Inducible Phosphorylation of NF-κB p65 at Serine 468 by T Cell Costimulation Is Mediated by IKKe

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Here we identify IKKe as a novel NF-κB p65 kinase that mediates inducible phosphorylation of Ser468 and Ser536 in response to T cell costimulation. In addition, the kinase activity of IKKe contributes to the control of p65 nuclear uptake. Serines 468 and 536 are evolutionarily conserved, and the surrounding amino acids display sequence homology. Down-regulation of IKKe levels by small interfering RNA does not affect inducible phosphorylation of Ser536 but largely prevents Ser468 phosphorylation induced by T cell costimulation. Ser536-phosphorylated p65 is found predominantly in the cytosol. In contrast, the Ser468 phosphorylated form of this transcription factor occurs mainly in the nucleus, suggesting a function for transactivation. Reconstitution of p65 with the coactivating acetylase CREB-binding protein/p300, allows the regulation of NF-κB transcriptional activity (9, 10). Thus far, eight different phosphorylation sites have been mapped for the strongly NF-κB p65 subunit. Three sites are contained in the N-terminal Rel homology domain, whereas five sites (Ser468, Thr505, Ser529, Ser535, and Ser536) are contained within both C-terminal TADs. Inducible phosphorylation of Ser536 and threonine 311 promotes the interaction of p65 with the coactivating acetylase CREB-binding protein/p300, thus leading to p65 acetylation and stimulating NF-κB-driven transcription. Various experimental approaches revealed that phosphorylation of serines 529, 535, and 536 serve to stimulate NF-κB-dependent transcription. Expression of a p65 protein mutated in Ser529 in p65−/− cells revealed only a minor role of Ser529 in transactivation, since it only contributes to achieve the Tax-induced maximal transcriptional response (11). Phosphorylation of Ser535 is mediated by the calmodulin-dependent protein kinase IV, which results in an increase of NF-κB-dependent transcription, as revealed by a phosphomimetic mutation where Ser535 was replaced by glutamic acid (12). Basal phosphorylation of Ser468, a recently discovered phosphorylation site within TAD2 (13), is exerted by GSK3β (14). The same site can also be phosphorylated by IKKβ in response to TNFα or IL-1 stimulation (15). We have previously shown that phosphorylation of p65 Ser468 can be induced by T cell costimulation (13), but the responsible kinase(s) is not yet known. Phosphorylation of p65 NF-κB at Ser536 couples p65 to TAFIIS1-mediated transcription and is mediated, dependent on the stimulus, by various kinases, including IKKe/β, RSK1, TBK1 (TANK-binding kinase-1)/NAK (NF-κB-activating kinase)/T2K (TRAF2-associated kinase), and IKKe (also called IKKa) (16, 17).

TBK1 and IKKe show sequence homology to IKKa/β but are not components of the IKK complex (18, 19). Both kinases have been recently mainly recognized for their ability to phosphorylate interferon regulatory factor proteins in response to viral infection (20, 21). IKKe overexpression promotes dimerization and nuclear translocation of interferon regulatory factor-3 but also enhances the DNA binding activity of C/EBPβ (22). IKKe is mainly regulated via inducible, NF-κB-dependent expression but is prominently expressed in T cells (19, 23). Its pathways and the DNA damage-induced NF-κB pathway. All NF-κB activating events have in common that they lead to the proteasome-dependent generation of DNA-binding dimers (5). NF-κB signals activating the canonical pathway funnel into the IKK complex, which is composed of the enzymatically active subunits IKKα and IKKβ and the regulatory subunits IKKγ/NEMO (6, 7) and ELKS (8). IKKβ-mediated IκB phosphorylation allows subsequent ubiquitination and proteolytic destruction of this inhibitory protein. This leads to an unmasking of the p65 nuclear localization sequence and results in NF-κB nuclear immigration, DNA binding, and gene expression.

Once activated, inducible post-translational modifications, including phosphorylation, acetylation, ubiquitination, or prolyl isomerization, allow the regulation of NF-κB transcriptional activity (9, 10). Thus far, eight different phosphorylation sites have been mapped for the strongly NF-κB p65 subunit. Three sites are contained in the N-terminal Rel homology domain, whereas five sites (Ser468, Thr505, Ser529, Ser535, and Ser536) are contained within both C-terminal TADs. Inducible phosphorylation of Ser536 and threonine 311 promotes the interaction of p65 with the coactivating acetylase CREB-binding protein/p300, thus leading to p65 acetylation and stimulating NF-κB-driven transcription. Various experimental approaches revealed that phosphorylation of serines 529, 535, and 536 serve to stimulate NF-κB-dependent transcription. Expression of a p65 protein mutated in Ser529 in p65−/− cells revealed only a minor role of Ser529 in transactivation, since it only contributes to achieve the Tax-induced maximal transcriptional response (11). Phosphorylation of Ser535 is mediated by the calmodulin-dependent protein kinase IV, which results in an increase of NF-κB-dependent transcription, as revealed by a phosphomimetic mutation where Ser535 was replaced by glutamic acid (12). Basal phosphorylation of Ser468, a recently discovered phosphorylation site within TAD2 (13), is exerted by GSK3β (14). The same site can also be phosphorylated by IKKβ in response to TNFα or IL-1 stimulation (15). We have previously shown that phosphorylation of p65 Ser468 can be induced by T cell costimulation (13), but the responsible kinase(s) is not yet known. Phosphorylation of p65 NF-κB at Ser536 couples p65 to TAFIIS1-mediated transcription and is mediated, dependent on the stimulus, by various kinases, including IKKe/β, RSK1, TBK1 (TANK-binding kinase-1)/NAK (NF-κB-activating kinase)/T2K (TRAF2-associated kinase), and IKKe (also called IKKa) (16, 17).

TBK1 and IKKe show sequence homology to IKKa/β but are not components of the IKK complex (18, 19). Both kinases have been recently mainly recognized for their ability to phosphorylate interferon regulatory factor proteins in response to viral infection (20, 21). IKKe overexpression promotes dimerization and nuclear translocation of interferon regulatory factor-3 but also enhances the DNA binding activity of C/EBPβ (22). IKKe is mainly regulated via inducible, NF-κB-dependent expression but is prominently expressed in T cells (19, 23). Its
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kinase activity is triggered in response to T cell stimulation or the phorbol ester phorbol-12-myristate-13-acetate (PMA) but not by the cytokines TNFα or IL-1 (19). Whereas NF-κB regulates IKKe, the role of IKKe for NF-κB activation remains elusive, as IKKe 

+ cells show unchanged inducible IkBα phosphorylation and DNA binding. On the other hand, lipopolysaccharide-induced expression of some late NF-κB target genes, including COX-2, regulated on activation, normal T cell expression and secreted (RANTES), and interferon-

α (IP-10) is lost in IKKe-deficient cells (22), suggesting a role of IKKe for the regulation of NF-κB at a later step.

Here we reveal an additional function for IKKe and show that it serves as a novel p65 kinase that mediates inducible phosphorylation at serines 468 and 536 in vitro and in vivo, thus stimulating gene expression.

MATERIALS AND METHODS

Cell Culture, Transfections, and Stimulations—Mouse embryonic fibroblasts (MEFs) lacking p65 (24) and human 293T and HeLa cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, and 1% (v/v) penicillin/streptomycin. Adherent cells were transfected using Rotifect (Roht) according to the manufacturer’s instructions. Jurkat T leukemia cells and Jurkat T-ON cells were grown in supplemented RPMI 1640 medium. Jurkat cells (1.5 × 10^6) were transfected by electroporation using a gene pulser (Bio-Rad) at 250 V/950 μF and 20 μF with constant capacitance.

Subcellular Fractionation—Human 293T cells were transiently transfected to express the hemagglutinin-tagged p65 variants. 36 h later, cells were lysed in Nonidet P-40 lysis buffer and one aliquot was used to confirm correct expression of the proteins. Equal amounts of protein contained in the supernatant were subjected to Western blot analysis using the Amersham Biosciences enhanced chemiluminescence system.

Purification of GST-p65 and Kinase Assays—The GST-p65 (354–551) fusion protein was expressed in Escherichia coli BL21 cells and purified by affinity chromatography on glutathione-Sepharose 4B according to standard protocols. The immune complex kinase assays were done by immunoprecipitation of FLAG-tagged IKKe using α-FLAG antibodies or by immunoprecipitation of endogenous IKKe.

The precipitate was washed three times in Nonidet P-40 lysis buffer and two times in kinase buffer (20 mM Hepes/KOH, pH 7.4, 25 mM β-glycerophosphate, 2 mM dithiothreitol, 20 mM MgCl₂). The kinase assay was performed in a final volume of 20 μl of kinase buffer containing 40 μM ATP and 2 μg of the purified GST-p65 substrate protein. After incubation for 20 min at 30 °C, the reaction was stopped, separated by SDS-PAGE, and analyzed by immunoblotting with phosphospecific antibodies.

Co-immunoprecipitation Experiments—Human 293T cells were transiently transfected to express the hemagglutinin-tagged p65 variants. 36 h later, cells were lysed in Nonidet P-40 lysis buffer, and one aliquot was used to confirm correct expression of the proteins. Equal amounts of protein contained in the remaining supernatants were immunoprecipitated either with α-hemagglutinin or with control antibodies and 25 μl of protein A/G-Sepharose and rotated for 4 h on a spinning wheel at 4 °C. The immunoprecipitates were washed five times in Nonidet P-40 buffer and then boiled in 1× SDS sample buffer prior to SDS-PAGE and further analysis by Western blotting.

Subcellular Fractionation—Nuclear and cytosolic proteins were separated upon resuspending pelleted cells in 400 μl of cold buffer A (10 mM Hepes/KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithioreitol, and 0.5 mM phenylmethylsulfonyl fluoride) by gentle pipetting. After incubation for 20 min on ice, 10 ml of 10% Nonidet P-40 was added, and cells were lysed by vortexing. The homogenate was centrifuged for 30 s in a microcentrifuge. The supernatant representing the cytosolic fraction was collected, and the pellet containing the cell nuclei was dissolved in 100 μl of buffer C (20 mM Hepes/KOH, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithioreitol, 0.5 mM phenylmethylsulfonyl fluoride). The Eppendorf tubes were incubated for 15 min on ice and centrifuged for 10 min with 13,000 rpm at 4 °C. The supernatant representing the nuclear fraction was collected.
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RESULTS

IKKe Directly Phosphorylates NF-κB p65 at Ser468—Basal phosphorylation at Ser468 is mediated by GSK3β, whereas TNFα- and IL-1-induced phosphorylation of this site is maximal already 7.5 min after stimulation and is mediated by IKKβ (15). We have previously shown that Ser468 is inducibly phosphorylated by T cell costimulation (13), but the kinase mediating inducible phosphorylation in response to this stimulus is not known. Ser468 is contained in a sequence motif called TAD1 that shows homology to TAD1 (4). The relative positions of Ser536 and Ser468 within the sequence motif are conserved (Fig. 1A), raising the possibility that both sites may employ the same kinase. From all of the kinases known to mediate Ser536 phosphorylation, only IKKa/IKKB and IKKe are known to be induced by T cell costimulation or by PMA and ionomycin, which mimic T cell costimulation upon protein kinase C activation and calcium release, respectively (32). To compare the relative roles of IKKa, IKKB, and IKKe for p65 phosphorylation, Jurkat T cells were transfected with expression vectors encoding these three IKKs together with very low amounts of a vector directing expression of a GFP-tagged p65 protein, allowing expression of this fusion protein at physiological levels. This experimental approach was taken, because the low transfection efficiency of Jurkat T leukemia cells hampers the analysis of the endogenous p65 protein, thus enabling the detection of the slower migrating GFP-p65 fusion protein, which is fully regulated and functional (25). Whereas expression of IKKa only slightly induced p65 Ser536 phosphorylation, IKKB strongly triggered Ser536 phosphorylation of GFP-p65 and also of the endogenous p65 protein (Fig. 1B). IKKe also caused a slight induction of Ser468 phosphorylation. In contrast, even faint amounts of IKKe potently stimulated p65 phosphorylation at serines 536 and 468 and even caused the appearance of a slower migrating p65 form (Fig. 1B). In contrast to IKKB, the overexpression of IKKe failed to cause the phosphorylation of the endogenous p65, which is in complex with IKKα proteins. A similar experimental approach revealed that this strong IKKe-induced phosphorylation could not be further enhanced by PMA/ionomycin stimulation (Fig. 1C). Is the slower migrating form of p65 due to IKKe-mediated phosphorylation, or is it also caused by other modifications? To address this question, extracts from cells coexpressing GFP-p65 and IKKe were incubated with λ-phosphatase. This treatment completely converted the slower migrating form of p65 into the faster migrating version (Fig. 1D), showing that both sites can be phosphorylated independently from each other. To test this hypothesis experimentally, IKKe-mediated phosphorylation of each individual site was determined with a substrate GFP-p65 protein, where the respective other phosphorylation site was mutated to alanine. These experiments revealed full Ser468 phosphorylation in the presence of a mutated Ser536 and vice versa (Fig. 2A), showing that both sites can be phosphorylated independently from each other. Mutation of the phosphorylatable serines to alanine precluded binding of the phosphospecific antibodies, thus confirming their specificity. Next, we wanted to test whether IKKe phosphorylates p65 directly or causes phosphorylation upon activation of a downstream kinase. Cells were transfected to express epitope-tagged wild type or
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**FIGURE 2.** IKKe directly phosphorylates p65 in vitro and induces the occurrence of a slower migrating p65 form. **A** Jurkat cells were transfected to express GFP-p65 or various GFP-p65 mutants, where Ser^{468} and/or Ser^{536} were changed to alanine together with FLAG-tagged IKKe at the indicated combinations. Phosphorylation of p65 and expression of IKKe was analyzed by immunoblotting as shown. **B** Jurkat cells were transfected with expression vectors for epitope-tagged forms of IKKα, IKKβ, and kinase-inactive point mutants of these kinases, respectively. After 36 h, cells were lysed, and the IKK proteins were immunoprecipitated (IP) and purified from cell lysates with polyclonal FLAG or α-Myc antibodies, respectively. Subsequently, kinase activity was monitored by immune complex kinase assays (IKKα) using recombinant GST-p65-(354–551) as substrate. Phosphorylation of p65 was determined by immunoblotting using phosphospecific antibodies as shown. Note that IKKe causes the upshift also in these in vitro assays. In the control immunoblot, the α-FLAG antibody was detected first, followed by visualization of IKKβ with α-Myc antibodies.

kinase-inactive forms of IKKe or the control protein IKKβ, followed by purification via immunoprecipitation and in vitro kinase assays. The GST-p65-(354–551) protein was efficiently phosphorylated at Ser^{468} and Ser^{536} by IKKβ wild type but not by the kinase-inactive IKK point mutant, as revealed by immunoblotting with phosphospecific antibodies (Fig. 2B). These in vitro experiments also revealed that IKKe can cause the induction of a slower migrating p65 form. We also found a direct phosphorylation of p65 at both sites when IKKβ was used as a kinase source. In summary, these results identify IKKe as a kinase directly mediating p65 phosphorylation at Ser^{468} and Ser^{536}.

**NF-κB p65 Phosphorylated at Ser^{468} is Found Predominantly in the Nucleus—Following T cell costimulation, p65 phosphorylated at Ser^{468} is predominantly found in the cytosol (33). We thus asked whether the same holds true for p65 phosphorylated at Ser^{468} and stimulated Jurkat cells for various time periods with PMA/ionomycin, followed by subcellular fractionation into cytosolic and nuclear extracts (Fig. 3A). The Ser^{468}-phosphorylated p65 protein was found predominantly in the nucleus, which is in contrast to the Ser^{536}-phosphorylated p65 occurring mainly in the cytosol. Also, the kinetics revealed differences, since Ser^{536} phosphorylation started to vanish already 30 min after stimulation, whereas Ser^{468} phosphorylation displayed a delayed kinetics and was unchanged even 45 min after PMA/ionomycin treatment.

To test whether the same intracellular distribution occurs when p65 phosphorylation is triggered by IKKe, Jurkat cells were transfected to express GFP-p65 in the absence or presence of cotransfected IKKe. Two days after transfection, nuclear and cytosolic extracts were prepared and p65 phosphorylation was analyzed by immunoblotting (Fig. 3B). Also in this setting, Ser^{468}-phosphorylated p65 was found predominantly (but not exclusively) in the nucleus. Overexpressed IKKe was found in the nucleus and in the cytosol, which reflects the distribution of the endogenous kinase that is also found in both fractions (Fig. 3A).

To address the question of whether IKKe-mediated phosphorylation of p65 Ser^{468} can also occur in the nucleus, we tested the effects of IKKe on phosphorylation of the Gal4-p65 protein, which is constitutively nuclear (26). IKKe-triggered Ser^{468} phosphorylation was further augmented by treatment with PMA/ionomycin (Fig. 3C). These results show that IKKe phosphorylates nuclear p65 that is not in complex with IκB but do not exclude the possibility that IKKe-mediated p65 phosphorylation can also happen in the cytoplasm. In contrast, coexpression of a kinase-inactive IKKe point mutant completely inhibited this inducible phosphorylation, pointing to the relevance of IKKe for this pathway.

**The Kinase Activity of IKKe Is Activated in Response to T Cell Costimulation—**To address the question of whether the kinetics of IKKe activation parallels that of p65 Ser^{468} phosphorylation, Jurkat cells either containing or lacking the IKKγ/NEMO protein were stimulated with PMA/ionomycin for various periods. Immune complex kinase assays using the immunoprecipitated endogenous IKKe protein as a kinase source revealed phosphorylation of the p65 substrate protein 20 and 45 min after stimulation (Fig. 4A). These data show that induction of IKKe kinase activity parallels p65 Ser^{468} phosphorylation and that IKKγ/NEMO, which is essential for the function of the IKK complex, is not important for primary IKKe activation at the time points analyzed. The amount of IKKe protein is unchanged in response to T cell costimulation and not influenced by the presence of IKKγ/NEMO (Fig. 4B).

**Phosphorylation of p65 at Ser^{468} Requires Its Release from IκBα—**We next asked whether free or IκBα-bound p65 is phosphorylated at Ser^{468}. To address this question, Jurkat cells lacking the IKKγ/NEMO protein and thus being unable to phosphorylate IκBα and to release the p65 protein from the cytosol (6) were stimulated for various periods with PMA/ionomycin. IKKγ/NEMO-deficient cells failed to induce p65 phosphorylation at Ser^{468} and Ser^{536}, whereas IKKγ/NEMO-reconstituted control cells showed full phosphorylation at both sites (Fig. 5A), suggesting that only free and untrapped p65 can be phosphorylated.

To substantiate this finding by an independent experimental approach, Ser^{468} phosphorylation was tested in cells where IκBα phosphorylation was blocked by the IKKβ inhibitor AS602868 (34). Also, this experimental approach confirmed that the prevention of IKK activation precluded nuclear translocation of p65 and its phosphorylation at Ser^{468} (Fig. 5B). Similarly, IKKe-mediated Ser^{468} phosphorylation of GFP-p65 was strongly diminished upon coexpression of IκBα (Fig. 5C). This experiment also showed strongly diminished IκBα levels in the presence of overexpressed IKKe, which is presumably due to the function of IKKe as an IκBα Ser^{36} kinase (19).

Given the ability of IKKe to trigger IκBα decay, we tested whether IKKe expression can cause p65 Ser^{468} phosphorylation by compensating the inactivity of the classical IKK complex in IKKγ/NEMO-deficient Jurkat cells. Expression of IKKe caused Ser^{468} and Ser^{536} phosphorylation of coexpressed GFP-p65 in the absence and presence of IKKγ/NEMO (Fig. 5D). This suggests that this kinase can liberate and phosphorylate the p65 protein independent from the IKK complex, a process that may be facilitated by the possibility that not all of the expressed GFP-p65 protein is bound by IκBs.

In summary, all of these experiments show that IKKe-mediated Ser^{468} phosphorylation does not occur when the release of IκB-bound p65 is inhibited. Accordingly, stabilization of the IκB-NF-κB complex by the proteasome inhibitor MG132 enhanced PMA/ionomycin-induced and IKKβ-mediated phosphorylation of IκB and p65 Ser^{468} within this complex (Fig. 5E). In contrast, T cell costimulation-induced p65 phospho-
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rylation at Ser468 was diminished in the presence of MG132, corroborating the concept that this phosphorylation does not take place within the IκB-NF-κB complex. Accordingly, co-immunoprecipitation experiments showed no influence of the p65 Ser468 phosphorylation status on its ability to associate with IκBα (supplementary Fig. 1).

IKKε Serves to Modulate p65 Nuclear Import—To investigate the effects of IKKε on p65, we generated stable Jurkat cell lines allowing doxycycline-dependent expression of the wild type or kinase-inactive IKKε. To measure the impact of IKKε on nuclear import of NF-κB, cells were induced with doxycycline to trigger IKKε expression and stimulated for various periods with PMA/ionomycin. Nuclear and cytosolic extracts were tested for the occurrence of NF-κB by immunoblotting. Control cells and cells induced to express IKKε behaved similarly and showed nuclear immigration starting from 7.5 min and reaching its maximum 30 min post PMA/ionomycin treatment (Fig. 6). In contrast, cells expressing IKKε kinase-active showed a strongly impaired accumulation of nuclear p65 (Fig. 6), suggesting that IKKε contributes to the control of p65 nuclear import. Decreased nuclear import was not only seen for p65 but also for its dimerization partner p50.

T Cell Costimulation-induced p65 Ser468 Phosphorylation Depends on IKKε—The relative contribution of IKKε for p65 phosphorylation in response to T cell costimulation was investigated by testing the effect of small interfering RNAs specific for IKKε. Jurkat cells were transfected either with the control vector pSUPER or with pSUPER-IKKε, a vector that directs the synthesis of small interfering RNAs specific for IKKε together with a plasmid conferring resistance to puromycin. After selection of stably transfected Jurkat cell pools, p65 phosphorylation was investigated in untreated and PMA/ionomycin-stimulated cells. Whereas Ser536 phosphorylation was not significantly affected by impaired IKKε levels, phosphorylation at Ser468 was significantly reduced (Fig. 7A), thus showing an important contribution of IKKε for the phosphorylation at this site. Since GSK3β mediates basal p65 Ser468 phosphorylation in unstimulated cells (14) and AS602868 displays some inhibitory effects on GSK3β, a possible contribution of this kinase for
PMA/ionomycin-induced phosphorylation was tested. Jurkat cells were left untreated or stimulated in the absence or presence of the GSK3/β/H9252 inhibitor LiCl (35). Detection of Ser468 phosphorylation by immunoblotting revealed no significant changes in the presence of LiCl (Fig. 7B), thus excluding a role of GSK3/β for Ser468 phosphorylation induced by T cell costimulation.

**Phosphorylation at Ser468 and Ser536 Enhances the Transcriptional Activity of p65**—The impact of IKK/β-mediated Ser468 and Ser536 phosphorylation on gene expression was tested in transient reporter gene assays. p65−/− MEFs were cotransfected with a NF-κB-dependent luciferase gene and increasing amounts of either wild type p65 or variants thereof that are point-mutated in the phosphoacceptor sites. The phosphomimetic p65 S468E mutant, where the serine was changed to glutamic acid, boosted NF-κB-dependent gene expression more strongly than the wild type protein (Fig. 8A), indicating that phosphorylation of Ser468 serves to enhance transcription. An increased transcriptional activity was also observed for p65 S536E and the double mutant, where both serines were changed to glutamic acids. Whereas mutation of Ser536 to Ala did not significantly impair the transcriptional activity of the mutant protein, the Ser468 to Ala mutant showed a markedly decreased ability to trigger gene expression. Accordingly, a p65 mutant where both serines were changed to alanine showed a strongly reduced transcriptional activity. To test whether mutation of Ser468 has an impact on DNA binding (e.g., by changes in the intramolecular conformation), cells were transfected with GFP-p65 and FLAG-tagged IKKβ and IκBα at the indicated combinations. Total cell extracts were analyzed for p65 phosphorylation and the occurrence of the indicated proteins. D, IKKγ/NEMO-deficient and control cells were transfected to express the indicated combinations of GFP-p65 and IKKβ as shown, followed by the analysis of p65 phosphorylation by Western blotting. E, Jurkat T cells were left untreated or stimulated for 20 min with PMA/ionomycin in the absence or presence of MG132. Total cell extracts were analyzed by immunoblotting for p65 phosphorylation at Ser468 or Ser536 and also for the occurrence of phosphorylation and degradation of IκBα as shown.

**FIGURE 5.** Induced p65 Ser468 phosphorylation does not occur for cytoplasmically trapped p65. A, Jurkat cells lacking IKKγ/NEMO and control cells retransfected to express IKKγ/NEMO were stimulated for the indicated periods as shown. Cytoplasmic and nuclear extracts were analyzed by immunoblotting (IB) for the phosphorylation of p65 and the occurrence of control proteins as shown. B, Jurkat cells were preincubated for 60 min with AS602868 (1.2 μg/ml) and stimulated for 20 min with PMA/ionomycin. Equal amounts of protein contained in nuclear extracts were analyzed by Western blotting for phosphorylation of p65 Ser468 and the occurrence of p65 and HDAC-1. Note that the IKKβ inhibitor prevents nuclear entry of p65. C, Jurkat cells were transiently transfected with expression vectors encoding GFP-p65 and FLAG-tagged IKKβ and IκBα at the indicated combinations. Total cell extracts were analyzed for p65 phosphorylation and the occurrence of the indicated proteins. D, IKKγ/NEMO-deficient and control cells were transfected to express the indicated combinations of GFP-p65 and IKKβ as shown, followed by the analysis of p65 phosphorylation by Western blotting. E, Jurkat T cells were left untreated or stimulated for 20 min with PMA/ionomycin in the absence or presence of MG132. Total cell extracts were analyzed by immunoblotting for p65 phosphorylation at Ser468 or Ser536 and also for the occurrence of phosphorylation and degradation of IκBα as shown.
be conveniently distinguished from endogenous NF-κB, since it migrates more slowly. Both p65 forms displayed similar DNA binding, suggesting that the changed activity of p65 mutated in Ser468 is attributable to the transactivation potential rather than to altered DNA binding.

**DISCUSSION**

IKKe is important for the lipopolysaccharide-induced expression of a specific subset of NF-κB target genes, including TNFα, IL-1, IP-10, and RANTES, but not the early activated gene IL-8. On the other hand, IKKe−/− cells show normal lipopolysaccharide-induced phosphorylation and degradation of IL-8 and no changes in NF-κB DNA binding (22). These data point to the importance of IKKe for NF-κB-mediated transactivation, and accordingly an earlier study provided evidence that IKKe contributes to DNA binding activity of C/EBPβ, thus affecting the expression of target genes, which often depend on the coordinate binding of NF-κB and C/EBP (22). However, since CCAAT/enhancer-binding protein δ DNA binding is only partially compromised but target gene transcription is completely abolished in IKKe−/− cells, it can be assumed that further mechanisms contribute to the stimulatory effect of IKKe on NF-κB-dependent transcription. Here we provide evidence that IKKe serves to mediate p65 phosphorylation at Ser468 and Ser536, as revealed by *in vitro* and *in vivo* experiments. Whereas the p65 protein can enhance transcription of the IKKe gene (36), this study shows that IKKe in turn can control the phosphorylation of p65, thus establishing an autoregulatory loop.

Also, the IKKe homologue TBK1 was identified as a p65 Ser536 kinase that exerts its function together with the NAP1 (for NAK-associated protein 1) adapter protein (37). Accordingly, recombinant IKKe and TBK1 enzymes are enzymatically similar to each other, and both require phosphorylation of a critical Ser in their activation loops for kinase activity (38). Deletion of the TBK1 gene leads to embryonic lethality at approximately embryonic day 15 from massive hepatocyte apoptosis (39), which resembles the phenotype of p65−/− mice (40). Similar to IKKe, TBK1−/− MEFs show normal IκB degradation and DNA binding but defects in the transactivation of specific NF-κB target genes (39). Of note, these transcriptional effects are not seen when NF-κB-dependent transcription is measured with synthetic reporter genes (20), raising the possibility that the effects of IKKe and TBK1 are specific for individual genes. It will be interesting to learn in future studies whether these promoter specific effects are due to p65 phosphorylation or to other events such as NF-κB subunit exchange or chromatin remodeling (41).

Thus, we suggest that IKKe and TBK1 not only serve to mediate interferon-regulatory factor-3 phosphorylation following engagement of Toll-like receptors 3 and 4 or viral infection (20, 21) but also fulfill a function for NF-κB activation upon phosphorylation of p65 and controlling its nuclear import. The inhibitory effect of kinase-inactive IKKe on p65 nuclear import is not linked to p65 Ser468 phosphorylation, since this site is not relevant for its subcellular distribution (data not shown) (15). Therefore, it is plausible that these effects are due to phosphorylation of p65 at additional sites or by modification of further substrate proteins.

IKKe fails to induce an upshift for p65 that is mutated in serines 536 and 468, but since p65 phosphorylation must not necessarily result in the occurrence of an upshifted form (9), we cannot exclude the possibility that IKKe is also modifying p65 at additional sites. Here we show that IKKe mediates inducible p65 Ser468 phosphorylation in response to T cell costimulation, thus identifying the third kinase for this site. Whereas GSK3β mediates basal phosphorylation in unstimulated cells (14), IKKeβ was shown to mediate Ser468 phosphorylation in response to TNFα or IL-1 (15). However, these cytokines trigger Ser468 phosphorylation with a very fast kinetics, and this p65 modification cannot be detected even 15 min after stimulation. Since the kinase activity of IKKe cannot be augmented by these two cytokines (19), it can be assumed that...
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FIGURE 8. Functional analysis of p65 phosphorylation. A, p65-deficient MEFs were transiently transfected with a luciferase reporter gene controlled by three NF-κB sites along with the indicated amounts of expression vectors encoding wild type and point-mutated p65 proteins. 30 h post-transfection, cells were harvested and tested for luciferase activity. Fold induction of gene expression is shown; bars indicate S.D. from at least four independent experiments. Appropriate expression of transfected proteins was ensured by Western blotting (data not shown). B, HeLa cells were left untreated or transfected to express GFP-p65 or GFP-p65 S468A. One day later, cells were left untreated or stimulated for 20 min with TNFα. Cell extracts were prepared, and an aliquot was tested for NF-κB DNA binding by EMSAs. The positions of a constitutively DNA-binding protein (open arrow) and of NF-κB DNA complexes (arrows) are indicated (top). Another aliquot of the extract was assayed by immunoblotting (IB) for comparable expression of GFP-tagged p65 proteins (bottom). EMSA, electrophoretic mobility shift assay.

FIGURE 9. Evolutionary conservation of the p65 Ser468 and Ser536 phosphorylation sites. NF-κB p65 proteins from the indicated organisms were compared for conservation of Ser468 and Ser536. Homologous amino acids are indicated by stars, and homologous and related amino acids are indicated by dots.

IKKe will not play a relevant role for these stimulatory pathways. In costimulated T cells, Ser468 phosphorylation occurs with a significantly delayed kinetics, and a contribution of IKKβ for this process is unlikely, since the activity of this kinase already drops at these late time points (33), and various experimental approaches including small interfering RNA and a dominant negative IKKe mutant revealed the importance of this kinase for Ser468 phosphorylation triggered by costimulation. As several phosphorylation sites within p65 are modified by various kinases (9, 10), it remains to be seen whether other stimuli affecting Ser468 phosphorylation employ further kinases. The concept that various stimuli employ distinct kinases for a given p65 phosphorylation site also holds true for the Ser536 phosphorylation site. In this case, Ser536 phosphorylation triggered by TNFα or T-cell costimulation is mainly mediated by IKKe (27, 33), whereas DNA damage employs RSK1 to modify the same site (42). The stimulus preferentially employing IKKe for phosphorylation of Ser536 remains to be identified in future studies.

Both modified serines are evolutionarily conserved between mammals and amphibians (Fig. 9), arguing for their functional importance.

Our results indicate that p65 Ser468 phosphorylation, which occurs within the short TAD1’ sequence motif shared with TAD1 (4), primarily affects p65-dependent transactivation. The TAD1’ sequence motif was shown to activate transcription when fused as a multimer to the DNA binding domain of Gal4 (45), supporting a primary role in transactivation. The TAD1 region can form an inducible α-helix upon binding to other proteins (45), and accordingly secondary structure predictions suggest that also the homologous TAD1’ region containing Ser468 could fold in such an α-helix (46). In such a conformation, phosphorylation of Ser468 would increase the negative charge on one helix surface. This phosphorylation could then affect binding to corepressors or coactivators, such as CREB-binding protein/p300, which contact p65 in the adjacent region between amino acids 477 and 503 (47). In support of a possible role in the regulation of gene expression, Ser468-phosphorylated p65 is preferentially found in the nucleus. Phosphorylation of Ser468 induced by T cell costimulation does not occur alone and is accompanied by further p65 modifications. We suggest that distinct p65 modification patterns control the subset of induced target genes as well as the duration and amplitude of the NF-κB response.

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