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News and Commentary

The dsRNA-dependent protein kinase, PKR and cell death

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Cellular recognition of foreign dsRNA species is an important component of host defense, responsible for initiating innate immune responses to virus infection. Over the years, a number of cellular gene products have been demonstrated to directly bind dsRNA and to play a variety of roles in combating virus replication. This includes facilitating the induction of antiviral type I interferon (IFN), regulating translation and even inducing cellular apoptosis to debilitate virus replication and dissemination. A key player in these dsRNA-mediated events is PKR (protein kinase RNA-dependent), an IFN-inducible serine/threonine protein kinase that autophosphorylates following association with dsRNA to regulate the function of substrate targets. Indeed, PKR has been reported to play a number of roles in the cell, including influencing signal transduction, tumorigenesis and apoptosis in the event of virus infection and other forms of cellular stress. Here, we discuss some of the properties of PKR including its role as an accessory of cell death.

Virus infection of the cell leads to activation of innate immune responses that are accountable for impeding early virus replication and facilitating the establishment of adaptive immunity that comprise the generation of neutralizing antibodies and cytotoxic T cells.[1,2] These innate cellular sentinels include a number of molecules that recognize dsRNA species, usually generated by viruses following infection of the cell.[3] A major consequence of virus replication involves the activation of the interferons (IFNs), an event that can be triggered by dsRNA, and which leads to powerful antiviral responses being invoked.[3,4] Indeed, it is plausible that dsRNA species may be inadvertently generated by many types of virus.[5] For example, single-stranded viruses may generate dsRNA from replicative intermediates, that is, both positive and negative sense RNA. Alternatively, single-stranded RNAs are capable of forming dsRNA duplexes. In the case of viruses with dsRNA genomes, such as reovirus, the inducer of IFN may be the genome itself, as well as corresponding transcripts. Finally, the presence of dsRNA has also been documented for DNA viruses, including vaccinia virus, adenovirus (ADV) and herpes simplex virus (HSV).[3,6–8] In this situation, it has been reported that complementary mRNAs can be produced from overlapping genes transcribed from both directions.

The IFNs are now known to consist of two main families referred to as type I (α/β) and type II (γ), although other species have now been identified.[3,9] The type I IFNs which are induced by most cell types including plasmacytoid dendritic cells, leukocytes and fibroblasts, are grouped together on the short arm of chromosome 9 and consist of a number of α genes and pseudogenes, and one β gene.[3,10–12] In contrast, type II IFN consists of a single gene on chromosome 12 whose product is mainly secreted by Th-1 lymphocytes and NK cells. Over the past few years, the mechanisms of IFN induction by dsRNA/virus have been gradually unraveled and are now known to involve Toll Receptor activation by pathogen-associated molecules such as lipopolysaccharide (LPS) and even dsRNA.[13] For example, exogenous dsRNA species probably generated from lysed, virus-infected cells may bind to Toll Receptor 3 (TLR3) to stimulate type 1 IFN transcription via activation of IRF3 as well as members of the NF-κB and AP1 family.[14] Aside from TLR3, however, interest has fallen onto other dsRNA-binding molecules such as RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation associate gene 5), which are DExD/H box helicases that may function as intracellular dsRNA transducing counterparts to activate IFN via similar pathways.[15] In addition, the dsRNA-dependent protein kinase, PKR, has also been proposed to play a role in the induction of IFN following interaction with viral dsRNA, as discussed further below.[16] Collectively, it is likely that the cell has probably evolved a number of stress-sensing mechanisms that respond to virus infection in an attempt to either stabilize the cell until the crisis has been taken care of, or if not, by invoking cell death, in an attempt to eliminate the threat to the organism. Understanding the mechanisms of how a cell decides its fate, in effect survival or apoptosis, is the object of intense investigation and the decision to invoke cell death almost certainly requires that a number of events must occur, to condemn the cell.

While the exact mechanisms of dsRNA-mediated induction of IFN-β are being unraveled, it has been determined that the IFNβ gene and perhaps some IFNα genes are transcribed and the proteins secreted from infected cells, which bind to species specific cell-surface receptors.[3] This initiates the Janus protein kinase (JAK)/signal transducers and activators of transcription (STAT) pathway (specifically, Jak1/Tyk2 and Stat1/2 heterodimers complexed with IRF 9), which induces the transcription of genes that contain DNA elements in their promoter regions referred to as ISREs (interferon stimulated response elements).[17,18] Many hundreds of genes are induced by IFN, including IRF7 which is required to facilitate transcriptional upregulation of the IFNα gene family and enhance the IFN response.[19] Evidence indicates that IFN is able to inhibit virus replication by mechanisms that likely involve the early blocking of viral transcription/translation as well as genome replication which could activate stress-related cell death.[3] The importance of IFN antiviral action has been

best emphasized by demonstrating that mice lacking a functional IFN system are extremely sensitive to lethal infection with numerous viruses such as vesicular stomatitis virus (VSV), semliki forest virus, and vaccinia virus.[20,21] However, while it is clear that IFN can prevent the replication of a variety of viruses such as VSV without damaging the cell or host, evidence indicates that these cytokines can also greatly sensitize certain tissue-cultured cells to apoptosis, for instance in response to certain viruses and dsRNA.[22,23] Indeed, IFN-inducible genes are known to include proapoptotic TRAIL and PKR itself.[19] Thus, current models suggest that IFN is able to function in an autocrine or paracrine fashion to induce the death of a virus-infected cell, therefore impeding virus spread. Alternatively, secreted IFN can induce an antiviral state in an uninfected cell, effectively preventing virus replication in the absence of apoptosis.

PKR is a Member of the DsRNA-Binding Protein (DRBP) Family

Studies designed to understand the mechanisms of IFN induction in the early 1970s indicated that aside from being able to potently induce IFN, dsRNA also mediated considerable cytotoxicity.[24] This effect, which we now know is due to the cell undergoing apoptosis, was noted to be greatly exacerbated by actually pretreating cells with IFN.[25,26] That exposure to IFN greatly sensitized cells to dsRNA-induced cell death inferred that IFN-induced genes, potentially able to interact with dsRNA, played a key role in governing apoptosis. It was also noted during this period that viral-specific dsRNA could inhibit the initiation of translation *in vitro*.[3,7,27–29] This inhibitory effect was again more pronounced when the cell extracts were isolated from reticulocytes and IFN-treated cells, similarly implying a role for IFN-inducible dsRNA-interacting transducing molecules. Subsequently, purification studies using poly IC and antibody affinity columns eventually lead to the isolation of a kinase from IFN-treated cell extracts that could be phosphorylated, *in vitro*, following the addition of dsRNA.[27] The kinase, now referred to as PKR, was eventually cloned by screening expression libraries, was confirmed to be IFN-inducible and to potently inhibit protein synthesis *in vitro*.[30]

Following cloning, it was elucidated that PKR contained two dsRNA-binding domains (DRBDs) in its amino terminus. These domains were found to share homology with other dsRNA-binding proteins such as *Xenopus* rbpa and *Drosophila* Staufen, prototypes of the DRBP family.[31] Other members of the DRBP family are now known to include DICER, RNAse III and the ADAR family of adenosine deaminases.[31] The DRBDs do not recognize specific nucleotide sequences and have been reported as predominantly interacting with A-form double-helix RNAs. The DRBDs bind nonspecifically to dsRNAs, including ssRNAs with extensive secondary structures and do not associate with dsDNA or ssDNA.[31] Experimental data suggest that as little as 11–16 bp dsRNA can interact with a DRBD. Interestingly, *in vitro*, the concentration of activator does play a role in the autophosphorylation of PKR, and low levels of dsRNA potently activates the kinase, while higher concentrations of dsRNA are less effective (for a review, see Williams,[16] Hovanessian,[27] Samuel,[28] Fierro-Monti and Mathews[32]). Possibly, high concentrations of dsRNA prevent PKR activity by impeding intermolecular interactions and trans autophosphorylation. In humans, PKR, which exists as a 551 amino-acid protein encoded from a single gene, is located on chromosome 2p21 and contains a serine/threonine kinase domain located in the C-terminus. Substantial evidence now indicates that interaction with dsRNA causes PKR to form homodimers and to autophosphorylate, in trans, on multiple serine/threonine residues including threonine 446 and 451[16,28,32,33] (Figure 1).

PKR and the Control of Translation

Following autophosphorylation, PKR is able to catalyze the phosphorylation of target substrates, the most well characterized being the eIF2α subunit on Ser 51.[27,34,35] Phosphorylated eIF2α sequesters eIF2B, a rate-limiting component of translation, leading to an inhibition of protein synthesis in the cell. eIF2 is a heterotrimer composed of three subunits (α, β, γ), which functions by associating with guanisine tri-phosphate (GTP) and the initiator met-tRNA$_i$ to form a ternary complex (for a review, see Kapp and Lorschv[36] and Merrick[37]). The ternary complex delivers the met-tRNA$_i$ to the 40s ribosomal subunit and along with other translation factors, including eIF3, forms the 43s preinitiation structure. Newly assembled 43s ribosome/eIF complexes associate with an mRNA transcript near the 5′ m^{7}G cap, and advance along the transcript in a 3′ direction until an AUG start codon is located within the context of an appropriate Kozak sequence.[38] Once the AUG codon has been recognized, GTP bound by eIF2 is hydrolyzed in a reaction catalyzed, in part, by another initiating factor, eIF5. The met-tRNA$_i$ is subsequently released from the ternary complex to initiate nascent peptide chain synthesis, and eIF2 dissociates from the 43s initiation complex. The GDP associated with the free eIF2 is exchanged for GTP by the activity of the eIF2B complex, which is itself a heteropentamer comprised of α, β, γ, δ and ε subunits. Following GTP exchange, eIF2 is incorporated into a new ternary complex and the next round of initiation begins[36–38] (Figure 2). Phosphorylation on serine 51 of eIF2α by stress-responsive kinases such as PKR, however, causes eIF2 to acquire an increased affinity for, and functionally sequester, the GTP exchange factor eIF2B, which is required for maintaining eIF2 activity.[39,40] Thus, in response to stress, eIF2α kinases can depresses global translation rates by inhibiting eIF2-GTP recycling and, subsequently, initiation of translation.[39] Aside from dsRNA-mediated activation of PKR, accumulation of mis-folded proteins in the endoplasmic reticulum (ER) leads to activation of an ER resident eIF2α kinase named PKR-like endoplasmic reticular kinase (PERK) or pancreatic eIF2α kinase (PEK).[34,41,42] Similarly, the mammalian homologue of the yeast GCN2 eIF2α kinase functions as a cytoplasmic sensor of amino-acid levels via two His-tRNA-like domains in its carboxy terminus. GCN2 kinase activity is upregulated under starvation conditions during which the levels of charged tRNAs fall.[40] The activity of the heme-regulated inhibitor kinase (HRI), in contrast, is predominantly expressed in

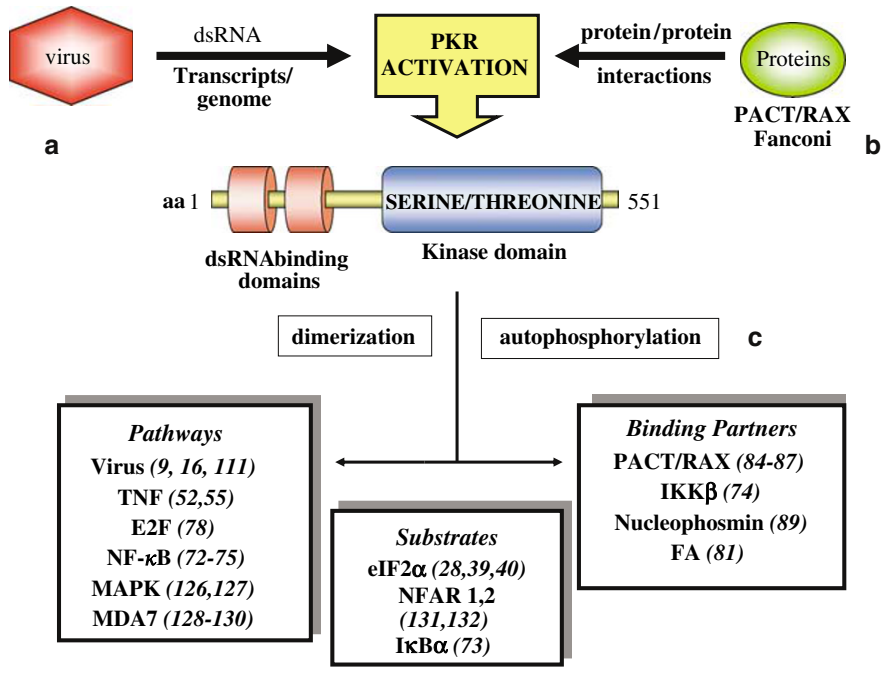

Figure 1 The functions of PKR: (**a**) dsRNA produced as a result of virus infection activates PKR, inducing dimerization and autophosphorylation and allowing the kinase to phosphorylate substrate targets. (**b**) In addition to dsRNA, however, proteins such as PACT/RAX have been reported to directly associate with PKR, similarly stimulating autophosphorylation. (**c**) Aside from targeting substrates, PKR is also been described as directly interacting with selected proteins without phosphorylating them. PKR has additionally been reported to be involved in regulating a number of pathways involving TNFα, E2F and MDA7, although the mechanisms of action remain to be determined

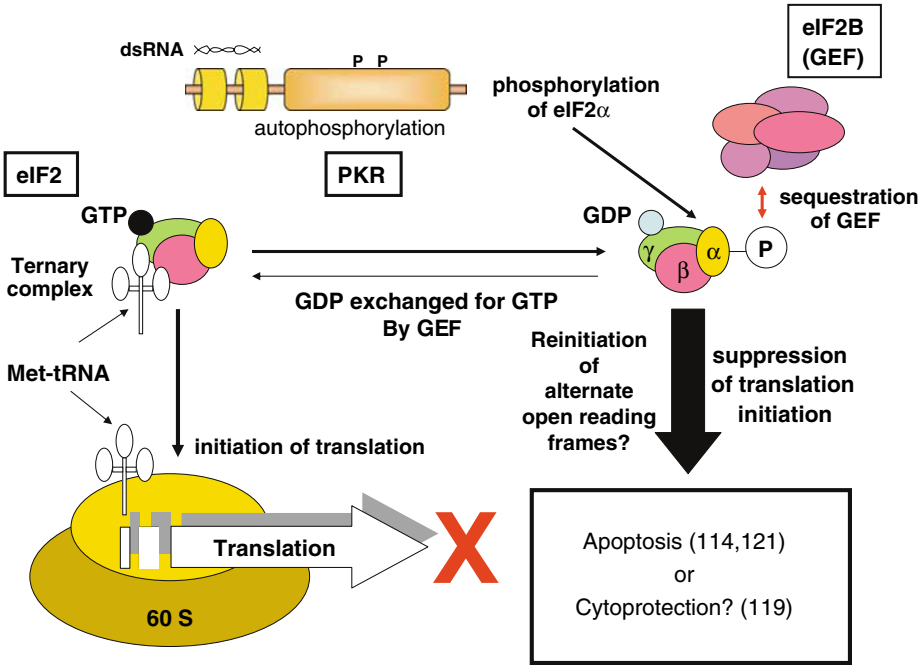

Figure 2 The regulation of eIF2 and translation by PKR. Under normal conditions, eIF2 associates with GTP and Met-tRNAi to form the ternary complex, which delivers Met-tRNAi to the 40S ribosomal subunit prior to the arrival of mRNA. Met-tRNA is then released on the initiating AUG codon and translation ensues. This event leads to the hydrolysis of GTP to GDP. Replacement of GDP with GTP is required for a fresh round of initiation to occur, an event carried out by the guanine nucleotide exchange factor, eIF2B. Following binding of dsRNA, PKR autophosphorylates and subsequently phosphorylates eIF2α on serine 51. This causes sequestration of eIF2B to eIF2 and prevents GDP exchange, inhibiting the initiation of protein synthesis and influencing apoptosis. Other eIF2α kinases that can carry out similar functions include PERK, GCN2 and HRI

erythroid cells, and is negatively regulated by hemin binding.[40,41] Disregulation of the eIF2α checkpoint by ER stress or by dsRNA has been indicated to lead to cell death, as described below.

Attempts to further elucidate the importance of PKR in IFN-mediated antiviral function triggered a number of experiments aimed at overexpressing this kinase in mammalian cells in the absence of other interferon-induced gene products. However, it rapidly became clear that PKR exerted a powerful growth suppressive and toxic effect on the host and few clones expressing recombinant PKR could be isolated.[43–45] One possibility for these observations could include that viral-specific sequences commonly used in eucaryotic expression plasmids could be activating PKR and subsequently eIF2α to inhibit translation within the host cell. Certainly, attempts to express PKR using baculoviruses and retroviruses were met with failure probably for these reasons.[44,45] In contrast, murine fibroblasts expressing a catalytically inactive dominant-negative PKR variant were readily isolated and became malignantly transformed, speculatively suggesting that PKR may be involved in tumor suppression.[44,46]

The first indications that PKR could mediate apoptosis came from studies with recombinant vaccinia viruses expressing wild-type PKR or a defective PKR variant.[47–51] Essentially, selected viruses expressing the functional gene but not the PKR variant were found to cause the rapid apoptosis of HeLa cells. Other data showed that cells expressing catalytically inactive PKR variants were more resistant to influenza-induced apoptosis, and that inactivation of PKR with antisense RNA similarly rendered U937 cells less susceptible to EMCV-induced apoptosis. Studies on murine fibroblasts lacking PKR also indicated resistance to TNF-induced apoptosis.[52–55] Collectively, these data provided powerful evidence that PKR is a proapoptotic gene that can mediate dsRNA and virus-induced programmed cell death. Further clues as to the mechanisms of PKR-mediated apoptosis has come from cells that inducibly expressed PKR. In this situation, using murine 3T3 L1 cells fibroblasts, overexpressed PKR was not overly toxic, but cells were rendered extremely susceptible to treatment with dsRNA or certain viruses such as influenza virus.[56] Activation of PKR was found to correlate with the expression of the death receptor Fas which appeared to be able to escape the translational block imposed by PKR.[56,57] Further investigations revealed that cells lacking FADD but not the mitochondria related Apaf-1 were more resistant to dsRNA and PKR-induced apoptosis, clearly implicating the importance of the FADD/caspase-8 death signaling process in this pathway.[22,58] In addition, HeLa cells expressing dominant-negative FADD variants appear resistant to dsRNA-induced cell death and specific inhibitors of caspase-8 block dsRNA-mediated cell death. Presently, the mechanism of PKR-induced Fas expression and FADD-mediated death is unclear. Since PKR has been proposed to play a role in mediating dsRNA-signaling, it is plausible that PKR may induce the transcription of several death-promoting molecules through signal transduction pathways such as those involving NF-κB and the p38 mitogen-activated kinase (MAP) pathway, which can strongly influence apoptosis.[16,59–61] In this regard, Fas and FasL are known to be induced by NF-κB.[62–64] However, NF-κB is also known to elicit the transcriptional induction of a number of antiapoptotic genes, such as the cIAPs.[65] One model of PKR-mediated cell death could, therefore, involve the inhibition of protein synthesis (in this case through activating eIF2α), and concomitant activation of NF-κB, a combination known to influence apoptosis.[66] For example, oligonucleotide array analysis using NIH 3T3 cells that inducibly expressed PKR indicated that the kinase, following expression, transiently induced NF-κB-induced survival genes including the c-IAPs or A20, an event which was found to delay PKR-mediated cell death.[67] NF-κB is known to activate a wide range of genes involved in regulating both the innate and adaptive immune responses (such as transcriptionally inducing numerous cytokines and chemokines, adhesion molecules and regulators of apoptosis).[68] The activation of NF-κB by PKR, however, was not found to require PKR autophosphorylation. Cell death was reported to occur through eventual phosphorylation of eIF2α by an activated PKR.[67] The authors propose that PKR thus serves as both a pro and antiapoptotic factor, first delaying an apoptotic response, perhaps in an effort to save the cell or to allow the secretion of warning factors that alert other cells. Later, cell death could occur, perhaps through the eIF2α or other pathways, to eliminate the infected cell and limit virus spread. Nevertheless, a recent report indicated that while HSV-1 infection could indeed activate NF-κB in a PKR-dependent manner, this event did not result in any inhibition of cell death.[8,69] Certainly, activation of NF-κB by PKR has been well documented, yet still remains controversial, no doubt since the regulation of these transcription factors remains a complex issue.[70–72] For example, PKR has been reported to regulate NF-κB by directly phosphorylating IκB, which then becomes the target of degradation by the ubiquitin proteasomal pathway.[73] Phosphorylation of subunits of NF-κB then leads to translocation to the nucleus. Other groups, however, have reported that a nonphosphorylated PKR can activate the NF-κB pathway via directly interacting with IKKβ to cause phosphorylation of I$\kappa\beta$.[74] In contrast, alternate studies have shown that dsRNA-mediated activation of NF-κB activity does not require PKR.[75] Finally, recent data additionally demonstrate that NF-κB can be regulated through eIF2α activation.[76,77] Collectively, overwhelming evidence indicates that PKR can influence the regulation of NF-κB and perhaps apoptosis through this pathway, although further work will no doubt be required to exactly clarify this important and complicated issue.

PKR has also been documented to play a role in influencing apoptosis governed by a variety of cellular proteins. For example, the transcription factor E2F-1 is known to induce cell cycle progression and if disregulated can potently induce cell death. Recently identified E2F-1-induced genes have been reported to include PKR, which was further demonstrated to play a role in regulating E2F-1-mediated apoptosis.[78] PKR has similarly been implicated as a potential pathogenic factor of Alzheimer's, Parkinson's and Huntingtons' diseases.[79] That is, inadvertent activation of PKR may contribute towards neuronal cell death. Data indicate that the endoplasmic reticulum, important for post-translationally modifying and correctly folding proteins, may be damaged in neuronal degenerative disease.[80] Studies into this field lead to

the isolation of PKR as a protein that may facilitate ER stress-mediated cell death in these situations. PKR has also been found to be implicated in Fanconi anemia (FA), a disease characterized by leukemia.[81,82] FA protein variants have been reported to functionally interact with PKR, to activate the kinase and thus inadvertently induce cell death. Normal FA proteins, in contrast, may repress PKR activity. It has recently been further reported that PKR is involved in mediating macrophage apoptosis following TLR4 activation by LPS.[83] A lack of PKR, however, was not found to effect p38 or IKK activation in response to this stimuli.

It remains to be shown how PKR is exactly activated in the above examples. For instance, while the autophosphorylation of PKR by dsRNA is now well characterized, it has been reported that some cellular proteins can carry out similar activating functions. In one example, a protein referred to as PACT (or RAX in the mouse) has been demonstrated as being able to directly bind and activate PKR.[84–87] PACT/RAX is member of the DRBD family, as described earlier, and has been shown to induce apoptosis following activation of PKR, in response to stress such as serum starvation and arsenite treatment.[31,88] Similar studies aimed at seeking novel PKR-interacting proteins lead to the isolation of nucleophosmin (NPM), a protein often expressed at elevated levels in tumors.[89] NPM was shown to bind to PKR and inhibit its activity, thus preventing apoptotic responses and potentially promoting sporadic malignancies. Tumor-specific activation of PKR has also been used to inhibit malignant disease in animal models using RNA activators unique to the cancer.[90] Given that expression of a catalytically inactive PKR variant leads to malignant transformation of NIH 3T3 cells and tumorigenicity in nude mice, this data would further imply that suppression of PKR function could contribute towards cancer of the cell.[44]

The Regulation of PKR by Viruses

It is now known that many viruses encode products that directly block the apoptotic signaling cascade to promote their survival.[91–93] For example, adenovirus encodes E1B-19K, a product functionally analogous to Bcl-2 that can influence mitochondrial induced cell death as well as apoptosis mediated through Fas and TNFR-1.[94] Other viruses that encode products to prevent caspase activation include cowpox virus crmA and insect baculovirus-encoded p35.[93,95,96] EBV encodes a Bcl-2 homologue, BHRF1 as well as LMP1, which may induce expression of endogenous Bcl-2 and NF-κB.[93,97,98] Finally, viral homologues of inhibitors of apoptosis referred to as Flips have similarly been reported.[99] Considering this data, it is unsurprising that viruses have also devised mechanisms to inhibit PKR, thus preventing the inhibition of protein synthesis, which would be disadvantageous to their replication or cell death.[100,101] Examples include vaccinia virus, which has been reported to encode two products that impede PKR activity.[102,103] One of the proteins, referred to as E3L, itself contains a DRBD and is primarily detected in the nucleus where it competes for dsRNA activators and may even bind to and inhibit the kinase through dsRNA bridging. E3L appears to interact with both the DRBDs and the catalytic domain of PKR, and dsRNA appears to enhance E3L association with the DRBDs and reduce its interaction with PKR's catalytic domains. A second vaccinia protein, referred to as K3L, shares homology to the known PKR substrate eIF2α and is thought to function by competitively sequestering the kinase.[102,103] Other viral proteins shown to inhibit PKR-mediated apoptosis include Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded LANA2, as well as Epstein–Barr virus (EBV)-encoded RNAs (EBERs), which may inhibit PKR by preventing correct activation.[104–106] Influenza virus protein NS1 has been similarly reported to inhibit PKR, as have selected hepatitis C virus (HCV), herpes simplex 1 (HSV) and human papilloma virus (HPV) proteins.[107–110] Certainly, studies using PKR-null mice or MEFs lacking PKR activity have confirmed that this kinase is a key component of the host early defense system, which acts in innate immunity prior to the activation of the IFN system and the acquired immune response.[111] PKR-null mice are susceptible to usually nonlethal doses of vesicular stomatitis virus (VSV) and show increased sensitivity to influenza infection.[111–113] Indeed, cells and animals lacking PKR and translational restraints exhibited more apoptosis in response to virus infection, presumably since unbridled virus replication exerted a cytolytic effect independent of PKR activity. Aside from their susceptibility to certain viruses, however, PKR knockout mice are developmentally normal.[71] There is no significant impairment of type I IFN gene induction by dsRNA or virus in many mouse organs, perhaps since the Toll Receptors or RIG-I like molecules compensate for loss of PKR. The induction of type I IFN has been reported to be impaired in PKR$-/-$ MEFs in response to dsRNA, although this effect can be corrected if the cells are primed with IFNα.[71] Nevertheless, it is not yet clear whether PKR-deficient animals are defective in apoptosis signaling in response to cellular stress. PKR appears to be present in most mammalian species, although it is less clear whether the kinase exists in lower order vertebrate species or indeed invertebrates. The considerable amounts of data indicating that numerous viruses suppress PKR clearly indicate that this kinase has potent antiviral activity, and presumably its inhibition would have profound impact on the regulation of viral translation and/or viral-induced apoptosis.

Mechanisms of PKR Action

Elucidating the mechanisms of PKR-mediated apoptosis has been difficult to clarify, although obviously, one potential mechanism could involve the eIF2α pathway and the inhibition of proteins synthesis. For example, transient expression of a variant of eIF2α that mimics phosphorylated eIF2α (Ser$\rightarrow$51Asp) was reported to cause apoptosis in COS-1 cells.[114] Conversely, cells infected with recombinant vaccinia viruses expressing an eIF2α variant (Ser 51–Ala), unable to be phosphorylated by PKR, have been shown to be protected from virus-induced apoptosis.[48] It is plausible that PKR-mediated inhibition of protein synthesis may deplete the cell of short-lived proteins involved in suppressing cell death. However, treatment of cells with levels of cycloheximide capable of inhibiting protein synthesis does not necessarily

induce rapid apoptosis unless another apoptotic signal such as Fas or TNF receptor ligation is performed.[115,116] In contrast, PKR-mediated apoptosis may work in a manner similar to the GCN2 paradigm in yeast, where the amount of unphosphorylated eIF2α governs whether translation of certain mRNAs will occur at an authentic initiation codon or at alternative upstream noncoding open reading frames.[34] The mechanism of transcript-specific translational upregulation has been reported to be, in part, dependent upon upstream open reading frames (uORFs) in the 5′ untranslated region (UTR) of the mRNA. Under normal physiologic conditions, these short uORFs lower the efficiency of translation, presumably by impeding the progress of the scanning ribosome. However, under conditions in which the levels of phosphorylated eIF2α rise, and levels of available ternary complex fall, these uORFs may favor the association of the transcript with active ribosomes.[34,38] Examples to date of transcripts that are regulated in this manner include the transcription factors GCN4 in yeast, and ATF4 in mammalian cells.[117–119] Both of these proteins are responsible for upregulating the transcription of genes whose products are essential for an effective response to a specific stress. This view is supported by a recent knock-in mouse model of eIF2α which lacks the ability to regulate key enzymes such as PEPCK, and which are involved in gluconeogenesis.[120] These results indicate that eIF2α functions as a key switch in modulating the cellular response to stress by regulating levels of transcription factors and effector molecules such as ATF4 which in turns can transcriptionally activate molecules such as the C/EBP homologous protein CHOP (also referred to as GADD153). CHOP may sensitize cells to ER stress-mediated death by directly regulating target genes in the nucleus such as GADD34, which has been reported to dephosphorylate activated eIF2α and induce death by actually repromoting protein synthesis. Accordingly, such regulation can decide whether a cell lives or dies.[121,122]

To further clarify the role of eIF2α in apoptosis, transformation and gene regulation, our own laboratory developed inducible, murine cell lines expressing a phospho-mimetic eIF2α variant (eIF2α-S51D) and the phosphorylation-insensitive eIF2α-S51A variant.[123] Through this approach, we observed a reproducible and distinct change in the cellular morphology of both the eIF2α-S51D- and the eIF2α-S51A-expressing cell lines, for reasons that remain unclear.[124] Expression of the eIF2α-S51A variant resulted in a very mild increase in growth rates.[67] Similarly, fibroblasts isolated from a knockin mouse model carrying two copies of the S51A allele exhibited translation rates that were elevated 18–35%.[120] Such knockin mice die within hours after birth due to hypoglycemia caused by deterioration of pancreatic beta islet cells.[120] The eIF2α-S51D-expressing cells demonstrated a reduction in growth rate, suggesting a connection between cell cycle regulation and translation initiation and were more resistant to virus infection. However, cells inducibly expressing eIF2α-S51D did not undergo rapid apoptosis.[123] Thus, it appears that at least in a 3T3 L1-inducible system, the eIF2α-S51D mutant causes a modest elevation in 'background' cell death, but does not induce global cell death. Further, apoptosis in response to well-characterized stimuli such as TNFα was also unaltered by expression of either of the eIF2α variants.[123] This data may indicate that while the phosphorylation of eIF2α can influence cell death, it is not sufficient on its own to induce apoptosis and perhaps requires other stress stimuli. Thus, while eIF2α has indeed been proposed to play a role in cell death, this initiation factor has also been implicated in the activation of cytoprotective gene expression pathways such as those involving NF-κB, which may actually potentially suppress apoptosis.[76,77,119] Therefore, eIF2α phosphorylation may exert completely contrasting effects, depending on other forms of costimuli occurring within the cell.

As mentioned earlier, experiments using 3T3L1 cell lines inducibly overexpressing human PKR has also shown that dsRNA transfection results in an apparent induction in the levels of the Fas death receptor protein. This PKR-dependent induction did not strongly correlate with a commensurate increase in the levels of Fas transcription as measured by RNase protection assay.[56] However, Fas or other apoptotic genes were not significantly upregulated in cells expressing the phosphomimetic version of eIF2α. While further work is clearly required to clarify this issue, it is plausible that the dsRNA-dependent regulation of Fas and apoptosis occurs independent of eIF2α and through other dsRNA signaling pathways.[76,77,117,119]

Finally, evidence indicates that PKR may have a plethora of targets other than eIF2α. Thus, these pathways may play a significant role in governing cell death. As discussed, PKR has been reported to be able to influence the regulation of NF-κB.[16] While this may involve PKR directly associating with members of the NF-κB family and even via eIF2α, as discussed, other reports have indicated that this may occur through PKR associating with members of the TRAF family.[125] Aside from NF-κB, PKR has also been shown to phosphorylate mitogen-activated protein kinase 6 (MKK6) and to regulate p38 mitogen-activated protein kinase (MAPK) activation in response to dsRNA stimulation.[126,127] The p38 MAPK cascade regulates a variety of cellular responses to stress, inflammation and cytokines.[59] Activation of these stress-activated kinases has been reported as being defective in cells lacking PKR following exposure to LPS, dsRNA and proinflammatory cytokines. Phospho-MAPK is known to activate a number of transcription factors such as ATF-2 and CHOP as well as influence the regulation of P53 and p73 and cell death.[59] Another molecule shown to interact with PKR is melanoma differentiation-associated gene (mda-7) also referred to as IL-24, a potent tumor suppressor protein.[128–130] Adenoviral-mediated expression of mda-7 has been shown to cause growth suppression and apoptosis in a wide variety of cancer cells and does not harm normal cells. This effect was reported to be through promoting mitochondrial dysfunction and reactive oxygen species (ROS) production. Recent data indicated that mda-7 may activate PKR to induce apoptosis, an effect that correlated with eIF2α phosphorylation.[130] Unraveling the mechanisms of mda-7 function, while complex are considered key issues since it is clear that this cytokine holds considerable promise as an antitumor agent. Yet another series of molecules that have been reported to interact with PKR and serve as a substrate for this kinase are referred to as the NFAR proteins (Nuclear factors associated with dsRNA although also referred to as NF-90/DRBP76 and in the mouse as mILF3).[31,131] It has been determined that a

single NFAR gene exists on chromosome 19, which generates the two alternatively spliced variants. NFAR-1 is a 90kDa protein that is 99% identical to DRBP76, while NFAR-2, exists as a 110kDa protein.[131,132] Aside from demonstrating that the NFARs associate with PKR in yeast, both NFAR-1 and 2 share homology with eIF2α, the known PKR cytoplasmic substrate, and appear to be substrates for PKR at least *in vitro*, indicating that they may function in PKR-mediated signaling events in the cell.[131] One model for this association would include that an activated PKR, perhaps following virus infection, would phosphorylate the NFARs to regulate their function, an effect detrimental to virus infection. Given this, the vaccinia virus DBRP E3L has been found to interact with the NFAR proteins, perhaps as a mechanism to counter the apoptotic action of PKR. Additionally, while adenovirus VA RNA_I has been shown to bind to and inhibit PKR, another adenovirus structural RNA, VA RNA_{II}, was identified as binding to NFAR2/NF90.[133] However, what is the function of the NFARs? In transfected cells, both NFARs were found to regulate the expression of cotransfected reporter genes, probably at the post-transcriptional level and it is possible that the NFARs play a key role in mRNA transport.[134] However, the exact functions of the NFARs remain to be determined. Finally, PKR has been proposed to play a role in signaling pathways involving STAT-1, p53, cyclin B1, PDGF, IRF-1, ASK-1, most of which are known to strongly influence cell death. Thus, it is plausible that PKR may be involved in a number of signaling pathways and may facilitate apoptosis in response to a number of stimuli. Determining the importance of these interactions as well as exact mechanisms of PKR-mediated cell death, however, remain key issues to be concluded.

Summary

PKR has been shown to potentially regulate cell death in response to a number of stimuli such as dsRNA, and may even be activated following direct interaction with other proteins. What is less clear is how PKR may mediate these effects. While some data indicate that PKR can facilitate cell death through eIF2α and the regulation of translation, other data indicate that this may not be sufficient on its own to trigger apoptosis. Almost certainly a combination of other stress-related events would need to occur to condemn the cell to die. Presently, it is clear that PKR is important in preventing virus translation, through eIF2α phosphorylation, following infection of the cell. This event would allow time for IFN to be produced, to fortify an antiviral state. However, it is also extremely likely that PKR functions to regulate pathways other than those involving eIF2 that may similarly facilitate host defense against virus infection or which govern cell death. This could involve signaling mechanisms involving the FADD, NF-κB or the p38, MAPK stress kinase pathways. Elucidating the exact mechanisms of PKR-associated cell death remains an important issue since this kinase plays a key role in antiviral host defense and perhaps tumorigenesis. In addition, recent studies show that PKR may play a role in neurogenerative diseases. The potential value of understanding how PKR may regulate cell growth and apoptosis may thus provide therapeutic opportunities that could be exploited in strategies designed to combat viral, malignant and neurodegenerative disease.

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