The Multisubunit IκB Kinase Complex Shows Random Sequential Kinetics and Is Activated by the C-terminal Domain of IκBα*

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The multisubunit IκB kinase (IKK) catalyzes the signal-inducible phosphorylation of N-terminal serines of IκB. This phosphorylation is the key step in regulating the subsequent ubiquitination and proteolysis of IκB, which then releases NF-κB to promote gene transcription. As measured by [32P]incorporation into a GST-IκBα fusion protein, varying both the concentration of GST-IκBα and [γ-32P]ATP resulted in a kinetic pattern consistent with a random, sequential binding mechanism. Values of 55 nM and 7 μM were obtained for the dissociation constants of GST-IκBα and ATP, respectively. The value of α, a factor by which binding of one substrate changes the dissociation constant for the other substrate, was determined to be 0.11. This indicates that the two substrates bind in a cooperative fashion. Peptides corresponding to either amino acids 26–42 (N-terminal peptide) or amino acids 279–303 (C-terminal peptide) of IκBα inhibited the IKK-catalyzed phosphorylation of GST-IκBα; the C-terminal peptide, unexpectedly, was more potent. The inhibition by the C-terminal peptide was competitive with respect to GST-IκBα and mixed with respect to ATP, which verified the sequential binding mechanism. The C-terminal peptide was also a substrate for the enzyme, and a dissociation constant of 2.9–6.2 μM was obtained. Additionally, the N-terminal peptide was a substrate (K_m = 140 μM). Competitive inhibition of the IKK-catalyzed phosphorylation of the C-terminal peptide by the N-terminal peptide indicated that the peptides are phosphorylated by the same active site. Surprisingly, the presence of the C-terminal peptide greatly accelerated the rate of phosphorylation of the N-terminal peptide as represented by a 160-fold increase in the apparent second-order rate constant (k_cat/K_m). These results are consistent with an allosteric site present within IKK that recognizes the C terminus of IκBα and activates the enzyme. This previously unobserved interaction with the C terminus may represent an important mechanism by which the enzyme recognizes and phosphorylates IκBα.

In unstimulated cells, NF-κB normally resides in the cytoplasm as an inactive complex with an IκB inhibitory protein. This class of protein includes IκB-α, IκB-β, and IκB-ε, which all contain ankyrin repeats necessary for complexation with NF-κB (for a review, see Ref. 3). In the case of IκB-α, which is the most carefully studied member of this class, stimulation of cells with agents that activate NF-κB-dependent gene transcription results in a phosphorylation of IκB-α at Ser-32 and Ser-36 (4). This is critical for subsequent ubiquitination and proteolysis of IκB-α, which then leaves NF-κB free to translocate to the nucleus and promote gene transcription (5–7). Indeed, a mutant in which both Ser-32 and Ser-36 have been changed to alanine prevents signal-induced activation of NF-κB and results in an IκB-α that is neither phosphorylated, ubiquitinated, nor proteolytically digested (7). Analogous serines have been identified in both IκB-β and IκB-ε, and phosphorylation at these residues appears to regulate the proteolytic degradation of these proteins by a mechanism similar to that of IκB-α (8, 9). Because of its important role in the activation of NF-κB, the inhibition of this signal-inducible phosphorylation of IκB is an important target for novel anti-inflammatory agents.

A high molecular mass (500–900 kDa) multisubunit IκB kinase (IKK)1 that phosphorylates at Ser-32 and Ser-36 of IκB-α has been isolated from HeLa cells (10–12). The IKK also phosphorylates at Ser-19 and Ser-23 in the N terminus of IκB-β (13). The kinase activity is greatly enhanced if the cells are first treated with tumor necrosis factor-α, which appears to activate a kinase cascade leading to the phosphorylation of the IKK (11, 13).

Recently, two catalytic subunits (termed IKK-α and IKK-β) of IKK have been identified, cloned, and shown to be widely expressed in human tissues (12–16). IKK-α and IKK-β form homo- and heterodimers with each other, but the active complex appears to be the heterodimer (12, 14). Demonstration that IKK is the kinase involved in the signal-inducible degradation of IκB-α was accomplished by both antisense inhibition of IKK-α and the use of dominant negative, catalytically inactive mutants of IKK-α and IKK-β (12, 15, 16). Both approaches abrogated cytokine-induced activation of NF-κB. The signal-induced activation of IKK appears to proceed through phosphorylation of the IKK-α and/or IKK-β subunits by a mitogen-activated protein kinase kinase (such as mitogen-activated protein kinase/ERK kinase kinase-1 or NF-κB-inducing kinase), which greatly enhances the enzymatic activity.

A knowledge of the mechanism is central to the understanding of any enzyme. To this end, we report here a kinetic investigation of the multisubunit IKK that demonstrates, surpris-

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† The abbreviations used are: IKK, IκB kinase; GST, glutathione S-transferase; Fmoc, N-(9-fluorenylmethoxycarbonyl); HPLC, high performance liquid chromatography.

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The NF-κB/Rel family of transcriptional activators regulates the expression of numerous genes involved in inflammatory and immune responses, such as the cytokines tumor necrosis factor-α, interleukin-6, interleukin-8, and interleukin-1β; the adhesion molecules E-selectin and VCAM-1; and the enzyme nitric oxide synthase (for reviews, see Refs. 1 and 2).
ingly, that the enzyme recognizes and is stimulated by elements of the C terminus of IκBα.

**EXPERIMENTAL PROCEDURES**

**Materials—**GST-IκBα was purchased from Santa Cruz Biotechnology, and the purity was estimated to be 34% by SDS-polyacrylamide gel electrophoresis and Coomassie staining. HeLa S3 cells were obtained from ATCC. Tumor necrosis factor-α was purchased from R&D Systems, and [γ-32P]ATP (1000 Ci/mmol) was purchased from Amersham Pharmacia Biotech.

**Peptide Synthesis—**An N-terminal peptide corresponding to amino acids 26–42 of IκBα (LDDHDSGLDSMKDEEY) was prepared along with a C-terminal peptide corresponding to amino acids 279–303 of IκBα (MLPESEDEYDSTDESTFEETEDEEL). Each peptide was synthesized on Fmoc-Knorr amide resin (Midwest Biotech, Fishers, IN) with an Applied Biosystems (Foster City, CA) model 433A synthesizer and the FastMoc chemistry protocol (0.25 mmol scale) supplied with the instrument. Amino acids were double coupled as their N-Fmoc derivatives, and reactive side chains were protected as follows: Asp and Glu, t-butyl ester; Ser and Thr, t-butyl ether; His, triphenylmethyl; Lys, t-butyloxycarbonyl; and Arg, pentamethylcroman-sulfonyle. After the final double coupling cycle, the N-terminal Fmoc group was removed by the two-step treatment with piperidine in N-methylpyrrolidine as described by the manufacturer. The N-terminal free amines were then treated with 10% acetic anhydride, 5% diisopropylamine in N-methylpyrrolidine to yield the N-acetyl derivative. The protected peptide tetrabutylammonium salts were purchased from Santa Cruz Biotechnology (Foster City, CA) and used as a 100 mM stock in 100 mM EDTA, 100 mM NaCl, pH 7.5. Protease inhibitor mixture (Calbiochem-Novabiochem Corp. La Jolla, CA) was prepared as a 100 mM stock (50 mM AEBSF, 50 mM benzamidine, 1 mM EDTA, 100 μM E-64, 100 μM leupeptin, 100 μM aproitin) and diluted to 1× in ice-cold lysis buffer.

**Cell Culture—**HeLa S3 cells were grown as a suspension culture in minimum essential medium (Life Technologies, Inc.). Medium was supplemented with 10% FBS and 50 μg/ml gentamicin.

**Isolation of IKK—**A procedure adapted from the one described by Lee et al. (11) was used. HeLa S3 cells that had been collected and resuspended at a concentration of 6 × 10^6 in 25 ml of medium were treated with 20 ng/ml tumor necrosis factor-α for 10 min at 37 °C and then pelleted at 600 × g for 10 min at 4 °C. Cells were washed once with ice-cold PBS and resuspended in ice-cold lysis buffer (50 mM Tris, 1 mM EGTA, pH 7.5). Mixtures of IKK were added to solutions containing peptide and [γ-32P]ATP (1000 Ci/mmol) in 50 mM Tris-HCl, 5 mM MgCl₂, containing 3.1 μM okadaic acid at pH 8. After 60–90 min, the kinase reactions were frozen until analyzed by HPLC.

**Kinetic Mechanism of IKK—**There are several kinetic mechanisms that have been described for two substrate enzyme systems. Fig. 1 shows a Hanes plot of IKK velocity as a function of the concentration of IκB-α at different ATP concentrations. This analysis is best fit to a random, sequential binding mechanism in which the enzyme binds both substrates prior to product release. This is in contrast to a ping-pong mechanism, in which one product is released before the second substrate binds, which would give a Hanes plot with lines intersecting at the y axis (17). Verification of a random versus ordered binding mechanism comes from the use of inhibitors and is presented below. This sequential mechanism is defined in Scheme 1, where K_{iBα} and K_{iBα} are the dissociation constants for IκB-α in the absence and presence, respectively, of ATP in the active site; K_{ATP} and αK_{ATP} are the dissociation constants for ATP in the absence and presence, respectively, of IκB-α in the active site. Using a nonlinear regression analysis of the data (18), values of 55 ± 25 nM, 7.3 ± 3.4 μM, and 0.11 were obtained for K_{iBα}, K_{ATP}, and α, respectively. A value of α<1 demon-
strates that the binding of one substrate increases the affinity for the second substrate (17).

**Peptide Analogs of IκB-α as Inhibitors and Substrates of IKK**—In an effort to verify the sequential mechanism represented in Scheme 1, peptides corresponding to either amino acids 26–42 of IκB-α (termed the N-terminal peptide) or amino acids 279–303 of IκB-α (a C-terminal peptide) were tested as inhibitors of the IKK-catalyzed phosphorylation of IκB-α. With the N-terminal peptide, a dose-dependent inhibition was obtained that yielded a linear Dixon plot, as shown in Fig. 2. This is not surprising because this N-terminal peptide corresponds to the amino acid sequence around the phosphorylation sites (Ser-32/Ser-36) of IκB-α. Indeed, this peptide is also phosphorylated by the enzyme (see below). Unexpectedly, a C-terminal peptide also inhibited the enzyme, and Fig. 2 demonstrates that it was even more potent than the N-terminal peptide.

As represented in Fig. 3, the inhibition of the IKK-catalyzed phosphorylation of IκB-α by the C-terminal peptide was shown to be competitive with respect to IκB-α. This would be expected from a mechanism detailed in Scheme 2, in which the binding of the inhibitor (i.e., peptide) competes with the binding of IκB-α but not with the binding of ATP. Verification of this mechanism comes from the inhibition observed with the C-terminal peptide while keeping the concentration of IκB-α fixed and varying the concentration of ATP. As shown in Fig. 4, the inhibition pattern observed in this case is mixed type. As expected from Scheme 2, infinitely large concentrations of ATP are unable to completely overcome the inhibition produced by the C-terminal peptide, an observation that unequivocally rules out an ordered binding mechanism that would have shown competitive inhibition (17, 19).

Values for $K_I$ and $\beta$ were estimated from the data represented in Figs. 3 and 4 and from the values of $K_{\text{ATP}}$, $K_{\text{IκB^α}}$, and $\alpha$ determined above. First, by obtaining from Fig. 4 a value of $20.5 \pm 8.9 \mu M$ for the apparent $K_i$ ($K_{\text{app}}'$), the value of the intrinsic $K_i$ was calculated to be $6.2 \pm 3.4 \mu M$ from Equation 1 (17). Then, the apparent $K_i$ ($K_{\text{app}}''$) of $4.4 \pm 0.9 \mu M$ from Fig. 3 was used in Equation 2 (taken from Ref. 17) to obtain a value for $\beta$ of $0.3 \pm 0.5$. This value, within experimental error, was the same as that obtained for $\alpha$. Because the value for $\beta$ is less than 1, it demonstrates that the binding of the peptide also increases the affinity for ATP.

$$K_{\text{app}}' = K_i \left(1 + \frac{[\text{IκB}]}{K_{\text{IκB^α}}} \right) \quad \text{(Eq. 1)}$$

$$K_{\text{app}}'' = \frac{\beta K_i \left(1 + \frac{K_{\text{ATP}}}{[\text{ATP}] + \beta K_{\text{ATP}}} \right)}{\left(1 + \frac{[\text{IκB}]}{K_{\text{IκB^α}}} \right)} \quad \text{(Eq. 2)}$$

Even more surprisingly, the C-terminal peptide was phosphorylated by IKK. A hyperbolic plot (not shown) of rate versus peptide concentration was obtained that yielded an apparent $K_m$ of $2.9 \pm 1.0 \mu M$ for the peptide under these conditions.

Because the concentration of ATP used was only 8 nM, this apparent $K_m$ is probably a close approximation of the true $K_m$. Indeed, this value agrees well with the $K_i$ value of $6.2 \pm 3.4 \mu M$ calculated for the C-terminal peptide as an inhibitor.

Although the N-terminal peptide was also a substrate for the IKK, it bound with considerably less affinity to the enzyme under these conditions, giving a $K_m$ of $140 \pm 28 \mu M$. The $V_{\text{max}}$ for phosphorylation of this N-terminal peptide is approximately $1/\beta$ of the $V_{\text{max}}$ for phosphorylation of IκB-α under identical conditions (results not shown). An analog of the N-terminal peptide, which had residues corresponding to Ser-32 and Ser-36 of IκB-α changed to alanines, was not phosphorylated under these conditions. However, this mutant peptide inhibited the IKK-catalyzed phosphorylation of IκB-α with a potency...
roughly equal to that shown by the N-terminal peptide (results not shown).

In an attempt to verify that the C-terminal and N-terminal peptides are binding to (and being phosphorylated by) the same active site, the inhibition by the N-terminal peptide of C-terminal peptide phosphorylation was measured. As shown in Fig. 5, the data fit to competitive inhibition, giving an apparent $K_I$ value ($K_{app}$) of 12 ± 2 μM for the N-terminal peptide.

Surprisingly, the C-terminal peptide did not inhibit the IKK-catalyzed phosphorylation of the N-terminal peptide. Instead, the presence of the C-terminal peptide greatly increased the rate of phosphorylation of the N-terminal peptide as shown in Fig. 6. As shown in Fig. 6 and Table I, this effect resulted from both a decrease in the apparent $K_m$ and an increase in the $k_{cat}$ ($V_{max}$).

**DISCUSSION**

The results presented here clearly demonstrate that the IKK proceeds through a random sequential binding mechanism in which both substrates bind before the first product is released (Scheme 1). During the course of this work, however, a number of surprising observations were made, including the finding that the enzyme recognizes a peptide corresponding to amino acids 279–303 of the C terminus of IkB-α. Indeed, this C-terminal peptide inhibited the IKK-catalyzed phosphorylation of IkB-α. Recognition by the enzyme of the C terminus of IkB-α...
is surprising because IKK has been shown to phosphorylate at Ser-32 and Ser-36 within the N terminus of IκB-α (13). The inhibition shown by this peptide, however, does explain the findings of Kuno et al. (20), in which this same C-terminal peptide was shown to block both LPS-induced NF-kB activation and phosphorylation of IκB-α in a cell-free system.

Our additional finding that the C-terminal peptide is phosphorylated by IKK corroborates a recent report that showed that a small amount of IκB-α phosphorylation catalyzed by IKK occurs within residues 264–314 of the C terminus (12). The present results also show that the enzyme phosphorylated an N-terminal peptide corresponding to amino acids 26–42 of IκB-α. Competitive inhibition of C-terminal peptide phosphorylation by the N-terminal peptide (Fig. 5) indicated that these peptides are not phosphorylated by two different enzymes within the IKK preparation but, instead, are binding to the same active site.

The most surprising observation was that the rate of IKK-catalyzed phosphorylation of the N-terminal peptide was greatly increased in the presence of the C-terminal peptide. This effect resulted from both an increase in the \( k_{cat} \) (\( V_{max} \)) and a decrease in the apparent \( K_m \) (Fig. 6 and Table I). A small but discernible increase in the rate of IκB-α phosphorylation was also observed at low concentrations of the C-terminal peptide (results not shown). The activation by the C-terminal peptide presumably occurs through its binding to a regulatory site other than the active site. The activation of the active site may result from a conformational change in the enzyme induced by C-terminal peptide binding to this putative regulatory site.

This observation that the C terminus of IκB-α activates the IKK-catalyzed phosphorylation of the N terminus can be used to reconcile the variable dissociation constants measured for the N-terminal peptide. For instance, when the N-terminal peptide was used as a substrate in the absence of the C-terminal peptide, a \( K_m \) of 140 ± 28 \( \mu M \) was determined. When the N-terminal peptide was used as an inhibitor of C-terminal peptide phosphorylation, a considerably smaller dissociation constant (apparent \( K_f \) of 12 ± 2 \( \mu M \) was obtained. This is consistent with the C-terminal peptide increasing the affinity of the enzyme for the N-terminal peptide. In addition, the data presented in Fig. 2 can be used with the \( K_f \) determined for the C-terminal peptide to estimate a dissociation constant (\( K_f \)) of 43 ± 27 \( \mu M \) for the N-terminal peptide when used as an inhibitor of IκB-α phosphorylation. This dissociation constant for the N-terminal peptide is also smaller than the \( K_m \) value of 140 \( \mu M \) because the C terminus of IκB-α is able to activate the IKK to bind the N-terminal peptide.

Although the apparent \( K_m \) for the N-terminal peptide in the absence of the C-terminal peptide is 140 \( \mu M \), the apparent \( K_m \) in the presence of the C-terminal peptide is reduced to a value of 21 \( \mu M \) (Table I). In fact, this apparent \( K_m \) in the presence of the C-terminal peptide is over an estimate of the “true” dissociation constant under these conditions. This is because the C-terminal peptide is competitively inhibiting the binding of the N-terminal peptide at the active site even though C-terminal binding to the putative regulatory site is enhancing N-terminal peptide binding. Thus, the value of the true dissociation constant (\( K_m \)) for the N-terminal peptide is approximately 4 \( \mu M \) when activated by C-terminal peptide binding to a regulatory site (i.e., \( K_{m,app} = K_m(1 + [I]/K_I) \), where \([I]\) and \(K_I\) equal the concentration and active-site dissociation constant, respectively, of the C-terminal peptide (21)). Therefore, the presence of the C-terminal peptide increases the apparent second-order rate constant \( (k_{cat}/K_m) \) for the phosphorylation of the N-terminal peptide by a factor of at least 160 as compared with the value in the absence of the C-terminal peptide (i.e., 291/4 versus 64/140).

The \( V_{max} \) values for N-terminal peptide phosphorylation were plotted at various C-terminal peptide concentrations to give a hyperbolic dependence (Fig. 7). This implies that the dissociation constant for the binding of the C-terminal peptide to the regulatory site is approximately 1 \( \mu M \). Thus, binding of the C terminus to this putative regulatory site may provide most of the overall free energy of binding of IκB-α to the enzyme. Moreover, this model may account for the specificity of phosphorylation of IκB-α at Ser-32/Ser-36 because activation of the active site would only occur when the C terminus of IκB-α is bound to the enzyme. In fact, when the C terminus of IκB-α is bound to the regulatory site, the N terminus may then be optimally positioned to interact with the now-activated active site.

This finding that the IKK recognizes and is activated by the C terminus of IκB-α also has important ramifications for our understanding of both the regulation of the degradation of IκB-α and the activation of NF-κB. Indeed, the presence and phosphorylation state of the C terminus of IκB-α has been shown to play a role in the signal-induced degradation of IκB-α (22–24). An analogous C-terminal region rich in proline, glut-
mate, aspartate, serine, and threonine residues (termed the PEST domain) has also been identified in IκB-β (8, 25). Interestingly, a recent report has indicated that recombinantly transferring the C terminus (along with the N terminus) of IκB-α to an unrelated protein, such as GST, enables GST to be phosphorylated and degraded in a signal-responsive way (27), although this may result from a recognition of the C terminus by the proteasome (26). Transferring only the N terminus of IκB-α to GST did not result in a recombinant protein that underwent signal-induced proteolysis.

Because the IKK is composed of multiple subunits, it is not clear whether activation by the C terminus of IκB-α results from binding to an allosteric site on the IKK-α and/or IKK-β catalytic subunits or through binding to another subunit within the complex, which then allosterically affects these catalytic kinase subunit(s). Because it has been reported that IKK-α and IKK-β need to form a heterodimer for maximal enzyme activity (15), an intriguing possibility is that the binding of the C terminus of IκB-α to one of the catalytic subunits (IKK-β in this example) allosterically affects the other subunit to accelerate the N-terminal phosphorylation of a second IκB-α molecule. We are currently addressing these questions through the use of purified IKK-α and IKK-β catalytic subunits.

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