The catalytic activity of dsRNA-dependent protein kinase, PKR, is required for NF- κ B activation

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The double stranded RNA-dependent protein kinase (PKR), in addition to its role as a translational controlling factor, is a key transcriptional regulator exerting antiviral and antitumoral activities. We have previously shown that induction of NF- κ B by PKR is involved in apoptosis commitment and this process is mediated through activation of the IKK complex. To gain insights into the mechanism of activation of NF- κ B by PKR, we have analysed the domains of PKR involved in IKK activation and subsequent NF- κ B induction. In PKR^{0/0} cells infected with a collection of vaccinia virus (VV) recombinants expressing different mutant forms of PKR, we found that only PKR forms conserving the catalytic activity are able to activate NF- κ B. An inactive PKR mutant (K296R), was unable to induce NF- κ B activation despite full expression of the protein in a wide range of concentrations, as defined by Western blot, EMSA, IKK kinase activity and NF- κ B transactivation assays. Moreover, the mutant PKR (K296R) acts as a dominant negative of PKR-induced eIF-2 α phosphorylation and NF- κ B activation. However, PKR mutants unable to activate NF- κ B still retain their ability to associate with the IKK complex, as confirmed by immunoprecipitation analysis. We conclude that the catalytic activity of PKR and not only a protein-protein interaction with the IKK complex, is needed for activation of the transcription factor NF- κ B. *Oncogene* (2001) 20, 385–394.

Keywords: PKR; IKK; NF- κ B; dsRNA

Introduction

Viral infection induces a complex protective response in the infected cell that is in some aspects shared by different viruses. One of these mechanisms is mediated through the accumulation of dsRNA as a by-product of viral replication, that acts as a second messenger, triggering activation of different signal transduction

pathways and switching on dsRNA dependent enzymes to induce a cellular antiviral response (Jacobs and Langland, 1996). Among dsRNA-triggered enzymes, the best known are the 2-5-A oligosynthetases, key partners of a pathway of RNA degradation together with ribonuclease L (reviewed in Rebouillat and Hovanessian, 1999), and the protein kinase, PKR (Clemens and Elia, 1997; Meurs *et al.*, 1990). PKR controls different cellular processes such as cell growth (Chong *et al.*, 1992), differentiation (Petryshyn *et al.*, 1984), apoptosis (Der *et al.*, 1997; Lee and Esteban, 1994) and also exerts antiviral (Lee *et al.*, 1996; Lee and Esteban, 1993) and antitumor activities (Meurs *et al.*, 1993). PKR is a serine threonine kinase, functionally divided in an N-terminal regulatory region, and a C-terminal kinase domain. The N-terminal regulatory region is composed of three clusters of basic residues. The first two act as dsRNA binding motifs (dsRBMs). Although PKR can be activated by binding of heparin and other polyanions (George *et al.*, 1996; Hovanessian and Galabru, 1987), or by the activator protein PACT/RAX (Ito *et al.*, 1999; Patel and Sen, 1998), the main mechanism of PKR activation is mediated by its interaction with dsRNA (Clemens and Elia, 1997). After dsRNA binding, PKR dimerizes, and it is activated through autophosphorylation in residues located in the autoactivation loop (Romano *et al.*, 1998) and in the third basic domain (TBD) (Taylor *et al.*, 1996). The TBD is a key regulatory region of PKR. It is involved in dimerization of PKR, and autophosphorylation sites are also located in its sequence (Tan *et al.*, 1998; Taylor *et al.*, 1996). Both dimerization and autophosphorylation are key events involved in PKR activation. Thus, deletion of TBD, or point mutations into this region results in inactive PKR mutants (Lee *et al.*, 1994). Once activated, PKR inhibits translational initiation by phosphorylating the α subunit of the eukaryotic initiation factor 2 (eIF-2 α , Levin and London, 1978) and also regulates the activation of several transcription factors such as IRF-1, p53, and NF- κ B (Kumar *et al.*, 1997; Cuddihy *et al.*, 1999). Among these factors the induction of NF- κ B is known to have a significant impact on PKR functions. NF- κ B activation by PKR is involved in IFN- β induction in response to dsRNA (Chu *et al.*, 1999; Visvanathan and Goodbourn, 1989) and has also been shown to play a role in the induction of cell death triggered by PKR (Gil *et al.*, 1999).

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The Rel/NF- κ B family of transcription factors upregulates the expression of genes involved in immune and inflammatory responses, cell differentiation and control of apoptosis, among other important processes (review by Ghosh *et al.*, 1998). The main regulatory mechanism of NF- κ B is mediated through its interaction with inhibitory molecules of the I κ B family that retain NF- κ B in the cytoplasm (Verma *et al.*, 1995). In response to a variety of activators, including dsRNA (Visvanathan and Goodbourn, 1989), the prototypic member of this family, I κ B α , is phosphorylated by the I κ B kinase complex (IKK) at serines 32 and 36, rendering the factor susceptible to proteolysis via the ubiquitin-proteasome pathway (Roff *et al.*, 1996). This event unmasks a nuclear localization sequence allowing NF- κ B translocation to the nucleus, where it upregulates the transcription of many genes. The IKK signalsome (DiDonato *et al.*, 1997; for a review see Zandi and Karin, 1999; Karin, 1999) is a 900 kDa protein kinase complex consisting of at least two catalytic subunits (IKK1 or IKK α and IKK2 or IKK β) (Zandi *et al.*, 1997; Mercurio *et al.*, 1997) and a scaffold protein (IKK γ or NEMO) (Yamaoka *et al.*, 1998). Given the fact that IKK is a common transducer of many pathways of NF- κ B activation, one of the major questions to be addressed is how the IKK complex is activated. Studies have focused on IKK activation in response to cytokines such as IL-1 and TNF- α . From initial work, it seems that MAP3K such as NIK and MEKK1 (and others of the family) are key transducers in these pathway (Lee *et al.*, 1997; Nemoto *et al.*, 1998). In addition, the role of several pathway specific-adaptor proteins (such as members of the TRAF family or MyD88) in IKK activation has been clearly shown (Hsu *et al.*, 1995; Arch *et al.*, 1998; Wesche *et al.*, 1997). Interestingly, protein kinases such as RIP (in the TNF- α pathway) or IRAK (in the IL-1 pathway) also are needed for NF- κ B induction, but their role is limited to be scaffold proteins, and their kinase activity is dispensable for IKK activation (Knop and Martin, 1999; Maschera *et al.*, 1999; Ting *et al.*, 1996).

Recently, we and others have shown that NF- κ B activation by PKR involves the I κ B kinase complex (IKK) (Chu *et al.*, 1999; Gil *et al.*, 2000; Zamanian *et al.*, 2000), but the question of how PKR activates IKK is a subject of controversy. It has been shown that PKR-mediated activation of NF- κ B can be inhibited by blocking PKR catalytic activity using the chemical inhibitor 2-aminopurine (Kumar *et al.*, 1994; Cheshire *et al.*, 1999). However, more recently another study suggested that expression of a PKR K296R point mutant lacking catalytic activity, was able to activate the IKK complex, (Chu *et al.*, 1999). Thus, to clarify the mechanism of IKK activation by PKR it is important to define if PKR catalytic activity is needed for NF- κ B activation, or if indeed, PKR acts as a mere scaffold protein. Using a collection of vaccinia virus (VV) recombinants that express PKR wild type and different mutant forms, in this investigation, we have defined the induction of NF- κ B by these constructs in

PKR^{0/0} cells to avoid interference caused by endogenous PKR. Our findings revealed that interaction of PKR with the IKK complex is not sufficient and that kinase activity is needed for PKR-mediated NF- κ B activation.

Results

Catalytic activity of PKR is necessary to trigger NF- κ B activation

To get a better insight into the mechanism of NF- κ B induction by PKR we have measured the ability of PKR wild type (WT) and different deletion mutants to activate NF- κ B. We used VV recombinants that express in an IPTG dependent manner different PKR mutants. A scheme of PKR mutant forms is shown in Figure 1a. Expression from VV recombinants in PKR^{0/0} cells of PKR WT and different mutants by VV recombinants is shown in panel b. PKR mutants accumulate in cells according to their ability to inhibit protein synthesis. Thus, expression levels of VTL-8 and VTL-7 are higher than WT-PKR, whereas VTL-23 expression is lower, as previously described (Rivas *et al.*, 1999; Lee *et al.*, 1994).

The NF- κ B activation induced by the different PKR-expression vectors in PKR^{0/0} cells was assessed by electrophoretic mobility shift assay (EMSA) (Figure 1c). Expression of PKR WT resulted in nuclear NF- κ B translocation and this effect was also induced by the VTL-23 mutant and to a lesser extent by the VTL-7 mutant. However, VTL-8 expression vector, that lacks the third basic domain, a key regulatory region involved in PKR dimerization and autophosphorylation (Taylor *et al.*, 1996; Tan *et al.*, 1998), was not able to activate NF- κ B. Thus, activation of NF- κ B by the PKR mutants appeared to correlate with the kinase activity of the enzyme (Rivas *et al.*, 1999; Lee *et al.*, 1994). To provide further evidence that induction of NF- κ B correlated with catalytic activity of PKR mutants, we measured the levels of eIF-2 α phosphorylation on serine 51, by using an antibody that specifically recognizes phosphorylated eIF-2 α . As shown in panel d, eIF-2 α was found to be phosphorylated by the same PKR mutants able to activate NF- κ B.

PKR WT but not a catalytically inactive point mutant (K296R, VTL9) activates NF- κ B

To provide further evidence for the requirement of catalytic PKR activity in NF- κ B activation, we infected PKR^{0/0} cells with VV recombinants expressing either PKR WT or a noncatalytic point mutant VTL9 (K296R). As we can observe in Figure 2a, expression of wild type PKR but not the VTL9 mutant was able to activate NF- κ B, as measured by EMSA. A Western blot showing PKR expression is presented in Figure 2b. As previously described (Rivas *et al.*, 1999; Lee *et al.*, 1994), higher levels of VTL9 mutant than PKR WT

Figure 1 Activation of NF- κ B by a collection of PKR mutants correlates with their catalytic activity. (a) Schematic representation of PKR wild type and different mutants used in this study. All the proteins are expressed by VV recombinants under the control of the same VV/operator hybrid promoter. Kinase domain of PKR is shown in black, dsRNA binding domains (aa 18–80 and 107–159) and third basic domain (aa 232–267) are shown. (b) PKR^{0/0} cells were infected with 5 pfu/cell of the indicated viruses, and expression of proteins was induced by adding 5 mM IPTG. At the indicated times (16 or 20 hpi), cells were collected and samples were used to analyse levels of accumulation of PKR mutants by Western blot using PKR directed antibodies. (c) Nuclear extracts of the same samples prepared as described in Materials and methods were used to perform EMSA using an NF- κ B probe. The relative intensities of the shifted bands were quantified and expressed as fold, arbitrarily assigning the value of one to extracts of cells infected with VV and collected at 16 hpi. (d) *In vivo* phosphorylation of eIF-2 α induced by PKR and different mutants was measured in cell extracts by immunoblotting using antibodies directed against the phosphorylated form of eIF-2 α and total eIF-2 α . The relative intensities of the bands were quantified and expressed as the ratio between phosphorylated and total eIF-2 α signals. The unit was defined as the value obtained for mock treated cells

Figure 2 Expression of wild type PKR, but not of a PKR kinase dead mutant (K296R) activates the transcription factor NF- κ B. (a) PKR^{0/0} cells were infected with 5 pfu/cell of the indicated VV recombinants. Expression of PKR proteins was induced by adding 5 mM IPTG, and at times indicated (16 and 20 hpi) cells were collected, nuclear extracts prepared and used to perform a NF- κ B EMSA assay as described in Materials and methods. As control, NF- κ B activation induced by serum addition (S) is shown. Arrow point to transcriptionally active NF- κ B (p50/p65 and p50/c-rel) complexes as defined by competition with unlabelled probe, and supershift experiments (data not shown). (b) Cytoplasmic extracts from same samples were prepared and analysed by SDS-PAGE followed by immunoblotting using PKR specific antibodies

were found in infected cells, because this mutant does not inhibit protein synthesis, thus leading to accumulation of the mutated protein.

Since expression levels of foreign proteins produced from VV recombinants can be efficiently regulated through the concentration of IPTG, which modulate the operator/VV hybrid promoter system (Melkova and Esteban, 1995; Rodriguez and Smith, 1990), next we evaluated the effect of IPTG concentration over PKR WT expression and activation of NF- κ B under these regulated conditions. As observed in Figure 3a the levels of phosphorylated PKR (as shown by the partial shift of the PKR band shown in Western blot), changed significantly in an IPTG dependent manner as PKR WT levels increased with repression of LacI by adding different concentration of IPTG. In agreement with this observation, *in vivo* phosphorylation of eIF-2 α , and NF- κ B activation was seen only when PKR WT was induced with concentration of IPTG equal or above 0.04 mM (Figure 3a,b).

Because activation of the IKK complex involves multiple protein-protein interactions (Karin and Ben-Neriah, 2000; Karin and Delhase, 2000), the high levels of expression of VTL9 mutant protein could introduce a bias in the system, due to a potential interference in NF- κ B induction through sequestration of other proteins. Thus, we analysed by EMSA the induction of NF- κ B in the presence of different concentrations of the VTL9 protein. In contrast to that observed for PKR WT expression, VTL9 was unable to activate NF- κ B regardless of the levels of expression (Figure 3d). In agreement with EMSA results, only PKR WT, and not different doses of VTL9 mutant induced PKR and eIF-2 α phosphorylation (Figure 3c). From the findings of Figure 3, a clear correlation between PKR

activation and induction of NF- κ B was also established by modulating the levels of expression of PKR WT.

As a further approach to establish the ability of PKR WT and VTL9 mutant to activate NF- κ B activation, we measured the transactivation potential of both proteins through the use of an NF- κ B reporter assay. As shown in Figure 4, PKR WT but not VTL9 mutant was able to induce NF- κ B, in a dose-dependent manner.

Previous findings have established that NF- κ B activation by PKR is mediated through the IKK

Figure 4 PKR WT, but not VTL9 mutant is able to activate NF- κ B as measured by a reporter assay. PKR^{0/0} cells were infected with 5 pfu/cell of VV PKR or VTL-9 and at 1 hpi, IPTG was added at concentrations indicated to induce expression of PKR. Then cells were transfected with 2 μ g of 3enh- κ B-ConA-luc plasmid. At 20 hpi, cells were collected and luciferase activity measured as indicated in Materials and methods. Gray shadow indicates luciferase background levels obtained in cells infected with 5 pfu/cell of VV

Figure 3 Effect of regulated expression of PKR WT and kinase dead mutant over NF- κ B activation *in vivo*. PKR^{0/0} cells were infected with 5 pfu/cell of VV PKR or VTL-9 and at 1 hpi, IPTG was added at concentrations indicated to induce expression of PKR (a,c). Cytoplasmic extracts, were analysed by Western blot using anti PKR antibodies and antibodies directed against the phosphorylated form of eIF-2 α . Unspecific band recognized by anti-PKR antibodies is marked with an asterisk (*) (b,d). At 16 or 20 hpi, nuclear extracts were prepared and analysed by gel shift assay using a NF- κ B specific probe

complex (Chu *et al.*, 1999; Gil *et al.*, 2000; Zamanian *et al.*, 2000). In order to further demonstrate that PKR catalytic activity is involved in this process, we evaluated the ability of PKR WT and VTL9 to induce IKK activity. To this end, the IKK complex was immunoprecipitated with anti-IKK1 antibodies at different times post-infection from cells expressing PKR WT or VTL9 (Figure 5a) and a kinase assay using GST-I κ B α (aas 1–70) as substrate was performed to evaluate its activation state. A representative result is shown in Figure 5b. Upon PKR WT expression, IKK activity was induced and increased with time. However when mutant PKR (K296R) was expressed, no induction of IKK activity over background levels was observed. Densitometric analysis of IKK kinase assay (Figure 5c) remarked these results. IKK kinase activity increased with time in VV PKR infected cells, whereas this increase was not observed in VTL9 infected cells. At 21 hpi, IKK kinase activity was 7.5 times higher in PKR than in K296R expressing cells.

A catalytically inactive PKR mutant (K296R, VTL9) acts as a dominant negative mutant for NF- κ B activation and eIF-2 α phosphorylation

Although the precise mechanism of action is controversial (Proud, 1995), it has been firmly established that expression of PKR (K296R) mutant interferes with WT PKR catalytic activity in a dominant negative fashion (Sharp *et al.*, 1993). Thus, we reasoned that if PKR (K296R) mutant is not able to activate NF- κ B, it should also interfere with WT PKR-triggered NF- κ B activation. To test this possibility, we decided to infect PKR^{0/0} cells with VV PKR together with increasing amounts of VTL9 (Figure 6). To check the effectiveness of this strategy of inhibiting PKR catalytic activity, we analysed by Western blot the degree of phosphorylation of eIF-2 α . As shown in panel b, by increasing the VTL9/VV PKR ratio, the degree of eIF-2 α phosphorylation was reduced. The decrease of eIF-2 α phosphorylation as a consequence of PKR (K296R) dominant negative role, correlated with a diminished NF- κ B activation observed at higher ratios of PKR (K296R) expression (Figure 6a). Hence, PKR (K296R) acts as a dominant negative mutant not only of PKR catalytic activity, but also of NF- κ B activation induced by PKR.

PKR mutants interact with the IKK complex irrespective of their ability to activate NF- κ B

PKR physically interacts with the IKK complex, as it has been demonstrated in different studies (Chu *et al.*, 1999; Zamanian *et al.*, 2000). In addition, it has been shown that several protein kinases that are upstream activators of IKK, such as IRAK or RIP activate the IKK complex irrespective of their catalytic activity, through their interaction with IKK (Knop and Martin, 1999; Maschera *et al.*, 1999; Ting *et al.*, 1996). To analyse whether or not PKR binding and IKK activation are different processes, we decided to test by immunoprecipitation analysis the ability of different

Figure 5 PKR WT but not VTL9 mutant induces IKK activity. HeLa cells were infected (5 pfu/cell) with indicated viruses, and 1 hpi 5 mM IPTG was added to induce PKR proteins. Cells were collected at times indicated, and extracts prepared. (a) Immunoblot of total cell extracts reacted with anti-PKR antibody is shown. (b) These extracts were subjected to immunoprecipitation with anti-IKK1 antibodies. Immunoprecipitates were thoroughly washed and subjected to kinase assay as explained in Materials and methods. A Western blot with anti-IKK1 antibody as a control of immunoprecipitation is shown in lower panel. The reaction products were separated by a 13% SDS-PAGE, gel fixed, dried, and autoradiographed (upper panel). (c) Kinase assay shown in (b) was scanned and densitometric analysis performed. IKK activity is shown as fold times. The value of one was arbitrarily assigned to IKK activity observed in VV PKR infected cells at 12 hpi

PKR mutants to interact with the IKK complex. Three PKR mutants (VTL7, VTL8 and VTL9) were chosen based on their high levels of expression. We expressed these mutants together with IKK1 in PKR^{0/0} cells by infecting these cells with the indicated VV recombinants. The levels of expression of PKR mutants and IKK1 proteins in total extracts are shown in Figure 7a. These samples were immunoprecipitated using anti-PKR antibodies and the interaction of PKR with co-expressed IKK1 was shown by Western blot analysis of immunoprecipitates (Figure 7b). This result was specific as an unrelated antibody was not able to coimmunoprecipitate PKR and IKK1 (data not shown). Remarkably, both the VTL9 (K296R) mutant and the VTL8 mutant (that harbours a deletion in the third basic domain), interacted with IKK1 despite their inability to

Figure 6 Catalytically inactive PKR mutant (K296R, VTL9) acts as a dominant negative mutant for NF- κ B activation. **(a)** PKR^{0/0} cells were infected with the indicated viruses, and collected at 16 and 20 hpi. Nuclear extracts were prepared and analysed by gel shift assay using a NF- κ B specific probe. **(b)** Cell extracts from cells collected 16 hpi were analysed by SDS-PAGE followed by Western blot using antibodies recognizing the phosphorylated form of eIF-2 α (upper panel) or total eIF-2 α (lower panel)

activate NF- κ B. In addition, from this experiment it is also deduced that the PKR domain responsible of PKR/IKK association must be located in a region different than the TBD or dsRBM2 domains.

Analysis of IKK1 and IKK2 phosphorylation by PKR

Phosphorylation of both IKK1 and IKK2 occurs during IKK activation (Karin and Ben-Neriah, 2000), a process that is at least in part dependent on the autocatalytic activity of IKKs (Karin and Ben-Neriah, 2000). As we have demonstrated that PKR kinase activity is required to activate IKK, we analyse if IKK1 or IKK2, the catalytic subunits of the IKK complex, were the targets of PKR kinase activity. To circumvent troubles associated with autophosphorylation activity of the IKK complex, we took advantage of cells lacking both IKK1 and IKK2 (IKK 1,2^{0/0} MEFs, a kind gift from Dr I Verma), and of VV expressing kinase dead versions of these proteins (VV IKK1 DN and VV IKK2 DN). We have previously shown that the IKK complex is detected associated with PKR immunoprecipitates (Gil *et al.*, 2000, and Figure 7). Thus, as a first assay PKR was immunoprecipitated from IKK 1,2^{0/0} MEFs infected with VV recombinants expressing WT PKR and IKK kinase dead subunits, and *in vitro* phosphorylation of immunoprecipitated proteins was analysed. As shown in Figure 8a, PKR was phosphorylated in this *in vitro* assay due to its autocatalytic activity, but no band corresponding to neither IKK1 nor IKK2 was specifically phosphorylated. Next we decided to test if IKKs were phosphorylated by PKR, or by downstream PKR target by an *in vivo* phosphorylation assay

Figure 7 PKR mutants interact with the IKK complex irrespective of their ability to activate NF- κ B. **(a)** PKR^{0/0} cells were infected with 5 pfu/cell each of VV IKK1 and indicated viruses expressing PKR mutants: VTL9 (9), VTL7 (7), VTL8 (8), or control VV (VV). At 20 hpi cells were collected, extracts prepared and analysed by SDS-PAGE followed by Western blot against PKR and IKK1. Unspecific band recognized by anti-PKR antibodies is marked with an asterisk (*). **(b)** Extracts were immunoprecipitated using anti-PKR antibodies and immunoprecipitates were analysed by SDS-PAGE followed by Western blot using antibodies directed against PKR and IKK1. Unspecific band corresponding to immunoglobulins is marked with two asterisks (**)

(Figure 8b). This assay was performed in IKK 1,2^{0/0} MEFs infected with VV recombinants. Cells were labelled with γ -³²P-ATP between 12 and 16 hpi, because optimal IKK activation by PKR has been previously observed at these times (Figure 5). As a control of labelling, PKR autophosphorylation was analysed. As shown in Figure 8b, PKR was phosphorylated in the immunoprecipitates, but again, neither IKK1 nor IKK2 were found to be phosphorylated. In order to

Figure 8 IKK1 and IKK2 are not phosphorylated by PKR in IKK knock-out cells. **(a)** IKK 1, 2^{0/0} MEFs were mock infected (M) or infected with 10 pfu/cell of VV (V), 3 pfu/cell of VV IKK1DN and 3 pfu/cell of IKK2 DN (I), 4 pfu/cell of VV PKR (P), or 3 pfu/cell of VV IKK1DN and 3 pfu/cell of IKK2 DN and 4 pfu/cell of VV PKR (I+P). VV was used to maintain a total multiplicity of infection of 10 pfu/cell when required. After 1 h of viral adsorption 5 mM IPTG was added and cells were collected 24 hpi. Cell extracts were immunoprecipitated using an anti-PKR antibody. Immunoprecipitates were extensively washed and an *in vitro* kinase reaction was performed. Extracts were separated by SDS-PAGE, gel dried and autoradiographed. **(b)** IKK 1, 2^{0/0} MEFs were mock infected (M) or infected with same viruses as in **(a)**. After 1 h of viral adsorption 5 mM IPTG was added and at 12 hpi cells were *in vivo* labelled with 100 μ Ci/ml of [32 P- γ] ATP, as described in Materials and methods. After 4 h of *in vivo* labelling, cells were collected and extracts prepared for immunoprecipitation using anti PKR antibodies (left panels) and anti-IKK1 antibodies (right panels). Immunoprecipitates were analysed by SDS-PAGE electrophoresis followed by Western blot analysis (top panels) or autoradiography (bottom panels)

analyse if there was some phosphorylation of the IKK DN upon PKR expression, we decided to immunoprecipitate the IKK complex using an anti-IKK1 antibody. This experiment also failed to show *in vivo* phosphorylation of IKK kinase dead mutants by PKR.

Discussion

In recent years, significant progress has been made in understanding the mechanisms of signalling pathways regulating NF- κ B activity, particularly, those responding to the proinflammatory cytokines TNF- α and IL-1 (Karin and Ben-Neriah, 2000). Induction of NF- κ B by these and other stimuli converge in a core pathway involving activation of the IKK complex. However, whether the activation of the IKK complex is channelled by common upstream activators or by

different molecules, depending on the nature of different stimuli which induces NF- κ B activation remains undefined.

Involvement of IKK complex in transducing the NF- κ B activation signal induced by PKR has been shown by several independent studies (Chu *et al.*, 1999; Gil *et al.*, 2000; Zamanian *et al.*, 2000), but the mechanisms involved in this activation are not understood. To clarify the relationship among PKR and the IKK complex, we have evaluated the role of different PKR mutants on NF- κ B activation. From our results, it is clear that PKR catalytic activity is needed for NF- κ B activation, since a point mutation that disrupts PKR catalytic activity makes PKR unable to activate NF- κ B as measured by IKK kinase assay, EMSA and a transactivation reporter assay. This has also been confirmed by NF- κ B activation studies with PKR deletion mutants. The requirement of kinase activity of PKR in this process is in contrast with the role of other kinases such as IRAK and RIP, that are able to activate NF- κ B irrespective of their catalytic activity (Knop *et al.*, 1999; Maschera *et al.*, 1999; Ting *et al.*, 1996). RIP and IRAK kinases have been shown to act as a scaffold making possible for other proteins to activate the complex, or as an alternative, they could promote activation of IKK triggering its autophosphorylation by physical interaction. However, PKR association with the IKK complex is not sufficient requirement for the activation of NF- κ B, as deduced from the observation that PKR mutants unable to activate NF- κ B can be found associated with the IKK complex (Figure 7).

The need of PKR kinase activity for NF- κ B activation is also supported by the previous observation that 2-aminopurine, an inhibitor of PKR catalytic activity, is able to inhibit NF- κ B activation induced by PKR (Kumar *et al.*, 1994; Cheshire *et al.*, 1999). However, another study has suggested that PKR kinase dead mutant is able to activate NF- κ B (Chu *et al.*, 1999). These discrepancies could be due to the different experimental model used. In particular, we have carried out our studies in PKR^{0/0} cells in order to rule out the effects caused by heterodimerization between endogenous PKR and exogenously expressed forms. In other studies (Chu *et al.*, 1999), the role of K296R mutant in NF- κ B activation has been assessed in WT MEFs, containing endogenous PKR. In contrast, in our cell system the effect of PKR was assessed by measuring NF- κ B activation in cells infected with VV expressing PKR WT and increasing amounts of mutant PKR (K296R). We have found that K296R acts as a dominant negative mutant of NF- κ B activation triggered by PKR. These results confirm previous results using *in vitro* expression systems in which the PKR-dependent protein synthesis inhibition was reverted by a PKR mutant (Sharp *et al.*, 1993). However, it should be emphasized that high amounts of K296R expression were needed to significantly inhibit NF- κ B translocation, thus suggesting that WT mutant PKR heterodimers could still retain catalytic activity. On the other hand, our VV-

based system allow us to express PKR and mutants in a 100% of cells, thus avoiding limitations derived from transient expression. VV-based, IPTG-inducible expression of proteins, has been shown to be a useful system for studies on the role of several IFN-induced enzymes (Melkova and Esteban, 1995; Lee *et al.*, 1993, 1994; Diaz-Guerra *et al.*, 1997a,b). By taking advantage of regulated expression of proteins, here we show that PKR K296R mutant is unable to activate NF- κ B irrespective of levels of expression. Regulation of expression levels of PKR WT protein showed that NF- κ B is only induced under conditions that allow PKR activation (as observed by PKR autophosphorylation and *in vivo* measurement of eIF-2 α phosphorylation). Taking together, these findings further remark the need of PKR activation for IKK induction.

While PKR kinase activity is needed for IKK activation, an obvious question is which protein is the target of PKR-induced phosphorylation in the complex. In this context, phosphorylation of IKK2 is known to regulate both activation and inhibition of IKK. Phosphorylation sites involved in IKK2 activation are known to be in the context of a consensus MAP3K site (Delhase *et al.*, 1999). In addition, the same IKK2 residues seem to be phosphorylated during IKK activation triggered by PKR and other kinases (Chu *et al.*, 1999). Thus, a common kinase (or several related kinases), probably of the MAP3K family appeared to be involved in IKK2 phosphorylation. We decided to test if PKR could phosphorylate either IKK1 or IKK2, the catalytic subunits of the IKK complex. Experiments described in this investigation, and additional kinase assays performed with *in vitro* translated immunopurified proteins (data not shown) failed to demonstrate PKR phosphorylation of IKK1 and IKK2 kinase dead mutants. These mutants are unable to autophosphorylate themselves but still retain the MAP3 kinase site. Since several studies have suggested that IKK complex is autophosphorylated upon signals that trigger its activation (Karin and Ben-Neriah, 2000), and knowing that IKK mutants lacking kinase activity were not phosphorylated by PKR, one possibility is that PKR activation could induce, by yet unknown mechanisms, autophosphorylation of the IKK complex.

From association studies between PKR mutants and IKK complex, it has been shown that mutants lacking either dsRBM2 or TBD are still able to interact with IKK (Figure 7). This indicate that the domain of PKR involved in interaction with IKK should be other than the dsRBM. The ability of VTL-23 mutant (lacking both dsRBMs) to activate the IKK complex, suggests that the catalytic domain of PKR is most probably the region involved in interaction with the IKK complex. The activation of NF- κ B by other eIF-2 α kinases (such as HRI) has been suggested previously (Ghosh and Baltimore, 1990). Thus, interaction between the catalytic domain of eIF-2 α kinases and IKK complex could be a common framework for NF- κ B activation by this family of kinases.

In summary, in this investigation we have defined the requirements of PKR to activate the IKK complex. We

showed that interaction between PKR and the IKK complex and ability of PKR to activate NF- κ B are two separated functions. Clearly, PKR catalytic activity is needed for NF- κ B activation, although precise target of PKR catalytic activity in this signaling pathway remains elusive. Thus, the biological role of PKR is not limited to be a scaffold protein during NF- κ B activation such as IRAK or RIP, but rather PKR it is required as an active kinase.

Materials and methods

Materials

Monoclonal antibody recognizing total eIF-2 α was a gift from César de Haro. Polyclonal antibodies directed against phosphorylated eIF-2 α were from Biosource. For IKK *in vitro* kinase assay IKK complex were immunoprecipitated using a polyclonal antibody generously donated by RT Hay. For immunoblot analysis anti-IKK1 antibody from Santa Cruz was used. For PKR immunoprecipitation and detection of PKR by Western blot analysis a polyclonal antibody previously described (Gil *et al.*, 1999) was used. HRP-conjugated antibodies were purchased from Cappel.

The 3enh- κ B-ConA-luc (Arenzana-Seisdedos *et al.*, 1993) carries a luciferase gene under the control of three synthetic copies of the κ B consensus of the immunoglobulin κ -chain promoter cloned into the *Bam*HI site located upstream of the conalbumin transcription start site.

Cells and viruses

Mouse 3T3-like fibroblasts derived from homozygous PKR knockout mice (PKR^{0/0}) (Yang *et al.*, 1995) were obtained from C. Weissmann (University of Zurich, Switzerland) and grown in Dulbecco's modified medium (DMEM) supplemented with 10% fetal calf serum. Mouse embryo fibroblast derived from knockout mice for both IKK1 and IKK2 (IKK1, 2^{0/0} MEFs) were a kind gift from I Verma (Salk Institute, USA). Cells were cultivated at 37°C with 5% CO₂. Recombinant VVs expressing the following PKR forms: VV PKR (the wild-type enzyme), VTL-9 (the catalytically inactive point mutant Lys 296 Arg); and the deletion mutants VTL-7 (Δ 104–157), VTL-8 (Δ 233–271) or VTL-23 (Δ 1–97, 104–157, 186–222) were as described (Lee *et al.*, 1993, 1994). All viruses express PKR mutant forms under regulated control of a isopropyl β -thiogalactoside piranoside (IPTG) inducible promoter.

Immunoblotting

For immunoblot analysis, total cell extracts were fractionated by gel electrophoresis (SDS–PAGE) and proteins were transferred to nitro-cellulose paper. Filters were incubated with antiserum overnight at 4°C incubated with secondary antibody and detected using ECL Western blotting reagents (Amersham). Exposure of filters to Kodak X-OMAT films was performed for times varying from 3 s to 5 min, as needed.

NF- κ B luciferase reporter assays

Semiconfluent PKR^{0/0} cells grown in 6-well plates were infected with the viruses indicated and transfected after 1 h of virus adsorption with 2 μ g of DNA per well of 3enh- κ B-

ConA-luc plasmid using Lipofectamine reagent (GIBCO–BRL) according to manufacturer's direction. Where indicated, isopropyl β -D-thiogalactopyranoside (IPTG) at the indicated concentrations was added to the cultures at the time of transfection. Cells were harvested 20 h after infection, lysed using Reporter Lysis Buffer (Promega), and luciferase activity determined.

Gel retardation assay

Nuclear extracts from PKR^{0/0} cells were prepared as previously described (Arenzana-Seisdedos *et al.*, 1995). Three μ g of nuclear extracts were analysed using α -³²P-dCTP labelled double-stranded synthetic wild type HIV enhancer oligonucleotide 5'-AGCTTACAAGGGACTTTCCGCTGG-GGACTTTCCAGGGA-3' containing the two κ B consensus motifs.

Immunoprecipitation of PKR and IKK complexes

Confluent PKR^{0/0} cells grown in 60 mm plates were infected for 20 h with the recombinant viruses indicated and then scraped, lysed using cell lysis buffer (Gil *et al.*, 2000) and clarified supernatant was mixed with 150 μ l of protein A sepharose previously incubated with specific antibodies, and further incubated overnight. Immunoprecipitates were analysed by SDS–PAGE followed by immunoblot with anti-PKR and anti-IKK1 specific antibodies.

IKK kinase assay

HeLa cells (10⁷ cells) were infected with the indicated viruses. Cell lysates were subjected to immunoprecipitation using anti-IKK1 C-terminal antibody, as described above. The immunoprecipitates were further washed with kinase buffer (20 mM HEPES pH 7.7, 2 mM MgCl₂, 2 mM MnCl₂, 10 mM NaF, 300 mM Na₃VO₄, 2 μ M PMSF, 1 μ g/ml leupeptin, 1 mM DTT). *In vitro* kinase reaction was allowed to proceed at 30°C for 30 min in 40 μ l of kinase buffer supplemented with 3 μ Ci [γ -³²P]ATP and 2 μ g bacterially expressed GST-I κ B α (1–70). The reaction was stopped with 20 μ l SDS

sample buffer, boiled for 5 min, fractionated on 13% SDS–PAGE and visualized by autoradiography.

Analysis of putative PKR phosphorylation of IKK1 or IKK2

MEFs derived from mouse lacking IKK1 and IKK2 genes (a gift from Dr I Verma), were infected with the indicated viruses described in the figure legends. For *in vitro* labelling experiment, cells were collected at 24 hpi and immunoprecipitated with a PKR antibody as described above. The immunoprecipitates were further washed (three times) with kinase buffer. *In vitro* kinase reaction was allowed to proceed at 30°C for 30 min in 40 μ l of kinase buffer supplemented with 3 μ Ci [γ -³²P]ATP until the reaction was stopped with 20 μ l SDS sample buffer, boiled for 5 min, fractionated on 7% SDS–PAGE, and visualized by autoradiography.

For *in vivo* labelling experiment, at 12 hpi cells were washed (three times) with DMEM lacking phosphates and maintained for 30 min in the same medium before adding 100 μ Ci/ml [γ -³²P]ATP for an additional 4 h. Afterwards, cells were collected and extracts were immunoprecipitated using either anti-PKR or anti-IKK1 antibodies. Immunoprecipitates were analysed by 7% SDS–PAGE followed by Western blot analysis using anti-PKR or anti-IKK1 antibodies. In addition, gels were dried, exposed to X-Omat films (KODAK) and autoradiograms performed.

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