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IKK-*i*, a novel lipopolysaccharide-inducible kinase that is related to IκB kinases

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Abstract

Using the suppression subtractive hybridization technique, we isolated a novel kinase, IKK-*i*, whose message is drastically induced by lipopolysaccharide (LPS) in the mouse macrophage cell line RAW264.7. The predicted protein contains the kinase domain in its N-terminus, which shares 30% identity to that of IKK-α or IKK-β. The C-terminal portion contains a leucine zipper and a potential helix-loop-helix domain, as in the case of IKK-α and IKK-β. IKK-*i* is expressed mainly in immune cells, and is induced in response to proinflammatory cytokines such as tumor necrosis factor-α, IL-1 and IL-6, in addition to LPS. Overexpression of wild-type IKK-*i* phosphorylated serine residues Ser32 and Ser36 of IκB-α (preferentially Ser36), and significantly stimulated NF-κB activation. These results suggest that IKK-*i* is an inducible IκB kinase which may play a special role in the immune response.

NF-κB plays an important role in the regulation of a variety of genes involved in immune, acute phase and inflammatory responses. The active form of NF-κB is composed of homo- and heterodimers of the NF-κB/Rel family members. In the majority of mammalian cells, NF-κB exists as an inactive form in the cytoplasm by an association with a member of the family of inhibitory molecules (IκB), IκB-α, IκB-β or IκB-ε (1). NF-κB is activated by a variety of signals, including cytokines such as tumor necrosis factor (TNF)-α and IL-1, bacterial products such as lipopolysaccharide (LPS), oxidative stress, viruses, and DNA-damaging agents. The activation of NF-κB complexes is achieved through the degradation of IκB and subsequent dissociation of the NF-κB–IκB complexes (2–6). In the case of IκB-α, the N-terminal serine residues, Ser32 and Ser36, are phosphorylated in response to signals, followed by polyubiquitination and degradation (7–9). The released NF-κB complexes then translocate to the nucleus, where they up-regulate expression of many genes involved in the immune and inflammatory responses.

Recently, two closely related IκB kinases (IKKs) have been identified and cloned (10–14). One of the kinases is identical to a previously cloned serine/threonine kinase of unknown function, named CHUK. The structural characteristic of CHUK is that it contains helix-loop-helix and leucine zipper sequences as is often seen in transcriptional factors. The second kinase is highly related to CHUK. CHUK and its relative are now referred to as IKK-α and IKK-β respectively. IKK-α and IKK-β are 52% identical in amino acids. Both kinases directly phosphorylate Ser32 and Ser36 of IκB-α, and overexpression of each wild-type kinase leads to NF-κB activation. The activity of IKK-α and IKK-β is stimulated by TNF-α and IL-1 treatment. IKK-α and IKK-β form a heterodimer that can interact directly with the upstream kinase, NIK (12). These three kinases are considered to be present in the large 700 kDa IκB kinase complex (15,16).

In an attempt to isolate novel genes that are induced in activated macrophages, and are responsible for immune and

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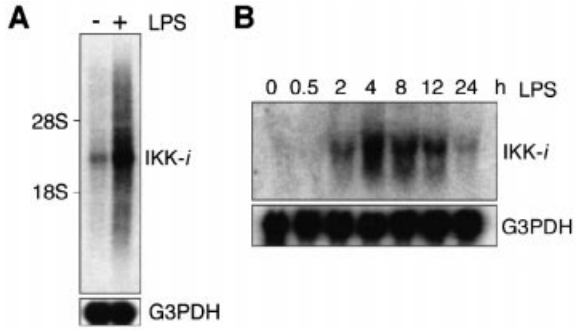

Fig. 1. Induction of IKK-*i* expression by LPS in RAW264.7 cells. (A) RAW264.7 cells were left unstimulated (–) or stimulated (+) with 100 ng/ml LPS (Difco, Detroit, MI) for 4 h. Two micrograms of poly(A)$^+$ RNA was subjected to Northern blot analysis using the IKK-*i* cDNA fragment obtained from subtraction as probe. The same membrane was rehybridized with G3PDH cDNA probe. (B) RAW264.7 cells were stimulated with 100 ng/ml LPS for the indicated periods. Total RNA was extracted and subjected (25 μg/lane) to Northern blot analysis. The membrane was hybridized with IKK-*i* cDNA. The same membrane was rehybridized with the G3PDH cDNA probe.

inflammatory responses, we prepared a cDNA library from cultures of the LPS-stimulated mouse macrophage cell line, RAW264.7, and screened the library by the suppression subtractive hybridization technique (17). Using this technique, we previously identified a novel LPS-inducible chemokine receptor (18). In addition to this gene, we obtained several clones with a novel sequence. This gene was only slightly expressed in non-stimulated RAW264.7 cells, but was dramatically induced by stimulation with LPS (Fig. 1A). In order to analyze LPS-induced expression of this gene, we stimulated RAW264.7 cells with LPS for various periods and subjected the cells to Northern blot analysis (Fig. 1B). Expression of mRNA for this gene was induced within 2 h after stimulation and reached the peak level at 4 h in RAW264.7 cells. The full-length cDNA of this gene was obtained from the cDNA library of RAW264.7 cells that were activated by LPS for 4 h as described previously (18).

The obtained cDNA has an open reading frame of 2151 bp, encoding a protein of 717 amino acids. The deduced amino acid sequence of this cDNA showed 82.3% identity with the human cDNA clone (KIAA0151) of unknown function, indicating that this gene is a murine homologue of KIAA0151. The N-terminal portion of this polypeptide contains a putative serine/threonine kinase domain, whereas the C-terminal portion contains a leucine zipper domain and a potential helix-loop-helix domain (Fig. 2). The catalytic domain of this LPS-inducible protein kinase has a significant similarity (30% identical) with that of IKK-β, a recently identified serine/threonine kinase that contains a catalytic domain in its N-terminus, and a leucine zipper domain and a helix-loop-helix-domain in its C-terminus (Fig. 2). Thus, this LPS-inducible kinase has a striking structural similarity with a member of the IKK family. Based on its similarity and the novel function of this kinase that we are going to describe in this paper, we designated this kinase IKK-*i* (for inducible IκB kinase).

We examined the tissue distribution of IKK-*i* transcripts by Northern blot analysis in various human tissues (Fig. 3A). The major IKK-*i* transcript was 4.0 kb in length. High expression of IKK-*i* mRNA was observed in thymus, spleen, peripheral blood leukocytes, pancreas and placenta, with the highest expression in spleen. Low expression was observed in lung, kidney, prostate, ovary and colon. We examined LPS-induced expression of IKK-*i* mRNA in various murine cell lines (Fig. 3B). IKK-*i* mRNA expression was induced by LPS stimulation in the NK cell line (5E3) and monocytic leukemia cell line (M1). A mouse pre-B cell line (70Z3) and mature B cell line (WEHI231) also showed an increase in IKK-*i* mRNA in response to LPS. We next examined whether other stimuli induce IKK-*i* expression. Stimulation of RAW264.7 cells with IL-6 and IFN-γ induced IKK-*i* mRNA expression (data not shown). IKK-*i* mRNA expression was up-regulated upon stimulation of thioglycollate-elicited peritoneal macrophages with TNF-α, IL-1β, IFN-γ and IL-6 in addition to LPS (Fig. 3C). In contrast, mRNA expression levels of IKK-α or IKK-β were not augmented in response to these stimuli.

As described above, IKK-*i* mRNA is constitutively expressed in spleen. We next examined which cell population expresses IKK-*i* mRNA in spleen. T and B cell populations were prepared from splenocytes and IKK-*i* expression was analyzed by Northern blot analysis (Fig. 3D). The T cell population, but not B cell population, expressed IKK-*i* mRNA constitutively. In the case of B cells, IKK-*i* expression was induced in response to LPS.

Taken together, IKK-*i* is predominantly expressed in immune cells and is inducible in response to LPS or other inflammatory cytokines.

We next investigated whether IKK-*i* phosphorylates IκB-α *in vitro*. To express IKK-*i* in mammalian cells, an N-terminal FLAG epitope-tagged wild-type IKK-*i* (FLAG-IKK-*i* WT) was cassetted into the pEF-BOS mammalian expression vector. Human embryonic kidney 293 cells were transiently transfected with FLAG-IKK-*i* WT. The cells were lysed and immunoprecipitated with anti-FLAG mAb. The kinase activity in the immunoprecipitates was measured by *in vitro* kinase assay with GST–IκB-α (residues 1–72) fusion protein or GST–IκB-α (1–72) mutant with substitution of Ser32 and/or Ser36 to alanine (S32A, S36A and S32,36A) as the substrates. As shown in Fig. 4(A), IKK-*i* phosphorylated GST-IκB-α WT and S32A but not GST-IκB-α S32,36A. S36A was weakly phosphorylated by IKK-*i*, suggesting that IKK-*i* phosphorylated preferentially Ser36 of two serine residues, which are important for degradation of IκB-α and NF-κB activation.

We prepared a series of mutant IKK-*i* to investigate the regulation of the kinase activity of IKK-*i*. The K38A mutant carries a point mutation in which the lysine residue at 38 is substituted by alanine. The substitution of this residue is known to block the phosphotransfer reaction and results in a kinase-negative mutant in a number of protein kinases, including IKK-α and IKK-β. Phosphorylation of the serine residue at 176, which is present in the activation loop between kinase subdomains VII and VIII, is essential for activation of IKK-α (19). Mutation studies indicated that the changes of Ser177 and Ser181 of IKK-β by alanines decreased its activity, whereas the changes by glutamates enhanced it. Unlike for IKK-α and IKK-β, the residue of IKK-*i* that corresponds to Ser176 of IKK-α is glutamate. Therefore, we prepared three mutants in which the corresponding residues were changed to

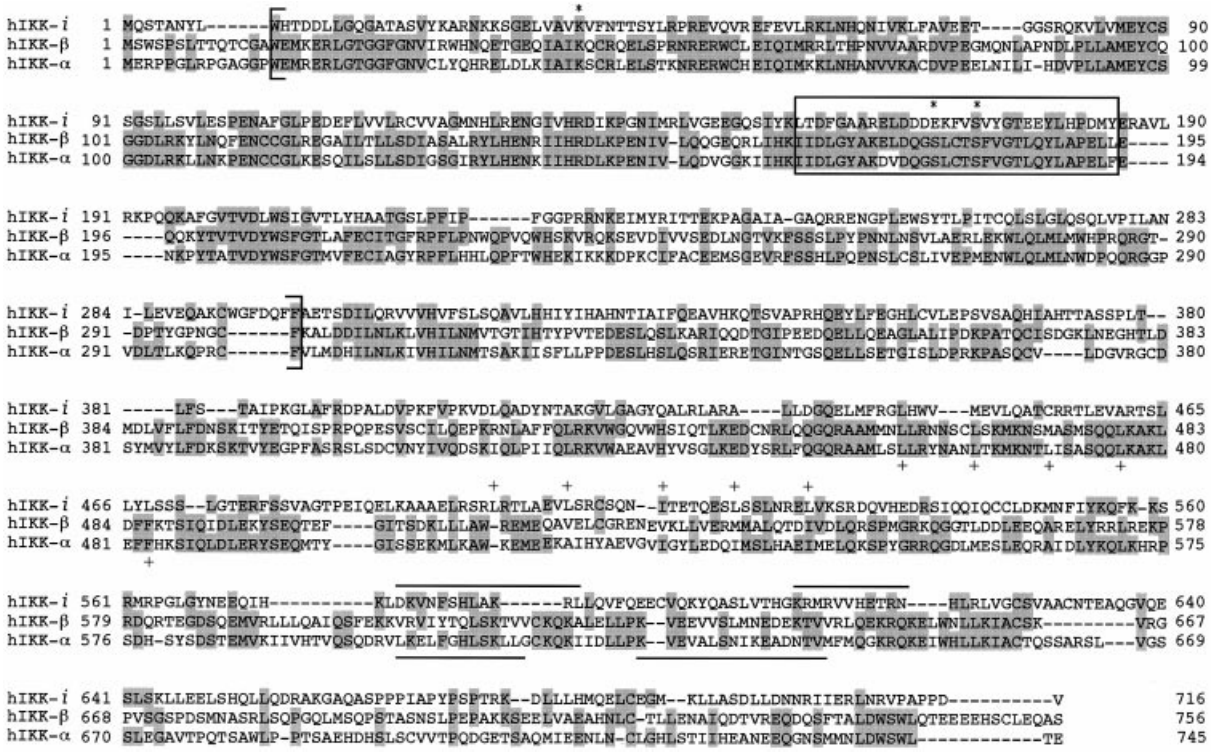

Fig. 2. Amino acid sequence of IKK-*i*. Alignment of the amino acid sequences of human IKK-*i* with IKK-α and IKK-β. The solid backgrounds indicate identical amino acid residues. Brackets delineate the boundaries of the kinase domain. The activation loop is boxed. Amino acid residues mutated for some experiments in this paper are asterisked. Two helices of the potential helix-loop-helix domain of IKK-*i* are overlined, and two helices of the helix-loop-helix domain of IKK-α and IKK-β are underlined. The heptad repeats of hydrophobic residues within the leucine zipper domain of IKK-*i* are indicated with + on top of the letters and of IKK-α and IKK-β under the letters. The nucleotide and amino acid sequence data reported in this paper are available from DDBJ, EMBL and GenBank under accession numbers AB016590 (human IKK-*i*) and AB016589 (mouse IKK-*i*).

alanine or glutamate (E168A, S172A and S172E). In addition, a deletion mutant lacking the C-terminal region (ΔC, encoding amino acids 1–543) was also constructed. As shown in Fig. 4(B), both auto- and IκB-α-phosphorylations were completely abolished by the mutation in K38A. The kinase activity of E168A was only slightly decreased compared to that of wild-type. In contrast, the mutation of Ser172 to alanine resulted in the loss of kinase activity. Contrary to our expectation, change of Ser172 to glutamate did not enhance the kinase activity of IKK-*i*, but rather lost it. Phosphorylation of IκB-α was not detected in the ΔC mutant, although autophosphorylation was similar to that of wild-type. These results suggest that Ser172 of IKK-*i* is a major autophosphorylation site and that the C-terminal region is necessary for phosphorylating IκB-α.

The ability of IKK-*i* to phosphorylate IκB-α allowed us to test whether IKK-*i* induces NF-κB activation. Human 293 cells were transiently co-transfected with the indicated amounts of FLAG-IKK-*i* WT or K38A mutant along with the NF-κB-dependent luciferase reporter gene plasmid and luciferase activity was measured. As shown in Fig. 4(C), the luciferase activity was significantly increased by expression of IKK-*i* WT in a dose-dependent manner. On the other hand, NF-κB activation was not altered in K38A mutant-transfected cells. These results indicate that IKK-*i* mediates NF-κB activation through phosphorylation of the N-terminal regulatory region of IκB-α.

The kinase activity of IKK-α and IKK-β is stimulated by TNF-α or IL-1 treatment (10–14). To determine whether the kinase activity of IKK-*i* is also enhanced by these cytokines, we transiently transfected FLAG-tagged IKK-*i* in 293 cells or COS-7 cells. Thirty-six hours later, 293 cells were treated with TNF-α and COS-7 cells were treated with IL-1β for 3 or 7 min (Fig. 4D). Then cells were lysed and anti-FLAG immunoprecipitates were examined by an *in vitro* kinase assay. To prove that the cells were stimulated by these cytokines, Western blot analysis was performed for the cell lysate of TNF-α-treated 293 cells with anti-phosphorylated p38 MAP kinase (p-p38) antibody, and for the cell lysate of IL-1-treated COS-7 cells with anti-phosphorylated extracellular signal-regulated kinase (p-ERK)-1 antibody. p-p38 and p-ERK-1 increased by treating the cells with these cytokines, but both TNF-α and IL-1 did not change the degree of phosphorylation of exogenous IκB-α, indicating that the kinase activity of IKK-*i* is not enhanced by these cytokines.

In the present study, we describe the cloning and initial characterization of a novel IκB kinase that shows homology with IKK-α and IKK-β. NF-κB activation depends on the signal-induced phosphorylation of IκB proteins at two specific serine residues: Ser32 and Ser36 in the case of IκB-α, and Ser19 and

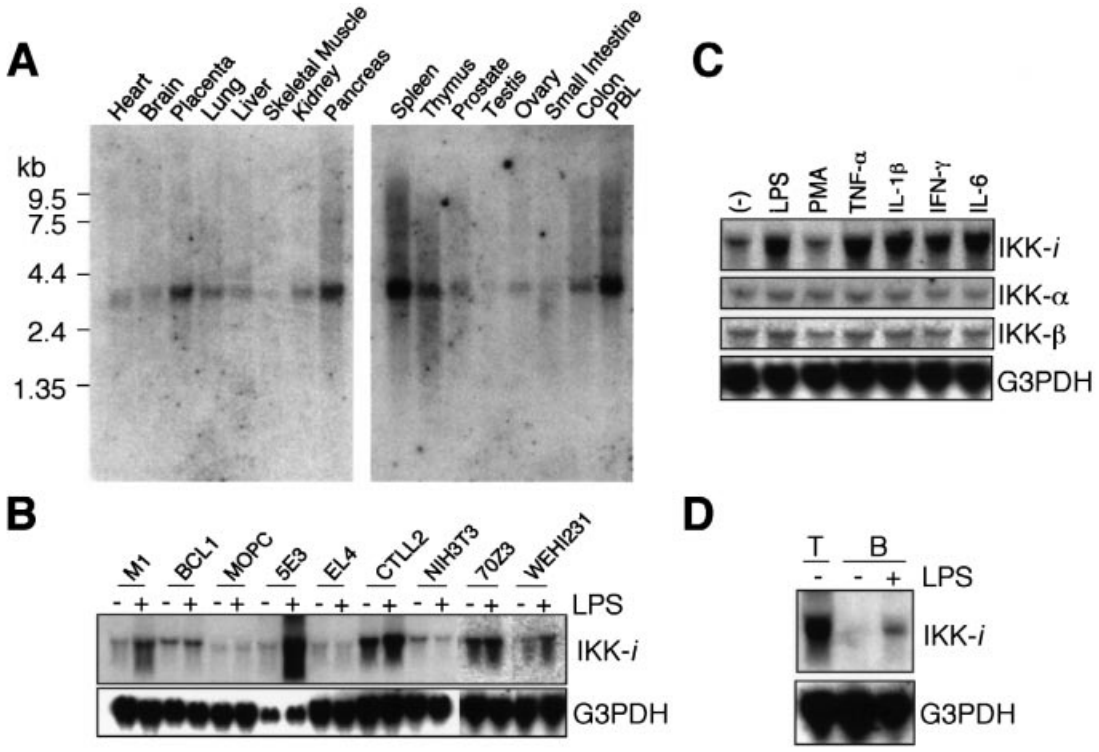

Fig. 3. Expression of IKK-*i* mRNA in various tissues. (A) A filter containing 2 μg of poly(A)$^+$ RNAs from various human tissues was hybridized with human IKK-*i* cDNA probe (Clontech, Palo Alto, CA). PBL; peripheral blood leukocytes. (B) Various murine cell lines were left unstimulated or stimulated with 100 ng/ml LPS (100 μg/ml for 70Z3 and WEHI231) for 4 h. Total RNA was extracted and subjected to Northern blot analysis for IKK-*i* expression (upper panel). The same membrane was rehybridized with G3PDH cDNA probe (lower panel). (C) C57BL/6 mice were i.p. injected with 2 ml of 4% thioglycollate. Three days later, peritoneal exudate cells were isolated and cultured for 2 h. Adherent cell monolayers were used as peritoneal macrophages. Then, the peritoneal macrophages were stimulated with 250 U/ml IFN-γ (Genzyme, Cambridge, MA), 100 ng /ml TNF-α (Genzyme), 1000 U/ml IL-6 (Genzyme) or 100 ng /ml IL-1β (Genzyme) for 4 h. Total RNA was extracted, and analyzed for expression of IKK-*i*, IKK-α, IKK-β and G3PDH by Northern blot. Probes for IKK-α and IKK-β were generated by PCR from a mouse peritoneal exudate cell cDNA library. (D) T or B cells were purified from splenocytes of C57BL/6 mice by magnetic cell sorting (MACS; Miltenyi Biotec, Bergish Gladbach, Germany) using microbeads–Thy-1 or microbeads–B220 respectively. Purified T and B cells were >98% positive for CD3 and B220 as determined by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA). The purified B cells were stimulated with 100 mg/ml LPS. Total RNA was extracted and expression of IKK-*i* was analyzed by Northern blot (upper panel). The same membrane was rehybridized with the G3PDH cDNA probe (lower panel). T, T cell; B, B cell.

Ser23 in IκB-β (20). The proteosome-dependent destruction of IκB requires dual phosphorylation of the serine residues. Although IKK-*i* primarily phosphorylates the second serine residue of IκB-α, transient overexpression of IKK-*i* is able to significantly stimulate NF-κB activation, suggesting that the phosphorylation of the second serine residue may be sufficient for the ubiquitination and destruction of IκB-α. In fact, the IKK complex is shown to have some preference for the second serine residue as compared with the first one of IκB proteins (20). Alternatively, the dual phosphorylation of IκB proteins may be mediated by the combined action of phosphorylation of the second serine residue by IKK-*i* and phosphorylation of the first serine residue by other kinases such as mitogen-activated 90 kDa ribosomal S6 kinase and casein kinase II (20,21).

The kinase activities of IKK-α and IKK-β are augmented in response to TNF-α and IL-1 stimulation (10,11,13,14), and are regulated by two upstream kinases, NIK and MEKK1 of the MAP3K family (12,19,22,23). MAP3K is shown to activate MAP2K by phosphorylating serine and threonine residues in the activation loop between kinase subdomain VII and VIII (24). Both IKK-α and IKK-β contain a canonical MAP2K activation loop motif (Ser-X-X-X-Ser, where X is any amino acid) (11). In addition to the position of the serine residues, the surrounding amino acid sequence is also conserved between IKK-α and IKK-β. It has been demonstrated that phosphorylation of IKK-α by NIK occurs specifically on Ser176 (19). The phosphorylation of IκB-α by IKK-α was greatly impaired when Ser176 of IKK-α was mutated to alanine. In contrast, mutation of Ser176 to glutamate significantly enhanced the IKK-α activity (19). Similarly, mutation of two corresponding serine residues of IKK-β to alanine residues abolished the kinase activity, while mutation to glutamate residues enhanced it (11). In the case of IKK-*i*, the corresponding region is not conserved and the amino acid corresponding to Ser176 of IKK-α is replaced by glutamate. Mutation of the Ser172 to alanine abolished the autophosphorylation of IKK-*i* as well as IκB-α phosphorylation. The replacement of the critical serine residue of IKK-*i* with glutamate did not augment IκB-α kinase activity, but rather decreased it. Neither NIK nor

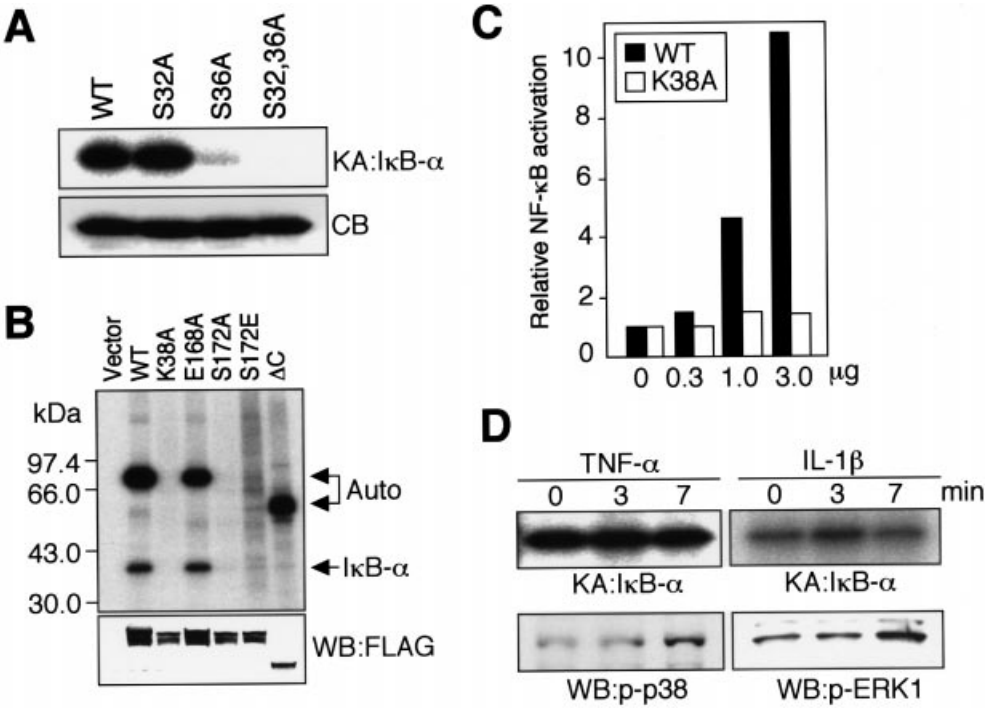

Fig. 4. IKK-*i* phosphorylates IκB-α *in vitro* and activates NF-κB. (A) Human 293 cells were transiently transfected with 10 μg of FLAG-tagged IKK-*i* WT by lipofection (Qiagen, Hilden, Germany). Thirty-six hours after transfection, cell lysates were immunoprecipitated with anti-FLAG mAb (Sigma, St Louis, MO). The *in vitro* kinase assay was carried out in the presence of 1.0 μg of GST–IκB-α WT, S32A, S36A or S32,36A. Proteins were dissolved by SDS–PAGE and visualized by autoradiography. Phosphorylation of GST–IκB proteins were indicated (upper panel). The lower panel shows Coomassie blue (CB) staining of the gel. The GST–IκB-α fusion protein was expressed in *Escherichia coli* BL-21 in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and purified with glutathione–Sepharose beads (Amersham Pharmacia, Uppsala, Sweden). (B) Human 293 cells were transiently transfected with 10 μg of FLAG-IKK-*i* WT, K38A, E168A, S172A, S172E or ΔC. After 36 h, the cell lysates were immunoprecipitated with anti-FLAG mAb and the *in vitro* kinase reaction was carried out in the presence of GST–IκB-α (1–72) (upper panel). The lower panel shows Western blot analysis of the same lysates with anti-FLAG mAb (Sigma). (C) Effect of overexpression of IKK-*i* on NF-κB-dependent reporter gene activity. Human 293 cells were transiently co-transfected with 1.0 μg of reporter plasmid (pNF-κB Luc) (Stratagene, La Jolla, CA) together with the indicated amounts of the expression plasmid for FLAG-IKK-*i* WT or K38A by lipofection. The total DNA dose was kept constant to 4 μg by supplementation with empty vector (pEF-BOS). Twenty-four hours after transfection, relative luciferase activity was determined and normalized on the basis of sea-pansy luciferase activity (pRL-SV40) (Promega, Madison, WI). Similar results were obtained from five independent experiments in which each transfection was performed in duplicate. (D) 293 and COS-7 cells were transiently transfected with FLAG-IKK-*i*. Thirty-six hours after transfection, 293 cells (left panels) and COS-7 cells (right panels) were left untreated (–) or treated with 40 ng/ml of TNF-α or 40 ng/ml of IL-1β respectively. Cell lysates were immunoprecipitated with anti-FLAG mAb and the *in vitro* kinase reaction was carried out in the presence of GST–IκB-α (1–72) (upper panels). The same lysates were examined by Western blot analysis with anti-phosphorylated p38 MAP kinase (p-p38) (New England Biolabs, Beverly, MA) for TNF-α and anti-p-ERK-1 (New England Biolabs) for IL-1 (lower panels). KA, kinase activity; WB, Western blot.

MEKK1 phosphorylated IKK-*i* and co-transfection with NIK or MEKK1 did not augment IKK-*i* kinase activity (our unpublished data). Immunoprecipitation experiments showed that IKK-*i* did not associate with either IKK-α or IKK-β, indicating that IKK-*i* may not be involved in the formation of the 700 kDa IKK complex (our unpublished data).

Taken together, these results suggest that phosphorylation of Ser172 of IKK-*i* is essential for autophosphorylation and IκB kinase activity, but is not augmented by the upstream kinases, NIK and MEKK1. This may be also consistent with the fact that IKK-*i* autophosphorylation and IκB kinase activity is not augmented in response to either IL-1 or TNF-α. The fact that overexpression of IKK-*i* is enough for autophosphorylation as well as NF-κB activation suggests that IKK-*i* activity may be mainly regulated at the level of mRNA induction, but not at the level of phosphorylation by stimuli-activated kinases. At present, the kinase that phosphorylates serine residues of IKK-*i* remains unknown. IKK-*i*, itself, may autophosphorylate Ser172.

Finally, although T cells express IKK-*i* mRNA constitutively, we could not detect NF-κB activation in T cells (our unpublished data), just that IKK-*i* mRNA induction is not sufficient for NF-κB activation. There may be translational regulation of IKK-*i* mRNA or an inhibitor(s) present in T cells. Further work including expression of IKK-*i* protein and generation of IKK-*i*-deficient mice will reveal the role of IKK-*i* in the immune and inflammatory responses.

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Abbreviations

IKK	IκB kinase
LPS	lipopolysaccharide
p-ERK	phosphorylated extracellular signal-regulated kinase
TNF	tumor necrosis factor

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