

# Caspase 9 activation by the dsRNA dependent protein kinase, PKR: molecular mechanism and relevance

Jesús Gil<sup>1,2</sup>, Maria Angel García<sup>1</sup>, Mariano Esteban\*

Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Campus Universidad Autónoma, 28049 Madrid, Spain

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**Abstract** The double-stranded RNA-dependent protein kinase (PKR) induces apoptosis by activation of the FADD/caspase 8 pathway. Here we show that upon PKR expression, caspase 9 is processed and activated, correlating with the translocation of cytochrome *c* to the cytoplasm and breakdown of mitochondrial potential upon Bax insertion. However, treatment of cells with an inhibitor of caspase 9 could not prevent PKR-induced apoptosis. During PKR-induced apoptosis, caspase 9 is activated downstream of caspase 8. Our findings revealed that caspase 9, although dispensable, is a mediator of PKR-induced cell death.

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**Key words:** Apoptosis; Double-stranded RNA-dependent protein kinase; Caspase 9; Cytochrome *c*; Mitochondria

## 1. Introduction

The double-stranded (ds) RNA-dependent protein kinase PKR [1,2] controls cell growth [3], cell differentiation [4], viral clearance [5] and induction of apoptosis [6]. Characterisation of the mechanism by which PKR induces apoptosis is important for fully understanding PKR function. Events immediately downstream of PKR involved in apoptosis are phosphorylation of eIF2 $\alpha$  by PKR and activation of NF- $\kappa$ B transcription factors [7,8]. Activation of these pathways results in the subsequent transcriptional induction of Fas, p53 and Bax [9–11]. Eventually, induction of all these mediators results in the triggering of caspase activation. Studies performed by others and us have highlighted the importance of the FADD/caspase 8 pathway in PKR-induced apoptosis [9,10]. Caspase 8 is specifically activated and initiates cell death execution in response to death receptors [12] but not

to other stimuli that, in contrast, activate caspase 9 as the initiator caspase [13].

Caspase 9 activation occurs through the formation of a complex called apoptosome requiring at least two additional partners, Apaf-1 and cytochrome *c* [14]. Apaf-1 is an adapter protein that, upon oligomerisation induced by cytochrome *c* and dATP, provides the basis for the initiation of the apoptotic cascade common to many insults [15]. Cytochrome *c* is located in the mitochondria, where it is a key component of the respiratory chain. In the onset of some types of apoptosis, cytochrome *c* is translocated from the mitochondria to the cytoplasm [16] in a process strictly regulated but not yet fully understood (reviewed in [17]). Both apoptotic pathways (initiated by caspases 8 or 9) converge in the final activation of terminator caspases (caspases 3, 6, 7) [12]. Crosstalk between caspase 8 and caspase 9 pathway occurs mainly through cleavage of cytosolic Bid by caspase 8 [18]. Truncated Bid (tBid) recruits BH3-only molecules like Bad or Bax [19,20]. This complex translocates from the cytosol to the mitochondria, thus mediating caspase 8-induced mitochondrial damage.

In order to gain insights into the role of caspase 9 on the triggering of apoptosis by PKR, we first analysed the effect of PKR expression on the pathway. PKR expression resulted in caspase 9 activation preceded by Bax insertion into the mitochondria, this results in a decrease in the mitochondrial potential and translocation of cytochrome *c* to the cytoplasm. In addition, we addressed the impact and importance of caspase 9 activation by using caspase-specific chemical inhibitors.

## 2. Materials and methods

### 2.1. Reagents

Caspase inhibitors were from Calbiochem. Antibodies recognising specifically cleaved caspase 9 were from Biosource. Human antiserum recognising mitochondria was a kind gift from Miguel Torres and has been described previously [21]. Other antibodies were from Pharmingen or Sigma.

### 2.2. Cell culture and viruses

HeLa cells (ECACC 85060701) were grown in Dulbecco's modified Eagle's medium supplemented with 10% normal calf serum. Wild type and recombinant vaccinia virus (VV) expressing isopropyl  $\beta$ -thio-galactopyranoside (IPTG)-inducible PKR (VV PKR) have been described previously [22].

### 2.3. Caspase and apoptosis assays

Caspase 9 activity was measured using a colorimetric assay (R&D systems) with LEDH-pNA as substrate. Free pNA was determined by measuring absorbance at 405 nm. The Cell Death Detection kit (Roche) was used for apoptosis quantification. All samples were analysed in duplicate.

\*Corresponding author. Fax: (34)-91-585 4506.

E-mail address: [mesteban@cnb.uam.es](mailto:mesteban@cnb.uam.es) (M. Esteban).

<sup>1</sup> Both authors contributed equally to the manuscript

<sup>2</sup> Present address: Wolfson Institute for Biomedical Research, University College of London, Gower Street, London WC1E 6BT, UK.

**Abbreviations:** PKR, dsRNA-dependent protein kinase; VV, vaccinia virus; VV PKR, recombinant VV expressing PKR; dsRNA, double-stranded RNA; FACS, fluorescence-associated cell sorter; IPTG, isopropyl  $\beta$ -thio-galactopyranoside; TNF, tumour necrosis factor; CHX, cycloheximide; hpi, hours post infection

#### 2.4. Detection of changes in mitochondrial potential

Changes in mitochondrial potential were measured using the JC-1 dye (InterGen). After staining, cells were washed and analysed by confocal microscopy or trypsinised and analysed by fluorescence-associated cell sorter (FACS). For FACS analysis duplicate samples were analysed.

#### 2.5. Analysis of protein expression in mitochondrial and cytosolic fractions

HeLa cells were incubated on ice in digitonin lysis buffer (75 mM NaCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 250 mM sucrose and 190 µg digitonin (Sigma)). After 10 min, cytosolic supernatant was obtained by centrifugation at 10 000 × g for 5 min. Mitochondrial pellets were resuspended in 0.1% Triton X-100, 25 mM Tris (pH 8.0) prior to SDS-PAGE analysis.

#### 2.6. Confocal microscopy

HeLa cells were subjected to immunofluorescence staining as described in [23]. Appropriate isotype-specific secondary antibodies conjugated to fluorescein or Texas red (Molecular Probes) were used. Cells were visualised using a Bio-Radiance 2000 confocal laser microscope using a 73 × magnification objective.

### 3. Results

#### 3.1. Caspase 9 is activated during PKR-induced apoptosis

PKR was expressed in an inducible manner using recombinant VV that provides the necessary environment for PKR activation [6,22,24]. Caspase 9 activity was measured in extracts of HeLa cells infected with VV PKR under regulation of IPTG. Cells were collected at 12, 16, 20 and 24 h post infection (hpi) and caspase 9 activity was quantified by LEHD-pNA cleavage. As shown in Fig. 1A, PKR expression induces caspase 9 activity. The timing of caspase 9 activation was similar to that of caspase 3 activation, as measured by cleavage of DEVD-pNA (data not shown). In addition, after VV PKR infection of HeLa cells, we observed caspase 9 processing by confocal microscopy using a cleavage-specific antibody (Fig. 1B). A clear localised signal with a strong and discrete cytoplasmic punctate pattern was observed. Processing was first seen at 16 hpi and increased with time. No signal was observed in control HeLa cells and only a diffuse signal in VV-infected cells.

#### 3.2. Release of cytochrome *c* to cytoplasm and mitochondrial depolarisation during PKR-induced apoptosis

Translocation of cytochrome *c* from the mitochondria to the cytosol is a regulated step triggering caspase 9 activation [16]. As shown in Fig. 2, cytochrome *c* is located in the mitochondria of untreated cells (panel a) or cells infected with VV (panel b). However, upon PKR expression, cytochrome *c* is translocated to the cytosol, as observed in more than 35% of cells at 20 hpi and nearly all cells by 24 hpi (panels c, d).

A key event correlating with apoptosis initiation is the decrease in mitochondrial polarisation potential [17]. We therefore analysed mitochondrial polarisation upon PKR expression using the JC-1 dye. As shown in Fig. 3A, cells expressing PKR exhibit a diffuse dye distribution when compared with uninfected (mock) and VV-infected cells. This was also confirmed by FACS analysis (Fig. 3B). JC-1 diffuses to the cytoplasm as the mitochondrial potential falls as revealed by a shift from red to green fluorescence. JC-1 FACS profile of control apoptotic HeLa cells (treated with tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) plus cycloheximide (CHX), panel b) showed a clear increase in the green cytoplasmic JC-1 form, when compared with untreated cells (panel a). Cells infected with VV PKR at

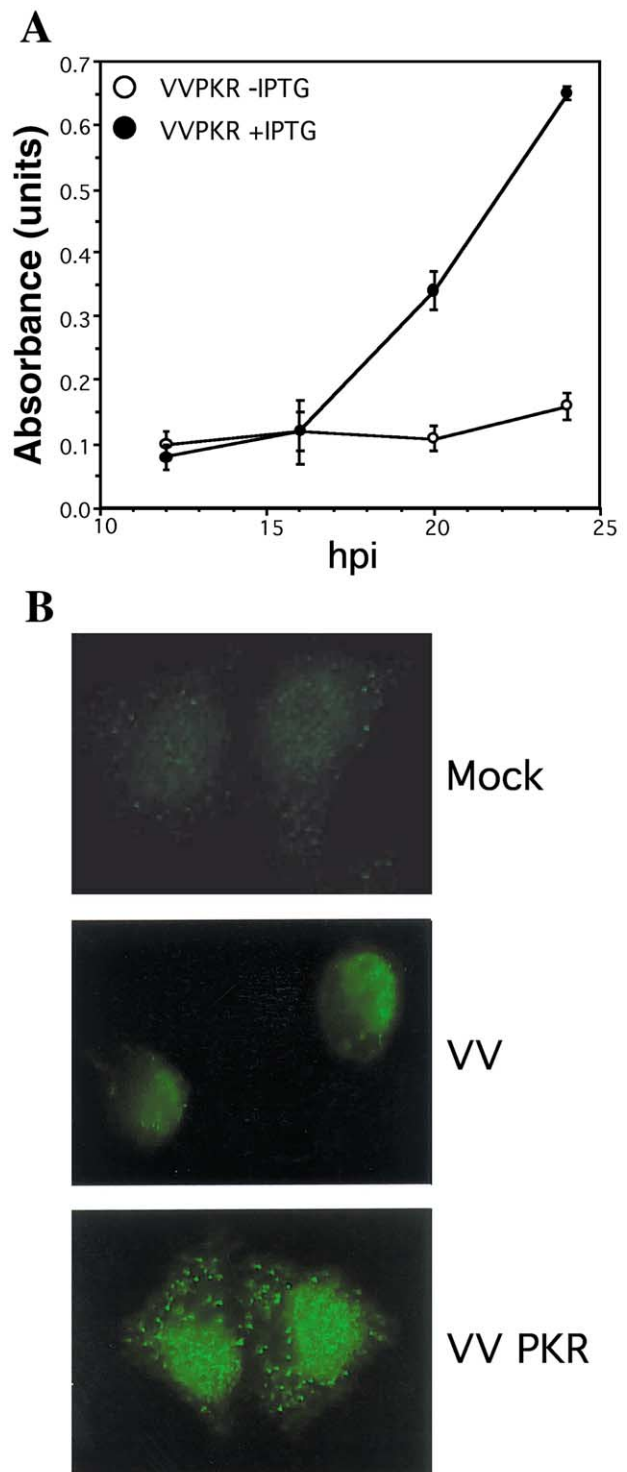


Fig. 1. PKR expression induces caspase 9 activation. A: HeLa cells were infected (5 pfu/cell) with VV PKR and incubated with IPTG (+IPTG) or without IPTG (–IPTG) after 1 h of viral adsorption. At indicated times cells were collected and caspase 9 activity was determined. B: Caspase 9 cleavage was analysed by confocal microscopy of HeLa cells at 20 hpi. Cells were either mock infected or infected with 5 pfu/cell of the indicated viruses. 5 mM IPTG was added 1 h after viral adsorption.

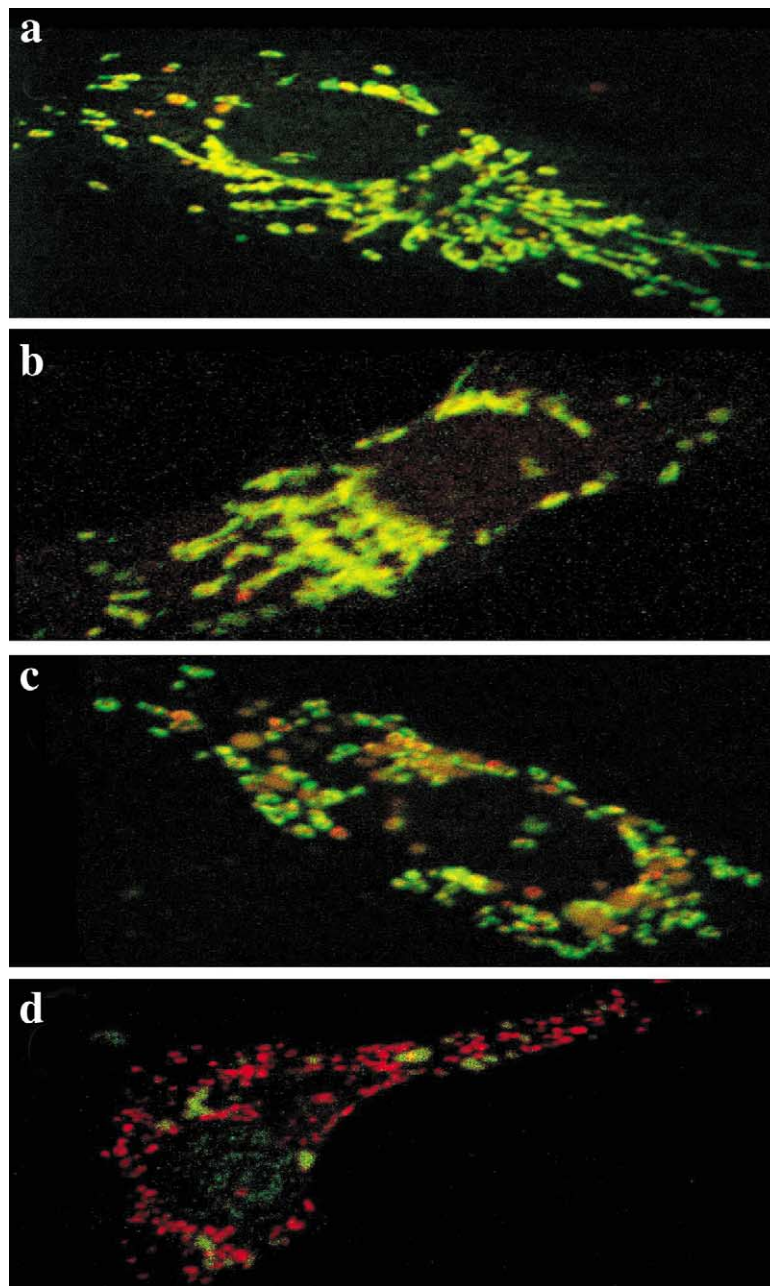


Fig. 2. Cytochrome *c* is translocated to the cytosol upon PKR expression. HeLa cells were infected at 5 pfu/cell and analysed by confocal microscopy using antibodies directed against cytochrome *c* (green) or mitochondria (red). Colocalisation of both antibodies is shown. a: Mock-infected cells; b: VV-infected cells for 24 h; VV PKR-infected cells for 20 h (c) and 24 h (d).

16 hpi (panel d) show a small fluorescent shift compared with cells infected with VV (panel c), marking the onset of mitochondrial depolarisation. At 24 hpi the shift in PKR-expressing cells was stronger (panel f) when compared with VV infected cells (panel e).

### 3.3. Bax translocates to the mitochondria during PKR-induced apoptosis

Balance between different Bcl-2 family proteins is responsible for regulating mitochondrial-based processes during apoptosis [17]. Therefore, we analysed the levels and subcellular distribution of different Bcl-2 family proteins upon PKR ex-

pression. No changes were observed in levels and distribution of Bcl-XL and Bcl-2 (Fig. 4A). Interestingly, although overall levels of Bax remained unchanged, PKR expression resulted in mitochondrial localisation of Bax (Fig. 4B).

### 3.4. Role of caspase 9 in PKR-induced apoptosis

To analyse the relevance of caspase 9 on PKR-induced apoptosis, we tested the effect of a specific caspase 9 inhibitor (z-LEHD) upon cell death. As observed in Fig. 5, treatment of cells with increasing doses of z-LEHD that abolished caspase 9 activity (data not shown) caused a small inhibition of PKR-induced apoptosis (lanes 2, 3 and 4) compared to un-

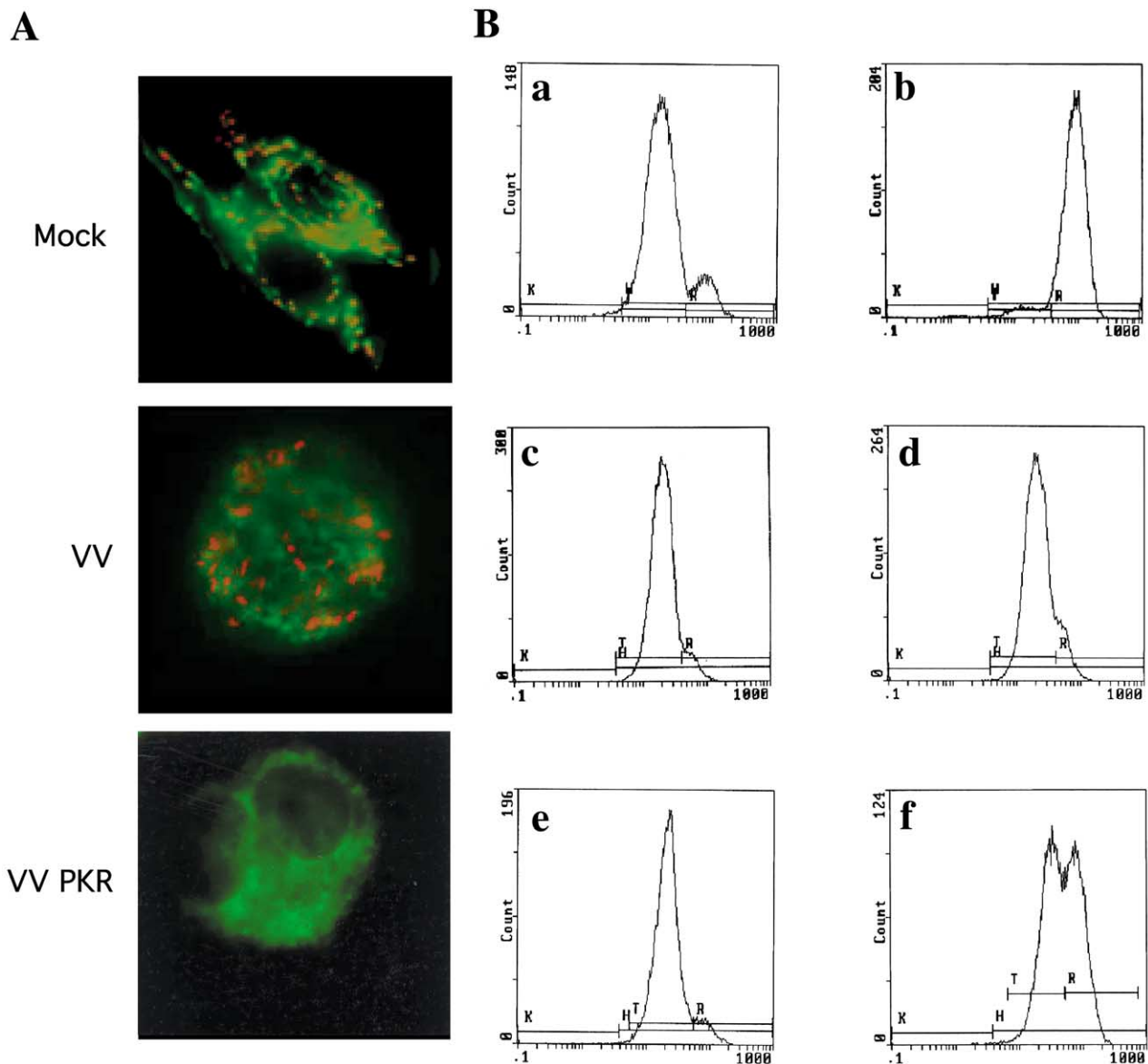


Fig. 3. PKR expression induces mitochondrial depolarisation. HeLa cells were mock-infected (a), treated with TNF- $\alpha$  (20 ng/ml) and CHX (100  $\mu$ g/ml) for 12 h (b) or infected with VV for 16 h (c) or 24 h (e), or VV PKR for 16 h (d) or 24 h (f) in the presence of 5 mM IPTG. Cells were pulsed with JC-1 as described and analysed by confocal microscopy (A) or by FACS (B). Profiles of FL-1 (green) channel are shown.

treated PKR-expressing cells (lane 1). This finding is in contrast to that observed in PKR-expressing cells treated with z-IETD, a caspase 8 inhibitor (lane 6).

In addition, we analysed the effect of caspase inhibitors on the translocation of Bax from the cytosol to the mitochondria. Treatment of PKR-expressing cells with the general inhibitor of caspases z-VAD or with a specific inhibitor of caspase 8 resulted in maintained Bax cytosolic levels (Fig. 6A). Interestingly, z-VAD treatment also prevented mitochondrial depolarisation (Fig. 6B, bottom panel), and prevented caspase 9 activation (Fig. 6C). When cells were treated with the caspase 8 inhibitor z-IETD, caspase 9 activity decreased significantly compared with untreated cells (Fig. 6C, lane 1). As a control, cells were treated with a caspase 9 inhibitor, completely abolishing caspase 9 activity. These results suggest that caspase 9 lies downstream of caspase 8 in PKR-induced apoptosis.

#### 4. Discussion

Induction of apoptosis is a common response to viral infections. Several proteins have been involved in initiating cell death in response to viral infections, and PKR is one of those host defense proteins specifically activated by dsRNA, an intermediate in the virus replication life cycle. PKR appears to control a programme involved in regulating the expression of a set of genes, by modulating transcription and translation [7,8,24]. As a consequence the caspase 8 pathway is activated [9,10]. However, the precise involvement of other major pathways of apoptosis, e.g. the caspase 9 pathway, has not been defined. In this report we have analysed whether PKR expression results in caspase 9 activation, and have established the biological relevance of this activation.

As shown here, caspase 9 is activated upon PKR expres-

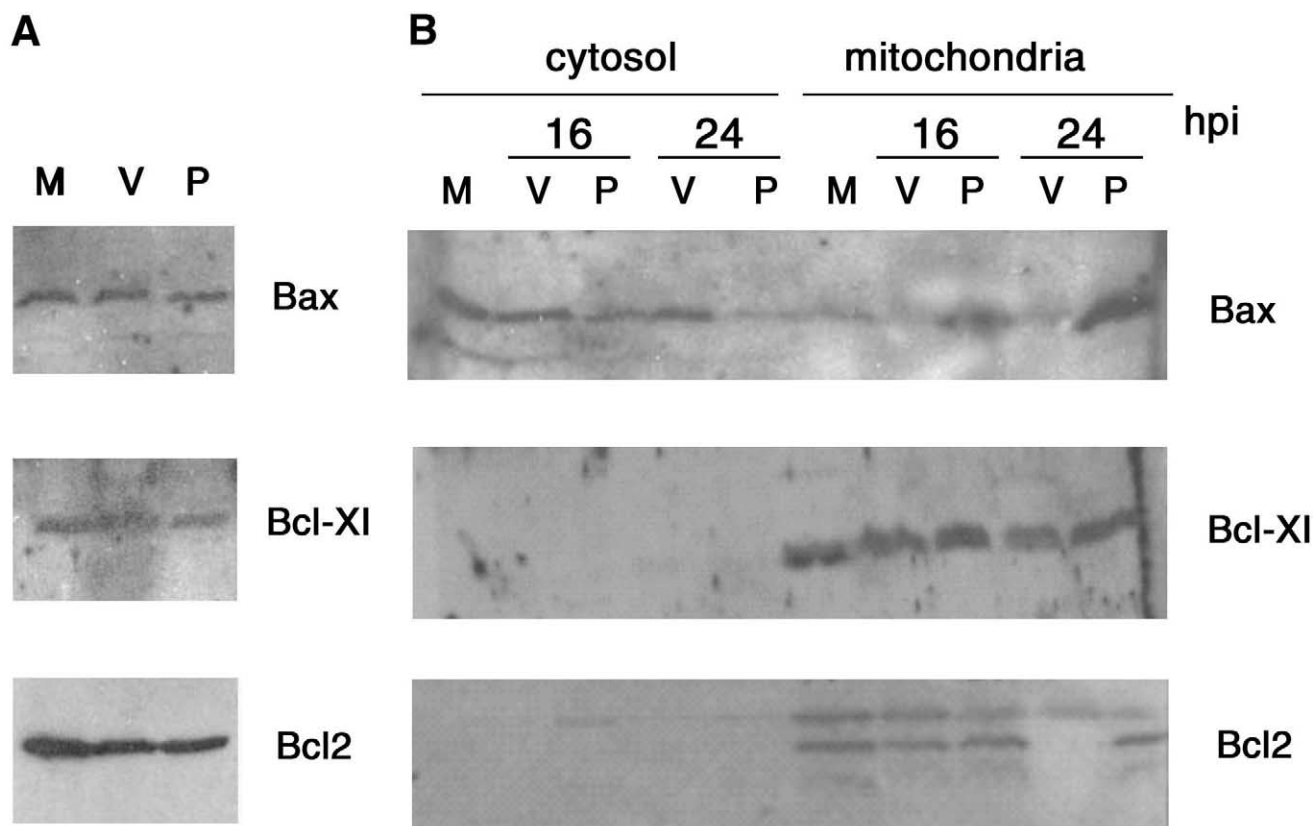


Fig. 4. Levels and distribution of Bcl-2 family proteins upon PKR expression. HeLa cells were mock-infected (M) or infected with VV (V) or VV PKR in the presence of 5 mM IPTG (P) during the times indicated. A: Total extracts were analysed by immunoblotting using antibodies to Bax (upper panel), Bcl-XL (middle panel) or Bcl2 (lower panel). B: Mitochondrial and cytoplasmic extracts of the same samples as in A.

sion, correlating with the translocation of Bax to the mitochondria and cytochrome *c* to cytoplasm, resulting in mitochondrial depolarisation. Although Bax had been previously shown to be transcriptionally upregulated during PKR-induced apoptosis [10], we cannot confirm these findings, instead we show a strong relocation to the mitochondria. Inhibition of caspase 9 by z-LEDH (at concentrations that completely abolished caspase 9 activity) led only to a small decrease in PKR-induced cell death. These findings suggest that caspase 9 has an accessory role in PKR-induced apoptosis. This was further supported by our data shown in Fig. 5 whereby treatment of cells with a caspase 8 inhibitor not only led to a significant protection against PKR-induced apoptosis but caspase 9 activity was significantly abolished. This correlates with the cytoplasmic localisation of Bax and no change in the mitochondrial potential. This observation strongly suggests that caspase 9 is activated downstream of caspase 8 during PKR-induced apoptosis. We hypothesised that cleavage of Bid by caspase 8 could be involved in Bax translocation, mitochondrial damage and caspase 9 activation upon PKR expression. Although we failed to detect tBid upon PKR expression using several antibodies (data not shown), an involvement of Bid cleavage in these processes still seems to be the most probable explanation. However, we cannot rule out that PKR can induce apoptosis by preferentially activating one or more pathways depending of the cell type.

In conclusion, we have found that caspase 9 is activated by PKR and this correlates with Bax localisation to the mito-

chondria, resulting in mitochondrial depolarisation and translocation of cytochrome *c* to the cytosol. These biochemical processes take place downstream of caspase 8 and are secondary to caspase 8 activation.

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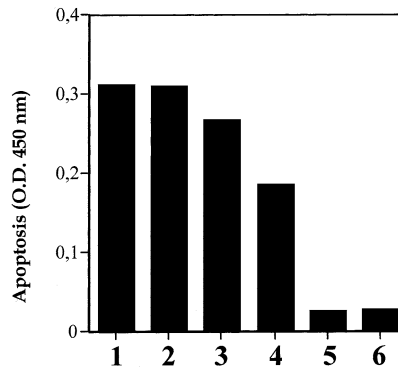


Fig. 5. Effect of caspase 9 inhibition on PKR-induced apoptosis. HeLa cells were mock-infected (lane 5) or infected with VV PKR for 20 h in the presence of 5 mM IPTG. After 1 hpi cells were left untreated (lanes 1 and 5) or treated with 25 μM of z-VAD (lane 6) or with different concentrations (2, 10 or 40 μM) of the caspase 9 inhibitor z-LEHD (lanes 2, 3 and 4 respectively).

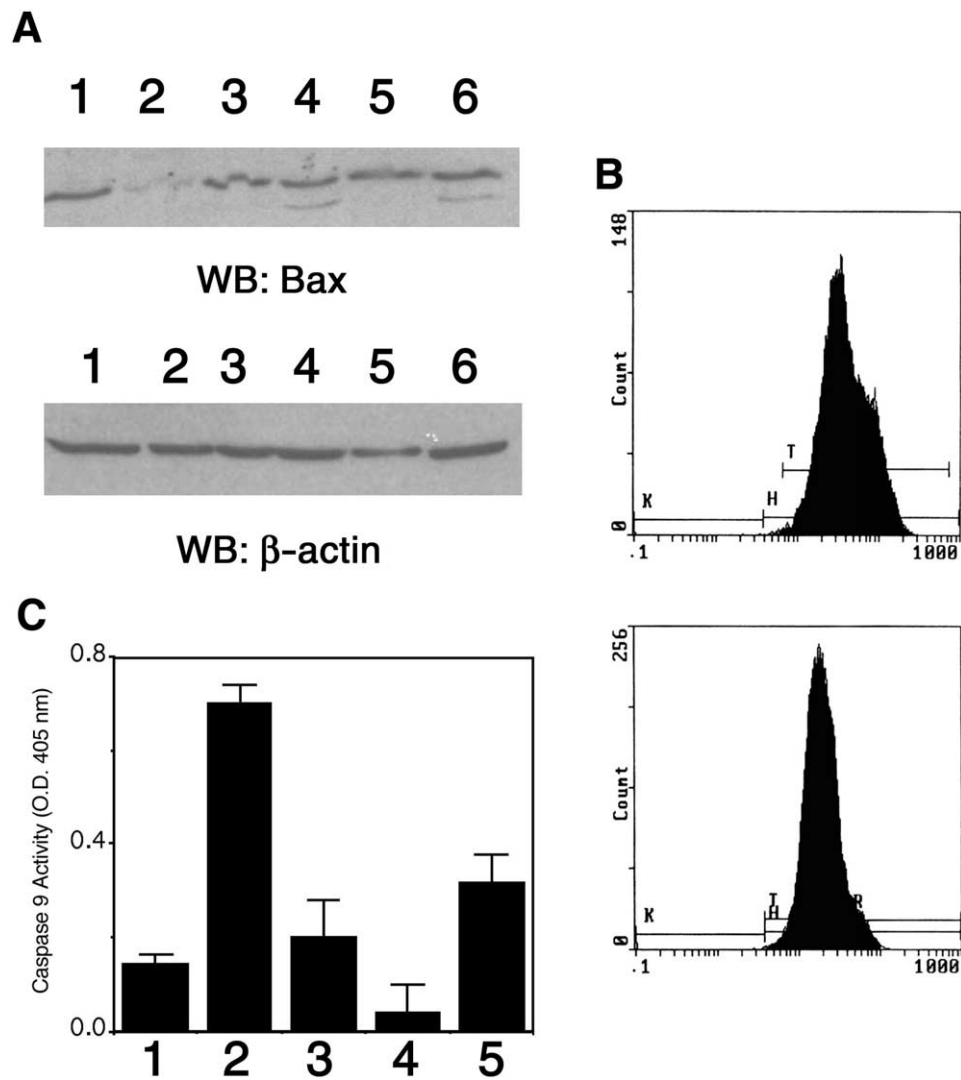


Fig. 6. Effect of caspase inhibitors on Bax translocation, mitochondrial potential and caspase 9 activity in PKR-induced apoptosis. A: HeLa cells were infected with VV (lanes 1, 3 and 5) or VV PKR (lanes 2, 4 and 6) in the presence of 5 mM IPTG. Cells were either left untreated (lanes 1 and 2) or treated with 40  $\mu$ M z-IETD-fmk (lanes 3 and 4) or 40  $\mu$ M z-VAD-fmk (lanes 5 and 6). Samples were collected at 20 hpi, cytosolic extracts prepared and analysed by immunoblotting. B: FACS analysis of HeLa cells pulsed with JC-1 after infection during 24 h with VV PKR in the presence of 5 mM IPTG. Profiles of VV PKR-infected cells untreated (top panel) or treated with 50  $\mu$ M z-VAD (bottom panel). C: HeLa cells were either mock-infected (lane 1) or infected with VV PKR at 5 pfu/cell in the presence of 5 mM IPTG for 24 h (lanes 2–5). Cells were mock-treated (lanes 1 and 2) or treated with z-VAD-fmk (50  $\mu$ M; lane 3), z-LEDH-fmk (40  $\mu$ M; lane 4) or z-IETD-fmk (50  $\mu$ M; lane 5).

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