Protein Kinase C-associated Kinase (PKK) Mediates Bcl10-independent NF-κB Activation Induced by Phorbol Ester*

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Protein kinase C-associated kinase (PKK) is a recently described kinase of unknown function that was identified on the basis of its specific interaction with PKCβ. PKK contains N-terminal kinase and C-terminal ankyrin repeats domains linked to an intermediate region. Here we report that the kinase domain of PKK is highly homologous to that of two mediators of nuclear factor-κB (NF-κB) activation, RICK and RIP, but these related kinases have different C-terminal domains for binding to upstream factors. We find that expression of PKK, like RICK and RIP, induces NF-κB activation. Mutational analysis revealed that the kinase domain of PKK is essential for NF-κB activation, whereas replacement of serine residues in the putative activation loop did not affect the ability of PKK to activate NF-κB. A catalytic inactive PKK mutant inhibited NF-κB activation induced by phorbol ester and Ca2+-ionophore, but it did not block that mediated by tumor necrosis factor α, interleukin-1β, or Nod1. Inhibition of NF-κB activation by dominant negative PKK was reverted by co-expression of PKCβι, suggesting a functional association between PKK and PKCβι. PKK-mediated NF-κB activation required IKKα and IKKβ but not IKKγ, the regulatory subunit of the IKK complex. Moreover, NF-κB activation induced by PKK was not inhibited by dominant negative Bimp1 and proceeded in the absence of Bcl10, two components of a recently described PKC signaling pathway. These results suggest that PKK is a member of the RICK/RIP family of kinases, which is involved in a PKC-activated NF-κB signaling pathway that is independent of Bcl10 and IKKγ.

NF-κB 1 is a transcription factor that mediates the activation of a large array of target genes that are involved in the regulation of diverse functions including inflammation, cell proliferation, and survival (1). During inflammatory responses NF-κB is activated in response to multiple stimuli including tumor necrosis factor (TNF), lipopolysaccharides (LPS), and interleukin-1 (IL-1) (1). These trigger molecules interact with surface receptors or specific intracellular sensors that lead to the activation of NF-κB through signal-specific mediators and common downstream effectors such as IkBα and IkB kinase (IKK) (1, 2). RICK and RIP are highly related kinases that mediate NF-κB activation in the Nod1 (or Nod2) and TNFR1 (or TRAIL) receptor signaling pathways, respectively (3–8). RICK and RIP contain N-terminal kinase domains linked to intermediate (IM) regions but the following different C-terminal domains: a caspase-recruitment domain (CARD) and a death domain (DD), respectively (9–13). These C-terminal domains mediate recruitment of RIP and RICK to upstream signaling components, whereas the IM regions link these kinases to the common regulator IKK (9–13). The IM region of both RIP and RICK is essential for NF-κB activation (9–13). Thus, RICK and RIP serve as bridging molecules connecting signal-specific components to common mediators of NF-κB activation. These observations suggest that proteins carrying kinase domains homologous to those of RIP and RICK, but different C-terminal domains, might be involved in the activation of novel NF-κB signaling pathways.

PKK, a mouse kinase composed of an N-terminal kinase domain, an IM region, and a C-terminal domain containing 11 ankyrin repeats was recently identified for its ability to interact with protein kinase C (PKC) isoform PKCβι, whereas its human counterpart named DJK was shown to associate with PKCβ (14, 15). PKCs mediate intracellular signals triggered by stimulation of a variety of extracellular ligands including those associated with G-coupled and antigen receptors (16). Classical and novel PKCs are known to be activated by phorbol ester and intracellular Ca2+ and by phorbol ester only, respectively, and to induce the activation of multiple transcription factors such as nuclear factor-κB, inhibitor of NF-κB; IKK, IkB kinase; AP-1, activator protein-1; PMA, phorbol myristate acetate; TNFα, tumor necrosis factor α; LPS, lipopolysaccharides; IL-1, interleukin-1; CARD, caspase-recruitment domain; DD, death domain; IM, intermediate; ARD, ankyrin repeats-containing domain; ECT, expressed sequence tag; HA, hemagglutinin; WT, wild type; MEF, mouse embryonic fibroblast; NF-AT, nuclear factors of activated T cell; NF-IL6, nuclear factor-interleukin-6; MHC, major histocompatibility complex; CIITA, MHC class II transcriptional activator; IRF-1, interferon regulatory factor-1.

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31871
PKK Is a Phorbol Ester-responsive Activator of NF-κB

RESULTS AND DISCUSSION

PKK Is Highly Related to RICK—To identify novel RICK-like molecules, public protein and nucleotide databases were searched for homologous proteins using the entire human RICK sequence (9). As expected, we identified RIP (E values: $4 \times 10^{-20}$ and $3 \times 10^{-20}$ for human and mouse RIP, respectively) and its homologue RIP3 (E values: $1 \times 10^{-31}$ and $5 \times 10^{-30}$ for human and mouse RIP3, respectively) as molecules with significant homology to RICK (Fig. 1). In addition the search identified PKK, a kinase of unknown function, as the most homologous protein to RICK in available databases (E = $4 \times 10^{-31}$ for mouse PKK and $4 \times 10^{-30}$ for human PKK). We also identified zebrabfish orthologues of PKK and RICK. The domain structure of the fish PKK and RICK was identical to that of their mammalian orthologues (Fig. 1A). Significantly, zebrabfish PKK was more homologous to human RICK (E = $5 \times 10^{-30}$) than human PKK to human RICK or RIP3 (Fig. 1B). As expected from the homology between RICK and RIP, PKK also exhibited significant similarity to RIP (E = $4 \times 10^{-31}$ and RIP3 (E = $5 \times 10^{-32}$ and $3 \times 10^{-30}$ for human and mouse, respectively) (Fig. 1B). These results indicate that PKK is a novel member of the RICK/RIP family of kinases. Further analysis of protein sequences revealed that the homology between PKK and RICK-related kinases was restricted to their kinase domains in that no significant similarity was identified in the IM and C-terminal domains. Consistent with these findings, RICK and RIP have C-terminal CARD and DD, respectively, whereas PKK contains 11 ankyrin repeats in its C terminus (Fig. 1A). The IM region of RICK and RIP is serine/threonine rich and essential for the interaction with IKK and NF-κB-inducing activity (8, 35). Interestingly, the IM region of PKK was also serine/threonine rich, but it did not exhibit any significant amino acid homology to that of RICK and RIP.

PKK Activates NF-κB and AP-1—Given the amino acid and
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Transfection of the wild-type (WT) PKK cDNA into HEK293T cells first tested whether expression of PKK activates NF-κB. The structural homology between PKK and RICK-related kinases, we first tested whether expression of PKK activates NF-κB. Transfection of the wild-type (WT) PKK cDNA into HEK293T cells induced activation of NF-κB in a dose-dependent manner as measured with a reporter luciferase construct (Fig. 2A). The induction of NF-κB by PKK was specific in that transfection of the PKK cDNA did not induce transactivation of NF-AT, NF-IL6, p53, CIITA, and IRF-1, and class II MHC-dependent promoters (Fig. 2B). In control experiments the transcriptional activity of the reporter constructs was stimulated by expression of proteins known to induce their activation (Fig. 2B). We also found that expression of PKK induced significant activation of AP-1 (Fig. 2B) as did expression of MEKK1, a known activator of AP-1 (26).

The Kinase Domain of PKK Is Essential for NF-κB Activation—To identify the domains of PKK that are required for NF-κB activation, a series of deletion mutants carrying each domain alone or in combination were constructed (Fig. 3A). Expression of PKK mutants containing the kinase domain resulted in NF-κB activation, whereas mutants containing the IM region and/or ankyrin-repeats-containing domain (ARD) alone were inactive (Fig. 3C). Immunoblotting analysis showed that the lack of activity of the mutants could not be explained by different expression levels of the mutant proteins (Fig. 3C, inset). Thus, the kinase domain of PKK is necessary and sufficient for NF-κB activation, suggesting that the catalytic region acts as an effector domain in PKK signaling. Consistent with this hypothesis, replacement of the conserved aspartate residue (D143) in the catalytic site for alanine rendered PKK inactive (Fig. 3C).

Human and mouse PKK contain a SXXXX motif (SHDLS) at positions 171 and 173 and tyrosine residues in its putative activation loop (Fig. 3B). This finding indicates that the canonical motif in the activation loop of kinases is not evolutionarily conserved in PKK. Together, these observations suggest that the ability of PKK to activate NF-κB is not regulated by phosphorylation of its putative activation loop.

PKK Is Involved in PMA/Ca2+-ionophore-induced NF-κB Activation—PKK is known to interact with PKCβ, suggesting that these proteins may function in a common signaling pathway (14). Recent studies have revealed that Bimp1, Bel10, and MALT1 are components of a receptor-mediated signaling pathway that links PKC activation to NF-κB induction (17, 18). Therefore, we next tested whether PKK regulates an NF-κB signaling pathway mediated by Bimp1, Bel10, and MALT1 in HEK293T cells that are known to express endogenous PKK (15). Treatment of HEK293T cells with PMA/Ca2+-ionophore induced NF-κB activation, which was inhibited by the PKK mutant carrying an alanine substitution at the catalytic aspartate residue (D143A) (Fig. 4A). The inhibitory effect was specific in that expression of PKK D143A did not block NF-κB activation induced by Bimp1, Bel10, oligomerized MALT1, TNFα (Fig. 4A), IL-1β, or Nod1.2 Additional control experiments shown in Fig. 4A revealed that activation of NF-κB induced by PKK, Bimp1, Bel10, activated MALT1, PMA/Ca2+-ionophore, or TNFα could be inhibited by a dominant interfering form of IKKβ but not by that of MyD88, an essential mediator of IL-1/Toll receptor signaling (32). Because PKK associates with PKCβ1 (14), we tested if the PKK D143A mutant inhibits PMA-induced NF-κB activation through a functional interaction with PKCβ. Expression of PKCβ1 reverted the effect of the PKK D143A mutant, whereas a kinase negative mutant of PKCβ1 (K371M) and PKCε did not (Fig. 4B). The mechanism by which PKCβ1 reverts the dominant negative effect of the PKK mutant is unclear. A possible explanation is that overexpressed catalytically active PKCβ1 competes out dominant negative PKK for cellular factor(s) necessary for function. The selective effect of PKCβ1 is consistent with the observation that PKK interacts with PKCβ1 (14) but not with

2 A. Muto, N. Inohara, and G. Núñez, unpublished results.
PKC. In addition, activation of AP-1 induced by PMA/Ca\(^{2+}\)-ionophore was specifically inhibited by PKK dominant negative (Fig. 4C), suggesting that PKK also acts in a PMA-induced AP-1 signaling pathway activated by PKC/βI.

**NF-κB Activation Induced by PKK Requires IKKα and IKKβ but Not IKKγ**—NF-κB activation by RICK and RIP is mediated by the IKK complex, a universal regulator that phosphorylates IκBα resulting in degradation of IκBα and nuclear transloca-
PKK Is a Phorbol Ester-responsive Activator of NF-κB

Fig. 5. PKK acts through the IKK complex and independently of Bcl10 to activate NF-κB. A. PKK-induced NF-κB activation is inhibited by dominant negative forms of IKKα and IKKβ but not by those of IKKγ, Bimp1, or MyD88. Induction of NF-κB activation was determined in triplicate cultures of HEK293T cells transfected with 1.6 ng of pcDNA3-Myc-PKK or stimulated with 50 ng/ml PMA and 0.7 μg/ml of A23187, 10 ng/ml IL-1β or 10 ng/ml TNFα for 4 h in the presence of pBVIx-Luc and pEF-BOS-β-gal. Results are presented as a percent of values obtained with PKK and control plasmid. In the experiment shown, PKK, PMA/Ca²⁺-ionophore, IL-1β and TNFα induced 55 ± 3, 196 ± 15, 423 ± 22, and 183 ± 55-fold activation of NF-κB, respectively. Values represent mean of normalized values ± S.D. of triplicate cultures. B. PKK-mediated NF-κB activation requires IKKα and IKKβ. Induction of NF-κB activation was determined in WT, IKKα−/−, IKKβ−/−, and IKKα−/−/IKKβ−/− MEFs transfected with 100 ng of pcDNA3-Flag-PKK, pcDNA3-Nod1-Flag, and pcDNA-IKKβ-Myc in the presence of pBVIx-Luc and pEF-BOS-β-gal. Results were normalized according to the value obtained with cells transfected with vector alone, which was considered as 1. In the experiment, relative κB-dependent activity of WT, IKKα−/−, IKKβ−/−, and IKKα−/−/IKKβ−/− MEFs with control vector was 1, 0.08, 0.11, and 0.006, respectively. C. PKK-induced NF-κB activation in both parental Rat-1 and IKKγ-deficient 5R cells. Induction of NF-κB activation was determined in Rat-1 and IKKγ-deficient 5R MEFs transfected with 330 ng of pcDNA3-Flag-PKK, pcDNA-IKKβ-Myc, and pcDNA3-Nod1-Flag, or stimulated with 10 ng/ml TNFα, 10 ng/ml IL-1β, or 1 μg/ml LPS in the presence of pBVIx-Luc and pEF-BOS-β-gal. D. PKK-mediated activation of NF-κB in the absence of Bcl10. Bcl10−/− and Bcl10−/− MEFs were transfected with 900 ng of the indicated expression plasmid: pcDNA3-Flag-PKK, pcDNA3-Nod1-HA, or pcDNA3-Bimp1-Flag.

To determine whether NF-κB activation by PKK is also dependent on IKKs, PKK was co-expressed with the catalytic inactive forms of IKKα and IKKβ. NF-κB activation induced by PKK as well as that induced by PMA/Ca²⁺-ionophore, IL-1β and TNFα, was inhibited by catalytic inactive IKKα and IKKβ (Fig. 5A). In control experiments, PKK-mediated NF-κB activation was not affected by dominant negative forms of Bimp1 or MyD88 (Fig. 5A). The ability of PKK to activate NF-κB was also determined in MEFs lacking IKKα and IKKβ. Whereas PKK activated NF-κB in WT fibroblasts, it was unable to induce NF-κB in cells lacking IKKβ or in cells lacking both the IKKα and IKKβ proteins (Fig. 5B). These results suggest that NF-κB activation induced by PKK requires catalytic IKKs. However, we found that purified PKK did not phosphorylate IKKα or IKKβ in vitro, suggesting that PKK does not function through direct phosphorylation and activation of the IKK complex.

Next we tested if NF-κB activation by PKK requires IKKγ, a regulatory component of the IKK complex (18, 33–35). PKK was co-expressed with a truncated mutant of IKKγ (residues 134–419) that inhibits NF-κB activation induced by RIP and RICK (8). Surprisingly, co-expression of the IKKγ mutant did not inhibit PKK-mediated NF-κB activation (Fig. 5A). To verify the latter result, we tested the ability of PKK to activate NF-κB in parental Rat1 fibroblasts and IKKγ-deficient 5R cells, a Rat1 derivative cell line that is defective in IKKγ (22). Expression of PKK induced NF-κB activity not only in parental Rat1 cells but also in 5R cells (Fig. 5C). As controls, stimulation with TNFα, IL-1β, or LPS, or expression of Nod1 (all of which require IKKγ) induced NF-κB activation in parental Rat1 but not in 5R cells (Fig. 5C). It was shown in Fig. 3 that the IM region of PKK is not essential for NF-κB activation. In contrast, the same region of RIP and RICK is essential for NF-κB activation and mediates the interaction with IKKγ (8, 35). Thus, unlike in RICK and RIP, the IM region of PKK and IKKγ are dispensable for NF-κB activation.

Bcl10 Is Not Required for PKK-mediated NF-κB Activation—Bimp1 and its interacting partners Bcl10 and MALT1 have been shown to act downstream of PKC in a signaling pathway leading to NF-κB activation (17, 18). In Fig. 4A we showed that NF-κB activation induced by expression of Bimp1, Bcl10, and activated MALT1 is unaffected by dominant negative PKK.

tion of NF-κB (2, 8).
Conversely, Fig. 5A demonstrated that a dominant negative form of Bimp1 had no effect on PKK-mediated NF-κB activation. To determine whether PKK could act upstream of Bcl10, we tested the ability of PKK to induce NF-κB in MEFs deficient in Bcl10 (18). Both PKK and Nod1 induced NF-κB activation in both Bcl10+/− and Bcl10−/− MEFs (Fig. 5D). In control experiments shown in Fig. 5D, Bcl10 was required for NF-κB activation induced by Bimp1, a protein that acts upstream of Bcl10 to activate NF-κB (17). Together with the results shown in Fig. 4A, these results suggest that PKK functions in a PKC signaling pathway of NF-κB activation that is independent from Bcl10.

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