Induction of NF-κB by the Akt/PKB kinase

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The serine/threonine kinase Akt (also known as protein kinase B, PKB) is activated by numerous growth-factor and immune receptors through lipid products of phosphatidylinositol (PI) 3-kinase. Akt can couple to pathways that regulate glucose metabolism or cell survival [1]. Akt can also regulate several transcription factors, including E2F, CREB, and the Forkhead family member Daf-16 [2–4]. Here, we show that Akt can regulate signaling pathways that lead to induction of the NF-κB family of transcription factors in the Jurkat T-cell line. This induction occurs, at least in part, at the level of degradation of the NF-κB inhibitor IκB, and is specific for NF-κB, as other inducible transcription factors are not affected by Akt overexpression. Furthermore, the effect requires the kinase activity and pleckstrin homology (PH) domain of Akt. Also, Akt does not act alone to induce cytokine promoters and NF-κB reporters, because signals from other pathways are required to observe the effect. These studies uncover a previously unappreciated connection between Akt and NF-κB induction that could have implications for the control of T-cell growth and survival.

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Results and discussion

The promoter of the interleukin-2 (IL-2) gene has provided a model system with which to study signals downstream of the T-cell receptor (TCR) and the CD28 co-stimulatory receptor [5–7]. To investigate the potential role of Akt in T-cell activation, Jurkat T cells were co-transfected with an expression vector encoding Akt and a reporter plasmid containing the luciferase gene driven by the IL-2 promoter. Overexpression of Akt in Jurkat cells led to a 7–10-fold increase in IL-2 promoter activity over that obtained by stimulation with phorbol myristate acetate (PMA) and ionomycin alone (Figure 1a). Intriguingly, the effect is similar to that seen when CD28 (but not the TCR) is crosslinked in conjunction with the sub-maximal stimulus of PMA and ionomycin (data not shown;

[6]). Overexpression of Akt had no effect on the basal level of IL-2 promoter activity (data not shown).

The IL-2 promoter contains a number of binding sites for transcription factors, including sites for NFAT, AP-1, NF-κB, and a CD28-responsive element/AP-1 composite site (termed RE/AP) [6,8]. Reporter plasmids containing individual binding sites from the IL-2 promoter were assayed using soluble anti-TCR antibody and PMA as stimuli, as they provide an efficient but sub-maximal stimulus. Little if any effect of Akt overexpression was seen on luciferase reporter plasmids driven by AP-1- or NFAT-binding sites (Figure 1a), but when a reporter driven by the NF-κB consensus site from the human immunodeficiency virus long terminal repeat (HIV LTR) or the RE/AP-binding site from the IL-2 promoter was examined, a dramatic potentiation was noted upon Akt overexpression. As with the IL-2 promoter, Akt overexpression did not affect the basal level of transcription from any of these individual elements (data not shown). Akt was expressed at comparable levels in all transfections (Figure 1a, inset). The selective enhancement by Akt of NF-κB and RE/AP reporters suggested that Akt was specifically influencing NF-κB induction. The consequences of Akt overexpression for other NF-κB-dependent reporter plasmids were investigated. Only certain responses of reporters driven by NF-κB elements from the major histocompatibility complex (MHC) class I promoter (Figure 1b), the RE/AP site (see above) or the IL-2 receptor α chain promoter (data not shown) were strongly augmented by Akt. The effect of Akt was most striking in conjunction with a low concentration of PMA.

The NF-κB reporter assays were repeated with various mutants of Akt. An inactivating mutation of the kinase domain or deletion of the PH domain prevented Akt from potentiating NF-κB-dependent transcription (Figure 2a). A myristylated version of Akt, which is targeted to the plasma membrane and is constitutively active, was also capable of increasing transcription from NF-κB-binding sites. Thus, Akt augmentation of NF-κB activity requires Akt kinase activity and the Akt PH domain.

As Akt is activated in part by products of PI 3-kinase, we determined whether the effect of Akt on NF-κB induction was dependent upon cellular PI 3-kinase activity, using the PI 3-kinase inhibitors LY294002 and wortmannin [9,10]. Both LY294002 (Figure 2b) and wortmannin (data not shown) could effectively inhibit the potentiation of RE/AP reporter (or NF-κB reporter; data not shown) activity by wild-type Akt in the presence of PMA, demonstrating that endogenous PI 3-kinase activity is required.

Figure 1

Effect of Akt overexpression on inducible
transcription in T cells. Jurkat cells were
transfected by electroporation and stimulated
18 h later for 6 h (see Supplementary material
published with this paper on the internet).
Reporter plasmid (20 μg) was co-transfected
with 10 μg empty vector (Vector) or vector
containing Akt. PMA was used at 10 ng/ml,
ionomycin (iono) at 1 μM, and soluble anti-
TCR antibodies (C305 culture supernatant) at
a 1:1,000 dilution. Data shown are the average
of three experiments ± standard deviation
(SD). **(a)** Reporter plasmids containing
luciferase driven by the individual transcription
factor binding sites (NFAT, AP-1, NF-κB and
RE/AP) or the full-length IL-2 promoter (IL-2)
were assayed. The inset in (a) shows samples
of a representative transfection (10^6 cell
equivalents) analyzed for Akt expression
(upper band) by western blotting. **(b)** Reporter
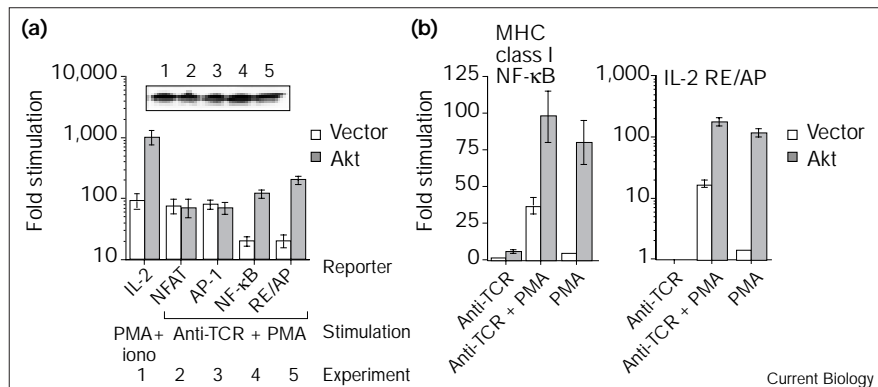
plasmids containing luciferase driven by the
NF-κB-binding site from the MHC class I
promoter or by the IL-2 RE/AP site, as
indicated, were assayed. The fold stimulation

refers to the fold stimulation over that obtained
in the absence of any stimulation.

The inhibitors did not block induction of reporter activity
by PMA and myristylated Akt (Figure 2b and data not
shown), however, consistent with reports that membrane-
targeted Akt is partially PI 3-kinase independent
[1,11,12]. An activated, membrane-targeted form of PI
3-kinase, p110–CAAX [13], potentiated the induction of
NF-κB activity by Akt and PMA (Figure 2c), but did not
affect RE/AP or NF-κB activity in the absence of Akt
expression, with or without PMA (Figure 2c and data not
shown). Thus, although PI 3-kinase activity is required for
NF-κB induction by Akt, PI 3-kinase overexpression
alone is not capable of mediating such an effect. However,
other potential targets of p110 include proteins such as
Itk, Vav, and phospholipase Cγ [14–16].

We examined whether stimulation of Jurkat cells with PMA
and Akt resulted in increased nuclear levels of NF-κB pro-
teins that could bind to NF-κB-binding sites. These experi-
ments were conducted with Jurkat cell lines stably
expressing myristylated Akt, which mediates stronger
NF-κB-mediated transcriptional activation than wild-type
Akt in stable cell lines (data not shown). Low levels of
nuclear NF-κB-binding factors were seen when a control
clone was stimulated with PMA alone, with significantly
higher levels when anti-CD28 antibody was also added as a
positive control. By contrast, clones expressing myristylated
Akt yielded significantly higher nuclear levels of NF-κB-
binding complexes. These results suggest that the strong
transcriptional upregulation by Akt is due, at least in part, to

Figure 2
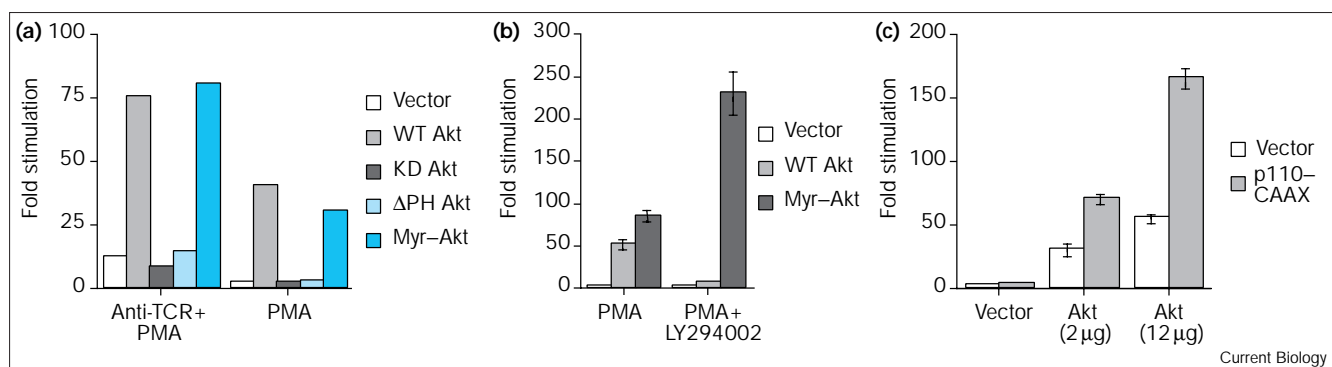
The kinase and PH domains of Akt are required to augment NF-κB
induction in conjunction with PI 3-kinase activity. Jurkat cells were
transfected and stimulated as described in Figure 1. **(a)** Cells were
transfected with 20 μg reporter driven by the NF-κB-binding site from
the HIV LTR and 10 μg of the indicated Akt expression plasmids: WT,
wild type; KD, kinase-deficient; ΔPH, PH domain deleted; Myr,

myristylated. Vector represents the empty vector control. **(b,c)** Cells
were transfected with 20 μg RE/AP-driven reporter and 10 μg (except
where otherwise noted) of the indicated Akt or p110 expression
plasmids. PMA was added at 10 ng/ml in all cases and LY294002
was added at 20 μM. Data presented are representative of three to five
independent experiments.

Figure 3
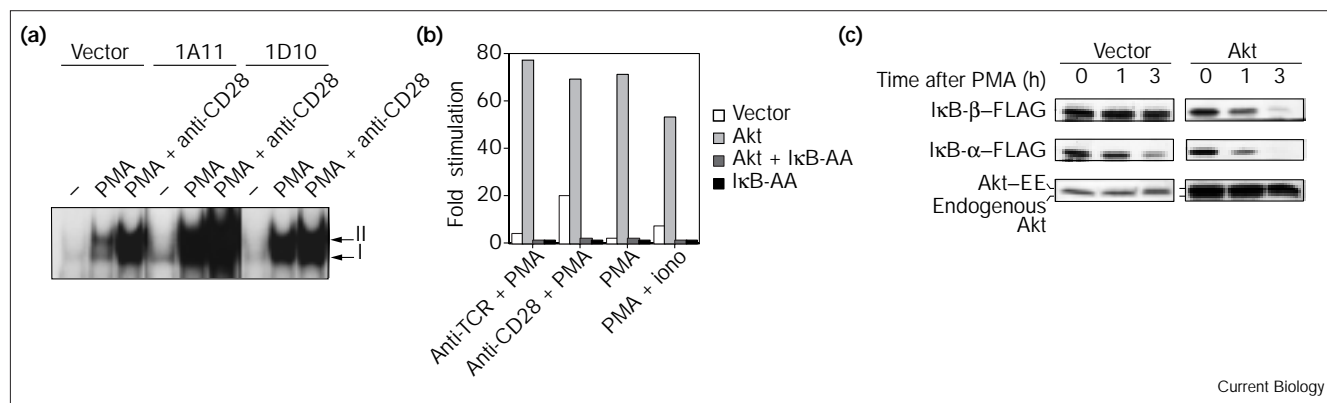
Induction of NF-κB DNA-binding activity and IκB degradation by Akt. (a) Jurkat cells expressing myristylated Akt (1A11 and 1D10) or cells carrying a control, empty vector were treated with the indicated stimuli for 4 h. Nuclear extracts were prepared and gel shift assays carried out (see Supplementary material). Complex I contains p50–p50 homodimers and complex II contains p50–p65 and p50–c-Rel heterodimers. (b) Jurkat cells were transfected with 20 μg HIV NF-κB-driven reporter and the indicated plasmids carrying Akt or IκB-AA (10 μg or 2 μg, respectively). Cells were stimulated as described in

Figure 1. Anti-CD28 ascites was used at 1:10,000 dilution. (c) Jurkat cells were transfected with 10 μg each of FLAG-tagged IκB-α and IκB-β and 10 μg empty pCDEF3 vector (vector) or 10 μg pCDEF3 carrying EE-tagged wild-type Akt (Akt). Cells were pretreated with 50 μg/ml cycloheximide for 15 min, then stimulated with 10 ng/ml PMA for the indicated times. Whole-cell lysates were analyzed by western blotting for expression of IκB-α, IκB-β, and Akt. Results are representative of three independent experiments.

increased NF-κB levels in the nucleus, which would be expected to result from degradation of IκB proteins.

The requirement for IκB degradation in NF-κB activation by Akt was first addressed with a mutant form of IκB-α (IκB-AA) that contains serine-to-alanine mutations of the sites required for phosphorylation and degradation. IκB-AA therefore cannot be phosphorylated and degraded and acts as a potent inhibitor of NF-κB activation [17]. IκB-AA potently inhibited the activation of a NF-κB-driven reporter by various stimuli, including PMA and Akt (Figure 3b). IκB-AA also inhibited activation of the RE/AP-driven reporter, but not of an NFAT-driven reporter (data not shown). Similar results were seen with MG-132, a proteasome inhibitor which prevents IκB degradation [17] (data not shown). These experiments implicate the involvement of IκB in Akt-mediated NF-κB induction. To examine this more directly, tagged forms of IκB-α and IκB-β were expressed in Jurkat cells, with or without Akt overexpression. The degradation of IκB was then followed, after treatment with PMA and cycloheximide, which was added to prevent NF-κB-mediated IκB re-expression. PMA treatment alone was sufficient to effect moderate to complete degradation of IκB-α, but this occurred more efficiently in cells overexpressing Akt (Figure 3c). The effect on IκB-β was even more dramatic, in that PMA did not induce significant degradation of IκB-β unless cells overexpressed Akt.
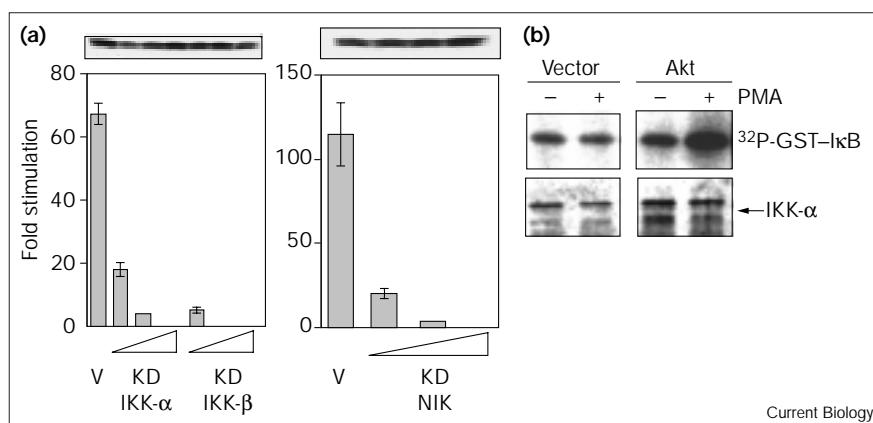
We tested whether known IκB kinases [18] were required for Akt-mediated NF-κB activation. The IκB kinases

IKK-α and IKK-β directly phosphorylate IκB-α and IκB-β on the two serine residues that are critical for IκB ubiquitination and degradation. Kinase-deficient IKK-α or IKK-β was co-transfected with Akt. These kinase-deficient forms of IKK-α or IKK-β inhibited the activation of an NF-κB-driven reporter by PMA and Akt, but did not affect Akt expression (Figure 4a) or transfection efficiency (data not shown). NF-κB-inducing kinase (NIK) has also been implicated in NF-κB activation (reviewed in [18]). A kinase-deficient form of NIK [19] was transfected into Jurkat cells with Akt and specific inhibition of NF-κB reporter activity was also observed in these cells after stimulation with PMA (Figure 4a), without a significant effect on expression of Akt or transfection efficiency (data not shown). Finally, the effect of Akt on IKK activation was assessed directly. Empty vector or an Akt expression vector was co-transfected with a vector encoding epitope-tagged IKK-α. As shown in Figure 4b, PMA alone (at 10 ng/ml) was unable to activate IKK-α in cells transfected with empty vector, but could activate IKK-α when Akt was overexpressed.

The results presented here are consistent with a model whereby Akt and other signals act upstream of the IKKs in effecting NF-κB induction. Akt may contribute to NF-κB induction by a subset of receptors in T cells, such as the TCR, CD28, or the IL-1 receptor [20]. We are currently investigating whether these or other receptors require Akt activation in order to mediate inducible transcription. We are also interested in identifying the direct target(s) for Akt in the NF-κB pathway. The IKKs, IκBs and NIK do

Figure 4

Activation of NF-κB by Akt requires the IκB kinases and NIK. **(a)** Jurkat cells were transfected with 20 μg HIV NF-κB-driven reporter and empty pCDNA3 vector (V) or increasing amounts of the indicated kinase-deficient (KD) kinases, along with 10 μg wild-type Akt. Kinase-deficient IKKs were transfected at 2, 8 and 20 μg, whereas kinase-deficient NIK was transfected at 1, 5 and 10 μg. Cells were stimulated with 10 ng/ml PMA. The insets show representative examples of levels of Akt expression for each of the experiments, as analyzed by western blotting. **(b)** Jurkat cells were co-transfected with FLAG-tagged IKK-α and either empty vector or Akt. The next day, cells were either stimulated with PMA alone (10 ng/ml) or left untreated, and IKK activity (upper panels) was determined (see Supplementary material). The protein levels of transfected IKK-α are also shown (lower panels). Results are representative of three independent experiments.

not contain a consensus sequence for phosphorylation by Akt, as defined by Alessi *et al.* [21]. Akt may therefore lead to IKK activation through a MAPKKK family member other than NIK or, alternatively, Akt may target a previously unknown pathway for IKK activation.

Supplementary material
Additional methodological detail is published with this paper on the internet.

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Supplementary material

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Supplementary materials and methods

Plasmids
The IL-2 promoter reporter contains 275 bp upstream of the IL-2 transcriptional start site [S1]. NF-κB reporters were from the following sources: HIV, from Jorge Moscat and MHC class I from Albert Baldwin. The IL-2 receptor α NF-κB and IL-2 RE/AP reporters were described previously [S2,S3]. NFAT and AP-1 luciferase reporters have been described previously [S2]. The pCMV4 vectors encoding FLAG-tagged IκB-α and IκB-β were from Dean Ballard; kinase-deficient NIK, and IKKs were from Warner Greene; p110–CAAX was from Julian Downward; FLAG–IKK-α was from David Goeddel.

Antibodies and biochemicals
Polyclonal antibodies directed against Akt (C-20) and IκB-β (C-20) were obtained from Santa Cruz Biotechnology. Antiserum specific for IκB-α was from Joseph Didonato. Murine IgM (clone C305) specific for the Jurkat TCR was produced in a Miniperm apparatus and used at a dilution of 1:1,000. Anti-human CD28 ascites (clone 9.3) was obtained from Bristol-Myers Squibb, and used at a 1:2,000 dilution. Anti-FLAG antibody M2 was obtained from Sigma Chemical Co. PMA, cyclohex-imide, LY294002, and wortmannin were obtained from Calbiochem.

Transfections and luciferase assays
Jurkat cells were transfected by electroporation. Cells grown to a density of $0.5–1.0 \times 10^6$ per ml were washed into room-temperature, serum-free RPMI at a final density of 3×10^7/ml. Cells (0.4 ml) were added to a 0.4 cm gap cuvette (Invitrogen) containing plasmid DNA, as indicated. Electroporation was carried out at 250 V, 960 μF. Cells were immediately transferred to a small tissue culture plate with 10 ml RPMI containing 5% FCS and cultured for 16–18 h at 37°C, 5% CO_2. For generation of stable cell lines, cells were plated at various dilutions in 96-well plates, in complete medium containing 2 mg/ml G418. Several weeks later, clones were transferred and expanded. Expression of tagged Akt was confirmed with a polyclonal antiserum specific for Akt.

For luciferase assays, the cells were washed and resuspended at 2×10^6 per ml in RPMI containing 5% FCS. Cells were aliquotted into round-bottom 96-well plates (50 μl) with RPMI+FCS containing 2× concentration of the indicated stimuli (50 μl). After 6 h at 37°C, 5% CO_2, cells were permeabilized with 11 ml luciferase harvest buffer (10% Triton X-100, 1 mM DTT, 0.2 M KPO_4 pH 7.8). After 5 min at room temperature, samples were transferred to an opaque 96-well plate, and 100 μl of luciferase assay buffer was added (10 mM ATP, 20 mM $MgCl_2$, 0.2 M KPO_4 pH 7.8). Luciferase activity was determined in a MicroLumat luminometer (EG&G Berthold), with a 10 sec reading, after injection of 50 μl 1 mM luciferin.

Gel shift assays
Before preparation of nuclear extracts, Jurkat cells were treated for 4 h in RPMI+FCS, as indicated. Cells (10^7) were pelleted and washed twice with PBS. The final pellet was resuspended in 0.8 ml buffer A (10 mM HEPES-KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and left on ice for 15 min. NP-40 was added to 0.5% and samples vortexed for 20 sec, then spun for 30 sec at maximum speed. The supernatant was discarded and the pellet washed with 0.5 ml buffer A. The pellet was solubilized with 0.1 ml buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) by gently agitating for 20 min. Samples were spun for 10 min at maximum speed in a microfuge and the supernatants saved and quantitated for protein levels.

Nuclear extracts (6 μg) were mixed with buffer, ^{32}P-labeled NF-κB probe [S3], and poly(dl-dC) in a final volume of 20 μl. Reactions were incubated for 20 min at room temperature. Half of each reaction was loaded on a 4% acrylamide–TBE gel. Gels were dried and exposed to X-OMAT film for 8–24 h.

SDS–PAGE and western blotting
Whole-cell lysates were prepared by lysing 10^6 cells in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris pH 7.5 + protease inhibitors) at 4×10^7 cells/ml. After incubation on ice for 10 min, lysates were spun for 10 min at maximum speed in a microcentrifuge. Post-nuclear supernatants were mixed with 4× reducing sample buffer and boiled for 5 min. Samples were separated on a 10% polyacrylamide gel, which was then transferred to PVDF membrane (Millipore) using a semi-dry blotting apparatus. Blots were blocked with 5% non-fat milk in wash buffer (250 mM NaCl, 20 mM Tris pH 7.5, 0.05% Tween-20) for 2 h at room temperature. Primary antibody was incubated for 1 h at room temperature in wash buffer, followed by three 10 min washes. Horseradish-peroxidase-conjugated secondary antibody was diluted to 1:10,000 in wash buffer and incubated with blots for 1 h at room temperature, followed by three 15 min washes, and development by enhanced chemi-luminescence (Amersham).

IKK kinase assays
Jurkat T cells were transfected, as above, with FLAG-tagged IKK and indicated additional plasmids. Cells were harvested 20 h later and washed with PBS. A total of 10^7 cells per condition were resuspended in RPMI and stimulated for 20 min at 37°C. Cells were washed once in ice-cold PBS and lysed in 800 μl lysis buffer (1% NP-40, 250 mM NaCl, 1 mM EDTA, 50 mM Hepes pH 7.4) + protease inhibitors. IKK was immunoprecipitated from post-nuclear supernatants with M2 antibody coupled to protein-G beads, with rotation for 2 h. Beads were washed three times with lysis buffer and once with kinase buffer (1 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM HEPES pH 7.4) + phosphatase inhibitors. Reactions were carried out for 30 min at 30°C in 20 μl kinase buffer with 1 μg GST–IκB and 5 μCi [γ-^{32}P]ATP. Reactions were stopped by the addition of SDS sample buffer. Samples were boiled for 5 min, separated by SDS–PAGE and blotted to PVDF. Blots were exposed to film overnight to determine kinase activity, then blocked and probed for IKK levels with a polyclonal IKK antibody.

Supplementary references
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