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IκB Kinase (IKK)-Associated Protein 1, a Common Component of the Heterogeneous IKK Complex

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Activation of the transcription factor NF-κB is controlled by the sequential phosphorylation, ubiquitination, and degradation of its inhibitory subunit, IκB. We recently purified a large multiprotein complex, the IκB kinase (IKK) signalsome, which contains two regulated IκB kinases, IKK1 and IKK2, that can each phosphorylate IκBα and IκBβ. The IKK signalsome contains several additional proteins presumably required for the regulation of the NFκB signal transduction cascade in vivo. In this report, we demonstrate reconstitution of IκB kinase activity in vitro by using purified recombinant IKK1 and IKK2. Recombinant IKK1 or IKK2 forms homo- or heterodimers, suggesting the possibility that similar IKK complexes exist in vivo. Indeed, in HeLa cells we identified two distinct IKK complexes, one containing IKK1-IKK2 heterodimers and the other containing IKK2 homodimers, which display differing levels of activation following tumor necrosis factor alpha stimulation. To better elucidate the nature of the IKK signalsome, we set out to identify IKK-associated proteins. To this end, we purified and cloned a novel component common to both complexes, named IKK-associated protein 1 (IKKAP1). In vitro, IKKAP1 associated specifically with IKK2 but not IKK1. Functional analyses revealed that binding to IKK2 requires sequences contained within the N-terminal domain of IKKAP1. Mutant versions of IKKAP1, which either lack the N-terminal IKK2-binding domain or contain only the IKK2-binding domain, disrupt the NF-κB signal transduction pathway. IKKAP1 therefore appears to mediate an essential step of the NF-κB signal transduction cascade. Heterogeneity of IKK complexes in vivo may provide a mechanism for differential regulation of NF-κB activation.

Transcription factors of the NF-κB/Rel family are critical regulators of genes involved in inflammation, cell proliferation, and apoptosis (reviewed in reference 6). The prototype member of the family, NF-κB, is composed of a dimer of p50 NF-κB and p65 RelA (3). NF-κB is present in the cytoplasm of resting cells but upon activation enters the nucleus in response to multiple stimuli, including viral infection, UV irradiation, and exposure to proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (reviewed in references 5 and 6). NF-κB is also activated by various chemical stimuli, including phorbol esters, chemotherapeutic agents, oxidizing agents, and inhibitors of serine and tyrosine phosphatases (4, 5, 22).

NF-κB exists in the cytoplasm in an inactive form by virtue of its association with inhibitory proteins termed IκB, of which the most important may be IκBα, IκBβ, and IκBε (4, 7, 17, 18). The IκB family members, which have common ankyrin-like repeat domains, regulate the DNA binding and subcellular localization of NF-κB/Rel proteins by masking a nuclear localization signal located near the C terminus of the Rel homology domain (8, 9). NF-κB activation is achieved through the signal-induced proteolytic degradation of IκB in the cytoplasm. Extracellular stimuli initiate a signaling cascade leading to activation of two IκB kinases, IKK1 (IKKα) and IKK2 (IKKβ), which phosphorylate IκB at specific N-terminal serine residues (S32 and S36 for IκBα, S19 and S23 for IκBβ) (9, 10, 16, 30, 31, 37, 40, 43). Phosphorylated IκB is then selectively ubiquitinated, presumably by an E3 ubiquitin ligase, the terminal member of a cascade of ubiquitin-conjugating enzymes (20, 33, 42). In the last step of this signaling cascade, phosphorylated and ubiquitinated IκB, which is still associated with NF-κB in the cytoplasm, is selectively degraded by the 26S proteasome (2, 11, 14, 15, 32). This process exposes the nuclear localization signal, thereby freeing NF-κB to interact with the nuclear import machinery and translocate to the nucleus, where it binds its target genes to initiate transcription.

We, and others, recently identified a high-molecular-weight multiprotein complex containing an inducible IκB kinase activity (13, 16, 30, 31, 40, 43). Two kinases contained in this complex, termed IκB kinases 1 (IKK1, IKKα) and 2 (IKK2, IKKβ), were cloned and demonstrated to play a key role in NF-κB activation by a variety of stimuli (16, 30, 31, 40, 42). IKK1 and IKK2 are related members of a new family of intracellular signal transduction enzymes, containing an N-terminal kinase domain and a C-terminal region with two protein interaction motifs, a leucine zipper, and a helix-loop-helix motif. These motifs mediate heterodimerization of IKK1 and IKK2, which is essential for function. There is strong evidence that IKK1 and IKK2 are themselves phosphorylated and activated by one or more upstream activating kinases, which are likely to be members of the mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK) family of enzymes (12, 21, 23). One such upstream kinase, NIK, was identified by its ability to bind directly to TRAF2, an adapter protein thought to couple both TNF-α and IL-1 receptors to NF-κB activation (27). A second MAPKKK, MEKK-1, has been shown to copurify with IKK activity (30). Coexpression of either NIK or MEKK-1 enhances the ability of the IKKs to phosphorylate IκB and activate NF-κB (16, 30, 31, 40, 43). The likely sites of activating phos-

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phorylation on the IKKs have been identified as two serine residues within the kinase activation loop, which lie within a short region of homology to the MEK (MAP kinase kinase) family of proteins (30). Phosphorylation of these two serine residues in the MEKs is required for their activation. In IKK2, mutation of the two corresponding serine residues to alanine yields an inactive, dominant negative protein capable of blocking the activation of endogenous NF-κB (30). Conversely, mutation of these residues to glutamate yields a constitutively active kinase, presumably because the glutamate residues mimic to some degree the phosphoserines obtained after phosphorylation by the upstream activating kinase (30).

IKK1 and IKK2 were identified as components of a high-molecular-weight complex termed the IκB kinase (IKK) signalsome. The IKK signalsome displays all the expected characteristics of the cytokine-inducible IκB kinase, including rapid induction in response to known inducers of NF-κB, the ability to phosphorylate specifically both N-terminal serine residues of IκBα and IκBβ, and inhibition by known inhibitors of NF-κB activation. It is unclear whether IKK1 and IKK2 require the presence of other components of the IKK signalsome for IκB kinase activity and, if not, what specific function these additional components may play in NF-κB activation. Here we report the production and characterization of recombinant forms of IKK1 and IKK2. Whereas dimerization appears required for activity, complex formation with other proteins is not essential for full IκB kinase activity. IKK proteins can form homo- or heterodimeric complexes in vitro, suggesting the possibility that heterogeneity in IκB kinase complexes exists in vivo. We identified discrete IKK-containing complexes in vivo, which display differing levels of IκBα kinase activity. To understand the potential role of additional components of these complexes, we identified and cloned a novel component of the IKK signalsome, which we named IKK-associated protein 1 (IKKAP1). This protein interacts specifically with IKK2 and appears to mediate an essential step of the NF-κB signal transduction cascade.

MATERIALS AND METHODS

Cell culture. HeLa cells were maintained in Dulbecco modified Eagle medium (Mediatech) containing 10% fetal calf serum (Hyclone), antibiotics, and 2 mM L-glutamine (Mediatech). SLB cells were cultured in RPMI 1640 (Mediatech) containing fetal calf serum, antibiotics, and 2 mM L-glutamine.

Antibodies. Rabbit anti-IKK2, anti-N-IKKAP1, and anti-C-IKKAP1 antibodies were raised against QTEEEEHSCLEQAS, DQDVLGEESPLGKPAMC, and CLALPSQRRSPPEEPPDF synthetic peptides, respectively (Alpha Diagnostics Inc.). Anti-IKK2 antibodies were affinity purified on specific peptide columns prior to use. IKKα (IKK1)-specific antibodies were obtained from PharMingen, La Jolla, Calif., and anti-RelA antibodies were obtained from Santa Cruz Biotechnology, Inc. Of the antibodies to epitope-tagged proteins, GluGlu monoclonal antibody was raised against the synthetic peptide, EEEEYMPME (Berkeley Antibody Co.), and the Flag monoclonal antibody was raised against the synthetic peptide, MDYKDDDDK.

Library screening. By using a Prime It kit (Stratagene) and [α-^{32}P]dCTP (Amersham), a ^{32}P-labelled DNA probe was generated from an expressed-sequence tag (EST) clone identified while searching a comprehensive EST database with peptide sequences obtained from the isolated protein. The resulting DNA probe was used to screen 5×10^5 plaques lifted from a human HeLa cell lambda cDNA library (Stratagene) as described by the manufacturer. The filters were washed to a final stringency of 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS) at 50°C and exposed to X-ray film overnight at −70°C with intensifying screens. The films were developed, and hybridizing plaques were identified and isolated. Phage were eluted from the agarose plugs in SM and stored at 4°C (primary plaque pools). Secondary and tertiary plaque purifications were performed in a similar fashion to that for the primary pools. Individual clones were excised to generate subclones in the pBluescript SK(−) phagemid vector and subsequently sequenced with the *Taq* Dye Terminator cycle-sequencing kit (Applied Biosystems) on an automated DNA sequencer (model 377; Applied Biosystems). One full-length (pBS hIKKAP1) and several partial human IKKAP1 cDNA clones were identified.

Plasmids. pCR-Script IKK1 was digested with the restriction endonucleases *Eco*RI and *Not*I, and the IKK1 insert was subcloned into the baculovirus transfer vector pAcSG-His NT-C (PharMingen), creating pAcSG-His-IKK1. pCR-Script IKK2 WT and S177/181→E was digested with *Not*I, and the IKK2 insert was subcloned into the baculovirus transfer vector pAcSG-His NT-A (PharMingen), creating pAcSG-His-IKK2 WT and pAcSG-His-IKK2EE. pSport 1-761011 (Genome Systems), an EST clone encoding mouse IKKAP1, was sequenced on an automated DNA sequencer (model 377; Applied Biosystems). Oligonucleotide primers were designed to generate PCR products that encode full-length (FL) and N- (ΔC IKKAP1) and C (ΔN IKKAP1)-terminal IKKAP1 proteins (see Fig. 5A), with pBS hIKKAP1 as a template. The respective PCR products were subsequently subcloned into a pcDNA3-EE expression vector, which encodes an N-terminal GluGlu epitope tag. RelA and IκBα baculovirus expression vectors and the glutathione *S*-transferase (GST)-IκBα/GST-IκBβ bacterial expression vectors were described previously (30).

Kinase assay. Samples from column fractions, immunoprecipitates, or baculovirus-expressed IKK protein were subjected to an in vitro kinase assay. Kinase assays analyzed via SDS-polyacrylamide gel electrophoresis (PAGE) analysis were performed with kinase buffer (20 mM HEPES [pH 7.7], 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride [PMSF], 10 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 mM dithiothreitol [DTT]) at 30°C for 30 to 60 min in the presence of 1 to 3 μCi of [γ-^{32}P]ATP and the indicated substrate. The kinase reaction was terminated by the addition of 6× SDS-PAGE sample buffer, subjected to SDS-PAGE analysis and visualized by autoradiography. Kinase assays for kinetic analysis of the baculovirus-expressed IKKs were performed in a 96-well microplate format as described in each figure legend.

Recombinant-protein production. Sf9 cells (monolayer) were infected at a multiplicity of infection of 5 to 10 with recombinant baculovirus encoding His-tagged IKK1 (BAC-His-IKK1), His-tagged IKK2 (BAC-His-IKK2), or His-tagged IKK2EE (BAC-His-IKK2EE) either alone or in combination (PharMingen). The cells were harvested 72 h postinfection. Whole-cell lysate was prepared, and the His-tagged IKK proteins purified on a nickel-nitrilotriacetic acid resin (Qiagen) as specified by the manufacturer. In some instances, the His-IKK protein was further purified by fractionation on a Mono Q column (Pharmacia). The resulting protein was purified to near homogeneity. Purifications of baculovirus-expressed RelA and IκBα and bacterially expressed GST-IκBα/GST-IκBβ were as previously described (30).

Identification of IKKAP1. Coomassie blue-stained bands were excised and digested in situ with trypsin (Boehringer Mannheim) as described previously (38). The unseparated pool of tryptic peptides was subjected to analysis by nanoelectrospray tandem mass spectrometry as described previously (38, 39). Analysis was performed with an API III triple-quadrapole mass spectrometer (PE Sciex). Peptide sequence tags were assembled by using tandem mass-spectrometric data (28, 29). Searching comprehensive protein and EST databases was performed using PeptideSearch v.3.0 software and specific search algorithms.

In vitro translation studies. GluGlu-tagged IKKAP1, ΔN IKKAP1, and ΔC IKKAP1 were prepared by coupled in vitro transcription and translation in wheat germ lysate (Promega). Reactions were performed as described in the manufacturer's protocol.

Large-scale IKK signalsome purification. (i) Anti-MKP1 immunoprecipitation and purification. IKK signalsome protein was prepared as previously described (30). Briefly, HeLa S3 cells were stimulated for 7 min with 20 ng of TNF-α (R&D Systems) per ml and harvested by scraping, and whole-cell lysate was prepared (1.2 g of total protein) by resuspending the cells in two packed-cell pellet volumes of WCE lysis buffer (20 mM Tris [pH 8.0], 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 mM DTT). Cell suspensions were gently rotated at 4°C for 45 min and centrifuged at 60,000 × *g* for 60 min in a Ti50.1 rotor. Approximately 5 mg of anti-MKP-1 antibody (Santa Cruz Biotechnology) was added to the lysate, and the mixture was incubated at 4°C for 2 h with gentle rotation. Then 15 ml of protein A-agarose (Calbiochem) was added, and the mixture was incubated for an additional 2 h. The immunoprecipitate was then sequentially washed with 2× PD buffer (40 mM Tris [pH 8.0], 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 mM DTT), 1× 1.5 M urea–PD buffer, and 2× PD buffer. The immunoprecipitate was then made into a thick slurry by the addition of 8 ml of PD buffer and 25 mg of the specific MKP-1 peptide to which the antibody was generated (Santa Cruz Biotechnology) and incubated overnight at 4°C with gentle rotation. The eluted IKK signalsome was then desalted on PD10 desalting columns (Pharmacia), equilibrated with 50 mM Q buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.025% Brij 35, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 10 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 mM DTT), and chromatographed on a Mono Q column (Pharmacia). Fractions containing IκB kinase activity were pooled, concentrated, and subjected to preparative SDS-PAGE, and protein bands were visualized with colloidal blue stain (Novex), excised, and submitted for sequence determination by mass spectrometry (see above).

(ii) Anti-IKK2 immunoprecipitation and purification. HeLa S3 cells were stimulated for 7 min with 20 ng of TNF-α per ml and harvested, and whole-cell lysate was prepared (1.2 g of total protein). Approximately 5 mg of anti-IKK2-specific antibodies was added to the lysate, and the mixture was incubated at 4°C for 2 h with gentle rotation; subsequently, 15 ml of protein A-agarose (Calbiochem) was added, and the mixture was incubated for an additional 2 h. The immunoprecipitate was then washed extensively with 2× PD buffer. The immunoprecipitate was then made into a thick slurry by the addition of 8 ml of PD buffer and 25 mg of the specific IKK2 peptide to which the antibody was generated (Alpha Diagnostics Inc.) and incubated overnight at 4°C with gentle rotation. The eluted IKK signalsome was then concentrated and subjected to chromatography on a Hi Load 16/60 Superdex 200 prep grade gel filtration column that was equilibrated in GF buffer (20 mM Tris HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 0.025% Brij 35, 1 mM benzamidine, 2 mM PMSF, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 10 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 mM DTT). Isolated fractions were analyzed by Western blot analysis with either anti-IKK1- or anti-IKK2-specific antibodies. The high-molecular-mass fractions (approximately 550 to 800 kDa) containing IKK1 and IKK2 protein were pooled and subjected to Mono Q column chromatography (Pharmacia). Fractions containing either the IKK2 homodimeric signalsome or the IKK1-IKK2 heterodimeric signalsome were pooled, concentrated, and subjected to preparative SDS-PAGE. Protein bands were visualized with colloidal blue stain, excised, and submitted for sequence determination by mass spectrometry (see above).

Immunoprecipitations. For small-scale immunoprecipitations, HeLa cells were either stimulated with TNF-α or not stimulated, and 300 μg of whole-cell lysate was prepared and diluted to 0.5 ml with PD buffer and 0.5 to 2.0 μg of the indicated antibody. This reaction mixture was incubated on ice for 1 to 2 h, and then 10 μl of protein A or G beads was added and the mixture was left to incubate with gentle rotation for an additional 1 h at 4°C. The immunoprecipitate was then washed three times with PD buffer and once with kinase buffer without ATP and subjected to a kinase assay as described above.

Immunocytochemistry. HeLa cells were transiently transfected with either Flag-tagged IKK2 or GluGlu-tagged IKKAP1 as previously described (30). At 36 h after transfection, the cells were fixed for 30 min with paraformaldehyde and permeabilized with 0.5% Triton. For immunofluorescence staining, the cells were incubated with primary antibody in phosphate-buffered saline containing 5% donkey serum and 0.5% Triton X-100 for 30 min followed by fluorescein-conjugated or Texas red-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc.) (used at 1:100 dilution) for 30 min at room temperature. The glass slides were rinsed and covered with a glass coverslip sealed with Vectashield (Vector Laboratories) before being scored, and representative fields were photographed. Primary antibodies used for immunofluorescence staining included antibodies against RelA (Santa Cruz Biotechnology), GluGlu tag peptide (Berkeley Antibody Co.), and Flag tag peptide (IBI-Kodak).

RESULTS

Recombinant IKK1 and IKK2 display IκB kinase activity. The IKK signalsome contains a number of protein components in addition to IKK1 and IKK2. It is unclear whether the IκB kinase activity associated with the IKK signalsome is completely attributable to IKK1 and IKK2 or whether this activity requires the presence of proteins in addition to IKK1 and IKK2. To investigate this possibility, we produced recombinant versions of IKK1 and IKK2 by using a baculovirus expression system. In addition to the wild-type enzymes, we produced a recombinant form of IKK2, designated IKK2EE, in which the serine residues at positions 177 and 181 within the MEK-related activation loop were mutated to glutamic acid. We previously demonstrated that this form of IKK2 was constitutively active in vivo and could induce NF-κB nuclear translocation in the absence of any other stimulus (30). Recombinant IKK1, IKK2, and IKK2EE were expressed at high level in Sf9 insect cell cultures. Purified IKK1, IKK2, and IKK2EE proteins, when analyzed by gel filtration chromatography, were present as either homodimers, in singly infected cells, or heterodimers, in IKK1- and IKK2-coinfected cells (data not shown). SDS-PAGE analysis of purified recombinant proteins revealed the presence of only IKK1 or IKK2EE, and no other proteins were associated with these dimeric complexes (Fig. 1A). IKK2EE underwent limited C-terminal proteolysis in that IKK2-specific antibodies directed against an internal region of IKK2 react with all forms of IKK2EE (Fig. 1A) whereas antibodies directed to the extreme C-terminal region of IKK2 identify only the full-length IKK2 protein (data not shown). Recombinant forms of IKK1 and IKK2EE displayed IκB kinase activity as detected by γ-^{32}P transfer (Fig. 1B). Recombinant IKK1-IKK2 heterodimer was capable of phosphorylating GST fusion proteins containing either the N-terminal 54 residues of IκBα or the N-terminal 44 residues of IκBβ (Fig. 1B, lanes 1 and 3). IKK1-mediated phosphorylation was specific for serines 32 and 36 of IκBα and serines 19 and 23 of IκBβ as demonstrated by the lack of phosphate transfer to substrates where these serines were mutated to threonines (lanes 2 and 4). Essentially identical results were obtained for recombinant IKK2EE or IKK1 homodimers (lanes 5 to 8 and 10 to 14, respectively). IKK1 and IKK2EE were also capable of phosphorylating full-length IκBα bound within a RelA complex (lanes 9 and 14, respectively). As previously seen for the IKK signalsome, recombinant IKK1 and IKK2 complexes processed IκBα substrate more efficiently than they processed IκBβ and also were capable of efficiently phosphorylating RelA. However, the IKKs were not found to be a general kinase for all Rel-related proteins, in that cRel and NF-κB p52 are not phosphorylated by IKK2 EE (Fig. 1C).

Titration of IKK1 and IKK2 protein in a microtiter plate-based IκB kinase assay demonstrated that the IKK2 enzyme is a more efficient IκB kinase than IKK1. In addition, the mutation of serines 177 and 181 to glutamic acid in IKK2EE resulted in an enzyme with dramatically enhanced IκB kinase activity (Fig. 1D). The specific activity of IKK2EE was at least 10-fold greater than that of IKK2, while IKK2 displayed a 4-fold greater specific activity than IKK1. The substrate specificities and kinetic characteristics of the recombinant IKK proteins were investigated (Table 1). Consistent with data presented in Fig. 1D, the k_{cat}, a measure of the rate of turnover of the enzyme-substrate complex, for the IKK2EE mutant was 10-fold greater than that for the IKK2 enzyme. The K_m for ATP, GST-IκBα, and GST-IκBβ were very similar for each enzyme, with IκBα being preferred as a substrate relative to IκBβ. The apparent substrate selectivity constants (k_{cat}/K_m) of IKK2EE showed a 25-fold preference for full-length IkBα (positions 1 to 317) (k_{cat}/K_m = 220 h^{-1} μM^{-1}) compared to the truncated form (positions 1 to 54) (k_{cat}/K_m = 9 h^{-1} μM^{-1}). A similar trend was observed in K_m values: K_m for 1 to 317 = 0.05 μM, and K_m for 1 to 54 = 1.1 μM.

We previously reported the presence of a RelA kinase activity within the IKK signalsome (30). We observed that recombinant IKK1 and IKK2 homodimers could mediate RelA phosphorylation in vitro (Fig. 1B). Similar findings were observed for the IKK1-IKK2 heterodimer (data not shown). The time course of IKK2EE phosphorylation of the RelA-IκBα complex revealed that RelA was phosphorylated similarly to IκBα (Fig. 1E). Detailed kinetic analysis revealed that IKK2EE processed IkBα and RelA equally as demonstrated by similar specificity constants (k_{cat}/K_m): 93 h^{-1} μM^{-1} for IkBα and 76 h^{-1} μM^{-1} for RelA. Subsequent analysis of phosphopeptides derived from RelA by limited trypsin digestion revealed only one peptide with significant phosphate incorporation (data not shown). Therefore, in addition to phosphorylation of IκB proteins, IKK1 and IKK2 mediate site-specific phosphorylation of RelA.

Heterogeneity of the IKK signalsome in vivo. From analysis of recombinant proteins, only IKK1 and/or IKK2 is required for IκB kinase activity in vitro. In addition, recombinant IKK1 and IKK2 could form homo- and heterodimeric complexes. To investigate the possibility that such heterogeneity of IKK2-containing complexes exists in vivo, we immunoprecipitated IKK2-containing complexes from HeLa cells, subjected this

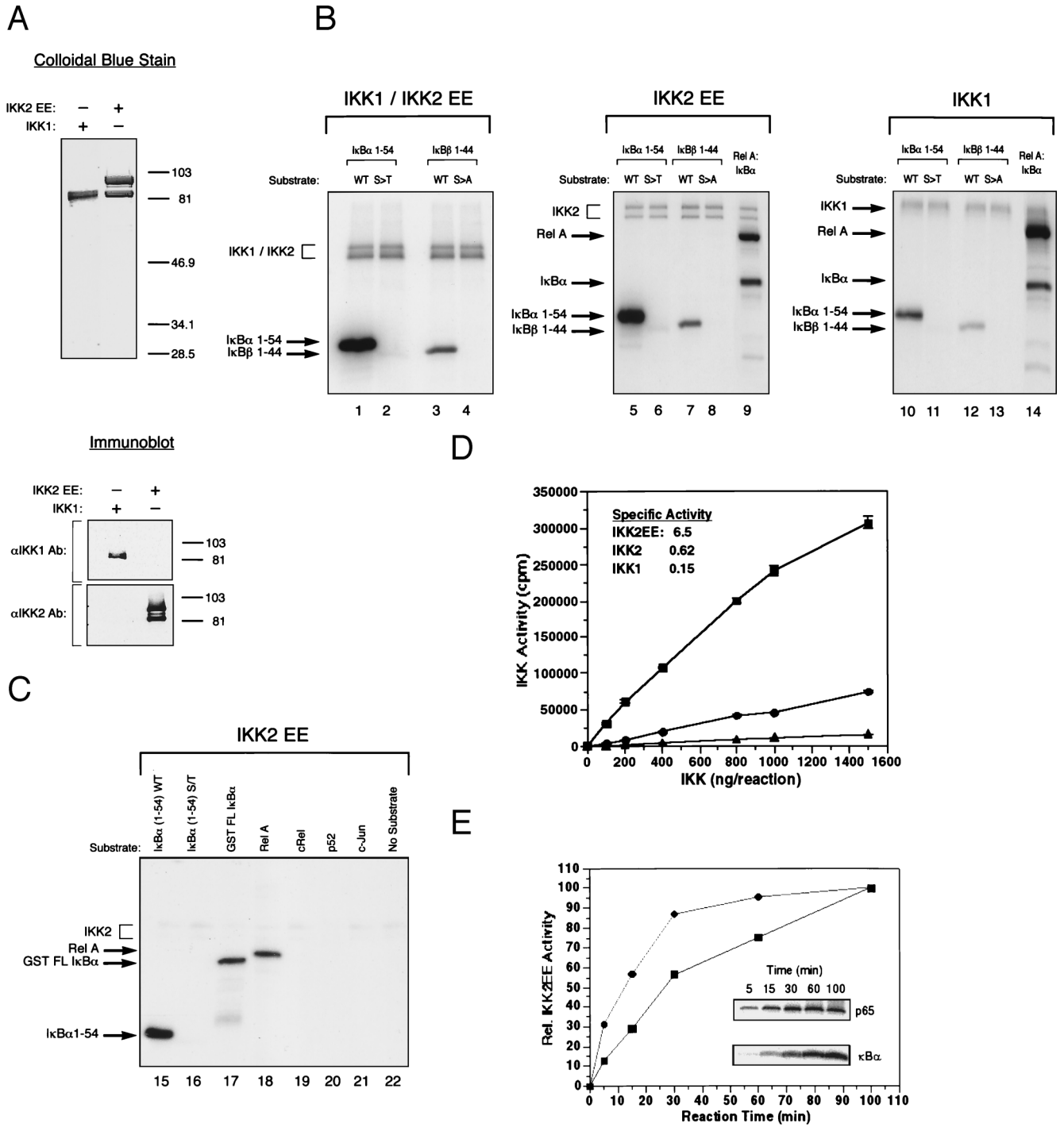

FIG. 1. IKK1 and IKK2 are bona fide IκB kinases. (A). Detection of baculovirus-expressed IKK1 and IKK2EE proteins. Sf9 cells were infected with either IKK1 or IKK2EE baculovirus as indicated. Whole-cell lysate was prepared, and proteins were purified by standard Ni-chelate chromatography, resolved by SDS-PAGE, and visualized with colloidal blue stain (top) or probed in a Western blot analysis with IKK1-specific or IKK2-specific antibodies (bottom). (B) Purified baculovirus-expressed IKK1 and IKK2EE display substrate specificity identical to the IKK signalsome. Purified, baculovirus-expressed IKK1/IKK2EE, IKK2EE, or IKK1 were examined for their ability to phosphorylate wild-type substrates IκBα 1–54 (lanes 1, 5, and 10), IκBβ 1–44 (lanes 3, 7, and 12), and RelA-IκBα complex (lanes 9 and 14). The substrate specificity of the recombinant IKK proteins was also examined against the mutant IκBα and IκBβ substrates in which the serine phosphorylation sites were mutated as follows: IκBα 1–54 S32/36→T (lanes 2 and 6) and IκBβ 1–44 S19/23→A (lanes 4 and 8). (C) Determined substrate specificity of IKK2EE for Rel-related proteins. Purified baculovirus-expressed IKK2EE was examined for its ability to phosphorylate control and Rel-related substrates as indicated. (D) Quantitative comparison of the IκB kinase activity of the recombinant IKK proteins. Titration of IKK1 (▲), IKK2 (●), and IKK2EE (■) is shown. Kinase reactions were performed in a 96-well microplate format with 25 μM ATP and 4.5 μM GST IκBα 1–54 WT (1 h at room temperature in a 0.1-ml volume). The specific activity (nanomoles per minute per milligram) of recombinant IKK was determined as a function of the GST IκBα 1–54 concentration. (E) Time course of the phosphorylation of RelA-IκBα complex by IKK2EE. ^{32}P phosphate incorporation into RelA (●) and IkBα (■) was plotted as a function of the reaction time. Kinase reactions were performed at room temperature for the indicated times. Individual kinase reactions were from a common reaction where 32-μl aliquots were removed at each time point and quenched with SDS-PAGE loading buffer. Kinase reaction mixtures were run on SDS-PAGE, and the gels were dried and subjected to autoradiography (see RelA and IκBα insets as indicated). The mixture at each time point contained 50 ng of IKK2EE, 0.37 μg of p65-IκBα complex, and 3 μM ATP.

TABLE 1. Kinetic analysis of recombinant IKK proteins[a]

Protein	k_{cat} (h^{-1})	K_m (μM) for:			
		IκBα 1–54	IκBα 1–317	IκBβ 1–44	ATP
IKK2	3.3	0.5	ND[b]	1.1	0.56
IKK2EE	33.8	1.1	0.1	3.0	0.63
IKK1	0.78	1.4	ND	2.2	0.63

[a] Kinase reactions were performed with purified baculovirus-expressed IKK proteins in a 96-well microplate format (for 1 h at room temperature in a 0.1-ml volume). In all cases, GST IκBα 1–54 WT was used as the substrate.
[b] ND, not done.

protein to gel filtration chromatography, and examined the distribution of IKK1 and IKK2 in column fractions. In unstimulated cells, IKK1 and IKK2 proteins were detected in the high-molecular-mass fraction of approximately 700 kDa (Fig. 2A, lanes 2 to 4). Of note, a smaller amount of IKK2 not associated with IKK1, was also present as a distinct complex of approximately 300 kDa (lanes 7 and 8). IκB kinase activity was not detected in any of these fractions (data not shown). In TNF-α-stimulated cells, a similar distribution of IKK1 and IKK2 complexes was observed. IκBα kinase activity was associated predominantly with fractions containing the 700-kDa complex. These data demonstrate that at least two pools of IKK2 exist in HeLa cells, a prominent 700-kDa pool and a minor pool of approximately 300-kDa. Because the IKK complexes were first purified by immunoprecipitation with anti-IKK2 antibodies, it is clear that IKK1 associates directly with IKK2 in the 700-kDa complex.

Further fractionation of the high-molecular-mass IKK-containing complexes was performed by anion-exchange chromatography (Fig. 2B). Two distinct pools of IKK2-containing fractions were identified, those containing IKK2 only (Fig. 2B, top panel, lanes 3 to 5) and those containing IKK2 and IKK1 (lanes 6 to 12). The IKK1-IKK2 heterodimeric complexes exhibited higher levels of IκB kinase activity than did complexes containing IKK2 only (bottom panel).

The existence of multiple IKK complexes within cells suggests the possibility for dynamic rearrangements of components of these complexes. To determine if the relative amount of IKK1 associated with IKK2 changes following cell activa-

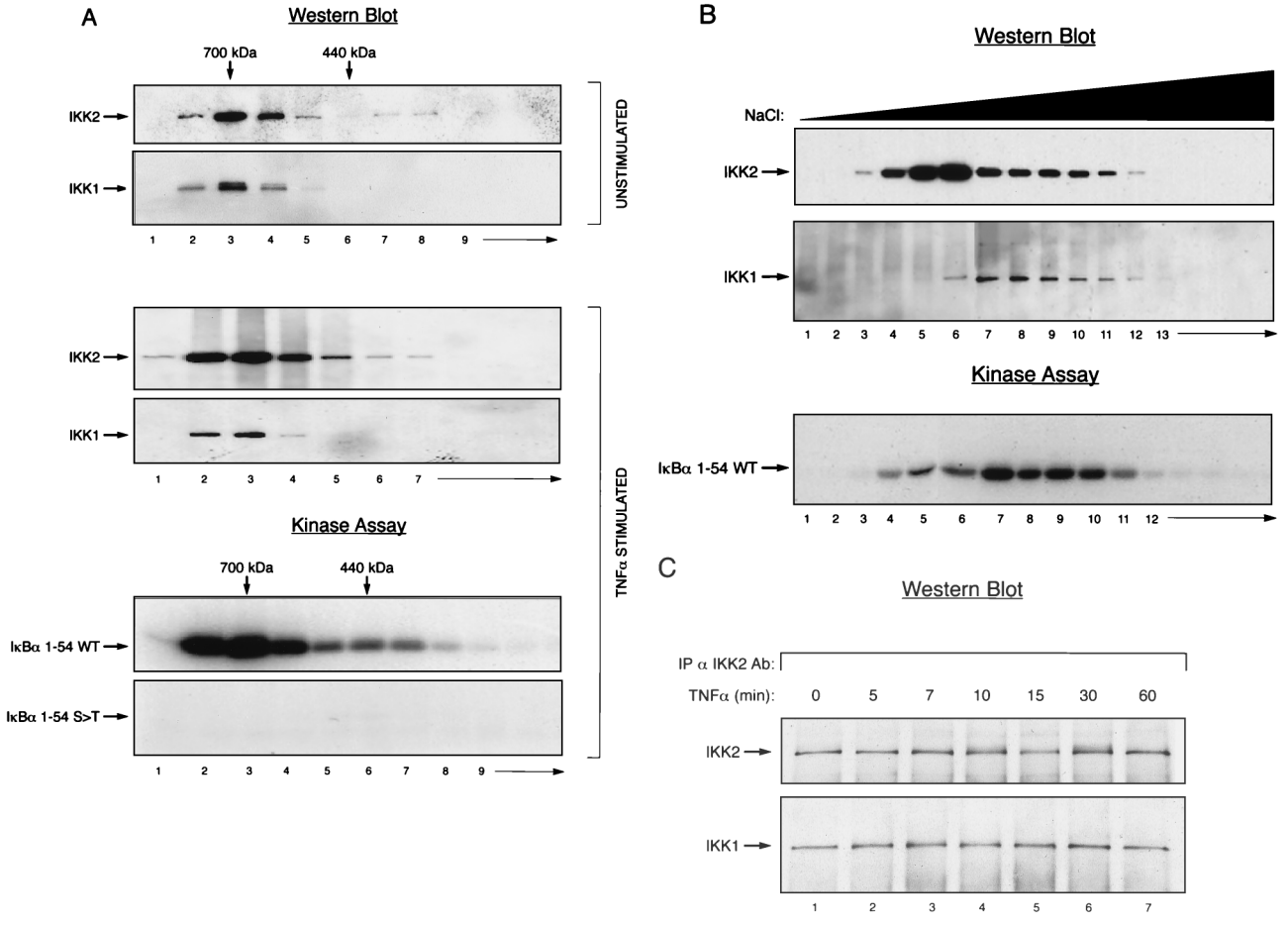

FIG. 2. The IKK signalsome exists as distinct heterogeneous complexes. (A) Preparative-scale immunoprecipitations were performed with whole-cell lysates prepared from HeLa cells that were stimulated with TNF-α or not stimulated, using IKK2-specific antibodies. The immunocomplex was eluted with IKK2-specific peptides and fractionated by gel filtration chromatography. Aliquots from the fractions were analyzed by Western blot analysis with anti-IKK2 and anti-IKK1 antibodies as indicated and for IκBα kinase activity by using GST IκBα 1–54 WT and GST IκBα (S32/36→T) as substrates where indicated. (B) The high-molecular-mass gel filtration fractions corresponding to lanes 1 through 4 of the TNF-α-stimulated lysate were pooled and fractionated further on a Mono Q column. The Mono Q fractions were analyzed by Western blot analysis with anti-IKK2 and anti-IKK1 antibodies and for IκBα kinase activity with GST IκBα WT as indicated. (C) The amount of IKK1 associated with IKK2 is not altered upon cell stimulation. Immunoprecipitations were performed from whole-cell lysates prepared from HeLa cells stimulated with TNF-α or not stimulated, using IKK2-specific antibodies (Ab). The immune complex was divided into two aliquots, and the proteins were resolved on SDS-PAGE and subsequently subjected to Western blot analysis with either anti-IKK2-specific or anti-IKK1-specific antibodies, as indicated.

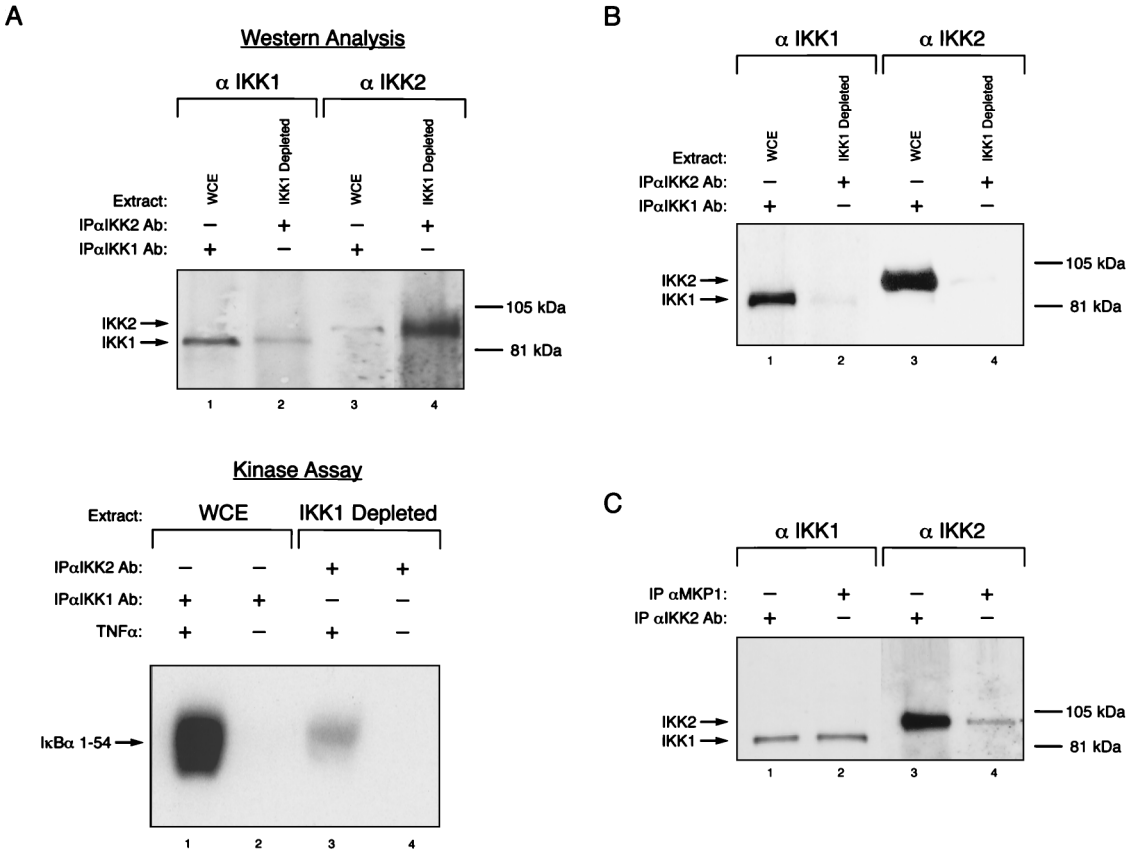

FIG. 3. The IKK signalsome displays cell-type-specific heterogeneity in subunit composition. (A) IKK1 depletion studies further substantiate the existence of distinct IKK-containing pools. (Top) Whole-cell extract (WCE) from TNF-α-stimulated HeLa cells was depleted of IKK1-containing complexes by immunoprecipitation with an excess of anti-IKK1-specific antibodies (Ab) (lanes 1 and 3). The IKK1-depleted supernatant (IKK1 Depleted) was subsequently immunoprecipitated with anti-IKK2 antibody (lanes 2 and 4). Immunoprecipitates were resolved by SDS-PAGE and subjected to Western blot analysis with anti-IKK1-specific and anti-IKK2-specific antibodies, as indicated. (Bottom) Immunoprecipitations were performed with WCEs of HeLa cells that were stimulated with TNF-α (lanes 1 and 3) or not stimulated (lanes 2 and 4) as described for the top panel. The immunoprecipitates were then analyzed for IκBα kinase activity with GST IκBα 1–54 as the substrate. (B) SLB cells do not contain detectable levels of the IKK2 homodimeric complex. Whole-cell extracts were prepared from unstimulated SLB cells and analyzed for the presence of IKK1-IKK2 and IKK2-only complexes exactly as described for panel A above. (C) The IKK signalsome component recognized by the anti-MKP antibody is specific for the IKK1-IKK2 heterodimeric complex. Immunoprecipitations were performed from whole-cell extracts prepared from TNF-α-induced HeLa cells by using either anti-MKP1 (lanes 2 and 4) or anti-IKK2-specific (lanes 1 and 3) antibodies. The immunoprecipitates were resolved by SDS-PAGE and subjected to Western blot analysis with either IKK1-specific or IKK2-specific antibodies, as indicated.

tion, we used Western blot analysis to examine IKK1 and IKK2 levels following immunoprecipitation of TNF-α-stimulated HeLa cell extracts by using specific anti-IKK2 antibodies (Fig. 2D). This analysis revealed no apparent stimulation- or time-dependent changes in the association of IKK1 with IKK2. IKK1-IKK2 heterodimeric complexes therefore appear to be relatively stable in vivo.

To further explore the existence of distinct IKK complexes, we used an alternative approach to analyze the presence and composition of distinct IKK complexes in HeLa cells. Using sequential immunoprecipitation with IKK1- and IKK2-specific antibodies, we identified a pool of IKK2 not associated with IKK1 (Fig. 3A). Whole-cell extracts were subjected to immunoprecipitation with IKK1-specific antibodies, and the remaining proteins were then subjected to immunoprecipitation with IKK2-specific antibodies. Immunoprecipitated proteins were analyzed by Western blotting for IKK1 and IKK2 protein. IKK1 immunoprecipitates contained both IKK1 and IKK2 protein (Fig. 3A, top panel, lanes 1 and 3). IKK2 immunoprecipitates prepared from anti-IKK1 immunodepleted extracts contained high levels of IKK2 and very little IKK1 (top panel, lanes 2 and 4). The relative ratios of IKK1 to IKK2 were dramatically different between the two fractions. We examined the relative kinase activity associated with each pool of IKK complex (Fig. 3A, bottom panel). The majority of the IκB kinase activity was associated with the IKK1-containing pool (lanes 1 and 2), and significantly less was associated with the IKK2-only-containing pool (lanes 3 and 4). To determine if similar IKK complexes are present in other cell types, we performed similar immunodepletion experiments in the SLB cell line (Fig. 3B). In contrast to HeLa cells, IKK1 immunoprecipitates contained high levels of IKK2 and immunoprecipitation with IKK2-specific antibodies did not identify a second, IKK2-only pool.

To further delineate the composition of IKK1-IKK2 and IKK2-only complexes in HeLa cells, we examined the ability of an anti-MKP-1 antibody, which was previously demonstrated to be capable of binding and purifying an IKK1-IKK2 heterodimeric complex, to bind the respective IKK complexes (30). Equal amounts of whole-cell extract from TNF-α-induced HeLa cells were subjected to immunoprecipitation with either anti-MKP-1 or anti-IKK2 antibodies. The respective immunoprecipitates were subjected to SDS-PAGE and Western blot analysis for the presence of IKK1 and IKK2 proteins

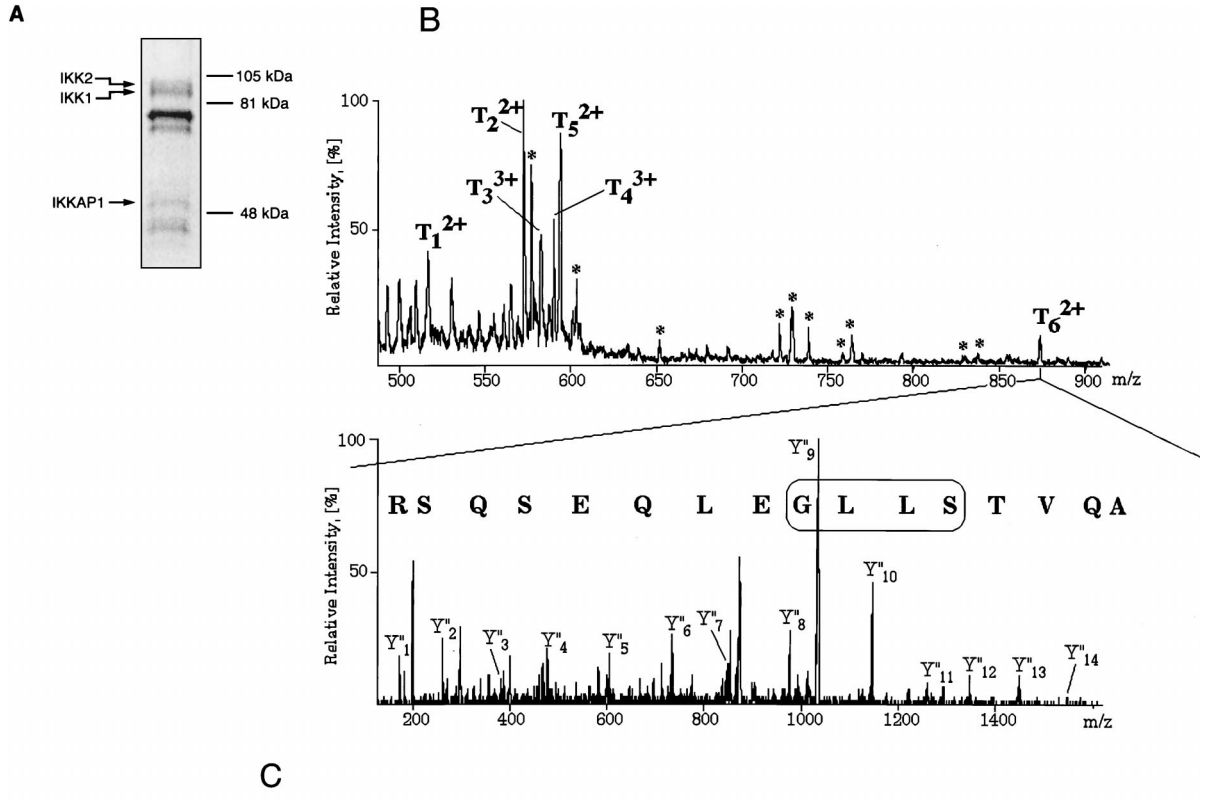

FIG. 4. Identification of IKKAP1, a novel component of the IKK signalsome. (A) Whole-cell extract was prepared from TNF-α-stimulated (20 ng/ml, 7 min induction) HeLa cells (1.4 g of total protein. The IKK signalsome was then immunoprecipitated from the HeLa extract with anti-MKP-1 antibodies and eluted overnight at 4°C in the presence of excess MKP1-specific peptide. Eluted IKK signalsome was then fractionated on a Mono Q column, IκB kinase-active fractions were pooled, concentrated, and subjected to preparative SDS-PAGE, and the protein bands were visualized by colloidal blue staining. Individual protein bands were excised and submitted for peptide sequencing. The identities of the respective protein bands, as determined by sequence analysis (see below), are indicated to the left of the figure. (B) Sequencing of IKKAP1 by nanospray mass spectrometry. (Top) Part of the spectrum of unseparated in-gel tryptic digest of IKKAP1. The peptide ions designated by T were in turn isolated by the first quadruple mass analyzer of a quadruple instrument and fragmented in the collision cell, and their tandem mass spectra were acquired. Peaks designated by asterisks belong to trypsin autolysis products. (Bottom) Tandem mass spectrum of the doubly charged ion T6 with *m/z* 873.4. A continuous series of C-terminal-containing fragments (Y″ ions) were used to construct a peptide sequence tag. In the region of the spectrum above the parent ion, a short stretch of sequence could be deduced unambiguously (boxed). This sequence stretch, combined with the peptide molecular mass of 1,744.8 and the masses of the correspondent Y″ ions, were assembled in a peptide sequence tag (977.2)glls(1,347.2). Searching of the comprehensive database did not produce any hits. However, search of an EST database identified the peptide sequence AQVTSLLGELQESQSR in several EST clones. After the full-length sequence of IKKAP1 was obtained, other peptide ions, (T1 to T5 in the top panel) were matched to the corresponding tryptic peptides. (C) Amino acid comparison of human and mouse IKKAP1. The initial peptide sequence identified by nanoelectrospray mass spectrometry is underlined; the leucine zipper motif is boxed; amino acid identities not conserved between human and mouse IKKAP1 are shown by boldface type; gaps inserted to optimize alignment are indicated by dashes.

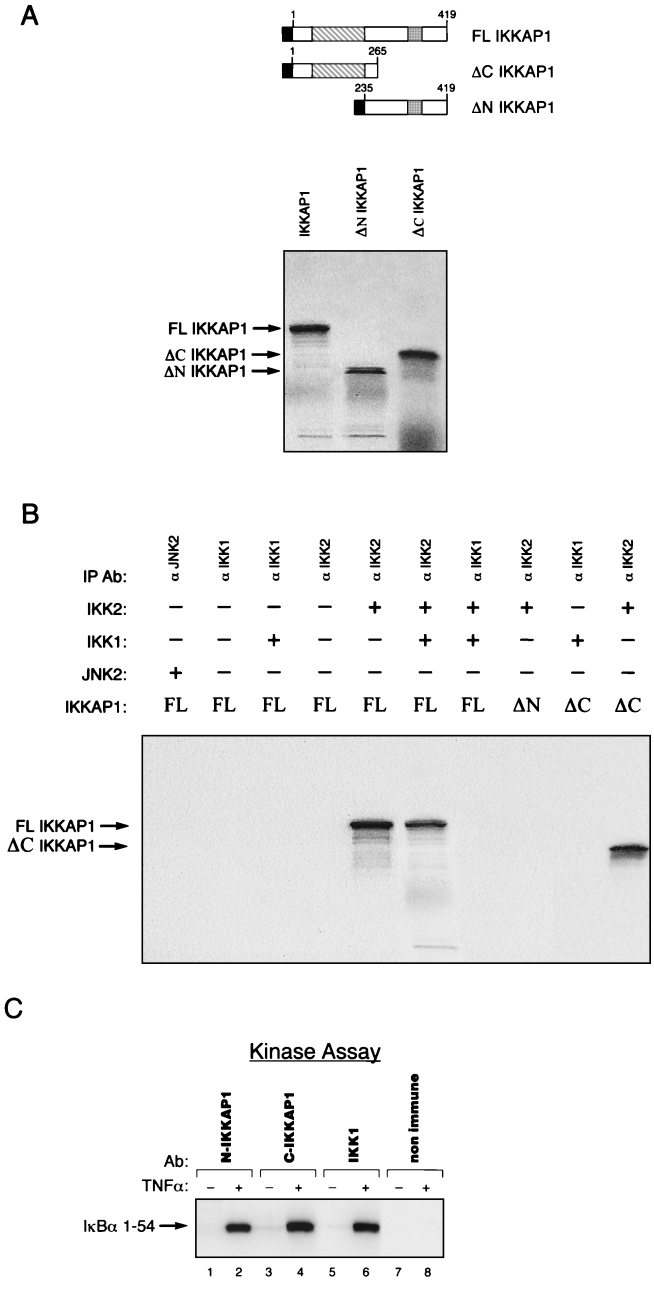

FIG. 5. IKKAP1 interacts specifically with IKK2. (A) A schematic representation of the various IKKAP1 proteins that were translated in vitro for examination in the IKK association studies is shown at the top. Symbols: solid box, GluGlu epitope tag; checked box, leucine zipper motif; diagonal stripes, coiled-coil repeat domain. Below this diagram is an autoradiograph obtained after all three IKKAP1 proteins were translated in vitro in the presence of [^{35}S]methionine using wheat germ extract. The proteins were resolved by SDS-PAGE, the gel was dried, and the IKKAP1 proteins were visualized by autoradiography. The IKKAP1 products are indicated to the left. (B) In vitro-translated IKKAP1 interacts with recombinant IKK2. [^{35}S]methionine labeled IKKAP1 proteins were mixed with IKK1, IKK2, or JNK, as indicated (37°C for 30 min). IKK1, IKK2, and JNK were then immunoprecipitated with specific antibodies (Ab) as indicated. The immune complex was washed extensively and resolved by SDS-PAGE, and the gel was dried and subjected to autoradiography to monitor association with the respective IKKAP1 protein. (C) Immunoprecipitation of endogenous IKKAP1 brings down TNF-α-induced IκBα kinase activity. Immunoprecipitations were performed with whole-cell extracts of HeLa cells that were stimulated with TNF-α or not stimulated, using antibodies directed against peptides derived from either the N or C terminus of IKKAP1. Additional immunoprecipitations were included as controls with anti-IKK1 antibodies or nonimmune sera. The immunoprecipitates were then analyzed for IκBα kinase activity with GST IκBα 1–54 as the substrate.

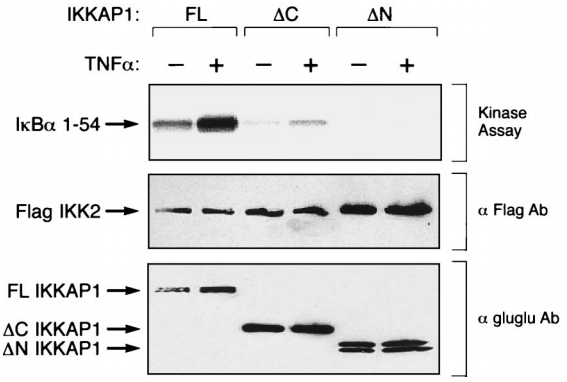

FIG. 6. Mutant versions of IKKAP1 block TNF-α-induced activation of IKK2 in vivo. HeLa cells were cotransfected with Flag-tagged IKK2 and either GluGlu-tagged FL (full length), ΔC, or ΔN IKKAP1 expression vectors. Immunoprecipitations with anti-Flag antibodies were performed with whole-cell lysates of the transfected HeLa cells, as indicated at the top of the figure. The immunoprecipitates were then analyzed for IκBα kinase activity with GST IκBα 1–54 as the substrate (top). Whole-cell lysates were subjected to Western blot analysis to determine the level of expression of Flag-IKK2 (middle) and GluGlu-IKKAP1 proteins (bottom). The proteins are indicated to the left of the figure, and the antibody (Ab) used for Western analysis is indicated to the right.

(Fig. 3C). Immunoprecipitates using the IKK2-specific antibody contained IKK1 and IKK2 protein; however, there were significantly greater amounts of IKK2, consistent with the presence of IKK1-IKK2 heterodimers and IKK2 homodimers in HeLa cells. Immunoprecipitates obtained with the MKP-1 antibody contained an amount of IKK1 equal to that observed with the IKK2 antibody. In contrast, the level of IKK2 was dramatically lower than that for anti-IKK2 immunoprecipitation, suggesting that this antibody recognizes only IKK1-IKK2 heterodimers.

Cloning of IKKAP1, a novel component of the IKK signalsome. In an attempt to identify additional components of the IKK complexes, proteins from whole-cell lysates of TNF-α-induced HeLa cells were immunoprecipitated with anti-MKP-1 antibodies, thus isolating the IKK1-IKK2 heterodimeric pool. The protein complex was eluted with an MKP-1 peptide and fractionated further by anion-exchange chromatography as described previously (30). Fractions displaying IκB kinase activity were pooled and subjected to preparative SDS-PAGE (Fig. 4A). Protein bands were excised, digested with trypsin, and analyzed by high-mass-accuracy matrix-assisted laser deposition and ionization (MALDI) peptide mass mapping (see Materials and Methods). As expected, protein species of 85 and 87 kDa were identified as IKK1 and IKK2, respectively. A single peptide sequence was obtained from a protein species of approximately 50 kDa (Fig. 4B). This peptide was found to be an identical match to several mouse and human ESTs. Multiple cDNA clones were isolated from a human cDNA library by using probes generated from the human EST clones, and the deduced polypeptide sequences were determined (Fig. 4C). A single open reading frame of 1,257 bp was identified, which encoded a protein of 419 amino acids. The cDNA sequence encoded an initiation codon matching Kozak's rule, and we therefore predict that this is the N terminus of the polypeptide. The murine EST cDNA that encoded a polypeptide with high identity to that of the human sequences was obtained; however, the cDNA clone lacked the first 68 amino acids identified in the human clones. The protein encoded by these cDNAs was named IKKAP1 (IKK-associated protein 1). IKKAP1 sequence matched NEMO, which was recently identified based on its ability to complement an

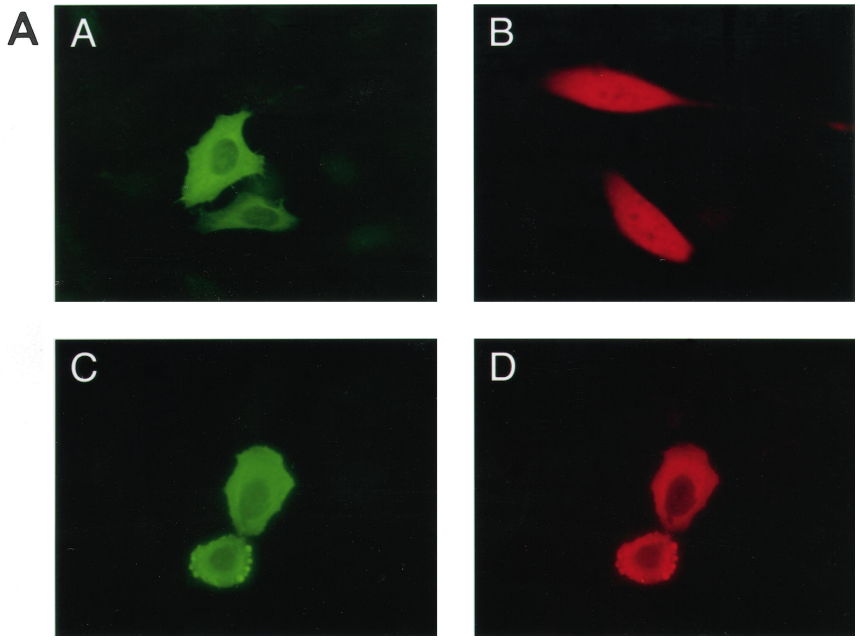

FIG. 7. IKKAP1 mediates an essential step in the NF-κB activation pathway. (A) IKKAP1 localizes to the cytoplasm by virtue of its association with IKK2. HeLa cells were transiently transfected alone, with IKK2 (panel A) or GluGlu-tagged IKKAP1 (panel B) or with the two in combination (panels C and D). At 36 h after transfection, the cells were subjected to immunocytochemical analysis. IKK2 and GluGlu-tagged IKKAP1 were visualized by fluorescein isothiocyanate-conjugated (panels A and C) and Texas red-conjugated (panels B and D) secondary antibodies, respectively. (B) Overexpression of mutant versions of IKKAP1 blocks stimulus-dependent RelA nuclear translocation. HeLa cells were transiently transfected with GluGlu-tagged ΔN IKKAP1 (panels A to D) or ΔC IKKAP1 (panels E to H). At 36 h after transfection, the cells were subjected to immunocytochemical analysis. Endogenous IKK2 (panels A, C, E, and G) and GluGlu-tagged ΔN IKKAP1 (panels B and D) and ΔC IKKAP1 (panels F and H) were visualized by fluorescein isothiocyanate- and Texas red-conjugated secondary antibodies, respectively.

NF-κB activation-deficient cell line (41). IKKAP1 contains several recognizable protein motifs, including a carboxy-terminal leucine zipper motif and several N-terminal coiled-coil repeat motifs known to function in protein-protein interactions.

IKKAP1 interacts directly with IKK2. IKKAP1 was purified based upon its association with IKK1-IKK2 heterodimer complexes. To determine whether IKKAP1 interacts directly with IKK1 or IKK2, recombinant IKK1 or IKK2 was incubated with [^{35}S]methionine-labeled IKKAP1, which was produced by in vitro transcription and translation (Fig. 5A). The resulting complexes were analyzed by immunoprecipitation with IKK-specific affinity-purified antibodies. Immunoprecipitated complexes were washed extensively and subjected to SDS-PAGE analysis (Fig. 5B). Interestingly, IKKAP1 was found to interact with IKK2 but not IKK1. As expected, IKKAP1 did not associate with JNK2. We then addressed whether IKK1 and IKK2 homodimers, when in the presence of IKKAP1, undergo subunit exchange or higher-order complex formation. To this end, equal amounts of IKK1 and IKK2 protein were mixed along with [^{35}S]methionine-labeled IKKAP1 and subsequently immunoprecipitated, as described above, with either IKK1- or IKK2-specific antibodies. IKK2-specific antibodies, but not IKK1-specific antibodies, were capable of immunoprecipitating IKKAP1, further demonstrating the stability of the IKK dimerization interaction. We then examined whether the IKKAP1 N-terminal coiled-coil repeat domain, ΔC IKKAP1, or the C-terminal leucine zipper domain, ΔN IKKAP1, was sufficient to mediate IKK2 interaction (Fig. 5B). Expression vectors encoding the N-terminal domain, ΔC IKKAP1, or the C-terminal domain, ΔN IKKAP1, of IKKAP1 were tested in the IKK2 association assay. ΔC IKKAP1, but not ΔN IKKAP1, was capable of stable complex formation with IKK2. Therefore, it is likely that IKKAP1 associates with the IKK complex through its ability to specifically bind IKK2 via the N-terminal coiled-coil repeat domain. To determine if IKKAP1 is also a component of the IKK2 homodimer complex in HeLa cells, we purified this complex and identified a protein species of 50 kDa, which upon analysis by nanoelectrospray mass spectrometry was identified as IKKAP1 (data not shown). IKKAP1 is therefore a common component of both IKK complexes in HeLa cells. To further demonstrate that IKKAP1 is a bona fide component of the IKK signalsome, we sought to immunoprecipitate IκB kinase activity with antibodies to endogenous IKKAP1 (Fig. 5C). Immunoprecipitations were performed from whole-cell lysates of HeLa cells that were stimulated with TNF-α or not, using antibodies directed against peptides derived from either the N- or C-terminal region of IKKAP1. As expected, anti-IKKAP1 and anti-IKK1 immunoprecipitates contained similar levels of stimulus-dependent IκBα kinase activity. Immunoprecipitates with nonimmune sera contained no detectable IκBα kinase activity.

IKKAP1 mediates an essential step in the NF-κB activation pathway. Because IKKAP1 does not possess any motif associated with enzymatic function, it is unclear what role this protein may play in NF-κB activation. We postulated that through its ability to associate with IKK2, IKKAP1 may influence IKK2 subcellular localization, association with other IKK signalsome components, interaction with upstream activators or recruitment of the IκB substrate. To examine the effect of wild-type and mutant versions of IKKAP1 on stimulus-dependent IKK activation, HeLa cells were cotransfected with Flag-tagged IKK2 and either FL, ΔN, or ΔC GluGlu-tagged IKKAP1 expression vectors (Fig. 6). Whole-cell lysates of cells treated with TNF-α or not treated were immunoprecipitated with anti-Flag antibodies and subsequently assayed for IKK activity. We

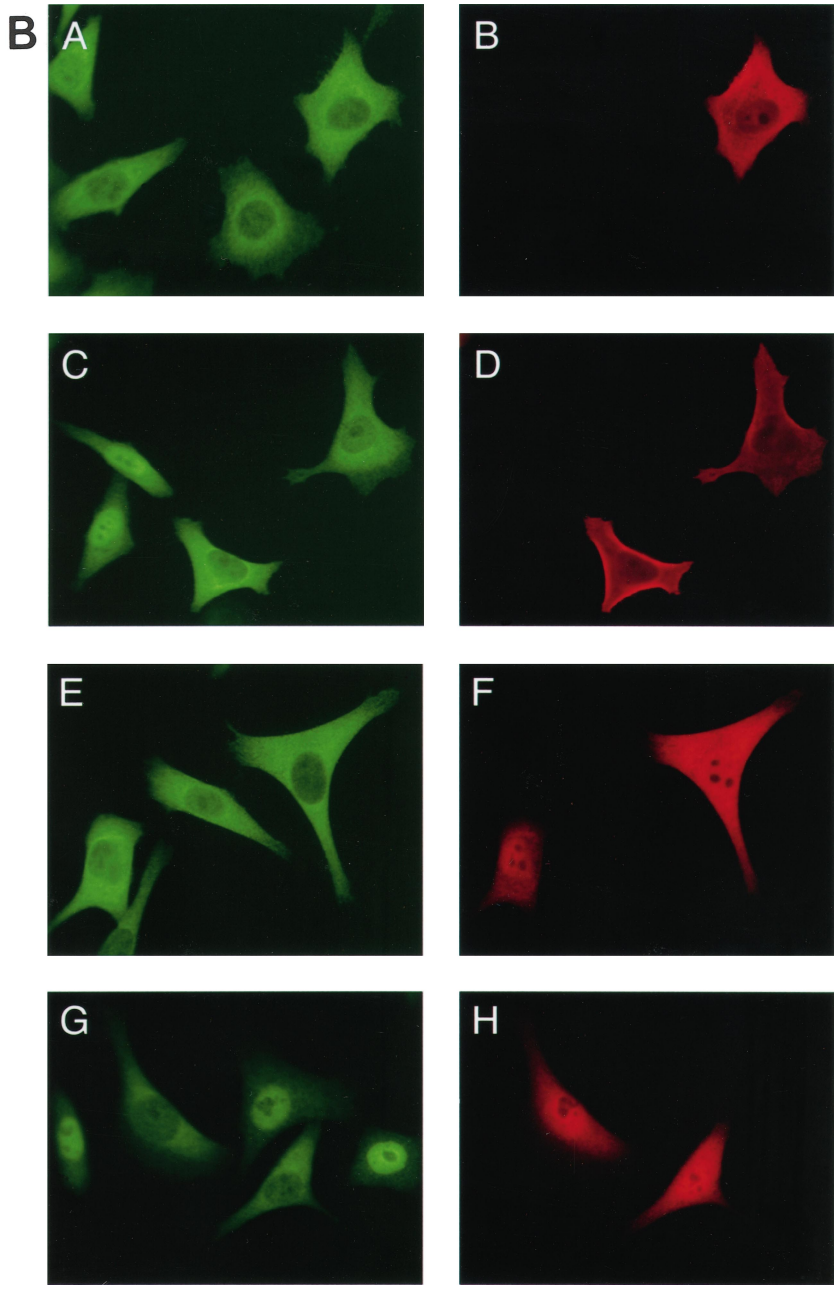

FIG. 7—*Continued.*

observed strong stimulus-dependent IkBα kinase activity in the presence of FL IKKAP1. In contrast, both ΔN IKKAP1 and ΔC IKKAP1 potently inhibited IKK2 activation. The relative levels of expression of Flag-IKK2 or GluGlu-IKKAP1 proteins were comparable (Fig. 6, lower panels as indicated). Interestingly, although ΔN IKKAP1 retains no IKK2 binding properties it still functions as a potent inhibitor of IKK2 activation.

These results suggest that the N- and C-terminal domains of IKKAP1 mediate distinct, essential, regulatory events. The N-terminal coiled-coil domain would be predicted to localize IKKAP1 to IKK2, whereas the C-terminal domain may mediate interaction with upstream components of the NF-κB activation cascade. We used immunocytochemical techniques to explore the effect of IKK2 and IKKAP1 overexpression on their respective subcellular localization. Consistent with previous observations, IKK2, when transiently expressed in HeLa cells, was localized exclusively to the cytoplasm (Fig. 7A, panel A). Transfection of cells with IKKAP1 resulted in localization of IKKAP1 in both the cytoplasm and nuclear compartments (panel B). To determine whether overexpression of IKKAP1 affected IKK2 localization, HeLa cells were transiently transfected with Flag-tagged IKK2 and GluGlu-tagged IKKAP1. Immunocytochemical analysis revealed that IKKAP1 expression had no effect on IKK2 subcellular localization (panels C and D). Rather, we observed that IKK2 expression dramatically altered the subcellular localization of IKKAP1, excluding IKKAP1 from the nucleus and colocalizing with IKK2 in the cytoplasm. Hence, consistent with the results of in vitro binding

experiments, it appears that IKK2 functions to directly bind IKKAP1 and localize it to the cytoplasm.

Since ΔC IKKAP1 and ΔN IKKAP1 blocked TNF-α-induced activation of IKK2 (Fig. 5), we would expect these mutant versions of IKKAP1 to have a profound effect on stimulus-induced NF-κB nuclear translocation. Immunocytochemical studies were performed to determine whether overexpression of IKKAP1 mutants could block stimulus-dependent RelA nuclear translocation. GluGlu-tagged ΔN IKKAP1 or ΔC IKKAP1 was transiently transfected in HeLa cells that were stimulated with TNF-α or not stimulated, and the subcellular localization of endogenous RelA was monitored (Fig. 7B). Neither ΔN IKKAP1 nor ΔC IKKAP1 had any effect on the subcellular localization of RelA in unstimulated HeLa cells (Fig. 7B, panels A and B and panels E and F, respectively). In contrast, we observed a potent inhibition of TNF-α-induced RelA nuclear translocation upon overexpression of ΔN IKKAP1 and ΔC IKKAP1 (panels C and D and panels G and H, respectively). These results strongly suggest that IKKAP1 mediates an essential step in the NF-κB activation pathway.

DISCUSSION

In this study, we demonstrated that IKK1 and IKK2 represent bona fide IκB kinases and that distinct IKK complexes composed of different proteins exist in vivo. In addition, we have purified and cloned a novel component of these complexes which specifically interacts with IKK2 and participates in NF-κB activation.

Although IKK1 and IKK2 were identified as kinase components of the IKK signalsome, formal confirmation of their identities as bona fide IκB kinases is complicated by the fact that functional analysis was performed by transfection experiments in mammalian cells. It is possible that overexpressed proteins associate with other cellular proteins which themselves represent the authentic IκB kinase. For this reason, we expressed and purified IKK1 and IKK2 by using a baculovirus expression system and analyzed in detail the protein species obtained and the kinase activities associated with them. We also expressed a mutant form of IKK2 (IKK2EE) in which two serine residues contained within the MEKK-related activation loop were mutated to glutamic acid. In our previous studies, we reported that this mutant displayed constitutive kinase activity and was capable of inducing NF-κB translocation to the nucleus of transfected HeLa cells in the absence of any other stimuli (30). We sought to determine if this mutation truly resulted in elevated levels of IκB kinase activity. Purified recombinant IKK1 and IKK2, expressed alone or together, associated as dimers in the absence of other proteins and exhibited IκB kinase activity with similar selectivity and kinetic parameters to those found from analysis of the endogenous IKK signalsome (30). Detailed kinetic analysis revealed that both IKK1 and IKK2 display a preference for IκBα over IκBβ as a substrate. In addition, IKK2 showed a marked preference for phosphorylation of full-length IκBα compared to the truncated form, IκBα 1–54. Further support for this finding was provided by Burke et al., who demonstrated that a peptide corresponding to the C-terminal region of IκBα enhanced IKK signalsome phosphorylation of a peptide containing Ser32 and Ser36 (10). In this study, the K_m for IκBα 1–317 was similar to that determined for recombinant IKK2EE. The K_ms of IKK2EE for free IκBα compared to that for IκBα in the context of a RelA-IκBα complex were also similar. We did not observe any significant difference in substrate selectivity for each of the IKK dimers formed, either IKK1-IKK2 heterodimers or IKK1 or IKK2 homodimers. Complexes containing the IKK2EE mutant consistently displayed greater levels of kinase activity, confirming a key role for the activation-loop serines in regulation of IKK activity. Based upon these characteristics, we conclude that IKK1 and IKK2 are bona fide IκB kinases and that full kinase activity can be reconstituted in vitro without the requirement for additional proteins.

In addition, recombinant IKK1 and IKK2 exhibited strong RelA-phosphorylating activity, again consistent with previous results demonstrating stimulus-dependent phosphorylation of IκBα and RelA by the endogenous IKK signalsome (30). The residues of RelA targeted for phosphorylation by the IKKs are unknown, as is the potential physiologic role of this event. The level of RelA kinase activity associated with IKK1 and IKK2 is comparable to that observed for IκBα as determined by detailed kinetic analysis. These findings suggest that IKK-mediated RelA phosphorylation may play a physiologic role. Moreover, the IKKs do not appear to be general kinases for all Rel-related proteins in that they do not phosphorylate cRel or NF-κB p52. We are currently identifying the sites on RelA which are phosphorylated by the IKKs. Recently, inducible phosphorylation of RelA was demonstrated to be mediated by the catalytic subunit of protein kinase A, and this phosphorylation enhanced the transactivating potential of RelA-containing complexes (44). In addition, RelA was found to undergo TNF-α-induced phosphorylation on Ser529 (36). The relationship of these events, if any, to that mediated by IKK1 and IKK2 is under investigation.

IKK1 and IKK2 can form homo- and heterodimers in vitro (16, 30, 31, 40, 43), and our finding of similar complexes in vivo is consistent with these kinases being able to variably associate. Whereas the HeLa cell line used in these studies contained both the IKK1-IKK2 heterodimer and the IKK2 homodimer, SLB cells contained only the IKK1-IKK2 heterodimer. Therefore, mechanisms must exist for the regulated assembly of the IKK complexes in different cells. The mechanism which regulates complex assembly remains unclear. Perhaps the relative levels of IKK1 and IKK2 expression dictate complex formation. Alternatively, IKK-associated proteins could influence the nature of complex formation, whereby selective protein-protein interactions facilitate the assembly of specific complexes. Interestingly, IKK1-IKK2 and IKK2-only complexes are subject to distinct modes of activation in that they display markedly different levels of activation in response to TNF-α treatment. The IKK1-IKK2 heterodimeric complex was potently activated by TNF-α, in contrast to the IKK2 homodimeric complex, which exhibited only a modest increase in activation. There may be physiologic conditions that preferentially activate the IKK2 homodimer. We did not observe any change in the composition or relative amounts of IKK1-IKK2 heterodimer in stimulated cells, suggesting that a dramatic reorganization of these complexes does not occur upon cellular activation. However, we cannot discount the possibility that other components of these complexes are dynamically regulated and affect IKK function upon cellular activation.

In an effort to better understand IKK regulation, studies were initiated to further elucidate the subunits comprising the respective IKK complexes. The IKK signalsome was originally purified with an anti-MKP-1 antibody; however, we were unable to identify MKP-1 as a component of the IKK complex, either by direct sequence determination or by using a panel of antibodies recognizing distinct epitopes on MKP-1. The identity of the MKP-1 epitope remains elusive, although we have been able to exclude IKK1, IKK2, and IKKAP1 as candidates. We determined that this antibody specifically immunoprecipitates the IKK1-IKK2 heterodimer complex but not the IKK2 homodimer complex. This finding suggests the presence of a

protein in the IKK1-IKK2 complex that is not present in the IKK2-homodimeric complex. Studies to identify the IKK signalsome component that is recognized by the MKP-1 antibody are under way. In contrast, by virtue of its ability to bind IKK2, IKKAP1 was identified as a component of both the IKK1-IKK2 heterodimeric and IKK2 homodimeric complex in cells. IKKAP1 associates with IKK2 in vitro and in vivo via sequences contained within the N-terminal coiled-coil repeat region of IKKAP1. IKK2 binding studies established that the IKK2 binding domain of IKKAP1 resides within amino acids 68 through 235. In HeLa cells, transient overexpression of either the IKKAP1 N-terminal (ΔC IKKAP1) or C-terminal (ΔN IKKAP1) domain potently inhibited both IKK2 activation and RelA nuclear localization. These studies suggest that the C- and N-terminal domains of IKKAP1 play distinct and essential roles in IKK activation.

Yamaoka et al. recently described the identification of NEMO (NF-κB essential modulator) via genetic complementation studies of cells unresponsive to NF-κB activating stimuli (41). NEMO is essential for activation of the NF-κB activation pathway. We report independent data showing the biochemical purification and cloning of a novel component of the IKK signalsome, IKKAP1, which is the human homolog of murine NEMO. Blast search analysis of the available gene databases identified two additional proteins related to IKKAP1: FIP-2, which displays significant sequence similarity to IKKAP1, and FIP-3, which is identical to IKKAP1 (24, 26). FIP-2 and FIP-3 were identified as E3 14.7-kDa interacting proteins, which are adenovirus proteins encoded by the early transcription region 3 (E3) and function to inhibit the cytolytic effects of TNF-α (25, 26). Interestingly, FIP-3 (IKKAP1/NEMO) associates with components of the TNF-α receptor complex including RIP (25, 26). Our immunocytochemical studies provide an intriguing observation where ΔN IKKAP1 displays stimulus-dependent subcellular localization to the cell membrane, perhaps mediated by direct association with the TNF-α receptor complex. We postulate that IKKAP1 provides a scaffold upon which IKK2-containing complexes could be localized to the upstream components of the NF-κB activation cascade. Indeed, JIP-1 (JNK-interacting protein 1) was recently demonstrated to function as a mammalian scaffold protein for the JNK signaling pathway. JIP-1 binds specific upstream components of the JNK pathway and facilitates signal transduction mediated by the bound proteins (38). JIP-1 is highly selective for a given MAP kinase module, namely, MLK, MKK7, and JNK. This suggests that different scaffold proteins facilitate activation of JNK mediated by other MAP kinase modules. IKKAP1/FIP-3 and FIP-2 may play a similar role in the activation of NF-κB by diverse upstream signaling cascades.

The studies described herein begin to address issues regarding the functional divergence of IKK1 and IKK2. A preference for TNF-α-induced activation of IKK1-IKK2 heterodimers relative to IKK2 homodimers suggests that either IKK1 or IKK1-specific associated proteins are required for full activation of the IKK complex. Conversely, IKKAP1-mediated interaction with upstream activators can be achieved only if IKK2 is present. Thus, the IKK signalsome, by virtue of the functional diversity of IKK1 and IKK2 and their respective associated proteins, provides the potential to integrate the diverse array of signaling pathways known to activate NF-κB in different cell types.

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