

The inhibitor of κ B kinase β (IKK β) phosphorylates I κ B α twice in a single binding event through a sequential mechanism

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Running title: *IKK β phosphorylates I κ B α twice using a sequential mechanism*

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Keywords: NF-kappa B (NF- κ B); Inhibitor of NF- κ B α (I κ B α); Inhibitor of κ B kinase β (IKK β); pre-steady-state kinetics; protein kinases; protein phosphorylation; enzyme mechanisms

ABSTRACT

Phosphorylation of Inhibitor of κ B (I κ B) proteins by I κ B Kinase β (IKK β) leads to I κ B degradation and subsequent activation of nuclear factor κ B (NF- κ B) transcription factors. Of particular interest is the IKK β -catalyzed

phosphorylation of I κ B α residues Ser³² and Ser³⁶ within a conserved destruction box motif. To investigate the catalytic mechanism of IKK β , we performed pre-steady-state kinetic analysis of the phosphorylation of I κ B α protein substrates catalyzed by constitutively active, human IKK β .

Phosphorylation of full-length I κ B α catalyzed by IKK β was characterized by a fast exponential phase followed by a slower linear phase. The maximum observed rate (k_p) of IKK β -catalyzed phosphorylation of I κ B α was 0.32 s⁻¹ and the binding affinity of ATP for the IKK β •I κ B α complex (K_d) was 12 μ M. Substitution of either Ser³² or Ser³⁶ with Ala, Asp or Cys reduced the amplitude of the exponential phase by approximately 2-fold. Thus, the exponential phase was attributed to phosphorylation of I κ B α at Ser³² and Ser³⁶, whereas the slower linear phase was attributed to phosphorylation of other residues. Interestingly, the exponential rate of phosphorylation of the I κ B α (S32D) phosphomimetic amino acid substitution mutant was nearly twice that of wild-type I κ B α and 4-fold faster than any of the other I κ B α amino acid substitution mutants, suggesting that phosphorylation of Ser³² increases the phosphorylation rate of Ser³⁶. These conclusions were supported by parallel experiments using GST-I κ B α (1-54) fusion protein substrates bearing the first 54 residues of I κ B α . Our data suggest a model wherein, IKK β phosphorylates I κ B α at Ser³² followed by Ser³⁶ within a single binding event.

Introduction

The nuclear factor κ B (NF- κ B)² family of transcription factors are evolutionarily conserved master regulators of cell proliferation, innate immunity, inflammation, cell differentiation, and apoptosis (1,2). Dysregulation of NF- κ B is associated with many disorders, including: cancer, autoimmune disease, neurodegenerative diseases, arthritis, and diabetes (3-5). Thus, investigation of the molecular mechanisms of NF- κ B activation is important for our understanding of human disease.

The mammalian NF- κ B family consists of RelA (p65), RelB, c-Rel, p50/p105 (NF- κ B1) and p52/p100 (NF- κ B2), which form fifteen separate homodimeric or heterodimeric complexes (6). In resting cells, NF- κ B dimers containing RelA, RelB and/or c-Rel are sequestered in the cytoplasm through interactions with Inhibitor of κ B (I κ B) proteins I κ B α , I κ B β , or I κ B ϵ . In contrast, NF- κ B dimers containing p100 and p105 are localized to the cytoplasm through a C-terminal inhibitory domain containing multiple

ankyrin repeat motifs. In response to various stimuli, NF- κ B is activated through one of two separate pathways: the canonical or the noncanonical pathway. Within the canonical pathway, a Ser/Thr-specific I κ B kinase (IKK) complex phosphorylates I κ B proteins within a conserved DSGXXS destruction box motif, leading to I κ B polyubiquitination, 26S proteasome-mediated degradation and subsequent NF- κ B release. Free NF- κ B dimers then translocate to the nucleus to regulate transcription. In the noncanonical pathway, the IKK complex phosphorylates p100 to induce proteolytic processing of p100 to the activated NF- κ B2 subunit p52, which also localizes to the nucleus. The IKK complex also catalyzes the phosphorylation of several protein substrates within alternative signaling pathways, and this activity is thought to coordinate the functions of the NF- κ B pathways with other cellular pathways (reviewed in reference 7).

The IKK complex consists of the non-enzymatic protein NEMO (NF- κ B essential modulator, also called IKK γ) and a homodimer or heterodimer of the catalytic subunits IKK α and IKK β (8-12). Although IKK α and IKK β share 54% amino acid sequence identity, these kinases possess distinct substrate specificities. For example, IKK α predominantly catalyzes phosphorylation of p100 within the noncanonical pathway (13,14). In contrast, IKK β is primarily responsible for phosphorylation of I κ B α , I κ B β , I κ B ϵ within the canonical pathway (15-17).

Structural studies indicate that IKK β possesses a tri-modular architecture (Fig. 1A) consisting of an N-terminal kinase domain (KD), a ubiquitin-like domain (ULD) and a C-terminal scaffold/dimerization domain (SDD) (18-20). The KD contains an activation loop with the MEK consensus motif SxxxS (S177 and S181 in human IKK β , Fig. 1A). This activation loop is essential for the activity of IKK β as mutational analysis indicates that changing these Ser residues to Ala prevents IKK β activation, whereas substitution of these critical Ser residues with phosphomimetic Glu residues renders the kinase constitutively active (12,19,21,22).

Although the phosphorylation of I κ B proteins by IKK β is a critical step within the canonical pathway of NF- κ B activation, the molecular mechanism of IKK β is poorly

understood. Here, we used pre-steady-state kinetic analysis to investigate the molecular mechanism of I κ B α phosphorylation catalyzed by constitutively active IKK β . Our data indicate that IKK β phosphorylates full-length I κ B α twice within the conserved DSGXXS destruction box motif (Fig. 1C) during a single binding event. Our study also suggests that IKK β preferentially phosphorylates I κ B α sequentially at Ser³², followed by Ser³⁶. We conclude that these aspects of the kinetic mechanism of IKK β may be shared among the IKK and IKK-related kinases.

Results

Determination of the pre-steady-state kinetic parameters of IKK β phosphorylation of full-length I κ B α - The activity of human IKK β (Fig. 1A) is significantly increased upon phosphorylation of activation loop residues Ser¹⁷⁷ and Ser¹⁸¹ within the kinase domain (21,22). In order to measure the catalytic rates of activated IKK β , we chose to utilize a constitutively active IKK β (S177E, S181E) phosphomimetic amino acid substitution mutation (12,19,21) for our study. Within the canonical pathway of NF- κ B activation, IKK β specifically phosphorylates I κ B α at residues Ser³² and Ser³⁶ (Figs. 1B and C) to induce the proteolytic degradation of I κ B α and the subsequent release of NF- κ B dimers. Importantly, I κ B α is also phosphorylated at other sites *in vivo*, particularly within the C-terminal PEST domain (Fig. 1B) (23,24). However, unlike phosphorylation of residues within the destruction box motif, phosphorylation of residues within the PEST domain does not specifically target I κ B α for ubiquitination and 26S proteasome-mediated degradation (24).

Steady-state kinetic analysis has demonstrated that IKK β phosphorylates I κ B α by using a random sequential mechanism (25), indicating that IKK β can bind I κ B α and ATP in any order prior to catalysis. However, because IKK β exhibits autophosphorylation (Fig. 2), we chose to measure the pre-steady-state kinetic parameters of IKK β -catalyzed phosphorylation of I κ B α by first incubating IKK β with the I κ B α protein substrate to generate the IKK β •I κ B α complex, and subsequently initiating the reaction by the rapid addition of ATP. To initially characterize the molecular mechanism of IKK β -catalyzed phosphorylation of full-length I κ B α ,

the ground-state binding affinity of ATP (K_d) for the IKK β •I κ B α complex and the maximum observed rate (k_p) of IKK β -catalyzed phosphorylation were determined by using pre-steady-state kinetic analysis. To this end, we measured the ATP concentration dependence of the phosphorylation rate of IKK β under conditions where IKK β was in a 4-fold molar excess compared to the I κ B α substrate to ensure that nearly all of the I κ B α substrate was initially bound by the kinase. IKK β has previously been shown to bind I κ B α with an equilibrium dissociation constant (K_d) of 56 nM (26), and thus, we calculated that >98% of the I κ B α substrate was initially bound by IKK β under our experimental conditions.

A preincubated solution of full-length I κ B α and IKK β (S177E, S181E) was rapidly mixed with a solution containing increasing concentrations of [γ -³²P]ATP for various times. The reaction products were then separated by SDS-PAGE and phosphorylation of I κ B α was quantified by using autoradiography (Fig. 2). A [γ -³²P]-labeled, linearized DNA vector was included as a loading control because it was easily separated from both IKK β and I κ B α by SDS-PAGE, and it provided a consistent signal after incubation with IKK β , I κ B α and ATP (Fig. 2). The amount of phosphorylated I κ B α detected at each time point was normalized to the loading control and plotted as a function of reaction time (Fig. 3A). The resulting plots indicated that IKK β (S177E, S181E) phosphorylated I κ B α with an initial, fast exponential phase rate (k_e), followed by a significantly slower linear phase rate (k_l) and therefore, these data were fit to biphasic Equation 1 (see Experimental Procedures). The biphasic nature of the plot can be clearly observed in Fig. 4A when longer reaction time points were included in the plot. We hypothesized that the initial exponential rate corresponded to phosphorylation of I κ B α at residues Ser³² and Ser³⁶ within the destruction box motif, and the significantly slower linear rate resulted from either the phosphorylation of I κ B α at sites outside of the destruction box motif, such as the PEST domain (Fig. 1B), or phosphorylation of I κ B α substrate that was not initially bound in a productive complex by IKK β , or both. The exponential rates of IKK β -catalyzed phosphorylation of I κ B α were then plotted as a

function of ATP concentration and fit to hyperbolic Equation 2 (Fig. 3B), yielding a k_p of $0.32 \pm 0.01 \text{ s}^{-1}$ for the maximum observed rate of IKK β -catalyzed phosphorylation of full-length I κ B α and a K_d of $12 \pm 1 \text{ }\mu\text{M}$ for ATP binding to the IKK β •I κ B α complex.

IKK β phosphorylates I κ B α twice within a single binding event - To further investigate the molecular mechanism of IKK β phosphorylation, we sought to test the ability of IKK β (S177E, S181E) to phosphorylate either Ser³² or Ser³⁶ within the destruction box motif of I κ B α (Figs. 1B and 1C). To this end, we evaluated the phosphorylation of seven separate full-length I κ B α amino acid substitution mutants (Fig. 1C) in which either residue Ser³² or Ser³⁶ were individually changed to i) Ala to eliminate the phosphorylation site, ii) Asp to mimic the size and charge of a phosphorylated Ser, or iii) Cys to mimic the size and hydrophilic character of Ser while eliminating the phosphorylation site. An I κ B α (S32A, S36A) double alanine substitution mutant was also tested in which both Ser residues within the destruction box motif were eliminated. The amount of phosphorylated I κ B α product was plotted as a function of reaction time and fit to biphasic Equation 1 (Fig. 4). Kinetic data derived from the best fit curves are reported in Figs. 4C-4E and Table 1.

Comparing the kinetic parameters of IKK β -catalyzed phosphorylation of wild-type (wt) I κ B α and the I κ B α amino acid substitution mutants, the amplitude of the exponential phase produced by phosphorylation of each of the I κ B α single amino acid substitution mutants was reduced approximately 2-fold when compared to the exponential phase amplitude of wt I κ B α (Fig. 4C and Table 1). Additionally, the observed exponential phase amplitude produced by phosphorylation of the I κ B α (S32A, S36A) double amino acid substitution mutant was reduced by 17-fold when compared to wt I κ B α (Fig. 4C and Table 1). These data indicate that the exponential phase of IKK β -catalyzed phosphorylation of I κ B α corresponds to phosphorylation of I κ B α at Ser³² and Ser³⁶. Interestingly, the exponential phase of wt I κ B α phosphorylation can be fit to a single rate (Fig. 4), indicating that IKK β phosphorylated I κ B α at Ser³² and Ser³⁶ rapidly, without a significant

intervening step. This finding strongly suggests that IKK β phosphorylated wt I κ B α twice, without complete disassociation and reassociation of the IKK β •I κ B α complex. Thus, we concluded that IKK β phosphorylates I κ B α processively within a single binding event.

Interestingly, the exponential rate of phosphorylation of the I κ B α (S32D) phosphomimetic substitution mutant was increased by approximately 1.8 fold when compared to the exponential rate of phosphorylation of wt I κ B α (Fig. 4D and Table 1). In contrast, the exponential rate of phosphorylation for all of the other I κ B α single amino acid substitution mutants, including the I κ B α (S36D) phosphomimetic amino acid substitution mutant, was reduced by approximately 1.5-fold when compared to wt I κ B α phosphorylation (Fig. 4D and Table 1). These data suggest that if I κ B α is phosphorylated at Ser³², the rate at which IKK β catalyzes the second phosphorylation event at Ser³⁶ is increased, while phosphorylation of I κ B α Ser³⁶ does not increase the rate of phosphorylation at Ser³².

The linear rate of IKK β -catalyzed phosphorylation of the I κ B α single amino acid substitution mutants was virtually identical (within 2-fold) to the linear rate of IKK β -catalyzed phosphorylation of wt I κ B α (Fig. 4E and Table 1). These results are consistent with the hypothesis that the linear phase of phosphorylation of I κ B α and the I κ B α amino acid substitution mutants results primarily from phosphorylation of I κ B α at sites outside of the destruction box motif (Figs. 1B and 1C) by IKK β . Interestingly, the linear rate of IKK β -catalyzed phosphorylation of the I κ B α (S32A, S36A) double amino acid substitution mutant was 2 to 4-fold faster than for wt I κ B α and the I κ B α single amino acid substitution mutants (Fig. 4E and Table 1), suggesting that IKK β -catalyzed phosphorylation of Ser³² and Ser³⁶ may have slowed the linear PEST domain phosphorylation rate in these experiments.

Interactions between IKK β and the C-terminal region of I κ B α do not significantly influence the molecular mechanism of IKK β -catalyzed phosphorylation - Although IKK β preferentially phosphorylates I κ B α within the destruction box

motif (Figs. 1B and 1C), we predicted that IKK β is also capable of phosphorylating I κ B α at sites within the C-terminal region (residues 55-317), such as within the PEST domain (Fig. 1B). To rule out the possibility that the observed rates of IKK β -catalyzed phosphorylation of full-length I κ B α were significantly influenced by phosphorylation of I κ B α residues outside of the destruction box motif, we evaluated the phosphorylation of GST-I κ B α (1-54) fusion proteins (Fig. 1B and C). These GST-I κ B α (1-54) protein substrates lack the C-terminal region of I κ B α . Thus, we predicted that non-specific substrate phosphorylation by IKK β would be reduced when testing these protein substrates. We assessed the IKK β -catalyzed phosphorylation of GST-I κ B α (1-54) bearing the first 54 residues of I κ B α , including the destruction box motif, and seven separate amino acid substitution mutations of GST-I κ B α (1-54) (Figs. 1B and C) under pre-steady-state conditions. The amount of phosphorylated I κ B α substrate detected was plotted as a function of reaction time (Figs. 5A and 5B). In contrast to phosphorylation of the full-length I κ B α substrates (Figs. 4A and 4B), only a single exponential phase of GST-I κ B α (1-54) was observed (Figs. 5A and 5B). Therefore, these data were fit to single-exponential Equation 3 (see Experimental Procedures). Kinetic data derived from the best fit curves are reported in Figs. 5C and 5D as well as Table 2. The absence of a clear linear phase was likely due to either a reduction in non-specific phosphorylation of the GST-I κ B α (1-54) fusion protein at sites outside of the destruction box motif when compared to phosphorylation of the full-length I κ B α substrates and/or a reduction in the number of initial, unproductive protein complexes. Consistently, the rate of GST-I κ B α (1-54)(S32A, S36A) phosphorylation was too slow to be accurately measured (Figs. 5A and 5B).

It has been previously demonstrated that the C-terminal region (residues 55-317) of I κ B α (Fig. 1B) interacts with the ULD and SSD of IKK β (Fig. 1A) (19). Such interaction may help to properly position the N-terminal destruction box motif of I κ B α for phosphorylation by the KD of IKK β (Fig. 6) and thus, influence the molecular mechanism of IKK β . Interestingly, in agreement with our observations of IKK β -catalyzed phosphorylation of the full-length I κ B α

substrates, the amplitude of the exponential phase of GST-I κ B α (1-54) wt phosphorylation was approximately 2-fold greater than the exponential phase amplitude of all six of the GST-I κ B α (1-54) single amino acid substitution mutants (Fig. 5C and Table 2). Thus, we concluded that interactions between IKK β and the C-terminal region of I κ B α are not required for IKK β to phosphorylate I κ B α twice within a single binding event. Furthermore, the full-length I κ B α and the GST-I κ B α (1-54) wt substrates were both phosphorylated at similar exponential rates (Figs. 4D and 5D, Tables 1 and 2), suggesting that interactions between IKK β and the C-terminal domain of I κ B α do not significantly alter the overall exponential rate of IKK β -catalyzed phosphorylation. However, in contrast to the results obtained with the full-length I κ B α amino acid substitution mutants (Fig. 4D and Table 1), the GST-I κ B α (1-54)(S32D) phosphomimetic amino acid substitution mutant was phosphorylated at a similar rate to the GST-I κ B α (1-54) wt substrate and the remaining GST-I κ B α (1-54) single amino acid substitution mutants were phosphorylated at a rate that was only reduced by approximately 1.5-fold when compared to GST-I κ B α (1-54) wt (Fig. 5D and Table 2). Thus, substitution of destruction box residues Ser³² or Ser³⁶ of the GST-I κ B α (1-54) substrates altered the exponential rate of phosphorylation of these truncated substrates to a lesser extent than substitution of Ser³² or Ser³⁶ within the full-length I κ B α substrates. We concluded that although interactions between IKK β and the C-terminus of I κ B α do not significantly influence the overall exponential rate of IKK β -catalyzed phosphorylation, these interactions may influence the individual rates of phosphorylation of I κ B α Ser³² and Ser³⁶.

Discussion

The pathways of NF- κ B regulation are widely viewed as potential therapeutic targets (27,28). Thus, an understanding of the molecular mechanisms of the enzymes involved in the regulation of these critical pathways, such as IKK β , may be advantageous for the development of novel therapies for the treatment of a wide array of human diseases, such as cancer. The IKK β -catalyzed phosphorylation of I κ B protein substrates is an essential step within the canonical

pathway of NF- κ B activation. Thus, the IKK β signal transduction pathway has been the subject of intensive study (29). However, despite recent crystal structures of IKK β (18-20), the molecular mechanism of IKK β -catalyzed phosphorylation remains poorly understood. Here we employed a pre-steady-state kinetic approach to investigate the phosphorylation of I κ B α by a constitutively active form of human IKK β . Importantly, we chose to study the phosphorylation of both full-length I κ B α and GTS-I κ B α (1-54) fusion protein substrates (Fig. 1B and C) to investigate potentially important interactions between IKK β and the C-terminal domain of I κ B α .

We concluded that IKK β phosphorylates I κ B α twice within a single binding event and that phosphorylation of Ser³² of I κ B α increases the rate of phosphorylation of Ser³⁶. Thus, we propose a model (Fig. 6) wherein IKK β binds I κ B α and processively phosphorylates I κ B α first at Ser³², followed by a second phosphorylation event at Ser³⁶. Reciprocal GST-pull down experiments have indicated that the ULD and SSD of IKK β bind strongly to the C-terminal region (residues 55-317, Fig. 1B) of I κ B α (19). Such a binding event may orient the N-terminus of I κ B α for proper phosphorylation at Ser³² and Ser³⁶ by the kinase domain of IKK β . Consistently, deletion of the ULD-SDD of IKK β results in the shifting of phosphorylation specificity from the cognate sites Ser³² and Ser³⁶ of I κ B α to its C-terminal PEST domain (Fig. 1B) (19). Besides the model in Fig. 6, IKK β may phosphorylate Ser³⁶ first, followed by Ser³², or randomly on either Ser³² or Ser³⁶ first. Our kinetic data (Fig. 4 and Table 1) indicate that the model in Fig. 6, relative to the two competing models, will allow IKK β to achieve the highest I κ B α phosphorylation efficiency.

Furthermore, of the two cognate I κ B α phosphorylation sites, the IKK β kinase domain may preferentially phosphorylate I κ B α at Ser³² first by virtue of a preference for phosphorylation of the 27-DDXXDS-32 amino acid sequence (Fig. 7). After phosphorylation of I κ B α at Ser³², the phosphorylated Ser³² residue will possess a similar size and charge as an aspartic acid residue. Thus, after phosphorylation of Ser³², the amino acid sequence surrounding Ser³⁶ will also resemble the potentially preferred amino acid sequence (Fig. 7). Binding of the I κ B α C-terminal

region by IKK β may be important for this preferential phosphorylation step as the exponential rate of phosphorylation of the full-length I κ B α (S32D) phosphomimetic substitution mutant was increased by approximately 2-fold with respect to the I κ B α wt substrate (Fig. 4D and Table 1), whereas this increase in the exponential rate of phosphorylation was not observed with the GST-I κ B α (1-54)(S32D) fusion protein substrate, which lacks the C-terminal region of I κ B α (Fig. 5D and Table 2). Importantly, this sequential phosphorylation of Ser³² followed by Ser³⁶ is not a requirement for IKK β activity, as phosphorylation of Ser³² and Ser³⁶ can occur independently of one another (Figs. 4 and 5). However, it is clear that IKK β is sensitive to even minor changes in the amino acid sequence of the I κ B α destruction box motif as evidenced by the fact that the exponential rate of phosphorylation of every I κ B α amino acid substitution mutant, except for the phosphomimetic S32D substitution, was slower than the exponential rate of phosphorylation of I κ B α substrates bearing the wild-type destruction box motif (Tables 1 and 2).

IKK β must phosphorylate I κ B α at both Ser³² and Ser³⁶ to induce the ubiquitination and degradation of I κ B α , and the subsequent release of NF- κ B dimers. Kinases are known to phosphorylate protein substrates at multiple sites through one of two separate mechanisms (reviewed in reference 30): i) a distributive mechanism, in which a kinase phosphorylates a substrate only once per binding event, or ii) a processive mechanism, in which a kinase phosphorylates a substrate two or more times per binding event. If IKK β utilized a distributive mechanism to phosphorylate I κ B α , then we would predict that pre-steady-state analysis would reveal separate rates for the first and second phosphorylation events due to the intervening disassociation and reassociation steps. Therefore, our finding that the exponential phase of IKK β -catalyzed phosphorylation of full-length I κ B α can be fit to a single rate (Fig. 4) is most consistent with the processive phosphorylation of Ser³² and Ser³⁶ without a significant intervening step. In support of this conclusion, the amplitude of the exponential phase produced by phosphorylation of each of the single amino acid substitution mutants in the I κ B α destruction box motif was reduced

approximately 2-fold when compared to the exponential phase amplitude of wt I κ B α (Fig. 4C and Table 1).

Single Ser to Ala amino acid substitutions at Ser³² or Ser³⁶ allow I κ B α to escape polyubiquitination and degradation, and render I κ B α a constitutive inhibitor of NF- κ B *in vivo* (31-34). Thus, a single phosphorylation event catalyzed by IKK β is insufficient to activate NF- κ B. The catalysis of two phosphorylation events within a single binding event is an efficient mechanism by which IKK β may activate NF- κ B. Strikingly, IKK β is known to catalyze two separate phosphorylation events with multiple substrates, including I κ B α , I κ B β and I κ B ϵ (reviewed in reference 35), suggesting that the processive catalysis of two phosphorylation events may be a general mechanism by which IKK β phosphorylates protein substrates. Additionally, IKK α is known to phosphorylate p100/NF- κ B2 at multiple residues to induce the proteolytic activation of this NF- κ B subunit (13) and the IKK-related kinases, IKK ϵ (also called IKK-i) and TANK-binding kinase 1 (TBK1), are known to phosphorylate the interferon regulatory factor (IRF) proteins IRF-3, IRF-5 and IRF-7 at two or more sites to activate these critical regulators of the innate immunity response (reviewed in reference 36). Thus, the processive catalysis of multiple phosphorylation events during a single binding event may be a conserved mechanism of both the IKK and IKK-related kinases.

Experimental Procedures

Materials - Reagents were purchased from the following companies: [γ -³²P]ATP from MP Biomedicals, ATP from Thermo Scientific, Optikinase from USB corporation, and plasmid pBluescript II KS + from Agilent Technologies.

Expression and purification of constitutively active human IKK β (S177E, S181E) - A recombinant baculovirus was generated by using the Bac-to-Bac system (Invitrogen) to express full-length, human IKK β (S177E, S181E) (residues 1-756) bearing a C-terminal hexahistidine affinity purification tag. The recombinant kinase was produced by infection of Hi5 insect cells in SF-900 II serum-free media (Gibco) in suspension. At 48 hours post-

infection, the cells were harvested and recombinant IKK β (S177E, S181E) was purified as previously described (19). Briefly, Hi5 cell pellets expressing IKK β were suspended in Buffer A (20 mM Tris, pH 8.0, 200 mM NaCl, 10 mM imidazole, and 0.25 mM TCEP), disrupted by sonication and cleared by centrifugation at 40,000 x g for 1 h. The cleared cell lysate was then incubated with Ni-NTA resin (Qiagen) and unbound proteins were removed by extensive washing with Buffer A. Bound proteins were subsequently eluted with a gradient of 10 mM to 250 mM imidazole with Buffer B (20 mM Tris, pH 8.0, 200 mM NaCl, 250 mM imidazole and 0.25 mM TCEP). IKK β -containing fractions were then pooled and applied to a Resource-Q anion exchange column (GE healthcare). The column was washed with Buffer C (40 mM Tris, pH 8.0, 0.1 M NaCl, 2 mM EDTA, 5 mM DTT) and bound proteins were eluted using a linear gradient of 0.1 M to 1 M NaCl with buffer D (40 mM Tris, pH 8.0, 2 mM EDTA, 5 mM DTT, 1 M NaCl). Fractions containing IKK β were pooled, concentrated and applied to a gel filtration column equilibrated to Buffer F (20 mM Tris, pH 7.6, 150 mM NaCl, 10 mM DTT). The gel filtration fractions containing IKK β were then concentrated and finally dialyzed against Storage Buffer (50 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1 mM DTT, 0.2 mM EDTA, 50% glycerol). Full-length IKK β (S177E, S181E) was determined to be >95% pure on the basis of Coomassie staining of SDS-PAGE gels.

Expression and purification of full-length I κ B α protein substrates - Full-length, human I κ B α was produced as a GST-fusion protein in *E. coli* by using vector pGEX4T3/I κ B α wt (19). The GST-I κ B α fusion protein possessed an N-terminal GST tag, followed by a tobacco etch virus (TEV) protease cleavage site and residues 1-317 of I κ B α . GST-tagged I κ B α was expressed in BL21 (DE3) pLysS cells at 20°C for 16 h. The cells were then harvested, suspended in GST Binding Buffer (25 mM NaH₂PO₄, pH 7.3, 140 mM NaCl, 2.7 mM KCl, and 5 mM DTT), lysed by French press and cleared by centrifugation at 40,000 x g for 30 min. The cleared cell lysate was incubated with glutathione sepharose resin (GE healthcare) and unbound proteins were removed by extensive washing with GST Binding Buffer. Bound

Uncovering the kinetic mechanism of IKK β -catalyzed phosphorylation of I κ B α

proteins were eluted by using a linear gradient of 0 mM to 15 mM reduced glutathione with GST Elution Buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM DTT, 15 mM reduced glutathione). Fractions containing GST-tagged I κ B α were then dialyzed against TEV Cleavage Buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM DTT) and the GST tag was removed from full-length I κ B α by incubation with TEV protease prepared in house as described previously (37). After cleavage, the protein fractions were applied to a MonoQ anion exchange column (GE healthcare) and washed with MonoQ Binding Buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 2 mM DTT). I κ B α was then eluted by using a linear gradient of 100 mM to 1.5 M NaCl with MonoQ Elution Buffer (50 mM Tris, pH 7.6, 1.5 M NaCl, 2 mM DTT). Fractions containing I κ B α were then applied to a Superdex 200 gel filtration column (GE healthcare) equilibrated with Size Exclusion Buffer (100 mM Tris, pH 7.6, 200 mM NaCl, 2 mM DTT, 0.4 mM EDTA). Fractions containing full-length I κ B α were pooled, concentrated, and dialyzed against Storage Buffer. All full-length I κ B α amino acid substitution mutants were expressed and purified by using the same methods. Full-length I κ B α and all I κ B α amino acid substitution mutants were determined to be >95% pure on the basis of Coomassie staining of SDS-PAGE gels (38,39).

Expression and purification of GST-I κ B α (1-54) fusion protein substrates - The GST-I κ B α (1-54) fusion protein was expressed from plasmid pDBhisGST(TEV)-I κ B α (1-54) in *E. coli*. This fusion protein possessed an N-terminal hexahistidine affinity tag, followed by a GST tag, a TEV protease cleavage site and residues 1-54 of I κ B α . The GST-I κ B α (1-54) fusion protein was expressed in BL21 (DE3) pLysS at 37°C for 3 h. The cells were then harvested, suspended in Ni Binding Buffer (50 mM Na₂HPO₄, pH 8.0, 300 mM NaCl), lysed using a French press, and cleared by centrifugation at 40,000 x g for 30 min. The cleared cell lysate was then incubated with Ni-NTA resin and unbound proteins were removed by washing with Ni Binding Buffer. Bound proteins were eluted by using a linear gradient of 0 mM to 250 mM imidazole with Ni Elution Buffer (50 mM Na₂HPO₄, pH 8.0, 300 mM NaCl, 250 mM imidazole, 2 mM DTT). Fractions containing GST-I κ B α (1-54) were

concentrated and applied to a Superdex 200 gel filtration column equilibrated with Size Exclusion Buffer. Fractions containing GST-I κ B α (1-54) were then pooled, concentrated, and dialyzed against Storage Buffer as described for full-length I κ B α above. The GST-I κ B α (1-54) fusion protein substrate and all GST-I κ B α (1-54) amino acid substitution mutants were determined to be >95% pure on the basis of Coomassie staining of SDS-PAGE gels.

Pre-steady-state kinetic measurements of full-length I κ B α phosphorylation catalyzed by constitutively active IKK β - All kinetic assays were performed in Kinase Reaction Buffer (50 mM Tris-Cl, pH 7.6 at 30°C, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 10% glycerol, 0.1 mM EDTA) at 30°C. The reported concentrations are final after mixing all components. A pre-incubated solution containing IKK β (S177E, S181E) (3 μ M) and full-length I κ B α (0.75 μ M) was rapidly mixed with a solution containing [γ -³²P]ATP (2.5 μ M to 500 μ M, 125 Ci/mole) and a linearized, 5'-[³²P]-radiolabeled pBlueScript loading control (250 CPM/ μ L). The reaction mixtures were quenched at various times by the addition of EDTA to a final concentration of 375 mM. Reactions were carried out by using a rapid-chemical quench flow apparatus (KinTek). Reaction products were resolved by using SDS-PAGE (12% polyacrylamide) and quantified by using a Typhoon TRIO (GE Healthcare). The detected amount of [³²P]-radiolabeled I κ B α was normalized to the radiolabeled pBlueScript internal standard at each time point and the normalized data was plotted as a function of time. The time courses of product formation at each nucleotide concentration were fit to biphasic Equation 1 (Eq. 1) by using the nonlinear regression program, KaleidaGraph (Synergy Software),

$$[\text{product}] = A/[1 - \exp(-k_e t)] + k_l t \quad (\text{eq. 1})$$

where A is the exponential phase amplitude, t is the reaction time, k_e is the exponential phase rate and k_l is the linear rate (40-42). The k_e values were then plotted as a function of ATP concentration and hyperbolic Equation 2 (Eq. 2) was used to acquire the maximum observed rate (k_p) of I κ B α phosphorylation catalyzed by IKK β (S177E,

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S181E) and the binding affinity of ATP for the IKK β •I κ B α complex (K_d) (43).

$$k_e = k_p [ATP]/([ATP] + K_d) \quad (\text{eq. 2})$$

Comparison of IKK β -catalyzed phosphorylation of I κ B α and I κ B α amino acid substitution mutants

- A pre-incubated solution containing IKK β (3 μ M) and either full-length I κ B α wt (0.75 μ M) or the indicated I κ B α amino acid substitution mutant (0.75 μ M) was rapidly mixed with a solution containing [γ - 32 P]ATP (500 μ M, 125 Ci/mole) and a linearized, 5'-[32 P]-radiolabeled pBlueScript loading control (250 CPM/ μ L). The reaction mixtures were quenched and product

formation was quantified as described above. The time courses of full-length I κ B α phosphorylation were then fit to biphasic Equation 1 (Eq. 1).

The pre-steady-state kinetic parameters of the GST-I κ B α (1-54) substrates were determined as described for the full-length I κ B α substrates above. However, the IKK β phosphorylation of the GST-I κ B α (1-54) substrates lacked a clearly defined linear rate. Therefore, the time courses of GST-I κ B α (1-54) product formation were fit to single-exponential Equation 3 (Eq. 3), where A is the exponential phase amplitude, t is the reaction time and k_e is the observed exponential phase rate.

$$[\text{product}] = A[1 - \exp(-k_e t)] \quad (\text{eq. 3})$$

Data availability

The manuscript contains all data described within the text.

Acknowledgements

We would like to thank Drs. Walter Zahurancik and Brian Maxwell for critical reading of the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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FOOTNOTES

This work was supported by National Institutes of Health Grant (R01GM122093) and National Science Foundation Grant (MCB-1716168) to Z.S.

The abbreviations used are: I κ B, Inhibitor of NF- κ B; IKK, Inhibitor of κ B kinase; IRF, interferon regulatory factor; NEMO, NF- κ B essential modulator; NF- κ B, Nuclear factor κ B; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TBK1, Tank-binding kinase 1; TCEP, tris(2-carboxyethyl)phosphine

Figure Legends

Figure 1. Schematic diagrams of human IKK β , I κ B α and I κ B α mutations. (A) Human IKK β . The boundaries of the kinase domain (KD), Ubiquitin-like domain (ULD), Scaffold/Dimerization domain (SDD) and NEMO binding domain (NBD) are shown. The positions of Ser¹⁷⁷ and Ser¹⁸¹ within the kinase activation loop are also indicated. (B) Full-length I κ B α and the GST-I κ B α (1-54) fusion proteins. I κ B α is composed of the signal response domain (SRD) (residue 1-66), the ankryin repeat domain (ARD) (residue 67-280), and the PEST domain (residue 281-317). (C) I κ B α amino acid substitution mutants. The destruction box motif (residues 31 to 36) of wild-type (wt) I κ B α and each I κ B α amino acid substitution mutation are shown. The specific sites of I κ B α phosphorylation are highlighted in grey.

Figure 2. Example autoradiogram demonstrating I κ B α phosphorylation catalyzed by IKK β . A pre-incubated solution of IKK β (3 μ M) and full-length I κ B α (0.75 μ M) was rapidly mixed with a solution containing a [γ -³²P]-labeled loading control and 500 μ M [γ -³²P]ATP. After various times, the reactions were stopped by the addition of EDTA to a final concentration of 375 mM. The reaction products were then resolved by using SDS-PAGE and visualized by autoradiography. The positions of the radiolabeled loading control, IKK β and I κ B α are indicated.

Figure 3. ATP concentration dependence of the pre-steady-state rate of full-length I κ B α phosphorylation catalyzed by constitutively active IKK β . (A) A pre-incubated solution of IKK β (3 μ M) and full-length I κ B α (0.75 μ M) was rapidly mixed with a solution containing a [γ -³²P]-labeled loading control and increasing concentrations of [γ -³²P]ATP for various times. The solid lines represent the best fits to biphasic Equation 1 (see Experimental Procedures). (B) The exponential rates (k_e) obtained from the data fitting above were plotted as a function of ATP concentration and fit to hyperbolic Equation 2, yielding a k_p of 0.32 ± 0.01 s⁻¹ and a K_d of 12 ± 1 μ M.

Figure 4. IKK β phosphorylation of full-length I κ B α and full-length I κ B α amino acid substitution mutants. A pre-incubated solution of IKK β (3 μ M) and the indicated full-length I κ B α (0.75 μ M) was rapidly mixed with a solution containing a [γ -³²P]-labeled loading control and [γ -³²P]ATP (500 μ M) for various times. Phosphorylation of the full-length I κ B α substrates was then normalized to the loading control and plotted as arbitrary phosphorylation units (a.u.) as a function of time from (A) 0 to 500 sec or (B) 0 to 50 sec. The solid lines represent the best fits to biphasic Equation 1 (see Experimental Procedures). The exponential phase amplitudes (C), the exponential phase rates (D), and the linear phase rates (E) were separately plotted against wild-type I κ B α and its various mutants.

Figure 5. IKK β phosphorylation of GST-I κ B α (1-54) and GST-I κ B α (1-54) amino acid substitution mutants. A pre-incubated solution of IKK β (3 μ M) and the indicated GST-I κ B α (1-54) fusion protein (0.75 μ M) was rapidly mixed with a solution containing a [γ -³²P]-labeled loading control and [γ -³²P]ATP (500 μ M) for various times. Phosphorylation of the full-length I κ B α substrates was then normalized to the loading control and plotted as arbitrary phosphorylation units (au) as a function of time from (A) 0 to 500 sec or (B) 0 to 50 sec. The solid lines represent the best fits to single-exponential Equation 3 (see Experimental Procedures). The exponential phase amplitudes (C) and the exponential phase rates (D) were separately plotted against wild-type I κ B α and its various mutants.

Figure 6. Proposed model of double I κ B α phosphorylation catalyzed by IKK β within a single binding event. The C-terminal region of I κ B α is first bound by the ULD and SDD of IKK β , which serves to coordinate the N-terminal region of I κ B α for phosphorylation by the KD. After recognition and binding of the I κ B α phosphorylation site by the KD, IKK β catalyzes the first phosphorylation of I κ B α . The N-terminal region of I κ B α is then rapidly repositioned within the kinase active site and the second phosphorylation event is catalyzed while the C-terminal region of I κ B α remains bound to IKK β .

Figure 7. Model of sequential I κ B α phosphorylation catalyzed by IKK β . Residues 27 to 36 of I κ B α are shown above with the proposed, preferred recognition site of IKK β shown below. After the first phosphorylation event,

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the size and charge of phosphorylated Ser³² mimics Asp, thereby changing residues 31 to 36 into a second preferred recognition site.