Phosphorylation of IκB by the IκB kinase (IKK) complex is a critical step leading to IκB degradation and activation of transcription factor NF-κB[1]. The IKK complex contains two catalytic subunits, IKKα and IKKβ, the latter being indispensable for NF-κB activation by proinflammatory cytokines [2-7]. Although IKK is activated by phosphorylation of the IKKβ activation loop[8], the physiological IKK-kinases mediating responses to extracellular stimuli remain obscure[1,9]. Here we describe a novel IKK-related kinase, named NAK (NF-κB-Activating Kinase), capable of activating IKK through direct phosphorylation. NAK induces IκB degradation and NF-κB activity through IKKβ. Endogenous NAK is activated by phorbol ester tumor promoters and growth factors, whereas catalytically inactive NAK specifically inhibits activation of NF-κB by protein kinase C-ε (PKCε). Thus, NAK is an IKK–kinase that may mediate IKK and NF-κB activation in response to growth factors that stimulate PKCε activity.

A portion of human NAK cDNA was amplified by PCR using degenerate primers based on sequences common to IKKα and IKKβ. A 240 bp PCR product was used to isolate a cDNA, whose sequence predicts a translation product of 730 amino acids with a calculated molecular mass of 84 kDa (Fig. 1a). NAK exhibits 61 % overall identity to IKK-i, that was recently described as a lipopolysaccharide (LPS)-inducible IκB kinase[10], and its catalytic domain

exhibits 30 % identity to IKKα/β. Like IKKα/β, NAK contains leucine-zipper (LZ) and helix-loop-helix (HLH) motifs within its C-terminal half. However, whereas IKKα/β contain two serines in their activation loop, whose phosphorylation is required for activation[1,8,11], NAK contains glutamic acid (Glu 168) instead of one of these serines. A 2.2kb NAK transcript is ubiquitously expressed with highest expression found in testis (Fig. 1b).

IκBα phosphorylation at serines 32 and 36 is required to trigger its ubiquitination and subsequent degradation[12-15]. Thus, we determined whether NAK induces IκBα phosphorylation and ubiquitination *in vivo*. Expression of NAK alone induced phosphorylation of IκBα at Ser 32 (Fig. 2a, lane 3), and coexpression of NAK and IKKβ enhanced this modification (lane 6). Although overexpression of IKKβ induces Ser 32 phosphorylation on its own[1] (data not shown), modest expression of IKKβ alone was insufficient (lane 4). When FWD1(E3RS$^{IκB}$/β-TrCP), the specificity component of the SCF ubiquitin ligase for IκBα[16,17], was coexpressed with NAK, FWD1 bound phosphorylated IκBα and stimulated its ubiquitination (lanes 7 and 10). Consistent with this, NAK overexpression resulted in nuclear translocation of RelA(p65) NF-κB subunit (Fig. 2b). However, NAK-induced IκBα phosphorylation was inhibited by

coexpression of catalytically inactive IKKβ(KM), in a dose-dependent manner (Fig. 2c, top panel), suggesting that NAK functions upstream of IKK.

We also investigated whether NAK can directly phosphorylate IκBα or IKKβ. We expressed wild-type (wt) and catalytically inactive (KM) NAK in insect cells, as C-terminal Myc and 6xHis tagged proteins. Wt NAK, but not NAK(KM), phosphorylated GST-IκBα(1-54)( Fig. 2d, compare lanes 2 and 3). Autophosphorylation of NAK was also observed. GST-IκBα with alanine at position 32 was phosphorylated by NAK (lane 5), but GST-IκBα with alanine at position 36 was not (lane 6). Thus, at least *in vitro*, NAK phosphorylates IκBα only at Ser-36, but not at Ser-32. Using similar mutants of IκBβ we found that NAK only phosphorylates Ser-23, but not Ser-19 (data not shown). Wt NAK, but not NAK(KM), also phosphorylated GST-IKKβ(132-206) (compare lanes 8 and 9), but not an alanine substituted mutant GST-IKKβ(S177A/S181A) (lane 10). Therefore, NAK can phosphorylate either or both serines in the activation loop of IKK-β. Importantly, wt NAK, but not NAK(KM), stimulated the IκBα kinase activity associated with IKKβ, without changing IKKβ expression level (Fig. 2e). Wt NAK also reduced the electrophoretic mobility of IKKβ (Fig. 2a, compare lanes 10 and 11 and Fig. 2e, compare lanes 3 and 4). Since phosphatase treatment reversed this mobility shift (data not shown), it is most likely due to

IKKβ phosphorylation.

As phosphorylation of IκBα at both Ser32 and Ser36 is required for induction of IκBα degradation[15-17], NAK is unlikely to function as an IκBα kinase. Instead it is more likely to function as an IKK-kinase (IKK-K). To establish this concept, we examined whether recombinant NAK expressed in insect cells can activate the physiological IKK complex purified from non-stimulated HeLa cells. Wt NAK, but not NAK(KM), enhanced IKK activity, measured by phosphorylation of GST-IκBα(full length; S36A)(Fig. 2f). NIK and MEKK1 can activate JNK and/or p38MAPK when overexpressed[9]. However, neither p38MAPK nor JNK were activated by overexpression of NAK (Fig. 2g). Thus, NAK may be a specific upstream regulator of IKK.

Next, we tested whether NAK stimulates the transcriptional activity of NF-κB through IKK. When modest and comparable levels of NAK and IKKβ were expressed, NAK caused approximately a 6-fold increase in luciferase expression, whereas the effect of IKKβ alone was weaker (Fig. 3a). Neither NAK(KM) nor IKKβ(KM) stimulated NF-κB activity. NF-κB activation by NAK was further confirmed by electrophoretic mobility shift assay (Fig. 3b). Overexpression of IKKβ, which results in its activation through autophosphorylation[9] also enhanced NF-κB DNA-binding activity (Fig. 3b) and caused a seven-fold

increase in luciferase expression, which was not inhibited by NAK(KM) (Fig. 3c). In contrast, stimulation of NF-κB-Luc expression by NAK was inhibited by IKKβ(KM) (Fig. 3c), indicating that NAK acts upstream to IKKβ. Consistent with this, activation of NF-κB-Luc by NAK was reduced in *IKKα-/-* and *IKKβ-/-* fibroblasts in comparison to fibroblasts derived from wt mouse embryo (Fig. 3d, left panel). The reduction in NF-κB activation by NAK was more pronounced in *IKKβ-/-* cells than in *IKKα-/-* cells, in agreement with the more important role of IKKβ in NF-κB activation by an external stimulus, such as TNFα (Fig. 3d, right panel)[2-8].

Although NF-κB is activated by a plethora of external stimuli [18-21], it remains unclear how these versatile stimuli activate a common downstream target, the IKK complex. To identify in which pathway NAK operates, we tested the ability of NAK(KM) to interfere with NF-κB activation. Induction of NF-κB-Luc by a phorbol ester tumor promoter (PMA) was inhibited in a dose-dependent manner by either NAK(KM) or IKKβ(KM) (Fig. 3e, left panel), therefore NAK may act downstream of protein kinase C (PKC)[22]. In contrast, induction of NF-κB-Luc by TNFα, IL1-β, LPS, and ionizing irradiation were inhibited by IKKβ(KM), but not by NAK(KM) (Fig. 3e, right panel and data not shown). Induction of AP-1 transcriptional activity by PMA was also not inhibited by

NAK(KM) (Fig. 3f). Thus the dominant inhibitory activity of NAK(KM) is stimulus- and NF-κB-specific. Interestingly, NAK(KM) inhibited NF-κB activation by PKCε, but not by PKCα or PKCθ, suggesting that it may be activated only by PKCε or closely related isozymes (Fig. 3g). In this regard, it should be noted that PKCε was recently implicated in protection of cells from apoptosis[23,24]. This anti-apoptotic function of PKCε could be mediated via NAK, as NF-κB is a well established anti-apoptotic factor[25].

We also examined the activity and regulation of endogenous NAK. Like the recombinant protein, endogenous NAK phosphorylates IκBα only at Ser-36, but not at Ser-32 (Fig. 4a). Endogenous NAK was not present in IKK complexes purified from nonstimulated or TNFα-stimulated HeLa cells (Fig. 4b). In addition NAK was not coprecipitated with IKKα, IKKβ, and IKKγ/NEMO either in non-treated or PMA treated cells (Fig. 4c). Therefore, NAK is not a component of the classical IKK complex[1] and does not become part of it upon PMA treatment. However, NAK activity was stimulated by PMA with kinetics that matched those of IKK activation (Fig. 4d) and de novo protein synthesis was not required for this stimulation (data not shown). By comparison, there was only a minor and rather late increase in NAK activity in TNFα-stimulated cells that did not correlate with IKK activation. While this work being

completed, NAK was described as a kinase (TBK1) capable of forming a ternary complex with TRAF2 and TANK when overexpressed[26]. However, since kinase defective NAK does not block physiological TRAF-dependent NF-κB activation, it is questionable whether NAK is involved in TRAF-mediated signaling. We asked whether physiological stimuli, such as PDGF which activates PKCε[27], could activate NAK. PDGF induced a time-dependent increase in NAK activity, which was abrogated by Ro 31-8220, a specific PKC inhibitor (Fig. 4e). Hence, PDGF enhances NAK activity through PKC. In addition, NF-κB activation by PDGF was inhibited by NAK(KM) (Fig. 4f). Taken together, NAK is likely to function downstream of PKCε or related isozymes and upstream to IKK in the signaling pathway through which PDGF or other growth factors stimulate NF-κB activity.

In conclusion, our data suggest that although NAK has similar general organization to IKKα/β with especially high similarity within its kinase domain, it functions as an IKK-kinase rather than an IκB-kinase. Although NAK can phosphorylate one of the two regulatory serines required for induction of IκB degradation, this is insufficient for NF-κB activation and NAK does not stably associate with other IκB kinases. While NAK may activate only a small fraction of IKK because of its relatively low abundance, it is possible that through a positive feedback mechanism NAK-activated IKKβ can

phosphorylate an adjacent IKK subunit through intramolecular trans-autophosphorylation and thus propagate a higher level of IKK activation[1].

## Materials and Methods

### Molecular Cloning of NAK

Degenerate primers, 5′-AT(TCA)AT(TCA)CA(TC)(CA)GNGA(TC)ATCAAACC-3′ and 5′-GG(AGT)ATNCCNA(AG)(TC)TT(TC)TCAAAGAT-3′, based on conserved motifs in the kinase domains of IKKα and IKKβ, were used to synthesize cDNAs by reverse-transcription (RT) PCR of RNA derived from MDAH041 cells. Additional 5′ sequences were obtained by 5′ rapid amplification of cDNA ends (RACE), and 3′ sequences were obtained by screening human fetal brain cDNA library (Stratagene).

### Preparation of recombinant NAK protein and anti-NAK antibody

Baculoviruses expressing Myc/6xHis-tagged NAK were generated by cotransfection of pVL1393-NAK with linearized baculovirus DNA (BaculoGold: Pharmingen) into Sf9 cells. After amplification of the virus, cell lysates were prepared 48h post-infection and Myc/His-NAK was purified on ProBond Resin (Invitrogen). GST-NAK protein expressed in E. coli was purified on glutathione Sepharose beads (Pharmacia), and used to immunize rabbits. Antisera were precleaned on HiTrap NHS column coupled with GST protein and then affinity purified on HiTrap NHS column coupled with GST-NAK protein. The protein concentration was adjusted to 0.6 mg/ml.

**Kinase assay**

Wt NAK and NAK(KM) proteins expressed in Sf9 cells were immunoprecipitated with anti-Myc antibody (MBL). The kinase activity was determined at 30 °C for 30 min in a 30 μl reaction mixture containing 50 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 10 μM β-glycerophosphate, 1 mM NaF, 0.1 mM $Na_3VO_4$, 0.1 mM PMSF, 10 μM ATP and 185 kBq[$\gamma$-$^{32}$P]ATP (222 TBq/mmol; NEM). Reaction products were separated by SDS-PAGE, and phosphorylated proteins were detected by autoradiography. For immunecomplex kinase assay, serum-starved HeLa and A172 cells ($2x10^6$ cells) were treated with PMA(20 nM) and PDGF(50 ng/ml PDGF-AA plus 50 ng/ml PDGF-BB), respectively. The treated cells were then lysed in an IP-kinase buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 25 mM EGTA, 1 mM EDTA, 0.1 % Tween 20, and 10 % glycerol) containing a cocktail of protease inhibitors (20 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 100 μg/ml PMSF) and phosphatase inhibitors (50 mM NaF, and 0.1 mM $Na_3VO_4$). The cell lysates (2 mg protein) were immunoprecipitated with anti-NAK antibody or anti-IKKα antibody (Santa Cruz; M280), and the immunoprecipitates were assayed for kinase activity using GST-IκBα or GST-IKKβ as substrates. The

immunoprecipitates were also subjected to Western blotting to determine the amount of precipitated proteins.

**Transfections, immunoblot analysis, reporter assays, EMSAs, and immunostaining**

293T cells on 35 mm plates were transfected with pcDNA3-Flag-NAK, pCR3-Flag-IKKβ, pcDNA3-Flag-FWD1, or pcDNA3-myc-IκBα using FuGENE 6 transfection kit (Boehringer). After 36 h, cells were lysed in IP-kinase buffer. Immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting.

Luciferase activity was determined 36 h post-transfection, using an enhanced luciferase assay kit and a luminometer. All transfections included pmiwZ[28] to normalize transfection efficiencies based on β-galactosidase activity. The amounts of NAK and IKKβ expressed in 293T cells were determined by Western blotting using anti-Flag antibody (M5, IBI).

Electrophoretic mobility shift assays (EMSAs) were performed on lysates of transiently transfected 293T cells. NF-κB DNA binding was assayed using a $^{32}$P-labelled κB probe.

Immunostaining of COS-7 cells transfected with expression vectors of Flag-NAK was performed as described previously[17].

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**Figure legends**

**Fig. 1 Structure and expression of NAK**

(a) Optimized alignment of the human NAK, IKK-i, IKKα and IKKβ protein sequences is shown using single letter codes. Identical and similar amino acids are black-boxed and shadow-boxed, respectively. The HLH is marked by dotted lines and the leucine zipper is indicated by asterisk (*). (b) Northern blot analyses of polyA$^+$ RNA from various human tissues with NAK (upper panel) and β-actin (lower panel) cDNA probes.

**Fig. 2 NAK induces phosphorylation and ubiquitination of IκBα through activation of IKKβ**

(a) 293T cells were transfected with the indicated expression vectors and lysed 36 hrs latter. Anti-Myc immunoprecipitates were analyzed by immunoblotting with antibodies specific to phospho-IκBα(ser32)[α-P-IκBα; NEB 9241S], ubiquitin (α-Ub), or the Flag epitope. (b) NAK facilitates nuclear translocation of NF-κB. Cos7 cells transfected with Flag-NAK were fixed and stained with anti-p65RelA (middle), anti-Flag (left), and Hoechst33258 (right). Arrowheads indicate the transfected cells. (c) 293T cells were cotransfected with Flag-NAK, increasing amounts (μg) of IKKβ(KM), FWD1, and Myc-IκBα. Phospho-IκBα(Ser 32) was detected as described above (upper panel). Expression levels of NAK, IKKβ (middle panel) and IκBα (bottom panel) were determined by immunoblotting.

(d) NAK phosphorylates IκBα and IKKβ *in vitro*. Myc-tagged wt NAK (Wt) or a K38M (KM) expressed in Sf9 cells were immunoprecipitated with anti-Myc or normal rabbit IgG (N), and assayed for phosphorylation of the indicated substrates. Autoradiography (KA, upper panel) and Coomassie brilliant blue staining (CBB, lower panel) are shown. (e) NAK enhances IKKβ kinase activity. After transfection with the indicated expression vectors, anti-Myc immunoprecipitates were assayed for phosphorylation (upper panel; KA) of GST-IκBα(1-54, S36A), whose presence is shown by CBB staining (second panel; CBB). Expression of NAK (third panel; IB) and IKKβ (fourth panel; IB) were determined by immunoblotting. (f) NAK directly activates IKK. IKK, chromatographically purified[29] from nonstimulated HeLa cells, was incubated with purified NAK or NAK(KM) from Sf9 cells , or buffer (control), and was assayed for phosphorylation of GST-IκBα(Full-length, S36A; KA). The amount of IKK was determined by immunoblotting (IB) with anti-IKKα. IκBα phosphorylation was quantitated by phosphoimaging and used to calculate relative specific activities (RA). (g) NAK does not stimulate JNK or p38MAPK activities. After transfection with NAK or NAK(KM), JNK and p38 activities were determined by immunecomplex kinase assay as described previously[9]. Positive controls included osmotic shock (0.5 M NaCl) for p38 and UV irradiation (40 J $m^{-2}$) for JNK.

**Fig. 3 NAK activates NF-κB**

(a) 293T cells were cotransfected with the indicated expression vectors, together with β-galactosidase expression vector driven by an actin promoter(pmiwZ) and NF-κB-luciferase(Luc) reporter. After 36h, luciferase activities were determined, and normalized for β-gal galactosidase expression. The values are shown as mean±SD (n=5). (b) The indicated expression vectors were transfected into 293T cells and nuclear extracts were analyzed by EMSAs with either wt or mutant κB oligonucleotide probes. Some samples were preincubated with unlabelled wt or mutant κB oligonucleotides, or anti-RelA antibody as indicated. IKKβ0.2 and IKKβ2.0 refer to the amounts of DNA(in μg/plate) used for transfections. (c) Increasing amounts of NAK(KM)(1.4 and 5.6 μg/plate) or IKKβ(KM)(0.5 and 2.0 μg/plate) were cotransfected with wt IKKβ or NAK, respectively, and the NF-κB-Luc and actin-β gal reporters. Normalized luciferase activities were determined as described above. (d) Fibroblasts derived from wild-type, *IKKα-/-*, or *IKKβ-/-* mouse embryos were transfected with NAK and the 2xNF-κB-Luc reporters (NAK), or 2xNF-kB-Luc reporters followed by TNFα stimulation (TNFα). Luciferase activities were determined as described above. Results shown are averages of two experiments done in triplicates. (e) The NF-κB-Luc and actin-β gal reporters were

cotransfected with increasing amounts of NAK(KM)(1.0, 2.0, and 3.0 μg/plate) or IKKβ(KM)(0.5, 1.0, and 2.0 μg/plate). After 30 h, cells were treated with either PMA (left panel) or TNF-α (right panel) for additional 6h. Luciferase activity was determined as described above. (f) AP-1-Luc reporter was cotransfected with increasing amounts of NAK(KM)(1.0, 2.0, and 3.0 μg/plate) and luciferase activities were determined after treatment with PMA. (g) Expression vectors for several PKC isozymes (α, θ, ε)[30] were cotransfected with the NF-κB-Luc and actin-β gal reporters and increasing amounts of NAK(KM)(0.5, 1.0, 2.0, and 3.0 μg/plate). Luciferase activities were determined as described above.

**Fig. 4 Endogenous NAK is activated by PMA and PDGF and is not part of the IKK complex.**

(a) Serum starved-HeLa cells were treated with PMA for 30 min. Cell lysates (2 mg) were subjected to immunecomplex kinase assay with the indicated IκBα substrates after immunoprecipitation with anti-NAK. NRS represents normal rabbit serum. (b) S-100 cytoplasmic extract(L) and purified IKK complex(P) from nonstimulated(-) or TNFα-stimulated(+) HeLa cells were immunoblotted with antibodies specific to NAK, IKKα, IKKβ, and IKKγ. (c) Non-stimulated and PMA-stimulated HeLa cell extracts were subjected to immunoprecipitation-immunoblot analysis using the indicated antibodies. NRS represents normal