Protein kinase C-associated kinase regulates NF-κB activation through inducing IKK activation

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Abstract. Activation of the transcription factor NF-κB induced by extracellular stimuli requires IKKα and IKKβ kinase activity. How IKKα and IKKβ are activated by various upstream signaling molecules is not fully understood. We previously showed that protein kinase C-associated kinase (PKK, also known as DIK/RIP4), which belongs to the receptor-interacting protein (RIP) kinase family, mediates the B cell activating factor of the TNF family (BAFF)-induced NF-κB activation in diffuse large B cell lymphoma (DLBCL) cell lines. Here we have investigated the mechanism underlying NF-κB activation regulated by PKK. Our results suggest that PKK can activate both the classical and the alternative NF-κB activation pathways. PKK associates with IKKα and IKKβ in mammalian cells and induces activation of both IKKα and IKKβ via phosphorylation of their serine residues 176/180 and 177/181, respectively. Unlike other members of the RIP family that activate NF-κB through a kinase-independent pathway, PKK appears to activate IKK and NF-κB mainly in a kinase-dependent manner. Suppression of PKK expression by RNA interference inhibits phosphorylation of IKKα and IKKβ as well as activation of NF-κB in human cancer cell lines. Thus, PKK regulates NF-κB activation by modulating activation of IKKα and IKKβ in mammalian cells. We propose that PKK may provide a critical link between IKK activation and various upstream signaling cascades, and may represent a potential target for inhibiting abnormal NF-κB activation in human cancers.

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Introduction

The transcription factor NF-κB regulates the expression of hundreds of target genes involved in immune response, inflammation, cell proliferation and survival (1-3). Mammals express five NF-κB family proteins: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel. NF-κB1 and NF-κB2 are produced as precursor proteins, p105 and p100, which are then processed into p50 and p52, respectively. While the processing of p105 to p50 appears to be constitutive, the processing of p100 to p52 is regulated. The NF-κB proteins form various homo- and hetero-dimers to generate functional NF-κB. In most normal unstimulated cells, NF-κB dimers are retained in the cytoplasm by IκB proteins. The IκB proteins interact, through their ankyrin repeats, with the Rel homology region (RHD) of NF-κB dimers. Such ankyrin repeats are also present in unprocessed NF-κB1 (p105) and NF-κB2 (p100), which act as IκB proteins that retain the Rel proteins in the cytoplasm (2,4,5).

Two NF-κB activation pathways, the classical and the alternative pathways, have been extensively characterized. In the classical NF-κB pathway (also known as the canonical pathway), the activated IκB kinase (IKK) complex, consisting of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ (also called NEMO), phosphorylates IκB proteins. This triggers IκB polyubiquitination and its subsequent degradation by the 26S proteasome. IKKβ and IKKγ are essential for the classical NF-κB activation pathway, while IKKα seems dispensable for the activation of this pathway in response to most tested stimuli. On the other hand, activation of the alternative NF-κB pathway (also known as the non-canonical pathway) depends on IKKα, but neither IKKβ nor IKKγ. In this alternative pathway, the active IKKα homodimer induces phosphorylation of NF-κB2/p100. The phosphorylated p100 is then polyubiquitinated and processed into p52. Activation of the classical NF-κB pathway predominantly results in active p50/RelA and p50/c-Rel dimers, while the alternative pathway leads to selective release of the p52/RelB complex. The released NF-κB dimers then translocate into

the nucleus, bind target gene promoters and activate gene transcription (2,4,5).

Numerous NF-κB-activating cascades induced by extracellular stimuli converge on the activation of IKKα and IKKβ (2,4,5). This activation is regulated by the phosphorylation of two conserved serine residues ($Ser^{176\ and\ 180}$ in IKKα and $Ser^{177\ and\ 181}$ in IKKβ) located in their activation loops (6-8). Despite great progress in the understanding of signaling pathways controlling NF-κB activation, the molecular mechanisms underlying activation of IKK complexes induced by various signaling molecules remain to be fully elucidated.

Protein kinase PKK (also known as DIK and RIP4) was originally identified as a protein kinase Cβ and δ interacting protein (9,10). We previously showed that PKK physically interacts with co-transfected PKCβ and can be phosphorylated by PKCβ *in vitro*, suggesting that it may be a downstream target of PKCβ (10). We also reported that PKK affects cell survival and BAFF-mediated IKK phosphorylation and NF-κB activation in DLBCL (34). PKK belongs to the RIP kinase family and shares high sequence homology at the N-terminal kinase domain with other members of this gene family (9-11). Similar to other members of the RIP family, PKK was shown to activate NF-κB in transient transfection assays (12-14). Moreover, catalytically inactive mutants of PKK can block NF-κB reporter activation (13,14). These results suggest that PKK regulates NF-κB activation. It was reported that PKK-induced NF-κB reporter activation is inhibited by dominant-negative IKKα and IKKβ and that PKK failed to activate the NF-κB reporter in mouse embryonic fibroblasts (MEFs) deficient in IKKβ, suggesting that NF-κB activation by PKK requires IKK activity (14). How PKK regulates NF-κB activation has, however, remained elusive.

Here we have investigated the molecular mechanism by which PKK regulates NF-κB activation. Our results indicate that PKK activates NF-κB through both the classical and the alternative pathways by inducing phosphorylation of both IKKα and IKKβ. In addition, we show that PKK induces IKK activation primarily in a kinase-dependent manner.

Materials and methods

Cell culture, antibodies, and expression plasmids. HEK293, HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum. Human diffuse large B cell lymphoma (DLBCL) SUDHL-6 cells were cultured in RPMI with 10% fetal bovine serum. Antibodies against IKKα, IKKβ, p65, Rel-B, Bcl2, Mcl-1 and MCM7 were from Santa Cruz Biotechnology, Inc. Antibody specific for human p100/p52 was from Upstate Biotechnologies. Antibodies against IκBα, phospho-IκBα (p-Ser32), phospho-IKKα (p-Ser^{180})/IKKβ (p-Ser181), and phospho-IKKα (p-$Ser^{176/180}$)/IKKβ (p-$Ser^{177/181}$) were from Cell Signaling Technology. Anti-HA tag antibody was from Covance Research Products. Antibodies specific for γ-tubulin and Flag-tag were from Sigma. Rabbit polyclonal anti-PKK antibody was raised against the peptide AHINLQSLKFQGGHGPAATLL (amino acids 759-779 of human PKK).

The pCMV5 plasmids expressing Flag-tagged human PKK, PKK-N and the catalytically inactive mutants of Flag-PKK (D143A) and Flag-PKK (K51R) were described previously (10,13). Expression plasmid for the N-terminal HA-tagged p100 was kindly provided by Dr Shao-Cong Sun. Expression plasmids for Flag-IKKα, Flag-IKKβ, Flag-IKKα (KA) and Flag-IKKβ (KA) were kindly provided by Dr David Goeddel (15). Plasmids for HA-IKKα and HA-IKKβ were generous gifts from Dr Michael Karin (8).

Transfection, immunoprecipitation, western blot analysis and in vitro kinase assays. The transfection, preparation of cell lysates, immunoprecipitation and western blot analysis were performed as previously described (10,16). For *in vitro* kinase assays, HEK293T cells were transfected with plasmids expressing the indicated Flag-tagged proteins. Forty hours post-transfection, the cell lysates were immunoprecipitated with the anti-Flag antibody (M2). The *in vitro* kinase assays were carried out in the presence of [γ-^{32}P]ATP essentially as previously described (10,16). The kinase reaction products were analyzed by autoradiography following SDS-polyacrylamide gel electrophoresis.

RNA interference. To knockdown the expression of PKK in cultured human cell lines, we employed RNA interference with small hairpin RNAs (shRNAs) (17,18). Two targeting sequences (shPKK-a: 5'-GCTAGTGGATGCCATCATATC; and shPKK-b: 5'-TACCTCCACTCACGAAGGA) were selected and the PKK-specific shRNAs were expressed from the pRetro-H1 vector (Cellogenetics Inc.). The control shRNA construct (shControl) carries a random hairpin sequence: 5'-GTTCTCCGAACGAACGTGTCACG. To transiently suppress PKK expression, HEK293 and HeLa cells were transfected with an shPKK and shControl. Twenty-four hours after transfection, puromycin (2 μg/ml, the vector carries a puromycin-resistant gene) was added into the culture medium for 4 days to select transfected (PKK knocked-down) cells. To generate SUDHL-6 cells with PKK expression being stably suppressed, we infected SUDHL-6 cells with retroviruses expressing an shPKK or shControl. The transduced cells were selected with puromycin (0.5 μg/ml) for at least two weeks.

Results

Expression of PKK can activate the alternative NF-κB pathway as well as the classical NF-κB pathway. It was previously shown that overexpression of PKK activated NF-κB reporters and induced IκBα phosphorylation, suggesting that PKK can activate the classical NF-κB pathway (12-14). Whether PKK activates the alternative NF-κB pathway has not been explored. To address this issue, we transfected Flag-tagged PKK alone or together with IKKα, which is known to be involved in activation of the alternative pathway, into HEK293T cells and analyzed the processing of p100 (NF-κB2) to p52. As shown in Fig. 1A, while overexpression of PKK modestly increased level of endogenous p52, co-expression of PKK with IKKα greatly increased level of endogenous p52 with a concomitant decrease in the level of endogenous p100 (Fig. 1). Co-expression of PKK and IKKα also resulted in significant processing of the exogenous p100 into p52 (Fig. 1B). These results indicate that PKK, together with IKKα, can activate the alternative NF-κB pathway. Consistent with the activation

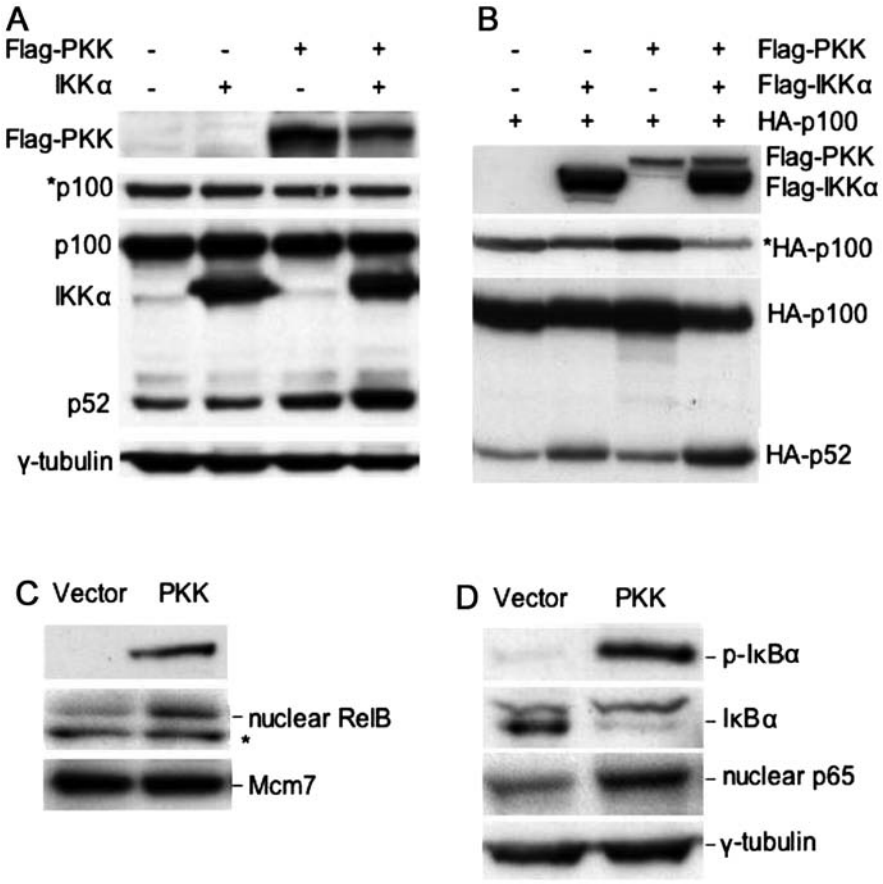

Figure 1. PKK can activate the alternative NF-κB pathway as well as the classical NF-κB pathway. (A) Overexpression of PKK, together with IKKα, induces processing of endogenous p100 to p52. HEK293T cells were transfected with plasmids expressing the indicated proteins, together with a plasmid carrying a puromycin-resistant gene. Twenty-four hours post-transfection, puromycin (1.5 μg/ml) was added into the culture medium to select transfected cells for 2 days. Expression or phosphorylation of the indicated proteins was analyzed on western blots. *p100, a shorter exposure of the p100/p52 blot, showing a decrease in the level of p100 following transfection of PKK and IKKα. (B) PKK cooperates with IKKα to induce processing of the co-transfected p100. HEK293T cells were transfected with the plasmid expressing N-terminal HA-tagged p100 alone or together with the indicated expression plasmids. Twenty-four hours post-transfection, the protein levels of p100 and p52 were analyzed using an anti-HA antibody. *HA-p100, a shorter exposure of the HA-p100/HA-p52 blot, showing a decrease in the level of HA-p100 following co-transfection with PKK and IKKα. (C) Overexpression of PKK induces nuclear accumulation of RelB. HEK293T cells were transfected with the plasmid vector pCMV5 or pCMV5-Flag-PKK. Forty-eight hours post-transfection, total cellular extracts or nuclear extracts were prepared. The levels of transfected Flag-PKK in total cellular extracts, and the levels of RelB and Mcm7 (which was used as the loading control for nuclear proteins) were analyzed by western blotting. *A protein that cross-reacts with the RelB antibody. (D) Overexpression of PKK activates the classical NF-κB pathway. Expression of the indicated proteins from total or nuclear extracts prepared as described in (C) was analyzed by western blotting. p-IκBα, phosphorylated IκBα (phospho-Ser^{32}).

of the alternative pathway, nuclear accumulation of endogenous RelB was increased following PKK overexpression (Fig. 1C). We also examined the effect of PKK overexpression on activation of the classical NF-κB pathway in our assays. Overexpression of PKK resulted in phosphorylation of endogenous IκBα (Fig. 1D), consistent with the previously reported results (12). In addition, we observed a concurrent decrease in the protein level of the endogenous IκBα and an increase in nuclear translocation of p65 (Fig. 1B), hallmarks of activation of the classical NF-κB pathway. Together, these results indicate that PKK can induce NF-κB activation through both the classical and the alternative pathways.

PKK interacts with IKKα and IKKβ. A crucial regulatory step in the activation of NF-κB pathways is the activation of IKK complexes (4,5,19). It was previously reported that activation of an NF-κB reporter by PKK overexpression was inhibited by dominant-negative mutants of IKKα and IKKβ and that PKK-mediated activation of the NF-κB reporter was diminished in mouse embryo fibroblasts deficient in IKKβ protein (14). These results suggest that NF-κB activation induced by PKK requires IKK activity. These observations, however, did not address directly whether PKK works upstream of IKKs or in parallel with IKKs to induce NF-κB activation. To investigate the relationship between PKK and IKKα and IKKβ, we examined whether PKK interacts with IKKα and IKKβ. We transfected HEK293T cells with the plasmid expressing Flag-tagged full-length PKK. The interaction between Flag-PKK with the endogenous IKKα and IKKβ was determined by co-immunoprecipitation experiments. Data presented in Fig. 2A show that both IKKα and IKKβ are present in the PKK immunoprecipitates, suggesting that PKK interacts with IKKα and IKKβ in mammalian cells. While PKK-N, which contains only the N-terminal kinase domain of PKK protein (aa1-320) and is capable of activating NF-κB (13), interacts with IKKα or IKKβ (Fig. 2B), the C-terminal domain

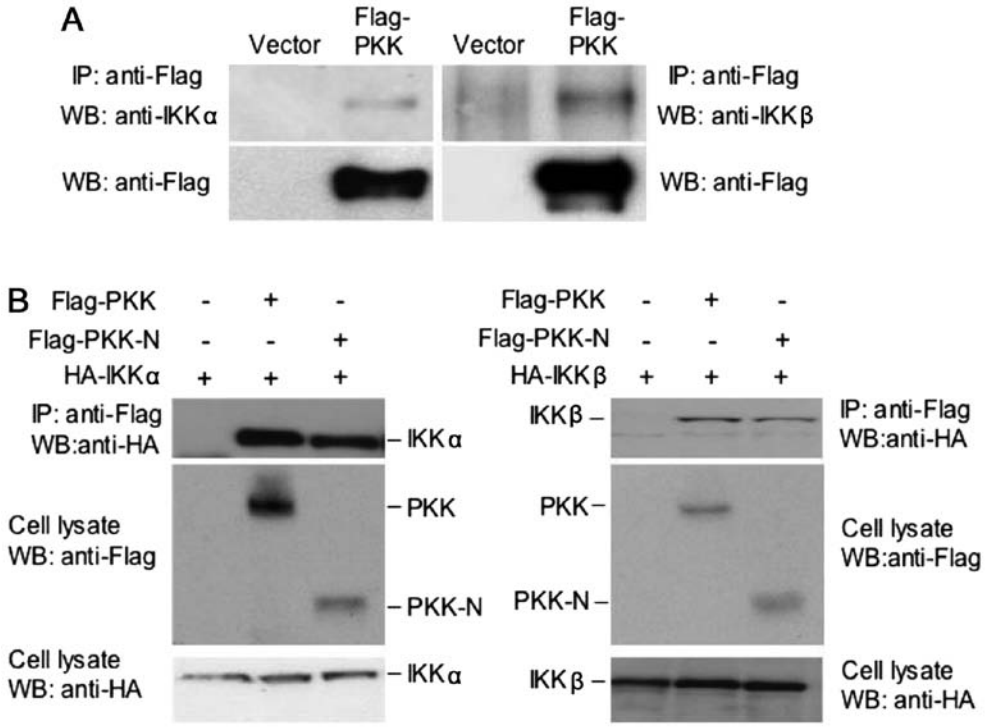

Figure 2. PKK interacts with IKKα and IKKβ. (A) HEK293T cells were transfected with pCMV5 or pCMV5-Flag-PKK as indicated. Forty hours post-transfection, lysates from transfected cells were immunoprecipitated with the anti-Flag antibody (M2). The presence of the endogenous IKKα and IKKβ, as well as Flag-PKK, in the immuno-complex was analyzed on western blots. (B) HEK293T cells were transfected with plasmids expressing the indicated proteins. Forty hours post-transfection, the Flag-PKK or the Flag-PKK-N complex was immunoprecipitated with the M2 antibody. The presence of co-expressed IKKα (the top left panel) or IKKβ (the top right panel) in the immunoprecipitates was analyzed on western blots. The expression levels of Flag-PKK, HA-IKKα and HA-IKKβ in different transfections were also analyzed by western blotting (middle and bottom panels).

of PKK (aa461-786) showed no interaction with IKK proteins (data not shown). These results indicate that the kinase domain of PKK mediates its association with IKKα and IKKβ.

PKK induces phosphorylation of IKKα and IKKβ in vitro and in vivo. The observation that PKK associates with IKKα and IKKβ prompted us to explore whether PKK acts upstream of IKKs. We first examined whether PKK can induce phosphorylation of IKKα and IKKβ *in vitro*. We transfected Flag-tagged PKK with a Flag-tagged kinase-inactive mutant of IKKα [IKKα (KA)] or IKKβ [IKKβ (KA)] (15) in HEK293T cells, and immunoprecipitated both PKK and IKKs with the antibody specific for the Flag-tag. Phosphorylation of the kinase-inactive IKK mutants in the immunoprecipitates was then carried out *in vitro* in the presence of [γ-^{32}P]ATP. The kinase-inactive IKK mutants were used in this experiment to avoid their autophosphorylation so that phosphorylation of IKKs induced by PKK could be easily and unambiguously detected. As shown in Fig. 3, both PKK and PKK-N induced phosphorylation of IKKα and IKKβ *in vitro*, in addition to their autophosphorylation as we previously reported (10). In contrast, the catalytically-inactive PKK (D143A), although expressed at similar levels as PKK and PKK-N, was unable to induce the phosphorylation of co-immunoprecipitated IKKs (Fig. 3, and data not shown). Thus, PKK induces IKK phosphorylation in a kinase-dependent manner *in vitro*.

We next investigated whether expression of PKK activates IKK *in vivo*. Phosphorylation of IKKα and IKKβ at serine residues 176/180 and 177/181 (Ser$^{176/180}$ and Ser$^{177/181}$), respectively, controls their activation, and the levels of phosphorylation at these residues reflect the relative activity of these kinases (6-8,20,21). To analyze IKK activation, we examined the phosphorylation of IKKα and IKKβ at these serine residues following PKK overexpression. We expressed IKKα or IKKβ alone or together with PKK in HEK293T cells, and assayed phosphorylation of Ser$^{176/180}$ and Ser$^{177/181}$ of IKKα and IKKβ, respectively, using a commercially available antibody that is specific for both phospho-Ser$^{176/180}$ of IKKα and phospho-Ser$^{177/181}$ of IKKβ. While overexpression of IKKα or IKKβ alone showed some basal phosphorylation at Ser$^{176/180}$ or Ser$^{177/181}$, co-expression of PKK dramatically increased phosphorylation of IKKα and IKKβ at these serine residues (Fig. 4A). Overexpression of PKK also induced phosphorylation of the endogenous IKKα and IKKβ while it had no effect on the expression levels of IKK proteins (Fig. 4B). Taken together, these results indicate that PKK acts upstream of IKKs by inducing their phosphorylation, and thus activation.

PKK induces IKK activation in a kinase-dependent manner. It has been shown that several members of the RIP family, including RIP, RIP2 and RIP3, activate NF-κB through a kinase-independent mechanism (11,22-26). It was also reported that certain kinase-inactive PKK were capable of activating an NF-κB reporter, but at much lower efficiency than the wild-type PKK (13). Since we observed that a catalytically-inactive kinase failed to induce IKK phosphorylation *in vitro* (Fig. 3), we investigated whether PKK induces endogenous IKK phosphorylation *in vivo* in a kinase-dependent manner.

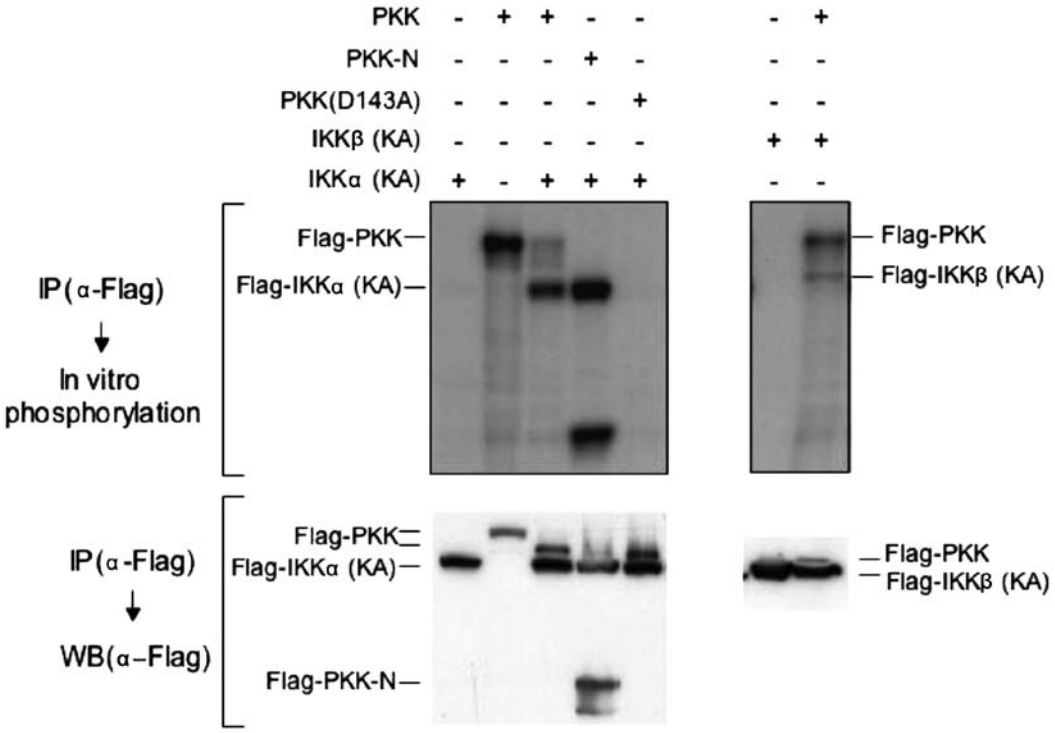

Figure 3. PKK induces phosphorylation of IKKα and IKKβ *in vitro*. HEK293T cells were transfected with plasmids expressing the indicated proteins. Forty hours post-transfection, the Flag-tagged PKK, PKK-N, IKKα and IKKβ proteins were immunoprecipitated with the anti-Flag antibody. Phosphorylation of the immunoprecipitated proteins *in vitro* was carried out in the presence of [γ-^{32}P]ATP, and analyzed by autoradiography (top panels). The expression levels of transfected PKK and IKK proteins were analyzed on western blots (bottom panels).

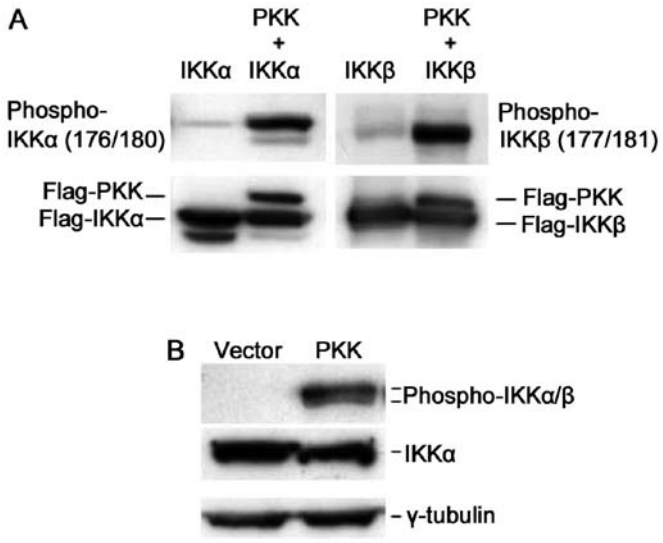

Figure 4. PKK induces IKK phosphorylation *in vivo*. (A) HEK293T cells were transfected with plasmids expressing the indicated Flag-tagged proteins. Twenty-four hours post-transfection, phosphorylation of transfected IKKα and IKKβ *in vivo* was determined by western blot analysis using the antibody specific for phospho-Ser$^{176/180}$ and phospho-Ser$^{177/181}$ of IKKα and IKKβ, respectively. (B) HEK293T cells were transfected with either pCMV vector or pCMV-Flag-PKK. Phosphorylation of endogenous IKKα and IKKβ *in vivo* was analyzed using the antibody specific for phospho-Ser$^{176/180}$ and phospho-Ser$^{177/181}$ of IKKα and IKKβ, respectively.

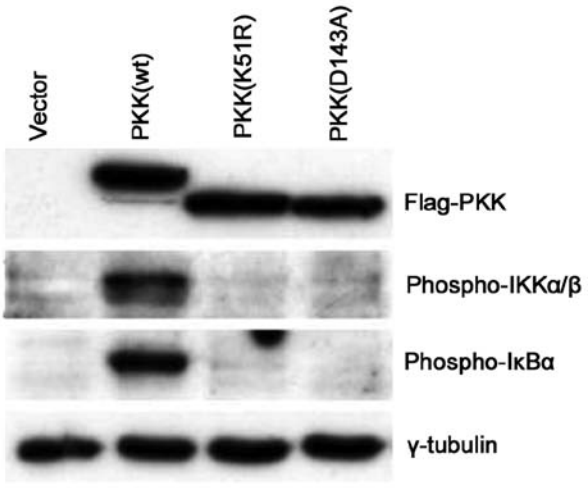

Figure 5. PKK induces IKK phosphorylation in a kinase-dependent manner. HEK293T cells were transfected with plasmids expressing the Flag-tagged wild-type or mutant PKK as indicated. Twenty-four hours post-transfection, phosphorylation of the endogenous IKKα and IKKβ, as well as IκBα, *in vivo* was analyzed by western blotting.

In contrast to the effect of wild-type PKK on IKK activation, expression of two catalytically-inactive mutants of PKK (D143A and K51R) were unable to induce IKK phosphorylation or IκBα phosphorylation (Fig. 5). These data suggest that PKK induces endogenous IKK activation primarily in a kinase-dependent manner.

Suppression of PKK expression inhibits NF-κB activation via inhibition of IKK phosphorylation. To determine whether endogenous PKK is involved in IKK activation,

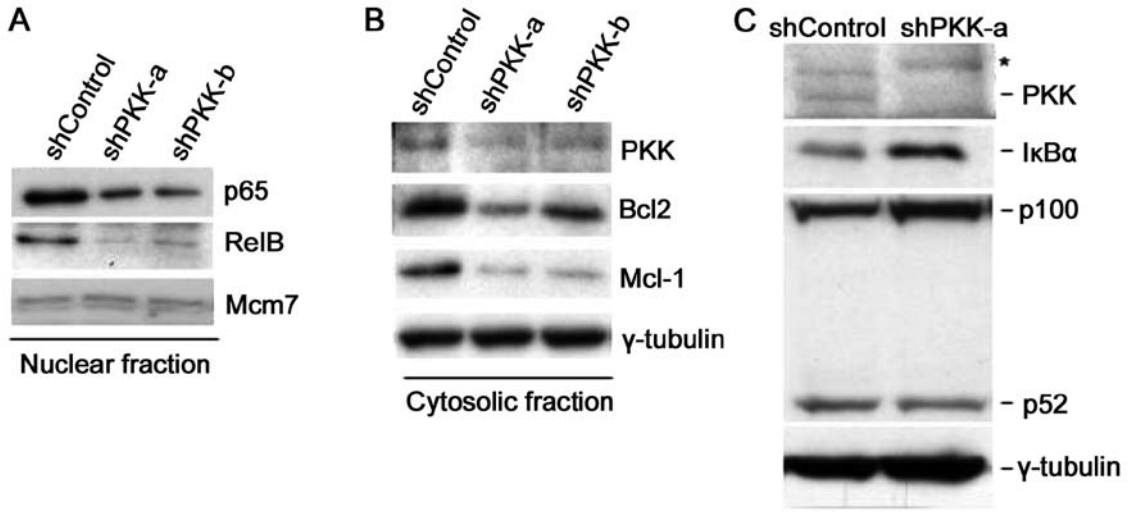

Figure 6. Suppression of PKK expression by RNA interference inhibits activation of both the classical and the alternative NF-κB pathways. HeLa cells were transfected with shControl, shPKK-a or shPKK-b as indicated. Twenty-four hours post-transfection, puromycin (2 μg/ml) was added into culture medium to select for the transfected cells for 2 days. Nuclear (A) and cytosolic (B) fractions were prepared, and expression of the indicated proteins was analyzed on western blots. (C) HEK293 cells were transfected with shControl or shPKK-a. Twenty-four hours post-transfection, the transfected cells were selected with puromycin for 4 days. Expression of the indicated endogenous proteins was analyzed on western blots. *A protein that cross-reacts with the anti-PKK antibody.

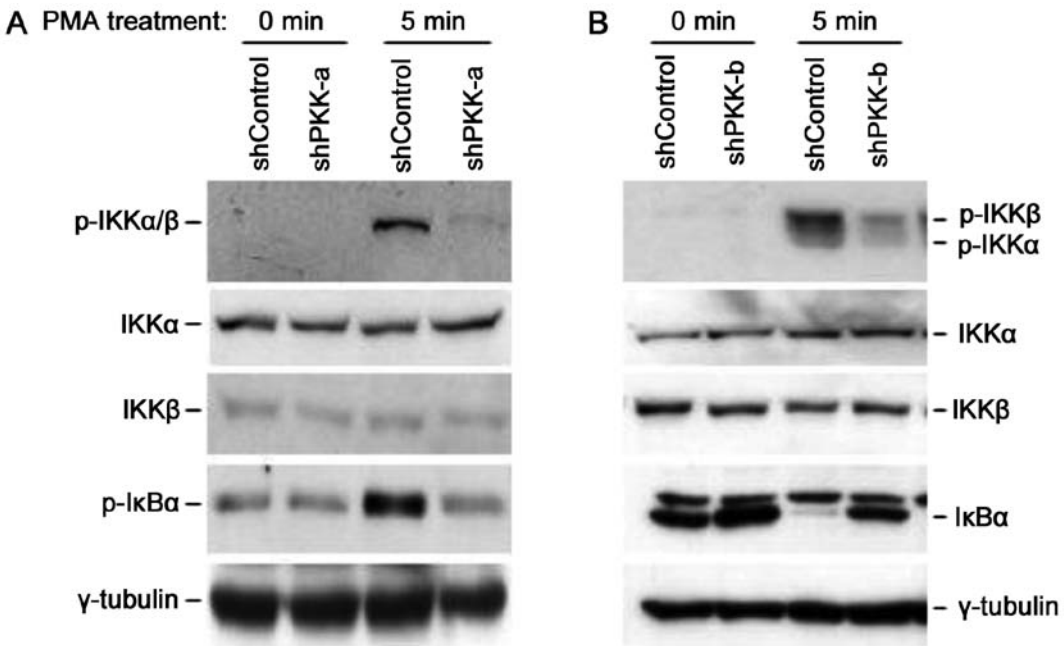

Figure 7. PKK is required for PMA-induced IKK activation. HeLa cells that express shControl or shPKK-a (A), and SUDHL-6 cells that stably express shControl or shPKK-b (B) were serum-starved overnight. The cells were then treated with PMA (100 ng/ml) and ionomycin (300 ng/ml) for the indicated times. The levels of expression and phosphorylation of IKKα, IKKβ and IκBα were analyzed.

we examined the effect of suppression of PKK expression by RNA interference on IKK activation. To knockdown the expression of PKK, we prepared two retroviral constructs that express small hairpin RNAs (shRNAs) (17,18) targeting two distinct sequences of human PKK (referred to as shPKK-a and shPKK-b). Suppression of PKK expression by either shPKK-α or shPKK-β in HeLa cells inhibited nuclear accumulation of p65 and RelB (Fig. 6A), indicating the requirement for PKK in activation of both the classical and the alternative NF-κB pathways. More importantly, knockdown of PKK resulted in decreased expression of Bcl2 and Mcl-1, known transcriptional targets of NF-κB (Fig. 6B). Suppression of PKK expression by shPKK in HEK293 cells also resulted in accumulation of IκBα and accumulation of p100 with a concomitant decrease in the level of p52 (Fig. 6C). Together, these results support the view that PKK regulates activation of both the classical and the alternative NF-κB pathways in mammalian cells.

Our data from *in vitro* IKK phosphorylation and PKK over-expression experiments suggest that PKK functions upstream of IKK (Figs. 3 and 4). To further test this idea, we investigated

whether knockdown of PKK has any effect on IKK phosphorylation. Using NF-κB reporter assays, it was previously shown that PKK mediates NF-κB activation induced by PMA and ionomycin, known activators of protein kinase C (PKC) in mammalian cells (13,14). Thus, we examined whether suppression of PKK expression inhibits IKK activation induced by these PKC activators. As shown in Fig. 7A, IKK phosphorylation at $Ser^{180/181}$ was rapidly increased upon PMA/ionomycin stimulation in HeLa cells expressing shControl. Knockdown of PKK, while having no effect on the expression levels of IKKα and IKKβ proteins, inhibited PMA/ionomycin-induced IKK phosphorylation at $Ser^{180/181}$ (Fig. 7A). As our previous studies with transgenic mice expressing a catalytically inactive PKK suggested that PKK may be involved in B lymphocyte development (27), we examined whether PKK plays a role in NF-κB activation in a B-cell line. Suppression of PKK expression in human B-cell lymphoma SUDHL-6 cells resulted in inhibition of phosphorylation of IKKα and IKKβ induced by PMA/ionomycin (Fig. 7B). These results demonstrate that PKK regulates PMA/ionomycin-induced NF-κB activation through modulating phosphorylation of IKKα and IKKβ in various mammalian cell lines.

Discussion

We investigated the function of PKK in NF-κB activation. We show that PKK regulates both the classical and the alternative NF-κB activation pathways. We also explored the mechanism by which PKK regulates NF-κB activation. We demonstrate that PKK induces phosphorylation of $Ser^{176/180}$ and $Ser^{177/181}$ of IKKα and IKKβ, respectively. Together, these results indicate that PKK functions upstream of IKK in NF-κB activation pathways, extending the previous observation that activation of an NF-κB reporter induced by PKK overexpression requires IKKβ (14). Our finding also provides an explanation for the earlier report that PKK-deficient keratinocytes had similar but not identical defects as IKKα-deficient cells in keratinocyte differentiation (28), as loss of PKK function would affect activation of both IKKα and IKKβ.

Several members of the RIP family, such as RIP, RIP2 and RIP3, function upstream of IKK in NF-κB activation (reviewed in ref. 11). This study identifies PKK (RIP4) as another RIP family member that acts as an upstream regulator of IKK activation. However, unlike the other RIP family members, which regulate NF-κB activation in a kinase-independent manner, PKK appears to regulate IKK and NF-κB activation predominantly in a kinase-dependent manner. It was previously reported that certain kinase-inactive PKK mutants could activate, albeit at much lower efficiency than the wild-type PKK, an NF-κB reporter in transient transfection experiments (13), suggesting that PKK may also be capable of activating NF-κB through a kinase-independent pathway under certain circumstances. It has been shown that RIP1 and RIP2 seem to activate NF-κB through the classical pathway (11). Here we show that PKK can regulate the activation of both the classical and the alternative NF-κB pathways. Thus, PKK possesses some unique features among the RIP family members. The physiological stimuli that require PKK function for NF-κB activation remain to be determined. As PKK interacts with protein kinase C β and δ (9,10) and regulates NF-κB activation induced by PMA/ionomycin (Fig. 7), known protein kinase C activators, it is reasonable to speculate that PKK functions downstream of protein kinase C β and δ and that PKK may play a critical role in NF-κB activation pathways involving PKC function. Given that PKK regulates the activation of both the classical and the alternative NF-κB pathways in a variety of mammalian cells (Figs. 6, 7, and data not shown), it is possible that PKK also plays a role in NF-κB activating pathways independent of PKC function.

Activation of IKKα and IKKβ, which is manifested by their phosphorylation at $Ser^{176/180}$ and $Ser^{177/181}$, respectively, is crucial for NF-κB activation induced by a variety of extracellular stimuli. The molecular mechanisms underlying IKK activation, i.e., the kinases that directly phosphorylate IKKα and IKKβ, in response to various extracellular signals remain to be clarified (5). We showed that PKK associates with IKKα and IKKβ *in vivo* (Fig. 2), and that PKK can induce IKK phosphorylation both *in vitro* and *in vivo* (Figs. 3 and 4). We have expressed and purified His-tagged IKK proteins from insect Sf9 cells and were unable to detect phosphorylation of the IKK proteins *in vitro* by purified PKK expressed from a baculoviral vector in insect Sf9 cells (data not shown). Muto *et al* also reported that, as an unpublished result, purified PKK did not phosphorylate IKKα and IKKβ *in vitro* (14). Thus, it is possible that PKK may activate IKKα and IKKβ through an indirect mechanism. Additional experiments are needed to elucidate how PKK induces activation of IKKα and IKKβ. TGF-β activating kinase 1 (TAK1) and NF-κB-activating kinase (NIK) are kinases that have been shown to function upstream of IKKs, possibly through directly phosphorylating IKK kinases (6,20). PKK may induce activation of IKK through activating either one of these kinases. We are currently testing these possibilities.

Overexpression of a catalytically inactive mutant of PKK in transgenic mice inhibited the generation of pro-B cells, suggesting a role for PKK in B cell development (27). However, mice deficient in PKK exhibit normal B cell populations in all examined compartments (28,29), indicating that PKK is dispensable for B cell development in mice. In addition, activation of the classical NF-κB pathway induced by BCR, CD40 or TLR in PKK deficient B cells appears to be normal (29). Here we show that the knockdown of PKK inhibits NF-κB activation in human B lymphoma cell lines (Fig. 7B, and unpublished data). One explanation for these apparently contradictory observations may be that the function of PKK in B-cells can be compensated by other members of the RIP family. Alternatively, PKK may have acquired an essential role in NF-κB activation in malignant B lymphocytes. Additional experiments are required to resolve these issues.

Deregulation of NF-κB activation has been associated with numerous human diseases including cancer, chronic inflammation and autoimmune diseases (30-33). As PKK plays a critical role in NF-κB activation pathways, molecular elucidation of the mechanism of PKK function may provide new insights into mechanisms leading to abnormal NF-κB activation in human diseases, and may also facilitate the development of therapeutic agents for human diseases resulting from aberrant NF-κB activation.

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