IKK-i and TBK-1 are Enzymatically Distinct from the Homologous Enzyme IKK-2

COMPARATIVE ANALYSIS OF RECOMBINANT HUMAN IKK-i, TBK-1, AND IKK-2*

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NF-κB is sequestered in the cytoplasm by the inhibitory IκB proteins. Stimulation of cells by agonists leads to the rapid phosphorylation of IκBs leading to their degradation that results in NF-κB activation. IKK-1 and IKK-2 are two direct IκB kinases. Two recently identified novel IKKs are IKK-i and TBK-1. We have cloned, expressed, and purified to homogeneity recombinant human (rh)IKK-i and rhTBK-1 and compared their enzymatic properties with those of rhIKK-2. We show that rhIKK-i and rhTBK-1 are enzymatically similar to each other. We demonstrate by phosphopeptide mapping and site-specific mutagenesis that rhIKK-i and rhTBK-1 are phosphorylated on serine 172 in the mitogen-activated protein kinase activation loop and that this phosphorylation is necessary for kinase activity. Also, rhIKK-i and rhTBK-1 have differential peptide substrate specificities compared with rhIKK-2, the mitogen-activated protein kinase activation loop of IKK-2 being a more favorable substrate than the IκBα peptide. Finally, using analogs of ATP, we demonstrate unique differences in the ATP-binding sites of rhIKK-i, rhTBK-1, and rhIKK-2. Thus, although these IKKs are structurally similar, their enzymatic properties may provide insights into their unique functions.

NF-κB1 is a ubiquitous transcription factor that plays an important role in the regulation of a wide variety of genes involved in immune, inflammatory, and stress responses (1–4). In resting cells, NF-κB is sequestered in the cytoplasm in an inactive state by association with members of the IκB family of inhibitory proteins (IκBα, IκBβ, or IκBe), the best characterized being IκBα (5–8). Stimulation of cells with an agonist results in phosphorylation, ubiquitination, and degradation of IκBs, thus releasing NF-κB for nuclear translocation and activation of gene transcription (9–11). Two IκB kinases (IKK-1 or IKKα and IKK-2 or IKKβ), which specifically phosphorylate the critical serines in IκBs, have been cloned and characterized by several laboratories (12–22). A third adapter protein, NEMO (NF-κB essential modulator, also called IKKγ) is necessary for IKK phosphorylation and activation by upstream kinases (23–25). IKK-1 and IKK-2 are ubiquitously expressed in most human tissues and appear to be the converging point in the activation of NF-κB by a diverse array of agonists (15, 19).

IKK-1 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 (13, 26). IKK-2 is an 87-kDa, 756-amino acid protein with the same overall structure as IKK-1. IKK-1 and IKK-2 are 52% identical overall, with 65% identity in the kinase domain and 44% identity in the C-terminal region, which contains the leucine zipper and helix-loop-helix domains. The kinase activities of IKK-1 and IKK-2 are regulated by phosphorylation. Both enzymes contain canonical mitogen-activated protein kinase kinase kinase (MAPKKK) activation loops, which are the targets for phosphorylation and hence activation by upstream kinases (14, 28, 29). Several experimental approaches from different laboratories have indicated that IKK-2, rather than IKK-1, is essential for NF-κB activation in response to a wide range of inflammatory and stress stimuli including tumor necrosis factor α and interleukin-1β (14, 21, 29). Additionally, IKK-2 also demonstrates a significantly more potent kinase activity using IκBα or IκBβ as substrates.

Recently, two homologs of IKK-1 and IKK-2 have been described, called IKK-i (also known as IKK-ε) and TBK-1 (also known as T2K or NAK), and activation of either of these kinases results in NF-κB activation (30–34). IKK-i and TBK-1 show 30% amino acid identity to IKK-2 in the N-terminal kinase domain and an overall similar topology in the C terminus including a leucine zipper-like domain as well as a helix-loop-helix region (30–34). Additionally, the canonical activation loop motif of IKK-i and TBK-1 differ from IKK-2 with a glutamic acid being present instead of a serine (ExxxExxxExxx) (30–32). IKK-i and TBK-1 also differ from IKK-2 in several other respects. First, they both specifically phosphorylate serine 36 in IκBα and not serine 32. Second, IKK-i is predominantly expressed in immune cells and tissues, including peripheral blood leukocytes, spleen, and thymus, respectively (30). TBK-1 is constitutively and ubiquitously expressed, with the highest level of expression in the testis (32). Third, mRNA for IKK-i, but not IKK-2, can be induced in multiple cell lines in response to several agonists including cytokines and lipopolysaccharide (30). Finally, IKK-i and TBK-1 show significant kinase activity
when overexpressed in and isolated from nonstimulated mammalian cells, and both associate with TRAF/TANK proteins within the cell (35, 36). In contrast, IKK-2 and IKK-1 demonstrate significant kinase activity only when isolated from mammalian cells that have been stimulated with agonists (14, 21). Taken together, it is clear that the IKK isoforms activate NF-κB via distinct mechanisms, have differential tissue distribution, associate within distinct signaling complexes in cells, and differ in their enzymatic properties as well as their substrate recognition.

Both homodimers and heterodimers of rhIKK-1 and rhIKK-2 have been purified to homogeneity and characterized enzymatically by a number of laboratories (14–22). However, neither rhIKK-i nor rhTBK-1 has been purified and characterized. In this report, we have cloned, expressed in a baculovirus expression system, and purified rhIKK-i and rhTBK-1 to compare and contrast their enzymatic properties to each other and rhIKK-1 and rhIKK-2 homodimers. We show that rhIKK-i and rhTBK-1 exhibit a significantly higher catalytic activity compared with rhIKK-1 or rhIKK-2 using IκB as the substrate. However, the $K_m$ for IκB peptide with respect to IKK-i and TBK-1 is >40-fold higher than the IκB $K_m$ for IKK-1 and >200-fold higher than that for IKK-2. In contrast, the $K_m$ for the MAPKK activation loop peptide of rhIKK-2 is significantly lower for both rhTBK-1 and rhIKK-1. We demonstrate that rhIKK-i and rhTBK-1, like IKK-i and IKK-2, are phosphorylated during their expression and that this phosphorylation is necessary for kinase activity. We demonstrate by peptide mapping that both rhIKK-i and rhTBK-1 are phosphorylated on serine 172 in the MAPKK activation loop and that substitution of this serine to either alanine or glutamic acid results in decreased kinase activity. Additionally, we show that, as previously reported for rhIKK-1 and rhIKK-2 (22), ADP is a competitive inhibitor of both rhIKK-i and rhTBK-1 but with unique IC$_{50}$ values of inhibition. Using a panel of ATP and ADP analogs, we further demonstrate that the ATP-binding sites for rhIKK-i and rhTBK-1 are distinct from each other and from either rhIKK-1 or rhIKK-2. These observations suggest the possibility of developing selective inhibitors of each kinase to better understand their roles in the NF-κB activation pathway. Thus, the characterization of rhIKK-i and rhTBK-1 defines unique biochemical properties that provide insight into their roles within the NF-κB activation pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**

All reagents used were of the highest grade commercially available. Anti-FLAG M2 affinity resin, FLAG peptide, antibodies specific for the FLAG peptide, ATP and ADP and their analogs, bovine serum albumin, Nonidet P-40, protease inhibitors, and dithiothreitol (DTT) were obtained from Sigma-Aldrich. Anti-TBK-1 antibody (M-375) was from Santa Cruz Biotechnologies. Recombinant λ protein phosphatase was from New England Biolabs. Peptides were either purchased from American Peptide Co. or made in the peptide synthesis laboratory at Pharmac. (γ-32)P-ATP (2500 Ci/mmol) was purchased from Amersham Pharmacia Biotech. cDNA used to obtain full-length cDNA of IKK-i and TBK-1 and Advantage cDNA polymerase mix for PCRs were from CLONTECH (Palo Alto, CA).

**Cloning and Expression**

*Cloning hIKK-i cDNA—*Human T cell Jurkat cDNA was used as a template to generate Advantage cDNA polymerase mix and primers (Sigma-Genosys, Houston, TX) homologous to the areas containing the initiation codon or the termination codon according to the published sequence of IKK-i (GenBank accession number AF018388). Those primers also contained a BamHI site at the 5’ end of the forward oligonucleotide or an EcoRI site at the 5’ end of the reverse oligonucleotide. The sequences of the oligonucleotides at the IKK-i site were: forward, 5’-AGCATCAGATCCATGAGACAGCAGCAAGCCTTATCCGACACAGGTTGCAGGAGCTTGCAATTTACTGCGT.

The PCR was run on a 1% agarose gel, and a band of ~2 kb was gel-purified. The purified DNA was cut with BamHI and EcoRI and cloned into pFASTBAC (Invitrogen) containing an N-terminal FLAG coding region (pMON46007) in frame with the initiator methionine. An isolate was obtained, fully sequenced, and identified as pMON48028.

*Site-directed Mutagenesis—*The serine residues at positions 172 in IKK-i and TBK-1 were mutated to alanine or glutamic acid residues by twostep-mediated mutagenesis. Briefly, the forward cloning oligonucleotide (described in the cloning sections) for each cDNA was used to prime a PCR along with the reverse mutant oligonucleotide containing the desired codon change. Simultaneously, the forward mutant oligonucleotide was used to prime a second PCR with the reverse cloning oligonucleotide as described in the cloning sections. Those products were gel-purified, and a fraction of each was used as a template in fresh PCRs containing the original cloning oligonucleotides. The products were gel-purified, restriction-digested with the appropriate enzymes, and ligated into the vectors described in the cloning sections. All oligonucleotides were from the Midland Certified Reagent Company, and the PCR reagents were from Invitrogen. The mutant oligonucleotides are shown below, where the letter following the letter S indicates the resulting amino acid residue at position 172. Entire cDNAs were sequenced to verify the mutations and to confirm that no other mutations had occurred. The mutant oligonucleotides are: IKK-i SER, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; IKK-i SAF, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’;

IKK-i SEF, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; IKK-i SAF, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; TBK-1 SAF, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; TBK-1 SEF, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; TBK-1 SER, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; TBK-1 SAF, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; TBK-1 SAF, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; TBK-1 SAF, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’; TBK-1 SER, 5’-TGAATACTAGTCGACGAGTCGACGAGGAGGA-3’.

*Insect Cell Expression—*TBK-1 and IKK-i were expressed in Sf9 insect cells using the commercially available Bac-to-Bac™ baculovirus expression system (Invitrogen). Following the manufacturer’s suggested protocols, donor plasmids pMON48028 and pMON48029 were used to generate recombinant baculovirus Bactmid DNAs containing human TBK-1 and IKK-i, respectively, via transposition in Escherichia coli. Purified bacmid miniprep DNAs were checked by restriction digestion for the presence of the correct inserts and then used to transfect Sf9 insect cells for production of recombinant viruses. Transfection was accomplished using Cell Fectin™ reagent (Invitrogen) following the standard protocol for Sf9 cells. Briefly, a transfection mixture containing 5 µl of the appropriate miniprep DNA and 5 µl of Cell Fectin in 1 ml of SF-900 serum-free medium was added to cells that had been seeded in a 6-well tissue culture plates at 9 × 10^4 cells/well. After 5 h, the mixture was removed, and the cells were fed 3 ml of complete IPL-41 medium (Invitrogen). After 3 days, the medium containing recombinant virus was cleared of cell debris and stored at 4°C. Infecting fresh Sf9 cells with a portion of each transfection medium made larger virus stocks, designated P1. The larger stocks were titered by plaque assay and used for production of recombinant proteins. To generate material for purification, fresh Sf9 cells at 1 × 10^6 cells/ml were infected with recombinant bacmid DNA at a MOI of 1. The infected cultures were allowed to proceed for 72 h, then the cells were harvested, and the resulting pellets were stored at −80°C until used.

*Enzyme Isolation*—All purification procedures were carried out at 4°C until otherwise noted. The buffers used were: buffer A (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM MgCl₂, 2 mM DTT, 0.1% Nonidet P-40, protease inhibitors, and dithiothreitol) and buffer B (20 mM Tris-HCl, pH 7.6, 750 mM NaCl, 2 mM MgCl₂, 2 mM DTT, 50% glycerol, 0.1% Nonidet P-40, protease inhibitors, and dithiothreitol). The buffers used were: buffer A (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM MgCl₂, 2 mM DTT, 0.1% Nonidet P-40, protease inhibitors, and dithiothreitol) and buffer B (20 mM Tris-HCl, pH 7.6, 750 mM NaCl, 2 mM MgCl₂, 2 mM DTT, 50% glycerol, 0.1% Nonidet P-40, protease inhibitors, and dithiothreitol).
Characterization of rhIKK-i and rhTBK-1

NaCl, 0.1% Nonidet P-40, 10% glycerol, 20 mM NaF, 20 mM β-glycerophosphate, 5 mM benzamidine, 2.5 mM sodium metabisulfite, 1 mM DTT, 1 mM EDTA, and 0.5 mM EGTA) and buffer B (same as buffer A with the addition of 500 mM NaCl).

*Isolation of rhIKK-i—*Cells from a 20-liter fermentation of baculovirus-infected insect cells expressing N-terminal FLAG-tagged IKK-i were microfluidized and centrifuged at 26,000 × g for 1 h. The supernatant was collected, and the pH was adjusted to 7.6 with sodium hydroxide. 25 ml of anti-FLAG M2 affinity gel, pre-equilibrated in buffer A, was added to the supernatant pool and allowed to mix overnight. The resin was washed with four resin volumes of buffer A/wash, then poured into a 26/20 column, and washed with 15 resin volumes of buffer B. The FLAG-tagged IKK-i was eluted using FLAG peptide in buffer A. Dithiothreitol was added to the pool to a final concentration of 5 mM, followed by concentration in a stirred cell using an Amicon YM 30 membrane. Bovine serum albumin was added to the concentrated pool to a final concentration of 0.1%, which was then frozen at −80 °C in aliquots. Recombinant hIKK-i (Ser → Glu and Ser → Ala) mutants from a 2-liter fermentation were isolated following procedures described above.

*Isolation of rhTBK-1—*Cells from a 20-liter fermentation of baculovirus-infected cells expressing FLAG-tagged TBK-1 were microfluidized, adjusted to pH 7.6, and centrifuged as described for IKK-i. 50 ml of anti-FLAG M2 antibody resin was added to the pool and allowed to mix overnight. The resin was washed using four bed volumes of buffer A/wash, then poured into a 26/20 column, and washed with 15 resin volumes of buffer B. The TBK-1 protein was eluted with FLAG peptide in buffer A. Dithiothreitol was added to the pool to a final concentration of 5 mM followed by concentration with an Amicon YM 30 membrane. Bovine serum albumin was added to the concentrated pool to a final concentration of 0.1%, and the sample was aliquoted and stored at −80 °C until use. Recombinant hTBK-1 (Ser → Glu and Ser → Ala) mutants from a 2-liter fermentation were isolated following similar procedures.

**SDS-PAGE and Western Blotting**

Purified samples of rhIKK-i and rhTBK-1 were analyzed by SDS-PAGE (12.5% Bis-Tris NuPAGE gel) run in MES buffer. The proteins were detected by Coomassie stain. For Western blot analyses, the proteins were transferred to nitrocellulose membranes (Novex) and detected by chemiluminescence (SuperSignal) using anti-FLAG antibody. For Western blot analyses, the proteins were transferred to nitrocellulose membranes (Novex) and detected by Coomassie stain. For Western blot analyses, the proteins were transferred to nitrocellulose membranes (Novex) and detected by chemiluminescence (SuperSignal) using anti-FLAG antibody.

**Phosphatase Treatment**

Phosphatase treatment was as previously described for rhIKK-1 and rhIKK-2 (22). Briefly, 4–8 μg of rhIKK-i or rhTBK-1 was immunoprecipitated with 40 μg of FLAG antibody followed by coupling to protein G-Sepharose beads. Immunoprecipitated rhIKK enzymes were washed and resuspended in 50 mM Tris-HCl, pH 7.6, containing 0.1 mM EDTA and 2 mM MgCl₂. Following an incubation with recombinant Aλ protein phosphatase (800 units) at room temperature for 30 min, cold lysis buffer (20 mM HEPES, pH 7.6, containing 0.5% Nonidet P-40, 50 mM NaCl, 0.1 mM EDTA, 1 mM EGTA, 5 mM DTT, 1 mM sodium orthovanadate, and 10 mM β-glycerophosphate) was added to the samples to stop the reaction. After several washes, 10% of the beads were removed for Western blot analysis, and the remaining material was pelleted and resuspended in 100 μl of the kinase buffer used for in vitro enzyme assay.

**Phosphopeptide Analysis**

IKK-i was concentrated and dialyzed into 6 mM guanidine, 0.5 mM Tris, pH 8.5, 5 mM dithiothreitol for 1 h. The free cysteines were alkylated with 15 mM iodoacetic acid on ice in the dark for 30 min as described (37). Alkylated protein was dialyzed against 2 mM urea/pH 8.0. Protein was digested with trypsin (Promega, Madison, WI) at a 60:1 protein:enzyme ratio. The resulting peptides were separated by reverse-phase HPLC using a 120 A microbore HPLC system (Applied Biosystems, Foster City, CA). 1 × 150 mm Vydac (Hesperia, CA) C-18, 300, a reverse-phase column was used for the separation, employing a linear gradient of acetonitrile/water with 0.1% trifluoroacetic acid. The fractions were collected and analyzed by matrix-assisted laser desorption ionization MALDI mass spectrometry using a Voyager DE-RI time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). A 0.25-μl aliquot of the collected fraction was mixed at a 1:4 ratio with matrix (50 mM solution of α-cyano-4-hydroxycinnamic acid) and allowed to dry. The MALDI mass spectrometry data were collected in the reflection mode, using an average of 100 scans. The measured masses of the tryptic peptides were compared with the expected masses based on the known amino acid sequence. Potentially phosphorylated peptides were identified by the increase of 80 Da or multiples of 80 Da over the expected mass of a given tryptic peptide. To confirm the identity of the phosphopeptides and to determine the site of phosphorylation, the phosphopeptide-containing fractions were subjected to tandem mass spectrometry using a Q-T mass spectrometer (Micromass, Inc., Beverly, MA) fitted with a nano-electrospray ionization source (38, 39). 1–3 μl of the sample was transferred into a PicoTip glass nanospray emitter with a 2 μm inner diameter tip (New Objective, Woburn, MA). Up to 20 min of fragmentation data were collected and averaged for each sample.

**Enzyme Assay**

Kinase activity was measured essentially as described previously for IKK-2 (22) using a 23-α-amino acid residue peptide derived from IxBa (Glu-Leu-Lys-Lys-Glu-Arg-Leu-Asp-Asp-Arg-His-Asp-Ser-Glu-Leu-Asp-Ser(Ser)-Met-Lys-Asp-Glu-Glu). The standard reaction mixture contained biotinylated IxBa (500 μM for IKK-i, 750 μM for TBK-1, or 5 μM for IKK-2), (γ-32P)ATP (10 μM for IKK-i, 15 μM for IKK-1, or 2 μM for IKK-2), 1 mM DTT, 50 mM KCl, 2 mM MgCl₂, 2 mM MnCl₂, 10 mM NaF, 25 mM Hepes buffer, pH 7.6, and 10 μl (15–20 ng of IKK-i or TBK-1 or 100 ng of IKK-2) of enzyme solution in a final volume of 50 μl. Following incubation at 25 °C for 30 min, 32P-IxBa was separated from unincorporated (32P)ATP by the transfer of a suitable aliquot of the reaction to a SAM 96 biotin capture plate, followed by sequential washes with 2 ml of 0.1% NaCl and 2 M NaCl containing 1% H₃PO₄ essentially as described (22). The plates were dried and counted in a Top-Count NXT after the addition of 25 μl of Microscint 20. Alternatively, the reaction was stopped by the addition of 150 μl of AG1X8 resin in 900 mM sodium formate buffer, pH 3 (the resin is in a slurry of 1 volume resin to 2 volume of sodium formate buffer). The resin was allowed to settle, and 50 μl of supernatant was transferred to a top count plate followed by the addition of 150 μl of Microscint 40, mixed well, and counted. Both assays gave results similar to those reported earlier (22), although the background was lower with the biotin capture method. The resin capture assay was used in experiments where nonbiotinylated peptide substrates were used. Biotinylated IxBa peptide was used in both assays and yielded similar results. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the transfer of 1 nmol of phosphate from ATP to the peptide substrate/min. Specific activity is expressed as units/mg of protein.

For Kₘ determination of purified enzymes, various concentrations of ATP or IxBa were used in the assay at a fixed concentration of the second substrate. For ATP Kₘ, the assays were carried out with 15 or 20 ng of IKK-i or TBK-1, respectively, with IxBa at 500 and 750 μM. Recombinant and ATP was varied from 0.31 to 10 μM. 32P-ATP was fixed at 10 μM for IKK-i and 15 μM for TBK-1, and IxBa concentration was varied from 31 to 1000 μM. Experiments involving IKK-2 were essentially as described earlier (22).

**RESULTS AND DISCUSSION**

IKK-i and TBK-1 have recently been described as isoforms of IKK-1 and IKK-2 based on similarities in their overall structure and sequence. Although rhIKK-1 and rhIKK-2 have been purified to homogeneity and enzymatically characterized by many investigators, the properties of rhIKK-i and rhTBK-1 have not been described thus far. To better understand the enzymatic similarities and differences between these four IKK isoforms, we have cloned, expressed, and purified rhIKK-i and rhTBK-1 and compared their enzymatic properties to those of rhIKK-1 and rhIKK-2.

Both rhIKK-i and rhTBK-1 containing a FLAG epitope tag at the N terminus were expressed in a baculovirus system and purified to homogeneity. Single bands at the expected molecular masses between 80–85 kDa were confirmed by SDS-PAGE followed by Coomassie stain, and the identities of rhIKK-i and rhTBK-1 were confirmed by Western blot analysis (Fig. 1A).

Bovine IxBa and rhTBK-1 exhibited a pH optimum between 7 and 8, similar to all other kinases. Purified enzymes were stable for at least 9 months at −80 °C when stored in buffer containing 0.1% bovine serum albumin, 0.1% Nonidet P-40, 10% glycerol, 5 mM DTT, and protease inhibitors (data not shown).

Like IKK-1 and IKK-2, TBK-1 and IKK-i are IκB kinases,
because they are able to phosphorylate IxBα as a substrate. We determined the kinetic parameters of rhIKK-i and rhTBK-1 using a 23-amino acid peptide encompassing residues 19–41 of IxBα as the phosphoacceptor peptide and compared them with those of rhIKK-1, rhIKK-2, and rhIKK-1/IKK-2 heterodimer (Fig. 1B and Table I). Both rhIKK-i and rhTBK-1 had a dramatically higher enzymatic activity compared with rhIKK-2 under comparable assay conditions (Fig. 1B). Table I compares the kinetic parameters of rhIKK-i and rhTBK-1 to our previously published findings on rhIKK-2 homodimer, rhIKK-1 homodimer, and rhIKK-1/IKK-2 heterodimer. The values for kinetic parameters shown in Table I depict data generated concomitantly with those of IKK-1 and TBK-1 to compare values under identical assay conditions. Note that the $K_{cat}$ and specific activity values reported here for IKK-1 and IKK-2 and IKK-1/IKK-2 heterodimer are 2–3-fold higher than our previously published values generated with early enzyme preparations. Further optimization of storage conditions have resulted in increased stability of all IKK isoforms.

Table I shows several key findings. First, the $K_{cat}$ for rhIKK-i and rhTBK-1 are $>40$–50-fold higher than either rhIKK-2 homodimer or rhIKK-2/IKK-1 heterodimer and $>300$-fold higher than rhIKK-1 homodimer. Second, IxBα is a significantly better substrate for rhIKK-2 homodimer and rhIKK-1/IKK-2 heterodimer with more than a 100-fold lower $K_m$ compared with rhIKK-i and rhTBK-1. Comparable results were obtained when IxBα-GST fusion protein was used as a substrate (data not shown). Thus, the catalytic efficiency ($K_{cat}/K_m$) using IxBα peptide as the substrate is 5–10-fold higher for rhIKK-2 and rhIKK-1/IKK-2 heterodimer compared with rhIKK-i and rhTBK-1. This is an intriguing result and may indicate that IxBα is not a preferred physiological substrate for either rhIKK-i or rhTBK-1. The data in Table I also confirm that the catalytic efficiency of rhIKK-1 is the lowest of the four isoforms by virtue of its low $K_m$ and its 5–10-fold higher $K_{cat}$ for the IxBα peptide compared with rhIKK-2. Interestingly, Zandi et al. (40) have shown that when full-length IxBα is used as a substrate, the $K_m$ values for IKK-1 and IKK-2 are comparable. However, the catalytic efficiency of both rhIKK-2 and rhIKK-1 is reported to be significantly higher when IxBα-NF-κB complex is used as a substrate instead of free IxBα. Thus, IxBα may be a significantly better substrate for rhIKK-i and rhTBK-1 when it is associated with other cellular proteins such as NF-κB.

The MAPKK activation loops of IKK-1 and IKK-2 are similar, each containing the SXXS motif with Ser$^{176}$ and Ser$^{180}$ and with Ser$^{177}$ and Ser$^{181}$ being the phosphoacceptor serines, respectively. This sequence differs from that in either IKK-i or TBK-1, which contain an EXXS motif, where the phosphoacceptor serine is Ser$^{172}$ (Fig. 2A). Thus, we wanted to evaluate the role of phosphorylation of the MAPKK activation loop in the regulation of IKK-i and TBK-1 kinase activity (Fig. 2). Both rhTBK-1 and rhIKK-i had phosphatase-sensitive kinase activity (Fig. 2B). These data are similar to that of rhIKK-2 shown here and previously reported for rhIKK-1, rhIKK-2, and rhIKK-1/IKK-2 heterodimer (22). Note that the decrease in kinase activity after phosphatase treatment was due to dephosphorylation because heat treatment of the phosphatase abolished its ability to inactivate rhIKK-i and rhTBK-1. Likewise, the constitutively active rhIKK-2 (S177E,S181E) did not demonstrate a loss of activity with phosphatase treatment, indicating a specific effect of phosphatase on the susceptible phosphorylated IKK isoforms. Similar results were obtained with the rhIKK-i (S172E) mutant enzyme, in that the kinase activity was not sensitive to phosphatase treatment (data not shown). The Western blot analysis confirms that the amount of kinase remained constant during the phosphatase treatment and the subsequent kinase assay. To identify the specific phosphorylated residue, we subjected rhIKK-i and rhTBK-1 to tryptic digestion followed by MALDI mass spectrometry to identify the phosphopeptide. Tandem mass spectrometry of the phosphopeptide-containing fractions identified Ser$^{172}$ as the phosphorylated residue in rhIKK-i (Fig. 2C) and rhTBK-1 (data not shown). Thus, like rhIKK-1 and rhIKK-2, rhTBK-1 and rhIKK-i are activated by phosphorylation of their MAPKK activation loops during expression.

To confirm the role of phosphorylation of IKK-i and TBK-1 in the regulation of kinase activity, we constructed mutants of IKK-i and TBK-1 in which serine 172 was replaced with either alanine or glutamic acid. Each mutant including rhIKK-i (S172A), rhIKK-i (S172E), rhTBK-1 (S172E), and rhTBK-1 (S172E) was cloned, expressed, and purified to homogeneity. The enzymatic properties of the purified mutant enzymes were compared with the respective wild type enzymes under comparable assay conditions (Fig. 3). Neither rhIKK-i (S172A) nor rhTBK-1 (S172A) had kinase activity (data not shown). These results are in agreement with the results reported by Shimada et al. (30) and Peters et al. (31), who showed that IKK-i (S172A), when overexpressed and immunoprecipitated from a mammalian system, had no kinase activity. Our results further extend these observations to TBK-1 (S172A), which has not been reported thus far. In contrast to the S172A construct, S172E mutants of rhIKK-i and rhTBK-1 had kinase activity, although the specific activity was reduced $>100$-fold compared with wild type rhIKK-i (Fig. 3A) or wild type rhTBK-1 (Fig. 3B). However, the $K_m$ values for ATP and IxBα of the rhIKK-i (S172E) and rhTBK-1 (S172E) mutants were comparable with the respective wild type enzymes (Table II). Thus, the reduced specific activity of the mutant (S172E) enzymes is mainly due to the rate of catalysis rather than altered substrate binding. These results are similar to our previous finding with rhIKK-2 (S177E,S181E) reported earlier and shown in Table II for comparison. Although our results show significant kinase activity.
of rhIKK-i (S172E), these results may still be in agreement with those reported by Shimada et al. (30), who showed a loss of kinase activity of the S712E mutant, and Peters et al. (31), who showed a highly reduced kinase activity of the IKK-i (S172E) mutant enzyme. Note that purified rhIKK-i (S172E), these results may still be in agreement with those reported by Shimada et al. (30, 31), because of its low specific activity and thus low signal to noise ratio in the resin assay. First, rhIKK-i and rhTBK-1 specifically phosphorylate serine 36 of IκBα, whereas IKK-1 and IKK-2 phosphorylate both serine 32 and serine 36 (15, 30–34). This suggests that the peptide substrate requirements for the IKK isoforms may be different.

Table III shows several unique differences in the peptide substrate specificity of rhIKK-i and rhTBK-1 compared with rhIKK-2. We did not evaluate these substrates against IKK-1 because of its low specific activity and thus low signal to noise ratio in the resin assay. First, rhIKK-i and rhTBK-1 specifically phosphorylate serine 36 of IκBα with \( K_m \) and \( K_{cat} \) values comparable with the parent IκBα peptide (Table III, peptide series A). Thus, peptide variants in which serine 36 was substituted with alanine (Ser\(^{36}\)-Ala\(^{36}\)) or with a phosphoserine (Ser\(^{36}\)-Ser(P)\(^{36}\)) were not substrates for rhIKK-i and rhTBK-1 but were comparable substrates to the parent (Ser\(^{36}\)-Ser\(^{36}\)) peptide with respect to rhIKK-2. In contrast, serine 32 peptide variants containing alanine (Ala\(^{32}\)-Ser\(^{36}\)) or phosphoserine (Ser(P)\(^{32}\)-Ser(P)\(^{36}\)) were efficient substrates for rhIKK-i, rhTBK-1, and rhIKK-2 with catalytic efficiency comparable with that of the wild type (Ser\(^{32}\)-Ser\(^{36}\)) peptide. Interestingly, peptide variants containing a phosphoserine at either position 32 or 36 had comparable catalytic efficiency with respect to rhIKK-2, because the \( K_{cat}/K_m \) ratio were 94 h\(^{-1}\) μM\(^{-1}\) and 62 h\(^{-1}\) μM\(^{-1}\), respectively. These data suggest that the phospho-
Characterization of rhIKK-i and rhTBK-1

(A) rhIKK-i activity: WT vs S172E
(B) rhTBK-1 activity: WT vs S172E

Fig. 3. Comparison of enzymatic activity of wild type and S172E mutant rhIKK-i and rhTBK-1. A, [γ-32P]ATP and biotinylated IκBα peptide were incubated with increasing amounts of either rhIKK-i (S172E) (dashed lines) or rhIKK-i wild type and kinase activity determined as described under “Experimental Procedures.” B, [γ-32P]ATP and IκBα peptide were incubated with increasing concentrations of rhTBK-1 (S172E) (dashed line) or rhTBK-1 wild type (solid line), and the kinase activity was measured as for IKK-i, WT, wild type.

Table II
Kinetic parameters of activation loop mutants

| Kinase          | Activity | $K_m$ (units/mg) | ATP | $K_m$ (μM) | IκBα | $K_{cat}$ (h⁻¹) | $K_{cat}$/$K_m$ (h⁻¹ μM⁻¹) |
|-----------------|----------|------------------|-----|------------|------|-----------------|-----------------------------|
| rhIKK-i (S172E)|          | 5.8 ± 1.2        | 3.7 ± 0.4 | 346 ± 107 | 30 ± 7 | 0.08            |
| rhTBK-1 (S172E)|          | 1.5 ± 0.3        | 10.1 ± 2 | 328 ± 16  | 8.4 ± 0.1| 0.02            |
| rhIKK-2 (S171E, S181E) | | 0.6 ± 0.1 | 0.2 ± 0.04 | 2.6 ± 0.3 | 3.1 ± 0.7 | 1.2 |

* Published observations (22).

Table III
Differential peptide substrate specificities of rhIKK-i, rhTBK-1, and rhIKK-2

| Peptide | rhIKK-i | rhTBK-1 | rhIKK-2 |
|---------|---------|---------|---------|
|         | $K_m$ (μM) | $K_{cat}$ (h⁻¹) | $K_m$ (μM) | $K_{cat}$ (h⁻¹) | $K_m$ (μM) | $K_{cat}$ (h⁻¹) |
| A       | GLKERRRLLDRHDSGLDSMKDDE | 202 ± 39 | 1966 ± 601 | 273 ± 26 | 1759 ± 241 | 0.8 ± 0.01 | 63 ± 3 |
| B       | DSGLDSM | 174 ± 60 | 1521 ± 55 | 537 ± 176 | 1377 ± 282 | NS |
| C       | AKELDQSLCTSFSVTGDLQ | 19.2 ± 3.4 | 657 ± 83 | 24 ± 6 | 1007 ± 43 | 0.75 ± 0.3 | 33 ± 3.6 |

* NS, not a substrate up to 1 mM.

...and rhTBK-1 maintained their specificity for serine 36, even in the truncated peptide. However, unlike peptide series A, when phosphoserine was substituted for serine 36 in peptide series B, it was no longer a substrate for rhIKK-i or rhTBK-1. This may be because the additional negative charge contributed by the phosphoserine residue in the truncated peptide may interfere with binding or phosphotransfer to the acceptor serine 36. In contrast to the 7-amino acid truncated IκBα peptide, the larger 23-amino acid IκBα peptide has several positively charged amino acids such as lysines and arginines that could compensate for a negatively charged phosphoserine residue.

To further define peptide substrate specificity, we tested a dozen diverse peptides containing serines that were substrates for other kinases (e.g., c-Jun peptide, HSP-27, CREB-tide, Fos peptide, etc.), and none of these peptides were substrates for either rhIKK-i, rhTBK-1, or rhIKK-2 (data not shown). Finally, we examined a 17-amino acid peptide (residues 170–187) derived from the activation loop of IKK. This peptide was indeed a more efficient substrate than IκBα for both rhIKK-i and rhTBK-1 (Table III, peptide C). Tojima et al. (32) have reported that overexpressed TBK-1 activates the IKK complex in mam-
malian cells by phosphorylating IKK-2 in the activation loop and thus acting as an upstream kinase. Note that the $K_{cat}/K_{m}$ values obtained for the IKK-2 loop peptide are 34, 41, and 43 $\mu$M$^{-1}$ $\text{cat/} \mu\text{M}$ for rhIKK-i, rhTBK-1, and rhIKK-2, respectively. This suggests that the IKK-2 loop peptide is an equally efficient substrate for the three IKK isoforms. In contrast to the loop peptide, the $K_{cat}/K_{m}$ values for the IxBo peptide are 9.7, 6.4, and 63 $\mu$M$^{-1}$ for rhIKK-i, rhTBK-1, and rhIKK-2, respectively, thus confirming that the IKK-2 loop peptide is a better substrate for rhIKK-i and rhTBK-1, as compared with IxBo. Phosphorylation of the IKK-2 loop peptide by rhIKK-2 itself is not surprising because it has been postulated to undergo autophosphorylation (29). Note that the $K_{cat}/K_{m}$ value for rhIKK-i is comparable with the values measured for the IKK-2 loop peptide (20). Taken together, these results clearly demonstrate that the peptide substrate specificities of rhIKK-i and rhTBK-1 are similar to each other and clearly distinct from that of rhIKK-2.

We have previously demonstrated that ADP is a competitive inhibitor of rhIKK-2 at the ATP site with an IC$_{50}$ value of 2.5–3 times their respective $K_{m}$ values for each enzyme. Closed squares, IKK-2; closed triangles, IKK-i; closed circles, TBK-1. B, competitive inhibition of rhIKK-i by ADP with respect to the ATP site. The concentrations of ADP used were 6.25, 12.5, and 25 $\mu$M. C, competitive inhibition of rhTBK-1 by ADP with respect to the ATP site. The concentrations of ADP used were 50, 100, and 200 $\mu$M.

In summary, in the present report, we have cloned, expressed, and purified rhIKK-i and rhTBK-1 and compared further extend these observations, we determined the IC$_{50}$ values for various ATP and ADP analogs for rhIKK-i and rhTBK-1 and compared them to previously reported results for rhIKK-1 and rhIKK-2 (Table IV). Note that the IC$_{50}$ values were comparable between rhIKK-2 and rhIKK-1. Interestingly, selective inhibition of rhIKK-i, rhTBK-1, and rhIKK-2 was noted with minor differences in the ATP analog structure. For example, $\alpha,\beta$-methyleneadenosine 5'-triphosphate and 2'-deoxyadenosine 5'-triphosphate showed comparable IC$_{50}$ values against rhIKK-2, rhIKK-i, and rhTBK-1, whereas 2,3-dideoxyadenosine 5'-triphosphate was >10-fold more potent against rhIKK-i and rhTBK-1 compared with rhIKK-2. In contrast, adenylylimidodiphosphate was >10-fold more potent against rhIKK-2 than either rhIKK-i or rhTBK-1. Interestingly, $\beta,\gamma$-methylene adenosine triphosphate, which only differs from the close analog $\alpha,\beta$-methylene adenosine triphosphate with respect to the position of the methylene bridge, gave selective inhibition of rhTBK-1. Additionally, ADP analogs such as adenosine 5'-O-(2-thiodiphosphate), like ADP, were 10-fold more potent against rhIKK-2 compared with rhIKK-i and 100-fold more potent compared with rhTBK-1. These observations demonstrate that the ATP-binding sites of rhIKK-2, rhIKK-i, and rhTBK-1 are indeed unique and amenable for the design of selective inhibitors.

### Table IV

| Analogs                                  | IC$_{50}$ (M) |
|------------------------------------------|--------------|
| Adenosine 5'-O-(3-thiotriphosphate)      | 4.9          |
| Adenosine 5'-(2-thiodiphosphate)         | 26           |
| Adenosine 5'-diphosphate                 | 19.6         |
| $\alpha,\beta$-Methyleneadenosine 5'-triphosphate | 3.4         |
| $\beta,\gamma$-Methyleneadenosine 5'-triphosphate | >200       |
| 2-Deoxyadenosine 5'-triphosphate         | 3.8          |
| 2,3-Dideoxyadenosine 5'-triphosphate     | 11           |
| 5'-Adenylylimidodiphosphate              | 142          |
| Adenosine 5'-monophosphate               | >200         |

$^a$ Published observations (22).
their enzymatic properties with those of rhIKK-2 and rhIKK-1. Despite the overall structural similarity to IKK-1 and IKK-2, IKK-i and TBK-1 are enzymatically distinct, rhIKK-i and rhTBK-1 share several similarities. The catalytic rate of rhIKK-i and rhTBK-1 are 50–100-fold higher than that of rhIKK-2. They clearly differ from rhIKK-2 with respect to their peptide substrate specificity. The fact that the best substrate identified for rhIKK-i and rhTBK-1 is the activation loop peptide of IKK-i and TBK-1 are enzymatically distinct. rhIKK-i and TBK-1 interact with complexes of TANK and TRAF proteins (35, 36). Baud et al. (41) previously demonstrated that the oligomerization of TRAF proteins was sufficient to activate the IKK signalosome complex.

The ATP analog studies show distinct differences in the ATP sites of the three isoforms. This is indeed intriguing because the ATP $K_m$ values do not reflect these active site differences. Differential inhibition of rhIKK-2, rhIKK-i, and rhTBK-1 is particularly interesting, because ADP is a product inhibitor. The unique $IC_{50}$ values may reflect differences in enzyme mechanism, with potential differences in the product inhibition patterns. IKK-1 and IKK-2 have been demonstrated to exhibit a classical random sequential mechanism (17, 18), but IKK-i and TBK-1 deviate from the classical random sequential mechanism. Finally, the selective inhibition of the IKK isoforms with analogs of ATP opens up the possibility of using selective inhibitors of IKK-i and TBK-1 to understand their roles in NF-$\kappa$B activation.

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IKK-i and TBK-1 are Enzymatically Distinct from the Homologous Enzyme IKK-2: COMPARATIVE ANALYSIS OF RECOMBINANT HUMAN IKK-i, TBK-1, AND IKK-2

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