Activation of NF- κ B by the dsRNA-dependent protein kinase, PKR involves the I κ B kinase complex

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Besides its known role as a translational controlling factor, the double stranded RNA-dependent protein kinase (PKR) is a key transcriptional regulator exerting antiviral and antitumoural activities. We have recently described that induction of NF- κ B by PKR is involved in apoptosis commitment. To define how PKR mediates NF- κ B activation by dsRNA, we have used two different approaches, one based on expression of PKR by a vaccinia virus (VV) recombinant and the other based on induction of endogenous PKR by poly I:C (pIC) treatment. We found that NF- κ B complexes induced by PKR are composed primarily of p50-p65 heterodimers and also of c-rel-p50 heterodimers. As described for other stimuli, following pIC treatment, PKR phosphorylates the NF- κ B inhibitor I κ B α at serine 32 before degradation. Expression by VV recombinants of IKK1 or IKK2 dominant negative mutants together with PKR showed inhibition of PKR-induced NF- κ B activation, as measured both by gel shift and luciferase reporter assays. Immunoprecipitation analysis revealed that PKR interacts with the IKK complex. Our findings demonstrate that physiological function(s) of PKR involve activation of the I κ B kinase complex. *Oncogene* (2000) 19, 1369–1378.

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Introduction

When viruses interact with cells they trigger multiple signalling pathways by diverse mechanisms. One stimuli by which many viruses induce common pathways of signal transduction is the production of double stranded RNA (dsRNA) (Jacobs and Langland, 1996). dsRNA has been proposed to act at several cellular levels, such as binding to scavenger receptors in macrophage cells (Brown and Goldstein, 1983; Yoshida *et al.*, 1992), triggering activation of different transcription factors like IRF-3 and IRF-7 (Braganca and Civas, 1998), and switching on different dsRNA dependent enzymes (Jacobs and Langland, 1996). Among these dsRNA-triggered enzymes the best characterized 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 serine-threonine kinase, PKR (Meurs *et al.*, 1990; for review Clemens and Elia, 1997). PKR controls different cellular processes such as cell growth (Chong *et al.*, 1992), differentiation (Petryshyn *et al.*, 1984), apoptosis (Lee and Esteban, 1994; Der *et al.*, 1997), is differentially regulated during cell cycle (Zamanian-Daryoush *et al.*, 1999), and also exerts antiviral (Lee and Esteban, 1993; Lee *et al.*, 1996) and antitumor activities (Koromilas *et al.*, 1992; Meurs *et al.*, 1993). All of these biological processes are the consequence of PKR-interaction with different targets. Upon activation, PKR inhibits translational initiation by phosphorylating the alpha subunit of the eukaryotic initiation factor 2 (eIF2 α , Levin and London, 1978) and also regulates activation of several transcription factors such as IRF-1, p53, and NF- κ B (Kumar

et al., 1997; Cuddihy *et al.*, 1999). Both transcriptional and translational regulation mediated by PKR are involved on PKR induced apoptosis (Gil *et al.*, 1999; Srivastava *et al.*, 1998; Balachandran *et al.*, 1998). Remarkably, abolition of NF- κ B activity by different approaches resulted in a significant decrease on PKR-induced apoptosis without affecting translational block (Gil *et al.*, 1999).

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 human family includes, NF- κ B1 (p50), NF- κ B2 (p52), RelA (p65), RelB, c-Rel, and the proteins p100 and p105. Different Rel/NF- κ B subunits associate forming homo- or heterodimers that have different affinities and transcriptional activities on the different κ B consensus sequences (Fujita *et al.*, 1992; Kretzschmar *et al.*, 1992; Perkins *et al.*, 1992). 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, the prototypic member of this family I κ B α , is phosphorylated 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. There, the complex binds to κ B consensus motifs in the DNA, upregulating the transcription of many genes. Recently, the kinase complex responsible for this phosphorylation upon different conditions was identified as the I κ B kinase signalsome (IKK) (DiDonato *et al.*, 1997; for a review see Zandi and Karin, 1999; Karin, 1999). This 900-kDa protein kinase complex consist of at least two catalytic subunits (IKK1 and IKK2) (Zandi *et al.*, 1997; Mercurio *et al.*, 1997) and a scaffold protein (named NEMO, IKKAP1 or IKK γ) (Yamaoka *et al.*, 1998). Biochemical analysis have shown that the predominant form of IKK is an IKK1-IKK2 heterodimer associated with a dimer or trimer of IKK γ (Rothwarf *et al.*, 1998). However, complexes containing IKK2 homodimers without IKK1 presence are also found *in vivo* (Mercurio *et al.*, 1999). Mice harbouring disruption of IKK1 (Hu *et al.*, 1999; Takeda *et al.*, 1999) or IKK2 genes (Li *et al.*, 1999a,b; Tanaka *et al.*, 1999) have been generated (reviewed in Karin, 1999) and it has been shown that IKK2 is necessary for NF- κ B activation induced by cytokines, and IKK1 is necessary for diverse developmental processes but dispensable for inducible NF- κ B activation. There are only minor exceptions to the IKK pathway of NF- κ B activation, such as I κ B phosphorylation-independent degradation in UV-treated cells (Bender *et al.*, 1998; Li and Karin, 1998) and I κ B α phosphorylation in tyrosine 42 in response to anoxia (Imbert *et al.*, 1996).

The mechanism involved in dsRNA-dependent NF- κ B activation remains to be elucidated. PKR was invoked as a kinase directly phosphorylating I κ B α in response to dsRNA as deduced from both *in vivo* and *in vitro* experiments (Kumar *et al.*, 1994). However, although PKR appears necessary for transducing this signal (Yang *et al.*, 1995; Maran *et al.*, 1994; Kumar *et al.*, 1997), other evidences point to an indirect role for PKR in NF- κ B

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activation. Mutant cells lacking IKK γ were unable to induce NF- κ B in response to pIC treatment (Yamaoka *et al.*, 1998). Additionally, a mutant cell line defective in dsRNA-induced NF- κ B activation (Leaman *et al.*, 1998) has been isolated. This cell line is not defective in NF- κ B activation triggered by other stimuli or in dsRNA-induced PKR autophosphorylation, suggesting that mediator molecules are involved in NF- κ B activation in response to dsRNA. The aim of this investigation was to define the PKR-mediated pathway of NF- κ B activation induced by dsRNA. We have addressed this issue using two strategies: the first takes advantage of a VV recombinant that expresses PKR under regulation of the *E. coli* *lacI* operator/repressor system, the second is based on treating cells with a synthetic dsRNA polymer composed of alternating riboinosinic and ribocytidilic subunits (pIC), usually used as a model of viral infection or dsRNA-triggered processes. Characterizing this apoptosis-involved pathway is of particular importance, since pIC is being used in different clinical trials for tumour treatments (Robinson *et al.*, 1976; Salazar *et al.*, 1996) and PKR has been invoked as a tumour suppresser gene (Meurs *et al.*, 1993; Koromilas *et al.*, 1992).

Results

Characterization of NF- κ B complexes activated by PKR
After infection of cells with a VV recombinant expressing PKR, NF- κ B was translocated to the cell nucleus by 9–12 h post infection as determined by gel-shift assay (Figure 1a). This delay is because expression of PKR is under the control of a late virus promoter, and after expression PKR needs to be autophosphorylated to become activated (Lee *et al.*, 1993). The activator of PKR produced from the recombinant VV is probably the viral dsRNA produced during the course of infection, since pIC treatment of the cells leads to activation of NF- κ B (Figure 1b). Because NF- κ B activation plays an important role on PKR-induced apoptosis (Gil *et al.*, 1999) and different complexes have been involved in apoptosis induction (Abbadie *et al.*, 1993), it was of interest to characterize the nature of the complexes mobilized by PKR. As shown in Figure 1c, competition with anti-p50 antibodies completely supershifted the NF- κ B complex. However, the use of anti-p65 or anti-c-rel antibodies leads to a partial supershift of the bands. Therefore, the NF- κ B complexes induced by PKR consist, primarily of p50-p65 heterodimers and of a fraction of p50-c-rel heterodimers. This was further confirmed in Western blot of nuclear extracts obtained at 12 and 14 hpi, when a NF- κ B binding was observed in samples expressing PKR. We observed that in cells expressing PKR, proteins c-rel, p65 and p50 accumulated in the nucleus coincident in time with the NF- κ B binding activity (Figure 1d). NF- κ B induction was further confirmed by immunofluorescence analysis of the subcellular distribution of NF- κ B p65 in response to pIC treatment or PKR expression. As shown in Figure 2a, in untreated HeLa cells most of the p65 labelling stains perinuclear and in cytoplasmic regions, clearly excluding the nucleus. As a control, we used cells treated with TNF α for 2 h to show that p65 was clearly translocated to the nucleus. Treating cells with pIC resulted also in a nuclear localization of p65 protein (Figure 2a), correlating with the results obtained in the NF- κ B binding assay (Figure 1b). In cells infected with VV or with VV PKR there is p65 localization in the nucleus only upon PKR expression (Figure 2b). In cells infected with VV, clumps of NF- κ B protein were localized in perinuclear regions, excluding nuclei from staining.

Kinetics of I κ B α degradation and phosphorylation in response to pIC treatment

Since translocation of NF- κ B to the nucleus occurs concomitantly with degradation of I κ B α , we next analysed the extent and kinetics of degradation of I κ B α in response to pIC in comparison with the degradation observed in response to TNF α . As shown in Figure 3a, after TNF α treatment of HeLa cells, total I κ B α levels decreased at 30 min but at later times the levels accumulated again due to resynthesis. The levels of a control protein, β -actin remain unchanged. When the inhibitor of protein synthesis, cycloheximide (CHX), is added prior to TNF α treatment, I κ B α protein cannot be resynthesized and I κ B α was not detected at 30 min and thereafter. When I κ B α stability was analysed after pIC treatment, we observed a delayed kinetic of degradation in comparison with TNF α , as I κ B α was degraded by 2 h post treatment (Figure 3b). Similar experiments were performed in cells derived from PKR knockout (PKR^{0/0}) or control mice (PKR^{+/+}). These experiments showed that I κ B α degradation in response to TNF α treatment follows similar kinetics, regardless of the presence or absence of PKR (Figure 3c). However, the presence of PKR is needed to find I κ B α degradation in response to pIC treatment (Figure 3d), pointing to the role of PKR in the dsRNA-dependent pathway of NF- κ B activation. Although atypical mechanism of NF- κ B activation also exist (Bender *et al.*, 1998; Li and Karin, 1998; Imbert *et al.*, 1996), the usual mechanism of NF- κ B induction involves phosphorylation of I κ B α on serines 32 and 36 by the IKK complex as a first step (Mercurio and Manning, 1999). Thus we analysed if I κ B α phosphorylation occurs on serine 32 after treatment of HeLa cells with pIC. To this aim, we treated HeLa cells with 10 μ g/ml of pIC for different times and analysed phosphorylation of I κ B α protein on serine 32 by immunoblot analysis with a specific antibody. As shown in Figure 4, I κ B α phosphorylation on serine 32 upon pIC treatment is clearly observed by 2 h after treatment (Figure 4a). Similar analysis using PKR^{0/0} and PKR^{+/+} cells showed that phosphorylation on serine 32 of I κ B α occurs only in the presence of PKR (Figure 4b). Additionally, in PKR^{+/+} cells, phosphorylation of I κ B α on serine 32 was observed from 30 min after treatment. Remarkably, this kinetic is compatible with the one observed for NF- κ B activation upon pIC treatment (Figure 1b). Since phosphorylation of I κ B α on serine 32, and concomitant degradation of this protein are hallmarks of the pathway of NF- κ B activation mediated by the IKK complex, we decided to study the involvement of this complex in pIC-induced NF- κ B activation.

PKR activation of NF- κ B involves the IKK complex

The IKK complex (Zandi and Karin, 1999), composed of at least two catalytic subunits, IKK1 and IKK2, and of at least one scaffold protein (IKK γ or NEMO), is a key player in NF- κ B activation upon many different stimuli. To check the role of the IKK complex in NF- κ B activation induced by PKR, we expressed IKK1 and IKK2 transdominant negative mutants, shown in diverse studies to interfere with IKK dependent activation (Zandi *et al.*, 1997), together with PKR. We generated two VV recombinants expressing IKK1 dominant negative mutant (DN) or a Flag tagged version of IKK2 DN protein. As shown by Western blot using specific antibodies, the recombinant viruses efficiently expressed both proteins (Figure 5a). In cells infected with VV recombinants coexpressing IKK and PKR, expression of both IKK1 DN or IKK2 DN, reduced the NF- κ B binding activity induced upon PKR expression to at least 50% in a reproducible manner ($n=4$, Figure 5b). This was further confirmed in a transient transfection assay using a 3enh- κ B-ConA-luc plasmid as a reporter. Clearly, expression of either IKK1 or IKK2 mutants together with PKR leads to a block of PKR-induced NF- κ B activity (Figure 5c). The findings of

Figure 1 Characterization of NF- κ B complexes induced by PKR. Time course of NF- κ B induction through pIC treatment or PKR expression. HeLa cells were mock treated (H) or infected with 10 p.f.u. of wild-type VV (lanes V) per cell or 5 p.f.u. per cell of VV PKR plus 5 p.f.u. of VV per cell in the presence of 5 mM IPTG (lane P) for the times indicated. (a) or were treated with pIC as described under Materials and methods. (b) Nuclear extracts were analysed by gel shift with a α - 32 P-dCTP-labelled double-stranded probe containing two κ B consensus sites. NF- κ B complexes are indicated by arrows. (c) Supershift analysis. Nuclear extracts from HeLa cells infected with 5 p.f.u./cell of VV PKR plus 5 p.f.u./cell of VV per cell in the presence of 5 mM IPTG for 14 h, were competed for 30 min with cold probe (com), or with the indicated antibodies or recombinant proteins before incubation with radioactive probe. As a reference, nuclear extracts without any competitor (–) were also included. Supershifted bands are marked with an asterisk. (d) Western blots of nuclear extracts. 25 μ g of same nuclear extracts were analysed by immunoblot analysis with antibodies directed against p50, p65 and c-rel

Figure 5 demonstrate that expression of both of the IKK mutants strongly inhibited NF- κ B activation triggered by PKR, proving the involvement of these IkB kinases in mediating the signal induced by PKR.

PKR interacts with the IKK complex *in vivo*

To gain further insights into the involvement of the IKK complex in PKR-induced NF- κ B activity, we next tested if PKR is associated with this complex *in vivo*, as it has been shown for other proteins that activate NF- κ B through the IKK signalsome (Regnier *et al.*, 1997; Lee *et al.*, 1997; Lallena *et al.*, 1999; Sakurai *et al.*, 1999). To this end, PKR and IKK1 noncatalytic mutants either singly or together were expressed from VV recombinants in PKR^{0/0} cells and after 20 h, cells were harvested and the lysates were immunoprecipitated with anti-PKR antibodies. Western blot analysis of total extracts reacted with anti-PKR and IKK antibodies revealed levels of expression of both PKR and IKK1 (Figure 6a). Immunoprecipitation analysis with anti-PKR antibodies followed by Western blot of the immunoprecipitates with either anti-PKR or anti-IKK1 antibodies revealed that IKK1 co-immunoprecipitates with PKR (Figure 6b). These findings showed physical association between PKR and the IKK complex, further supporting the involvement of IKK complex in transducing the signal of NF- κ B activation triggered by PKR.

Discussion

In the last few years, a number of studies on the role of the transcriptional factor NF- κ B as a dual pro- or anti-apoptotic protein (reviewed in Baeuerle and Baltimore, 1996; Foo and Nolan, 1999), and mechanisms explaining its inducible activation (reviewed in Zandi and Karin, 1999; Karin, 1999) have appeared. Most of these studies are centred in NF- κ B activation triggered by different cytokines or LPS (O'Connell *et al.*, 1998; Mercurio *et al.*, 1997; Li *et al.*, 1999a,b). However, little is known of the PKR-mediated pathway of dsRNA-induced NF- κ B activation. In a recent report, we described that NF- κ B activation has an important biological role in PKR-induced apoptosis (Gil *et al.*, 1999). Thus, the aim of this study was to gain further insights into the mechanism of NF- κ B activation induced by PKR, using biochemical, immunological and genetic approaches. Analysis of the composition of the PKR-induced NF- κ B complexes by Western blot of nuclear extracts and supershift assays, showed that p50-p65 heterodimers are the main components of the NF- κ B complexes. However, a fraction of c-rel-p50 complexes was also present, although reduced in abundance. In this regard, c-rel has been demonstrated to act as a proapoptotic factor during development (Abbadie *et al.*, 1993), in spite of the fact that c-rel can also act as an antiapoptotic factor to protect B cells from antigen receptor ligation-induced apoptosis (Grumont *et al.*, 1999). Considering the nature of the NF- κ B subunits induced by PKR which could be involved in the upregulation of specific subset of genes, our findings point out to a correlation between nuclear localization of p50-p65 heterodimer complexes and apoptosis induction. Interestingly, a recent report (Chan *et al.*, 1999)

Figure 2 Subcellular distribution of NF- κ B p65 subunit upon pIC treatment or PKR expression. HeLa cells grown overnight in 8-well chambered coverslips were mock-treated, treated with TNF α for 2 h or treated with pIC for 4 h (**a**) or infected with VV or with VV PKR (5 p.f.u./cell) for 16 h (**b**) and thereafter fixed and subjected to immunofluorescence analysis, as described under Materials and methods

has shown that NF- κ B p50-p65 recruitment is required for activation-dependent transcriptional regulation of the human Fas promoter, suggested to be involved in PKR-dependent apoptosis. It has been previously shown that the role of NF- κ B respect to apoptosis induction is dependent of apoptotic stimuli, but not of the composition of NF- κ B complexes

composition (Lin *et al.*, 1998). Thus other factors such as temporal or persistent activation of NF- κ B (Lin *et al.*, 1998) or even presence of different transcriptional coactivators together with NF- κ B (Sheppard *et al.*, 1999) could also be involved in the effect over apoptosis triggered by NF- κ B.

Figure 3 Kinetics of I κ B α degradation induced upon TNF α and pIC treatment. HeLa cells (**a,b**) or PKR^{0/0} and PKR^{+/+} cells (**c,d**) grown in 12-well plates were treated or not with 100 μ g/ml CHX during 45 min. After this time (time 0), cells were treated with (**a,c**) 20 ng/ml of TNF α or, (**b,d**) 10 μ g/ml of pIC, in the continuous presence of CHX for the cells pre-treated with CHX. At indicated times post treatment, cells were collected, extracts prepared and immunoblot analysis using anti-I κ B α antibodies were performed. As a control the same extracts were blotted with anti- β -actin antibodies to check equal loading and integrity of samples

In order for NF- κ B complexes to be translocated into the nucleus, the repressor I κ B α has to be degraded (Roff *et al.*, 1996). PKR was proposed to activate NF- κ B by directly phosphorylating I κ B α in response to dsRNA-induced activation (Kumar *et al.*, 1994). However, only recently kinases responsible for I κ B α phosphorylation upon different stimuli have been cloned and the function of these kinases have been defined by the generation of knockout animals (for review see Zandi and Karin, 1999; Karin, 1999). To analyse the mechanism of NF- κ B activation triggered by PKR, we have characterized the effect of pIC treatment over I κ B α protein. In a previous work we showed that I κ B α is phosphorylated on serine 32 in response to pIC treatment in a process involving PKR (Gil *et al.*, 1999). Here we found that phosphorylation of I κ B α begins 30 min after pIC treatment,

before NF- κ B binding or I κ B α degradation (Figure 4). Following I κ B α phosphorylation, this protein is degraded by the proteasome correlating with previous observations (Mellits *et al.*, 1993). I κ B α protein was degraded after pIC treatment with a slower kinetics than upon TNF α treatment (Figure 3). This delay could be explained by the need for mediators or additional steps in the NF- κ B activation pathway triggered by PKR compared with a direct triggering effect of NF- κ B activation in response to TNF α .

Phosphorylation of I κ B α on serines 32 and 36 is one of the hallmarks of IKK complex activation (Mercurio and Manning, 1999). Since the IKK complex has been characterized as a broad mediator of NF- κ B action (Karin, 1999), it was important to test the involvement of this complex in PKR-mediated activation of NF- κ B. To prove functional relationship between PKR and the IKK complex we used different approaches. One approach is based on the use of transdominant negative mutants of IKK1 and IKK2, previously shown to interfere with IKK activation (Zandi *et al.*, 1997). We expressed IKK1 DN and IKK2 DN from VV recombinants under control of an early/late strong promoter, which allowed constitutive expression of high levels of the IKK proteins, and to accumulate proteins before PKR was produced from a late inducible promoter. We expected that if IKK mutants were able to interfere with PKR the result will be inhibition of PKR effects. Indeed we observed inhibition of NF- κ B activity triggered by PKR, as measured by gel shift and by a luciferase reporter assays (Figure 5). It is interesting to note that in our system IKK1 DN acted as an inhibitor of the PKR-mediated NF- κ B activation. From biochemical studies and gene knockout approaches, it has been shown that IKK2 subunit plays a crucial role in NF- κ B activation induced by cytokines (Karin, 1999). The role of IKK1 seems to be restricted to morphogenesis during development (Hu *et al.*, 1999; Takeda *et al.*, 1999). The major biological importance of IKK2 over IKK1 is remarked by the fact that IKK1 exist only as heterodimers with IKK2, while IKK2 is able to also form homodimers (Mercurio *et al.*, 1999), and that regulation of the IKK complex resides in phosphorylation of certain residues present in IKK2, but not in IKK1 (Delhase *et al.*, 1999). Thus, our observation showing an inhibition of PKR-mediated NF- κ B activation by IKK1 DN could be interpreted as a specific targeting of IKK1-IKK2 heterodimers by PKR. By immunoprecipitation analysis we demonstrate a physical interaction between PKR and the IKK complex (Figure 6). Importantly, other kinases acting upstream of IKK complex such as NIK (Regnier *et al.*, 1997), MEKK1 (Lee *et al.*, 1997), TAK1 (Sakurai *et al.*, 1999) or atypical PKCs (Lallena *et al.*, 1999) have also been shown to coimmunoprecipitate with the IKK complex. Additionally, another activator of NF- κ B, the Tax protein of HTLV has been shown to activate the IKK complex through a direct interaction with the scaffold protein IKK γ (Uhlir *et al.*, 1998; Geleziunas *et al.*, 1998; Chu *et al.*, 1999; Jin *et al.*, 1999; Harhaj *et al.*, 1999) remarking the functional significance of physical association between upstream activators and the IKK complex. To discover which are the proteins responsible for interaction between PKR and the IKK complex will need further studies.

In conclusion, in this investigation, we provide new information on the pIC-induced, PKR-mediated pathway of NF- κ B activation. A scheme of the PKR-mediated pathway of NF- κ B activation is presented in Figure 7. Upon dsRNA activation of PKR, this protein acting directly or through other molecules, activates the IKK complex. Once activated, the IKK complex is responsible for I κ B α phosphorylation on serine 32 and 36, and this is followed by degradation of I κ B α by the proteasome. This degradation provides NF- κ B translocation to the nucleus. There, NF- κ B upregulates, among other genes, the transcription of pro-apoptotic genes, which will be involved in activation of apoptosis by PKR.

Figure 4 Kinetics of I κ B α phosphorylation on serine 32 induced upon pIC treatment. HeLa cells (a) or PKR^{0/0} and PKR^{+/+} cells (b) grown in 6-well plates were treated with 10 μ g/ml of pIC. At indicated times post treatment, cells were collected, extracts prepared and immunoblot analysis using antibodies that specifically recognize the form of I κ B α phosphorylated on serine 32, total I κ B α , or β -actin were performed

Figure 5 Expression of IKK1 and IKK2 dominant negative mutants by VV recombinants interferes with NF- κ B activation induced by PKR. (a) Expression of IKK1 and IKK2 dominant negative mutants driven from VV recombinants as detected by immunoblot using anti-IKK1 or M2 anti-FLAG antibodies. (b) NF- κ B binding assay of nuclear extracts of HeLa cells infected with the indicated viruses. As a control of expression level of IKK1 DN, FLAG-IKK2 DN and PKR, Western blots using antibodies directed against IKK1, FLAG epitope and PKR were performed. (c) NF- κ B reporter assay from duplicate extracts of HeLa cells infected with the indicated viruses

Materials and methods

Materials

Polyribonucleosinic polyribocytidilic acid (pIC, Roche) was prepared according to manufacturer's instruction as 10 mg/ml stock solution and stored at -20°C . Human TNF α (Sigma) was prepared as a 5 μ g/ml stock solution, aliquoted and stored at -80°C until use. Cycloheximide (CHX, Roche) was prepared as a 10 mg/ml stock solution and stored at -20°C . All other reagents, except when indicated were from Sigma.

Antibodies directed against p50, p65, c-rel and IKK1 were from Santa Cruz. For c-rel competition experiments, a polyclonal anti-c-rel antibody (kindly provided by Dr Nancy Rice) was used together with the Santa Cruz antibody. For Western blot, an anti-I κ B α polyclonal antibody (New England Biolabs) was used. For analysis of I κ B α phosphorylation on serine 32, an antibody that specifically recognizes the I κ B α phosphorylated form (New England Biolabs) was

used. For PKR immunoprecipitation, a PKR polyclonal antibody generated in rabbits using PKR expressed in *E. coli* was used. For detection of PKR by Western blot analysis the assays were carried out with a polyclonal antibody previously described (Gil *et al.*, 1999). Monoclonal antibody (AC-74) for mouse β -actin and anti-FLAG (M2) monoclonal antibody were from Sigma. Rhodamine-conjugated goat anti mouse IgG were from Jackson Laboratory. HRP-conjugated antibodies were purchased from Cappel.

Plasmids

pHLZ (Vázquez *et al.*, 1998) derived plasmids coding for IKK1 or Flag tagged IKK2 dominant negative mutants (coding for an alanine instead of the catalytic lysine residue) were generated by usual recombinant DNA techniques, using primers deduced from published IKK1 and IKK2 sequences (Zandi *et al.*, 1997; Mercurio *et al.*, 1997). 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

Figure 6 PKR co-immunoprecipitates with the IKK complex. (a) Western blot analysis using anti-PKR and anti-IKK1 antibodies of total cell extracts used for immunoprecipitation. (b) Western blot analysis of anti-PKR immunoprecipitates using anti-PKR and anti-IKK1 antibodies are shown. Bands corresponding to PKR and IKK1 are indicated with an arrow. Cross-reacting proteins probably corresponding to immunoglobulins are marked with an asterisk (*)

cloned into the *Bam*HI site located upstream of the conalbumin transcription start site.

Cells and viruses

HeLa cells (ECACC 85060701) were grown in DMEM supplemented with 10% newborn calf serum (NCS). After mock treatment or viral adsorption, cells were maintained with DMEM supplemented with 2% NCS. 3T3-like cells derived from homozygous PKR knockout mice (PKR^{0/0}) or wild-type animals with the same genetic background (PKR^{+/+}) (both a generous gift of C Weissmann, University of Zurich, Switzerland) were grown in DMEM supplemented with 10% FCS. The recombinant VV expressing IPTG inducible PKR (called WR 68K before, and VV PKR herein) was described previously (Lee et al., 1993). VV IKK1 DN and VV IKK2 DN were generated by recombination of their pHLZ based vectors pIKK1 DN and pIKK2 DN respectively with VV (Western Reserve Strain, WR) following standard procedures (Mackett et al., 1984).

pIC treatments

Semiconfluent cells seeded the night before, were serum starved for 2 h as previously described (Kumar et al., 1997) thereafter 10 μ g/ml of pIC were transfected for the times indicated using Lipofectamine (Gibco) following the supplier's instructions.

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

Figure 7 Model of NF- κ B activation triggered by dsRNA. A model summarizing the findings obtained in this report is shown. Upon dsRNA activation, PKR associates with the IKK complex and activates it directly or indirectly. This provokes I κ B α phosphorylation on serine 32, and concomitant degradation that makes it possible for NF- κ B to translocate to the nuclei and upregulate the transcription of κ B dependent genes

(Amersham). Exposure of filters to Kodak X-OMAT films was performed for times varying from 3 s to 5 min, as needed.

Immunofluorescence microscopy

HeLa cells were subjected to immunofluorescence staining according to procedures previously described (Alconada et al., 1996). Hoechst stain (0.5 μ g/ml) was added together with the secondary antibody. Samples were visualized in a Leica DM RXA microscope.

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 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.

NF- κ B luciferase reporter assays

Semiconfluent HeLa 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, 5 mM isopropyl β -D-thiogalactopyranoside (IPTG) was added to the cultures at the time of transfection. Cells were harvested 24 h after infection, lysed, and luciferase activity determined.

Gel retardation assay

Nuclear extracts from HeLa cells were prepared as previously described (Arenzana-Seisdedos *et al.*, 1995). Three μ g of nuclear extracts were analysed using α^{32} -P-dCTP labelled double-stranded synthetic wild-type HIV enhancer oligonucleotide 5'-AGCTTACAAGGGACTTTCCGCTGGGGAC-TTCCAGGGA-3' containing the two κ B consensus motifs. Binding competition was assessed by pre-incubating the extracts with a 40-fold excess of unlabelled oligonucleotide. When indicated, 30 or 60 ng recombinant I κ B α or specific polyclonal antibodies directed against p50, p65 or c-rel were added to samples 20 min before addition of the radiolabelled probe.

Acknowledgments

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