The protein kinase PKR: a molecular clock that sequentially activates survival and death programs

Olivier Donzé^{1,5,*}, Jing Deng², Joseph Curran³, Robert Sladek⁴, Didier Picard^{1,6} and Nahum Sonenberg^{2,6}

¹Département de Biologie Cellulaire, Université de Genève, Sciences III, Genève, Switzerland, ²Department of Biochemistry and McGill Cancer Centre, McGill University, Montreal, Quebec, Canada, ³Département de Génétique et de Microbiologie, Université de Genève, Centre Médical Universitaire (CMU), Genève, Switzerland and ⁴McGill University and Genome Quebec Innovation Centre, 740 Avenue Docteur Penfield, Montreal, Quebec, Canada

Cell death and survival play a key role in the immune system as well as during development. The control mechanisms that balance cell survival against cell death are not well understood. Here we report a novel strategy used by a single protein to regulate chronologically cell survival and death. The interferon-induced protein kinase PKR acts as a molecular clock by using catalysis-dependent and -independent activities to temporally induce cell survival prior to cell death. We show that the proapoptotic protein PKR surprisingly activates a survival pathway, which is mediated by NF- κ B to delay apoptosis. Cell death is then induced by PKR through the phosphorylation of eIF-2 α . This unique temporal control might serve as a paradigm for other kinases whose catalytic activity is not required for all of their functions.

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Introduction

PKR is an interferon-induced serine/threonine protein kinase that is activated by double-stranded RNA (dsRNA) (Proud, 1995; Robertson and Mathews, 1996; Williams, 1999). It belongs to a family of kinases that phosphorylate the α -subunit of the eucaryotic translation initiation factor 2 (eIF-2 α) (de Haro *et al.*, 1996), resulting in a dramatic inhibition of protein synthesis (Williams, 1999). PKR plays an important role in the antiviral defense by interferon (Katze, 1995; Tan and Katze, 1999; Balachandran *et al.*, 2000), has been implicated in cell growth control and differentiation

(Petryshyn *et al.*, 1988; Koromilas *et al.*, 1992; Donzé *et al.*, 1995), and might function as a tumor suppressor (Koromilas *et al.*, 1992; Meurs *et al.*, 1992; Barber *et al.*, 1995). Overexpression of PKR leads to apoptosis (Balachandran *et al.*, 1998; Donzé *et al.*, 1999). In addition to its role in translation, PKR participates in several signaling pathways to transcription. For example, PKR contributes to the induction of early genes such as *c-fos* and *c-jun* by platelet-derived growth factor (Mundschau and Faller, 1995) and modulates the transcription functions of STAT1 (Wong *et al.*, 1997). Other studies clearly demonstrated a role for PKR in the activation of NF- κ B (Maran *et al.*, 1994; Yang *et al.*, 1995).

The transcription factor NF- κ B is central to immune and inflammatory responses as well as virus replication (Baldwin, 1996; Perkins, 2000; Hiscott *et al.*, 2001). It is activated by a variety of stimuli including cytokines, mitogens, cellular stress and bacteria or virus infection (Miyamoto and Verma, 1995; Ghosh *et al.*, 1998). Several labs reported, using an antisense strategy (Maran *et al.*, 1994) or PKR null cells (Yang *et al.*, 1995), that PKR is required in NF- κ B activation by dsRNA. PKR acts upstream of IKK and of NF- κ B-inducing kinase (Zamanian-Daryoush *et al.*, 2000). It has recently been reported that activation of the IKK/NF- κ B pathway by PKR is independent of its kinase and of its dsRNA-binding activities and that PKR acts through IKK β binding (Chu *et al.*, 1999; Bonnet *et al.*, 2000; Ishii *et al.*, 2001). However, the role of PKR in NF- κ B activation has been questioned (Iordanov *et al.*, 2001).

We studied the role of the different PKR-induced signaling pathways, by analyzing the kinetics of activation of NF- κ B and eIF-2 α , and by performing an oligonucleotide array analysis using our NIH3T3 cell line that expresses PKRwt under the control of an inducible promoter (Donzé *et al.*, 1999). Surprisingly, we found that PKR induces NF- κ B and eIF-2 α signaling pathways in a chronological manner by using kinase-dependent and -independent strategies. Strikingly, NF- κ B-induced survival genes such as c-IAPs or A20 are induced by PKR (Wang *et al.*, 1998; Lee *et al.*, 2000; Karin and Lin, 2002). We also demonstrate that NF- κ B functions to delay PKR-induced apoptosis. Thus, a single protein, PKR, sequentially activates two conflicting programs: cell survival through NF- κ B signaling and apoptosis through phosphorylation of eIF-2 α .

Results

Sequential activation of NF- κ B and eIF-2 α by PKR

PKR has been reported to activate both eIF-2 α and NF- κ B (Williams, 2001); however, the kinetics of activation of these signaling pathways by PKR has never been addressed. To investigate this issue, the two pathways were dissected following the induction of the expression of PKRwt in NIH3T3 PKRwt cells. These inducible cells undergo apoptosis upon PKR expression without the requirement for dsRNA (Donzé *et al.*, 1999). The activation of NF- κ B and eIF-2 α was

*Corresponding author. Département de Biologie Cellulaire, Université de Genève, Sciences III, 30, Quai E. Ansermet, CH-1211 Genève 4, Switzerland. Tel.: +41-22-702-6812; Fax: +41-22-781-1747; E-mail: odonze@apotech.ch

⁵Present address: Apotech Corporation, Ch. des Croisettes 22, Epalinges CH-1066, Switzerland

⁶These authors contributed equally to the work

assayed by using an electrophoretic mobility shift assay (EMSA) and an immunoblot analysis using a phosphospecific anti-eIF-2 α antibody, respectively (Figure 1A) (Barber, 2001). Induction of NF- κ B occurs within 3 h upon tetracycline removal (Figure 1A, gel shift panel), when levels of PKR are still barely detectable by immunoblot analysis (Figure 1A, panel PKR antibody, lane 3 h). The activation of NF- κ B peaks around 6 h following tetracycline withdrawal. To confirm the EMSA data, we ascertained by Western blot analysis that I κ B β levels are reduced at early times following PKR induction (Supplementary Figure 1) (Zamanian-Daryoush *et al*, 2000). Interestingly, in this time period, there is no detectable kinase activity (Figure 1A; compare 4 and 8 h in the panels PKR antibody and eIF-2 α phosphorylation). The phosphorylation of eIF-2 α becomes detectable only 8 h after tetracycline removal. These data indicate that by using its core body and later its catalytic activity, PKR imposes a temporal separation and activates NF- κ B prior to eIF-2 α phosphorylation.

To demonstrate the significance of the temporal separation between NF- κ B activation and eIF-2 α phosphorylation in a physiological system, we studied the kinetics of activation of both signaling pathways during virus infection. Note that such a comparative and kinetic study has never been performed. Vesicular stomatitis virus (VSV) has been reported to activate NF- κ B and eIF-2 α in a PKR-dependent manner (Chu *et al*, 1999; Balachandran *et al*, 2000). In our studies, VSV infection of HeLa cells causes NF- κ B activation within 1 h, confirming an earlier observation (Boulares *et al*, 1996), while phosphorylation of eIF-2 α is detected at 4–6 h post-infection (Figure 1B), when VSV proteins become detectable (Stojdl *et al*, 2000). Measles virus (MeV) also triggers PKR and NF- κ B activation (Dhib-Jalbut *et al*, 1999). Infection of HeLa cells with MeV results in an early activation of NF- κ B at 4–6 h p.i., as observed in a previous study (Helin *et al*, 2001), while eIF-2 α phosphorylation occurs much later at 16–18 h postinfection (Figure 1C). Interestingly, the kinetics of the phosphorylation of eIF-2 α during Measles virus infection parallels the synthesis and accumulation of viral RNA and proteins (Helin *et al*, 2001; tenOever *et al*, 2002). Since the requirement of PKR for activation of NF- κ B and eIF-2 α during MeV infection has never been assessed, PKR autophosphorylation was studied and was found to parallel eIF-2 α phosphorylation at 16–18 h postinfection (Figure 1C, compare PKR-P and eIF-2 α -P). These results are in accordance with the data obtained using the inducible system (Figure 1A) and show that, in a more complex situation (during virus infection), PKR temporally separates NF- κ B and eIF-2 α . Moreover, the kinetics observed here agrees with a model where the early NF- κ B activation might be dsRNA-independent while the activation of eIF-2 α correlates with the initiation of the viral replicative cycle.

The proapoptotic factor PKR induces the expression of NF- κ B target genes encoding survival proteins

The finding of the temporal separation between the two PKR-triggered signaling pathways *a priori* appears puzzling. Moreover, the function of PKR-activated NF- κ B signaling remains unclear. To characterize this issue further, expression microarray studies were performed using RNA extracted from NIH3T3 cells expressing PKRwt under the control of a tetracycline-regulated promoter. Our screen identified 683 probe

Figure 1 Sequential activation of NF- κ B and eIF-2 α by PKR. (A) Sequential activation of NF- κ B and eIF-2 α in NIH3T3 cells inducibly expressing PKRwt. Nuclear extracts from NIH3T3 PKRwt were processed at different periods of time after tetracycline removal to analyze NF- κ B activity by EMSA. Cell extracts were examined by immunoblotting with an anti-PKR antibody, a phosphospecific anti-eIF-2 α antibody, and an anti-eIF-2 α antibody. (B) Sequential activation of NF- κ B and eIF-2 α signaling pathways in HeLa cells infected with VSV. Nuclear extracts from HeLa infected with VSV or mock-infected were processed at different periods of time after infection. NF- κ B DNA-binding activity was measured by EMSA. Lysates were subjected to Western blot analysis for phosphorylated eIF-2 α and total eIF-2 α . (C) Sequential activation of NF- κ B and eIF-2 α in HeLa cells infected with measles virus. NF- κ B activation and eIF-2 α phosphorylation were measured as described in (B). PKR activation was monitored by an autophosphorylation assay in the presence of γ - 32 P-ATP. Total PKR was checked by immunoblot analysis.

sets whose expression changed following PKR induction. Of these, approximately half varied in expression by at least 1.4-fold (Supplementary Table A). Importantly, the microarray data show that a statistically significant number ($P < 0.001$, see Materials and methods) of differentially regulated tran-

Figure 2 The proapoptotic factor PKR induces the expression of NF-κB target genes encoding survival proteins. (A) Analysis of several PKR-regulated genes by RT-PCR. Total RNA from NIH3T3 cells expressing PKRwt (wt) or the kinase-defective mutant PKR^{K296R} (K296R) were subjected to RT-PCR analysis with primers specific for the different indicated genes. Tet: tetracycline. The numbers indicate the time in hours (h) after tetracycline removal. (B) Nuclear extracts after different periods of time following tetracycline removal from NIH3T3 cells expressing the kinase-defective mutant PKR^{K296R} were used to analyze NF-κB activity by EMSA. PKR^{K296R} levels were examined in cell extracts by immunoblotting with an anti-PKR antibody.

scripts are known NF-κB targets (Figure 2A and Table 1), indicating that PKR is sufficient to induce the expression of endogenous NF-κB target genes. Note that NF-κB activation is specifically controlled by PKR in this system, since NF-κB is not activated upon tetracycline removal in the NIH3T3 cell line containing the empty vector (Supplementary Figure 2).

To confirm the microarray data, the expression of several mRNAs was examined by RT-PCR. As shown in Figure 2A, the expression of these genes is increased in accordance with the microarray data. These included Gadd34/Myd116 and Gadd153/Chop, whose induction is known to be dependent on eIF-2α phosphorylation (Novoa *et al*, 2001) (Supplementary Table A). Importantly, these results suggest the existence of distinct PKR-mediated signaling pathways, as the differentially expressed genes show different kinetics of induction and different requirements for PKR kinase activity (see below and Figure 2A, part K296R). Surprisingly, the expression of several key survival genes, known to be dependent on NF-κB, such as c-IAP1, c-IAP2, A20 (Wang *et al*, 1998; Lee *et al*, 2000; Karin and Lin, 2002) (Table 1), is induced by PKR, suggesting that PKR might also play a role in cell survival.

In the light of the data obtained by EMSA (Figure 1A), the kinase-independent activation of NF-κB is expected to occur also for endogenous targets *in vivo*. To test this hypothesis, a cell line expressing a kinase-defective mutant (Donzé *et al*, 1999) was used. NF-κB is also activated by PKR^{K296R} using EMSA (Figure 2B), confirming the data from Figure 1A. The kinase-defective mutant PKR^{K296R}, like PKRwt, increases the expression of NF-κB target genes such as c-IAP1 or IκBα (Pahl, 1999) *in vivo* as shown by RT-PCR analysis (Figure 2A), indicating that NF-κB target genes are induced in a kinase-independent manner. It is noteworthy that the

kinase-defective PKR mutant does not act through the endogenous wild-type PKR, since PKR autophosphorylation is inhibited by the expression of PKR^{K296R} in our inducible NIH3T3 cell lines (Donzé *et al*, 1999). However, although PKR kinase activity is not required for the upregulation of some NF-κB target genes, it potentiates the expression of others, since induction of these genes by the PKR mutant is less robust than by PKRwt. For example, the mRNA for A20, which acts in the termination of the NF-κB response and is required for cell survival (Lee *et al*, 2000), shows a stronger induction by PKRwt (Figure 2A). Importantly, other mRNAs such as Gadd34/myd116 or Gadd153/Chop, whose induction is dependent on eIF-2α phosphorylation, are not induced upon PKR^{K296R} expression (Figure 2A). Taken together, these data indicate that PKR, without a requirement for its kinase activity, induces survival genes such as c-IAPs or A20, which are NF-κB target genes.

The PKR-triggered survival response causes a delay in PKR-induced cell death

Since the activation of PKR ultimately leads to apoptosis, the upregulation of the antiapoptotic genes suggests that PKR may trigger NF-κB-mediated cell survival in order to delay cell death. To further substantiate the role of NF-κB as an inhibitor of PKR-mediated apoptosis, we modified NIH3T3 PKRwt cells to constitutively express a human IκBα super-repressor that blocks NF-κB activation (wt/IκBα^{sr}) (Kothny-Wilkes *et al*, 1999), as well as control cells that expressed the vector plasmid alone (wt/C). The human IκBα super-repressor (hIκBα^{sr}) is expressed in two analyzed stable transformants, and migrates more slowly compared to its mouse counterpart (mIκBα) (Figure 3A). The inhibition of NF-κB by the super-repressor was verified: (i) by the dramatic down-regulation of endogenous mouse IκBα itself a target gene of NF-κB (Ito *et al*, 1994), (ii) by the lack of degradation of IκBα upon TNFα treatment (Figure 3A), and (iii) by the inhibition of NF-κB-dependent transactivation, in response to TNFα of a luciferase reporter gene under the control of a NF-κB promoter (data not shown). The inhibition of the expression of antiapoptotic genes, such as c-IAP1, upon PKR activation in clones expressing the IκBα super-repressor was confirmed by RT-PCR (Figure 3B). As expected, upon PKR activation, expression of c-IAP1 is increased in NIH3T3 PKRwt cells with the vector alone (wt/C7, Figure 3B), as demonstrated for the parental NIH3T3 PKRwt cells (Figure 2A, wt), while expression of the same gene is inhibited in the presence of the super-repressor (wt/IκBα^{sr} 3).

The role of NF-κB during PKR-triggered cell cytotoxicity was monitored by a cell viability assay (Figure 4A). Following tetracycline removal, cells remain alive for 12 h for all clones (wt5, wt/C7, and both wt/IκBα^{sr} 3 and 7). The parental wt cells and the clones with the vector alone (wt/C7) do not begin to die until 20–24 h after tetracycline removal. In the two clones expressing the IκBα super-repressor (wt/IκBα^{sr} 3 and 7), cell death occurs earlier, starting already at 12 h after tetracycline removal. Cell death requires PKR kinase activity, since no cytotoxicity was observed in cells expressing PKR^{K296R} with or without functional NF-κB signaling (data not shown). In addition, the phosphorylation of eIF-2α by PKRwt is observed in cells expressing the IκBα super-repressor (Figure 4B). The cells die by apoptosis, as demonstrated by the binding of annexin V-EGFP to exposed

Table 1 PKR activates NF- κ B target genes

Probe set	Baseline	PKR +	MFC	Accession	Unigene	Gene	Description
Msa.1406.0_at	10.8	901.4	83.5	U19463	Mm.116683	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3 (A20)
X54149_s_at	3163.1	12825.6	4.1	X54149	Mm.1360	Gadd45b	Growth arrest and DNA-damage-inducible 45 beta
u20735_s_at	665.3	1903.8	2.9	u20735	Mm.1167	Junb	Jun-B oncogene
u88909_s_at	220.3	616.4	2.8	u88909	Mm.14483	Birc3	Baculoviral IAP repeat-containing 3 (c-IAP2)
M83380_s_at	1230	3165.2	2.6	M83380	Mm.1741	Relb	Avian reticuloendotheliosis viral (v-rel) on-cogene related B
Msa.1690.0_at	4233.2	10290.9	2.4	U36277	Mm.170515	Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha
Msa.6119.0_s_at	325.3	678.3	2.1	AA008681	Mm.87720	Arfrp1	ADP-ribosylation factor-related protein 1
U88908_s_at	327	637.7	2.0	U88908	Mm.2026	Birc2	Baculoviral IAP repeat-containing 2 (c-IAP1)
x12761_s_at	819.8	1621.8	2.0	x12761	Mm.482	Jun	Jun oncogene
Msa.16507.0_f_at	859.3	1614.2	1.9	W97190	Mm.399	Ier2	Immediate early response 2
J03168_s_at	351.1	616.2	1.8	J03168	Mm.1149	Irf2	Interferon regulatory factor 2
Msa.38650.0_s_at	1511.2	2671.1	1.8	AA144430	Mm.20225	Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100
D30782_s_at	661.7	1141.2	1.7	D30782	Mm.4791	Ereg	Epiregulin
k02782_s_at	3925.4	6364.6	1.6	k02782	Mm.19131	C3	Complement component 3
l00039_s_at	1133.6	1705	1.5	l00039	Mm.2444	Myc	Myelocytomatosis oncogene
x04972_s_at	5296.5	7970.8	1.5	x04972	Mm.2597	Sod2	Superoxide dismutase 2, mitochondrial
d14636_s_at	541.4	783.2	1.4	d14636	Mm.4509	Runx2	Runt-related transcription factor 2
j04596_s_at	3695	4927.9	1.3	j04596	Mm.21013	Cxcl1	Chemokine (C-X-C motif) ligand 1
M21065_s_at	2006.7	2657.5	1.3	M21065	Mm.1246	Irf1	Interferon regulatory factor 1
L28117_s_at	4867.8	6347.4	1.3	L28117	Mm.3420	Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105
Msa.1160.0_at	5434.9	6643.6	1.2	X03505	Mm.14277	Saa3	Serum amyloid A 3
X67644_s_at	14001.8	14841.1	1.1	X67644	Mm.25613	Ier3	Immediate early response 3
aa212865_at	1269.6	1406.1	1.1	aa212865	Mm.27557	Cdk9	Cyclin-dependent kinase 9 (CDC2-related kinase)
x51438_s_at	12981.7	11404.4	-1.1	x51438	Mm.7	Vim	Vimentin
X53476_s_at	21468.6	17799.4	-1.2	X53476	Mm.2756	Hmgn1	High-mobility group nucleosomal-binding domain 1
X53068_s_at	3908.9	3109.3	-1.3	X53068	Mm.7141	Pcna	Proliferating cell nuclear antigen
Z31334_s_at	7991	4871.2	-1.6	Z31334	Mm.168712	Col1a2	Procollagen, type I, alpha 2
d00208_s_at	16724.2	9318.4	-1.8	d00208	Mm.3925	S100a4	S100 calcium-binding protein A4

Transcript expression levels are shown at the baseline (in the presence of tetracycline) and following the induction of PKR synthesis (PKR + ; in the absence of tetracycline).

phosphatidylserine (Figure 4C) and by the condensation of chromatin (Figure 4D). Thus, PKR-induced cell death is delayed by a PKR-triggered survival response, which is mediated by NF- κ B activation.

Discussion

Using catalysis-dependent and -independent activities, the dsRNA-activated interferon-induced protein kinase PKR functions both as a pro- and antiapoptotic factor. PKR first triggers an NF- κ B survival response to delay a later apoptotic response, which relies on the phosphorylation of the translation factor eIF-2 α . These data are consistent with two reports showing that NF- κ B has an essential role in protecting cells against dsRNA or virus-induced apoptosis (Schwarz *et al*, 1998; Li *et al*, 2001). A recent study demonstrates that TNF α signaling uses a similar kinetics to activate an early NF- κ B antiapoptotic response, followed by a later death program triggered by caspase-8 (Micheau and Tschopp, 2003).

Temporal separation between survival and apoptosis by distinct molecular mechanisms

The mechanism by which these two opposite effects of PKR are exerted is novel. PKR kinase activity is not required for the induction of NF- κ B, and is therefore not required for the survival response. Recently, it was demonstrated that PKR

could activate NF- κ B by binding to the IKK β (Bonnet *et al*, 2000; Zamanian-Daryoush *et al*, 2000), independently of its catalytic and dsRNA-binding properties (Bonnet *et al*, 2000; Ishii *et al*, 2001). In contrast, induction of apoptosis is dependent on eIF2 α phosphorylation by PKR and requires kinase activity (Lee *et al*, 1997; Balachandran *et al*, 1998; Donzé *et al*, 1999). The separate kinase-dependent and -independent mechanisms endow PKR with an important property: the chronological activation of the survival and apoptosis responses. NF- κ B and survival signaling activation occurs several hours prior to the beginning of eIF2 α -mediated cell death, which in turn is dependent on dsRNA accumulation (Figure 5). This hiatus prevents a possible competition between the two conflicting programs. Another intriguing aspect revealed by our work is the need of PKR kinase activity to potentiate the expression of some NF- κ B genes, such as A20, involved in the termination of the NF- κ B response. NF- κ B controls the expression of different classes of genes, each of which might be required at different times, depending on the need of the cells. We posit that the interplay between kinase-independent and -dependent strategies might allow fine-tuning of the expression of NF- κ B target genes that are required later (such as inflammatory genes found in our array data). This requirement of the kinase activity of PKR and thus of dsRNA for the potentiation of NF- κ B signaling is consistent with the reported role of PKR in the dsRNA-mediated

Figure 3 Overexpression of an IκBα super-repressor blocks NF-κB activation. (A) Inhibition of NF-κB activity by expression of the human IκBα super-repressor. NIH3T3 cells expressing PKRwt (wt), PKR^{K296R} (K296R), PKRwt and the human IκBα super-repressor (wt/IκBα^{sr}3), or PKRwt and the empty plasmid (wt/C7), grown in the presence of tetracycline (1 μg/ml), were treated for 30 min with TNFα (10 ng/ml) where indicated. IκBα levels were monitored by immunoblotting using an anti-IκBα antibody. hIκBα^{sr} and mIκBα represent the human and mouse IκBα, respectively. Hsp90 antibody was used as a loading control. (B) Expression of c-IAP1 is inhibited in wt/IκBα^{sr} cells. Total RNA was subjected to RT-PCR analysis with primers specific for c-IAP1. Actin was used as control.

activation of NF-κB (Maran *et al*, 1994; Zamanian-Daryoush *et al*, 2000) (Figure 5).

How is cell survival regulated by PKR?

NF-κB-mediated survival involves several gene products (c-IAPs, TRAF1, 2, but also A1 or Bcl-x_L) (Karin and Lin, 2002). The best-studied NF-κB-induced antiapoptotic proteins are the c-IAPs, which directly bind and inhibit effector caspases, such as caspase-3 and -7 (Wang *et al*, 1998). Here we show that PKR induces the c-IAP1 and c-IAP2 genes in an NF-κB-dependent manner. However, the action of the survival genes is transient, and ultimately cells die by PKR-triggered apoptosis, which is activated by eIF-2α phosphorylation-dependent genes, possibly including Gadd153/Chop (Maytin *et al*, 2001) (Figure 5). It is highly likely that the transient nature of the effects of the survival proteins is probably due to the subsequent inhibition of their translation by PKR-mediated eIF-2α phosphorylation. This is somewhat reminiscent of the response to TNFα, which induces both apoptosis through caspase-8 activation and survival via NF-κB, but which is a poor inducer of apoptosis unless a protein synthesis inhibitor is added (Van Antwerp *et al*, 1998).

dsRNA-independent activation of NF-κB by PKR during virus infection

During virus infection, two conflicting cellular programs are triggered: apoptosis to eliminate infected cells, and cell survival to delay cell death in order to alert naive cells by producing antiviral cytokines (Sha *et al*, 1995; Chu *et al*, 1999; Iordanov *et al*, 2001; Li *et al*, 2001). Virus-induced apoptosis is dependent on PKR and eIF-2α (Der *et al*, 1997).

Figure 4 Activation of NF-κB by PKR delays the proapoptotic activity of PKR. (A) Cell viability assay. NIH3T3 cells expressing PKRwt (wt), PKRwt and the human IκBα super-repressor (wt/IκBα^{sr}3), or PKRwt and the empty plasmid (wt/C7) were seeded at 5×10^4 cells/plate in the presence of tetracycline. The number of viable cells is plotted as a function of time following tetracycline removal. Viable cells remaining after tetracycline removal are shown as a percentage of viable cells grown in the presence of tetracycline. (B) eIF-2α phosphorylation is functional in the absence of NF-κB activity. Cell extracts were processed at different periods of time after tetracycline removal to analyze phosphorylation of eIF-2α by immunoblotting with a phosphospecific anti-eIF-2α antibody and an anti-eIF-2α antibody. (C) wt/IκBα^{sr}3 clone dies by apoptosis. Cells were grown in the presence of tetracycline or without for 12 h and stained for the presence of phosphatidylserine by Annexin V-EGFP. (D) Chromatin condensation in wt/IκBα^{sr}3 following tetracycline removal. Cells grown in the presence or absence of tetracycline for 15 h were stained with the DNA dye Hoechst 33342 and visualized with a fluorescence microscope. Chromatin condensation is indicated by arrows.

Cell survival and cytokine production triggered during the infection are mediated by NF-κB (Schwarz *et al*, 1998; Li *et al*, 2001). The protein that senses virus infection to induce the

Figure 5 Model for sequential activation of survival and death programs by PKR. See text for details. The dotted line represents translational inhibition mediated by eIF-2 α phosphorylation. The dashed arrow represents potentiation of NF- κ B signaling by PKR kinase activity. Apoptosis triggered by eIF-2 α phosphorylation is delayed by the transient NF- κ B-induced survival response.

NF- κ B survival pathway was unknown. In this study, we provide evidence that cell survival mediated by NF- κ B and cell death triggered by eIF-2 α are regulated by the same protein, PKR. Here, we show for the first time a sequential activation of both NF- κ B and eIF-2 α pathways during the course of viral infection. According to our model, the early NF- κ B activation implies that some virus-triggered process other than dsRNA accumulation could be responsible for the induction of the PKR/NF- κ B pathway (Figure 5). Several studies reported that NF- κ B can be induced independently of the dsRNA production (Hiscott *et al*, 2001; Servant *et al*, 2002; Santoro *et al*, 2003). For example, virus binding to its extracellular receptor is sufficient for NF- κ B activation (Harrop *et al*, 1998; Bossis *et al*, 2002). Elucidation of the mechanism leading to dsRNA-independent activation of PKR for the early activation of NF- κ B will shed light on our understanding of the antiviral innate immune response.

In conclusion, we report that the dsRNA-activated, interferon-induced protein kinase PKR has a dual function. It chronologically activates cell survival through NF- κ B and cell death through eIF-2 phosphorylation, using kinase-independent and -dependent strategies, respectively. This property of PKR may be shared by other kinases involved in the immune response, such as RIP, IRAK, JAK1, and Tyk2 (Briscoe *et al*, 1996; Gauzzi *et al*, 1996; Malinin *et al*, 1997; Li *et al*, 1999), which do not require their kinase activity to mediate all of their functions. Thus, PKR might serve as a molecular clock to time the sequential events of survival and death following virus infection.

Materials and methods

Microarray analysis

Probes for microarray analysis were prepared using 20 μ g of total RNA obtained from control (+tetracycline) or from PKR-over-expressing (–tetracycline for 8 h) cells. The probes were hybridized to Affymetrix MU11K Gene Chips (Mu11K set, Affymetrix, Santa Clara, CA, USA) containing ~11 000 probes representing both ESTs as well as approximately 7000 known genes. Protocols for the probe

synthesis reaction, the probe hybridization as well as the post-hybridization array processing have been previously described (Novak *et al*, 2002). The arrays were scanned and analyzed using the Microarray Analysis Suite 5.0 (MAS5, Affymetrix, Santa Clara, CA, USA). MAS5 estimates the significance of changes in gene expression using a nonparametric statistical method applied to the measured intensities of each of 20 oligonucleotide probes contained in each probe set (Liu *et al*, 2002). In this study, the manufacturer's default parameters, including the default change P -value ($P < 0.005$), were used to analyze the data and identify differentially regulated probe sets. MAS5 analysis files for the microarray data set have been submitted to the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/; accession numbers GSM10411–GSM10414) and to ArrayExpress (www.ebi.ac.uk/arrayexpress/; accession number E-MEXP-50).

The significance of the overlap between PKR- and NF- κ B-regulated genes was calculated as follows. In total, expression measurements were obtained for 4427 probe sets, which include 75 probe sets representing 54 distinct NF- κ B-regulated transcripts. Our experiments detected 683 probe sets that were differentially expressed following PKR induction (Supplementary Table A). PKR induction altered the expression of 39 probe sets representing 28 transcripts (Table 1) known to be regulated by NF- κ B ($P < 0.001$ by χ^2 analysis).

Antibodies

For immunoblot analysis, cells were lysed as described previously (Donzé *et al*, 2001). PKR was detected using the polyclonal anti-PKR antibody K17 directed against the C-terminus (Santa-Cruz). The antibody against the phosphorylated form of eIF-2 α (Research Genetics) was used as specified by the manufacturer. Monoclonal anti-Hsp90 (H90-10) antibody was a kind gift from Dr David O Toft, Mayo Clinic.

Reverse transcriptase-PCR

Total RNA (1 μ g) was reverse-transcribed with MuLV reverse transcriptase (Gibco) using poly(dT) primer, as described by the manufacturer. Semiquantitative PCR was performed on 2- μ l aliquots from each cDNA reaction, using primer sets for detecting A20 (5'-GAGAGGCGCCAAAAGAATCAGAG-3', 5'-CCGGTGGCAA GAGTGTGGACT-3'), Gadd34 (5'-CCTACCCCTGTCTCTGGTAAC-3', 5'-ACCACCCTCCAGCTGTGATGT-3'), c-IAP1 (5'-GCGGCCGAGGAG GAGGAGT-3', 5'-ATGGCCACAGGAATGAACACGA-3'), IkB α (5'-GCACACCCAGCATCTCCAC-3', 5'-GGCGGCCCGAGTAAGC-3'), Gadd45 α (5'-GCCAAGCTGCTCAACGTAGACCCC-3', 5'-CACGGG CACCCACTGATCCAT-3'), Gadd153/CHOP (5'-GAGTCCCTGCCTTT CACCTT-3', 5'-AGCCGCTCGTTCTCTCTCAG-3'), β -actin (5'-GACG ATGCTGGGCCCCGGGCTGTATT-3', 5'-CATGGCTGGGGTGTGAAGG TCTC-3').

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 μ g) were incubated with a 32 P-labeled NF- κ B-specific oligonucleotide probe (AGTTGAGGGGACTTTCCAGGC). Reactions were analyzed on a 5% nondenaturing polyacrylamide gel in 0.5 $\times$ TBE. Dried gels were subjected to autoradiography.

Kinase assays

PKR from HeLa cells was immunoprecipitated as described above in buffer A. The immune pellet bound to protein G-sepharose beads was washed twice in activity buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM MgCl $_2$, 2 mM MnCl $_2$, 5% glycerol). PKR bound to beads was tested for kinase activity in 40 μ l of activity buffer containing 10 μ M ATP and 10 μ M of (γ - 32 P)ATP. Reactions were incubated for 20 min at 30°C.

Viral infections

Vesicular stomatitis virus (VSV). Mudd-Summers strain of the Indiana serotype was grown on BHK cells and titered on LLC-MK2 cells. For the infections, HeLa cells were grown to 50% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. Cells were infected with an m.o.i. of 4 in DMEM supplemented with 2% serum.

Measles virus. The MeV strain was Hu3 (human 3). Stocks were grown and titered in Vero cells. Infections of HeLa were as above,

except in serum-free medium for 1 h. This was replaced with medium containing 2% serum.

Cell culture

NIH3T3 cells expressing PKR (NIH3T3 PKR) under a tetracycline-repressed promoter have been described (Donzé *et al*, 1999). They were grown in DMEM (Gibco) supplemented with 5% fetal calf serum (FCS; Gibco) in the presence of tetracycline (1 µg/ml). To induce the expression of PKR, cells were washed twice with Tris-buffered saline (TBS) to remove tetracycline, and fed with fresh DMEM containing 5% FCS.

Generation of NIH3T3 PKRwt cells constitutively expressing the IκB super-repressor

NIH3T3 PKRwt cells were transfected with the plasmid pSVK3/IκB super-repressor (Algarde *et al*, 1999) or the empty vector pSVK3, and selected for resistance to hygromycin (Invitrogen; 100 µg/ml). Cells were kept in the presence of tetracycline (1 µg/ml) until induction. Cells were screened for the expression of IκB by immunoblotting. Seven clones expressing the IκB super repressor (wt/IκB^{sr}) and five with the control vector (wt/C) were generated, but two wt/IκB^{sr} clones and one wt/C clone were further characterized. All wt/IκB^{sr} clones start dying 12 h after tetracycline withdrawal, while the wt/C clones behave like the parental NIH3T3 PKRwt cells, with the first sign of cell death at 24 h.

Cell viability experiments

Approximately 5×10^4 cells were plated on each well of a six-well plate 1 day before the experiments. Cells were washed twice with TBS to remove tetracycline. After the indicated periods, the cells

were trypsinized and viable cells were counted by Trypan blue exclusion.

Nucleus morphology

Cells were incubated with a Hoechst labeling solution (5 µg/ml) for 30 min and examined under a fluorescence microscope.

Annexin V binding

Cells were assayed for annexin V using the Annexin V-EGFP apoptosis detection kit (Alexis Biochemicals) according to the manufacturer's instructions and examined by microscopy.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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