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Selective Inhibition of NF-κB Activation by a Peptide That Blocks the Interaction of NEMO with the IκB Kinase Complex

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Activation of the transcription factor nuclear factor (NF)–κB by proinflammatory stimuli leads to increased expression of genes involved in inflammation. Activation of NF-κB requires the activity of an inhibitor of κB (IκB)-kinase (IKK) complex containing two kinases (IKKα and IKKβ) and the regulatory protein NEMO (NF-κB essential modifier). An amino-terminal α-helical region of NEMO associated with a carboxyl-terminal segment of IKKα and IKKβ that we term the NEMO-binding domain (NBD). A cell-permeable NBD peptide blocked association of NEMO with the IKK complex and inhibited cytokine-induced NF-κB activation and NF-κB–dependent gene expression. The peptide also ameliorated inflammatory responses in two experimental mouse models of acute inflammation. The NBD provides a target for the development of drugs that would block proinflammatory activation of the IKK complex without inhibiting basal NF-κB activity.

The regulatory protein NEMO (also named IKKγ) is required for proinflammatory activation of the IκB-kinase (IKK) complex (*1*–*5*). We surmised that prevention of the NEMO-IKK interaction would inhibit signal-induced NF-κB activation and, therefore, attempted to identify the mechanism of interaction between NEMO and IKKβ. We analyzed the interaction of NEMO fused at its NH_2-terminus to glutathione *S*-transferase (GST-NEMO, see Fig. 1A) with IKKβ mutants lacking the catalytic, leucine zipper, and helix-loop-helix (HLH) domains [Fig. 1A and (*6*)]. None of the mutants interacted with GST, whereas all three COOH-terminal fragments (307–756, 458–756, and 486–756) interacted with GST-NEMO [Fig. 1A and (*7*)]. None of the NH_2-terminal fragments (1–458, 1–605, or 1–644) precipitated with GST-NEMO, demonstrating that NEMO interacts with the COOH-terminus of IKKβ distal to the HLH. An IKKβ mutant consisting of only residues 644 to 756 associated with GST-NEMO, confirming that this region mediates interaction between the molecules (Fig. 1B). Furthermore, IKKβ(644–756) dose-dependently inhibited cytokine-induced NF-κB activation in transfected HeLa cells [Fig. 1C and (*6*, *8*)]. The most likely explanation for this result is that overexpressed IKKβ(644–756) associates with endogenous NEMO and prevents recruitment of regulatory proteins to the IKK-complex.

To identify the domain of NEMO (*1*–*3*, *9*) required for association with IKKβ, we analyzed the interaction of GST-IKKβ(644–756) with truncation mutants of NEMO (Fig. 1D). IKKβ(644–756) associated with NEMO fragments 1–196, 1–302, and 44–419 but not 197–419 or 86–419, indicating that the interaction domain lies between residues 44 and 86. A deletion mutant lacking this α-helical region (residues 50–93, del.αH) did not interact with IKKβ(644–756) (Fig. 1E) and inhibited tumor necrosis factor–α (TNF-α)–induced NF-κB activity (Fig. 1F), confirming the dominant-negative effects of the NEMO COOH-terminus (*2*, *3*). These findings suggest that the NH_2-terminus of NEMO anchors it to the IKK-complex, leaving the remainder of the molecule accessible for interacting with regulatory proteins.

The IKKβ COOH-terminus contains a region with identity to IKKα (denoted α_1), a serine-rich domain (*10*), and a serine-free region (Fig. 2A). Analysis of IKKβ mutants omitting each of these segments indicated that NEMO associates with the COOH-terminus after residue 734 (Fig. 2A). The region of IKKβ from F734 to T744 [α_2 in Fig. 2B (*11*)] contains a segment that is identical to the equivalent sequence in IKKα. The IKKβ sequence then extends for 12 residues forming a glutamate-rich region (Fig. 2B) that we speculated would be the NEMO interaction domain. However, a truncation mutant omitting this region (1–744) associated with GST-NEMO (Fig. 2C). Thus, the NEMO-interaction domain of IKKβ appears to be within the α_2-region of the COOH-terminus.

We next used the IKKβ(1–744) and (1–733) mutants to determine the effects of NEMO association on IKKβ activity and found that IKKβ(1–733) induced NF-κB activation that was approximately 1.5 to 2 times that induced by wild-type IKKβ (Fig. 2D). Furthermore, NF-κB activity induced by IKKβ(1–744) was identical to that induced by wild-type IKKβ. Thus, NEMO may maintain basal IKKβ activity as well as regulate its signal-induced activation.

Because the α_2-region of IKKβ resembles the COOH-terminus of IKKα (Fig. 2B), we tested the ability of IKKα to interact with NEMO (*7*). IKKα and IKKβ expressed in wheat germ extract both associated with GST-NEMO demonstrating that the individual interactions are direct (Fig. 3A). Further analysis revealed that IKKα interacts with NEMO through the COOH-terminal region containing the six amino

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acids shared with the α_2-region of IKKβ (Fig. 3B). In contrast, the IKK-related kinase IKK*i* (*12*), which does not contain an α_2-homologous region, failed to interact with NEMO (*7*). Gene-targeting has demonstrated a profound difference between IKKα and IKKβ activation by TNF-α (*13*). Our findings suggest that this difference is not due to differential interaction with NEMO.

A mutant of IKKβ lacking the six α_2-region residues did not associate with GST-NEMO (Fig. 3C). Therefore, we have named this sequence the NEMO-binding domain (NBD) (*7*). We examined the effects of point mutations within the NBD and found that replacement of D738, W739, or W741 with alanine prevented association with NEMO (Fig. 3D). In contrast, replacement of L737, S740, or L742 with alanine did not affect NEMO binding (Fig. 3D). To test the effects of these mutations on IKKβ function, we measured NF-κB activation in transfected HeLa cells. Consistent with previous results (Fig. 2D), mutants that did not bind NEMO activated NF-κB to a greater extent than did wild-type IKKβ or IKKβ(1–744), whereas NEMO-binding mutants activated to the same level as the controls (Fig. 3E). These data strongly support the hypothesis that NEMO plays a role in the down-regulation of IKKβ activity.

The ability to selectively inhibit NF-κB activation induced by proinflammatory cytokines may be crucial for the treatment of inflammation. However, inhibition of the catalytic activity of the IKKs may block basal NF-κB activity and impair its function as a survival factor, leading to potentially toxic side effects. We reasoned that a more effective anti-inflammatory drug might result from blocking the interaction of NEMO with the IKK complex. Therefore, we designed cell-permeable peptides (*11*, *14*) spanning the IKKβ NBD and determined their ability to disrupt the IKKβ-NEMO interaction. The wild-type NBD peptide (Fig. 4A) consisted of the region from T735 to E745 of IKKβ fused with a sequence derived from the Antennapedia homeodomain that mediates membrane translocation (*15*). The mutant peptide was identical except that W739 and W741 in the NBD were mutated to alanines (Fig. 4A). Only the wild-type NBD peptide dose-dependently inhibited in vitro interaction of IKKβ with NEMO (Fig. 4B). Furthermore, after incubating HeLa cells with the peptides, the wild-type, but not the mutant, NBD peptide disrupted formation of the endogenous IKK complex (Fig. 4C).

The effects of the NBD peptides on IKK activation were determined by immune-complex kinase assays (*16*) by using IKK complexes precipitated from TNF-α–stimulated HeLa cells pretreated with peptides. The wild-type, but not the mutant, peptide de-

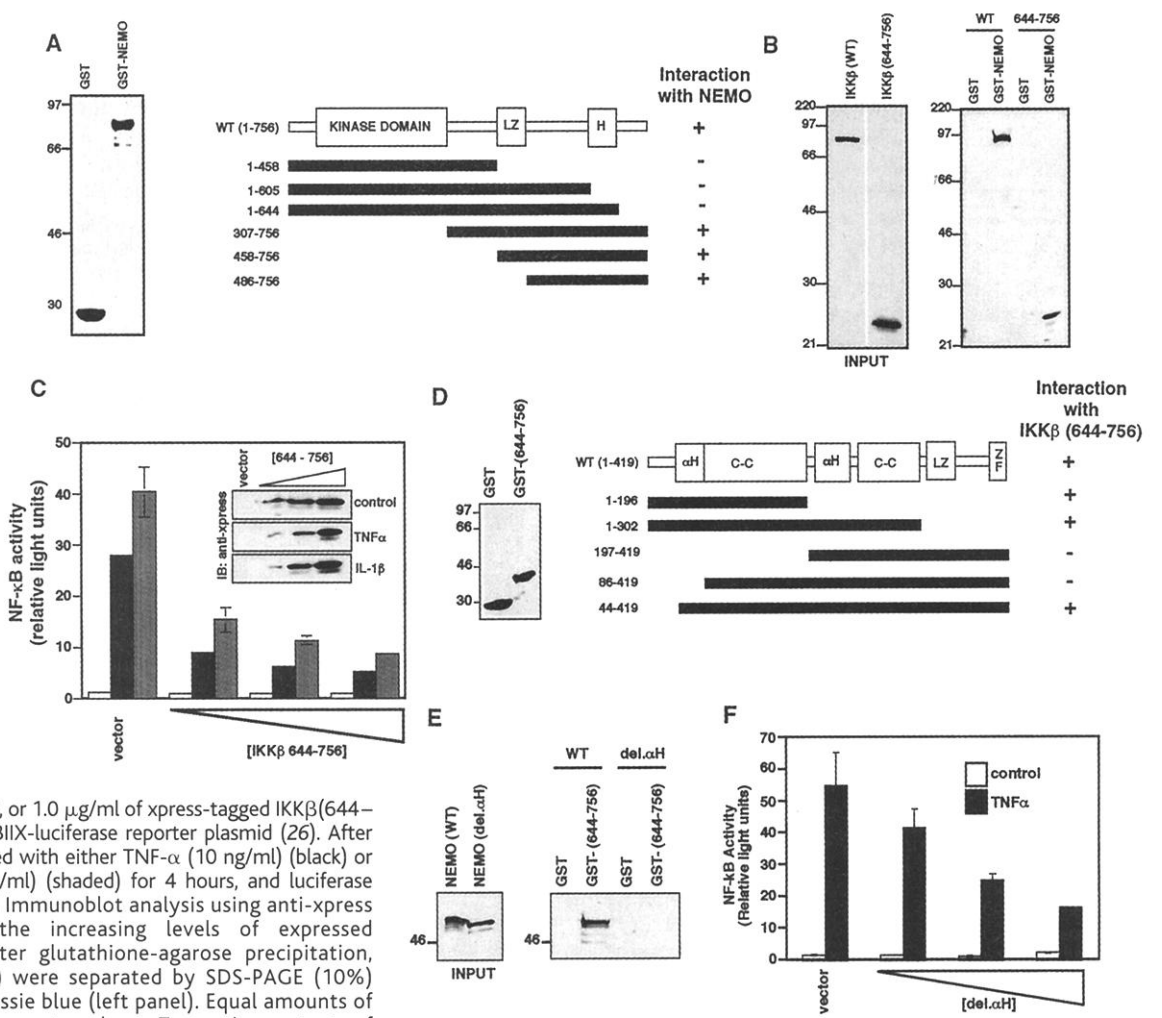


Fig. 1. Interaction of the COOH-terminus of IKKβ with the first α-helical region of NEMO. (**A**) GST and GST-NEMO were precipitated with glutathione-agarose, separated by SDS-PAGE (10%), and stained with Coomassie blue (left panel). Equal amounts of each were used in subsequent pull-down experiments. The truncation mutants of IKKβ (WT, wild-type; LZ, leucine zipper; H, HLH) were expressed (*6*) and used for GST pull-down [right panel, see (*7*)] as previously reported (*24*). NEMO-interacting mutants are indicated (+). (**B**) In vitro translated IKKβ and IKKβ(644–756) (left panel) were used for pull-down analysis (right panel). (**C**) HeLa cells were transiently transfected (*8*) with pcDNA-3.1-xpress (vector) or 0.25, 0.5, or 1.0 μg/ml of xpress-tagged IKKβ(644–756) together with the pBIIX-luciferase reporter plasmid (*26*). After 48 hours, cells were treated with either TNF-α (10 ng/ml) (black) or interleukin (IL)-1β (10 ng/ml) (shaded) for 4 hours, and luciferase activity was measured (*8*). Immunoblot analysis using anti-xpress (inset) demonstrates the increasing levels of expressed IKKβ(644–756). (**D**) After glutathione-agarose precipitation, GST and GST(644–756) were separated by SDS-PAGE (10%) and stained with Coomassie blue (left panel). Equal amounts of each were used for subsequent analyses. Truncation mutants of NEMO were constructed (C-C, coiled coil; LZ, leucine zipper), expressed, and used for pull-down analysis [right panel, see (*6*, *24*)], and interacting mutants are indicated (+). None of the mutants interacted with GST (*17*). (**E**) Wild-type NEMO and a mutant lacking the first α-helical region (del.αH) were expressed (input) and used for pull-down analysis by using the proteins in (D, left). (**F**) NF-κB activity (*8*) in HeLa cells transfected with pBIIX-luciferase and either pcDNA-3 (vector) or del.αH (0.25, 0.5, or 1.0 μg/ml) for 48 hours then treated for 4 hours with TNF-α (10 ng/ml).

creased TNF-α–induced IKK activity [Fig. 4D and (*7*)], whereas neither peptide inhibited TNF-α–induced phosphorylation of c-Jun (*7*). Electrophoretic mobility shift analysis (EMSA) demonstrated that only the wild-type peptide inhibited TNF-α–stimulated nuclear translocation of NF-κB in HeLa cells (Fig. 4E), whereas neither peptide affected DNA binding of the transcription factor Oct-1 (*17*). Furthermore, the wild-type NBD peptide inhibited TNF-α–induced NF-κB activity (Fig. 4F, upper panel). Basal NF-κB activity was enhanced approximately twofold by the wild-type peptide (Fig. 4F, lower panel), suggesting that removal of NEMO slightly increases the basal, intrinsic activity of the IKK complex while abolishing its responsiveness to TNF-α.

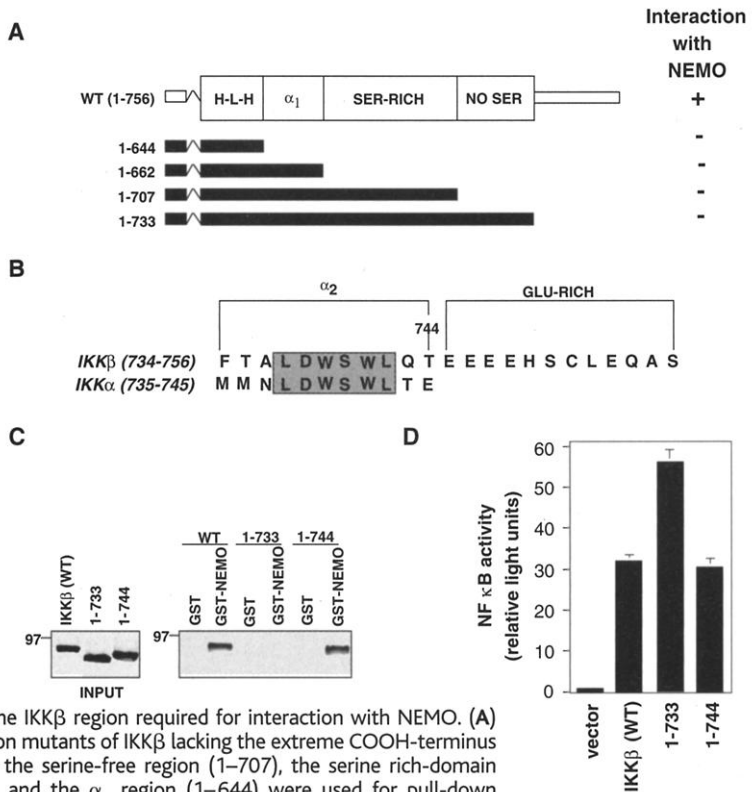
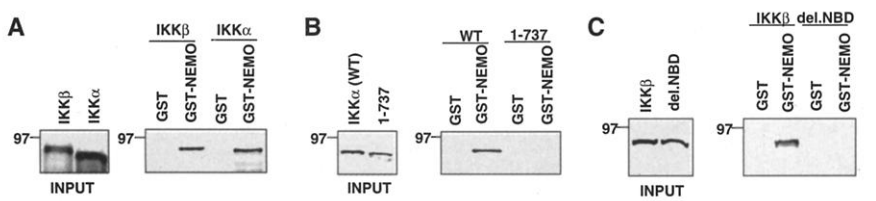
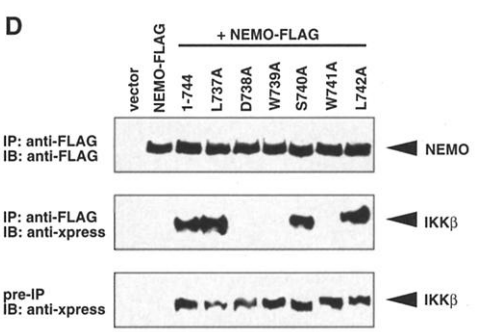


Fig. 2. The IKKβ region required for interaction with NEMO. (**A**) Truncation mutants of IKKβ lacking the extreme COOH-terminus (1–733), the serine-free region (1–707), the serine rich-domain (1–662), and the α_1 region (1–644) were used for pull-down analysis by GST-NEMO (Fig. 1A). None of the mutants interacted with GST (*17*). (**B**) Comparison of the COOH-termini of IKKα and IKKβ indicating the α_2 and glutamate-rich regions and the six identical amino acids (shaded). (**C**) Wild-type IKKβ and the truncation mutants (1–733 and 1–744, input) were used for in vitro pull-down analysis with either GST or GST-NEMO. (**D**) NF-κB activity in HeLa cells transfected with 1 μg/ml of the indicated constructs or vector (pcDNA-3) together with pBIIX-luciferase (*8*).

Many genes involved in inflammation are regulated by NF-κB (*18*). E-selectin is a leukocyte adhesion molecule expressed by vascular endothelial cells after activation by proinflammatory cytokines (*19*). To assess the anti-inflammatory potential of the NBD peptides, we pretreated human umbilical vein endothelial cells with the peptides then induced E-selectin expression with TNF-α. The wild-type peptide caused low-level expression of E-selectin (Fig. 5A). However, TNF-α–induced E-selectin was diminished in cells treated with wild-type, but not mutant, peptide (Fig. 5A). The wild-type NBD peptide also inhibited LPS-induced nitric oxide (NO) release from a macrophage cell line (*7*).

The effects of the NBD peptides in vivo were tested in two distinct experimental mouse models of acute inflammation. Ear edema induced with phorbol 12-myristate 13-acetate (PMA) (*20*, *21*) was reduced by the wild-type peptide (77 ± 3% inhibition) as effectively as dexamethasone (82 ± 9% inhibition), whereas the mutant was less effective (27 ± 9%) (Fig. 5C). Neither peptide had





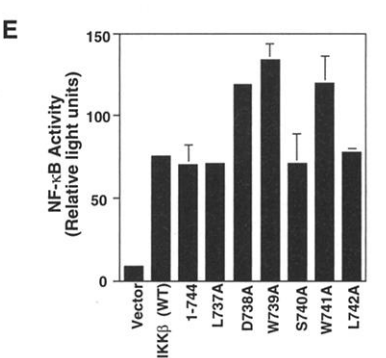


Fig. 3. The NBD consists of six COOH-terminal amino acids. Pull-down analysis was done by using GST-NEMO and either (**A**) IKKα and IKKβ in vitro translated in wheat germ extract, (**B**) wild-type IKKα and IKKα(1–737), or (**C**) a deletion mutant of IKKβ lacking the NBD (del.NBD). (**D**) COS cells were transfected for 48 hours with 1 μg/ml of pcDNA3.1-xpress (vector), vector plus NEMO-FLAG, or NEMO-FLAG plus xpress-tagged IKKβ(1–744) containing NBD point mutations (*25*). After lysis, samples were immunoprecipitated by using anti-FLAG (M2) and immunoblotted with anti-FLAG or anti-xpress. The expression level in lysates before immunoprecipitation was determined by immunoblotting with anti-xpress (lower panel). (**E**) HeLa cells were transfected for 48 hours with the indicated constructs together with pBIIX-luciferase, and NF-κB activity was measured (*8*).

any effect in the absence of PMA (*17*). In another model, peritonitis was induced in mice by intraperitoneal injection of zymosan, either alone or in combination with dexamethasone or the NBD peptides (*22*, *23*). Zymosan injection caused accumulation of inflammatory exudate fluids and migration of polymorphonuclear cells into the peritoneum that was inhibited by dexamethasone and the wild-type, but not the mutant, NBD peptide (Fig. 5D). Dexamethasone and wild-type peptide also reduced NO accumulation in the peritoneum of these animals (*7*). We therefore conclude that the wild-type NBD peptide is an effective inhibitor of inflammation in these experimental models.

In summary, we have identified the structural requirements for the association of NEMO with the IKKs and revealed that NEMO not only functions in the activation of IKKβ (*1*–*5*), but may also suppress the intrinsic, basal activity of the IKK complex. Drugs targeting the IKK-NEMO interaction may be of clinical importance for the control of inflammation, and as the NBD is only six amino acids long, it should be possible to design peptidomimetic compounds that disrupt the NEMO-IKK interaction. Such drugs would prevent activating signals from reaching the IKK complex, yet maintain a low level of NF-κB activity that may be required to avoid potential toxic side effects.

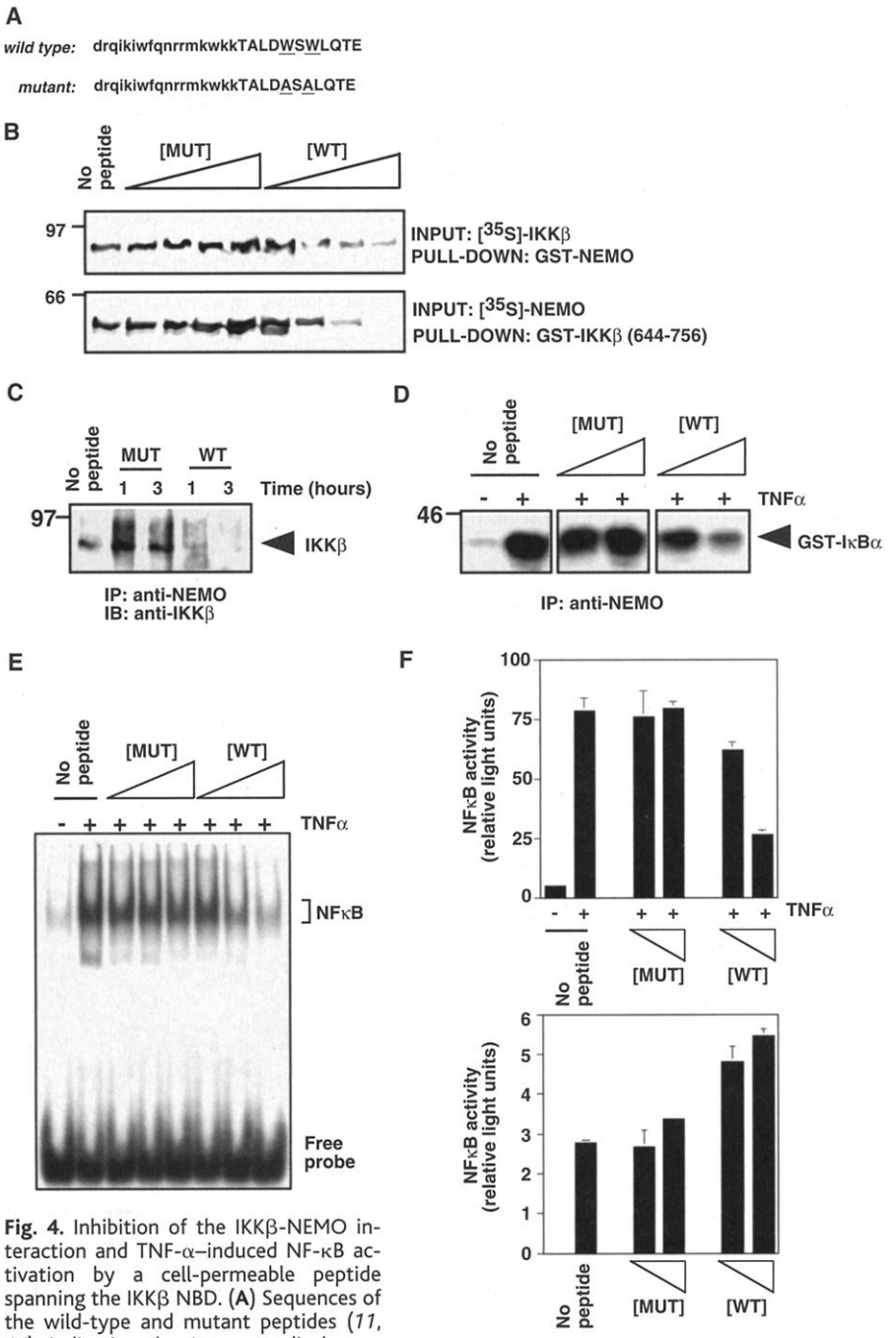


Fig. 4. Inhibition of the IKKβ-NEMO interaction and TNF-α–induced NF-κB activation by a cell-permeable peptide spanning the IKKβ NBD. (**A**) Sequences of the wild-type and mutant peptides (*11*, *14*), indicating the Antennapedia homeodomain (*15*) (lower case) and IKKβ (upper case) segments. Positions of the W→A mutations are underlined. (**B**) GST pull-down analysis was done with either GST-NEMO with in vitro translated IKKβ (upper panel) or GST-IKKβ(644–756) with in vitro translated NEMO (lower panel) in the absence (no peptide) or presence of 125, 250, 500, or 1000 μM of either mutant (MUT) or wild-type (WT) NBD peptide. (**C**) HeLa cells were incubated with either peptide (200 μM) for the times indicated and the IKK complex was immunoprecipitated by using anti-NEMO. The resulting immunoblot was probed with anti-IKKβ. (**D**) The IKK complex was immunoprecipitated by using anti-NEMO from HeLa cells treated with the peptides (100 or 200 μM) for 3 hours followed by incubation with TNF-α (10 ng/ml) (+) for 5 min and used for immune-complex kinase assay (*16*). (**E**) HeLa cells were incubated for 3 hours with 50, 100, or 200 μM of each peptide followed by treatment for 15 min with TNF-α (10 ng/ml) (+). EMSA was performed by using nuclear extracts and a specific κB-site probe as previously described (*26*). (**F**) NF-κB activity (*8*) in HeLa cells transfected with pBIIX-luciferase and incubated for 2 hours in the absence or presence of NBD peptides (100 and 200 μM) then treated for 4 hours with TNF-α (+ in upper panel) or untreated (lower panel).

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6. IKKα and IKKβ were gifts from M. Karin (University of California, San Diego). All subcloning and mutagenesis were done by polymerase chain reaction (PCR) using cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA) (*7*). Human NEMO cDNA was obtained by reverse transcriptase (RT)–PCR from HeLa cell mRNA using the Expand Long Template PCR System (Boehringer Mannheim, Mannheim, Germany) (*7*). For in vitro pull-down assays (*24*), proteins were expressed and [35S]methionine-labeled by in vitro transcription and translation using rabbit reticulocyte lysates or wheat germ extracts (Promega, Madison, WI).
7. Supplementary material is available to *Science* Online subscribers www.sciencemag.org/feature/data/1052581.shl.
8. HeLa and COS cells were seeded into either 24-well (10-mm, 1×10^5 cells/well) or 6-well (35-mm, 5×10^5 cells/well) plates and grown for 24 hours before transfection of DNA (1 μg/ml) by using Fugene6 (Roche, Basel, Switzerland). After 48 hours, cells were lysed with TNT (200 mM NaCl, 20 mM Tris, pH 8.0, 1% Triton X-100), and samples were used for immunoprecipitation (*25*) or luciferase assay (Promega luciferase assay system).
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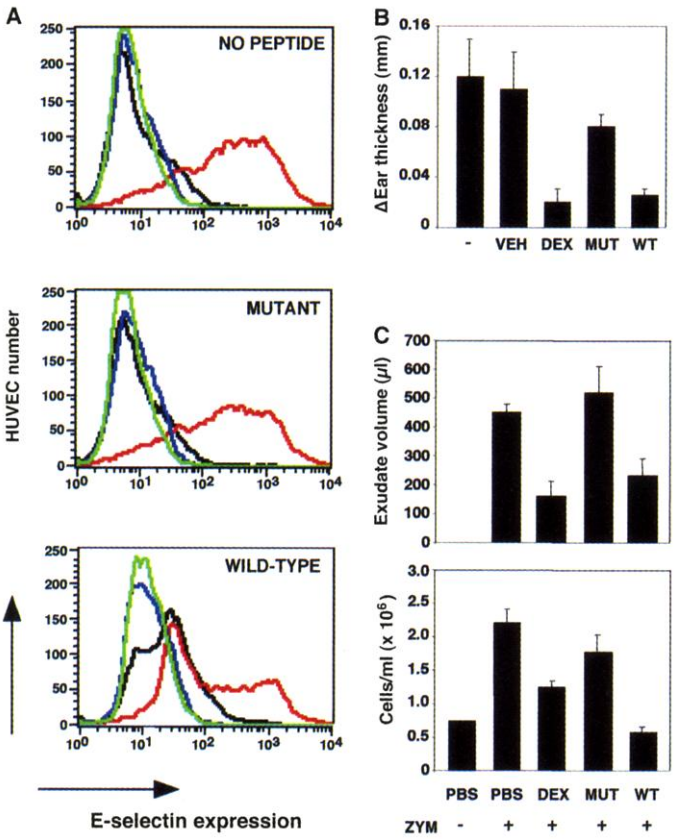

Fig. 5. The wild-type NBD peptide inhibits NF-κB–induced gene expression and experimentally induced inflammation. (**A**) Human umbilical vein endothelial cells were incubated for 2 hours with mutant (middle) or wild-type (bottom) NBD peptides (100 μM) then stimulated with TNF-α (10 ng/ml) for 6 hours. Control cells (top) received no peptide. Cells were stained with either anti–E-selectin (H4/18) or a nonbinding, isotype-matched control antibody (K16/16) and expression was measured by FACS [FACSort, Becton Dickinson, Paramus, NJ (*27*)]. The profiles show E-selectin staining in the absence (black) and presence (red) of TNF-α and control antibody staining under the same conditions (blue, no TNF-α; green, +TNF-α). (**B**) PMA-induced ear edema in mice topically treated with vehicle (VEH), dexamethasone (DEX) or NBD peptides was measured as described (*20*, *21*). (**C**) The effects of the NBD peptides and dexamethasone (DEX) on Zymosan (ZYM)-induced peritonitis in mice were determined as described (*22*). Control mice were injected with phosphate-buffered saline (PBS).

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14. The sequences of the NBD peptides are shown in Fig. 5A (*11*). Both peptides were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 20 mM. Unless shown, DMSO controls were no different from no peptide controls.
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16. Precipitated immune complexes were washed with TNT and kinase buffer (20 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 1 mM EDTA, 2 mM NaF, 2 mM β-glycerophosphate, 1 mM dithiothrietol, 10 μM ATP) then incubated for 15 min at 30°C in 20 μl of kinase buffer containing GST-IκBα(1–90) and 10 μCi [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Uppsala, Sweden). The phosphorylated substrate was precipitated by using glutathione-agarose (Amersham Pharmacia Biotech), separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and visualized by autoradiography.
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20. Ear edema was induced in replicate groups of mice (C57BL/6NCR) as described previously (*21*). Briefly, 20 μl of either NBD peptide (200 μg/ear), dexamethasone (40 μg/ear) or vehicle (DMSO:ethanol, 25:75 v/v) was applied to the right ear of mice 30 min before and 15 min after the application of 20 μl of PMA (5 μg/ear) dissolved in ethanol. Swelling was measured 6 hours after PMA application by using a microgauge (Mitutoyo America, Aurora, IL) and expressed as the mean difference in thickness between the treated and untreated ears.
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25. For immunoprecipitations, HeLa or COS cells grown in 6-well (35 mm) trays were lysed in 500 μl TNT. Transfected FLAG-tagged proteins were precipitated for 2 hours at 4°C by using 20 μl of anti-FLAG (M2)-conjugated agarose beads (Sigma). Endogenous IKKβ or NEMO were immunoprecipitated by using 1 μg of specific polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) plus 20 μl of Protein-A Sepharose (Amersham Pharmacia Biotech). For immunoblotting, precipitates were washed with TNT twice, and PBS then suspended in SDS–sample buffer. Proteins were separated by SDS-PAGE (10%), transferred to polyvinylidene diflouride membranes, and visualized by enhanced chemiluminesence (Amersham Pharmacia Biotech).
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Predictions of Biodiversity Response to Genetically Modified Herbicide-Tolerant Crops

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We simulated the effects of the introduction of genetically modified herbicide-tolerant (GMHT) crops on weed populations and the consequences for seed-eating birds. We predict that weed populations might be reduced to low levels or practically eradicated, depending on the exact form of management. Consequent effects on the local use of fields by birds might be severe, because such reductions represent a major loss of food resources. The regional impacts of GMHT crops are shown to depend on whether the adoption of GMHT crops by farmers covaries with current weed levels.

There is a growing research interest in the potential effects of the release of genetically modified (GM) crops (*1*) on biodiversity. This is prompted by concerns relating to the direct impact of GM crops on target organisms and the indirect effects on the wider environment. The environmental debate has to be set within a biodiversity landscape that is already affected by the intensification of agriculture (*2*). Although, in some senses, the introduction of GM crops may be no different than the introduction of any other technology that leads to the further intensification of agriculture, this new technology might offer a