Recombinant IkB Kinases α and β Are Direct Kinases of IkBa

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Activation of the transcription factor NF-κB is regulated by the phosphorylation and subsequent degradation of its inhibitory subunit, IkB. A large multiprotein complex, the IkB kinase (IKK), catalyzes the phosphorylation of IkB. The two kinase components of the IKK complex, IKKα and IKKβ, were overexpressed in insect cells and purified to homogeneity. Both purified IKKα and IKKβ specifically catalyzed the phosphorylation of the regulatory serine residues of IkBa. Hence, IKKα and IKKβ were functional catalytic subunits of the IKK complex. Purified IKKα and IKKβ also preferentially phosphorylated serine as opposed to threonine residues of IkBa, consistent with the substrate preference of the IKK complex. Kinetic analysis of purified IKKα and IKKβ revealed that the kinase activity of IKKβ on IkBa is 50–60-fold higher than that of IKKα. The primary difference between the two activities is the Km for IkBa. The kinetics of both IKKα and IKKβ followed a sequential Bi Bi mechanism. No synergistic effects on IkBa phosphorylation were detected between IKKα and IKKβ. Thus, in vitro, IKKα and IKKβ are two independent kinases of IkBa.

The transcription factor NF-κB plays a critical role in immune and inflammatory responses. It is regulated by the signaling of receptors for inflammatory cytokines such as TNF-α, IL-1, or other external stimuli. In resting cells, NF-κB is sequestered in the cytoplasm through its association with inhibitory proteins termed IkB. Two IkB proteins, IkBa and IkBβ, are rapidly phosphorylated at Ser residues in the N-terminal region upon stimulation by TNF-α and IL-1. The regulated phosphorylation is at Ser-32 and Ser-36 of IkBa and, correspondingly, Ser-19 and Ser-23 of IkBβ. The more recently cloned IκB protein, IκBe, also contains the two conserved Ser residues at the N terminus for signal-induced degradation. Phosphorylated IkBa and IκBe are subsequently ubiquitinated and undergo ubiquitin-dependent degradation by the 26 S proteasome. Degradation of IkBa results in the release of NF-κB, which then translocates to the nucleus, where it up-regulates the transcription of its target genes.

A 500–900-kDa protein complex that contains the TNF-α-induced IkBa kinase (IKK) has been purified and characterized independently by two groups. The IKK complex phosphorylates IkBa at the specific Ser residues that target the protein for ubiquitination and degradation. Two kinase subunits of the IKK complex, denoted IKKα and IKKβ, have been cloned (6–10). IKKα or IKKβ overexpressed in mammalian cells specifically phosphorylates IkBa and IkBβ after immunoprecipitation, and their kinase activities can be induced by TNF-α or IL-1. In HeLa cells, expression of antisense IKKα inhibited NF-κB activation by TNF-α or IL-1. Furthermore, overexpression of dominant-negative mutants of either IKKα or IKKβ blocked TNF-α/IL-1-induced NF-κB activation (8, 10). Thus, both IKKα and IKKβ contribute to the activity of the IKK complex and are involved in NF-κB activation.

An additional protein kinase, NF-κB-inducing kinase (NIK), has also been shown to be involved in the activation of IkB phosphorylation in both the IL-1 and TNF-α pathways. NIK has also been identified as an NIK-interacting protein by yeast two-hybrid analysis. Co-expression of NIK and IKKα (or IKKβ) stimulates the kinase activity of IKKα and IKKβ. NIK-dependent NF-κB activation is blocked by a dominant-negative IKKα mutant. These results indicate that NIK is an upstream regulator of IKKα and IKKβ.

Although the results using expressed and immunoprecipitated proteins are consistent with IKKα and IKKβ being the IkB kinases, it has not been demonstrated that IKKα or IKKβ directly phosphorylates IkBa. All phosphorylation experiments reported so far involve co-precipitation of other components of the high molecular mass complex from mammalian cells. Additionally, it has been reported that IKKα or IKKβ synthesized in wheat germ extracts was unable to phosphorylate IkBa. Thus, it is possible that IKKα and IKKβ may activate an as-yet-unidentified IkB kinase that co-immunoprecipitates with IKKα and IKKβ and phosphorylates IkBa (13). To resolve this issue, we have pursued in vitro studies with purified recombinant proteins. Recombinant IKKα and IKKβ were overexpressed in insect cells and purified. Here we demonstrate that purified IKKα or IKKβ alone is capable of phosphorylating specific Ser residues of IkBa in vitro, demonstrating that both IKKα and IKKβ are direct kinases of IkBa. We also show that both IKKα and IKKβ display a sequential Bi Bi mechanism. Kinetic parameters for both enzymes indicate that IKKβ is 50–60-fold more active than IKKα in catalyzing the phosphorylation of IkBa. Interestingly, IKKα and IKKβ showed no synergy in catalyzing the phosphorylation of IkBa in vitro. Such mechanism studies with purified IKKα and IKKβ provide new insights into the functions of these two kinase components of the multiprotein IKK complex.

EXPERIMENTAL PROCEDURES

Cloning—IKKα and IKKβ were cloned from a Jurkat cDNA library (CLONTECH) by polymerase chain reaction and were expressed as N-terminal Flag-tagged fusion proteins using the baculovirus expression vector pFastBac (Life Technologies, Inc.). IkBa was cloned into...
the RsrII and XhoI sites of pFastBac. IKKβ was cloned into the RsrII and KpnI sites of pFastBac. The protein sequences for the expressed IKKα and IKKβ are MDYKDDDDEK-IFKα (8) and MDYKDDDDEKKLAAANS-IFKβ (10), respectively. The K44M IKKβ mutant was constructed in the same vector as used for wild-type IKKβ. The Flag-tagged K44A IKKα mutant was constructed in the baculovirus expression vector pVL1393 (Invitrogen) at the BamHI and NotI sites. The above Flag-tagged recombinant proteins were expressed in SF9 cells. IKKα was also expressed as an N-terminal His₆-tagged protein in SF9 cells using pFastBacHta (Life Technologies, Inc.; EcoRI and XhoI sites). IkBa and IxBα(S32A/S36A) were expressed as a His₆-tagged threonine fusion protein (TRX-IxBα-(1–54)) in Escherichia coli BL21 (DE3). The sequences of all the constructed clones were verified by DNA sequencing.

Protein Expression and Purification—SF9 cells were grown in SF-900 II medium (Life Technologies, Inc.) with 1% fetal bovine serum and infected with baculoviruses following standard protocols (14). Cells were harvested 72 h after infection. Recombinant Flag-tagged IKKα, IKKβ, K44A IKKα, and K44M IKKβ were purified by affinity chromatography using anti-Flag M2 affinity gel (Eastman Kodak Co.). One liter of infected SF9 cells was lysed in 25 ml of 50 mM HEPES, pH 7.5, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail (Boehringer Mannheim and PharMingen). Cellular debris was removed by centrifugation at 16,000 × g for 30 min, and the NaCl concentration of the lysate was adjusted to 250 mM. The lysate was loaded onto a 5-ml column of anti-Flag M2 affinity gel; washed with 60 ml of 50 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride; and eluted with 250 μg/ml Flag peptide (Eastman Kodak Co.) in 50 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 1 mM EDTA, and 1 mM DTT. His₆-tagged IxBα was expressed and purified using standard protocols as described (15, 16). Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad) using bovine serum albumin as a standard. The purified proteins were stored in aliquots at −80 °C.

In Vitro Phosphorylation Assays—Kinase assays were performed in 20 mM HEPES, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 100 mM NaCl, 100 μM Na₃VO₄, 20 mM β-glycerophosphate, and 1 mM DTT. The amounts of the substrates ATP, [γ-³²P]ATP (2000 Ci/mmol; NEN Life Science Products), and IxBα are specified for each individual experiment. Reactions were performed at 23 °C for 10–30 min. Samples were analyzed by 4–20% SDS-polyacrylamide gel electrophoresis and autoradiography or by trichloroacetic acid precipitation on a microtiter plate (Millipore Corp.). For microtiter plate assays, 55 μl of reaction sample/well was quenched with 50 μl of 1 M sodium phosphate buffer, pH 8.0, and loaded onto a 96-well filter plate (Millipore Corp.). The column was washed three times with 0.6 ml of 0.1 M Na₂HPO₄, pH 8.0, 0.3 M NaCl, 20 mM imidazole, 10% glycerol, and 1 mM β-mercaptoethanol and eluted with 250 μl of 0.1 M Na₂HPO₄, pH 8.0, 0.3 M NaCl, 0.3 M imidazole, 10% glycerol, 1 mM β-mercaptoethanol, and protease inhibitor cocktail (Boehringer Mannheim).

RESULTS

IKKα and IKKβ Independently Phosphorylate IxBα—To investigate whether IKKα and IKKβ directly phosphorylate IxBα in vitro, we expressed IKKα and IKKβ in insect cells as N-terminal Flag-tagged proteins and purified them by affinity chromatography. Both IKKα and IKKβ were purified to apparent homogeneity and migrated on SDS-polyacrylamide gel with molecular masses predicted by their respective DNA sequences (Fig. 1). The predicted molecular masses of recombinant IKKα and IKKβ (86 kDa and 88 kDa, respectively) were confirmed by mass spectroscopic analysis (data not shown). Purified recombinant IKKα and IKKβ were tested for kinase activity by phosphorylation of IxBα. Either IKKα or IKKβ alone could phosphorylate IxBα (Fig. 2). Autophosphorylation of both IKKα and IKKβ was also observed. The phosphorylation of IxBα was specific for Ser-32 and Ser-36 of IxBα since there was no phosphorylation of the S32A/S36A mutant of IxBα (Fig. 2). To eliminate the possibility of trace kinase contaminants from the insect cells, two catalytically inactive kinase mutants (K44A IKKα and K44M IKKβ) were expressed and purified. The purified recombinant inactive mutants K44A IKKα and K44M IKKβ lacked activity in both the autophosphorylation and phosphorylation of IxBα (Fig. 2). Thus, both IKKα and IKKβ are direct IxBα kinases.

One distinguishing feature of IKKα as a Ser/Thr kinase is that it preferentially phosphorylates Ser versus Thr (6, 7). To test whether purified IKKα and IKKβ are Ser-selective kinases, we...
The Lineweaver-Burk plots of the data for both IKK and IKKβ showed significantly higher activity in the phosphorylation of Ser versus Thr residues of IκBα (Fig. 3). Quantitation of the phosphorylation by PhosphorImager analysis indicated that IKKα and IKKβ had 11- and 9-fold higher activities in the phosphorylation of Ser versus Thr residues of IκBα (1–54), respectively. The results indicate that the Ser selectivity of IKK is, at least in part, conveyed at the kinase subunit level.

**Kinetics of IKKα and IKKβ in the Phosphorylation of IκBα (1–54).**—Since both IKKα and IKKβ phosphorylate IκBα independently in vitro, we determined the kinetics of both in phosphorylating IκBα. Fig. 4 shows Michaelis-Menten plots of IKK activity as a function of the concentration of ATP at a constant concentration of IκBα. Both IKKα and IKKβ displayed standard Michaelis-Menten kinetics. The apparent $K_{m}$ values of IκBα and IKKβ were 126 and 136 nM, respectively (Fig. 4 and Table I). The kinase activities of IKKα and IKKβ were determined as a function of varying concentrations of IκBα (0.7–55 μM) at five fixed concentrations of ATP (30–1200 nM). The Lineweaver-Burk plots of the data for both IKKα and IKKβ followed Michaelis-Menten kinetics (Fig. 5 A and B). For both IKKα and IKKβ, a series of double-reciprocal straight-line plots intersected to the left side of the ordinates, indicating a sequential kinetic mechanism (18). At a saturating concentration of ATP (10-fold of $K_{m}$), the apparent $K_{m}$ value of IKKα for IκBα (23 μM) was 17-fold higher than that of IKKβ (1.3 μM). Under the same conditions, the apparent maximal turnover rates of IKKα and IKKβ were 0.08/min and 0.27/min at 23 °C, respectively (Fig. 5 A and B, and Table I). Based on the values of $k_{cat}$/$K_{m}$, IKKβ is 58-fold more potent than IKKα in the phosphorylation of IκBα (Table I). This result is consistent with the observation that when overexpressed in 293 cells, IKKβ is more potent in activating the NF-κB pathway than IKKα (10). One interesting observation is that the plots shown in Fig. 5 (A and B) intersected on the abscissa, indicating that the concentration of ATP has no effect on the apparent $K_{m}$ for IκBα. It was recently reported by Burke et al. (17) that a multisubunit IL-β kinase complex purified from TNF-α-stimulated HeLa cells showed random Bi Bi sequential kinetics with ATP and IκBα binding in a cooperative fashion ($α = 0.11$). To directly compare the kinetics of purified IKKα and IKKβ with those of the multisubunit IKK complex, the plots shown in Fig. 5 (A and B) were fitted to the random sequential model as described (17, 18). Accordingly, the data from Fig. 5 (A and B) were replotted as the slopes of the lines as a function of 1/ATP (Fig. 5C), and the $y$ intercepts as a function of 1/ATP (Fig. 5D) (18). For IKKα, values of 85 nm, 25 μM, 0.09/min, and 1.0 were obtained for $K_{ATP}$, $K_{IκBα}$, $k_{cat}$, and $α$, respectively (Fig. 5 C and D; and Table I). For IKKβ, values of 130 nm, 1.4 μM, 0.30/min, and 1.0 were obtained for $K_{ATP}$, $K_{IκBα}$, $k_{cat}$, and $α$, respectively (Fig. 5 C and D; and Table I). The 1.0 $α$ value for IKKα and IKKβ demonstrates that the binding of one substrate to the enzyme has no effect on the affinity for the other substrate. The obtained $K_{ATP}$ and $α$ values for IKKα and IKKβ are significantly different from those for the multisubunit IKK complex ($K_{ATP} = 7.3 μM$ and $α = 0.11$) (17). The $K_{m}$ for the N-terminal peptide (residues 26–42) of IκBα for the multisubunit IKK complex reported by Burke et al. (17) is 140 μM (14) in comparison with the $K_{m}$ values of 23 μM (IKKα) and 1.3 μM (IKKβ) for IκBα (1–54) (Table I). Although the kinetics for IKKα and IKKβ fit well to the random sequential mechanism, further inhibition studies are needed to distinguish between random and ordered Bi Bi mechanisms.

![Fig. 2. In vitro phosphorylation of IκBα by IKKα and IKKβ.](image-url)

Compared the phosphorylations of the peptides IκBα-(21–41), IκBα-(21–41)S32T/S36T, and IκBα-(21–41)S32pS36p by IKKα and IKKβ. Both IKKα and IKKβ phosphorylated IκBα-(21–41) and IκBα-(21–41)S32T/S36T, but not IκBα-(21–41)S32pS36p, which contained two phosphoserines (Fig. 3). Both IKKα and IKKβ showed significantly higher activity in the phosphorylation of Ser versus Thr residues of IκBα (Fig. 3). The $K_{m}$ and $V_{max}$ values for IκBα, 0.08/min and 0.27/min at 23 °C, respectively. The results indicate that the Ser selectivity of IKK is, at least in part, conveyed at the kinase subunit level.

![Fig. 3. IKKα and IKKβ show preference for Ser over Thr residues.](image-url)

Recombinant IKKα and IKKβ are Direct Kinases of IκBα. Michaelis-Menten plots were generated by varying [ATP] at a constant [TRX-IκBα] of 7 μM. Reactions (55 μl, plate assay) were performed at 23 °C for 15 min with [γ-32P]ATP (326 nCi). Kinase activities were recorded as incorporation of 32P (counts/min) into TRX-IκBα. Two-hundred nanograms of IKKα (closed circles) and 25 ng of IKKβ (closed triangles) were used in the assay. The initial rate $v$ was recorded as femtomoles of phosphate transferred to IκBα during the reaction period. The curves shown were obtained by fitting the data to a rectangular hyperbolic curve according to the Michaelis-Menten equation. The derived apparent $K_{cat}$/$ATP$, values were 128 and 136 μM for IKKα and IKKβ, respectively. Data are from a single experiment performed in duplicate and represent one of three experiments with similar results.
Recombinant IKKα and IKKβ Are Direct Kinases of IκBα

IKKα and IKKβ Show No Synergistic Effect on the Phosphorylation of IκBα—The native IKK complex contains both IKKα and IKKβ (6, 7). However, our in vitro studies showed that either IKKα or IKKβ alone could phosphorylate IκBα. We then investigated whether IKKα and IKKβ cooperate with each other in the phosphorylation of IκBα. When separately purified IKKα and IKKβ were mixed at equimolar concentrations, no synergistic effect on kinase activity was observed (Fig. 6A). In fact, if the kinase activities shown in Fig. 6A are normalized for the amount of IKKα and IKKβ, the activity of the IKKα/IKKβ mixture was ~20% less than the additive activity of IKKα and IKKβ (Fig. 6A). Because IKKβ was more active than IKKα, the activity of the IKKα/IKKβ mixture is probably due to the dominant activity of IKKβ. We further investigated whether the catalytically inactive mutant of IKKα would inhibit the kinase activity of wild-type IKKβ in vitro. When IKKβ was mixed with various amounts of the K44A IKKα mutant and then assayed for IκBα phosphorylation, no decrease in kinase activity was observed (Fig. 6B). Conversely, IKKα was not inhibited by the inactive K44M IKKβ mutant either (data not shown). These results are consistent with IKKα and IKKβ being independent kinases in vitro.

Kinase Activity of the Co-translated IKKα•IKKβ Complex Is Similar to the Additive Activity of IKKα and IKKβ—In mammalian cells, co-expression of IKKα and IKKβ yielded an IKKα•IKKβ heterocomplex (9, 10). To address whether IKKα and IKKβ form a stable complex when they are co-expressed in insect cells, Sf9 cells were infected with baculoviruses expressing various combinations of His8-IKKα, Flag-IKKα, and Flag-IKKβ. The resulting cell extract was subjected to co-immunoprecipitation analysis. Flag-IKKβ co-immunoprecipitated with His8-IKKα when they were co-expressed in Sf9 cells (Fig. 7), indicating formation of a stable IKKα•IKKβ heterocomplex. When His8-IKKα, Flag-IKKα, and Flag-IKKβ were co-expressed in Sf9 cells, the formation of both a His8-IKKα•Flag-IKKβ homocomplex and a His8-IKKα•Flag-IKKβ heterocomplex was detected (Fig. 7). However, more Flag-IKKβ was associated with His8-IKKα than Flag-IKKβ, despite similar expression levels of Flag-IKKα and Flag-IKKβ (Fig. 7). This suggests that assembly of the IKKα•IKKβ heterocomplex may occur more readily than assembly of the IKKα•IKKβ homocomplex in insect cells.

The co-expressed His8-IKKα•Flag-IKKβ complex was purified by anti-Flag M2 affinity chromatography, followed by a Nitrilotriacetic acid affinity column. The kinase activity of the purified complex was only ~20% more than the additive

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**TABLE I**

| Kinetic parameters of IKKα and IKKβ from data presented in Figs. 4 and 5
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| The value of **K_m**,**ATP** was obtained from Fig. 4. The remaining values were obtained from Fig. 5.
| **K_m**,**ATP** | **K_m**,**IκBα** | **k_cat** | **K_m**,**K_i**,**cat** | **k_cat** | **K_m**,**K_i**,**max** | **K_m**,**ATP** | **K_m**,**IκBα** | **a** |
|---|---|---|---|---|---|---|---|---|
| IKKα | 126 | 23 | 0.08 | 3.5 | 0.09 | 85 | 25 | 1.0 |
| IKKβ | 136 | 1.3 | 0.27 | 208 | 0.30 | 130 | 1.4 | 1.0 |

*a* The parameters were directly obtained from the Michaelis-Menten plots of 1/ν versus 1/[IκBα] at a fixed saturating concentration of ATP (10-fold of **K_m**,**ATP**).

*b* The parameters were obtained by fitting the two-substrate kinetics to a random sequential model as described under “Experimental Procedures.”

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**FIG. 5.** Kinetic patterns for IKKα and IKKβ with varying concentrations of IκBα. Double-reciprocal plots of 1/ν versus 1/[IκBα] were generated for IKKα (A) and IKKβ (B) at five fixed ATP concentrations of 30 nM (open squares), 60 nM (closed squares), 120 nM (×), 240 nM (open circles), and 1.2 μM (closed circles). Reactions (55 μl, plate assay) were performed at 23 °C for 15 min with 134 ng of IKKα (A) or 25 ng of IKKβ (B) using 411 nCi of [γ-**32**P]ATP. The initial rate ν was recorded as picomoles of phosphate transferred to IκBα during the reaction period. Data are from a single experiment performed in duplicate. In C, the slopes of the plots in A and B were replotted versus 1/[ATP]. In D, the y intercepts of the plots in A and B were replotted versus 1/[ATP]. Open circles, IKKα; closed circles, IKKβ. The x and y intercepts of the plots shown in C yielded −1/K_m,**ATP** and αK_m,**IκBα**/V_max, respectively. The x and y intercepts of the plots shown in D yielded −1/aK_m,**ATP** and 1/V_max, respectively (18).
FIG. 6. IKKα and IKKβ show no catalytic synergy. A, comparison of the activities of IKKα, IKKβ, the IKKα/IKKβ mixture, and the co-expressed IKKα/IKKβ complex. Reactions (55 μl, plate assay) were performed at 23 °C for 30 min with 50 ng of enzyme, 155 nCi of \([γ-32P]ATP\), 1 μM ATP, and 7 μM TRX-IκBα. To test the activity of the IKKα/IKKβ mixture, 25 ng each of purified IKKα and IKKβ were mixed on ice for 10 min before the assay. The co-translated IKKα/IKKβ complex was purified as described under "Experimental Procedures." Data represent one of two experiments with similar results. B, effect of the inactive K44A IKKα mutant on the kinase activity of IKKβ. Reactions (55 μl, plate assay) were performed at 23 °C for 30 min with 826 nCi of \([γ-32P]ATP\), 1 μM ATP, and 77 μM TRX-IκBα. The indicated amounts of enzymes were premixed before the assay. The amount of phosphate transferred to IκBα was recorded as counts/min. Data are presented as means ± S.E. of duplicate samples.

The activity of IKKα and IKKβ, based on normalized specific activities of each enzyme (Fig. 6A), is consistent with the data of the IKKα/IKKβ mixture, this result suggests that there was no significant synergy in IκBα phosphorylation between IKKα and IKKβ in the co-translated complex. Collectively, these data suggest that direct interactions between recombinant IKKα and IKKβ do not enhance their kinase activity.

**DISCUSSION**

Although IKKα and IKKβ are essential for IκBα phosphorylation (6–10), it has not been clear whether they directly phosphorylate IκBα. Here we have shown that purified recombinant IKKα or IKKβ (but not inactive kinase mutants of IKKα or IKKβ) phosphorylates IκBα in vitro, indicating that IKKα and IKKβ are direct kinases of IκBα. It is possible that an undetected co-purifying kinase could be activated by IKKα and, in turn, phosphorylates IκBα. However, the fact that there is a significant difference in \(K_m\) between purified IKKα and IKKβ strongly suggests that the two protein preparations contain different kinases. It is highly unlikely that purified IKKα and IKKβ contain two different contaminating kinases since they were expressed and purified identically. It has been previously shown that IKKα and IKKβ display distinct modes of regulation when overexpressed in mammalian cells (7, 9). Overexpressed IKKβ is constitutively active, whereas overexpressed IKKα is inactive unless cells are stimulated by TNF-α (7). As a consequence, overexpression of IKKβ results in greater activation of an NF-κB reporter gene than overexpression of IKKα (10). Our finding that purified recombinant IKKβ is 50–60-fold more active than IKKα toward IκBα is in agreement with previous results.

Recently, the enzyme kinetics of a multisubunit IκB kinase complex purified from TNF-α-stimulated HeLa cells were reported by Burke et al. (17). This kinase complex showed random sequential kinetics, and the two substrates (IκBα and ATP) bound in a cooperative fashion (\(α = 0.11\)). Although the kinetics of recombinant IKKα and IKKβ are consistent with a random sequential mechanism, both IKKα and IKKβ showed an \(α\) value of 1.0. Thus, unlike the IKK complex reported by Burke et al. (17), the affinity of IκBα for either IKKα or IKKβ is not affected by the binding of ATP. The reported dissociation constant of ATP for the IKK complex prepared by Burke et al. (17) was 7 μM. In contrast, the ATP dissociation constants for IKKα and IKKβ are 85 and 130 nM (Table I), respectively. Such notable differences in enzyme kinetics between IKKα (or IKKβ) and the IKK complex would probably suggest either that the kinase subunits in the IKK complex purified by Burke et al. (17) may be different from IKKα and IKKβ or that the activity of IKKα or IKKβ in the IKK complex was modified by other proteins in the complex. The IKK complex purified by Burke et al. (17) is apparently different from the IKK complex that contains IKKα and IKKβ (6, 7), since the former complex also phosphorylated the C-terminal peptide (residues 279–303) of IκBα (17), whereas the latter one did not phosphorylate the C-terminal region (residues 242–314) of IκBα (7). The \(K_m\) values of IKKα and IKKβ for ATP (130 nM) are considerably lower than those of other protein kinases, such as cAMP-dependent protein kinase (\(K_m = 10 \mu M\)) (19) and p38 mitogen-activated protein kinase (\(K_m = 23 \mu M\)) (20). This low \(K_m\) of IKKα and IKKβ, in combination with their Ser versus Thr

**FIG. 7. Interaction between IKKα and IKKβ.** Sf9 cells were infected with baculoviruses expressing the indicated recombinant proteins (His6-IKKα, Flag-IKKα, and Flag-IKKβ). Cells were lysed and immunoprecipitated with anti-His-tag antibodies (see "Experimental Procedures"). Crude cell lystate or immunoprecipitates (IP) were loaded on a 4–12% SDS gel, subjected to electrophoresis, and detected after electroblotting with anti-Flag M5 (Eastman Kodak Co.) or anti-His4 antibody. Three and 6 μl of the immunoprecipitates (total of 60 μl) were loaded for anti-Flag (upper) and anti-His-tag (middle) immunoblotting, respectively. Total cell lystate (1.5 μl; total of 250 μl) was loaded for anti-Flag immunoblotting (lower). The antibodies were visualized with the ECL kit (Amersham Pharmacia Biotech).
substrate selectivity, distinguishes IKKα and IKKβ as unique members of the Ser kinase family.

In addition to IkBα, both IkBβ and IkBe have also been reported to be phosphorylated by IKK (8, 10, 12). Our observation that IKKβ displayed a greater affinity (17-fold difference in K_m(IkBα)) for IkBα raises the possibility that the two kinases may also have different substrate preferences for phosphorylation of IkBα and IkBe. Thus, regulation of the degradation of different IkB isotypes could be achieved by two kinases with different preferences for the substrate IkB isomers. On the other hand, IKKα and IKKβ are also differentially regulated through upstream kinases; for example, MEKK1 has been shown to preferentially activate IKKβ (12, 21). Thus, differential regulation of IKKα and IKKβ could also regulate the degradation of different IkB proteins.

It has been reported that NIK activates IKKα and IKKβ and acts as an upstream kinase of IKKα (8, 10, 21). NIK co-expressed with IKKα in 293 cells preferentially phosphorylated the co-expressed IKKα at Ser-176, located in the activation loop of IKKα (22). However, these experiments were performed using immunoprecipitated proteins from crude cell lysate. We have not been able to show effects of recombinant NIK or catalytically inactive NIK on the activity of recombinant IKKα or IKKβ in phosphorylating IkBα in vitro.2 It is possible that although the baculovirus-expressed NIK showed reasonable activity in autophosphorylation, it was still less active or regulated differently than the NIK expressed in mammalian cells. Alternatively, an unidentified protein not found in insect cells may be required to mediate or regulate the action of NIK on IKKα.

It is puzzling that IKKα or IKKβ alone was active, yet they had no synergistic kinase activity. This indicates that in vitro, IKKα and IKKβ are two independent kinases, despite their coexistence in the 500–900-kDa IKK complex in cells (6, 7). In insect cells, co-expression of His6-IKKα and Flag-IKKβ yielded a stable His6-IKKα-Flag-IKKβ complex. Consistent with IKKα and IKKβ being two independent kinases in vitro, the co-expressed His6-IKKα-Flag-IKKβ complex displayed activity similar to the additive kinase activity of IKKα and IKKβ (Fig. 6A). It is possible that significant regulating effects on IKKα and IKKβ activity could occur in cells through interactions (catalytic or protein-protein) with other subunit(s) in the 500–900-kDa multiprotein IKK complex. Since the activation of NF-κB can be induced by various signals, other subunits in the IKK complex may also serve as signaling regulators or adapter molecules to allow selectivity in responding to different stimuli.

In summary, through these studies with purified recombinant IKKα and IKKβ, we have demonstrated that both IKKα and IKKβ are direct kinases of IkBα. It is therefore likely that IKKα and IKKβ are indeed catalytic subunits of the multiprotein IKK complex. The kinetics of both enzymes followed a sequential Bi Bi mechanism. The significantly higher activity of IKKβ in comparison with IKKα for IkBα may indicate their different roles in cellular function. There was no catalytic synergy between IKKα and IKKβ in the current in vitro studies, suggesting that further investigations into the functions of other components in the IKK complex are needed to understand the relative functional contributions of IKKα and IKKβ in cells.

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