeled ATP source. As can be seen in Figure 1C, wild-type recombinant PKR retrieved from the 3T3 L1 cells autophosphorylated in the presence of dsRNA, although some background activity was evident even in the absence of activator (Figure 1C, lanes 1–5). Further, PKR was able to catalyze phosphorylation of its substrate, eIF2α, which was added exogenously to the kinase reaction. In the presence of high amounts of dsRNA, characteristically, PKR activity was reduced as reported previously, arguably since intermolecular PKR phosphorylation events are inhibited (Mathews, 1993; Romano *et al*., 1995). In contrast, although PKRΔ6 was recognized by the anti-PKR antibody, no kinase activity was observed, as has been shown previously (Koromilas *et al*., 1992) (Figure 1C, lanes 6–10). The data conclusively show that wild-type PKR and the PKRΔ6 variant are being inducibly expressed to high levels in 3T3 L1 cells. Furthermore, the wild-type kinase is fully functional, being able to autophosphorylate as well as phosphorylate the substrate eIF2α.

Growth characteristics of 3T3 L1 cells inducibly expressing PKR

Following induction, the growth characteristics of the PKR-expressing cells were monitored closely. Surprisingly, following induction, the wild-type PKR-expressing

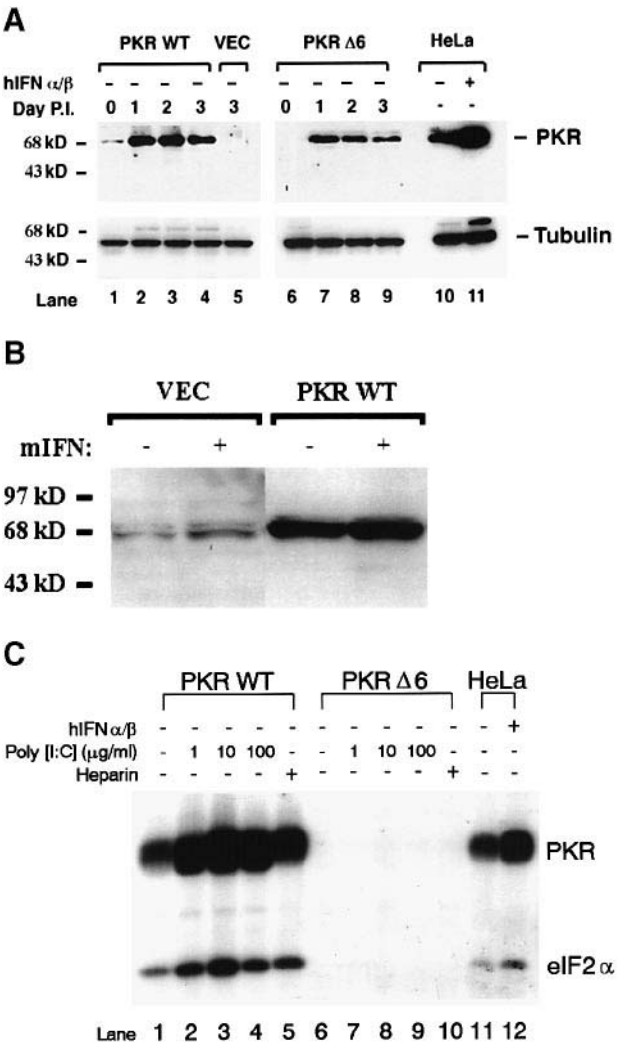


Fig. 1. (**A**) Inducible expression of PKR in 3T3-L1 cells. Cells carrying vector alone (pTRE), or tetracycline-controllable plasmids containing wild-type PKR (pTRE-PKR) or PKR variant (pTRE-PKRΔ6) were induced by withdrawal of Dox from the growth medium. The cells were harvested every 24 h post-induction (P.I.) for up to 3 days. Cell extracts (50 μg) were electrophoresed and analyzed by immunoblotting using a monoclonal antibody specific to human PKR as described in Materials and methods. Lane 5 is an extract from cells carrying the empty vector, pTRE, alone. IFN-treated and untreated HeLa cell extracts were used as a control to ensure PKR antibody specificity (lanes 10 and 11). Blots were stripped and re-probed with anti-tubulin antibody to ensure additionally that equal amounts of protein were being analyzed. (**B**) Expression of murine PKR in IFN-treated and untreated 3T3 L1 cells. 3T3 L1 cells were treated with 200 U of murine IFN-α (Sigma, MD) for 18 h. Extracts of the cells (50 μg) were electrophoresed and analyzed by immunoblotting using a polyclonal antiserum to PKR that recognizes the murine PKR species (Barber *et al*., 1992). Blots were also stripped and re-probed with anti-tubulin antibody. (**C**) Functional analysis of heterologous PKR expressed in 3T3 L1 cells. Extracts from cells expressing inducible wild-type PKR or PKRΔ6 proteins were immunoprecipitated using a human PKR-specific monoclonal antibody. Following washing, the immunopurified PKR was incubated with dsRNA [poly(I:C)] in the presence of [γ-^{32}P]ATP. To analyze whether PKR proteins could catalyze phosphorylation of substrate proteins, exogenous eIF2α was added to the reactions. Following electrophoresis, gels were subjected to autoradiography. Lanes 1–5, wild-type PKR; lanes 6–10, PKRΔ6; lanes 11–12, controls (immunoprecipitated PKR from IFN-treated HeLa cell extracts activated with 1.0 μg/ml dsRNA). Lanes 1 and 6, no dsRNA; lanes 2 and 7, 0.1 μg/ml dsRNA; lanes 3 and 8, 1.0 μg/ml dsRNA; lanes 4 and 8, 5 μg/ml dsRNA; lanes 5 and 10, heparin.

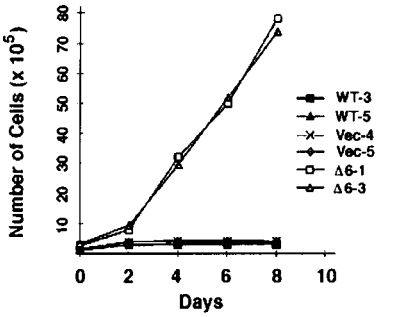
cells remained viable, even though high levels of functional PKR were being synthesized. Presumably this reflects the relatively low activity of the recombinant PKR in the 3T3 L1 cells. We observed that the PKR-expressing cells grew similarly to the vector control cells while the PKRΔ6-expressing cells grew much faster (Figure 2A). The doubling time of the PKRΔ6 cells was 24 h, in contrast to the wild-type PKR and control cells which doubled approximately every 37 h (Figure 2D). Morphologically, PKRΔ6 cells appeared to have a transformed phenotype, as has been described previously (Koromilas *et al*., 1992). To substantiate these observations, anchorage-independent growth studies were carried out. Accordingly, PKRΔ6 cells grew readily in soft agar, unlike cells expressing the wild-type PKR or controls, confirming the oncogenic nature of the PKR variant (Figure 2D). Inducing PKR in cells and then adding Dox after 6 days to reduce PKR synthesis had no effect on the growth rates of the control and wild-type PKR-expressing cells but reduced the growth rate of the PKRΔ6-expressing cells (Figure 2C). Thus the PKRΔ6 variant appeared to be mechanistically responsible for the transformed phenotype. However, the PKRΔ6 cells grew faster than the control cells even when maintained in the presence of Dox (Figure 2B) possibly because small amounts of the PKRΔ6 protein were being synthesized, even under repressed conditions as revealed by longer exposures of the immunoblots (data not shown).

As part of these studies, we examined the cell-cycle distribution of cells overexpressing wild-type or dominant-negative PKR proteins. Following induction, cells expressing heterologous PKR were harvested at 24 h intervals, for up to 10 days. DNA histogram analysis of the induced cells revealed that during exponential growth (day 1) ~56% of the control cells and 68% of the wild-type PKR-expressing cells were in G_1 compared with only 41% of cells expressing PKRΔ6 (Figure 2E). By day 6, in all the cell types, the number of cells in G_1 had increased slightly. However, the percentage of control and wild-type cells in G_1 was higher overall, suggesting that by this time a transient arrest had occurred in many of the cells. In contrast, a greater proportion of the PKRΔ6 cells were observed in S phase (16%) compared with the wild-type PKR-expressing (6.2%) or control cells (5.8%), consistent with the fact that the PKRΔ6 cells were undergoing constant cellular proliferation. In general, wild-type PKR-expressing cells retained a normal S-phase DNA content, though by day 6 markedly less cells were in G_2/M (16.9%) compared with the control cells (27.4%). These data are again consistent with the fact that a larger proportion of the wild-type PKR cells were arrested in G_1.

PKR induces apoptosis in 3T3 L1 cells

A number of studies have suggested that PKR plays a role in dsRNA-, viral- and stress-mediated apoptosis (Lee *et al.*, 1994; Yeung *et al*., 1996; Der *et al.*, 1997; Kibler *et al.*, 1997). However, several of these studies have only indirectly implied a role for PKR in programed cell death or have utilized viruses that express PKR, which complicates the interpretation of the results due to numerous other cellular and viral genes being induced at the same time. Thus, the precise role of PKR in cell lethality remains unclear. An advantage of the Tet-inducible system is that the physiological effects of a

A



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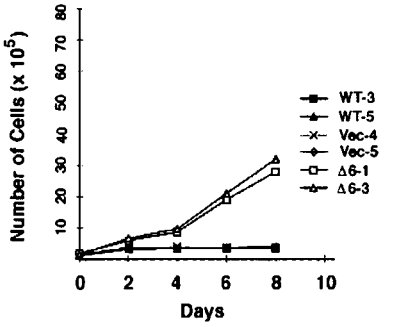

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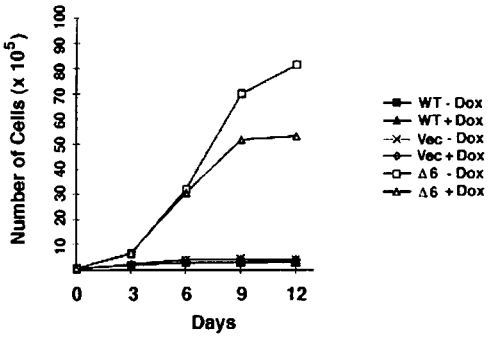


D

CLONE	CLONING EFFICIENCY (%)	DOUBLING TIME (HOURS)
VEC-4	0	37.5
VEC-5	0	38.1
WT-3	0	39.4
WT-5	0	40.5
Δ6-1	18.5	24.1
Δ6-3	17.7	25.4

E

	VEC-5		PKR WT-3		PKR Δ6-1	
DAY / PHASE	1	6	1	6	1	6
G_0-G_1	56.6	62.8	68.1	75.1	41.0	49.2
S	6.2	5.8	5.1	6.1	13.2	16.3
G_2-M	36.4	27.4	25.5	16.9	43.1	20.3

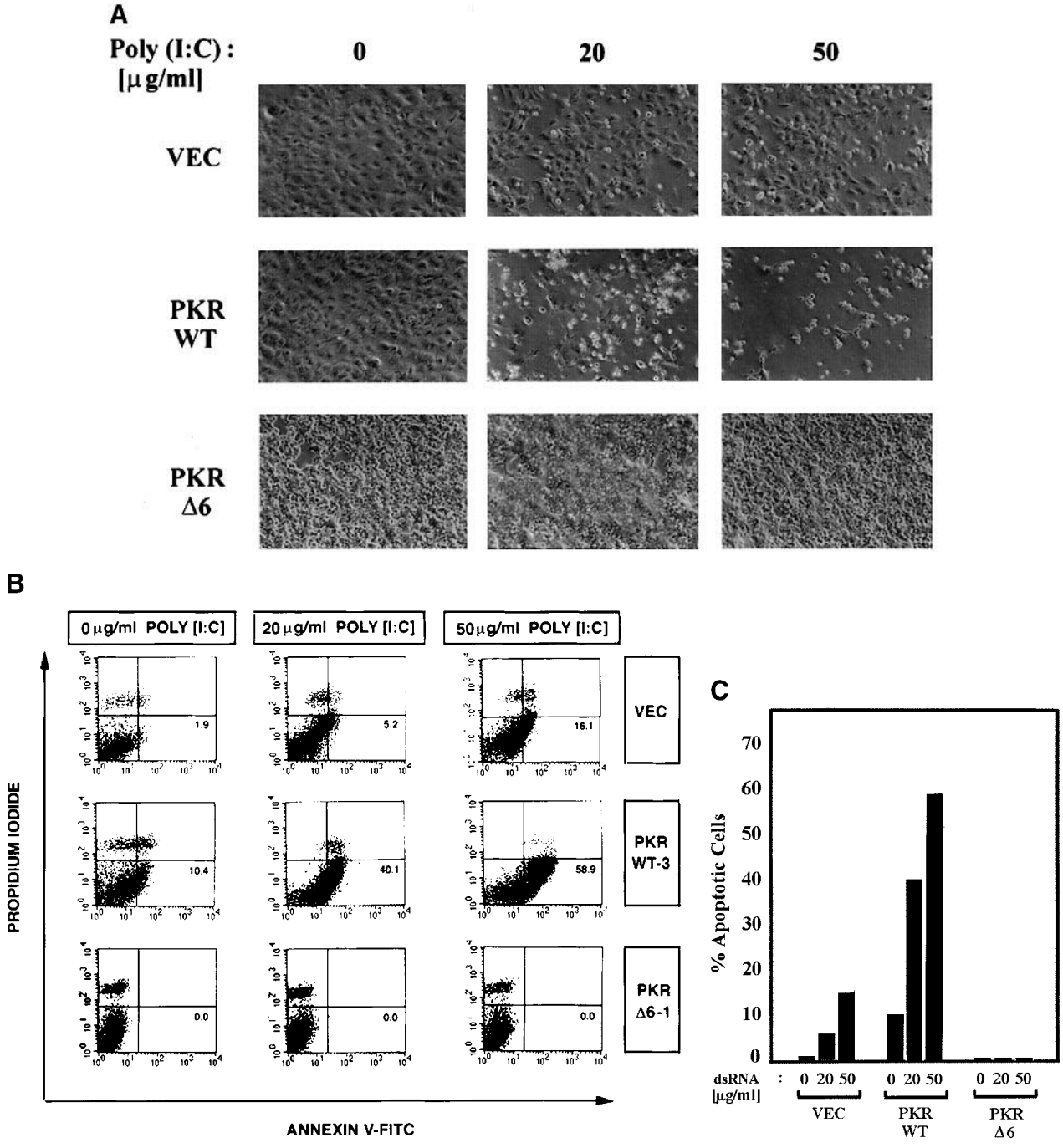
Fig. 2. Growth rates of 3T3 L1 cells expressing PKR proteins. Two independent cell lines expressing heterologous wild-type PKR, PKRΔ6 or cells carrying empty vector (pTRE) alone were seeded into culture dishes (5×10^4/dish). Cells were grown in the absence of Dox to induce expression of PKR (**A**) or in the presence of Dox to suppress expression of PKR (**B**). Control cells and cells overexpressing wild-type PKR or PKRΔ6 were also grown under inducing conditions (no Dox) for 3 days before Dox was added back to the medium. This had no effect on the control or wild-type PKR-expressing cells, but reduced the growth rate of cells expressing PKRΔ6 (**C**). The cells were trypsinized and counted every 2 days, in duplicate. The doubling time of the induced cells was determined (**D**) and the cells' ability to exhibit anchorage-independent growth determined as cloning efficiency as described in Materials and methods. (**E**) Cell cycle analysis of 3T3 L1 cells expressing wild-type PKR or PKRΔ6.

single gene can be observed in the absence of other ambiguous gene products. We were thus able to analyze for the first time the role of PKR in growth regulation, in the absence of other IFN- or viral-induced or -encoded genes. Accordingly, induced cells were transfected with dsRNA using Lipofectamine. Strikingly, within 24 h of dsRNA treatment, the PKR-expressing cells lost adherence to the tissue culture dish and exhibited all the characteristic hallmarks of apoptosis such as nuclear condensation and membrane blebbing (Figure 3A, middle row). This effect was not observed when the cells were treated with either Lipofectamine or dsRNA alone, which in the absence of Lipofectamine, did not appear to transfect these cells efficiently. Control cells containing empty vector were noted to be sensitive to dsRNA-mediated apoptosis, but much less so than the wild-type-expressing cells, as initially determined by morphological examination using phase-contrast microscopy (Figure 3A, top row). Furthermore, dsRNA induced apoptosis of the PKR-expressing cells in a dose-dependent manner, with greater amounts of dsRNA having a more dramatic effect on the cell. Significantly, dsRNA did not induce apoptosis of cells

expressing the PKRΔ6 variant (Figure 3A, bottom row). These cells remained completely resistant to apoptosis, regardless of the amount of dsRNA used in the experiments or time of incubation.

To confirm that PKR-overexpressing cells were undergoing apoptosis, annexin V staining was utilized, which detects phosphatidylserine (PS) loss to the outer membrane of apoptotic cells (Koopman *et al*., 1994). Propidium iodide staining additionally was utilized to determine the level of necrotic or late apoptotic cells. For these studies, cells in which PKR had been induced by Dox withdrawal were transfected with dsRNA and analyzed by fluorescein-conjugated annexin V staining using flow cytometry. Figure 3B and C shows that <2% of the untransfected control cells, carrying the vector alone, bound annexin V. This number increased to 16% following transfection with up to 50 μg/ml of dsRNA. In contrast, 10.4% of the untreated wild-type PKR-expressing 3T3 L1 cells showed evidence for annexin V staining, indicating that these cells have a slightly higher background incidence of apoptosis (Figure 3B and C). However, after transfection with up to 50 μg/ml dsRNA, nearly 60% of the wild-type PKR-overexpressing cells bound annexin V, demonstrating that these cells are acutely sensitive to dsRNA-mediated apoptosis (Figure 3B, middle row). These data were confirmed additionally by the TUNEL assay which detects DNA fragmentation in apoptotic cells (data not shown).

To establish whether endogenous PKR activation occurred during dsRNA-induced apoptosis, control or PKR-overexpressing 3T3 L1 cells were transfected with



dsRNA in the presence of ^{32}P-orthophosphate. Precipitation with poly(I:C) agarose allowed us to determine the activation state of the endogenous and heterologous PKR as well as the PKRΔ6. To ensure that we were measuring equal amounts of endogenous or recombinant PKR protein, including PKRΔ6, immunoblot analysis was carried out on the precipitated fractions using an anti-human PKR antibody (Figure 3D) or anti-murine PKR antibody (Figure 3E). Due to the relatively small amounts of murine PKR in the control cells, little activation of the endogenous kinase was observed following treatment with dsRNA (Figure 3D). Interestingly, not only was the PKRΔ6 inactive *in vivo*, but surprisingly the expression of the endogenous murine PKR was ablated in these cells (Figure 3E). In contrast, the recombinant PKR was clearly activated in the wild-type PKR-overexpressing cells following transfection of dsRNA (Figure 3D). Thus, dsRNA-induced apoptosis correlates with the *in vivo* activation of PKR.

To complement the above studies, endogenous levels of phosphorylated eIF2α were also measured in cells induced to express heterologous PKR using antibody that specifically recognizes the phosphorylated form of eIF2α [eIF2α is specifically phosphorylated on Ser51 (Choi *et al*., 1992; De Gracia *et al*., 1997)]. Figure 3E shows that following treatment with dsRNA, there are slightly higher levels of phosphorylated eIF2α in cells overexpressing PKR compared with control cells. Moreover, a significant proportion of eIF2α remained unphosphorylated in the wild-type PKR-overexpressing cells. In contrast, cells expressing the PKRΔ6 protein had 8-fold less phosphorylated eIF2α, even after dsRNA treatment.

PKR-mediated apoptosis may involve mechanisms other than the inhibition of protein synthesis

Conceivably, one potential mechanism of PKR-mediated apoptosis could involve phosphorylation of the PKR substrate eIF2α, which could lead to the inhibition of protein synthesis in the cell (Panniers and Henshaw, 1983). To determine whether the induction of apoptosis was simply a response to the inhibition of protein synthesis, the effects of cycloheximide (CHX) treatment in wild-type PKR, PKRΔ6 and control cells were determined. Treatment of cells with 2.5 μg/ml of CHX significantly inhibited protein synthesis in this cell type (Su *et al*., 1998; data not shown). However, despite 24 h of CHX treatment, control and wild-type PKR-expressing cells failed to show any signs of apoptosis (Figure 4A). Indeed,

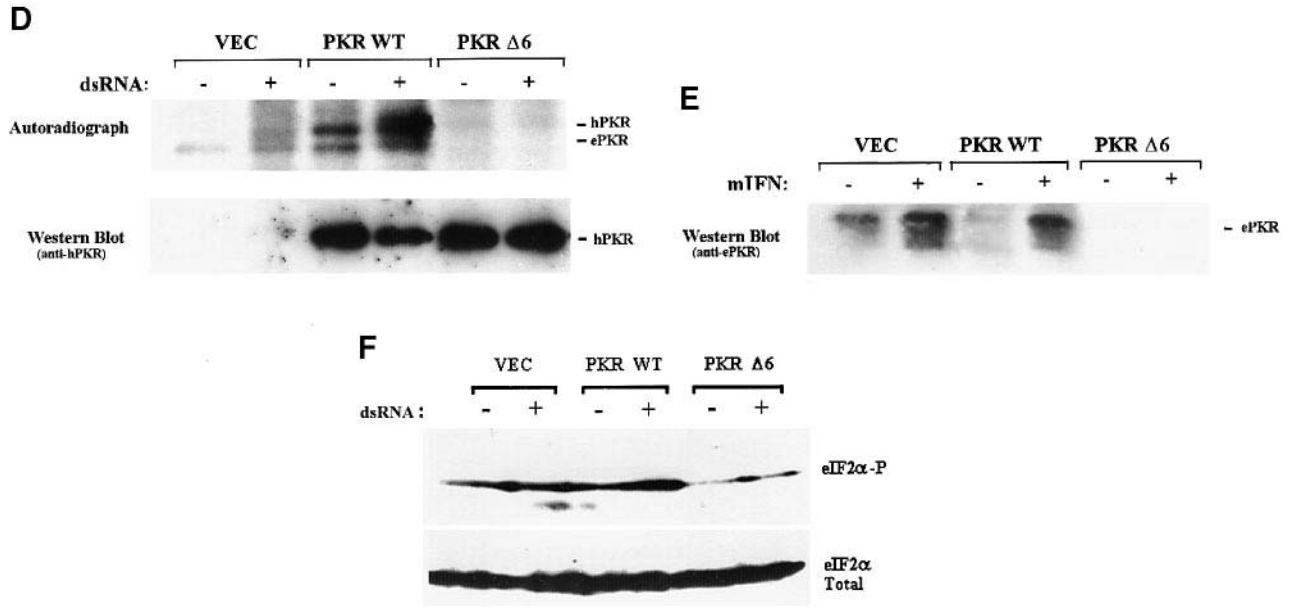


Fig. 3. 3T3 L1 cells inducibly expressing wild-type PKR but not PKRΔ6 are sensitive to dsRNA-mediated apoptosis. (**A**) Micrographs of 3T3 L1 cells treated with dsRNA. Cells inducibly expressing wild-type PKR, PKRΔ6 or carrying empty vector alone were seeded out into 35 mM culture dishes (2×10^5 cells/35 mM dish) and transfected 12 h post-induction with or without poly(I:C) in the presence of Lipofectamine. After 9 h, the transfection mix was replaced with fresh medium and the cells incubated for a further 16 h. Micrographs were taken at 40× magnification. (**B**) Annexin V staining of PKR-inducible cells. Cells inducibly expressing wild-type PKR, PKRΔ6 or carrying vector alone were transfected with or without dsRNA, as in (A). The cells were retrieved and stained with FITC-conjugated annexin V as well as propidium iodide and analyzed by flow cytometry. Cells undergoing apoptosis stain for annexin V and are shown in the lower right window. Cells in late apoptosis have compromised membranes and stain for both annexin V and propidium iodide (top right). Dead cells only incorporate propidium iodide (top left). Cells expressing PKRΔ6 were completely resistant to apoptosis induced by dsRNA. (**C**) Histogram of the number of PKR-inducible cells undergoing apoptosis compared with control cells or cells expressing PKRΔ6. (**D**) *In vivo* phosphorylation of PKR in cells treated with dsRNA. Cells expressing the inducible wild-type PKR, PKRΔ6 or carrying vector alone were labeled, 12 h post-induction, for 4 h, with [^{32}P]orthophosphate in the presence or absence of dsRNA/Lipofectamine. Cells were lysed and equal amounts precipitated with poly(I:C)–agarose. The heterologous, inducible, human PKR (M_r 68 kDa) is labeled hPKR, and the smaller endogenous murine kinase (65 kDa), ePKR (top panel). To ensure additionally that equivalent amounts of the precipitated extracts were measured, immunoblot analysis of equal fractions of the precipitated wild-type PKR and PKRΔ6 proteins was performed using an anti-human-specific monoclonal antibody (bottom panel). (**E**) Inhibition of endogenous PKR expression in cells containing PKRΔ6. Control cells carrying vector alone or cells overexpressing wild-type PKR or PKRΔ6 were treated with or without interferon, lysed and precipitated as above with poly(I:C)–agarose. Equal fractions were separated by SDS–gel electrophoresis and analyzed by immunoblot with a monoclonal antibody that recognizes the endogenous murine PKR. (**F**) Analysis of phosphorylated eIF2α levels in PKR-induced 3T3 L1 cells. Cells were lysed 24 h after treatment with dsRNA and analyzed by immunoblot using an antibody that recognizes total eIF2α, or with antibody that recognizes only the phosphorylated version of eIF2α (eIF2α-P).

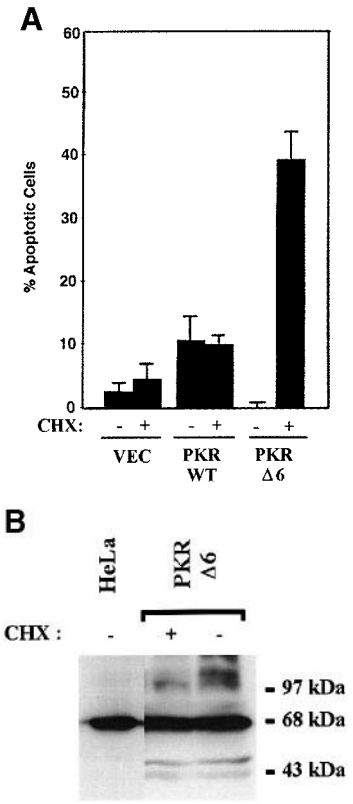


Fig. 4. Cycloheximide treatment of 3T3 L1 cells does not induce apoptosis. (**A**) Cells expressing wild-type PKR, PKRΔ6 or carrying vector alone were induced for 12 h and subsequently treated for up to 30 h with 2.5 μg/ml cycloheximide. The cells were analyzed by FACS using FITC-conjugated annexin V and propidium iodide. (**B**) Cycloheximide-treated and untreated 3T3 L1 cells (PKRΔ6) were lysed after 24 h, and cells extracts analyzed by immunoblot using a PKR-specific monoclonal antibody as described in Materials and methods.

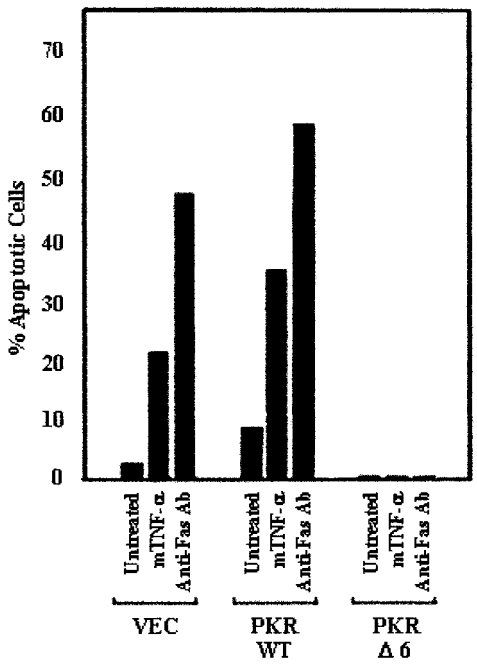


Fig. 5. Cells expressing the dominant-negative PKRΔ6 variant are resistant to TNF-α- and Fas-mediated cell death. PKR-induced cells were treated with 10 μg/ml TNF-α or anti-Fas Jo-2 antibody for 24 h and analyzed for apoptosis. The percentage of cells undergoing cell death was determined by annexin V staining.

the cells appeared to be in cytostasis, with a lower apoptotic percentage being detected by annexinV staining (Figure 4A). These data suggest that the observed mechanism of dsRNA/PKR-induced cell death is unlikely to be due solely to the global inhibition of protein synthesis.

In contrast to these observations, CHX induced apoptosis in ~38% of the PKRΔ6-expressing cells (Figure 4A). The mechanisms responsible for this remain unclear. However, to rule out the possibility that CHX treatment may inhibit synthesis of PKRΔ6, which may be necessary to prevent apoptosis, CHX-treated and untreated PKRΔ6-expressing cells were analyzed by immunoblot analysis to measure PKRΔ6 protein levels. As can be seen in Figure 4B, PKRΔ6 levels were not affected by CHX treatment, strongly suggesting that the mode of CHX-induced apoptosis is unlikely to be due to the inhibition of the production of the PKRΔ6 protein. It is possible that CHX may be inhibiting the production of an unidentified death repressor protein that is induced or perhaps activated in the PKRΔ6-transformed cells (Martin and Green, 1995).

Induction of the Fas (CD95/Apol) by PKR

It remained feasible that activated PKR could mediate apoposis by inducing known death agonists. As a start to investigating these possibilities, PKR-inducible cell lines were treated with a pro-apoptotic Fas-ligating antibody (Jo-2) or TNF-α. Our results indicate that in the absence of dsRNA treatment, wild-type PKR-overexpressing cells were not significantly more sensitive to Fas antibody or TNF-α than the control cells (Figure 5). However, a greater percentage of wild-type PKR-overexpressing cells, sequentially treated with dsRNA and anti-Fas Ab or TNF-α, were apoptotic compared with controls. (>20%, data not shown). Importantly, we found that cells expressing the PKRΔ6 variant were not only insensitive to dsRNA-mediated cell death, but were also completely resistant to apoptosis induced by either Fas ligand or TNF-α (Figure 5). Sensitivity to these apoptotic stimuli (~40%) was regained after suppression of the PKRΔ6 protein with Dox (data not shown).

To clarify further the mechanisms underlying these observations, we analyzed the protein levels of the death receptor Fas as well as anti-apoptotic Bcl-2 and pro-apopotic Bax in cells overexpressing the wild-type PKR or PKRΔ6 (Vaux *et al.*, 1988; Itoh *et al.*, 1991; Oltvai *et al.*, 1993; Nagata, 1997). Figure 6 demonstrates that following treatment with dsRNA, Fas protein levels were greatly increased in the wild-type PKR-overexpressing cells as compared with cells containing the vector alone. We estimate that Fas levels were induced ~10-fold more in the cells overexpressing wild-type PKR than in the controls (Figure 6B). The ability of PKR to regulate Fas expression was further reinforced since we found that this key death receptor was ablated dramatically in cells expressing the PKRΔ6 (Figure 6A). In addition to containing higher levels of Fas, wild-type PKR-overexpressing cells also contained >3.5-fold higher levels of Bax as compared with the control cells, although this protein did not seem to be induced as notably by dsRNA (Figure 6A). Interestingly, similarly to Fas, Bax was also undetectable in cells expressing PKRΔ6. Furthermore, levels of Bcl-2,

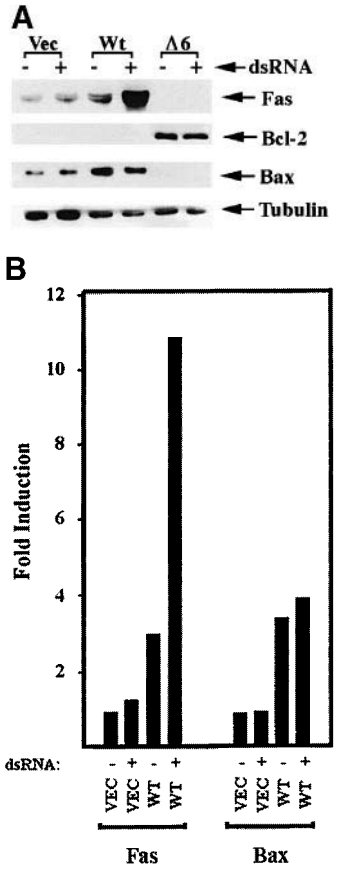


Fig. 6. Activation of PKR induces Fas/CD95 expression. (**A**) PKR-induced cells were treated with (+) or without (–) dsRNA. After 24 h, the cells were lysed and extracts analyzed by immunoblot using antibodies specifically recognizing Fas, Bax, Bcl-2 and tubulin. (**B**) Histogram showing the level of Fas and Bax induction. Measurements of Fas and Bax were determined by densitometric scanning, relative to tubulin.

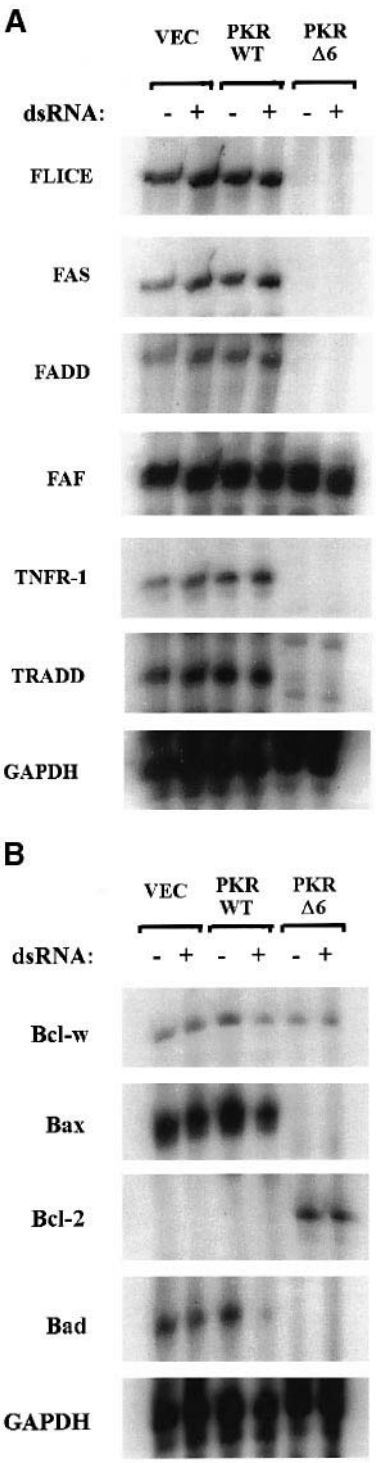


Fig. 7. RNase protection assay of cells inducibly expressing PKR. 3T3 L1 cells inducibly expressing wild-type PKR or PKRΔ6 were treated with dsRNA. Cells were collected during early apoptosis, after 18 h. Total RNA was prepared from the cells and mRNA species representing the above genes quantified using radiolabeled RNA probes as described in Materials and methods.

which inhibit apoptosis and promote cell survival, were significantly higher in the PKRΔ6-expressing cells than in control or wild-type PKR-overexpressing cells. These data demonstrate that activation of PKR augments the synthesis of key death agonists, Fas and Bax, that are involved in triggering apoptosis via two separate mechanisms, namely by (i) death receptor-triggered formation of the death-initiating signaling complex (DISC) and (ii) the induction of mitochondrial permeability transitions (MPTs) and cytochrome *c* release, respectively (Kluck *et al*., 1997; Li *et al*., 1997; Nagata, 1997; Yang *et al*., 1997; Pastorino *et al*., 1998; Scaffidi *et al*., 1998). Conversely, PKR variants that confer resistance to apoptosis are able selectively to abrogate the synthesis of Fas and Bax, as well as increase Bcl-2 expression.

To clarify whether the alterations in protein levels were mediated through translation or transcriptional regulation, we utilized the ribonuclease protection assay to measure the mRNA levels of several genes involved in signaling cell death. Figure 7A reveals that prior to treatment with dsRNA, there was a slightly higher level of Fas in the wild-type PKR-overexpressing cells compared with the controls carrying vector alone. Importantly, Fas mRNA levels increased 2-fold in both the control and wild-type PKR-expressing cells following transfection with dsRNA. In addition to these observations, we found that FADD, FLICE/caspase-8 and TNFR-1 mRNA levels also increased slightly in the control and wild-type-overexpressing cells following treatment with dsRNA (Figure 7A and B). Importantly, the mRNA of Fas ligand was undetectable in all cells analyzed in the presence or absence of dsRNA (data not shown).

It is also noteworthy that cells expressing the dominant-negative PKRΔ6 had dramatically reduced levels of not only Fas mRNA, but also mRNA representing TNFR-1, TRADD, FADD, FLICE/caspase-8, Bax and Bad. These data support the results of the protein analysis shown in Figure 6, although Fas protein levels in the wild-type PKR-overexpressing cells were induced more notably than the mRNA levels. This may be due to the greater stability of Fas protein versus its mRNA under dsRNA-induced apoptotic conditions. Interestingly, Bcl-2 mRNA was readily detectable in the PKRΔ6-expressing cells, though not in the control or wild-type PKR-overexpressing cells. FAF mRNA levels are the same in all the treated cells and served as an additional control to emphasize the specificity of the selective mRNA ablation mediated by the PKRΔ6 protein (Chu *et al*., 1995). Thus, our data clearly demonstrate that activation of PKR results in the transcriptional induction of Fas and possibly other molecules involved in death signaling, leading to apoptosis. Conversely, the expression of a dominant-negative PKR variant renders cells resistant to a variety of apoptotic stimuli by transcriptionally repressing the same genes and inducing others such as *Bcl-2*.

FADD-deficient cells are resistant to dsRNA-induced apoptosis

To elucidate further the mechanistic role of the death receptors in PKR-induced apoptosis, we determined whether FADD was involved in the signaling cascade leading to cell death. FADD is an essential downstream signal transducer required for apoptosis following ligation of Fas or TNFR-1, and possibly other as yet unknown death receptor molecules (Chinnaiyan *et al*., 1995; Yeh *et al*., 1998). We evaluated the susceptibility of murine fibroblasts lacking *FADD* ($FADD^{-/-}$) to dsRNA-induced cell death. Following treatment with dsRNA, *FADD*-deficient cells demonstrated markedly less apoptosis (<20%) in contrast to normal cells (>80%) (Figure 8). As a control, we treated the $FADD^{+/+}$ cells or the $FADD^{-/-}$ cells with TNF-α. More than 80% of the $FADD^{+/+}$ cells underwent apoptosis following this treatment, as compared with <20% of cells lacking *FADD*. Normal or $FADD^{-/-}$ cells were also doubly transfected with wild-type PKR-expressing Tet-inducible plasmids or empty vector alone, and then with dsRNA. Our results again indicated that cells deficient in *FADD* were significantly resistant to PKR-mediated cell death (<20%) compared with normal cells (>90%) (Figure 8).

The role of FADD in PKR-mediated apoptosis was confirmed further by demonstrating that PKR-overexpressing cell lines transiently transfected with the cDNA of dominant-negative *FADD* reduced dsRNA-mediated apoptosis ~20% less than empty vector control (data not shown). We postulate that the low level protection conferred by the dominant-negative *FADD* cDNA in the PKR-overexpressing cells was due to inefficient transfection, since HeLa cells stably transfected with dominant-negative *FADD* molecules are also nearly completely resistant to dsRNA-mediated apoptosis, similarly to the $FADD^{-/-}$ cells (data not shown).

To elucidate further the mechanistic role of the death receptors in PKR-mediated apoptsis, we utilized soluble Fc-Fas decoys and anti-Fas ligand antibodies to inhibit

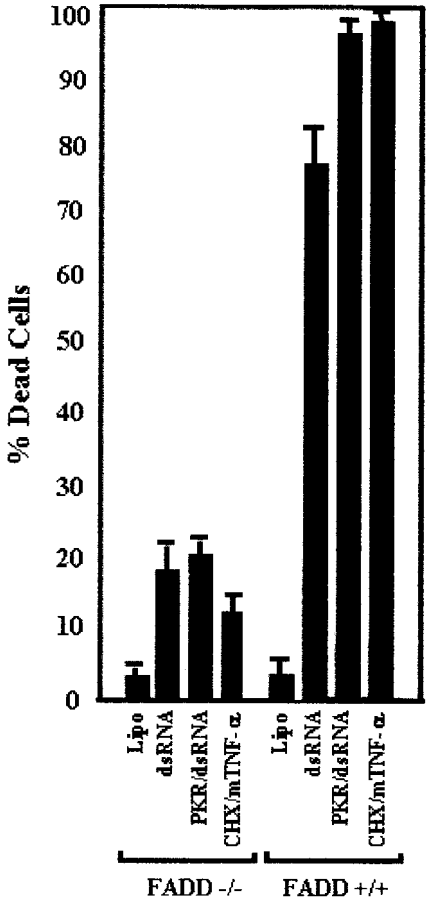


Fig. 8. Murine embryonic fibroblasts lacking the death receptor signal transducer FADD are resistant to dsRNA-mediated apotosis. Control cells (+/+) or cells lacking FADD (–/–) were transfected with dsRNA alone for 9 h or co-transfected with Tet-inducible vectors (pTRE) or vectors carrying the wild-type PKR (pTRE-PKR) and pUHD15 expressing the Tet-regulated transcriptional activator. As a control, cells were treated with 10 μg/ml TNF-α. After 24 h incubation, the amount of apoptotic cells was determined by trypan blue exclusion assay. As additional controls to assess the transfection efficiency (39%), cells were transfected with plasmids expressing green fluorescent protein and scored after 48 h using fluorescence microscopy.

putative Fas ligation on the cell surface (Kayagaki *et al*., 1997; MacFarlane *et al*., 1997). Interestingly, these inhibitors failed to protect the PKR-overexpressing cells from dsRNA-mediated cell death (data not shown). That Fas ligation via Fas–Fas ligand interactions may not be the cause of PKR-induced apoptosis would support our findings described above, that Fas ligand levels were undetectable in 3T3 L1 cells. It is therefore possible that these inhibitors are ineffective because they normally block upstream from PKR-induced mechanisms responsible for triggering apoptosis. Conceivably, the activation of PKR may promote the formation of DISCs (possibly by enhancing the levels of Fas, FADD and FLICE/caspase-8), as well as promote MPTs and cytochrome *c* release (by up-regulating Bax), as our protein and mRNA analysis suggests (Reed, 1997a; Scaffidi *et al*., 1998). It is noteworthy that the overexpresson of Fas, FADD and FLICE by themselves is sufficient to induce apoptosis (Chinnaiyan *et al*., 1995; Muzio *et al*., 1996; Yeh *et al*., 1998). Collectively, all of these data highlight that FADD and

the activity of the DISC play a crucial role in PKR-induced apoptosis.

Discussion

PKR is considered to function as a key effector molecule in the anti-proliferative and anti-viral properties mediated by IFN (Meurs *et al*., 1990). These conclusions are supported since a multitude of viruses specifically encode proteins to inhibit PKR, many of which have been shown to be necessary for viral resistance against IFN-mediated host defense mechanisms (Samuel, 1991; Mathews, 1993). Attempts to further resolve PKR's role in the many biological effects mediated by IFN have included trying to establish cell lines that functionally overexpress this kinase in the absence of any other IFN- or viral-induced genes. However, previous studies revealed that PKR exerts a growth-suppressive phenotype when heterologously synthesized in eukaryotic cells, thus preventing the establishment of clonal cell lines for study (Barber *et al*., 1992; Koromilas *et al*., 1992). One plausible explanation offered for these observations was that PKR may be phosphorylating eIF2α and inhibiting protein synthesis in the cell. For example, cellular RNAs containing extensive secondary structures may activate the heterologous PKR (Barber *et al*., 1993). Previous attempts to overexpress PKR in eukaryotic cells utilized baculoviruses, retroviral expression vectors or vectors that generated numerous transcripts of viral origin which could have contributed towards activation of the heterologous kinase (Barber *et al*., 1992, 1993; Koromilas *et al*., 1992). To circumvent these problems, we have developed constructs that contain the kinase under control of an inducible promoter. Surprisingly, following induction, the wild-type PKR-expressing cells did not undergo rapid cell death. One possible explanation for this may be that the recombinant PKR failed to kill the 3T3 L1 cells because insufficient endogenous cellular dsRNA was available to activate this kinase. It is also possible that the expression vectors, carrying the *PKR* gene, may not synthesize dsRNA of the optimal configuration or amount necessary for PKR activation (Kaufman *et al*., 1987, 1989; Manche *et al*., 1993). Our findings also imply that at least *in vivo*, PKR's own transcript is not a good activator of the kinase.

In contrast to PKR's growth-suppressive properties, expression of the catalytically inactive PKRΔ6 variant caused 3T3 L1 cells to become transformed. However, the mechanisms underlying the transforming properties of the PKRΔ6 remain unclear (Koromilas *et al*., 1992). It is highly likely that the PKR variant can compete with the endogenous PKR for target substrates such as eIF2α (Hershey, 1991; Donze *et al*., 1995). In agreement with this hypothesis, we demonstrate extremely low levels of eIF2α phosphorylation in PKRΔ6-expressing cells. Inhibiting eIF2α phosphorylation could result in the stimulation of protein synthesis in the cell (Kaufman *et al*., 1989). Consequently, genes that promote tumorigenesis may be overexpressed if their regulation is governed at the translational level (Weinberg, 1991; Sonenberg, 1994; Morris, 1995). In this context, it is noteworthy that enforced expression of an eIF2α (S51A) variant, resistant to phosphorylation, transforms NIH 3T3 cells (Donze *et al*., 1995). However, besides influencing the regulation of translation, it remains feasible that PKR could play a role in affecting other mechanisms controlling cell growth. PKR has been reported to function in a number of cellular signaling pathways, including those involving TNF-α, PDGF, NF-κB, STAT-1 and interleukin-3 (Ito *et al*., 1994; Munsdchau and Faller, 1994; Yang *et al*., 1995; Yeung *et al*., 1996; Kumar *et al*., 1997; Wong *et al*., 1997). It is, therefore, highly probable that physiological substrates of PKR other than eIF2α exist and, by competing for these targets, PKRΔ6 may affect dramatically the regulation of cellular growth. In addition, PKRΔ6 may neutralize the function of the wild-type kinase, either by forming inactive heterodimers with the endogenous PKR or by competing with the endogenous PKR for activators such as dsRNA. Conceivably, PKRΔ6 could also interact with other dsRNA-binding proteins that function in the regulation of cellular growth, such as those of the 2–5A system (St Johnston *et al*., 1992; Barber *et al*., 1995; Romano *et al*., 1995; Clemens and Elia, 1997; Zhou *et al*., 1997).

Although induction of the wild-type PKR in 3T3 L1 cells surprisingly did not induce rapid cell death in the absence of dsRNA, our studies clearly demonstrate that PKR is a key receptor/inducer in dsRNA-mediated apoptosis. For example (i) cells induced to overexpress only wild-type PKR were significantly more sensitive to dsRNA-mediated apoptosis than control cells, (ii) dsRNA-induced apoptosis correlated with the *in vivo* activation of PKR and (iii) the expression of a catalytically inactive PKR variant rendered cells completely resistant to dsRNA's effects. Importantly, PKR-overexpressing cells were also 3-fold more sensitive to apoptosis induced by influenza virus and parainfluenza virus, compared with control cells, while cells expressing PKRΔ6 were again resistant (data not shown). Our conclusions confirm a number of studies that have implicated a role for PKR in mediating programmed cell death in response to dsRNA and selected viral infections (Takizawa *et al*., 1996; Der *et al*., 1997; Kibler *et al*., 1997; Lee *et al*., 1997; King and Goodbourn, 1998; Srivastava *et al*., 1998).

The question thus arises as to how PKR induces apoptosis. One explanation is that PKR could inhibit the eIF2α pathway. This was supported by the observation that cells transiently transfected with an eIF2α (S51A) variant that is resistant to phosphorylation (and could enhance translation rates) were partially protected from apoptosis mediated by stimuli such as dsRNA and TNF-α (Srivistava *et al*., 1998). Furthermore, transient expression of PKR or a variant of eIF2α (S51D) which mimics a phosphorylated eIF2α molecule (both may conceivably inhibit translation) could reportedly induce apoptosis (Srivistava *et al*., 1998). It was proposed that eIF2α phosphorylation may suppress genes involved in promoting cell survival. Though we cannot entirely rule out that the expression of selected apoptotic genes may be regulated at the translational level, at least Bcl-2 expression in PKRΔ6-expressing cells appears to be induced largely at the level of transcription (Figure 7). It is also interesting to note that following treatment with CHX, PKRΔ6-expressing cells also demonstrated significant apoptosis. This is reminiscent of apoptosis of rapidly proliferating malignant cells that are treated with inhibitors of protein synthesis (Martin and Green, 1995). Possibly, the expression of anti-apoptotic genes is repressed following this

treatment. In contrast, inhibiting protein synthesis with CHX in PKR-overexpressing cells that are not transformed did not appear to enhance apoptosis.

Our findings indicate that activation of PKR need not necessarily lead to a global inhibition of protein synthesis in the cell, as demonstrated by the enhanced expression of Fas. Although it is possible that PKR activation may inhibit the translation of some cellular transcripts, it is plausible that activation of PKR triggers apoptosis via mechanisms independent of translational regulation. This is supported by the observation that Fas mRNA and protein levels as well as TNFR-1 and Bax levels are moderately higher in cells overexpressing the wild-type PKR. Conversely, in cells expressing PKRΔ6, Fas, TNFR-1 and Bax levels were found to be undetectable at both the protein and mRNA level. These observations are in contrast to the findings of Srivastava *et al*. (1998), who did not see any variation in Fas mRNA levels in control cells or cells expressing a PKR (K296P) variant (with a mutation in catalytic domain II) following treatment with dsRNA. This discrepancy may be explained by the fact that PKR molecules with mutations in catalytic domain II are able to be phosphorylated *in trans* by functional PKR and may not be *bona fide* transdominant-negative variants like the PKRΔ6 (Figure 3D; Barber *et al*., 1995; Romano *et al*., 1995).

Our data indicate that Fas and almost certainly other transcripts avoid activating PKR and being repressed at the level of translation. Conceivably, only mRNAs with extensive secondary structures such as certain viral-encoded mRNAs and perhaps some cellular mRNAs would cause the activation of PKR and inhibit their translation (Samuel, 1991). Although we cannot entirely rule out the possibility that activation of PKR may inhibit the translation of proteins involved in negatively regulating Fas expression or possibly proteases that facilitate Fas degradation, it is plausible that PKR may directly or indirectly activate proteins involved in Fas transcription. Alternatively, PKR-mediated Fas expression may be similar to the paradigm in yeast where the amount of phosphorylated eIF2α governs whether translation of the transcriptional activator GCN4 will occur at the authentic initiation codon or at alternative upstream open reading frames (Hinnebusch, 1996). In this situation, GCN4 translation is efficient only when significant amounts of eIF2α are phosphorylated. Although Bax as well as Fas contain upstream AUGs, further experiments will be required to test this hypothesis (Oltvai *et al*., 1993; Behrmann *et al*., 1994).

Our data demonstrating that PKR can induce Fas expression are in agreement with Takizawa *et al*. (1996), who implicated PKR in Fas induction following the infection of HeLa cells with influenza virus. However, our attempts to inhibit Fas ligation with soluble Fc-Fas decoys, or blocking antibodies to Fas or Fas ligand, did not prevent dsRNA-mediated cell death in the PKR-overexpressing cell lines. Conceivably, PKR could promote apoptosis by up-regulating the expression of a number of components of the DISC as our mRNA analysis suggests. That this mechanism may activate FLICE/caspase-8 without obvious up-regulation of the Fas ligand and/or Fas ligation is supported by two lines of evidence (i) enforced overexpression of FADD or TRADD alone was shown to induce apoptosis and (ii) oligomerization of caspase-8 with its adaptor molecule is sufficient to strongly trigger apoptosis (Chinnaiyan *et al*., 1995; Hsu *et al*., 1995; Muzio *et al*., 1998). Support for this paradigm also comes from the recent demonstration that autoactivation of pro-caspase-9 is possible by overexpression of Apaf-1 (Srinivasula *et al*., 1998). It is also noteworthy that PKR-mediated induction of the death receptors, such as Fas, may facilitate T-cell fratricide and additionally play a key role in the immune response to viral infection (Nagata, 1997).

The importance of the induction of the death receptors and DISC activation is underscored by demonstrating that cells lacking the key death receptor signal transducer FADD were resistant to dsRNA-mediated apoptosis. FADD is known to interact with Fas, as well as TNFR-1 and possibly other death receptors not yet characterized (Chinnaiyan *et al*., 1995). Additionally, the apoptosis-resistant phenotype of the PKRΔ6-expressing cells is associated with the complete down-regulation of mRNA representing Fas, TNFR-1, TRADD, FLICE/caspase-8 and FADD. It is not yet clear whether the transcriptional ablation of these pro-apoptotic genes and resistance to cell death are related to a loss of endogenous PKR activity or to a gain-of-function mechanism where PKRΔ6 could sequester PKR substrates involved in the control of apoptotic gene regulation. Since our preliminary data indicate that PKR null fibroblasts retain sensitivity to selected apoptotic stimuli compared with PKRΔ6-expressing 3T3 L1 cells, the PKRΔ6 product may indeed confer some gain-of-function phenotype (data not shown). Further analysis is underway to clarify this issue. Comparatively, other reports have shown that in the presence of actinomycinD, PKR-deficient fibroblasts are defective in their TNF-α-mediated apoptotic response, but not in TNF-α-mediated NF-κB signaling which promotes cell survival (Der *et al*., 1997).

The importance of FADD in mediating viral-induced apoptosis is also emphasized since, similarly to PKR, this death transducer is a key target for viral inhibitory proteins. Indeed, a new family of viral-encoded products referred to as FLIPs (FLICE inhibitory proteins) has evolved specifically to inhibit death receptor-mediated apoptosis (Bertin *et al*., 1997; Thome *et al*., 1997). FLIPs contain two death effector domains that interact with FADD to inhibit recruitment and activation of the protease FLICE/caspase-8. Cells expressing FLIPs, which are encoded by selected members of the herpesvirus family, were protected against apoptosis induced by Fas ligand or TRAIL-R (Bertin *et al*., 1997; Thome *et al*., 1997). In light of our observations, FLIPs may also prevent PKR-induced cell death, since dsRNA-induced apoptosis appears to be mediated largely through FADD-associated DISC activity.

It is also feasible that PKR's induction of Bax and of apoptosis could involve Bax-regulated MPTs and possible release of cytochrome *c*. Subsequent recruitment of Apaf-1 and sequential cleavage and activity of caspase-9 and -3 may contribute further to DISC-mediated apoptosis (Liu *et al.*, 1996; Li *et al.*, 1997; Salvesen and Dixit, 1997). Bcl-2 (death antagonist) and Bax (death agonist) reside in the outer mitochondrial membrane, and may act as channel proteins regulating ion and cytochrome *c* transport (Vaux *et al*., 1988; Oltvai *et al*., 1993; Knudson and Korsmeyer,

1997; Reed, 1997a; Yang *et al.*, 1997). While high Bax levels have been shown to facilitate MPT and efflux of cytochrome *c*, Bcl-2 and Bcl-XL inhibit the cytosolic accumulation of cytochrome *c* and the ensuing activity of Apaf-1 (Reed, 1997b; Vander Heiden *et al*., 1997; Hu *et al*., 1998; Pan *et al*., 1998). In our studies, we demonstrated that PKR-overexpressing cells contained high levels of Bax and low levels of Bcl-2, while in cells expressing PKRΔ6, Bax was ablated and Bcl-2 levels were high. Thus, high ratios of Bax to Bcl-2 in the PKR-overexpressing cells may disregulate MPTs, and facilitate the cytosolic accumulation of cytochrome *c*. Recently, Fas-activated FLICE/caspase-8 has been shown to recruit mitochondrial input into Apaf-1-mediated activation of the executioner, caspase-3, via cleavage of Bid, a BH3 domain-containing pro-apoptotic protein (Kluck *et al*., 1997; Vander Heiden *et al*., 1997; Yang *et al*., 1997; Amarante-Mendes *et al*., 1998; Luo *et al*., 1998; Scaffidi *et al*., 1998). However, using caspase-9-deficient mice, it has also been shown, at least in thymocytes and splenocytes, that activated caspase-8 can bypass the mitochondria and directly activate targets such as caspase-3 (Hakem *et al*., 1998; Kuida *et al*., 1998). Cleavage and activation of Bid and subsequent efflux of cytochrome *c* may depend on the levels of available caspase-8 and on the cell type, as recently proposed (Hakem *et al*., 1998; Kuida *et al*., 1998; Scaffidi *et al*., 1998). This may explain further why Bcl-2 blocks Fas-induced cell death in some cell types and not others (Adachi *et al*., 1997). Since cells lacking FADD are resistant to PKR-induced apoptosis, our data suggest that the activity of both pathways is interrupted in these cells.

While we have demonstrated that PKR is a key player in IFN-induced apoptosis, it is noteworthy that PKR is not the only IFN-inducible gene that may play a role in mediating cell death. Thymocytes and fibroblasts generated from IFN-regulated RNase L-deficient mice were shown to be less sensitive to apoptosis induced by staurosporine, Fas ligand and TNF-α (Zhou *et al*., 1997). Further, overexpression of RNase L in NIH 3T3 cells decreased cell viability and also induced an apoptotic phenotype (Castelli *et al*., 1997). The transcription factor IRF-1 is also known to confer growth-suppressive properties (Harada *et al*., 1993). These findings suggest that IFN and PKR probably influence several molecular determinants to induce apoptosis. These complex mechanisms have almost certainly led to the evolution of the formidable array of viral-encoded anti-apoptotic genes that prevent host defense-initiated cell death (Crook *et al*., 1993; Debbas and White, 1993; Tewari and Dixit, 1995; Bertin *et al*., 1997; Teodoro and Branton, 1997; Thome *et al*., 1997). PKR is a key target for viral inhibition, following infection of the cell (Samuel, 1991; Katze, 1993; Mathews, 1993). Our data indicate that viruses probably select PKR for repression, not only to escape blocks in translation, but also to avoid initiating host-mediated programmed cell death.

Materials and methods

Plasmid construction

Construction of PKRΔ6 has been described previously. Essentially, PKRΔ6 lacks six amino acids (361–366) between catalytic domains IV and V which render the PKR variant unable to autophosphorylate or activate substrate proteins (Koromilas *et al*., 1992). *Nde*I restriction sites (catATG) were introduced into the wild-type PKR and PKRΔ6 sites of translation initiation by site-directed mutagenesis as previously described (Barber *et al*., 1991). PKR genes were cut from pET11a vectors using *Nde*I and blunted using Klenow and dNTPs After heat inactivation, the constructs were cut with *Bam*HI and placed into *Sac*II- (blunted) and *Bam*HI-digested pUHD10-3 [pTRE; Clontech, CA (Gossen *et al*., 1995)]. The resultant plasmids contained PKR genes (wild-type and dominant-negative variant referred to as pTRE-PKR and pTRE-PKRΔ6, respectively) that were downstream of the Tet-responsive P_{hCMV-1} promoter.

Cell lines

3T3 L1 cells (Clontech, CA) transfected with a pTet-Off vector [pUHD15-1neo (Gossen *et al*., 1995)] subsequently were co-transfected with pTRE-PKR or pTRE-PKRΔ6 and pHyg using Lipofectamine (Gibco-BRL, MD). After 9 h incubation, the DNA mix was removed and replaced with fresh medium. After 24 h of recovery, cells containing PKR plasmids and pHyg were selected using 300 μg/ml hygromycin and 300 μg/ml G418 in the presence of 5 μg/ml Dox (Sigma, MI). Colonies were isolated and expanded. For protein induction, cells were washed three times with phosphate-buffered saline (PBS) and incubated with fresh medium lacking Dox. PKR-expressing cells were identified by immunoblot analysis of cell extracts using an anti-human PKR monoclonal antibody (Laurent *et al*., 1985).

Cell growth and cell cycle analysis

PKR-inducible cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco-BRL) in the presence or absence of 5 μg/ml Dox for up to 12 days. Medium was changed every other day. Cells were counted in duplicate using a hemocytometer and then fixed in cold 70% ethanol for cell cycle analysis. Cells were washed, treated with RNase, stained with 50 μg/ml propidium iodide, and analyzed by flow cytometry. The percentage of cells in G_1, S and G_2/M was calculated using CellQuest software.

Immunoblot analysis

Protein extracts from cell lines were prepared by disrupting in lysis buffer [10 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol (DTT), 2 mM EDTA, 1 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/ml aprotinin and 1% Triton X-100]. Supernatants were added to an equal volume of protein disruption buffer [5% SDS, 20% β-mercaptoethanol, 20% glycerol and 150 mM Tris–HCl (pH 6.8)] and boiled for 5 min prior to being loaded onto 10% SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose and after blocking in milk extract were incubated with monoclonal antibody specific to human PKR or with anti-PKR polyclonal antibody prepared as described elsewhere (Barber *et al*., 1992). After the membranes were washed and incubated with horseradish peroxidase-labeled anti-mouse antibody conjugate (Gibco-BRL), proteins were visualized using a chemiluminescence substrate (Pierce Chemicals, IL).

For analysis of Fas, Bcl-2, Bax and tubulin, antibodies were purchased from Santa Cruz, CA. Antibody directed to phosphorylated and unphosphorylated eIF2α has been described (Carrol *et al*., 1993; De Gracia *et al*., 1997).

Assay of protein kinase activity

Protein kinase activity was measured as described by Barber *et al.* (1991, 1995). Briefly, soluble extracts were diluted with 500 μl of buffer I [20 mM Tris–HCl (pH 7.5), 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 100 U/ml aprotinin, 1 mM DTT, 0.2 mM PMSF, 1% Triton X-100 and 20% glycerol] prior to the addition of monoclonal antibody specific to human PKR. After 1 h incubation at 4°C, protein G–Sepharose (Gibco-BRL) was added to the lysates and incubated for a further hour at the same temperature. Precipitates were then washed four times with buffer I and three times with buffer II [10 mM Tris–HCl (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 100 U/ml aprotinin and 20% glycerol]. Washed immunoprecipitates were incubated in kinase reaction buffer [20 mM Tris–HCl (pH 7.5), 0.01 mM EDTA, 50 mM KCl, 100 U/ml aprotinin, 0.3 mg of bovine serum albumin/ml, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1.25 μM [γ-^{32}P]ATP, 0.1 mM PMSF, 5% glycerol]. Purified eIF2α from rabbit reticulocytes (a gift from Rosemary Jagus) was then added to the mixtures. PolyI:polyC (Sigma) or 10 U/ml heparin was used as activator, and the mixtures were incubated at 30°C for 15 min. The reactions were stopped by adding protein disruption buffer containing 50 mM EDTA. After boiling for 2 min, proteins were analyzed by SDS–polyacrylamide gels and autoradiography.

RNA analysis

Cells were transfected with poly(I:C) (Pharmacia, NJ) and, after 24 h, lysed using TRIzol (Gibco-BRL) to collect total RNA. Following quantitation, ~10 μg of total RNA was incubated with [α-^{32}P]UTP-labeled mAPO-2 and mAPO-3 riboprobe sets (Riboquant; Pharmingen, CA). Following RNase treatment, protected probes were resolved using 5% acrylamide gels.

Apoptosis and cell viability analysis

Cells were transfected with dsRNA (Sigma, MI) using lipofectamine (Gibco-BRL). After 9 h incubation, the transfection mix was removed and fresh medium placed on the cells. After 12 h, cells were washed in cold PBS and incubated for 15 min with fluorescein-conjugated annexin V and propidium iodide (R & D Systems). Cell viability was determined by trypan blue exclusion analysis. Jo-2 and TNF-α were purchased from Pharmingen (CA) and Genzyme (CA), respectively.

Anchorage-independent cell growth

Six-well plates were coated with 1 ml of medium/0.5% agarose with or without 5 μg/ml Dox. Control or PKR-expressing cells were suspended in medium containing 0.25% agarose, again with or without Dox, and seeded onto coated wells. Medium/0.5% agarose was overlaid on the cells, followed by medium alone, in the presence or absence of Dox. The cells were replenished with fresh medium containing or lacking Dox every 2 days. Colonies with >30 cells (~5 cell doublings) were considered positive and experiments were carried out in duplicate.

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