

Novel Phosphorylations of IKK γ /NEMO

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ABSTRACT Central to NF- κ B signaling pathways is IKK γ /NEMO, a regulatory subunit of the cytoplasmic I κ B kinase (IKK) complex, which undergoes various posttranslational modifications, specifically phosphorylation, to regulate its function. Furthermore, Kaposi's sarcoma-associated herpesvirus (KSHV) FADD-like interleukin-1 β (IL-1 β) converting enzyme (FLICE) inhibitory protein (vFLIP) activates the NF- κ B signaling pathway by directly interacting with IKK γ /NEMO. However, the exact functions of IKK γ /NEMO phosphorylation and its KvFLIP interaction in NF- κ B activation remain elusive. Here, we report two novel phosphorylation sites of IKK γ /NEMO and their negative effect on the IKK γ /NEMO-mediated NF- κ B signaling pathway. First, the Src family protein tyrosine kinases (SF-PTKs), including Src, Fyn, Lyn, and Fgr, interact with and phosphorylate tyrosine residue 374 (Y374) of IKK γ /NEMO. Mutation of the Y374 residue to phenylalanine (Y374F) specifically abolished SF-PTK-mediated tyrosine phosphorylation, leading to increased tumor necrosis factor alpha (TNF- α)-induced NF- κ B activity. Moreover, our mass spectrometry analysis found that the serine 377 residue (S377) of IKK γ /NEMO underwent robust phosphorylation upon KvFLIP expression. Replacement of the IKK γ /NEMO S377 residue by alanine (S377A) or glutamic acid (S377E) resulted in a significant increase or decrease of NF- κ B activity and TNF- α -mediated IL-6 cytokine production, respectively. Our study thus demonstrates that the Y374 or S377 residue located at the C-terminal proline-rich domain of human IKK γ /NEMO undergoes phosphorylation upon TNF- α treatment or KvFLIP expression, respectively, resulting in the suppression of IKK γ /NEMO activity to induce NF- κ B activation. This study suggests the potential phosphorylation-mediated feedback negative regulation of IKK γ /NEMO activity in the NF- κ B signaling pathway.

IMPORTANCE Since unchecked regulation of NF- κ B has been linked to uncontrolled proliferation and cell death, the downregulation of the NF- κ B signaling pathway is as important as its activation. Specifically, the phosphorylation-mediated modification of IKK γ /NEMO is a critical regulatory mechanism of NF- κ B activity. Here, we report two novel phosphorylations of IKK γ /NEMO and their negative effects on the NF- κ B signaling pathway. First, the Src family protein tyrosine kinase interacts with and phosphorylates tyrosine residue 374 of IKK γ /NEMO, suppressing tumor necrosis factor alpha (TNF- α)-induced NF- κ B activity. Additionally, Kaposi's sarcoma-associated herpesvirus (KSHV) FADD-like interleukin-1 β (IL-1 β) converting enzyme (FLICE) inhibitory protein (KvFLIP) expression induces a robust phosphorylation of the serine 377 residue of IKK γ /NEMO, resulting in a significant decrease of NF- κ B activity. Our study thus demonstrates that the Y374 or S377 residue of IKK γ /NEMO undergoes phosphorylation upon TNF- α treatment or KvFLIP expression, respectively, resulting in the suppression of IKK γ /NEMO activity to induce NF- κ B activation. This also suggests the potential phosphorylation-mediated feedback negative regulation of IKK γ /NEMO activity in the NF- κ B signaling pathway.

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Members of the NF- κ B family are transcription factors involved in the regulation of diverse cellular processes, including inflammation, innate/adaptive immunity, apoptosis, and tumorigenesis (1). Under normal conditions, the NF- κ B complex is primarily sequestered in the cytoplasm by forming a complex with a family of inhibitory proteins or I κ Bs (2). A number of biological stimuli, including proinflammatory cytokines (tumor necrosis factor alpha [TNF- α] and interleukin-1 β [IL-1 β]), microbial infection, growth factors, and ligands for antigen receptors, converge at the activation of the I κ B kinase (IKK) complex, which is composed of two structurally related catalytic subunits, IKK α and IKK β , and a noncatalytic regulatory subunit, IKK γ /NF- κ B essential modulator (NEMO) (3). Activation of the IKK complex subsequently leads to phosphorylation and ubiquitination of I κ Bs for

proteasome-mediated degradation. Released NF- κ B translocates into the nucleus and induces transcription of numerous target genes involved in cell proliferation, survival, differentiation, immune responses, and inflammatory responses (2).

Inappropriate activation of the NF- κ B signaling pathway, however, is linked with several human diseases (4, 5). Thus, under normal conditions, the NF- κ B signaling pathway in response to a variety of stimuli has to be transient and tightly regulated by both positive and negative regulatory mechanisms. While a precise regulatory mechanism underlying IKK complex activation still remains unclear, various posttranslational modifications on the different subunits of the IKK complex appear to be clearly involved in NF- κ B activation (6, 7). IKK γ /NEMO has been reported to undergo phosphorylation, sumoylation, and ubiquitination by a

number of stimuli (3, 6, 7). IKK γ /NEMO is composed of a helical domain (HLX1), coiled-coil domain 1 (CC1), a second helical domain (HLX2), another coiled-coil domain (CC2) containing a minimum oligomerization domain, a NEMO ubiquitin (Ub) binding (NUB) domain, a leucine zipper (LZ) domain, a proline-rich domain, and a zinc finger (ZF) domain (8). Extensive characterization has found that IKK γ /NEMO undergoes K63-linked polyubiquitination at the lysine residues 285 and 399 upon TNF- α and T-cell receptor (TCR) stimulation, allowing the IKK complex to be recruited to TAK1, a kinase known to phosphorylate IKK β for its activation (9, 10, 11, 12). Sumoylation, followed by the monoubiquitination of IKK γ /NEMO, has been reported to play an important role in the activation of NF- κ B upon genotoxic stress (13, 14, 15). Phosphorylation of IKK γ /NEMO at multiple serine residues has been suggested to regulate NF- κ B activity in response to various stimuli (13, 16, 17, 18). IKK γ /NEMO phosphorylation at serine residues 85 and 141 by protein kinase C (PKC) is involved in the IKK β -mediated phosphorylation of I κ B (16). Phosphorylation of IKK γ /NEMO at serine residue 85 has been identified as a crucial site by ATM for IKK activation upon genotoxic stress (13). Several additional serine residues at positions 31 and 43 in the N terminus and at positions 376 and 377 in the proline-rich domain of the C terminus of IKK γ /NEMO have been identified to undergo phosphorylations upon stimulation with inflammatory cytokines (TNF- α and IL-1), by overexpression of TNF receptor I (TNFR1), or by Tax, an oncoprotein of the human T cell lymphotropic virus type 1 (HTLV-1) (18). Mutation of the S369 residue in mouse IKK γ /NEMO (equivalent to S376 in human IKK γ /NEMO) to alanine (S369A) has been reported to increase its ability to stimulate NF- κ B activity (17). In addition, phosphorylation of IKK α and IKK β at two serine residues of their activation T loop has been shown to activate their kinase activity upon cytokine treatments (19, 20, 21, 22). These studies strongly indicate that the posttranslational modifications of each subunit of the IKK complex can play either a positive or a negative role in activation of the NF- κ B signaling pathway.

Several viruses, including HIV-1, HTLV-1, Kaposi's sarcoma-associated herpesvirus (KSHV; human herpesvirus 8 [HHV-8]), and Epstein-Barr virus (EBV), are known to induce the activation of the NF- κ B signaling pathway (23). Among them, KSHV/HHV-8, which is a main causative agent of Kaposi's sarcoma (KS) and associated with rare lymphoproliferative diseases such as primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), expresses a protein called K13 during the latent stage of the viral life cycle (24). K13, encoded by ORF71 of the KSHV viral genome, is a viral homolog of cellular FLICE (FADD-like IL-1 β converting enzyme) inhibitory protein (FLIP) and is thus referred to as KSHV viral FLIP (KvFLIP). Similarly to cellular FLIPs (cFLIPs) and several vFLIPs encoded by herpesvirus saimiri (HVS), equine herpesvirus, and poxvirus, KvFLIP contains two tandem death effector domains (DEDs) (25, 26, 27). KvFLIP has been shown to directly interact with IKK γ /NEMO, leading to the constitutive activation of the NF- κ B signaling pathway and ultimately contributing to transformation and tumorigenesis (28, 29, 30, 31). However, the exact function of KvFLIP interaction with IKK γ /NEMO in the constitutive activation of NF- κ B remains elusive.

Here, we report that Fyn, a member of the Src family protein tyrosine kinases (SF-PTKs), interacts with and phosphorylates IKK γ /NEMO at the tyrosine 374 (Y374) residue, leading to the

negative effect on the TNF- α -mediated induction of the NF- κ B signaling pathway. Moreover, KvFLIP expression resulted in the substantial increase of S377 phosphorylation of human IKK γ /NEMO, regulating the activation of TNF- α -mediated NF- κ B activation. Thus, our study demonstrates that novel posttranslational modifications in the proline-rich domain of human IKK γ /NEMO have important negative regulatory effects on the activation of the NF- κ B signaling pathway induced by TNF- α or KvFLIP.

RESULTS

Effect of the phosphorylations of the IKK γ /NEMO proline-rich domain on TNF- α -mediated NF- κ B activation. To identify posttranslational modifications of IKK γ /NEMO by KvFLIP, IKK γ /NEMO was purified from HEK 293T cells transiently transfected with KvFLIP, followed by mass spectrometry analysis. The peptides derived from human IKK γ /NEMO revealed that IKK γ /NEMO was phosphorylated at serine residue 377 located at the C-terminal proline-rich domain. Furthermore, a large-scale proteomics analysis of the human tyrosine-phosphorylated proteins has suggested that IKK γ /NEMO undergoes tyrosine phosphorylation at residue 374 (Y374) in the same C-terminal proline-rich domain upon T cell receptor signal transduction (32). To examine if the phosphorylation sites of IKK γ /NEMO at the C-terminal proline-rich domain affect NF- κ B activation upon stimulation, amino acid residues of human IKK γ /NEMO at Y374 and S377 were individually mutated to either phosphoinhibitory phenylalanine (F), alanine (A), phosphomimetic aspartate (D), or glutamate (E) as depicted in Fig. 1A (upper panel). IKK γ /NEMO-knockout mouse embryonic fibroblasts (IKK γ /NEMO^{-/-} MEFs) were then stably reconstituted with the hemagglutinin (HA)-tagged IKK γ /NEMO wild type (WT) or each mutant, and their expressions were confirmed by immunoblotting with anti-HA antibody (Fig. 1A, lower panel). Interestingly, the phosphoinhibitory forms of IKK γ /NEMO mutants migrated faster than did WT and phosphomimetic forms of IKK γ /NEMO. Next, we examined whether these phosphorylation residue changes at the proline-rich domain of IKK γ /NEMO affected the inflammatory cytokine-induced NF- κ B signaling pathway. To do this, IKK γ /NEMO^{-/-} MEFs reconstituted with WT or each mutant of IKK γ /NEMO were stimulated with TNF- α and the levels of I κ B α phosphorylation were examined by immunoblotting. As shown in Fig. 1B, IKK γ /NEMO^{-/-} MEFs reconstituted with the Y374F or S377A mutant showed faster kinetics of I κ B α phosphorylation upon TNF- α stimulation than did those reconstituted with WT. In contrast, IKK γ /NEMO^{-/-} MEFs reconstituted with the Y374D or S377E mutant showed delayed or reduced I κ B α phosphorylation, respectively, compared with those reconstituted with WT (Fig. 1B). These results suggest that the Y374 and S377 phosphorylations at the proline-rich domain of IKK γ /NEMO appear to negatively regulate its function in TNF- α -mediated NF- κ B activation.

Since the NF- κ B signaling pathway induces the production of a number of inflammatory cytokines, we next measured the IL-6 levels from IKK γ /NEMO^{-/-} MEFs reconstituted with WT or each mutant of IKK γ /NEMO upon TNF- α stimulation. As shown in Fig. 1C, all cells, except for vector-reconstituted IKK γ /NEMO^{-/-} MEFs, produced IL-6 cytokine following TNF- α treatment. Interestingly, IKK γ /NEMO^{-/-} MEFs reconstituted with Y374F or S377A produced higher IL-6 levels upon TNF- α stimu-

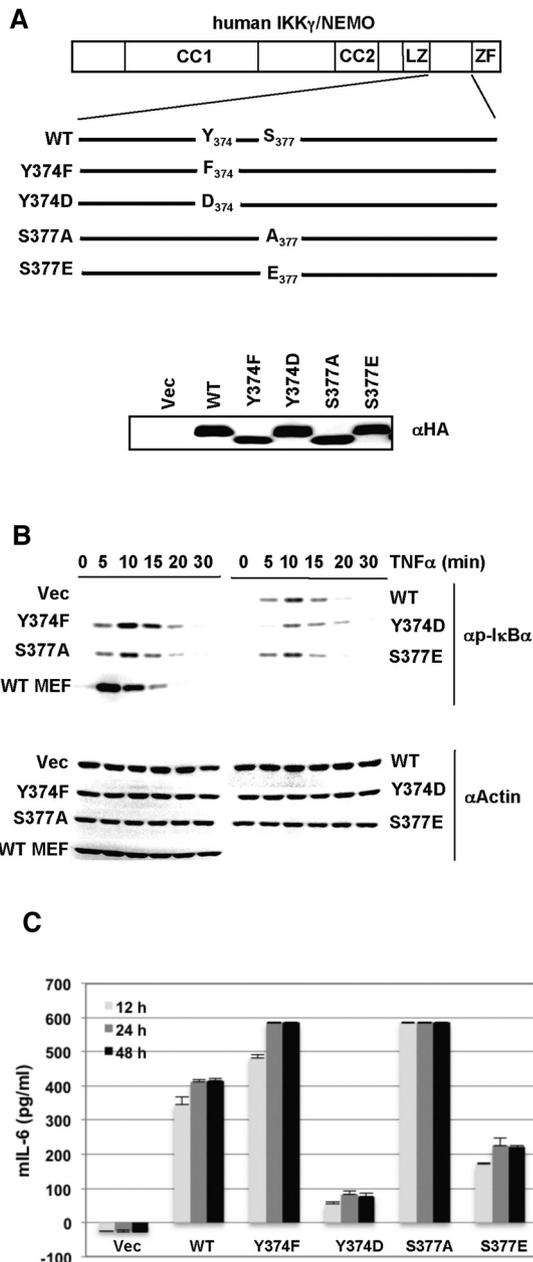


FIG 1 The Y374 or S377 phosphorylation of IKK γ /NEMO exerts negative effects on TNF- α -mediated NF- κ B activation. (A) A schematic representation of the location of Y374 and S377 in the C-terminal proline-rich domain of human IKK γ /NEMO. Individual mutations were generated by site-directed mutagenesis. IKK γ /NEMO-knockout (IKK γ /NEMO $^{-/-}$) mouse embryo fibroblasts (MEFs) were transfected with puromycin resistance (2 μ g/ml) and the HA-tagged IKK γ /NEMO WT or mutant gene. IKK γ /NEMO $^{-/-}$ MEFs stably expressing the IKK γ /NEMO WT or mutant were then established by puromycin resistance (2 μ g/ml). Expression of the IKK γ /NEMO WT or mutant was confirmed by immunoblotting with anti-HA antibody. (B) IKK γ /NEMO $^{-/-}$ MEFs stably expressing either HA-tagged IKK γ /NEMO WT or mutant were stimulated with mouse TNF- α (10 ng/ml) for indicated times. Whole-cell lysates (WCLs) were then analyzed by immunoblotting with anti-phospho-I κ B α (p-I κ B α) antibody. Immunoblotting with antiactin antibody served as a loading control. (C) IKK γ /NEMO $^{-/-}$ MEFs stably expressing either HA-tagged IKK γ /NEMO WT or mutant were stimulated with mouse TNF- α (10 ng/ml) for the indicated times. Supernatants were analyzed by ELISA for IL-6 cytokine production. Experiments were repeated twice, and the average of triplicate samples from one experiment is shown with the error bars denoting the standard error of the mean.

lation than did those reconstituted with WT (Fig. 1C). In contrast, IL-6 production was significantly attenuated in IKK γ /NEMO $^{-/-}$ MEFs reconstituted with the Y374D or S377E mutant compared to that in WT-reconstituted IKK γ /NEMO $^{-/-}$ MEFs; specifically, IKK γ /NEMO $^{-/-}$ MEFs reconstituted with the Y374D mutant showed the most severe attenuation of IL-6 production, consistent with the delayed and reduced phosphorylation of I κ B α as shown in Fig. 1B. Taken together, these results indicate that the phosphorylations of Y374 and S377 residues negatively affect IKK γ /NEMO activity.

Roles of the IKK γ /NEMO Y374 and S377 phosphorylations in IKK complex formation and IKK γ /NEMO ubiquitination. Previous studies have demonstrated that *in vivo* the IKK complex predominantly forms a large complex at least 700 to 900 kDa in size containing IKK α (85 kDa), IKK β (87 kDa), and IKK γ /NEMO (52 kDa) (33, 34). The interaction of IKK γ /NEMO with IKK α and IKK β , which bind at the beginning of CC1 of IKK γ /NEMO (amino acids 50 to 100) (6, 35, 36), is critical for the assembly of this high-molecular-mass IKK complex, leading to the IKK β stimulation and I κ B recruitment (1). Thus, we examined whether the Y374 or S377 phosphorylation at the proline-rich domain of IKK γ /NEMO affects its ability to assemble with IKK α and IKK β . To do this, whole-cell lysates (WCLs) of IKK γ /NEMO $^{-/-}$ MEFs reconstituted with the IKK γ /NEMO WT or each mutant were subjected to immunoprecipitation of IKK γ /NEMO, followed by immunoblotting for IKK α or IKK β . As shown in Fig. 2A, either IKK γ /NEMO WT or its mutant bound endogenous IKK α or IKK β at similar levels. It has been also suggested that the self-assembly activity of IKK γ /NEMO plays a critical role for activation of the IKK complex (1, 37, 38). In order to examine whether the Y374 or S377 phosphorylation at the proline-rich domain of IKK γ /NEMO influenced its oligomerization, HEK 293T cells were cotransfected with FLAG-tagged IKK γ /NEMO WT and HA-tagged IKK γ /NEMO WT or mutant, and whole-cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-HA antibody. This showed that IKK γ /NEMO WT and mutant showed similar levels of self-assembly activity (Fig. 2B). Our data collectively indicate that the Y374 or S377 phosphorylation at the proline-rich domain of IKK γ /NEMO does not affect its activity to assemble with IKK α and IKK β , nor to self-assemble.

To determine whether IKK γ /NEMO ubiquitination is affected by mutations of the Y374 and S377 phosphoacceptor residues, HEK 293T cells were transiently transfected with FLAG-tagged ubiquitin (Ub) and HA-tagged WT or each mutant of IKK γ /NEMO and subjected to pulldown with agarose beads conjugated with mouse anti-HA antibody, followed by immunoblotting with rabbit anti-FLAG antibody. The experiment showed that the WT and each mutant of IKK γ /NEMO carried similar levels of ubiquitination (Fig. 3A). These results were further confirmed in IKK γ /NEMO $^{-/-}$ MEFs reconstituted with the WT or mutant form of IKK γ /NEMO (Fig. 3B). These results imply that mutations of the Y374 and S377 phosphoacceptor residues of IKK γ /NEMO show little or no effect on its TNF- α -induced ubiquitinations.

SF-PTKs interact with and phosphorylate IKK γ /NEMO. A large-scale proteomics analysis of the human tyrosine-phosphorylated proteins has previously suggested that IKK γ /NEMO undergoes tyrosine phosphorylation at residue 374 (Y374) upon T cell receptor signal transduction (32, 39). To examine

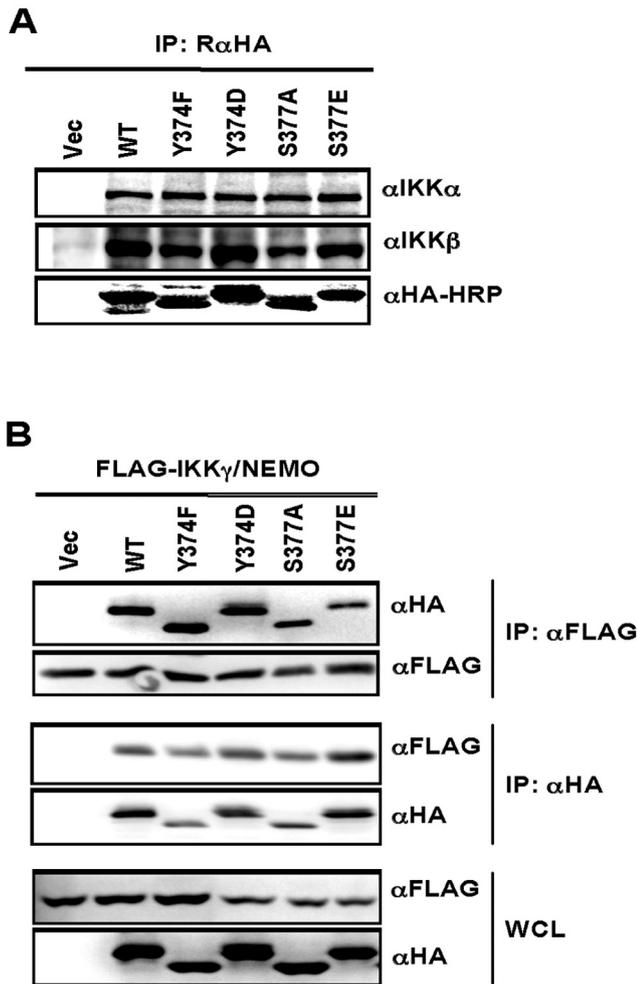


FIG 2 Mutation of the Y374 or S377 residue of human IKK γ /NEMO shows no effect on IKK complex formation or self-oligomerization. (A) IKK γ /NEMO^{-/-} MEFs stably expressing either HA-tagged IKK γ /NEMO WT or mutant were subjected to immunoprecipitation (IP) with rabbit anti-HA antibody, followed by immunoblotting with antibodies against IKK α and IKK β . (B) HEK 293T cells were transfected with HA-tagged IKK γ /NEMO WT or mutants together with FLAG-tagged WT IKK γ /NEMO. WCL was immunoprecipitated (IP) with anti-FLAG or anti-HA antibody, followed by immunoblotting with anti-HA or anti-FLAG antibody. WCL was analyzed by immunoblotting with the indicated antibodies to confirm the expression of IKK γ /NEMO.

whether members of the SF-PTKs phosphorylate IKK γ /NEMO, 293T cells were cotransfected with WT IKK γ /NEMO and various SF-PTKs, including Fyn, Lck, Fgr, Src, and Lyn. Whole-cell lysates were used for immunoblotting with phosphotyrosine (pY)-specific antibody. Besides the auto-tyrosine-phosphorylated Fyn (58 kDa), Lck (53 kDa), Fgr (55 kDa), Src (55 kDa), and Lyn (55 kDa), an additional tyrosine-phosphorylated 50-kDa protein, at a molecular mass equivalent to that of IKK γ /NEMO, was detected in cells expressing IKK γ /NEMO WT with Fyn, Fgr, Src, or Lyn, but not with Lck (Fig. 4A). The 50-kDa tyrosine-phosphorylated protein was not detected in cells expressing the IKK γ /NEMO Y374F mutant (Fig. 4A). Furthermore, the anti-pY immunoblotting of immunoprecipitated IKK γ /NEMO showed that the WT, but not the Y374F mutant, underwent tyrosine phos-

phorylation upon coexpression with Fyn, Fgr, Src, or Lyn (Fig. 4B). It should be noted that the levels of IKK γ /NEMO tyrosine phosphorylation were highest upon expression of Fyn, followed by Src, Fgr, and Lyn, whereas Lck expression did not induce the tyrosine phosphorylation of IKK γ /NEMO (Fig. 4B). Consistently, IKK γ /NEMO WT readily interacted with Fyn, Fgr, Src, or Lyn, but not with Lck, whereas the Y374F mutant failed to interact with any of these SF-PTKs (Fig. 4C). These results suggest that Fyn, Fgr, Src, and Lyn interact with and phosphorylate IKK γ /NEMO at its Y374 residue.

The Y374 and S377 phosphorylations of IKK γ /NEMO negatively affect TCR-mediated activation of NF- κ B activity and calcium (Ca²⁺) influx. To study whether the Y374 or S377 phosphorylation of IKK γ /NEMO plays a role in TCR-mediated NF- κ B activation, IKK γ /NEMO-deficient Jurkat T cells were stably reconstituted with HA-tagged IKK γ /NEMO WT or Y374 or S377 mutant and subsequently stimulated with the combination of phorbol myristate acetate (PMA) and ionomycin to mimic TCR stimulation. IKK γ /NEMO-deficient Jurkat T cells stably expressing empty vector failed to induce I κ B α phosphorylation upon PMA and ionomycin treatment, whereas IKK γ /NEMO-deficient Jurkat T cells stably expressing WT, Y374 or S377 mutant resulted in the robust induction of I κ B α phosphorylation (Fig. 5A). The I κ B α phosphorylation was detected as early as 5 min poststimulation and peaked at 20 min poststimulation in all cell lines (Fig. 5A). However, the levels of I κ B α phosphorylation were stronger in IKK γ /NEMO-deficient Jurkat T cells stably expressing Y374F or S377A mutant but weaker in IKK γ /NEMO-deficient Jurkat T cells stably expressing Y374D or S377E mutant than those in IKK γ /NEMO-deficient Jurkat T cells stably expressing WT (Fig. 5A). These results further suggest that the phosphorylations of Y374 and S377 residues negatively affect IKK γ /NEMO activity to induce I κ B α phosphorylation in either MEFs or Jurkat T cells.

To assess whether the Y374 and S377 phosphorylation of IKK γ /NEMO affected intracellular Ca²⁺ levels, each Jurkat T cell line was stimulated with PMA and ionomycin and the levels of intracellular Ca²⁺ influx were measured by flow cytometry analysis. Consistent with the I κ B α phosphorylation, stimulation-induced intracellular Ca²⁺ influx was significantly higher in IKK γ /NEMO-deficient Jurkat T cells reconstituted with the Y374F or S377A mutant than in IKK γ /NEMO-deficient Jurkat T cells reconstituted with the WT or the Y374D mutant (Fig. 5B). Interestingly, IKK γ /NEMO-deficient Jurkat T cells reconstituted with empty vector showed levels of intracellular Ca²⁺ increase similar to those of T cells reconstituted with the S377E mutant upon PMA/ionomycin treatment (Fig. 5B). Finally, the interactions of IKK γ /NEMO WT or mutant with endogenous IKK α or IKK β were not detectably altered (Fig. 5C). These results also suggest that the Y374 and S377 phosphorylations of IKK γ /NEMO negatively affect TCR-mediated Ca²⁺ influx.

DISCUSSION

It has been suggested that posttranslational modification of proteins can influence diverse functions of proteins, such as their activation, stability, localization, trafficking, and incorporation into higher complexes (40). A number of molecules involved in the induction of the NF- κ B signaling pathway are well known to undergo posttranslational modifications in response to diverse NF- κ B activators, such as TNF- α , IL-1, and microbial infection (3, 6, 7). A growing body of evidence indicates that IKK γ /NEMO

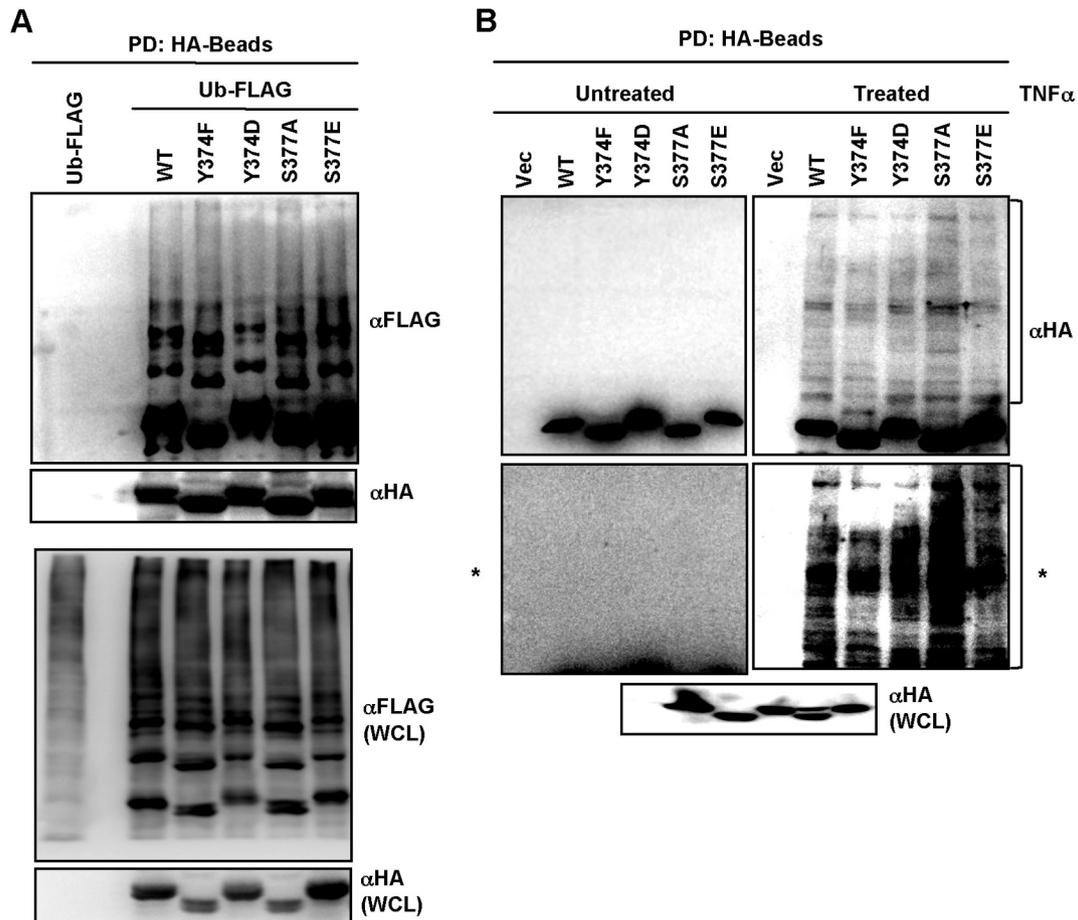


FIG 3 Mutation of the Y374 or S377 residue of human IKK γ /NEMO does not affect its ubiquitination. (A) HEK 293T cells were transfected with HA-tagged IKK γ /NEMO WT or mutants together with FLAG-tagged ubiquitin (Ub). WCL was used for the purification of the IKK γ /NEMO complex using agarose beads conjugated with mouse anti-HA antibody, followed by immunoblotting with rabbit anti-FLAG or rabbit anti-HA antibody. WCL was analyzed by immunoblotting with the indicated antibodies to confirm expression of IKK γ /NEMO. (B) IKK γ /NEMO^{-/-} MEFs stably expressing either HA-tagged IKK γ /NEMO WT or mutant were treated with TNF- α for 10 min, followed by immunoprecipitation with agarose beads conjugated with mouse anti-HA antibody and immunoblotting with rabbit anti-HA antibody (upper panel). A longer exposure of the blot marked with an asterisk is shown in the lower panel (both panels are marked with an asterisk). WCL was analyzed by immunoblotting with anti-HA antibody to confirm the IKK γ /NEMO expression.

is targeted for several posttranslational modifications, including ubiquitination, sumoylation, and phosphorylation. Thus, post-translational modifications confer crucial regulatory mechanisms to IKK γ /NEMO for the activation of IKK complex under most stimulation conditions (3, 6, 7). Previous studies have demonstrated that serine residue 369 (S369) in the C terminus of mouse IKK γ /NEMO (equivalent to serine residue 376 [S376] in that of human IKK γ /NEMO) is the major site of phosphorylation by IKK β (17). Moreover, the S376 residue of human IKK γ /NEMO was identified as the primary target for phosphorylation by IKK β and the vicinal S377 residue was suggested to serve a compensatory role for phosphorylation upon TNF- α stimulation or expression of HTLV-1 Tax (18, 41, 42). In this study, we found that the overexpression of KvFLIP, a viral oncoprotein known to interact with IKK γ /NEMO and to constitutively activate the NF- κ B signaling pathway (29, 30, 43), resulted in the phosphorylation of human IKK γ /NEMO at S377 located in its C-terminal proline-rich domain. Furthermore, we showed that mutation of the S377 residue of human IKK γ /NEMO to the phosphoinhibitory alanine (S377A) resulted in increased NF- κ B activity and IL-6 cytokine

production. These results are consistent with the previous study showing that the S369A mutation of mouse IKK γ /NEMO led to increased NF- κ B activity (17). Most biological processes require both positive and negative regulatory mechanisms to maintain equilibrium. While the NF- κ B pathway robustly induces host immune responses upon viral infection, it must slow down and ultimately stop when it reaches a late stage to avoid excessive production of inflammatory cytokines, which could lead to deleterious effects on host immunity. Thus, the S377 phosphorylation of IKK γ /NEMO may be one of these feedback inhibitory mechanisms to slow down NF- κ B-mediated immune responses.

Previous genome-wide analysis of tyrosine phosphorylation in combination with mass spectrometric analysis has shown that human IKK γ /NEMO undergoes tyrosine phosphorylation at the Y374 residue (32, 39). We also found that IKK γ /NEMO interacted with members of the SF-PTKs, including Fyn, Fgr, Src, and Lyn, resulting in the Y374 phosphorylation of IKK γ /NEMO. It should be noted that murine IKK γ /NEMO does not have a tyrosine residue corresponding to Y374 of human IKK γ /NEMO, suggesting that this tyrosine phosphorylation site is specific for human IKK γ /

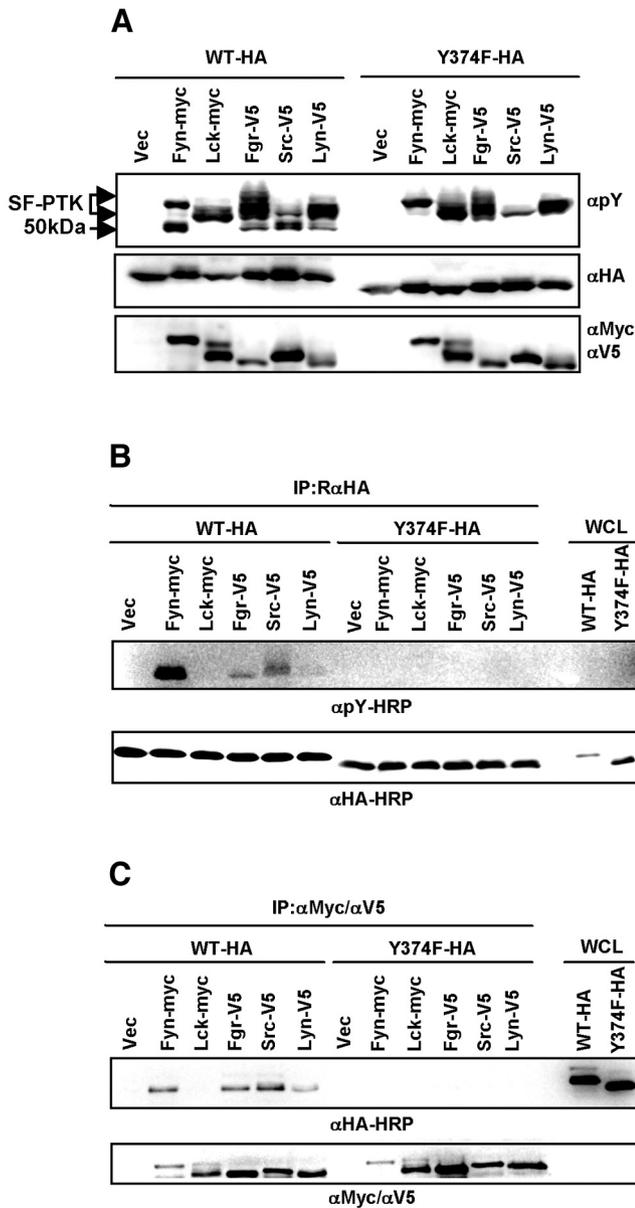


FIG 4 Expression of SF-PTKs leads to the Y374 phosphorylation of human IKK γ /NEMO. (A) Either IKK γ /NEMO WT or the Y374F mutant was coexpressed with each member of the Src family protein tyrosine kinases (SF-PTKs) in HEK 293T cells. WCL was then analyzed by immunoblotting with phosphotyrosine (pY)-specific antibody. HA (IKK γ /NEMO), Myc (Fyn and Lck), and V5 (Fgr, Src, and Lyn) tagging antibodies were used to demonstrate expression levels. (B) HA-tagged IKK γ /NEMO was overexpressed in HEK 293T cells along with SF-PTKs as described above and purified with rabbit anti-HA antibody in RIPA buffer, followed by immunoblotting with horseradish peroxidase (HRP)-conjugated anti-pY antibody. IP, immunoprecipitation. (C) HA-tagged WT IKK γ /NEMO was coexpressed with Myc-Fyn, Myc-Lck, V5-Fgr, V5-Src, or V5-Lyn in HEK 293T cells. WCL was subjected to immunoprecipitation with either anti-Myc or anti-V5 antibody, followed by immunoblotting with horseradish peroxidase-conjugated anti-HA antibody.

NEMO. IKK γ /NEMO has been shown to bind itself as well as a variety of cellular and viral proteins, resulting in the modulation of IKK complex activity in either a positive or a negative manner (44, 45). Despite its apparent negative function in NF- κ B activa-

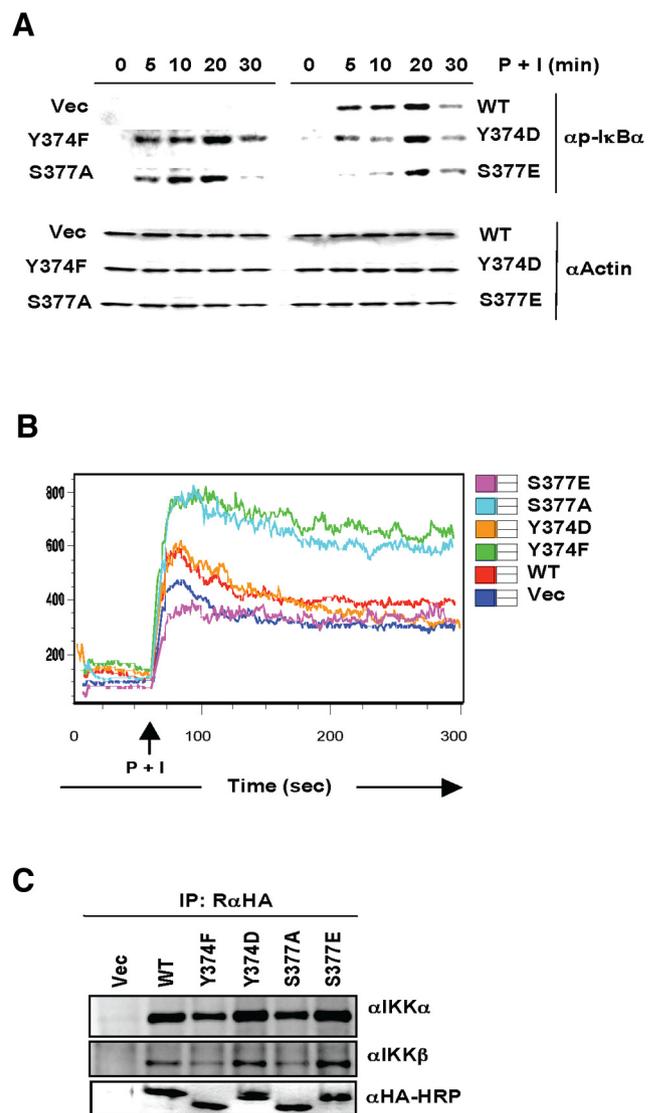


FIG 5 The Y374 or S377 phosphorylation of IKK γ /NEMO differentially contributes to NF- κ B activation and intracellular Ca²⁺ influx. (A) IKK γ /NEMO-deficient Jurkat T cells stably complemented with HA-tagged IKK γ /NEMO WT or mutant were stimulated with PMA (P) and ionomycin (I) for indicated times. WCL was analyzed by immunoblotting with anti-p-I κ B α antibody. Immunoblotting with antiactin antibody served as a loading control. (B) IKK γ /NEMO-deficient Jurkat T cells stably complemented with HA-tagged IKK γ /NEMO WT or mutant were loaded with Ca²⁺ indicator for 1 h at 37°C. Intracellular Ca²⁺ influx upon PMA and ionomycin (P + I) treatment was monitored for 5 min by flow cytometry analysis. (C) IKK γ /NEMO-deficient Jurkat T cells stably complemented with HA-tagged IKK γ /NEMO WT or mutant were subjected to immunoprecipitation (IP) with rabbit anti-HA antibody, followed by immunoblotting with antibodies against IKK α , IKK β , and HA.

tion, either the S377 or Y374 phosphorylation showed little or no effect on the ubiquitination, self-assembly, and IKK α /IKK β interaction of IKK γ /NEMO. However, we cannot exclude the possibility that multiple serine residues and a tyrosine residue located at the C-terminal proline-rich domain of IKK γ /NEMO temporally and differentially undergo phosphorylation/dephosphorylation under diverse stimulation conditions, leading to conformational changes at a given time that subsequently alter its affinity for itself

and other cellular proteins. Further detailed study is necessary to identify how the S377 and Y374 phosphorylation sites negatively regulate IKK γ /NEMO function to induce NF- κ B activity.

A variety of viral oncoproteins deregulate the NF- κ B signaling pathway by targeting IKK γ /NEMO (23). Earlier studies have suggested that KSHV KvFLIP is recruited to a ~700-kDa IKK complex and physically associates with IKK α , IKK β , and IKK γ /NEMO, thereby contributing to the constitutive activation of NF- κ B in KSHV-infected cells as well as in cells stably expressing KvFLIP (29, 30, 43). In addition, IKK γ /NEMO is essential for KvFLIP-mediated constitutive NF- κ B activation (29), and IKK α is primarily responsible for the KvFLIP-mediated constitutive activation of NF- κ B activity, whereas IKK β contributes to maximum induction of KvFLIP-mediated activation of NF- κ B activity (43, 46). A recent study showing that both KvFLIP and Tax activate the NF- κ B signaling pathway without involvement of the linear polyubiquitination of IKK γ /NEMO (47) implies a distinct mechanism by which these viral proteins can induce a cellular NF- κ B signaling pathway. Nevertheless, we are surprised that ectopic expression of the KvFLIP oncoprotein led to the increase of S377 phosphorylation of IKK γ /NEMO, which potentially suppresses NF- κ B activation. This raises the question of why KvFLIP induces the S377 phosphorylation of IKK γ /NEMO. While it remains unclear how IKK γ /NEMO S377 phosphorylation contributes to the KvFLIP-mediated induction of NF- κ B activation, it is possible that the S377 phosphorylation of IKK γ /NEMO functions as a physiological brake to keep the balance of host signaling pathways, and KvFLIP may utilize S377 phosphorylation as a decoy mechanism to deceive and evade host immune responses. Nevertheless, our study discovers novel posttranslational regulatory mechanisms to control the NF- κ B signaling pathway, and future study will be dedicated to answering the questions above.

MATERIALS AND METHODS

Plasmids. Human IKK γ /NEMO tagged with an HA epitope at the N terminus was cloned into the puoroIRES vector. For the construction of phosphoinhibitory IKK γ /NEMO mutants, phenylalanine residues and alanine residues were substituted for the tyrosine residue at 374 (Y374F) and serine residues at 377 (S377A) by site-directed mutagenesis. Similarly, aspartate and glutamate residues were substituted for the tyrosine residue at 374 (Y374D) and serine residues at 377 (S377E) for the construction of phosphomimetic IKK γ /NEMO mutants. The presence of these mutations was confirmed by sequencing. Myc-tagged Fyn and Lck were previously described (48). V5-tagged Fgr, Src, and Lyn were constructed in the puoroIRES vector.

Cell lines and transfection. HEK 293T cells (ATCC) and IKK γ /NEMO-knockout MEFs (obtained from Xin Lin, University of Texas, M. D. Anderson Cancer Center) were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). IKK γ /NEMO-deficient Jurkat T cells (obtained from Xin Lin, University of Texas, M. D. Anderson Cancer Center) were maintained in RPMI medium (Gibco) supplemented with 10% fetal bovine serum and antibiotics. To establish stable cell lines expressing WT IKK γ /NEMO and its mutants, IKK γ /NEMO^{-/-} MEFs were transfected with puoroIRES vector carrying the WT or mutant IKK γ /NEMO gene using Lipofectamine 2000 (Invitrogen), followed by selection in the presence of 2 μ g/ml of puromycin (Sigma) for 2 weeks. Similarly, IKK γ /NEMO-deficient Jurkat T cells stably expressing WT or mutant IKK γ /NEMO were established by electroporating cells with puoroIRES vector carrying the WT or mutant IKK γ /NEMO gene. Transient transfection of HEK 293T cells was per-

formed using polyethylenimine (PEI). At 36 h posttransfection, cells were harvested for immunoprecipitation.

Immunoprecipitation, immunoblotting, and reagents. Whole-cell lysate (WCL) was prepared in 1% NP-40 lysis buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing phosphatase inhibitor cocktail (Roche) at 36 h posttransfection and then pre-cleared for 2 h at 4°C in the presence of Sepharose beads. Precleared WCL was incubated for 2 h at 4°C with the indicated antibodies, followed by further incubation for 2 h at 4°C in the presence of protein A/G-conjugated beads. The immunoprecipitates were washed three times with 1% NP-40 lysis buffer. An SDS-PAGE gel was transferred onto a polyvinylidene difluoride (PVDF) membrane, followed by blocking in 5% non-fat milk and then immunoblotting with indicated antibodies. Antibodies against IKK α , IKK β (L570), I κ B α (L35A5), phospho-I κ B α (5A5), and recombinant mouse and human TNF- α were purchased from Cell Signaling. Antibody for V5 was purchased from Invitrogen. Antibodies for HA and Myc were obtained from Covance. Antibody for FLAG, HA-conjugated beads, PMA, and ionomycin were purchased from Sigma.

Ca²⁺ flux assay. IKK γ /NEMO-deficient Jurkat T cells were washed once with complete RPMI medium. Cells were then incubated with dye according to the manufacturer's instructions (BD). Ca²⁺ flux was measured by flow cytometry (FACS Canto; BD). Basal fluorescence was recorded for 1 min prior to stimulation with PMA and ionomycin for an additional 5 min.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed using supernatants from IKK γ /NEMO^{-/-} MEFs stimulated with mouse TNF- α (10 ng/ml) to quantify mouse IL-6 (BD OptEIA mouse IL-6 set) according to the manufacturer's instructions.

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