Characterization of the Recombinant IKK1/IKK2 Heterodimer

MECHANISMS REGULATING KINASE ACTIVITY

Received for publication, January 12, 2000, and in revised form May 19, 2000
Published, JBC Papers in Press, May 22, 2000, DOI 10.1074/jbc.M000296200

Q. Khai Huynh‡, Hymavathi Boddupalli, Sharon A. Rouw, Carol M. Koboldt, Troii Hall, Cindy Sommers, Scott D. Hauser, Jennifer L. Pierce, Rodney G. Combs, Beverly A. Reitz, Judy A. Diaz-Collier, Robin A. Weinberg, Becky L. Hood, Bryan F. Kilpatrick, and Catherine S. Tripp

From Discovery Research, G. D. Searle and Company, the Monsanto Life Science Company, St. Louis, Missouri 63167

Nuclear factor kappa B (NF-κB) is a ubiquitous, inducible transcription factor that regulates the initiation and progression of immune and inflammatory stress responses. NF-κB activation depends on phosphorylation and degradation of its inhibitor protein, IκB, initiated by an IκB kinase (IKK) complex. This IKK complex includes a catalytic heterodimer composed of IκB kinase 1 (IKK1) and IκB kinase 2 (IKK2) as well as a regulatory adaptor subunit, NF-κB essential modulator. To better understand the role of IKKs in NF-κB activation, we have cloned, expressed, purified, and characterized the physiological isoform, the rhIKK1/rhIKK2 heterodimer. We compared its kinetic properties with those of the homodimers rhIKK1 and rhIKK2 and a constitutively active rhIKK2 (S177E, S181E) mutant. We demonstrate activation of these recombinantly expressed IKKs by phosphorylation during expression in a baculoviral system. The $K_m$ values for ATP and IκBα peptide for the rhIKK1/rhIKK2 heterodimer are 0.63 and 0.60 μM, respectively, which are comparable to those of the IKK2 homodimer. However, the purified rhIKK1/rhIKK2 heterodimer exhibits the highest catalytic efficiency ($k_{cat}/K_m$) of 47.50 h$^{-1}$ μM$^{-1}$ using an IκBα peptide substrate compared with any of the other IKK isoforms, including rhIKK2 (17.44 h$^{-1}$ μM$^{-1}$), its mutant rhIKK2 (S177E, S181E, 1.18 h$^{-1}$ μM$^{-1}$), or rhIKK1 (0.02 h$^{-1}$ μM$^{-1}$). Kinetic analysis also indicates that, although both products of the kinase reaction, ADP and a phosphorylated IκBα peptide, exhibited competitive inhibitory kinetics, only ADP with the low $K_i$ of 0.77 μM may play a physiological role in regulation of the enzyme activity.

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Dept. of Discovery Pharmacology, Searle Discovery Research, c/o Monsanto Life Science Company, Mailbox T3M, 800 North Lindbergh Blvd., St. Louis, MO 63167. Tel.: 314-694-5360; Fax: 314-694-3415; E-mail: quang.k.huynh@monsanto.com.

1The abbreviations used are: NF-κB, nuclear factor kappa B; IKK, IκB kinase; IKK2 (S177E, S181E), a variant of IKK2 in which Ser$^{177}$ and Ser$^{181}$ are replaced by Glu; TNF, tumor necrosis factor; LPS, lipopolysaccharide; NEMO, NF-κB essential modulator; ERK, extracellular signal-regulated kinase; IKKAP1, IKK complex-associated protein 1; MAPK, mitogen-activated protein kinase; rh, recombinant human; wt, wild type; DTI, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; m.o.i., multiplicity of infection; IL-1β, interleukin 1β; BSA, bovine serum albumin; GST, glutathione S-transferase; λ PPase, recombinant λ protein phosphatase.

This paper is available on line at http://www.jbc.org

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
cloned by standard biochemical purification, copurifying with IKK1 from TNFα-stimulated HeLa S3 cells as well as by being identified in the public data base from an expressed sequence tag clone with sequence homology to IKK1 (6–8). IKK2 is an 87-kDa, 736-amino acid protein with the same overall topology as IKK1 except for the addition of an 11-amino acid extension at the C terminus. IKK1 and IKK2 are 52% identical overall with 65% identity in the kinase domain and 44% identity in the protein interaction domains in the C terminus. Data obtained using transient mammalian expression analysis, by in vitro translation experiments, and by coexpression in a baculoviral system reveal that IKK1 and IKK2 associate preferentially as a heterodimer through their leucine zipper motifs. Although homodimers have also been described in these systems, the heterodimer is thought to be the physiological form of the kinase in mammalian cells (7, 15). Finally, NEMO (also termed IKKγ) contains three α-helical regions, including a leucine zipper, interacts preferentially with IKK2, and is required for activation of the heterodimeric kinase complex perhaps by bringing other proteins into the signalsome complex (10–12).

The kinase activities of IKK1 and IKK2 are regulated by phosphorylation and require an intact leucine zipper for dimerization as well as an intact helix-loop-helix domain, which can exert a positive regulatory effect on kinase activity even when it is expressed in trans with the remainder of the IKK protein (4–8, 16). Both IKK subunits contain a canonical mitogen activated protein kinase kinase (MAPKK) activation loop motif near the N terminus, which is the target for phosphorylation and activation of kinase activity by MAPK/ERK kinase 1, although the physiological regulation by these two upstream kinases awaits further characterization (2–3, 17). Finally, phosphorylation of serines in the C terminus of IKK2 results in a decreased IKK activity and is postulated to be responsible for the transient kinase activity seen after stimulation of cells with an agonist (16).

IKK2 demonstrates a more potent kinase activity compared with IKK1 using IκBα or IκBβ as a substrate (6–8, 16). Mutations of the phospho-acceptor serine residues within the MAPKK activation loop alters IKK2 kinase activity; the serine to alanine substitutions result in decreased kinase activity, whereas the serine to glutamic acid substitutions result in a constitutively active kinase. Similar alanine mutations in IKK1 do not result in a decreased stimulation of total IKK activity in response to TNFα or IL-1β (16). IKK2 being the dominant kinase activity within the IKK complex is further supported by the analysis of fibroblasts from mice deficient in IKK1 or IKK2. Fibroblasts lacking IKK1 retain full IKK activity in response to cytokines and could activate NF-κB. In contrast, fibroblasts lacking IKK2 do not exhibit IKK activity when stimulated with cytokines nor do they activate NF-κB. Furthermore, the phenotype of each IKK knock-out is unique, with IKK1 deficiency resulting in skin and skeletal defects and IKK2 knock-out being embryonic lethal due to hematocyte apoptosis (18–22).

The IKK activity has been isolated and characterized from mammalian cells as well as from expression of recombinant IKK1 and IKK2 homodimers in baculovirus systems (9, 12, 15, 23–27). Both the isolated IKK complex from mammalian cells and the recombinant IKKs utilize all three isoforms of IκBs, α, β, and γ, as substrates equally well. However, there are differences in the kinetic data reported for the rhIKK homodimers. First, the $K_m$ for IκBa have varied in different publications, with the wide range of 1.4–23 μM being reported for rhIKK1 compared with more similar values of 0.5–1.3 μM being reported for rhIKK2. Second, most reports indicate that rhIKK2 phosphorylates truncated IκBα more efficiently than does rhIKK1 with the $k_{cat}$ (h⁻¹) being three to four times greater for rhIKK2 compared with rhIKK1. In addition, the rhIKK2 (S177E, S181E) mutant has a dramatically enhanced kinase activity, being approximately 10-fold higher than rhIKK2 (12).

Third, kinetic analysis using rhIKK2 also indicates that, in the presence of NF-κB, the $K_m$ for IκBα is decreased from 2.2 to 1.4 μM and the $V_{max}$ is increased by a factor of four, indicating that rhIKK2 phosphorylates IκBα bound to NF-κB more efficiently than it phosphorylates free IκBα (16). Although the physiological form described to be most abundant in mammalian cells is the IKK1/IKK2 heterodimer, its thorough kinetic characterization has not been described to date. In this paper we have characterized the heterodimer rhIKK1/rhIKK2 and compared its kinase activity to that of the rhIKK1 homodimer rhIKK2 and the mutant rhIKK2 (S177E, S181E) homodimer. Although all purified recombinant enzymes are capable of phosphorylating IκBα, the rhIKK1/rhIKK2 heterodimer exhibits the highest catalytic efficiency. This kinase activity is dependent on phosphorylation, because phosphatase treatment abolishes the ability of each rhIKK to phosphorylate IκBα. While characterizing the purified rhIKKs, we also found that both of the products of the kinase reaction, ADP and a phosphorylated IκBα peptide, exhibited inhibitory activity; however only ADP has a $K_i$ that may support a physiological role in the regulation the IKK activity.

While performing these kinetic analysis of rhIKK isoforms, it was noted that, unlike the IKK activity in mammalian cells, which is not present unless stimulated by an agonist, the rhIKKs expressed in a baculovirus system are catalytically active upon their isolation. Because this signaling pathway is remarkably conserved during evolution, with IKK activity being described in Drosophila, oysters, and Dicyostelium (1, 28–29), we propose that recombinantly expressed hIKKs can be activated by phosphorylation via a homologous signaling pathway in the baculoviral system. In this paper we also demonstrate that an anti-NEMO antibody can immunoprecipitate rhIKKs from insect cell lysates infected with baculovirus containing only recombinantly expressed IKK proteins, strongly suggesting the presence of a functional NEMO homologue in the insect cells mediating the phosphorylation and activation of rhIKKs during expression.

**EXPERIMENTAL PROCEDURES**

**Materials**

Biotin capture plates (SAM² 96) were from Promega. Anti-FLAG affinity resin, FLAG-peptide, Nonidet P-40, bovine serum albumin (BSA), ATP, ADP, AMP, LPS (Escherichia coli serotype 0111:B4), and dithiothreitol (DTT) were obtained from Sigma. Antibodies specific for NEMO (IKKγ) (FL-419), IKK1 (H-744), IKK2 (H-470), and IκBα (C-21) were purchased from Santa Cruz Biotechnology. Ni-NTA resin was purchased from Qiagen. Peptides were purchased from American Peptide Co. Protease inhibitor mixture tablets were from Roche Molecular Biochemicals. Sepharose S-300 column was from Amersham Pharmacia Biotech. Centriprep-10 concentrators with a molecular mass cut-off of 10 kDa, and membranes with a molecular mass cut-off of 30 kDa were obtained from Amicon. [γ-32P]ATP (2500 Ci/mmol) and [γ-32P]ATP (6000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. The other reagents used were of the highest grade commercially available.

**Cloning and Expression**

cDNAs of human IKK1 and IKK2 were amplified by reverse transcriptase-polymerase chain reaction from human placenta RNA (CLONTECH). hIKK1 was subcloned into pFastBac HTa (Life Technologies) and expressed as N-terminal His₆-tagged fusion protein. The hIKK2 cDNA was amplified using a reverse oligonucleotide primer that incorporated the peptide sequence for a FLAG-epitope tag at the C terminus of the IKK2 coding region (DYKDDDDK). The hIKK2:FLAG cDNA was subcloned into the baculovirus vector pFastBac. The rhIKK2 phosphorylates truncated IκBα more efficiently than does rhIKK1 with the $k_{cat}$ (h⁻¹) being three to four times greater for rhIKK2 compared with rhIKK1. In addition, the rhIKK2 (S177E, S181E) mutant has a dramatically enhanced kinase activity, being approximately 10-fold higher than rhIKK2 (12).
(S177, E177E) mutant was constructed in the same vector used for wild type rhIKK2 using a QuikChange mutagenesis kit (Stratagene). Viral stocks of each construct were used to infect insect cells grown in suspension culture. The cells were lysed at a time that maximal expression and rhIKK activity were demonstrated. Cell lysates were stored at −80 °C. Following centrifugation, the recombinant proteins were undertaken as described below.

**Enzyme Isolation**

All purification procedures were carried out at 4 °C unless otherwise noted. Buffer A for purification was used: buffer A (20 mM Tris-HCl, pH 7.6, containing 50 mM NaCl, 20 mM NaF, 20 mM β-glycerophosphate, 500 mM imidazole, 2.5 mM metalasulfite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 1 mM DTT, 1× Complete protease inhibitors, phosphatase inhibitors), buffer B (same as buffer A, except 150 mM NaCl), and buffer C (same as buffer A, except 500 mM NaCl).

**Isolation of rhIKK1 Homodimer**—Cells from an 8-liter fermentation of baculovirus-expressing IKK1 tagged with His peptide were centrifuged, and the pellet was resuspended in 50 ml of buffer A. The protein-resin slurry was poured into a 25-ml column, and the enzyme was eluted with 375 ml of buffer C containing 0.1% Nonidet P-40. The protein/resin mixture was poured into a 25-ml column, and the enzyme was eluted with buffer B containing FLAG peptide. Enzyme fractions (100 ml) were collected and incubated with anti-FLAG affinity resin on a rotator overnight in buffer B. The resin was washed in batch with 10–15 bed volumes of buffer A. The protein peak was collected and incubated with anti-FLAG affinity resin on a rotator overnight in buffer B. The resin was washed in batch with 10–15 bed volumes of buffer C. Washed resin was poured into a column, and the protein/resin slurry was centrifuged, and the resultant cell pellet (10.0 g) was suspended in 50 ml of buffer A. The protein-resin slurry was poured into a 25-ml column, and the enzyme was eluted using 5 bed volumes of buffer B containing FLAG peptide. A mixture, 5 mM DTT, 0.1% Nonidet P-40, and BSA, (concentrated to 0.1% in the final amount) was added to the eluted enzyme before concentrating in an Amicon membrane with a molecular mass cut-off of 30 kDa. Enzyme was aliquoted and stored at −80 °C.

**Isolation of rhIKK2 Homodimer and Its Mutant rhIKK2 (S177E, S181E)**—A 10-liter culture of baculovirus-expressing IKK2 tagged with FLAG peptide was centrifuged, and the resultant cell pellet (m.o.i. = 0.1; I = 72 h) was resuspended in 100 ml of buffer C. These cells were microfluidized and centrifuged at 100,000 × g for 45 min. The supernatant was collected, imidazole was added to the final concentration of 10 mM, and the mixture was incubated with 25 ml of Ni-NTA resin for 2 h. The supernatant was poured into a 25-ml column, and the enzyme was eluted using 250 ml of buffer C and then with 125 ml of 50 mM imidazole in buffer C. The rhIKK1 homodimer was eluted using 300 mM imidazole in buffer C. BSA and Nonidet P-40 were added to the enzyme fractions to the final concentration of 0.1%. The enzyme was dialyzed against buffer B, aliquoted, and stored at −80 °C.

**Phosphatase Treatment**—Immunoprecipitated rhIKKs were washed two times in 50 mM Tris-HCl, pH 7.2, containing 0.1 mM EDTA, 1 mM DTT, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 2 mM MgCl2 and resuspended in 50 μl of APPase (1000 units) was prediluted in the same buffer and added to the IKK samples. Following an incubation at room temperature for 30 min with intermittent mixing, cold lysis buffer was added to the tubes to stop the reaction. After several washes, the beads were removed for Western analysis, and the remaining material was pelleted and resuspended in 100 μl of the buffer used for the indicated kinase assay.

**Enzyme Assay**

Kinase activity was measured using a biotinylated IκBα peptide (Gly-Leu-Lys-Lys-Arg-Leu-Leu-Leu-Asp-Asp-His-Asp-Arg)28-Gly-Leu-Leu-Asp-Ser29-Met-Lys-Asp-Glu-Glu), a SAM96 biotin capture plate, and a vacuum system. The standard reaction mixture contained 5 μl of biotinylated IκBα peptide, 1 μl of [γ-32P]ATP (about 1 × 106 cpm), 1 mM DTT, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 2 μM MgCl2, and resuspended in 50 μl of lysis buffer, pH 7.6, and enzyme solution (1–10 μl) in a final volume of 50 μl. After incubation at 25 °C for 30 min, 25 μl of the reaction mixture was withdrawn and added to a SAM96 biotin capture 96-well plate. Each well was then washed successively with 800 μl of 0.1% NaCl, 12.0 ml of NaCl containing 1% H2PO4, 400 μl of H2O, and 200 μl of 95% ethanol. The plate was allowed to dry in a hood at 25 °C for 1 h, and then 25 μl of scintillation fluid (Microscint 20) was added to each well. Incorporation of [γ-32P]ATP was measured using a Top-Count NXT (Packard). Under each assay condition, the degree of phosphorylation of IκBα peptide substrate was linear with time and concentration for all purified enzymes. Results from the biotinylated peptide assay were confirmed by SDS-PAGE analysis of kinase reaction utilizing a glutathione bead assay. The resulting radiolabeled substrate was quantitated using a PhosphorImager (Molecular Dynamics). An ion exchange resin assay was also employed using [γ-32P]ATP and GST-IκBα-54 fusion protein as the substrates.28 Each assay system yielded consistent results in regard to results from the biotinylated peptide assay were confirmed by SDS-PAGE analysis of kinase reaction utilizing a glutathione bead assay. The resulting radiolabeled substrate was quantitated using a PhosphorImager (Molecular Dynamics). An ion exchange resin assay was also employed using [γ-32P]ATP and GST-IκBα-54 fusion protein as the substrates.28 Each assay system yielded consistent results in regard to results from the biotinylated peptide assay were confirmed by SDS-PAGE analysis of kinase reaction utilizing a glutathione bead assay. The resulting radiolabeled substrate was quantitated using a PhosphorImager (Molecular Dynamics). An ion exchange resin assay was also employed using [γ-32P]ATP and GST-IκBα-54 fusion protein as the substrates.28 Each assay system yielded consistent results in regard to the specific activities for each of the purified kinase isoforms. One unit of enzyme activity was defined as the amount required to catalyze the transfer of 1 nmol of phosphate from ATP to IκBα peptide per minute. Specific activity was expressed as units per milligram of protein. For experiments related to Kd determination of purified enzymes, various concentrations of ATP or IκBα peptide were used in the assay at a fixed concentration of either IκBα or ATP. For IκBα peptide K, assays were carried out with 0.4 μg of enzyme, 5 μM ATP, and IκBα peptide from 0.5 to 20 μM. For IκBα, assays K, were carried out with 0.4 μg of enzyme, 10 μM IκBα peptide, and ATP from 0.1 to 10 μM. For K, the affinity of the enzyme for the substrate was determined using the competitive inhibition assay.30

2 Q. K. Huynh, H. Boddupalli, C. M. Koboldt, B. L. Hood, B. F. Kilpatrick, and C. S. Tripp, unpublished data.
determination of rhIKK1 homodimer, due to its low activity and higher $K_m$ for IxBα peptide, rhIKK1 homodimer (0.3 μg) was assayed with 125 μM IxBα peptide and a 5-fold higher specific activity of ATP (from 0.1 to 10 μM) for ATP $K_m$ experiments and a 5-fold higher specific activity of 5 μM ATP and IxBα peptide (from 5 to 200 μM) for IxBα peptide $K_m$ experiments.

**Other Methods**

Protein was hydrolyzed for 24 h in 6 x HCl at 110 °C in vacuo and analyzed on a Beckman 6000 high performance amino acid analyzer. All analyses were performed after postcolumn derivatization of the hydrolysate with ninhydrin. Automated Edman degradation was carried out on an Applied Biosystems model 470 A protein sequencer as described (30). Protein concentrations were determined by the method of Bradford (31) or by SDS-PAGE with silver staining (32) using bovine serum albumin as the standard. Purity and molecular weights of the isolated enzyme were confirmed by SDS-PAGE with silver staining (32).

**RESULTS AND DISCUSSION**

Due to its prominent role in NF-κB activation, IKKs have been characterized by many groups. The native IKK complex has been isolated and biochemically analyzed from mammalian cells (4, 9, 27). In addition, rhIKK1 and rhIKK2 homodimers from baculovirus expression systems have been isolated and characterized (16–19). There has been a discrepancy in kinase activity between native and recombinantly expressed hIKKs. The native IKK complex did not exhibit kinase activity unless isolated from cells that had been stimulated by an agonist such as TNFα or IL-1β, but recombinantly expressed hIKKs from baculovirus systems have kinase activity when purified. The kinase activity seen with the native IKK complex was dependent on phosphorylation, because treatment with phosphatase abolished the kinase activity (5). Thus the IKK complex that was phosphorylated and activated in mammalian cells could be stably isolated in the presence of phosphatase inhibitors that prevented dephosphorylation, maintaining the kinase activity. We propose that this paradigm is also true for the rhIKKs expressed in a baculovirus system. Recombinant hIKKs when expressed in a baculovirus system are phosphorylated and require phosphorylation for their kinase activity, because it is abolished when these proteins were treated with APpase (Fig. 3A). All of the wild type rhIKKs have phosphorylation-dependent kinase activity, whereas the constitutively active mutant rhIKK2 (S177E, S181E) does not. Phosphatase treatment also results in a shift in mobility of each wild type rhIKK in SDS-PAGE/Western analysis, but this shift is not as dramatic for the mutant rhIKK2 (S177E, S181E). Preliminary data maps the phosphorylation of the rhIKK2 to the MAPKK activation loop. However, the exact phospho-acceptor sites on each rhIKK are unknown and currently being determined.

The mechanism by which this IKK phosphorylation and activation occurs in baculovirus-infected cells is unknown. In mammalian expression systems, NEMO is required for the activation of IKK. It is thought that NEMO brings other proteins such as MAP3Ks into the complex to phosphorylate the IKK catalytic subunits, primarily IKK2 (10–12). However, data to date have not described a need to coexpress NEMO with IKK1 and/or IKK2 to obtain kinase activity in baculovirus systems. Because the NF-κB signaling pathway is conserved in evolution (1), being described in *Drosophila*, oysters, and *Dictyostelium* (1, 28–29), we hypothesized that an endogenous

---

3 G. W. Lange, unpublished data.
functional NEMO homologue could function to activate the recombinant IKKs in the insect cells during viral infection. The role of NEMO in the phosphorylation and activation of native IKK2 is demonstrated in Fig. 1C. The pre-B cell line, 70Z, has been shown previously to activate IKK and NF-κB in response to LPS, whereas a mutant line, 1.2E3, does not contain NEMO and cannot activate NF-κB (10). Here we verify that NEMO can be detected by Western analysis in the 70Z line but not the mutant line, 1.2E3. Likewise, IKK can be phosphorylated and activated by LPS in the 70Z cells as demonstrated by a slower migrating band on IKK2 Western analysis (Fig. 1C). The kinetics of IKK2 phosphorylation and activation match the degradation of endogenous IκBα substrate. Note that, in the NEMO-deficient cells, neither IKK activation (gel shift) nor degradation of IκBα occurs in the presence of LPS. Next, using the anti-NEMO antibody, we could precipitate active, phosphorylated rIKK2 from the crude insect cell lysate but not from the affinity-purified rIKK2 preparation (Fig. 1B). Similar data were generated with baculovirus cell lysates containing rIKK1 and rIKK1/rIKK2 heterodimer. However, other proteins overexpressed in the baculovirus system could not be immunoprecipitated using the anti-NEMO antibody; hence, this is not a nonspecific interaction (data not shown). These data strongly suggest that a functional NEMO homologue from the insect cells binds to the rIKKs similar to the mammalian NEMO described in the signalling complex (5). We were unable to identify the functional NEMO homologue by Western blot in crude baculovirus cell lysates with this antibody. The anti-NEMO antibody could be used to precipitate all of the rIKKs, including rIKK1, which is thought not to bind NEMO in mammalian cells (data not shown). Note, rIKK1 is also phosphorylated during expression in the baculovirus system and this phosphorylation is required for its kinase activity as well (Fig. 1A). We did not detect phosphorylation of IKK1 isolated from mammalian cells stimulated with cytokines using the same methodology (data not shown). Thus there appears to be a difference in the regulation of IKK1 when overexpressed during the viral infection in the baculovirus system compared with endogenous IKK1 from stimulated mammalian cells. Nevertheless, these data indicate that the activated rIKKs expressed and purified from the baculovirus system are phosphorylated during their expression in a similar manner as described for mammalian IKKs isolated from cytokine-stimulated cells. Once the phosphorylation of rIKKs has occurred during expression, this activity can be preserved using the appropriate phosphatase inhibitors during the purification process, also similar to what is observed from mammalian expression.

Previously, many laboratories have expressed and characterized rIKK1 and rIKK2 homodimers, and these studies have produced varying kinetic results. Although IKK prefers heterodimer formation in both mammalian cells and when expressed in a baculovirus system, the kinetic properties of the purified, physiological IKK1/IKK2 heterodimer remain poorly described (7–9, 15). Here we have characterized the rIKK1/rIKK2 heterodimer isolated from coexpression in a baculovirus system. The purification procedure of each rIKK consisted of a combination of buffer extraction, gel filtration, and affinity chromatography. rIKK1 homodimer and rIKK2 homodimer and its mutant rIKK2 (S177E, S181E) were isolated to homogeneity as single bands on SDS-PAGE analysis (Fig. 2B) and found predominantly to be dimers by gel filtration analysis (data not shown). As expected, the purified rIKK1/rIKK2 heterodimer exhibited equal amounts of rIKK1 and rIKK2 by SDS-PAGE analysis (Fig. 2, A and B). Note that sequential affinity column chromatography was necessary to isolate rIKK1/rIKK2 heterodimers from each of the rIKK1 and rIKK2 homodimers produced during expression (Fig. 2A). Similar to other kinases, the rIKKs exhibited a narrow pH optimum centered around 7.6. All purified rIKKs were stable at −80 °C for at least 3 months in buffer containing 0.1% BSA, 0.1% Nonidet P-40, 10% glycerol, 5 mM DTT, and protease inhibitors.

The kinetic properties of the rIKK1/rIKK2 heterodimer are compared with those of the rIKK1 homodimer, rIKK2 homodimer, and rIKK2 (S177E, S181E) in Fig. 2C and in Table I. The kinetic parameters of purified rIKKs were determined using biotinylated IκBα peptide and SAM 96 biotin capture plates as described under “Experimental Procedures” and recently reported by Wisniewski et al. (24). These authors demonstrated similar results using either a biotinylated IκBα peptide or GST-IκBα peptide and recently reported by Wisniewski et al. (24). These authors demonstrated similar results using either a biotinylated IκBα peptide or GST-IκBα peptide and SAM 96 biotin capture plates as described under “Experimental Procedures.”
thermore, the phosphorylation of the 22-amino acid IκBα consensus peptide or a GST-IκBα1–54 fusion protein by purified rhIKKs reported herein was specific for Ser\(^{32}\) and Ser\(^{36}\), because there was no phosphorylation of either substrate in which the Ser\(^{32}\) and Ser\(^{36}\) were replaced by Ala (data not shown). Likewise, an irrelevant peptide, which is phosphorylated by p38 kinase, was not phosphorylated by the rhIKKs (data not shown).

The rhIKK1/rhIKK2 heterodimer has similar specific activity to the rhIKK2 homodimer (Fig. 2C). These data support similar findings when IKKs are overexpressed in mammalian cells in that the activation of IKK2 is responsible of the majority of the total kinase activity (16). Because the purified rhIKK1/rhIKK2 heterodimer produced by coexpression is highly active (Fig. 2 and Table I) compared with a lack of enhanced kinase activity from mixing rhIKK1 and rhIKK2 at equimolar concentrations after purification (15), it is reasonable to suggest that, during expression in insect cells, correct folding occurs between the rhIKK subunits, which results in higher kinase activity.

Results from Fig. 2C also indicated that our specific activity for rhIKK1 homodimer (about 0.1 nmol/min/mg) is in reasonable agreement with that for rhIKK1 homodimer (0.15 nmol/ min/mg of protein) reported by Mercurio et al. (16). It is interesting that, although the rhIKK1 is phosphorylated and that the kinase activity is dependent on this phosphorylation, the specific activity is still very low. These data support previous findings that IKK1 is not necessary for NF-κB activation in fibroblasts isolated from IKK1-deficient mice and that the phenotype of the IKK1 knock-out mice is unique compared with the IKK2-deficient mice. This suggests that this kinase has a unique function or that a unique substrate other than IκBα yields a higher specific activity for IKK1.

Our values for the rhIKK2 homodimer and its constitutive mutant rhIKK2 (S177E, S181E) were significantly different from those previously reported by Mercurio et al. (12) but similar to those reported for rhIKK2 by Li et al. (15). Although the specific activity of our purified rhIKK2 homodimer was at least 5-fold higher than that reported by Mercurio et al. (3.2 nmol/min/mg of protein compared with 0.62 nmol/min/mg (16), our mutant rhIKK2 (S177E, S181E) displayed 10-fold lower specific activity than their expressed kinase (0.63 nmol/min/mg of protein compared with 6.5 nmol/min/mg). Again, the expression conditions resulting in post-translational modifications such as phosphorylation could explain these differences, because phosphorylation both positively and negatively regulates the IKK kinase activity (16). We optimized our expression conditions to maximize specific activity rather than protein expression.

A comparison of the kinetic parameters (\(K_m\) and \(k_{cat}\) for ATP and IκBα peptide of the purified rhIKKs with those from other published results are also summarized in Table I. Note that the \(K_m\) values for each substrate for the rhIKK1/rhIKK2 heterodimer are comparable to those of the rhIKK2 homodimer. The \(K_m\) values previously reported for rhIKKs for ATP and for IκBα are shown for comparison. Despite different enzyme assays having been used, these values are in good agreement with each other and with our data (12, 15, 23, 24, 26). The \(K_m\) values for ATP of purified rhIKKs are lower than those of other protein kinases such as p38 kinase (\(K_m = 23.0 \mu M\)) and cAMP-dependent protein kinase (\(K_m = 10.0 \mu M\)) (33, 34). It is of interest that the native IKK complex isolated from HeLa S3 cells using a two-step purification procedure exhibited a very low dissociation constant for ATP (\(K_{diss} \approx 0.05 \mu M\)), as calculated by fitting the two substrate kinetics to a random sequential model (9). This finding is not surprising, because other component(s) in the IKK complex might induce conformational changes in the kinases such that substrate binding pockets become more accessible to the substrates.

For the apparent maximal turnover \(k_{cat}\), our value of 3.10 h\(^{-1}\) for the purified rhIKK2 (S177E, S181E) homodimer is rather low in comparison to the published value of 33.8 h\(^{-1}\) from Mercurio et al. (16) (Table I). However, our \(k_{cat}\) for the rhIKK2 homodimer is at least 4-fold greater than that for rhIKK2 homodimer reported by Mercurio et al. (16). Because different kinase assays have been used among various laboratories, we determined \(K_m\) and \(k_{cat}\) values of purified rhIKK2 homodimer and rhIKK1/rhIKK2 heterodimer by an ion-exchange resin-based assay\(^2\) as well. The \(K_m\) values for ATP of rhIKK2 and rhIKK1/rhIKK2 were 2.61 ± 0.70 and 0.63 ± 0.51 \(\mu M\), respectively.\(^2\) The \(K_m\) values for IκBα peptides of rhIKK2 and rhIKK1/rhIKK2 were 3.10 ± 1.53 and 0.60 ± 0.03 \(\mu M\), respectively.\(^2\) However, using this kinase assay, the \(k_{cat}\) values of rhIKK2 and rhIKK1/rhIKK2 were slightly lower at 8.6 ± 0.87 h\(^{-1}\) and 11.3 ± 1.31 h\(^{-1}\), respectively.\(^2\) Thus, the difference in \(K_m\) and \(k_{cat}\) values of purified rhIKKs from different groups may reflect differences in both the assay conditions as well as differences in the state of kinase activation by phosphorylation obtained from different expression conditions of each of the rhIKKs. In any case, data from Table I suggest that, although all four purified rhIKK enzymes are capable of phos-

---

### Table I  
Kinetic parameters of rhIKKs compared with those from recent publications

| Kinases                  | Specific activity | \(K_m\) | \(k_{cat}\) | \(k_{cat}/K_m\) |
|-------------------------|-------------------|--------|-------------|-----------------|
| rhIKK1/rhIKK2 heterodimer| 5.5 ± 0.47        | 0.63 ± 0.21 | 0.80 ± 0.30 | 28.50 ± 2.43    |
| rhIKK2 homodimer         | 3.2 ± 1.20        | 0.65 ± 0.15 | 0.94 ± 0.32 | 16.40 ± 6.20    |
|                          | 0.62               | 0.56<sup>a</sup> | 0.50<sup>d</sup> | 3.30<sup>d</sup> |
| rhIKK2 homodimer (S177E, S181E) | 0.63 ± 0.13 | 0.21 ± 0.04 | 2.62 ± 0.32 | 3.10 ± 0.70 |
|                          | 6.5<sup>c</sup>   | 0.63<sup>c</sup> | 1.10<sup>d</sup> | 33.80<sup>d</sup> |
| rhIKK1 homodimer         | 0.10 ± 0.03       | 0.90 ± 0.33 | 23.70 ± 1.50 | 0.51 ± 0.18 |
|                          | 0.15<sup>c</sup>  | 0.63<sup>c</sup> | 1.40<sup>d</sup> | 0.78<sup>d</sup> |
|                          | 0.13               | 0.13 ± 0.03 | 23.90 ± 4.80 | 4.80<sup>c</sup> |

<sup>a</sup> The apparent maximal turnover \(k_{cat}\) was expressed per hour (h\(^{-1}\)), and for comparison purposes published data are shown.

<sup>b</sup> Catalytic efficiency \(k_{cat}/K_m\) for IκBα peptide substrate.

<sup>c</sup> Values from Wisniewski et al. (24).

<sup>d</sup> Values from Mercurio et al. (12).

<sup>e</sup> Values from Li et al. (15).

<sup>f</sup> Values of dissociation constants for ATP \(K_{diss}\) and GST-IκBα \(K_{diss}\) from Burke et al. (9).
Inhibition of rhIKKs by ADP and its analogues

| Compounds                                                                 | IC_{50} (µM) |
|---------------------------------------------------------------------------|-------------|
| 1. Adenosine 5'-diphosphate                                               | 1.50        |
| 2. α,β-Methyleneadenosine 5'-triphosphate                                 | 1.80        |
| 3. Adenosine 5'-O-(3-thiotriphosphate)                                     | 3.03        |
| 4. Cordycepin 5'-triphosphate                                            | 8.00        |
| 5. Adenosine 5'-phosphosulfate                                            | 18.73       |
| 6. 5'-Adenylylimidophosphate                                              | 29.34       |
| 7. 2'-k3'-O-(4-Benzoyl)adenosine 5'-triphosphate                          | 23.70       |
| 8. Adenosine 5'-triphosphate, (1-1(2-nitrophenyl)ethyl)ester              | 66.06       |
| 9. Adenosine 5'-monophosphate                                             | >200        |
| 10. Adenosine 5'-diphospho morpholidate                                   | >200        |
| 11. Adenosine 5'-monophospho amide                                       | >200        |
| 12. Adenosine 5'-monophospho morpholidate                                 | >200        |
| 13. 8-Bromoadenosine 5'-diphosphate                                      | >200        |
| 14. N6-(6-aminohexyl)carbomethyl adenosine 5'-triphosphate                | >200        |
| 15. 2,3'-Dideoxyadenosine 5'-triphosphate                                | >200        |
| 16. α-Adenosine                                                           | >200        |
| 17. Adenosine 5'-triphosphate periodase, oxidized borohydride-reduced     | >200        |
| 18. Adenosine 5'-diphosphomannose                                         | >200        |
| 19. Adenosine 5'-monophosphate                                            | >200        |
| 20. Adenosine 5'-triphosphate periodase, oxidized                         | >200        |
| 21. Adenosine 5'-diphosphoribose                                          | >200        |
| 22. Adenosine 3'-phosphate 5'-phosphate                                   | >200        |
| 23. β,γ-Methyleneadenosine 5'-triphosphate                                | >200        |

During the characterization of the rhIKKs, we found that ADP strongly inhibited all isoforms with IC_{50} values in the range of 1.17 to 1.77 µM (Table II and Fig. 3). This inhibition is selective for ADP, because AMP shows a markedly decreased ability to inhibit rhIKK1/rhIKK2 heterodimer (Fig. 3A). Note that the other product of the kinase reaction, an IκBα peptide phosphorylated at Ser^{32} and Ser^{36} (Gly-Leu-Lys-Glu-Arg-Leu-Leu-Asp-Asp-Arg-His-Asp-Ser-P0$_{3}$H$_{2}$-Gly-Leu-Asp-Ser-P0$_{3}$H$_{2}$-Met-Lys-Asp-Glu-Glu), is not as effective at inhibiting rhIKK1/rhIKK2 as ADP is. Also, both products of the kinase reaction inhibit native IKK complex purified from mammalian cells similarly to rhIKK1/rhIKK2 but again, ADP is a far more effective inhibitor than the phosphorylated IκBα peptide (Fig. 3B). Kinetic analysis shows that ADP competitively inhibits rhIKK1/rhIKK2 heterodimer with respect to ATP (K_{i} value of 0.77 µM) and noncompetitively inhibits this kinase with respect to IκBα peptide (K_{i} value of 1.08 µM, Fig. 4). ADP does not inhibit p38 kinase α and β or c-Jun N-terminal kinase 2 in this concentration range, 4 most likely because the K_{m} for ATP for p38 kinase is so much higher than the IKKs. In the preparation of this manuscript, Peet and Li (26) reported the competitive inhibition by ADP of both the rhIKK1 and rhIKK2 homodimeric isoforms with similar K_{i} values of 0.15 µM as reported here. Thus, these data herein extend the observation to include the inhibition of the recombinant heterodimer and

---

4 R. P. Compton and J. L. Hirsch, unpublished data.
the mammalian IKK complex by ADP and support a potential physiological role for ADP in the feedback inhibition of endogenous IKK activity. Also the $K_i$ for ADP is not significantly changed as a result of dimerization of the two IKK subunits. To gain more insight regarding the ATP site, the effect of various ADP analogues on rhIKK activities were also examined. As shown in Table II, many ADP analogues, including adenosine 5'-O-(3-thiophosphate), adenosine 5'-phosphosulfate, α,β-methyleneadenosine 5'-triphosphate, 2'-&3'-O-(4-benzoyl)adenosine 5'-triphosphate, adenosine 5'-triphosphate, r-(1-2-nitrophenyl)ethyl)ester, and corydilicin 5'-triphosphate strongly inhibit all recombinant IKK isoforms. Among these analogues, α,β-methyleneadenosine 5'-triphosphate and adenosine 5'-O-(3-thiodiphosphate) are the strongest inhibitors with $IC_{50}$ values of 1.00 and 2.15 μM, respectively, and are comparable to ADP. However, no selectivity between rhIKK isoforms was identified with these compounds, indicating that the ATP sites in rhIKK1 and rhIKK2 are similar. This is not surprising, because these ADP analogs are relatively small compounds and the kinase sites are 65% homologous between the rhIKK1 and rhIKK2 isoforms. Note that many ADP analogs demonstrated no inhibition similar to AMP, thus defining some structural selectivity. Structural differences at this site, however, will no doubt be revealed once the crystal structures are solved. Further characterization of the rhIKK1/rhIKK2 heterodimer also indicates that the phosphorylated IκBα peptide competitively inhibits kinase activity with respect to IκBα ($K_i$ value of 263.74 μM) and noncompetitively inhibits the heterodimer with respect to ADP (Fig. 4). Similarly, Peet and Li (26) demonstrated that a nonphosphorylated IκBα peptide competitively inhibited rhIKK1 and rhIKK2 at the IκBα site with $K_i$ values of 139 and 90 μM, respectively. Thus, our results demonstrate that even a phosphorylated form of consensu IκBα peptide will not compete as well as ADP does at its site. These data suggest that, of the two products of the kinase reaction, ADP may significantly contribute to feedback inhibition of kinase activity in vivo. Inhibition by ADP could inhibit IKK in cellular situations where ATP reserves are low, given the fact that NF-κB, along with heat shock proteins, are the paradigm for stress response transcription factors. However, this is also dependent on the cellular concentration of phosphorylated IκBα and whether the $K_i$ for each phosphorylated IκB isoform would be lower when complexed with NF-κB, as reported for the $K_m$ of IκBα (23).

In summary, in the present study we have expressed, purified, and characterized the physiological form of the IKK kinase complex, the rhIKK1/rhIKK2 heterodimer, and compared its kinetic parameters with those of the rhIKK1 homodimer, rhIKK2 homodimer, and rhIKK2 (S177E, S181E) mutant by ADP and phosphorylated IκBα peptide (data not shown). $K_i$ values (in the text) were determined as described by Leatherbarrow (35).

FIG. 4. Inhibition by ADP and phosphorylated IκBα peptide on rhIKK1/ rhIKK2 heterodimer kinase activity. Competitive (A) and noncompetitive (B) inhibition of ADP on rhIKK1/rhIKK2 heterodimer kinase activity with respect to ATP and IκBα peptide, respectively. Competitive (C) and noncompetitive (D) inhibition of phosphorylated IκBα peptide on rhIKK1/rhIKK2 heterodimer kinase activity with respect to IκBα peptide and ATP, respectively. Concentration of inhibitors used were: (A and B) ADP: 0 μM (○), 0.4 μM (■), 1.0 μM (□), and 2.0 μM (▲) and (C and D) phosphorylated IκBα peptide: 0 μM (○), 0.5 μM (■), 1.0 μM (□), and 2.0 μM (▲). A similar pattern of inhibition was demonstrated for rhIKK2 homodimer, rhIKK1 homodimer, and rhIKK2 (S177E, S181E) mutant by ADP and phosphorylated IκBα peptide (data not shown). $K_i$ values (in the text) were determined as described by Leatherbarrow (35).
13. Hu, M. C. T., and Wang, Y. (1998) *Gene* **222**, 31–40
14. Connelly, M., and Marcu, K. (1995) *Cell. Mol. Biol. Res.* **41**, 537–549
15. Li, J., Peet, G. W., Pullen, S. S., Schembri-King, J., Warren, T. C., Marcu, K. B., Kehry, M. R., Barton, R., and Jakes, S. (1998) *J. Biol. Chem.* **273**, 30736–30741
16. Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999) *Science* **284**, 309–313
17. Karin, M., and Delhase, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9067–9069
18. Li, Q., Antwerp, D. V., Mercurio, F., Lee, K., and Verma, I. M. (1999) *Science* **284**, 321–325
19. Takeda, K., Takeda, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., and Akira, S. (1999) *Science* **284**, 313–316
20. Hu, Y., Baud, V., Delhase, M., Zhang, P., Deen, T., Ellisman, M., Johnson, R., and Karin, M. (1999) *Science* **284**, 315–320
21. Li, Q., Lu, Q., Hwang, J. Y., Buscher, D., Lee, K., Izpisua-Belmonte, J. C., and Verma, I. M. (1999) *Genes Dev.* **13**, 1322–1328
22. Tanaka, M., Fuentes, M. E., Yamaguchi, K., Durnin, M. H., Dalrymple, S. A., Hardy, K. L., and Goeddel, D. V. (1999) *Immunity* **10**, 421–429
23. Zandi, E., Chen, Y., and Karin, M. (1998) *Science* **281**, 1360–1363
24. Wisniewski, D., LoGrasso, P., Calaycay, J., and Marcy, A. (1999) *Anal. Biochem.* **274**, 220–228
25. Heilker, R., Freuler, F., Pulfer, R., Padova, F. D., and Edel, J. (1999) *Eur. J. Biochem.* **259**, 253–261
26. Peet, G. W., and Li, J. (1999) *J. Biol. Chem.* **274**, 32655–32661
27. Heilker, R., Freuler, F., Vanek, M., Pulfer, R., Koh, T., Peter, J., Zerves, H., Hofstetter, H., and Edel, J. (1999) *Biochemistry* **38**, 6231–6238
28. Escobas, J., Briant, L., Montagnani, C., Hez, S., Deveaux, C., and Roch, P. (1999) *FEBS Lett.* **453**, 293–298
29. Traincard, F., Ponte, E., Pun, J., Couch, B., and Veron, M. (1999) *J. Cell Sci.* **112**, 3529–3535
30. Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., and Hood, L. E. (1983) *Methods Enzymol.* **91**, 399–413
31. Bradford, M. M. (1986) *Anal. Biochem.* **72**, 248–254
32. Laemmli, U. K. (1970) *Nature* **227**, 680–685
33. Bhatnagar, D., Roskoski, R. J., Rosenthal, M. S., and Leonard, N. J. (1983) *Biochemistry* **2**, 6310–6317
34. LoGrasso, P. V., Frantz, B., Rubado, A. M., O’Keefe, S. J., Hermes, J. D., and O’Neil, K. A. (1997) *Biochemistry* **36**, 10422–10427
35. Leatherbarrow, R. J. (1992) *Grafit*, version 4.0, Erithacus Software Ltd., Staines, UK
Characterization of the Recombinant IKK1/IKK2 Heterodimer: MECHANISMS REGULATING KINASE ACTIVITY
Q. Khai Huynh, Hymavathi Boddupalli, Sharon A. Rouw, Carol M. Koboldt, Troii Hall, Cindy Sommers, Scott D. Hauser, Jennifer L. Pierce, Rodney G. Combs, Beverly A. Reitz, Judy A. Diaz-Collier, Robin A. Weinberg, Becky L. Hood, Bryan F. Kilpatrick and Catherine S. Tripp

J. Biol. Chem. 2000, 275:25883-25891. doi: 10.1074/jbc.M000296200 originally published online May 22, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000296200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 14 of which can be accessed free at http://www.jbc.org/content/275/34/25883.full.html#ref-list-1