The IκB-Kinase (IKK) complex is a multisubunit protein complex crucial for signal-induced phosphorylation of the IκB proteins and thus controls the activity of the transcription factor NF-κB. Besides the two kinases IKKα and IKKβ, the IKK complex contains NEMO/IKKγ, an additional subunit with regulatory and adaptor functions. NEMO not only confers structural stability to the IKK complex but also participates in the activation process of the IKK complex by linking the IKK subunits to upstream activators. In this study we analyze the IKKB-mediated phosphorylation of the IKK-binding domain of NEMO. In vitro, IKKB phosphorylates three serine residues in the domain of NEMO at positions 43, 68, and 85. However, mutational analysis revealed that only the phosphorylation of serine 68 in the center of the IKK-binding domain plays an essential role for the formation and the function of the IKK complex. Thus, Ser68 phosphorylation attenuates the amino-terminal dimerization of NEMO as well as the IKKB-NEMO interaction. In contrast, the NEMO-IKKα interaction was only mildly affected by the phosphorylation of Ser68. However, functional analysis revealed that Ser68 phosphorylation primarily affects the activity of IKKα. Furthermore, in complementation experiments of NEMO-deficient murine embryonic fibroblasts, a S68A-NEMO mutant enhanced, whereas a S68E mutant decreased, TNF-α-induced NF-κB activity, thus emphasizing the inhibitory role of the Ser68 phosphorylation on the signal-induced NF-κB activity. Finally, we provide evidence that the protein phosphatase PP2A is involved in the regulation of the Ser68-based mechanism. In summary, we provide evidence for a signal-induced phosphorylation-dependent alteration of the IKK complex emphasizing the dynamic nature of this multisubunit kinase complex.

The transcription factor NF-κB plays a crucial role in the initiation of innate and adaptive immune responses, in inflammation and tumorigenesis (1–3). In its inactive state NF-κB is bound to small cytoplasmic proteins, the IκB proteins. Stimulation with a wide variety of agonists, for example pro-inflammatory cytokines like TNF-α,2 bacterial components like lipopolysaccharide or by antigen receptors, funnels in the activation of a multisubunit IκB-kinase complex, which phosphorylates the IκB proteins at two specific serine residues. This phosphorylation marks the IκB proteins for proteasomal degradation setting NF-κB free, which then translocates into the nucleus and supports the expression of various pro-inflammatory or anti-apoptotic gene products. The IκB kinase (IKK) complex is composed of two catalytically active subunits, termed IKKα (IKK1) and IKKβ (IKK2), as well as NEMO/IKKγ, a subunit with regulatory and adaptor functions (4, 5). Analysis of mice deficient for either IKKα or IKKβ suggested that the IKKβ subunit is the major IKK regulating the canonical NF-κB pathway, and the IKKα subunit is crucial for a second, alternative NF-κB pathway leading to the activation of RelB-p52 heterodimers (6, 7). In contrast to the canonical NF-κB pathway, which depends on the presence of NEMO, the alternative pathway is NEMO-independent but requires the protein kinase NIK. However, recent data suggest that, instead of a clear assignment of IKKα and IKKβ to the alternative and the canonical NF-κB pathway, respectively, both kinases contribute to the activation of NF-κB by the canonical pathway with only gradual differences (8, 9).

Although a precise model regarding the molecular mechanism underlying IKK activation is still missing, it became clear that various post-translational modifications at different IKK subunits are involved in this process. Besides the phosphorylation of IKKα and IKKβ at two serine residues in their T-loop, the ubiquitination and occasionally the phosphorylation at a specific serine residue of NEMO seem to be required (10–12). This serine residue at position 85 of NEMO has been identified as a protein kinase Cθ, and more recently as an ATM target-site crucial for the IKK activation induced by genotoxic stress (12, 13). In addition, overexpression of the TNF-α-receptor 1 or the human T-cell lymphotrophic virus 1 Tax protein induces the IKKβ-mediated NEMO phosphorylation at several

1 To whom correspondence should be addressed. Tel.: 49-0731-502-2897; Fax: 49-0731-502-2892; E-mail: ralf.marienfeld@uni-ulm.de.

2 The abbreviations used are: TNF-α, tumor necrosis factor α; IKK, IκB kinase; EGS, ethylene glycol bis(succinimidylsuccinate); NEMO, NF-κB essential modulator; IP, immunoprecipitation; MOD, minimal oligomerization domain; MEF, murine embryonic fibroblast; WCE, whole cell extract; ERK2, extracellular signal-regulated kinase 2; GST, glutathione S-transferase; IBD, IKK-binding domain.

Phosphorylation of Serine 68 in the IκB Kinase (IKK)-binding Domain of NEMO Interferes with the Structure of the IKK Complex and Tumor Necrosis Factor-α-induced NF-κB Activity*

Received for publication, October 26, 2007 Published, JBC Papers in Press, October 31, 2007, DOI 10.1074/jbc.M708856200

Lyssan Palkowitsch†, Julia Leidner†, Sankar Ghosh§, and Ralf B. Marienfeld‖†

From the †Institute of Physiological Chemistry, Ulm University, Albert-Einstein-Allee 11, Ulm 89081, Germany and the ‡Department of Immunobiology and Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520-8011

The IκB-Kinase (IKK) complex is a multisubunit protein complex crucial for signal-induced phosphorylation of the IκB proteins and thus controls the activity of the transcription factor NF-κB. Besides the two kinases IKKα and IKKβ, the IKK complex contains NEMO/IKKγ, an additional subunit with regulatory and adaptor functions. NEMO not only confers structural stability to the IKK complex but also participates in the activation process of the IKK complex by linking the IKK subunits to upstream activators. In this study we analyze the IKKB-mediated phosphorylation of the IKK-binding domain of NEMO. In vitro, IKKB phosphorylates three serine residues in the domain of NEMO at positions 43, 68, and 85. However, mutational analysis revealed that only the phosphorylation of serine 68 in the center of the IKK-binding domain plays an essential role for the formation and the function of the IKK complex. Thus, Ser68 phosphorylation attenuates the amino-terminal dimerization of NEMO as well as the IKKB-NEMO interaction. In contrast, the NEMO-IKKα interaction was only mildly affected by the phosphorylation of Ser68. However, functional analysis revealed that Ser68 phosphorylation primarily affects the activity of IKKα. Furthermore, in complementation experiments of NEMO-deficient murine embryonic fibroblasts, a S68A-NEMO mutant enhanced, whereas a S68E mutant decreased, TNF-α-induced NF-κB activity, thus emphasizing the inhibitory role of the Ser68 phosphorylation on the signal-induced NF-κB activity. Finally, we provide evidence that the protein phosphatase PP2A is involved in the regulation of the Ser68-based mechanism. In summary, we provide evidence for a signal-induced phosphorylation-dependent alteration of the IKK complex emphasizing the dynamic nature of this multisubunit kinase complex.

The transcription factor NF-κB plays a crucial role in the initiation of innate and adaptive immune responses, in inflammation and tumorigenesis (1–3). In its inactive state NF-κB is bound to small cytoplasmic proteins, the IκB proteins. Stimulation with a wide variety of agonists, for example pro-inflammatory cytokines like TNF-α,2 bacterial components like lipopolysaccharide or by antigen receptors, funnels in the activation of a multisubunit IκB-kinase complex, which phosphorylates the IκB proteins at two specific serine residues. This phosphorylation marks the IκB proteins for proteasomal degradation setting NF-κB free, which then translocates into the nucleus and supports the expression of various pro-inflammatory or anti-apoptotic gene products. The IκB kinase (IKK) complex is composed of two catalytically active subunits, termed IKKα (IKK1) and IKKβ (IKK2), as well as NEMO/IKKγ, a subunit with regulatory and adaptor functions (4, 5). Analysis of mice deficient for either IKKα or IKKβ suggested that the IKKβ subunit is the major IKK regulating the canonical NF-κB pathway, and the IKKα subunit is crucial for a second, alternative NF-κB pathway leading to the activation of RelB-p52 heterodimers (6, 7). In contrast to the canonical NF-κB pathway, which depends on the presence of NEMO, the alternative pathway is NEMO-independent but requires the protein kinase NIK. However, recent data suggest that, instead of a clear assignment of IKKα and IKKβ to the alternative and the canonical NF-κB pathway, respectively, both kinases contribute to the activation of NF-κB by the canonical pathway with only gradual differences (8, 9).

Although a precise model regarding the molecular mechanism underlying IKK activation is still missing, it became clear that various post-translational modifications at different IKK subunits are involved in this process. Besides the phosphorylation of IKKα and IKKβ at two serine residues in their T-loop, the ubiquitination and occasionally the phosphorylation at a specific serine residue of NEMO seem to be required (10–12). This serine residue at position 85 of NEMO has been identified as a protein kinase Cθ, and more recently as an ATM target-site crucial for the IKK activation induced by genotoxic stress (12, 13). In addition, overexpression of the TNF-α-receptor 1 or the human T-cell lymphotrophic virus 1 Tax protein induces the IKKβ-mediated NEMO phosphorylation at several
additional serine residues, primarily at the serine residues 31 and 43 in the amino terminus and 376 and 377 in the carboxyl terminus of NEMO (14). However, no physiological functions for these IKKβ-mediated NEMO phosphorylation steps have been described. Ubiquitination of the carboxyl terminus of NEMO is another crucial step in the TNF-α or TCR-induced NF-κB activation process. One hypothesis is that the ubiquitination either leads to a structural alteration of the IKK complex, probably inducing a proximity-induced IKK activation (10, 16, 17), or might be important for the interaction of the IKK complex with upstream regulators for example the TNF-α-induced NEMO-RIK interaction (18, 19). However, post-translational modification of NEMO, like its phosphorylation or ubiquitination, might also positively influence its oligomerization status, which was for instance observed upon TNF-α stimulation or Tax overexpression (20–22). Two different domains mediate the oligomerization of NEMO, one domain, the minimal oligomerization domain (MOD), is located in the carboxyl terminus, spanning amino acids 246–365, and mediates a NEMO trimerization; the second domain is located in the amino terminus overlapping the IKK-binding domain of NEMO (23, 24). Importantly, the formation of both homotypic NEMO interactions is necessary for a functional IKK complex. Thus, peptides interfering with the carboxyl-terminal oligomerization like the BA-CC2 and BA-LZ peptides have been used to inhibit the signal–induced NF-κB activation (25). In contrast, peptides corresponding to the IKK-binding domain of NEMO have either no or at best a mild effect, probably due to the additional binding of these peptides to IKKα or IKKβ (24).

Here, we analyzed the impact of the phosphorylation of the amino-terminal IKK-binding domain of NEMO. Three serine residues are located in the α-helical part of the IKK-binding domain of NEMO at positions 43, 68, and 85. We show here that all three serine residues are IKKβ target sites in vitro. However, by using NEMO mutants with either phospho-inhibiting serine to alanine or phospho-mimetic serine to glutamic acid residues, we observed in the IKKβ-NEMO interaction. As a consequence we observed a significant reduction in the TNF-α-induced NF-κB activity in NEMO-deficient cells reconstituted with the phospho-mimetic S68E-NEMO mutant. Collectively, our data suggest that the activity of the IKK complex is negatively regulated by the NEMO phosphorylation at position Ser68.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Stable and Transient Transfection, and Reagents**—Human 293 cells (obtained from ATCC), NEMO-deficient murine embryonic fibroblasts (MEFs), and normal wild-type MEFs were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). For transient transfections of 2 × 10⁵ 293 cells, the CaPO₄-transfection method was used according to standard protocols. Stable transfection of MEFs was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Human recombinant TNFα was purchased by R&D Systems (Minneapolis, MN), and okadaic acid was from Calbiochem.

**Plasmids and Antibodies**—Expression vectors encoding FLAG-tagged human full-length NEMO, NEMO1–197, NEMO1–197 wild type and dominant negative IKKβ, Xpress-IKKα, and Xpress-IKKβ have been described elsewhere (26). The 3xFLAG firefly luciferase reporter plasmid and the Renilla luciferase reporter plasmid were described previously (27). The GST-NEMO expression vectors were generated by inserting the wild type or the mutated NEMO cDNA in-frame into the EcoRI and Xhol sites of the pGex 4T1 vector. Specific antibodies recognizing the FLAG-epitope or the Xpress-epitope were purchased from Sigma and Invitrogen, respectively. Antibodies specific for IKKα, IKKβ, 1xβ, ERK2, or NEMO were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The site-directed mutagenesis was performed using the Quick-Change site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol.

**Immunoprecipitation, GST Pulldown, and Immunoblotting**—The immunoprecipitation and immunoblotting procedures were performed as described previously (27). In brief, 0.5–1 μg of protein extracts were mixed with 25 μl of bovine serum albumin-blocked FLAG-antibody (M2, Sigma) linked to agarose beads. The samples were incubated for 1–12 h at 4 °C with agitation. After incubation, the precipitates were washed extensively in TnT buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1% Triton X-100, 1 mM PMSF, 2 μM leupeptin, 1 mM dithiothreitol). The resulting immunopurified proteins were used for immunoblotting experiments. In case of the GST pulldown analysis 1 μg of the GST fusion protein was mixed with 1 μl of the [35S]-labeled in vitro translated protein sample and incubated under agitation for 1 h prior to a pulldown assay with 25 μl of glutathione-conjugated beads (Sigma). The samples were washed extensively with TnT buffer and separated on a SDS-gel. The gel was stained with Coomassie, fixed, dried, and subjected to autoradiography. For the immunoblotting analysis, either the immunopurified protein complexes, or, as indicated, 50–100 μg of a protein extract were loaded on a standard SDS-polyacrylamide gel (PAA; polyacrylamide). SDS-PAGE and the transfer to nitrocellulose (Schleicher & Schuell) or nylon membranes (Immobilon polyvinylidene difluoride-membrane, Millipore) were performed using standard protocols. The membrane was blocked with 5% milk powder in TBS+Tween 20 prior to the incubation with the primary antibody (1:1000 in TBS+Tween 20), subsequently washed three times for 5 min each, and incubated in a TBS-Tween 20 solution containing either horseradish peroxidase-conjugated or IRDye700/800-conjugated secondary antibody (1:5000). The detection was performed using either ECL substrates from Amersham Biosciences or the Odyssey infrared scanning system (LICOR).

**In Vitro Kinase Assay and in Vivo Phosphorylation Studies**—For the in vitro kinase assay the indicated proteins were individually expressed in 293 HEK cells prior to an anti-FLAG immunoprecipitation. The resulting immunocomplexes were washed extensively with TnT and finally with kinase-assay buffer to equilibrate the samples. Beads carrying either the immunopurified IKK or the immunopurified NEMO proteins were mixed, and the kinase reaction was performed at 30 °C for 30 min after adding 10 μCi of [γ-32P]ATP in kinase reaction
buffer. The samples were subsequently washed extensively with TNT buffer and phosphate-buffered saline prior to a separation by SDS-PAGE. The separated proteins were transferred to nitrocellulose membrane, and the phosphorylation was monitored by autoradiography. For the in vivo phosphorylation studies 293 HEK cells were transiently transfected with the indicated expression vectors. After 24 h the cells were incubated for further 18 h in phosphate-free Dulbecco’s modified Eagle’s medium with 5% dialyzed calf serum prior to incubation with 100 \( \mu \text{Ci/ml} \) \( ^{32} \text{P} \) orthophosphate. The cells were treated as indicated, harvested, and lysed in TNT, and the resulting extracts were subjected to an anti-FLAG IP. The precipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and the resulting membrane was used for autoradiography to monitor the phosphorylation and subsequently subjected to immunoblot analysis.

**Luciferase Reporter Assay**—For the reporter gene assays, 293 cells were transiently transfected as described above. In general, we used 200 ng of the NF-κB-dependent reporter construct along with 15 ng of a harvested, and lysed in TNT, and the resulting extracts were subjected to an anti-FLAG IP. The precipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and further 18 h in phosphate-free Dulbecco’s modified Eagle’s medium with 5% dialyzed calf serum prior to incubation with 100 \( \mu \text{Ci/ml} \) \( ^{32} \text{P} \) orthophosphate. The cells were treated as indicated, harvested, and lysed in TNT, and the resulting extracts were subjected to an anti-FLAG IP. The precipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and the resulting membrane was used for autoradiography to monitor the phosphorylation and subsequently subjected to immunoblot analysis.

**RESULTS**

**IKKβ Phosphorylates the IKK-binding Domain of NEMO at Multiple Sites in Vitro**—Oligomerization of the adaptor protein NEMO is crucial for the function of the IKK complex (23, 25, 28). Yet, whether the activity of the IKK complex is regulated by an alteration of the oligomerization of NEMO described by Poyet et al. remains unclear (20). Interestingly, NEMO is a phospho protein targeted by different serine/threonine kinases in vivo and in vitro, and the identified serine target sites are located in both oligomerization domains (14, 29). In this study we analyzed the role of the IKKβ-dependent phosphorylation at different serine residues at positions 43, 68, and 85 within in the amino-terminal IKK-binding domain (IBD) of NEMO for the activity of the IKK complex. To determine which of the three serine residues are phosphorylated by IKKβ in vitro, we performed in vitro kinase assays using ectopically expressed wild-type FLAG-NEMO or NEMO mutants with serine to alanine or cysteine substitutions at the positions 43 (S43A), 68 (S68A), or 85 (S85C) either separately or combined as a triple mutant (SIIA/C/NEMO) alone or in conjunction with separately expressed FLAG-tagged IKKβ (for a schematic representation of the NEMO mutants used and the location of the serine residues see Fig. 1A). As depicted in Fig. 1B, we observed a high IKKβ-mediated phosphorylation of all FLAG-NEMO variants, which remained unchanged by the substitution of the single serine residues or by the combined substitution of all three serine residues (Fig. 1B, lanes 6–11). In contrast, none of the NEMO proteins were significantly phosphorylated in the absence of FLAG-tagged IKKβ (Fig. 1B, lanes 1–5) or in the presence of a dominant negative mutant of IKKβ (Fig. 1C, lanes 7–12). How-
ever, previous studies suggested that the phosphorylation of a specific IKK target site in NEMO might be masked by other phosphorylation events in NEMO due to the variety of IKK target sites in this protein (14, 29). Consistently, we observed a significant reduction of the IKKβ-mediated NEMO phosphorylation with all three single mutants (Fig. 1D) when we compared the different serine mutants with a wild-type version in the background of an amino-terminal FLAG-NEMO fragment, spanning the first hundred amino acids of NEMO in vitro kinase assay. However, the reduction in the in vitro phosphorylation of S85C-NEMO1–100 is less pronounced (for a quantification see Fig. 1D, middle panel). Moreover, the effect on the IKKβ-mediated NEMO phosphorylation was even more dramatic in case of the SIIIA/C-NEMO1–100 triple mutant (Fig. 1D, lane 5). Based on these data, we concluded that all three serine residues located in the IBD, Ser43, Ser68, and Ser85, are IKKβ target sites in vitro.

Phosphorylation of Serine 68 in the IKK-binding Domain of NEMO Attenuates the Amino-terminal NEMO Dimerization—Because the IKK-binding domain of NEMO is also crucial for the amino-terminal NEMO dimerization, we next compared the dimerization potential of the three single NEMO1–100 point mutants and the wild-type NEMO1–100. As shown in Fig. 2A, all mutants tested displayed an increased dimer formation, however, the dimerization of S68A-NEMO1–100 was significantly pronounced (Fig. 2A, lane 3), arguing for a critical role of Ser68 for the NEMO dimerization. The critical role of Ser68 was further supported by experiments with ectopic expressed full-length NEMO-proteins. Again, we observed an enhanced NEMO dimerization in case of S68A-NEMO and the SIIIA/C-triple mutant (Fig. 2B, lanes 4 and 6; right side: quantification of the dimer/monomer ratio in %). More strikingly, imitating the Ser68 phosphorylation by the substitution of Ser68 with a phospho-mimetic glutamic acid led to a significant decrease in NEMO dimerization (Fig. 2C, lane 4), further emphasizing the negative effect of the Ser68 phosphorylation on the amino-terminal NEMO dimerization. In order to analyze whether the Ser68 phosphorylation alters the global oligomerization status of NEMO, we treated whole cell extracts (WCEs) from 293 cells transfected with wild-type, S68A-, or S68E-NEMO with the cross-linker EGS. Without cross-linking S68A-NEMO slightly enhanced, and S68E-NEMO reduced, the formation of NEMO-dimers and higher molecular weight complexes (NEMOX) compared with wild-type NEMO (lanes 2–4). In addition, the NEMO-dimers mostly disappeared upon heating the samples for 10 min at 95 °C (lanes 6–8) with the exception of a remaining S68A-NEMO dimerization, thus once more demonstrating the positive effect of this mutation on the amino-terminal NEMO dimerization. However, cross-linking the proteins with EGS converted the different NEMO variants equally in NEMO-

FIGURE 2. The phosphorylation of Ser68 has a negative effect on the amino-terminal NEMO dimerization. Anti-NEMO immunoblot analysis of whole cell extracts from 293 cells ectopically expressing either NEMO1–100 mutants with serine to alanine/cysteine substitutions (A), S to A/C mutants (B), or S to E (C) mutants of full-length NEMO, as indicated. The NEMO triple mutant is indicated as SIIIA/C (B, right panel). NEMO-monomers (NEMO) and NEMO-dimers (NEMOX) are indicated. In addition, the NEMO dimer/monomer ratio (in %) for the different S to A mutants estimated in three independent experiments is shown in the right part of B, D; whole cell extracts from 293 cells ectopically expressing wild-type NEMO, S68A-NEMO, or S68E-NEMO were either left untreated (left and middle panels) or were subjected to a cross-linking reaction with EGS (right panel). In addition, a fraction of the untreated whole cell extracts was heated to 95 °C for 5 min (middle panel) prior to an anti-NEMO immunoblot assay. The location of the NEMO-monomer, -dimer (NEMO2) and higher molecular NEMO complexes (NEMOX) is indicated.
dimers and NEMO-based high molecular weight protein complexes (Fig. 2D, lanes 10–12), suggesting that only the amino-terminal dimerization, but not the global oligomerization status of NEMO is affected by the phosphorylation of Ser68, most likely due to an unaffected carboxyl-terminal oligomerization of NEMO.

Phosphorylation of Ser68 Has a Negative Effect on the IKKβ-NEMO Interaction—Having established the negative effect of the Ser68 phosphorylation on the NEMO dimerization, we next analyzed whether the interaction of NEMO with IKKα or IKKβ is also altered by the substitution of Ser43, Ser68, or Ser85. For this, we performed an in vitro interaction study with ectopically expressed FLAG-tagged NEMO proteins in combination with in vitro transcribed/translated IKKα or IKKβ. Similar to the NEMO dimerization, only the IKKβ interaction with S68E-NEMO was significantly reduced (Fig. 3A, lane 5, middle panel), whereas the IKKα interaction of S68E-NEMO was, if at all, only slightly affected (Fig. 3A, lane 5, upper panel). The specific negative effect of the S68E substitution on the IKKβ-NEMO interaction was also evident in in vivo interaction studies of IKKβ with the different NEMO variants (Fig. 3C, lane 4) in co-immunoprecipitation assays, whereas the S68E-NEMO-IKKα interaction was only slightly reduced (Fig. 3B, lane 4).

Interestingly, a similar negative effect on the IKKα interaction was also observed with the S85C-NEMO mutant (Fig. 3B, lane 8). Moreover, the S68A-NEMO mutant displayed an enhanced IKKβ interaction as shown in a head-to-head comparison of wild-type NEMO, S68E-, and S68A-NEMO (Fig. 3D, upper panel). However, because under these experimental conditions additional post-translational modifications of the ectopic expressed NEMO proteins could influence the interaction with the IKKs, we performed another in vitro interaction study where we compared the interaction of in vitro translated, 35S-labeled IKKα (left part), or IKKβ (right part).

Ser68 Is a Phospho-acceptor Site in Vivo—In a previous study serine 43, but not serine 68, has been identified as major in vivo IKKβ target site in NEMO (14). Given the specific importance
of the Ser68 phosphorylation for the NEMO dimerization and the NEMO-IKKβ interaction, we next asked whether Ser68 is also a phospho-acceptor site in vivo. Although the increased NEMO dimerization (Fig. 2) and IKKβ-NEMO interaction (Fig. 3E) observed with S68A-NEMO already suggested a basal in vivo phosphorylation of Ser68, we also wanted to prove the in vivo phosphorylation of NEMO at Ser68 more formally. For this, 293 HEK cells, transiently transfected with expression vectors for wild-type NEMO, S43A-NEMO, or S68A-NEMO, were metabolically labeled with [32P]orthophosphate and stimulated with TNF-α. As shown in Fig. 5A, all NEMO variants used display a high basal phosphorylation status. Interestingly, the basal phosphorylation of S68A-NEMO was even slightly enhanced. Because we observed in a similar in vivo phosphorylation experiment a strong reduction of the basal NEMO phosphorylation by co-transfection of a dominant negative mutant of IKKβ (Fig. 5B), we can conclude that the basal NEMO phosphorylation was caused by IKKβ. Furthermore, TNF-α stimulation augmented only the phosphorylation of wild-type NEMO, but not of S43A-NEMO or S68A-NEMO (Fig. 4A, lanes 5, 6, 9, 11, and 12). Based on the result from the in vitro kinase assay (Fig. 1) as well as previously published data (29) we assumed that an alteration in the phosphorylation of a single serine residue in NEMO could be masked by additional phosphorylation events. We used therefore NEMO1–100 mutants (wild-type, S43A, or S68A) in a similar in vivo phosphorylation study. Again, we observed a high basal phosphorylation of the three used NEMO1–100 variants. However, the basal phosphorylation of S43A-NEMO1–100 and of S68A-NEMO1–100 was significantly reduced in comparison to the wild-type NEMO1–100 (Fig. 4C, compare lanes 4, 7, and 10, a quantification of the relative phosphorylation is given in the right part). Moreover, we observed a decrease in the phosphorylation of all NEMO1–100 variants upon stimulation with TNF-α. Taken together, we concluded from these results that Ser68 is constitutively phosphorylated in vivo and that the amino terminus of NEMO is dephosphorylated upon TNF-α stimulation.

**NEMO Phosphorylation at Ser68 Exerts a Negative Effect on the Proximity-induced IKKα Activity**—To analyze the functional consequences of the Ser68 phosphorylation, we first analyzed the proximity-induced activation of IKKα and IKKβ. This assay is based on the augmentation of IKKα or IKKβ activity due to the trans-autophosphorylation of the IKK-moieties by the NEMO-mediated cross-linking of IKK-dimers (20). As shown in Fig. 5A, the addition of NEMO led to a significant increase in IKKα activity, whereas the already higher IKKβ activity is only mildly enhanced by wild-type NEMO (Fig. 5B) as monitored by a NF-κB-dependent luciferase assay. In addition, the mutation of Ser68 had different consequences for the NEMO-induced activity of IKKα and IKKβ. Whereas S68A-NEMO induced the IKKα activity even more and S68E-NEMO less efficiently then wild-type NEMO, thus suggesting a negative effect of Ser68 phosphorylation on the NEMO-induced IKKα activation (Fig. 5A), we observed a negative effect in case of both NEMO mutants, S68A-, and S68E-NEMO, on the NEMO-induced IKKβ activity (Fig. 5B). Furthermore, the positive effect on the activity of IKKα was specific for the alanine substitution at Ser68, because neither S43A- nor S85C-NEMO

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**FIGURE 4. Analysis of the Ser68 phosphorylation in vivo**. A, 293 cells transiently transfected with expression vectors for wild-type NEMO, S43A-NEMO, or S68A-NEMO were metabolically labeled with [32P]orthophosphate for 4 h prior to the stimulation with TNF-α as indicated. The resulting whole cell extracts were subjected to an anti-FLAG IP, and the precipitates were transferred to nitrocellulose membrane prior to autoradiography (upper panel) and a subsequent anti-FLAG IB (lower panel) to check for equal expression. Of note, the weak signals seen in lanes 2 and 3 are due to an incorrect transfer of the immunoprecipitated wild-type NEMO protein and don’t represent a specific signal in these samples. B, a similar in vivo labeling experiment was performed with whole cell extracts from 293 cells ectopically expressing FLAG-tagged NEMO alone (lane 2) or in combination with a dominant negative version of FLAG-IKKβ (lane 3). C, cells were transfected with expression vectors for wild-type NEMO1–100 (lanes 4–6), S43A-NEMO1–100 (lanes 7–9), or S68A-NEMO1–100 (lanes 10–12) prior to an in vivo labeling experiment similar to A. A quantification of the phospho-NEMO1–100 variants is depicted on the right side.
IKK Complex Alterations by NEMO Phosphorylation

FIGURE 5. Effect of Ser^{68} phosphorylation on the NEMO-induced activation of IKKα and IKKβ. For the proximity-induced IKK activation 293 HEK cells were transiently transfected with the indicated amounts of Xpress-tagged NEMO variants as indicated. After 24 h the cells were lysed, and a luciferase analysis was performed according to the manufacturer’s protocol. C, as control, the indicated amounts of the NEMO variants were used without IKK cotransfection in a similar reporter gene assay. D, the specificity of the Ser^{68A}-NEMO effect on IKKα activity was analyzed in a similar proximity-induced IKK activation assay performed with wild-type NEMO, S43A-NEMO, S68A-NEMO, and S85C-NEMO alone (left part) or in conjunction with IKKα (right part). E, 293 cells were transiently transfected with the indicated expression vectors in conjunction with the NF-κB reporter plasmid and a Renilla luciferase construct. After 24 h the cells were harvested, and the luciferase activity was determined.

had a similar effect (Fig. 5C), and no further increase in IKKα activity was observed by the SIIIIA/C-NEMO triple mutant (Fig. 5E). Moreover, neither the transfection of wild-type NEMO nor the transfection of the various NEMO mutants alone had a positive effect on the basal NF-κB activity (Fig. 5, C and 5D, left part). We recently demonstrated that the amino-terminal dimerization domain is sufficient to augment the IKK activity independently of the central MOD of NEMO. To further delineate whether the Ser^{68} phosphorylation in a deletion mutant of NEMO lacking the MOD (NEMO_{1–197}) has a similar effect on the IKK activity, we performed proximity-induced IKKα activity assays using wild-type, S68A-, and S68E-NEMO_{1–197}. Here, the effect of S68A-NEMO_{1–197} on the IKKα activity was even more pronounced compared with full-length NEMO (Fig. 6A). Furthermore, the co-transfection of S68E-NEMO_{1–197} had even a slight inhibitory effect on the IKKα activity (Fig. 6A), whereas the expression of the different NEMO mutants alone had no effect (Fig. 6C). In addition, the S68A-NEMO_{1–197}-mediated increase in NF-κB activity was paralleled by a significant increase in T-loop phosphorylation of IKKα as monitored by an anti-phospho-IKK immunoblot analysis (Fig. 6B, lanes 5 and 6), whereas wild-type NEMO_{1–197} induced only a modest IKKα phosphorylation and S68E-NEMO_{1–197} had no effect on the IKKα phosphorylation. Taken together, these data show that the phosphorylation of Ser^{68} has different effects on the activity of IKKα and IKKβ, and the amino terminus of NEMO is sufficient to mediate the Ser^{68} effect.

Phosphorylation of Ser^{68} Exerts a Negative Effect on the TNF-α-induced NF-κB Activity—To determine the functional consequences of Ser^{68} phosphorylation for the signal-induced NF-κB activity, we stably reconstituted NEMO-deficient MEFs with expression vectors encoding either murine wild-type NEMO, S68A-NEMO, S68E-NEMO, or S85C-NEMO alone (left part) or in conjunction with IKKα (right part). E, 293 cells were transiently transfected with the indicated expression vectors in conjunction with the NF-κB reporter plasmid and a Renilla luciferase construct. After 24 h the cells were harvested, and the luciferase activity was determined.
IKK Complex Alterations by NEMO Phosphorylation

Inhibition of PP2A Attenuates the NEMO Dimerization and the IKKβ-NEMO Interaction—Various protein phosphatases have been implicated in the regulation of the NF-κB system. Particularly PP2A has been demonstrated to interact with all three core subunits of the IKK complex, yet controversial results about the role of this phosphatase have been reported. Whereas several groups described a negative effect of PP2A on the IKK activity due to the dephosphorylation of the serine residues in the activation loop of IKKβ, another study reported a positive influence of PP2A on the IKK complex depending on the NEMO-PP2A interaction (30, 31). To analyze the impact of PP2A on the NEMO dimerization we treated 293 HEK cells with increasing concentrations of the PP2A-inhibitor okadaic acid (Fig. 8A). Indeed, we observed a significant reduction of NEMO dimerization with okadaic acid concentrations from 400 to 800 nM (Fig. 8A, upper panel, lanes 3–5). Furthermore, this loss of NEMO dimerization correlated with the induction of a slower migrating NEMO signal (Fig. 8A, middle panel, lanes 3–5), most probably a hyperphosphorylated form of NEMO, and a loss of IkBa (Fig. 8A, lower panel, lanes 3–5), indicating the activation of the IKK complex. To further investigate whether the combination of PP2A inhibition and IKK activity is required for the reduction of the amino-terminal NEMO dimerization, we treated cells with a suboptimal okadaic acid concentration of 200 nM in combination with the NF-κB agonist phorbol 12-myristate 13-acetate plus ionomycin (Fig. 8B). Consistent with our previously published result, the stimulation with phorbol 12-myristate 13-acetate plus ionomycin alone had no influence on the NEMO-dimer formation (Fig. 8B, lanes 1–5). However, in combination with the low concentration of okadaic acid, we observed a reduction in NEMO dimer formation, which correlated with the appearance of the slower migrating NEMO signal and the prolonged activation of the IKK complex as monitored by IkBa protein levels (Fig. 8B, lanes 6–10). To assess whether also the IKKβ-NEMO interaction is affected by the inhibition of PP2A by okadaic acid, we performed co-immunoprecipitation experiments with ectopically expressed NEMO wild-type or S68A-NEMO alone or in combination with FLAG-tagged IKKβ (Fig. 8C). The cells were either left untreated or were treated with 200 nM okadaic acid, and the level of IKK2-NEMO interaction was subsequently analyzed by an anti-FLAG IP. Upon treatment with okadaic acid, we observed a significant reduction in IKKβ-NEMO interactions with both wild-type and S68A-NEMO (Fig. 8B, upper panel, compare lanes 4 and 5 with lanes 11 and 12), and even with the endogenous NEMO (Fig. 8C, lanes 4 and 10). Consistent with the previous experiments, treatment with 200 nM okadaic acid alone had only a minor effect on the NEMO dimerization. However, the okadaic acid treatment in conjunction with the IKKβ cotransfection led to a distinct reduction in NEMO dimer formation with a remaining dimer formation only in case of S68A-NEMO (Fig. 8C, lane 12). Thus, these data support our hypothesis that the IKKβ-NEMO interaction and the NEMO dimerization are subject to a phosphorylation-dependent alteration. However, our results also suggest that Ser68 is not the only target site involved in this process.
FIGURE 7. Ser<sup>68</sup> phosphorylation has a negative influence on the TNF-α-induced NF-κB activity in vivo. A, 50 μg of whole cell extracts from NEMO-deficient MEFs stably reconstituted with either empty vector, murine wild-type NEMO, S68A-NEMO, or S68E-NEMO was used for an anti-NEMO immunoblotting analysis. The samples were either left untreated (lanes 1–4) or were heated to 95 °C for 5 min (lanes 5–8). B, for the anti-IKBα and anti-ERK2 immunoblot analysis 40 μg of cytoplasmic extracts of untreated or TNF-α-stimulated cells was analyzed by standard procedures. C, the different reconstituted cell lines as well as normal MEFs were transiently transfected with 200 ng of a B-dependent luciferase reporter-construct in conjunction with 30 ng of a Renilla luciferase reporter. After 24 h the cells were stimulated with 40 ng/ml TNF-α and further incubated for 4 h. The cells were subsequently lysed, and the luciferase activity was estimated. D, for the electrophoretic mobility shift assay experiment 10 μg of nuclear proteins from the different unstimulated or TNF-α-induced cell lines was incubated with a <sup>32</sup>P-labeled B-specific oligonucleotide. The samples were separated on a 4% native acrylamide gel, and the gel was dried and used for autoradiography. To determine the specificity of the NF-κB-signals (complexes I and II) nuclear protein of S68A-NEMO cells, stimulated with TNF-α for 40 min, were either subjected to a competition experiment using 100-fold excess of the cold B-specific probe (lane 21) or with 0.2 μg of an anti-RelA antibody (lane 22).

DISCUSSION

Although the current model envisions a relatively stable core IKK complex, composed of NEMO, IKKα, and IKKβ, largely unaffected by stimuli-induced alterations, several previously published studies suggest a more dynamic nature of this protein complex. For example, an increase in the molecular weight of the IKK complex upon stimulation with TNF-α, interleukin-1, or Tax cotransfection has been linked to an enhanced NEMO oligomerization (20, 21). In contrast, the biochemical analysis of the MOD or the amino-terminal NEMO dimerization revealed no signal-induced alteration of the NEMO oligomerization status (23, 24), although interference with NEMO oligomerization using peptides corresponding to the carboxy-terminal MOD of NEMO attenuated the IKK activity (25). Given the contradictory results regarding the signal-responsiveness of the NEMO oligomerization we set out to analyze the potential role of different serine phosphorylations in the IKK-binding domain of NEMO for the protein interactions in the IKK complex. The IBD encompasses amino acids 47–120 of NEMO and not only mediates the interaction of NEMO with IKKα and IKKβ, but is also crucial for a SDS-resistant NEMO dimerization (24). Previous studies identified Ser<sup>31</sup>, Ser<sup>43</sup>, and Ser<sup>85</sup> in the amino terminus and Ser<sup>376</sup> and Ser<sup>377</sup> in the carboxy terminus of NEMO as IKKβ, or protein kinase C, or ATM target sites (12, 14). But in contrast to the phosphorylation of Ser<sup>85</sup>, which is involved in the regulation of NF-κB upon genotoxic stress, a functional role for the phosphorylation of Ser<sup>31</sup> and Ser<sup>43</sup> has not been reported. Consistent with these findings we also observed only for the substitution of Ser<sup>68</sup>, but not of Ser<sup>31</sup> or Ser<sup>43</sup>, an effect on the amino-terminal NEMO dimerization and NEMO-IKKβ interaction (data not shown and Figs. 3 and 4). The reason for the negative effect on the NEMO dimerization might be the additional negative charge introduced by the phosphorylation or by the substitution of Ser<sup>68</sup> with the acidic glutamic acid in the amphiphilic α-helical coiled-coil domain, which weakens this protein-protein interaction. A similar molecular mechanism might account for the reduced IKK-NEMO interaction. Here, the additional negative charge at Ser<sup>68</sup> might act in concert with the phosphorylation of several previously described serine residues in the carboxyl terminus, including a serine residue in the NEMO-binding domain of IKKβ leading to a repulsion of the IKKβ-NEMO interaction (26, 32). Yet, it remains unclear whether the reduced IKKβ-NEMO interaction and amino-terminal NEMO dimerization caused by the Ser<sup>68</sup> phosphorylation of NEMO lead to a disassembly of the IKK complex. For instance, no striking difference was observed after cross-linking the different NEMO variants with EGS, which suggests that the global oligomerization status of NEMO remains unaffected by Ser<sup>68</sup>, which is probably due to the central MOD of NEMO. Furthermore, because the IKKα-NEMO interaction also remains unchanged, an IKKα-IKKβ heterodimer would most likely bind efficiently to NEMO regardless of the phosphorylation status at position Ser<sup>68</sup>. In this respect it is also important to note that a remaining IKKβ-NEMO interaction was still observed even with the S68E-NEMO mutant. Additional factors might con-
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NEMO (were subjected to immunoblot analysis with specific antibodies for either 293 HEK cells treated with the indicated concentrations of okadaic acid for 2 h of the IKK complex, which is in line with the reported positive effect of the PP2A on IKK activity, which is crucial during the initiation phase of the IKK activation (31).

The TNF-α-induced dephosphorylation of the amino terminus of NEMO in our in vivo phosphorylation studies implies that a phosphatase activity might be involved in the activation process of the IKK complex, which is in line with the reported positive effect of the PP2A on IKK activity, which is crucial during the initiation phase of the IKK activation (31). The reduced IKKβ-NEMO interaction and reduced NEMO dimerization observed in cells treated with okadaic acid, a potent PP2A inhibitor (Fig. 8), further support this hypothesis and thus an altered stability of the IKKβ-NEMO interaction or of the NEMO dimerization might contribute to the positive role of PP2A during the activation process of the IKK complex. Yet, the incomplete stability of the IKKβ interaction and dimerization of S68A-NEMO in okadaic acid-treated cells (Fig. 8C) as well as the inducible dephosphorylation of NEMO<sub>1-100</sub> (Fig. 4C) even after inactivation of Ser<sup>68</sup> suggests that a combination of different post-translational modifications in NEMO and the IKKs could account for a more pronounced effect. One example for such an additional post-translational modification, probably involved in the regulation of the IKK activity, is the already mentioned Ser<sup>740</sup> phosphorylation in the NEMO-binding domain in IKKβ. Although a negative role of Ser<sup>740</sup> phosphorylation for IKKβ activity has been described (26, 32), an alteration of the IKKβ-NEMO interaction was not observed. However, when we combined both phospho-mimetic mutants, S68E-NEMO and S740E-IKKβ, the negative effect on the IKKβ-NEMO interaction was even more pronounced than the effect achieved with the S68E mutation alone (data not shown). This opens the possibility that the fine-tuning of the activity of the IKK complex might be achieved by a set of accessory phosphorylation steps in both, the NEMO and the IKK proteins.

Given that only the IKKa but not the IKKβ activity was augmented by S68A-NEMO in the proximity-induced IKK activity assay, the increase in the TNF-α-induced NF-κB activity observed in the S68A-NEMO-reconstituted MEFs is somewhat surprising, because IKKβ but not IKKa is considered to be the major IκB kinase in the canonical NF-κB signaling pathway, based on the studies with IKKa- and IKKβ-deficient mice or with mice expressing exclusively a dominant negative mutant of IKKa (IKKaAA) (6, 7, 33). However, more recent studies using conditional knockout models or MEFs deficient in IKKa or IKKβ suggest that IKKa can at least partially compensate a lack of IKKβ (8, 34, 35). In addition, several studies implicated a regulatory role of IKKa for the activity of IKKβ (36, 37). In view of these studies, an induction of the IKKa moieties of the heterotrimeric IKK complex could account for the augmented NF-κB activity. One reason for the different effect of S68A-NEMO on the proximity-induced activation of IKKa and IKKβ might be the exclusive negative effect of the Ser<sup>68</sup> phosphorylation on the IKKβ interaction (Fig. 3A). Consistently, IKK mutants lacking the carboxy-terminal NEMO-binding domain and thus incapable to bind to NEMO display a significantly higher basal activity (26, 32). In contrast to the different NEMO interaction, the decrease in the amino-terminal NEMO dimerization by Ser<sup>68</sup> phosphorylation would pertain to the activity of both kinases, IKKa and IKKβ. As a consequence, the lack of Ser<sup>68</sup> phosphorylation in S68A-NEMO induced an increased IKKa activation and trans-autophosphorylation mediated by the strengthened amino-terminal NEMO dimerization (Fig. 6). On the other hand, additional post-translational modifications might be induced by the S68A substitution and might influence the IKKβ-NEMO relationship. For example, we always observed an increased basal in vivo phosphorylation of the S68A-NEMO (Fig. 4A). Importantly, the differential effect of the Ser<sup>68</sup> phosphorylation on the IKKa or IKKβ interaction could have variable effects on IKK complexes depending on whether they contain IKKa-IKKβ heterodimers or homodimers of IKKa or IKKβ. Indeed, differences in the activity and regulation of IKKa-IKKβ heterodimers and IKKβ-homodimers

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IKK Complex Alterations by NEMO Phosphorylation

have been observed (15). Furthermore, the recently described IKKa-NEMO and IKKB-NEMO heterodimers suggest the existence of different subpools of IKK complexes with a different composition regarding IKKa-homodimers, IKKB-homodimers, and/or IKKa-IKKB-heterodimers (22).

In conclusion our model of the role of the Ser<sup>68</sup> phosphorylation would predict that this serine residue is phosphorylated under basal conditions but will be dephosphorylated upon activation of the IKK complex by PP2A or another protein phosphatase leading to an enhanced amino-terminal NEMO dimerization and IKKB-NEMO interaction as part of the IKK activation program. The phosphorylation status of Ser<sup>68</sup> is later restored by the active IKKs. Importantly, the Ser<sup>68</sup> phosphorylation does not completely impair the activation of the IKK complex; hence, it rather seems to act as additional step in fine-tuning the NF-κB activity. Thus, several interesting questions remain to be answered in future studies. For example, the role of Ser<sup>68</sup> phosphorylation should be further explored, because we frequently observed a reduced IKKα interaction (Fig. 3B, lane 8) and IKKα activation (Fig. 5D) by S85C-NEMO. Furthermore, it is likely that several additional post-translational modifications, like the Ser<sup>740</sup> phosphorylation in the NEMO-binding domain of the IKKs, will intensify the Ser<sup>68</sup> effect. The analysis of these post-translational modifications and the identification of the participating phosphatase(s) are currently in progress in our laboratory.

Acknowledgments—We thank Prof. Michael Karin for the NEMO-deficient MEFs and Prof. Thomas Wirth and Bernd Baumann for reagents and critical reading of the manuscript.

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