

Characterization of the Recombinant IKK1/IKK2 Heterodimer

MECHANISMS REGULATING KINASE ACTIVITY*

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Nuclear factor kappa B (NF- κ B) is a ubiquitous, inducible transcription factor that regulates the initiation and progression of immune and inflammatory stress responses. NF- κ B activation depends on phosphorylation and degradation of its inhibitor protein, I κ B, initiated by an I κ B kinase (IKK) complex. This IKK complex includes a catalytic heterodimer composed of I κ B kinase 1 (IKK1) and I κ B kinase 2 (IKK2) as well as a regulatory adaptor subunit, NF- κ B essential modulator. To better understand the role of IKKs in NF- κ B 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 I κ B α peptide for the rhIKK1/rhIKK2 heterodimer are 0.63 and 0.60 μ M, 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 $\text{h}^{-1} \mu\text{M}^{-1}$ using an I κ B α peptide substrate compared with any of the other IKK isoforms, including rhIKK2 (17.44 $\text{h}^{-1} \mu\text{M}^{-1}$), its mutant rhIKK2 (S177E, S181E, 1.18 $\text{h}^{-1} \mu\text{M}^{-1}$), or rhIKK1 (0.02 $\text{h}^{-1} \mu\text{M}^{-1}$). Kinetic analysis also indicates that, although both products of the kinase reaction, ADP and a phosphorylated I κ B α peptide, exhibited competitive inhibitory kinetics, only ADP with the low K_i of 0.77 μ M may play a physiological role in regulation of the enzyme activity.

tion factor that plays a prominent role in the activation of the immune system and in stress responses by regulating the transcription of many early, inducible genes, including proinflammatory cytokines, adhesion molecules, growth factors, enzymes, and receptors (1–3). Specificity of gene expression is determined at a cellular level by a diverse array of external stimuli such as bacterial products, including lipopolysaccharide (LPS), as well as cytokines, most importantly tumor necrosis factor- α (TNF α) and interleukin 1 β (IL-1 β). Through the synergistic interaction with other transcription factors, further specificity can be achieved while maintaining enormous potential to coordinately induce a large number of functionally related genes. NF- κ B is composed of homo- and heterodimers of the Rel protein family and is sequestered in an inactive form in the cytoplasm by members of the I κ B family of inhibitory proteins (1–3). I κ Bs mask the nuclear localization signal on NF- κ B, preventing nuclear translocation and hence DNA binding to the promoter regions of responsive genes. Stimulation of cells with an agonist that activates NF- κ B leads to a series of biochemical signals, ultimately resulting in the phosphorylation, ubiquitinylation, and degradation of I κ Bs, thereby releasing NF- κ B for nuclear translocation (1–3). Recently, two I κ B kinases (IKK1 or IKK α and IKK2 or IKK β), which phosphorylate I κ Bs and thereby initiate their degradation, have been cloned and characterized by a number of laboratories (4–8). The catalytic subunits, IKK1 and IKK2, are similar structurally as well as enzymatically and exist as a heterodimer in a large protein complex referred to as the IKK signalosome (4–9). A third protein, NF- κ B essential modulator (NEMO; IKK γ , IKKAP1), is a regulatory adapter protein necessary for IKK activation and kinase activity (10–12). IKK1 and IKK2 are coexpressed in most human adult tissues as well as in different developmental stages of mouse embryos (4–8, 13). This kinase complex appears to represent a critical, common denominator in the activation of NF- κ B in a number of signal transduction pathways stimulated by a variety of agonists, including cytokines such as TNF α and IL-1 β , microbial products such as LPS, and viral proteins such as TAX, as well as phorbol esters, oxidizing agents, and serine/tyrosine phosphatases (1–3).

IKK1 (also termed IKK α (4–6)) was cloned simultaneously by standard biochemical purification of the I κ B kinase activity from TNF α -stimulated HeLa S3 cells and by its interaction with the mitogen-activated protein kinase, NF- κ B-inducing kinase, in a yeast two-hybrid screen. IKK1 was identified as the previously cloned serine/threonine kinase, CHUK (14). IKK1 (also termed IKK α) is an 85-kDa, 745-amino acid protein that contains an N-terminal serine/threonine kinase catalytic domain, a leucine zipper-like amphipathic helix, and a C-terminal helix-loop-helix domain. IKK2 (also termed IKK β) was also

Nuclear factor kappa B (NF- κ B)¹ is a ubiquitous transcrip-

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¹ The abbreviations used are: NF- κ B, nuclear factor kappa B; IKK, I κ B kinase; IKK2 (S177E, S181E), a variant of IKK2 in which Ser¹⁷⁷ and Ser¹⁸¹ are replaced by Glu; TNF, tumor necrosis factor; LPS, lipopolysaccharide; NEMO, NF- κ B essential modulator; ERK, extracellular signal-regulated kinase; IKKAP1, IKK complex-associated protein 1; MAPK, mitogen-activated protein kinase; rh, recombinant human; wt, wild type; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; m.o.i., multiplicity of infection; IL-1 β , interleukin 1 β ; BSA, bovine serum albumin; GST, glutathione S-transferase; λ PPase, recombinant λ protein phosphatase.

cloned by standard biochemical purification, copurifying with IKK1 from TNF α -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, 756-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 IKK γ) 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 MAP3Ks such as NF- κ B-inducing kinase and 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 I κ B α or I κ B β 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 TNF α or IL-1 β (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- κ B. In contrast, fibroblasts lacking IKK2 do not exhibit IKK activity when stimulated with cytokines nor do they activate NF- κ B. 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 hepatocyte 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 I κ Bs, 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 I κ B α have varied in different publications, with the wide range of 1.4–23 μ M being reported for rhIKK1 compared with more similar values of 0.5–1.3 μ M being reported for rhIKK2. Second, most reports indicate that rhIKK2

phosphorylates truncated I κ Bs 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- κ B, the K_m for I κ B α is decreased from 2.2 to 1.4 μ M and the V_{max} is increased by a factor of four, indicating that rhIKK2 phosphorylates I κ B α bound to NF- κ B more efficiently than it phosphorylates free I κ B α (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 I κ B α , 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 I κ B α . While characterizing the purified rhIKKs, we also found that both of the products of the kinase reaction, ADP and a phosphorylated I κ B α 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 *Dictyostelium* (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 (IKK γ) (FL-419), IKK1(H-744), IKK2(H-470), and I κ B α (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. Sephacryl 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 placental 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 (DYKDDDDKD). The hIKK2:FLAG cDNA was subcloned into the baculovirus vector pFastBac. The rhIKK2

(S177S, 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 until purification of the recombinant proteins was undertaken as described below.

Enzyme Isolation

All purification procedures were carried out at 4°C unless otherwise noted. Buffers used were: buffer A (20 mM Tris-HCl, pH 7.6, containing 50 mM NaCl, 20 mM NaF, 20 mM β -glycerophosphate, 500 μM sodium orthovanadate, 2.5 mM metabisulfite, 5 mM benzamide, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 1 mM DTT, 1 \times Complete protease 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-expressed IKK1 tagged with His peptide were centrifuged, and the cell pellet (multiplicity of infection (m.o.i.) = 0.1; $I = 72$ h) was resuspended in 100 ml of buffer C. The cells were microfluidized and centrifuged at $100,000 \times g$ 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 suspension was poured into a 25-ml column and washed with 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 .

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 buffer A. These cells were microfluidized and centrifuged at $100,000 \times g$ for 45 min. Supernatant was passed over a G-25 column equilibrated with 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 rhIKK2 homodimer 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 . rhIKK2 (S177E, S181E) homodimer mutant tagged with FLAG was isolated following the same method as described above for its wild type.

Isolation of rhIKK1/IKK2 Heterodimer—The heterodimer enzyme was produced by coinfection in a baculovirus system (FLAG IKK2/IKK1 His; m.o.i. = 0.1 and $I = 72$ h). Infected cells were centrifuged, and the resultant cell pellet (10.0 g) was suspended in 50 ml of buffer A. The protein suspension was microfluidized and centrifuged at $100,000 \times g$ for 45 min. Imidazole was added to the supernatant to a final concentration of 10 mM. The protein was allowed to bind 25 ml of Ni-NTA resin by mixing for 2 h. The protein-resin slurry was poured into a 25-ml column and washed with 250 ml of buffer A containing 10 mM imidazole followed by 125 ml of buffer A containing 50 mM imidazole. Buffer A, containing 300 mM imidazole, was then used to elute the protein. A 75-ml pool was collected, and Nonidet P-40 was added to a final concentration of 0.1%. The protein solution was then dialyzed against buffer B. The dialyzed heterodimer enzyme was then allowed to bind to 25 ml of anti-FLAG M2-agarose affinity gel overnight with constant mixing. The protein-resin slurry was then centrifuged for 5 min at 2000 rpm. The supernatant was collected, and the resin was resuspended in 100 ml of buffer C containing 0.1% Nonidet P-40. The resin was washed 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 using buffer B containing FLAG peptide. Enzyme fractions (100 ml) were collected and concentrated to 20 ml using an Amicon membrane with a molecular mass cut-off of 30 kDa. Bovine serum albumin was added to the concentrated enzyme to final concentration of 0.1%. The enzyme was then aliquoted and stored at -80°C .

Cell Culture—The wild type (wt) human pre-B cell line, 70Z/3, and its mutant, 1.3E2, were generously provided by Dr. Carol Sibley. The wt 70Z/3 and 1.3E2 cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 7% defined bovine serum (Hyclone) and 50 μM 2-mercaptoethanol. Human monocytic leukemia THP-1 cells, obtained from the ATCC, were cultured in RPMI 1640 supplemented with 10% defined bovine serum, 10 mM HEPES, 1.0 mM sodium pyru-

vate, and 50 μM 2-mercaptoethanol. For experiments, cells were plated in 6-well plates at 1×10^6 cells/ml in fresh media. Pre-B cells were stimulated by the addition of 10 $\mu\text{g}/\text{ml}$ LPS for varying lengths of time ranging from 0 to 4 h. THP-1 cells were stimulated by the addition of 1 $\mu\text{g}/\text{ml}$ LPS for 45 min. Cells were pelleted, washed with cold 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, and lysed at 4°C in 20 mM Hepes buffer, pH 7.6, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM β -glycerophosphate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 0.5% Nonidet P-40 (lysis buffer). The cytosolic fractions obtained following centrifugation at $10,000 \times g$ were stored at -80°C until use.

Immunoprecipitation and Western Blotting—Paste from SF9 cells containing rhIKKs was centrifuged ($100,000 \times g$, 10 min) to remove debris. rhIKKs were immunoprecipitated (100 μg of cell paste) from the cell supernatant using 3 μg of anti-NEMO antibody (FL-419) followed by coupling to protein A-Sepharose beads. rhIKKs were also immunoprecipitated from affinity chromatography-purified protein preparations (1 μg) using anti-FLAG, anti-His, or anti-NEMO antibodies (1–4 μg) followed by protein A-Sepharose coupling. The native, human IKK complex was immunoprecipitated from THP-1 cell homogenates (300 $\mu\text{g}/\text{condition}$) using the anti-NEMO antibody. Immune complexes were pelleted and washed three times with 1 ml of cold lysis buffer. Immunoprecipitated rhIKKs were chromatographed by SDS-polyacrylamide gel electrophoresis (PAGE) (8% Tris-glycine) and transferred to nitrocellulose membranes (Novex) and detected by chemiluminescence (SuperSignal) using specific anti-IKK antibodies (IKK2 H-470, IKK1 H-744). Native IKK2, I κ B α , and NEMO proteins from cytosolic lysates (20–80 μg) were separated by SDS-PAGE and visualized by chemiluminescence using specific antibodies.

Phosphatase Treatment—Immunoprecipitated rhIKKs were washed two times in 50 mM Tris-HCl, pH 8.2, containing 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 2 mM MnCl_2 and resuspended in 50 μl . APPase (1000 units) was precluded 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, 10% of the beads were removed for Western analysis, and the remaining material was pelleted and resuspended in 100 μl of the buffer used for the *in vitro* kinase assay.

Enzyme Assay

Kinase activity was measured using a biotinylated I κ B α peptide (Gly-Leu-Lys-Lys-Glu-Arg-Leu-Leu-Asp-Arg-His-Asp-Ser³²-Gly-Leu-Asp-Ser³⁶-Met-Lys-Asp-Glu-Glu), a SAM² 96 biotin capture plate, and a vacuum system. The standard reaction mixture contained 5 μM biotinylated I κ B α peptide, 1 μM [γ -³²P] ATP (about 1×10^5 cpm), 1 mM DTT, 50 mM KCl, 2 mM MgCl_2 , 2 mM MnCl_2 , 10 mM NaF, 25 mM Hepes 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 SAM² 96 biotin capture 96-well plate. Each well was then washed successively with 800 μl of 2 M NaCl, 1.2 ml of NaCl containing 1% H_3PO_4 , 400 μl of H_2O , 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 I κ B α 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 S-transferase (GST)-I κ B α _{1–54} fusion protein and [γ -³²P]ATP. 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-I κ B α _{1–54} fusion protein as the substrates.² Each assay system yielded consistent results in regard to K_m and 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 I κ B α peptide per minute. Specific activity was expressed as units per milligram of protein. For experiments related to K_m determination of purified enzymes, various concentrations of ATP or I κ B α peptide were used in the assay at a fixed concentration of either I κ B α or ATP. For I κ B α peptide K_m assays were carried out with 0.1 μg of enzyme, 5 μM ATP, and I κ B α peptide from 0.5 to 20 μM . For ATP K_m assays were carried out with 0.1 μg of enzyme, 10 μM I κ B α peptide, and ATP from 0.1 to 10 μM . For K_m

² Q. K. Huynh, H. Boddupalli, C. M. Koboldt, B. L. Hood, B. F. Kilpatrick, and C. S. Tripp, unpublished data.

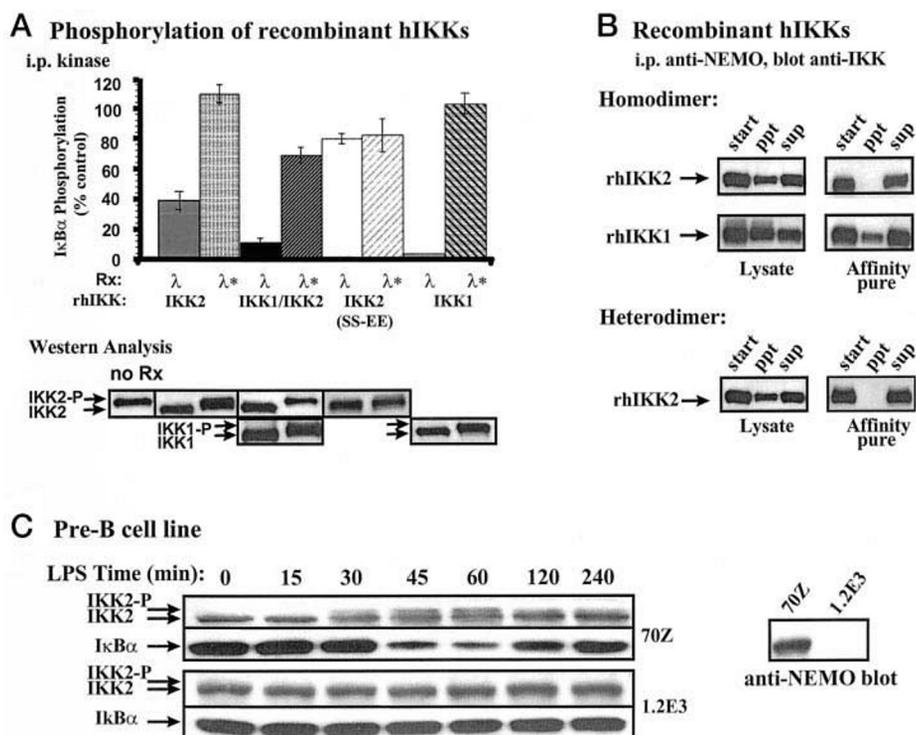


FIG. 1. Mammalian NEMO is required for endogenous IKK activation, and a functional NEMO homologue interacts with rhIKKs overexpressed in a baculovirus system. *A*, rhIKK proteins (His6-IKK1 and FLAG-IKK2 homodimers, His6-rhIKK1/FLAG -rhIKK2 heterodimer, and a FLAG -rhIKK-2 (S177E, SS181E) mutant) were immunoprecipitated using anti-FLAG or anti-His antibodies. The immunoprecipitated proteins were treated for 30 min with λ PPase (λ) or heat inactivated λ PPase (λ^*) followed by kinase assay or Western analysis. *B*, rhIKKs were immunoprecipitated from crude baculovirus cell paste extracts or from affinity-purified preparations using an anti-NEMO antibody. rhIKKs were separated by SDS-PAGE and visualized by Western analysis followed by chemiluminescence using specific antibodies. Identical results were seen using the anti-IKK1 antibody for the heterodimer rhIKK1/IKK2. There were no specific IKK bands detected in either SF9 cell lysates or in SF9 cell lysates expressing an irrelevant protein following anti-NEMO immunoprecipitation (data not shown). *C*, pre-B cells, 70Z wt and mutant 1.2E3, were plated in 6-well plates and stimulated with LPS for varying lengths of time ranging from 0 to 4 h. IKK2, I κ B α , and NEMO proteins were separated from cytosolic lysates by SDS-PAGE and visualized by Western analysis followed by chemiluminescence using specific antibodies.

determination of rhIKK1 homodimer, due to its low activity and higher K_m for I κ B α peptide, rhIKK1 homodimer (0.3 μ g) was assayed with 125 μ M I κ B α peptide and a 5-fold higher specific activity of ATP (from 0.1 to 10 μ M) for ATP K_m experiments and a 5-fold higher specific activity of 5 μ M ATP and I κ B α peptide (from 5 to 200 μ M) for I κ B α peptide K_m experiments.

Other Methods

Protein was hydrolyzed for 24 h in 6 N HCl at 110 $^{\circ}$ C *in vacuo* and analyzed on a Beckman 6000 high performance amino acid analyzer. All analyses were performed after postcolumn derivatization of the hydrolysates 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

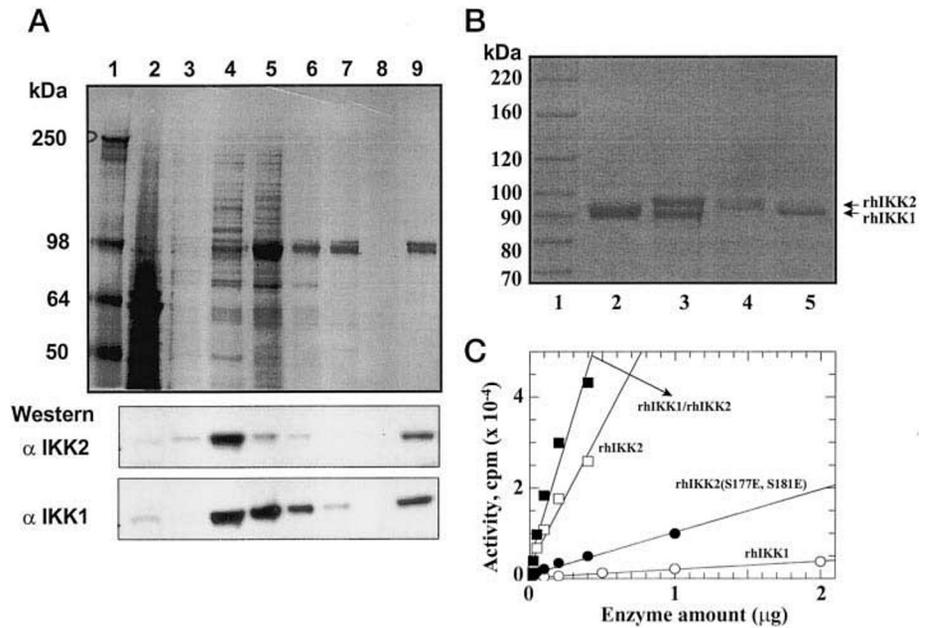
Due to its prominent role in NF- κ B activation, IKKs have been characterized by many groups. The native IKK complex has been isolated and biochemically analyzed from mammalian cells (4, 9, 27). In addition, rhIKK1 and rhIKK2 homodimers from baculovirus expression systems have been isolated and characterized (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 TNF α or IL-1 β , 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 λ PPase (Fig. 1A). 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.³ 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- κ B 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.

FIG. 2. Characterization of rhIKK isoforms. *A*, silver-stained SDS-PAGE and Western analysis of rhIKK1/rhIKK2 heterodimer. *Lane 1*, molecular mass standards; *lane 2*, Ni-NTA column flow-through fraction; *lane 3*, Ni-NTA column with 10 mM imidazole wash; *lane 4*, Ni-NTA column with 30 mM imidazole wash; *lane 5*, FLAG column flow-through fraction; *lane 6*, FLAG column with wash #1; *lane 7*, FLAG column with wash #2; *lane 8*, FLAG column with wash #3 and *lane 9*, FLAG column wash with FLAG peptide. *B*, silver-stained SDS-PAGE analysis of purified, rhIKK isoforms: *lane 1*, molecular mass standards; *lane 2*, purified rhIKK1 homodimer; *lane 3*, purified rhIKK1/rhIKK2 heterodimer; *lane 4*, purified rhIKK2 homodimer; and *lane 5*, purified rhIKK2 (S177E, S181E) mutant. *C*, comparison of the specific activities of purified rhIKKs. As indicated: rhIKK1 homodimer (○); rhIKK2 (S177E, S181E) (●); rhIKK2 homodimer (□); and rhIKK1/rhIKK2 heterodimer (■).



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- κ B in response to LPS, whereas a mutant line, 1.2E3, does not contain NEMO and cannot activate NF- κ B (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 I κ B α substrate. Note that, in the NEMO-deficient cells, neither IKK activation (gel shift) nor degradation of I κ B α occurs in the presence of LPS. Next, using the anti-NEMO antibody, we could precipitate active, phosphorylated rhIKK2 from the crude insect cell lysate but not from the affinity-purified rhIKK2 preparation (Fig. 1B). Similar data were generated with baculovirus cell lysates containing rhIKK1 and rhIKK1/rhIKK2 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 rhIKKs similar to the mammalian NEMO described in the signalsome 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 rhIKKs, including rhIKK1, which is thought not to bind NEMO in mammalian cells (data not shown). Note, rhIKK1 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 rhIKKs expressed and purified from the baculovirus system are phosphorylated during their expression in a similar manner as described for mammalian IKKs isolated from cytokine-stimu-

lated cells. Once the phosphorylation of rhIKKs 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 rhIKK1 and rhIKK2 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 rhIKK1/rhIKK2 heterodimer isolated from coexpression in a baculovirus system. The purification procedure of each rhIKK consisted of a combination of buffer extraction, gel filtration, and affinity chromatography. rhIKK1 homodimer and rhIKK2 homodimer and its mutant rhIKK2 (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 rhIKK1/rhIKK2 heterodimer exhibited equal amounts of rhIKK1 and rhIKK2 by SDS-PAGE analysis (Fig. 2, A and B). Note that sequential affinity column chromatography was necessary to isolate rhIKK1/rhIKK2 heterodimers from each of the rhIKK1 and rhIKK2 homodimers produced during expression (Fig. 2A). Similar to other kinases, the rhIKKs exhibited a narrow pH optimum centered around 7.6. All purified rhIKKs 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 rhIKK1/rhIKK2 heterodimer are compared with those of the rhIKK1 homodimer, rhIKK2 homodimer, and rhIKK2 (S177E, S181E) in Fig. 2C and in Table I. The kinetic parameters of purified rhIKKs were determined using biotinylated I κ B α peptide and SAM² 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 I κ B α peptide or GST-I κ B α _{1–54} fusion protein. Our results reported here support their findings that the streptavidin capture assay has a wide dynamic range, has a high signal-to-background ratio, and is much quicker and more quantitative than analysis of protein phosphorylation using the SDS-PAGE assay. Fur-

TABLE I
Kinetic parameters of rhIKKs compared with those from recent publications

Kinases	Specific activity	K_m		k_{cat}	k_{cat}/K_m^b
		ATP	I κ B α peptide		
	<i>units/mg</i>		μM	h^{-1}	
rhIKK1/rhIKK2 heterodimer	5.5 \pm 0.47	0.63 \pm 0.21 13 \pm 4 ^c	0.60 \pm 0.30 3.4 \pm 0.9 ^c	28.50 \pm 2.43	47.50
rhIKK2 homodimer	3.2 \pm 1.20 0.62 ^d	0.65 \pm 0.15 0.56 ^d 0.14 ^e 18 \pm 5 ^e	0.94 \pm 0.32 0.50 ^d 1.30 ^e 2.2 \pm 0.5 ^c	16.40 \pm 6.20 3.30 ^d 16.20 ^e	17.44
rhIKK2 homodimer (S177E, S181E)	0.63 \pm 0.13 6.5 ^d	0.21 \pm 0.04 0.63 ^d	2.62 \pm 0.32 1.10 ^d	3.10 \pm 0.70 33.80 ^d	1.18
rhIKK1 homodimer	0.10 \pm 0.03 0.15 ^d	0.90 \pm 0.33 0.63 ^d 0.13 ^e	23.70 \pm 1.50 1.40 ^d 23.0 ^e	0.51 \pm 0.18 0.78 ^d 4.80 ^e	0.02
I κ B kinase complex from HeLa S3 cells		7.0 \pm 3.4 ^f	0.05 \pm 0.02 ^f		

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

^b Catalytic efficiency (k_{cat}/K_m) for I κ B α peptide substrate.

^c Values from Wisniewski *et al.* (24).

^d Values from Mercurio *et al.* (12).

^e Values from Li *et al.* (15).

^f Values of dissociation constants for ATP (K_{ATP}) and GST-I κ B α ($K_{I\kappa B\alpha}$) from Burke *et al.* (9).

thermore, the phosphorylation of the 22-amino acid I κ B α consensus peptide or a GST-I κ B α_{1-54} fusion protein by purified rhIKKs reported herein was specific for Ser³² and Ser³⁶, because there was no phosphorylation of either substrate in which the Ser³² and Ser³⁶ 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- κ B 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 I κ B α 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 I κ B α 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 I κ B α are shown for comparison. Despite different enzyme assays having been used, these values are in good agreement with each another 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_{ATP} of 0.05 μM), 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 assay² as well. The K_m values for ATP of rhIKK2 and rhIKK1/rhIKK2 were 2.61 \pm 0.70 and 0.63 \pm 0.51 μM , respectively.² The K_m values for I κ B α peptides of rhIKK2 and rhIKK1/rhIKK2 were 3.10 \pm 1.53 and 0.60 \pm 0.03 μM , respectively.² However, using this kinase assay, the k_{cat} values of rhIKK2 and rhIKK1/rhIKK2 were slightly lower at 8.6 \pm 0.87 h^{-1} and 11.3 \pm 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 II
Inhibition of rhIKKs by ADP and its analogues

IC₅₀ values were determined as described by Leatherbarrow (35).

Compounds	IC ₅₀			
	rhIKK1	rhIKK2	rhIKK1/rhIKK2 heterodimer	rhIKK2 (S177E, S181E)
	μM			
1. Adenosine 5'-diphosphate	1.50	1.73	1.77	1.17
2. α,β -Methyleneadenosine 5'-triphosphate	1.80	2.50	1.00	3.52
3. Adenosine 5'-O-(3-thiotriphosphate)	3.03	2.42	2.15	1.64
4. Cordycepin 5'-triphosphate	8.00	7.40	3.00	10.09
5. Adenosine 5'-phosphosulfate	18.73	30.38	17.51	36.95
6. 5'-Adenylylimidophosphate	29.34	12.00	13.61	43.07
7. 2'-&3'-O-(4-Benzoyl)adenosine 5'-triphosphate	23.70	70.10	47.67	49.65
8. Adenosine 5'-triphosphate, <i>r</i> -(1-(2-nitrophenyl)ethyl)ester	66.06	58.43	54.86	48.40
9. Adenosine 5'-monosulfate	>200	>200	>200	>200
10. Adenosine 5'-diphospho morpholidate	>200	>200	>200	>200
11. Adenosine 5'-monophospho amidate	>200	>200	>200	>200
12. Adenosine 5'-monophospho morpholidate	>200	>200	>200	>200
13. 8-Bromoadenosine 5'-diphosphate	>200	>200	>200	>200
14. N6-(6-aminohexyl)carbomethyl adenosine 5'-triphosphate	>200	>200	>200	>200
15. 2,3'-Dideoxyadenosine 5'-triphosphate	>200	>200	>200	>200
16. α -Adenosine	>200	>200	>200	>200
17. Adenosine 5'-triphosphate periodase, oxidized borohydride-reduced	>200	>200	>200	>200
18. Adenosine 5'-diphosphomannose	>200	>200	>200	>200
19. Adenosine 5'-monophosphate	>200	>200	>200	>200
20. Adenosine 5'-triphosphate periodase, oxidized	>200	>200	>200	>200
21. Adenosine 5'-diphosphoribose	>200	>200	>200	>200
22. Adenosine 3'-phosphate 5'-phosphate	>200	>200	>200	>200
23. β,γ -Methyleneadenosine 5'-triphosphate	>200	>200	>200	>200

phorylating I κ B α peptide, the catalytic efficiency (k_{cat}/K_m) for the I κ B α peptide of rhIKK1/rhIKK2 heterodimer shows 2.7- and 2375-fold preference ($47.5 \text{ h}^{-1} \mu\text{M}^{-1}$) compared with either rhIKK2 homodimer ($17.44 \text{ h}^{-1} \mu\text{M}^{-1}$) or rhIKK1 homodimer ($0.02 \text{ h}^{-1} \mu\text{M}^{-1}$), respectively. These data are in agreement with the heterodimeric isoform being the physiological IKK. This increase in catalytic efficiency of the rhIKK1/rhIKK2 heterodimer compared with the rhIKK2 homodimer may also be the result of a differential regulation during expression and activation as a result of the presence of IKK1 in the complex, because mixing the isolated rhIKK subunits after expression did not yield an increase in catalytic efficiency.

During the characterization of the rhIKKs, we found that ADP strongly inhibited all isoforms with IC₅₀ 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 I κ B α peptide phosphorylated at Ser³² and Ser³⁶ (Gly-Leu-Lys-Lys-Glu-Arg-Leu-Leu-Asp-Asp-Arg-His-Asp-Ser-PO₃H₂-Gly-Leu-Asp-Ser-PO₃H₂-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 I κ B α 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 I κ B α 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

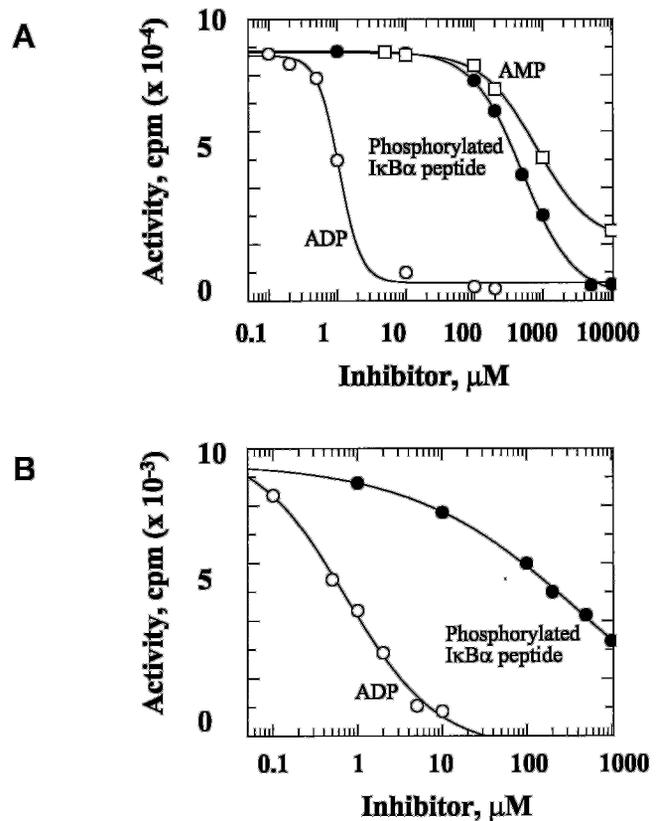
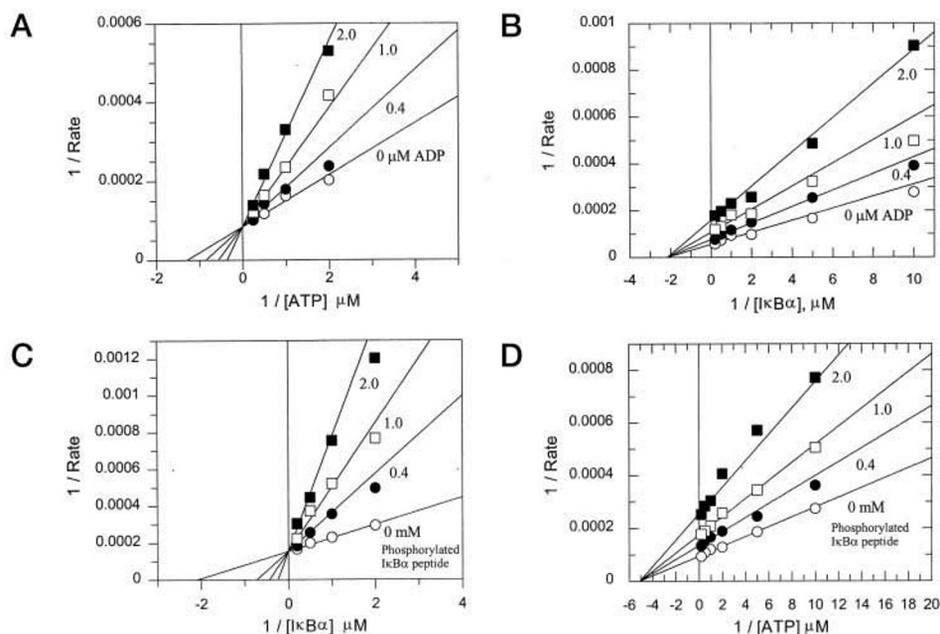


FIG. 3. Effects of the kinase products on activity of rhIKK1/rhIKK2 heterodimer (A) and on the activity of native IKK complex (B). IKK complex immunoprecipitated from LPS induced THP-1 monocytes using anti-NEMO antibody. ADP (\circ), phosphorylated I κ B α peptide (\bullet), and AMP (\square) were incubated with rhIKK1/rhIKK2 or native IKK complex, and kinase activities were measured as described under "Experimental Procedures." Similar effects of ADP, phosphorylated I κ B α peptide and AMP on IKK2 homodimer, rhIKK1 homodimer, and rhIKK2 (S177E, S181E) were seen (data not shown).

⁴ R. P. Compton and J. L. Hirsch, unpublished data.

FIG. 4. Inhibition by ADP and phosphorylated I κ B α 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 I κ B α peptide, respectively. Competitive (C) and noncompetitive (D) inhibition of phosphorylated I κ B α peptide on rhIKK1/rhIKK2 heterodimer kinase activity with respect to I κ B α peptide and ATP, respectively. Concentration of inhibitors used were: (A and B) ADP: 0 (\circ), 0.4 (\bullet), 1.0 (\square), and 2.0 μ M (\blacksquare) and (C and D) phosphorylated I κ B α peptide: 0 (\circ), 0.5 (\bullet), 1.0 (\square), and 2.0 mM (\blacksquare). A similar pattern of inhibition was demonstrated for rhIKK2 homodimer, rhIKK1 homodimer, and rhIKK2 (S177E, S181E) mutant by ADP and phosphorylated I κ B α peptide (data not shown). K_i values (in the text) were determined as described by Leatherbarrow (35).



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-thiotriphosphate), 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 cordycepin 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 μ M, 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 I κ B α peptide competitively inhibits kinase activity with respect to I κ B α (K_i value of 263.74 μ M) and noncompetitively inhibits the heterodimer with respect to ADP (Fig. 4). Similarly, Peet and Li (26) demonstrated that a non-phosphorylated I κ B α peptide competitively inhibited rhIKK1 and rhIKK2 at the I κ B site with K_i values of 139 and 90 μ M, respectively. Thus, our results demonstrate that even a phosphorylated form of consensus I κ B α 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- κ B, along with heat shock proteins, are the paradigm for stress response transcription factors. However, this is also dependent on the cellular concentration of phosphorylated I κ B α and whether the K_i for each phosphorylated I κ B isoform would be lower when complexed with NF- κ B, as reported for the K_m of I κ B α (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. The rhIKK1/rhIKK2 heterodimer exhibits the highest catalytic efficiency toward I κ B α -truncated substrates, supporting the current hypothesis that this is the physiological isoform found in the IKK signalsome. Although these purified rhIKKs are inhibited by both of their kinase products, ADP and phosphorylated I κ B α peptide, ADP by virtue of its low K_i may play a role in regulating kinase activity *in vivo*. Inhibition of IKK activity by ADP contributes mechanistically both to the transient kinase activity and NF- κ B activation demonstrated in cells. Furthermore, the rhIKKs expressed in a baculovirus system are activated by phosphorylation most likely via a homologous signaling pathway in insect cells, which is activated by viral infection similarly to mammalian cells. Thus the characterization of activated IKKs is facilitated by this apparent conservation of the NF- κ B signaling pathway.

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