IKKβ \{IkB [inhibitor of NF-\(\kappa\)B (nuclear factor \(\kappa\)B)] kinase \(\beta\)\} is required to activate the transcription factor NF-\(\kappa\)B, but how IKKβ itself is activated \textit{in vivo} is still unclear. It was found to require phosphorylation by one or more ‘upstream’ protein kinases in some reports, but by autophosphorylation in others. In the present study, we resolve this controversy by demonstrating that the activation of IKKβ induced by IL-1 (interleukin-1) or TNF (tumour necrosis factor) in embryonic fibroblasts, or by ligands that activate Toll-like receptors in macrophages, requires two distinct phosphorylation events: first, the TAK1 [TGF\(\beta\) (transforming growth factor \(\beta\))-activated kinase-1]-catalysed phosphorylation of Ser\(^{177}\) and, secondly, the IKK\(\beta\)-catalysed autophosphorylation of Ser\(^{181}\). The phosphorylation of Ser\(^{177}\) by TAK1 is a priming event required for the subsequent autophosphorylation of Ser\(^{181}\), which enables IKKβ to phosphorylate exogenous substrates. We also provide genetic evidence which indicates that the IL-1-stimulated, LUBAC (linear ubiquitin chain assembly complex)-catalysed formation of linear ubiquitin chains and their interaction with the NEMO (NF-\(\kappa\)B essential modulator) component of the canonical IKK complex permits the TAK1-catalysed priming phosphorylation of IKKβ at Ser\(^{177}\) and IKK\(\alpha\) at Ser\(^{176}\). These findings may be of general significance for the activation of other protein kinases.

**INTRODUCTION**

The canonical IKK \{IkB [inhibitor of NF-\(\kappa\)B (nuclear factor \(\kappa\)B)] kinase \(\beta\)\} complex, consisting of the protein kinases IKK\(\alpha\) and IKK\(\beta\) (also called IKK1 and IKK2) and a regulatory component called NEMO (NF-\(\kappa\)B essential modifier) \[1,2\], is one of the most studied of all protein kinases. It has been featured in over 10000 papers since its discovery in 1998 due to its essential role in activating NF-\(\kappa\)B, a ‘master’ transcription factor that regulates many physiological processes, including innate immunity and the cellular response to DNA damage \[3–5\]. Nevertheless, despite the vast number of publications that have focused on this protein kinase, its mechanism of activation is still controversial.

The activation of IKK\(\alpha\) and IKK\(\beta\) requires phosphorylation of the ‘activation loops’ of these protein kinases at Ser\(^{176}\) and Ser\(^{180}\) (IKK\(\alpha\)) or Ser\(^{177}\) and Ser\(^{181}\) (IKK\(\beta\)) \[4\]. The IKKs respond to many physiological stimuli, but are activated most powerfully by inflammatory stimuli, such as TLR (Toll-like receptor) agonists and the pro-inflammatory cytokines IL-1 (interleukin-1) and TNF (tumour necrosis factor). Genetic evidence indicates that the expression and activity of the TAK1 [TGF\(\beta\) (transforming growth factor \(\beta\))-activated kinase-1; also called MAP3K7 (mitogen-activated protein kinase kinase kinase 7)] is needed for the activation of the canonical IKK complex by IL-1 and TNF in MEFs (mouse embryonic fibroblasts). These agonists fail to activate the IKKs in MEFs that do not express the TAK1 catalytic subunit \[6\] or that express a truncated inactive form of TAK1 \[7\].

IL-1 and TNF trigger TAK1 activation within minutes, a speed compatible with a role in initiating the activation of the IKKs \[8\]. TAK1 is also reported to phosphorylate and activate the canonical IKKs \textit{in vitro} \[9\], activation being prevented by pharmacological inhibitors of TAK1 \[8,10,11\]. Similar lines of evidence indicate an essential role for TAK1 in activating the MKKs \[MAPK (mitogen-activated protein kinase) kinases\] that switch on the MAPK family members JNK1 (c-Jun N-terminal kinase 1) and JNK2 and p38 MAPKs in MEFs \[8–11\].

On the other hand, the canonical IKKs have been shown to be capable of phosphorylating and activating themselves \textit{in vitro} (reviewed in \[4\]). For example, Met\(^\gamma\)-linked (also called linear) ubiquitin oligomers \[12\] and other types of ubiquitin oligomers \[13\] have been reported to induce the activation of the canonical IKK complex \textit{in vitro}, apparently in the absence of any ‘upstream’ activating protein kinase. These observations raise the alternative possibility that the role of TAK1 \textit{in vivo} might be to stimulate the formation of these polyubiquitin chains, rather than to phosphorylate the canonical IKK complex directly. In addition, X-ray crystallographic analysis has revealed that human IKK\(\beta\) can adopt an open conformation that enables it to form oligomers, whereas mutagenesis studies have established that two of the surfaces that mediate oligomer formation are critical for the activation of IKK\(\beta\) in cells \[14\]. It has therefore been proposed that IKK\(\beta\) dimers transiently associate with one another through these interaction surfaces to promote \textit{trans} autophosphorylation as part of their activation mechanism. Consistent with an essential
role for autophosphorylation, we found that in IKKα-deficient MEFs the specific IKKβ inhibitor BI605906 prevented the IL-1- or TNF-stimulated conversion of IKKβ into the active di-phosphorylated species, i.e. phosphorylated at both Ser177 and Ser181 [8].

In the present study we report the unexpected finding that Tak1 and IKKβ phosphorylate different serine residues in the activation loop of IKKβ and demonstrate that the Tak1-catalysed phosphorylation of IKKβ at Ser177 is a priming event that enables IKKβ to activate itself by phosphorylating Ser181. We also provide genetic evidence showing that the formation of Met1-linked ubiquitin chains and their interaction with NEMO is needed for the Tak1-catalysed phosphorylation of Ser177 (IKKα) and Ser181 (IKKβ), and that Tak1 activity is not required for the formation of either Lys63-linked or Met1-linked ubiquitin chains.

**EXPERIMENTAL**

**Materials**

Murine IL-1α and TNF were purchased from Peprotech and mouse M-CSF (macrophage colony-stimulating factor) from R&D Systems. Pam3CSK4 was from Invivogen and LPS (lipopolysaccharide) from Sigma-Aldrich. The catalytic subunit of human PP1 γ purified by chromatography on nickel-nitrilotriacetate agarose. The monophosphorylated peptide KELDQGpSLCTSFVGTLQ (where pS is phosphoserine), corresponding to amino acids 171–187 of IKKβ with phosphoserine at Ser177 only or at both Ser177 and Ser181 respectively, were synthesized by Peptech. The IKKβ inhibitor BI605906 [8] was provided by Dr Natalia Shpiro (University of Dundee, Dundee, U.K.) and the TAK1 inhibitor NG25 by Dr Nathanael Gray (Harvard Medical School, Boston, MA, U.S.A.) [11], whereas the Tak1 inhibitor SZ-7-oxozaeanol was purchased from BioAustralis Fine Chemicals.

**Protein expression and purification**

The IKKβ (IKKβ[D166Å]) was expressed as a GST fusion protein in HEK (human embryonic kidney)-293T suspension cells and, after cell lysis, was purified from the cell extracts by chromatography on glutathione–Sepharose. The GST fusion protein was released from the glutathione–Sepharose by cleavage of the GST tag with PreScission protease. A catalytically active Tak1–TAB1 (TAK1-binding protein 1)-fusion protein [15] was expressed in insect Sf21 cells as a His-tagged protein and purified by chromatography on nickel-nitrilotriacetate agarose. The catalytic subunit of human PPIγ (protein phosphatase 1γ) was expressed in Escherichia coli as a GST-fusion protein, purified on glutathione–Sepharose and stored in a solution of 50 mM Tris/HCl, 0.15 M NaCl, 0.27 M sucrose, 0.03 % Brij35, 0.1 % 2-mercaptoethanol and 2 mM MnCl2.

**Antibodies**

An antibody recognizing the HOIP [HOIL1 (haem-oxidized IRP2 (iron regulatory protein 2) ubiquitin ligase 1)-interacting protein] component of LUBAC (linear ubiquitin chain assembly complex) was raised in sheep and purified as described in [16]. Antibodies recognizing IKKβ phosphorylated at Ser177 (catalogue number 2078S) or at both Ser177 and Ser181 (catalogue number 2697L) were obtained from Cell Signaling Technology, whereas the antibody recognizing IKKβ phosphorylated at Ser471 was from Abcam (catalogue number AB55341). Antibodies that recognize p105/NF-κB phosphorylated at Ser323 (catalogue number 4806S), GADPH (glyceraldehyde-3-phosphate dehydrogenase; catalogue number 2118S) and all forms of p38α MAPK (catalogue number 9212S) and JNK (catalogue number 9258S) were from Cell Signaling Technology. Antibodies recognizing NEMO (catalogue number 8C330; Santa Cruz Biotechnology), all forms of IKKβ (catalogue number DAM1774677; Millipore) and the HA (haemaglutinin) tag (catalogue number 12-013-819-001; Roche) were from the sources indicated. An antibody raised in sheep against the full-length human IKKβ catalytic subunit (S189C, bleed 1) was produced and affinity purified by the Antibody Production Team of the MRC Protein Phosphorylation and Ubiquitylation Unit at Dundee (co-ordinated by Dr James Hastie). An antibody recognizing Met1-linked ubiquitin chains was generously provided by Vishva Dixit, Genentech, U.S.A. and the antibody recognizing Lys63-linked ubiquitin chains was purchased from Merck-Millipore (catalogue number 05-1313).

**DNA constructs**

DNA encoding IKKβ (NCBI BAI45894.1) was amplified from total thymus RNA using the One Step RT PCR kit (Life Technologies). It was then cloned into pCR2.1 (Life Technologies), sequenced and sub-cloned into the NotI site of pTretox tight HA. Mutations were created following the QuikChange Site-Directed Mutagenesis method, but using KOD Hot Start DNA Polymerase (EMD Millipore).

**Cell culture, stimulation and immunoblotting**

MEFs and HEK-293 cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 2 mM glutamine, 10 % (v/v) FBS, and the antibiotics streptomycin (0.1 mg/ml) and penicillin (100 units/ml). DNA constructs were transfected into HEK-293 cells using polyethylenimine (Polysciences). BMDMs (bone-marrow-derived macrophages) were obtained by culturing bone marrow from the tibia and femurs of mice in the presence of mouse M-CSF and replating for 24 h before stimulation. Kinase inhibitors (10 mM) dissolved in DMSO, or an equivalent volume of DMSO for the control incubations, were added to the culture medium of cells grown as monolayers. After 1 h at 37°C, MEFs were stimulated with IL-1α or TNF and BMDM with LPS or Pam3CSK4 (see the Figure legends). Thereafter, cells were rinsed in ice-cold PBS and extracted in lysis buffer [50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium 2-mercaptoethanol, 1 mM DTT, 1 mM sodium orthovanadate, 0.27 M sucrose, 1 % (v/v) Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF]. Cell extracts were clarified by centrifugation (21 000 g for 10 min at 4°C) and protein concentrations determined by the Bradford assay. Cell extract protein (20 μg) was separated by SDS/PAGE (8 % gel), transferred on to PVDF membranes and proteins detected by immunoblotting and visualized by treating the blots with enhanced chemiluminescence (Amersham).

**Generation of MEFs from knockin mice**

Mice in which wild-type NEMO was replaced by the polyubiquitin-binding-defective mutant NEMO[D311N] were generated by Taconic-Artemis using conventional technology and their characterization will be reported elsewhere. Primary MEFs from NEMO[D311N] mice and wild-type littermates were generated at E11.5 (embryonic day 11.5), whereas MEFs from knockin mice expressing the inactive C879S mutant of HOIP were generated at E10.5 [16]. Immortalized IKKα-deficient MEFs and wild-type control MEFs were provided by Dr Inder Verma (Salk Institute, La Jolla, CA, U.S.A.). All animals were maintained in specific pathogen-free conditions consistent with EU and U.K.
regulations. All work was performed under a U.K. Home Office project license that was awarded after recommendation by the University of Dundee Ethical Review Committee.

Retroviral transduction of IKKα-knockout MEFs
IKKα-deficient MEFs stably expressing HA-tagged empty vector (EV), wild-type HA–IKKβ (WT), HA–IKKβ[S177A], HA–IKKβ[S177E] or HA–IKKβ[D166A/S177E] were generated by retroviral transduction using an MMLV (Moloney murine leukaemia virus)-based system prepared with the VSVG (vesicular-stomatitis-virus glycoprotein) envelope protein. Retroviral particles were prepared according to the manufacturer’s instructions (Clontech). Viruses encoding the gene of interest and Retroviral particles were prepared according to the manufacturer’s instructions (Clontech). Viruses encoding the gene of interest and

Assay of immunoprecipitated IKKβ
IKKβ immunoprecipitates were assayed for IKKβ activity in a 0.05 ml incubation containing 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA and 0.1 mg/ml microcystin (to inactivate any remaining traces of PP1γ). 0.3 mM of the peptide LDDRHDSGLDSMKDEEY (corresponding to amino acid residues 26–42 of IκBα), 10 mM magnesium acetate and 0.1 mM [γ-32P]ATP (5×106 c.p.m./nmol). After incubation for 10 min at 30°C on a shaking platform, the incorporation of 32P radioactivity into the peptide substrate was measured as described in [17].

RESULTS
IKKβ is activated by TAK1 and by autophosphorylation
We initially confirmed that IL-1 or TNF stimulate the dual phosphorylation of IKKβ at Ser177 and Ser181 in IKKα-deficient MEFs, and that this was prevented by the inclusion of the IKKβ inhibitor BI605906 in the culture medium (Figures 1A and 1B, top panel, compare lanes 1–3 with 10–12). In these and many earlier studies, the phospho-specific antibody used to monitor the phosphorylation of IKKβ recognizes the diphosphorylated species phosphorylated at both Ser177 and Ser181.
It was therefore possible that BI605906 and/or pharmacological inhibitors of TAK1 had suppressed the phosphorylation of just one of the serine residues in the activation loop. To address this possibility we therefore employed antibodies that recognize IKKβ phosphorylated at either Ser177 or Ser181. These studies led to the striking and surprising observation that BI605906 suppressed the IL-1- or TNF-stimulated phosphorylation of Ser181, but not the phosphorylation of Ser177 (Figures 1A and 1B, second and third panels from top, lanes 10–12). In contrast, two structurally unrelated inhibitors of TAK1, NG25 and SZ-7-oxozeaenol, prevented IL-1 or TNF from inducing the phosphorylation of IKKβ at both Ser177 and Ser181 in IKKα-deficient MEFs (Figures 1A and 1B, second and third lanes from top, lanes 4–9). Similar results were observed in BMDMs from knockin mice expressing the catalytically inactive IKKa[S176A/S180A] mutant (Figures 1C and 1D) [18].

The recognition of IKKβ by the Ser177 phospho-specific antibody appeared to be greatly enhanced when IKKα-deficient MEFs were incubated with BI605906 and then stimulated with IL-1 or TNF (Figures 1A and 1B, second panel from top, compare lanes 10–12 with 1–3). This observation is explained by the failure of the antibody to recognize IKKβ phosphorylated at Ser177 if Ser181 is also phosphorylated, and is not a reflection of a real increase in the phosphorylation of Ser177. This was shown by immunoblotting experiments with a synthetic mono-phosphorylated peptide corresponding to amino acid residues 171–187 of IKKβ containing phosphoserine at the position equivalent to Ser177, and a diphosphorylated form of this peptide with phosphoserine present at both Ser177 and Ser181 (Supplementary Figures S1A and S1B at http://www.biochemj.org/bj/461/bj4610531add.htm). We have encountered similar situations with other proteins in which the two sites of phosphorylation are separated by only four amino acid residues (e.g. [19]). In contrast, the antibody that recognizes the Ser181-phosphorylated form of IKKβ detected the di-phosphorylated form of the peptide (Supplementary Figure S1C), because this antibody recognizes the epitope Cys-Thr-pSer-Phε-Val (where pSer is phospho-Ser181), which does not contain Ser177. As expected, the antibody recognizing Ser181 of IKKβ did not detect the mono-phosphorylated peptide containing phosphoserine only at Ser177 (Supplementary Figure S1C).

The simplest interpretation of the results presented in Figure 1 was that the TAK1-catalysed phosphorylation of Ser177 was a prerequisite for the subsequent IKKβ-catalysed phosphorylation of Ser181. To investigate this hypothesis, we generated IKKα-deficient MEFs that stably expressed (under an inducible promoter) mutated forms of IKKβ in which Ser177 was changed to either glutamic acid (to mimic the effect of phosphorylation by introducing a negative charge) or to alanine (to prevent phosphorylation). The S177E mutant became phosphorylated at Ser181, even in MEFs that had not been stimulated with IL-1 or TNF, whereas the S177A mutant or wild-type IKKβ did not (Figure 2A). Moreover, under these conditions, incubation with the IKKβ inhibitor BI605906 induced substantial dephosphorylation of the S177E mutant at Ser181, whereas incubation with the TAK1 inhibitor NG25 had no effect. Furthermore, a catalytically inactive version of the S177E mutant, created by additionally mutating Asp196 in the Asp-Phe-Gly motif to alanine, failed to undergo phosphorylation at Ser181 (Figure 2B). Taken together, these experiments demonstrated, by two independent methods, that the phospho-mimetic S177E phosphorylation of a synthetic peptide comprising amino acid residues 26–42 of IκBα was suppressed by BI605906 similarly to wild-type IKKβ (Supplementary Figures S2 at http://www.biochemj.org/bj/461/bj4610531add.htm), establishing that the activity being measured was catalysed by IKKβ and not by another protein kinase present in the immunoprecipitates as a contaminant. Phosphatase treatment inactivated the IKKβ[S177E] mutant (Figure 2C), and this was accompanied by the dephosphorylation of Ser181 and a small increase in the electrophoretic mobility of IKKβ (Figure 2D). These experiments established that the phospho-mimetic S177E mutation had not activated IKKβ, but permitted IKKβ to auto-activate by phosphorylating Ser181.

BI605906 is a reversible inhibitor of IKKβ (Supplementary Figure S3 at http://www.biochemj.org/bj/461/bj4610531add.htm). To investigate whether the phosphorylation of Ser177 could activate IKKβ in the absence of Ser181 phosphorylation, we incubated IKKα-deficient MEFs with BI605906 to suppress the phosphorylation of Ser181 and assayed the endogenous IKKβ activity after its immunoprecipitation from the cells (Figure 2D). Taken together, the results presented in Figures 2 and 3 indicate that Ser177 is a priming event that enables IKKβ to auto-activate itself by phosphorylating Ser181.

**Activation of the canonical IKK complex**

The experiments presented above were carried out in IKKα-deficient MEFs or in BMDMs from knockin mice expressing the catalytically inactive IKKα[S176A/S180A] mutant, because IKKα activity is unaffected by BI605906 [8]. In contrast, the
phosphorylation of IKKβ at Ser\(^{181}\) was only decreased slightly by BI605906 in wild-type MEFs (Supplementary Figures S4A and S4B at http://www.biochemj.org/bj/461/bj4610531add.htm) and was not decreased significantly in Pam3CSK4- or LPS-stimulated BMDMs (Supplementary Figures S4C). This suggests that in wild-type cells, in which IKK\(\alpha\), IKK\(\beta\) and NEMO form a single ternary complex, phosphorylation of IKKβ at Ser\(^{181}\) can be catalysed \textit{in trans} by IKK\(\alpha\) if IKKβ is inhibited by BI605906.

The formation of Met1-linked ubiquitin chains and their interaction with NEMO is required for TAK1 to phosphorylate IKK\(\alpha\) and IKKβ at Ser\(^{176}/177\)

LUBAC is the only E3 ubiquitin ligase that catalyses the formation of Met1-linked (linear) ubiquitin chains in IL-1-stimulated MEFs, and the formation of these ubiquitin chains is required for robust activation of the canonical IKK complex by this agonist ([16], reviewed in [3]). To investigate whether Met1-linked ubiquitin chain formation was required for the phosphorylation of Ser\(^{177}\), Ser\(^{181}\) or both amino acid residues, we studied the phosphorylation of each of these sites in MEFs from knockin mice in which HOIP, the catalytic subunit of LUBAC, was replaced by the inactive HOIP\[C879S\] mutant [16]. These experiments demonstrated that the IL-1-stimulated phosphorylation of IKKβ at Ser\(^{177}\) or Ser\(^{181}\) or IKKα at Ser\(^{176}\) or Ser\(^{180}\) was greatly reduced in MEFs from HOIP\[C879S\]-knockin mice, as was the phosphorylation of p105/NFκB1 at Ser\(^{33}\), an established physiological substrate of IKKβ (Figure 4A) [20].

The Met1-linked ubiquitin chains formed by LUBAC bind to the NEMO component of the canonical IKK complex (reviewed in [3]). We therefore generated knockin mice expressing the NEMO\[D311N\] mutant of NEMO [21–23], and studied the phosphorylation of IKKβ in MEFs from these animals. We found that the IL-1-stimulated phosphorylation of IKKβ at Ser\(^{177}\) or Ser\(^{181}\), or IKKα at Ser\(^{176}\) or Ser\(^{180}\), was impaired in MEFs expressing the NEMO\[D311N\] mutant (Figure 4B), similar to the results obtained in MEFs from the HOIP\[C879S\]-knockin mice (Figure 4A).
The IL-1-stimulated phosphorylation of JNK1/JNK2 and p38 MAPK in MEFs from HOIP[C879S] or NEMO[D311N] mice was similar to wild-type MEFs (Supplementary Figure S5 at http://www.biochemj.org/bj/461/bj4610531add.htm), but was suppressed by the TAK1 inhibitors NG25 or 5Z-7-oxozeaenol (Supplementary Figure S6 at http://www.biochemj.org/bj/461/bj4610531add.htm). These control experiments indicated that activation of the TAK1 complex was unimpaired in MEFs from HOIP[C879S]- or NEMO[D311N]-knockin mice. The TAK1 inhibitor NG25 did not affect the IL-1-stimulated formation of Lys63-linked ubiquitin chains and NG25 does not suppress the phosphorylation of IKKβ by preventing formation of the ubiquitin chains. The enhanced formation of Met1-linked ubiquitin chains in the presence of NG25 implies the existence of a TAK1-dependent feedback control mechanism for restricting the formation of these ubiquitin chains.

Finally, it should be noted that although TAK1 phosphorylates the IKKα/β-NEMO complex at Ser177 in IKKa-deficient MEFs, the active TAK1 catalytic subunit is capable of phosphorylating a catalytically inactive mutant of the IKKβ catalytic subunit at Ser181, as well as Ser177, in vitro (Supplementary Figure S8 at http://www.biochemj.org/bj/461/bj4610531add.htm). It is therefore possible that the interaction of NEMO with IKKβ in the canonical IKK complex and/or the recruitment of the TAK1 complex to Lys63-linked ubiquitin chains are factors that prevent TAK1 from phosphorylating Ser181 in cells.

**DISCUSSION**

In the present study, we have clarified the mechanism by which the canonical IKK complex is activated. Unexpectedly, we discovered that the activation of IKKβ requires two sequential phosphorylation events. The activation process is initiated by the TAK1-catalysed phosphorylation of IKKβ at Ser177, which is a priming event that permits IKKβ to phosphorylate itself at Ser181, which is needed before IKKβ can phosphorylate exogenous substrates, such as IκBα (Figure 5). We have shown that this mechanism of activation operates in IL-1- or TNF-stimulated MEFs and in TLR-stimulated BMDMs indicating that it is likely to be of general significance. However, the identity of the ‘priming’ kinase may vary from cell to cell.

The mutation of Ser177 of IKKβ to glutamic acid (to mimic the effect of phosphorylation by introducing a negative charge) permitted the IKKβ catalytic subunit to autophosphorylate at Ser181 and this induced activation even in cells that had not been stimulated with IL-1 or TNF. Interestingly, the other two members of the IKK subfamily of protein kinases, IKKε and TBK1 (TANK [TRAF (TNF receptor-associated factor)-associated NF-κB activator]-binding kinase 1), both possess a glutamic acid at position 168 in their activation loops, which is the amino acid residue equivalent to Ser176/Ser177 of IKKα/β, and they are activated by the phosphorylation of Ser172, the site equivalent to Ser176/Ser177 of IKKα/β. These features explain why these IKK-related kinases are not activated directly by TAK1 in vitro and why they are instead activated by the canonical IKK complex and by autophosphorylation in response to IL-1 [8]. Once activated, IKK-related kinases restrict the activity of the canonical IKKs by phosphorylating inhibitory sites on the canonical IKKs, which is critical to prevent autoimmune nephritis in mice [8,24].

The activation of the canonical IKK complex by IL-1 does not require the phosphorylation of serine residues in the activation loop, but also the formation of a hybrid ubiquitin chain containing both Lys63-linked and Met1-linked ubiquitin oligomers [16]. The Lys63-linked ubiquitin chains interact with the TAB2 and TAB3 components of TAK1 complexes, inducing the auto-activation of TAK1 [9,13,25], whereas the Met1-linked ubiquitin chains formed by the action of the E3 ubiquitin ligase LUBAC [16,26] interact with NEMO [27,28] and are critical for activation of the canonical IKK complex [12,16,29]. Nearly all of the Met1-linked ubiquitin chains formed in response to IL-1 are activated covalently to Lys63-linked ubiquitin chains, which may facilitate the TAK1-dependent activation of canonical IKK complex by recruiting both protein kinases to the same ubiquitin chains [16]. In the present study, we found that the IL-1-stimulated phosphorylation of IKKα/IKKβ at Ser176/Ser177, and hence the phosphorylation of Ser180/Ser181, was suppressed in MEFs that were unable to produce Met1-linked ubiquitin chains or that expressed a ubiquitin-binding-defective mutant of NEMO (Figure 4). Thus the formation of Met1-linked ubiquitin chains and their interaction with NEMO are both needed for TAK1 to phosphorylate IKKα/IKKβ at Ser176/Ser177 and so enable the IKKs to complete the activation process by phosphorylating Ser180/Ser181 (Figure 5).

The activation of many protein kinases requires the phosphorylation of two amino acid residues within their activation loops. For example, similar to the canonical IKK complex, the seven members of the MKK family undergo dual phosphorylation at Ser/Thr-Xaa-Xaa-Xaa-Ser/Thr (where Xaa is any amino acid residue) sequences, enabling them to activate their cognate MAPKs. Similarly, most MAPKs are activated by the dual phosphorylation of a threonine and a tyrosine residue that are located in Thr-Xaa-Tyr within their activation loops. Although the activation of many MKKs and MAPKs is thought to be catalysed by a single protein kinase, the present study has shown that the requirement for one ‘upstream’ protein kinase does not exclude the possibility that a second protein kinase is also required. Indeed, we have shown that the activation of JNK requires the MKK7-catalysed phosphorylation of the threonine and the MKK4-catalysed phosphorylation of the tyrosine residue within...
the Thr-Xaa-Tyr motif [30]. The activation of a kinase by two different ‘upstream’ kinases provides additional opportunities for signal integration if each activating kinase responds to distinct physiological cues. We suggest that this situation may be a more frequent occurrence than has hitherto been realized, and that this is a neglected area that merits further attention.

**AUTHOR CONTRIBUTION**

Philip Cohen, Kristopher Clark and Jiazheng Zhang designed the experiments. Jiazheng Zhang performed the experiments. Philip Cohen and Jiazheng Zhang wrote the paper. Toby Lawrence provided the bone marrow from the knockin mice expressing catalytically inactive IKKα.

**ACKNOWLEDGEMENTS**

We thank Dr Natalia Shpiro for B6l05s96, Dr Nathanael Gray for N625, Dr Inder Verma for IKK-deficient MEFs, and Sambit Nanda and Christoph Emmerich for MEFs from NEMO(D311N) mice and HOIIP(C879S)-knockin mice respectively.

**FUNDING**

The work was supported by the Wellcome Trust [grant number WT100294], the U.K. Medical Research Council [grant number MRC-MR/K009851], AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Janssen Pharmaceutica, Merck-Serono and Pfizer.

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SUPPLEMENTARY ONLINE DATA
An unexpected twist to the activation of IKKβ: TAK1 primes IKKβ for activation by autophosphorylation

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Figure S1  Phosphorylation of Ser181 interferes with the recognition of phosphor-Ser177 by the phospho-specific antibody that recognizes this site

(A) The indicated amounts of phosphopeptides corresponding to amino acid residues 171–187 of IKKβ phosphorylated at Ser177 only or at both Ser177 and Ser181 were spotted on to nitrocellulose membranes and probed with the phospho-specific antibody recognizing phospho(p)-Ser177. (B) Same as (A), except that the peptides were immunoblotted with the antibody that only recognizes the di-phosphorylated form of IKKβ phosphorylated at both Ser177 and Ser181. (C) Same as (A), except that the phosphopeptides were immunoblotted with the antibody that recognizes IKKβ phosphorylated at Ser181.

Figure S2  Effect of BI605906 on the activity of wild-type and mutant IKKβ

HA-tagged wild-type IKKβ (WT) or the IKKβ[S177E] mutant (S177E) were expressed in HEK-293 cells, immunoprecipitated from the cell extracts using an anti-HA antibody and assayed for activity in the absence or presence of BI605906. The activities are plotted as a percentage of that obtained in the absence of inhibitor. Results are means±S.E.M. of duplicate determinations. Similar results were obtained in another independent experiment.

Figure S3  BI605906 is a reversible inhibitor of IKKβ

MEFs from IKKα-deficient mice were stimulated for 10 min with 5.0 ng/ml IL-1 and the cells were lysed. The endogenous IKKβ was immunoprecipitated from 0.2 mg of cell extract protein and incubated for 1 h at 30°C without (−, lane 1) or with (+ , lanes 2 and 3) 20 μM BI605906. In lane 3 only, the immunoprecipitates were washed extensively to remove BI605906. All the immunoprecipitates were then assayed for IKKβ activity. The Figure shows that IKKβ activity was fully restored after washing away the inhibitor. Results are means ± S.E.M. of duplicate determinations. Similar results were obtained in another independent experiment.

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Figure S4 Effect of inhibitors on agonist-stimulated phosphorylation of IKKβ at Ser177 and Ser181 in MEFs and BMDM

(A and B) MEFs from wild-type (WT) or IKKα-knockout (KO) mice were incubated for 1 h without (−) or with (+) 5.0 μM BI 605906 and then stimulated for 10 min with 5.0 ng/ml IL-1 (A) or 10 ng/ml TNF (B). Following cell lysis, cell extract (20 μg of protein) was denatured in SDS, subjected to SDS/PAGE, and immunoblotted with antibodies that recognize IKKα and IKKβ phosphorylated at Ser112 and Ser116 respectively, or with antibodies that recognize IKKα and IKKβ phosphorylated at Ser180 or Ser181 respectively. The membranes were also immunoblotted with antibodies that recognize all forms of IKKβ. (C) Same as (A and B), except that BMDMs from wild-type (WT) mice and knockin (KI) mice expressing the catalytically inactive mutant of IKKα were used and the cells were stimulated for 10 min with 1.0 μg/ml Pam3CSK4. An antibody recognizing GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control.

Figure S5 Phosphorylation of MAPKs is unimpaired in MEFs from HOIP[C879S] and NEMO[D311N] mice

(A) MEFs from wild-type mice (HOIP[WT]) or knockin mice expressing the inactive HOIP[C879S] mutant were stimulated with 5.0 ng/ml IL-1 for the times indicated. After cell lysis, 20 μg of cell extract protein was denatured in SDS, subjected to SDS/PAGE and immunoblotted with the antibodies indicated. (B) Same as (A), but using MEFs from mice expressing the polyubiquitin-binding-deficient mutant NEMO (NEMO[D311N]). The antibody recognizing pT183/Y185 of JNK1/2 was from Invitrogen and the antibody recognizing pT180/Y182 of p38α MAPK was from Cell Signaling Technology.

Figure S6 TAK1 inhibitors suppress the IL-1-stimulated phosphorylation of MAPKs in MEFs from HOIP[C879S]- and NEMO[D311N]-knockin mice

(A) As in Figure S5, except that before stimulation with IL-1, MEFs from mice expressing the HOIP[C879S] mutant were incubated for 1 h without (−) or with (+) the TAK1 inhibitors NG25 (1.0 μM) or 5Z-7-oxozaenol (1.0 μM) before stimulation with 5.0 ng/ml IL-1 for the times indicated. (B) Same as (A), except that the experiment was performed with MEFs from mice expressing the polyubiquitin-binding-deficient mutant NEMO[D311N].
Activation of IKKβ by TAK1 and autophosphorylation

Figure S7 Effect of TAK1 inhibition on the IL-1-stimulated formation of Lys63-linked and Met1-linked ubiquitin chains

MEFs were incubated for 1 h with (+) or without (−) 2 μM NG25 or 1 μM 5z-7-oxozaeanol, then stimulated for 10 min with 5 ng/ml IL-1α and lysed. The Met1-linked and Lys63-linked ubiquitin chains present in 2 mg of cell extract protein were captured on Halo-NEMO [1], released by denaturation in SDS and immunoblotted with antibodies that recognize Met1-linked or Lys63-linked ubiquitin chains specifically. The same cell extracts (20 μg of protein) were immunoblotted with an anti-GAPDH antibody as a loading control.

Figure S8 TAK1 phosphorylates IKKβ at Ser177 and Ser181 in vitro

Catalytically inactive IKKβ(D166A) (0.8 μM) was incubated for 3 min at 30°C with the indicated concentrations of the active TAK1–TAB1 fusion protein in 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 2 mM DTT, 10 mM magnesium acetate and 0.1 mM ATP. Reactions were terminated by denaturation in SDS and, after SDS/PAGE and transfer on to PVDF membranes, proteins were immunoblotted with antibodies that recognize IKKβ phosphorylated at Ser177 or Ser181 or antibodies recognizing all forms of IKKβ.

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Received 4 April 2014/20 May 2014; accepted 9 June 2014
Published as BJ Immediate Publication 9 June 2014, doi:10.1042/BJ20140444

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