The IκB Kinase (IKK) Complex Is Tripartite and Contains IKKγ but Not IKKAP as a Regular Component*

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Daniel Krappmann‡, Eunice N. Hatada‡‡, Sebastian Tegethoff‡, Jun Li†, Anke Klippel¹, Klaus Giese¶, Patrick A. Bauerle∗∗*, and Claus Scheidereit‡ ‡‡

From the *Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany, the †Department of Biology, Boehringer Ingelheim Pharmaceuticals, Ridgefield, Connecticut 06877-0368; ‡‡Åugen AG, Robert-Rössle-Str. 10, 13125 Berlin, Germany, and **Micromet GmbH, Am Klopferspitz 19, 82152 Martinsried, Germany

A critical step in the activation of NF-κB is the phosphorylation of IκBs by the IκB kinase (IKK) complex. IKKα and IKKβ are the two catalytic subunits of the IKK complex and two additional molecules, IKKγ/NEMO and IKKAP, have been described as further integral members. We have analyzed the function of both proteins for IKK complex composition and NF-κB signaling. IKAP and IKKγ belong to distinct cellular complexes. Quantitative association of IKKγ was observed with IKKα and IKKβ. In contrast IKAP was complexed with several distinct polypeptides. Overexpression of either IKKγ or IKAP blocked tumor necrosis factor α induction of an NF-κB-dependent reporter construct, but IKAP in addition affected several NF-κB-independent promoters. Whereas specific down-regulation of IKKγ protein levels by antisense oligonucleotides significantly reduced cytokine-mediated activation of the IKK complex and subsequent NF-κB activation, a similar reduction of IKAP protein levels had no effect on NF-κB signaling. Using solely IKKα, IKKβ, and IKKγ, we could reconstitute a complex whose apparent molecular weight is comparable to that of the endogenous IKK complex. We conclude that while IKKγ is a stoichiometric component of the IKK complex, obligatory for NF-κB signaling, IKAP is not associated with IκKs and plays no specific role in cytokine-induced NF-κB activation.

NF-κB transcription factors play a pivotal role in many cellular processes such as inflammation, immune response, cell proliferation, and apoptosis (1–5). The prototype of the NF-κB family is a heterodimer of the p50 and p65 (RelA) subunits. IκB proteins (IκBα, IκBβ, IκBε, p105, and p100) retain NF-κB in an inactive form in the cytoplasm. A conserved ankyrin repeat domain in these inhibitors masks nuclear translocation signals contained in the Rel homology domain of NF-κB.

In response to multiple stimuli, including TNFα,¹ IL-1β, phorbol ester, and lipopolysaccharides, NF-κB is liberated from IκB molecules and translocates to the nucleus (6). This critical step of NF-κB activation is initiated by phosphorylation of IκB proteins at conserved amino-terminal serine residues, e.g. at serines 32 and 36 of IκBα or serines 19 and 23 of IκBβ. Phosphorylated IκBα are bound by a βTrCP containing ubiquitin ligase (E3) complex, polyubiquitinated and subsequently degraded by the 26 S proteasome (7).

Most NF-κB-inducing stimuli trigger activation of an IκB kinase (IKK) complex with a high apparent molecular mass of 700–900 kDa (8, 9), which has specificity for the amino-terminal phosphoacceptor sites in IκBα or -β. The kinase complex contains two catalytic subunits termed IKKα (IKK1) and IKKβ (IKK2) (8, 10–13). IKKα and IKKβ are related molecules of 85 and 87 kDa, respectively, with an overall identity of about 44%. Both contain an NHa-terminal kinase domain, a leucine zipper and a COOH-terminal helix-loop-helix motif. IKKα and IKKβ form homo- or heterodimers via their leucine zipper (for review, see Ref. 14). Both kinases are stimulated by proinflammatory cytokines and their activation kinetics match that of IκBα phosphorylation. Highly purified recombinant IKKα and IKKβ can phosphorylate IκBα and IκBβ directly at the correct sites, thus no further downstream kinases are required for IκB phosphorylation (15, 16). IKKα and IKKβ also inducibly phosphorylate the NF-κB precursor protein p105 at three carboxy-terminal serines and thereby trigger proteolysis of the precursor (17). The IKK complex appears to contain a IKKαβ heterodimer (9), although in some cell types IKKβ homodimers are found as well (18). The recent generation of IKKα- or IKKβ-deficient mice has established the requirement of IKKβ for activation of NF-κB by proinflammatory stimuli (19–21). In contrast, IKKα was found to be dispensable for these stimuli but was essential for morphogenic functions, including differentiation and proliferation of epidermal keratinocytes and skeletal development (22–24). The IKKα knock-out model also demonstrated that the signal responsiveness and activity of the resulting IKKβ homodimer in these animals is fully functional. A predominant role of IKKβ for proinflammatory signaling is also evident from the observation that mutation of two amino acids in the activation loop of IKKβ, but not in IKKα, blocks IKK activation by NIK or cytokines (25).

In addition to the two kinases, the IKK complex has been reported to contain regulatory subunits. IKKγ (NEMO, IKKAP1) has been obtained by complementation cloning (26) and by microsequencing of the purified protein (18, 27). Murine IKKγ (NEMO) restored the defect of mutant cell lines which had lost the ability to activate NF-κB (26). IKKγ has an extended coiled-coil structure prediction, forms dimers and trimers in vitro (26, 27), and directly binds to IKKβ but not to IKKα (27). IKKγ is required for activation of NF-κB by TNFα, IL-1β, lipopolysaccharide, phorbol 12-myristate 13-acetate, double stranded RNA, or the human T-cell lymphotrophic virus...

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† Present address: Dept. of Pathology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.
‡ To whom correspondence should be addressed. Tel.: 49-30-9406-3816; Fax: 49-30-9406-3866; E-mail: scheidereit@mdc-berlin.de.

¹ The abbreviations used are: TNFα, tumor necrosis factor α; IL-1β, interleukin 1β; IκB, IκB kinase; IKAP, IKK-associated protein; RT-PCR, reverse transcriptase-polymerase chain reaction; DTt, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; pol II, polymerase II.

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cellular IKK expression of antisense cDNA (26, 27). Most, if not all of the IKK protein as a bait and was shown to interact with RIP and also contains coiled coils and a leucine zipper (29).

A further IKK-associated protein (IKAP) was isolated by affinity purification of the IKK complex using immobilized IgBo (30). IKAP is a 150-kDa protein with an amino-terminal WD40-like repeat domain. IKAP co-purified with IKKα, IKKβ, NIK, RelA, IgBo, and further proteins of 105, 100, 82, 80, 65, and 58 kDa. IKAP was reported to directly and independently interact with recombinant or transfected IKKα, IKKβ, and NIK. The ksequestration of IKKs and NIK suggested a function of IKAP as a scaffold protein (30).

It is not clear whether all endogenous IKK complexes associate with IKAP or only a subset of IKKs. Furthermore, it is not known whether IKK and IKAP are present in the same IKK complexes and how many other components are commonly associated with heterodimers of IKKα and IKKβ. In this study we have analyzed the composition of cellular IKKα+IKKβ complexes. We come to the conclusion that with regard to composition the major IKK complex is only tripartite and consists exclusively of IKKα, -β, and -γ. With in vitro reconstitution experiments we show that an IKKαβγ complex displays a gel filtration profile which, like that of endogenous IKK complexes, corresponds to a size range of more than 800 kDa relative to standard proteins. Various protein-protein interaction and functional assays demonstrate that cellular IKK complexes do not contain IKAP as an intrinsic component. IKAP appears as part of a novel complex containing additional proteins of 100, 70, 45, and 39 kDa. Overexpression of IKAP interferes with the activity of a set of different NF-κB-dependent as well as independent reporter genes, suggesting a function in a more general gene expression mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa and 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, and 100 units/ml penicillin/streptomycin. For stimulation, cells were treated with 20 ng/ml TNFα (Biomol) or 10 ng/ml IL-1β (Promega).

Transfection and Luciferase Assay—293 cells were transiently transfected by the calcium phosphate precipitation method as described previously (17). DNA constructs and amounts are indicated in the figure legend. Stimulation using 50 ng/ml TNFα was carried out 6 h prior to lysis. Cells were lysed 24 h after transfection and luciferase measurement was done with the dual luciferase reporter kit (Promega) according to the manufacturer’s protocol. For transfection of antisense oligonucleotides (GeneBlocks) HeLa cells were plated in 96-well dishes at 4000 cells/well the evening before transfection. Cells were transfected in triplicate with antisense or control oligonucleotides for up to 72 h in the presence of serum using a lipid-based delivery system (Atugen AG). Transfection efficiency was greater than 80% (data not shown). Cells were harvested and RNA or protein extracts were prepared 48 h after transfection. Relative amounts of mRNA were determined by Real Time TaqMan™ PCR analysis using the ABI Prism 7700 system (PE Applied Biosystems).

Plasmids and Oligonucleotides—IKKγ was amplified by PCR from a BJAB cDNA and cloned into the HindIII/BamHI pCDNA (Invitrogen), which contains a sequence coding for a HA epitope cloned into BamHI/ XbaI. For bacterial expression IKKγ cDNA was cloned into pGEX-6-P1 (Amersham Pharmacia Biotech). pRc/Flag-IKKα (Invitrogen) and pRc/Flag-IKKβ were used as sources.

Antisense oligonucleotides (GeneBlocks) complementary to IKKγ and IKKα were generated (Atugen AG): 17794:24058 GB3.3 (5’-GTTGTGAACCTCCGCTGCTT-3’); 17783:24047 GB3.3 (5’-AGCCTTGGAACATCCACACCATT-3’); 17785:24049 GB3.3 (5’-GACCTGTTGCTGCTAAGCACCACCAAGG-3’). A randomized control oligonucleotide GBC 5’-NIR-3’ was used as control.

Antibodies—Anti-IKKγ antibody was raised against a peptide comprising amino acids 57–72 of the human IKKγ molecule and anti-IKKα antibody was raised against a peptide comprising the very carboxyl terminus of human IKKα (amino acids 1313–1332). Peptide synthesis and immunization was done by Eurogentec. Furthermore, IKKγ antibody (FL-419) from Santa Cruz was used for Western blotting. Polyclonal antibodies against p65 (A), IκBo (C-21), IκBβ (N-20), HA (Y-11) were obtained from Santa Cruz. Monoclonal antibodies were as follows: IKKα antibody (B78-1) and mouse IgG1 isotype control, Pharmingen; IKKβ antibody, 10AG2, BIOSOURCE; Flag antibody, M5, Sigma.

Extracts, Electrophoretic Mobility Shift Assay, and Western Blotting—Whole cell extracts were analyzed by electrophoretic gel shift assay and Western blotting essentially as described previously (31). For the preparation of cytoplasmic, nuclear and chromatin extracts HeLa cells were washed with phosphate-buffered saline and swollen in buffer A (1 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol (DTT) plus protease inhibitors, 0.4 mM Pefabloc, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) for 10 min on ice. 0.15% Nonidet P-40 was added, thoroughly mixed, and spun down in a microcentrifuge. The supernatant was centrifuged at 14,000 rpm and after addition of 10% glycerol used as cytoplasmic extract. The nuclei were shaken for 15 min in buffer C (20 mM HEPES, pH 7.9, 20% glycerol, 0.4 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, plus protease inhibitors (see above)) and after centrifugation for 10 min at 14,000 rpm the supernatant was used as a nuclear extract. The pellet was washed once with ~10 volumes of buffer C and directly taken up and boiled for 15 min in SDS loading buffer to elute proteins that are tightly bound to chromatin.

Preparation of Extracts and Gel Filtration Analysis—Gel filtration analysis of endogenous proteins was either carried out from whole cell extracts of HeLa cells or cytoplasmic extracts of 70Z/3, 1.3E2 cells, or extracts transiently transfected 293 cells. Cells were lysed in a 300-μl volume of 100 mM Tris, pH 7.5, 300 mM NaCl, 0.3% Nonidet P-40, 2 mM EDTA, 1 mM DTT, 10 mM NaF, 8 mM β-glycerophosphate, 0.1 mM orthovanadate, 10% glycerol plus protease inhibitors (0.4 mM Pefabloc, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). 300 μl of low salt buffer (10 mM Tris, pH 7.5, 1 mM DTT plus protease inhibitors, see above) were added for dilution and 500 μl were added for gel filtration. Extracts from 70Z/3 cells or 1.3E2 cells were prepared as described (26). 6 mg of protein were analyzed by gel filtration. 100-mm plates of 293 cells were transiently transfected using calcium phosphate precipitation. Cells were lysed 4 h after transfection in 50 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 5% glycerol, 10 mM NaF, 8 mM β-glycerophosphate, 0.1 mM orthovanadate and protease inhibitors (see above). Equal volumes for each transfection were used for gel filtration analysis. Bacterially expressed glutathione S-transferase-IKKγ was bound to glutathione-Sepharose 4B and glutathione S-transferase was cleaved by PreScission protease (Amersham Pharmacia Biotech). Equal amounts (~200 ng) of purified baculovirally expressed IKKγ (15) and/or purified bacterially expressed IKKγ were mixed and incubated on ice for 45 min in 100 μl of BC-100 containing 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5 μg of bovine serum albumin before gel filtration analysis.

All gel filtration chromatography was carried out on a Superose 6 column (Amersham Pharmacia Biotech). 500-μl fractions were recovered and every fraction was analyzed by Western blotting. The column was calibrated with the molecular mass marker proteins thyroglobuline (689 Da), ferritin (440 KDa), catalase (232 KDa), and aldolase (158 KDa) (Amersham Pharmacia Biotech).

Co-immunoprecipitation—For in vitro co-immunoprecipitation proteins were translated in rabbit reticulocyte lysate in the presence of [35S]methionine using the in vitro transcription/translation kit from Promega. For immunoprecipitations, 3–10 μl of the translated products were mixed and preincubated for 1 h at 4 °C in HEPES, pH 7.9, 100 mM...
KCl, 0.5% Nonidet P-40, 0.5 mM EDTA, 0.5 mM DTT, 0.4 mM Pefabloc, and 1 μg/ml leupeptin, pepstatin, and aprotinin. After pre-clearance with protein A-Sepharose for 1 h at 4 °C, HA or Flag antibody and fresh protein A-Sepharose were added and incubated for another hour. The precipitates were washed three times with lysis buffer and boiled for 5 min with TNFα or IL-1β. Cellular lysis, immunoprecipitation was carried out overnight at 4 °C. After pre-clearing for 1 h with protein A-Sepharose the extracts were incubated for 4 h with the antibodies and protein A-Sepharose. Precipitates were washed four times with 50 mM Tris, pH 7.5, 150 mM NaCl, 0.15% Nonidet P-40, 1 mM EDTA, boiled in SDS loading buffer, and analyzed by Western blotting.

Metabolic Labeling and Immunoprecipitation—HeLa cells were prepared using 100 mM Tris, pH 7.5, 300 mM NaCl, 0.3% Nonidet P-40, 2 mM EDTA, 1 mM DTT, 10 mM NaF, 8 mM β-glycerophosphate, 0.1 mM orthovanadate, 10% glycerol plus protease inhibitors (see above). Extracts were diluted 1:1 with 10 mM Tris, pH 7.5, 1 mM DTT plus protease inhibitors (see above). After pre-clearing for 1 h with protein A-Sepharose the extracts were incubated for 4 h with the antibodies and protein A-Sepharose. Precipitates were washed four times with 50 mM Tris, pH 7.5, 150 mM NaCl, 0.15% Nonidet P-40, 1 mM EDTA, boiled in SDS loading buffer, and analyzed by Western blotting.

RESULTS

IKKγ and IKAP Are Part of Different Molecular Complexes—IKKγ and IKAP have each been suggested to stably associate with IKKα and IKKβ (26, 27, 30). To analyze the contribution of IKKγ and IKAP we tested if both proteins are part of the same complex with IKKα and IKKβ in whole cell extracts from HeLa cells fractionated by gel filtration (Fig. 1A). IKKα, IKKβ, and IKAP had an identical elution profile (fractions 12–16), with an apparent molecular mass between 700 and 900 kDa relative to molecular mass markers, as reported earlier (26). In contrast, IKAP was predominantly found in fractions containing proteins with lower apparent molecular mass (fractions 16–20), while only small amounts of IKAP co-eluted with the IKK complex and trailed into still much larger apparent sizes. In fact, IKAP peak elution roughly coincided with NF-κB-IκB complexes at an apparent molecular mass of 440–670 kDa (fractions 18–22). However, no association of IKAP and NF-κB-IκB complexes was observed after affinity purification with a p65 antibody (data not shown).

Next we asked, whether the IKK complex disruption seen in IKKγ-deficient cells (26) would affect the gel filtration elution profile of IKAP. We analyzed 70Z/3 cells and the 70Z/3-derived mutant cell line, 1.3E2, that exhibits impaired NF-κB activation and lacks IKKγ (26, 33, 34). In 1.3E2 cells, IKKα and -β elute at smaller apparent molecular masses, between 450 and 600 kDa (Fig. 1B). Thus, loss of IKKγ in 1.3E2 cells leads to a similar migration decrease of the IKKα-β complex compared with the parental 70Z/3 cells, as that observed between Tax-transformed Rat-1 fibroblasts and their IKKγ lacking variant (26).

Despite the shift of IKKα peak elution from fraction 14 to fraction 18 in the absence of IKKγ, the elution of IKAP complexes remained unchanged with maximal elution around fraction 18. This strongly suggests that IKAP is not part of a regular complex with IKKα and IKKγ.

To compare associations between IKKs and IKAP or IKKγ directly, we performed co-immunoprecipitations of in vitro
translated epitope-tagged proteins (Fig. 2A). HA-IKKγ was mixed with Flag-IKAP. Whereas HA antibody efficiently precipitated HA-IKKγ and Flag antibody pulled down Flag-IKAP, neither protein was co-precipitated with the other (lanes 1–4). We also added Myc-tagged IKKα and IKKβ to the IKKγ and IKAP mixture (lanes 5–8). IKKα and IKKβ could be efficiently co-precipitated with HA-IKKγ, but under these conditions no significant association was seen with IKAP after precipitation with either HA or Flag antibodies. Similar results were obtained in transiently transfected 293 cells, where we observed tight association between IKKα, IKKβ, and IKKγ, but only a very weak association between IKKα, IKKβ, and IKAP (data not shown). These observations raised the question whether association between cellular IKKs and IKAP could be detected in intact cells. Whole cell extracts of HeLa cells were used for immunoprecipitations and subsequent Western blotting (Fig. 2B). IKAP was specifically precipitated with the IKAP antibody (lane 3), but only very small amounts of IKAP were precipitated with antibodies directed against either IKKα or IKKγ (lanes 5 and 6) which were also seen with the IgG1 control (lane 4). Furthermore, the IKAP antibody did not co-precipitate IKKα (lane 3). Conversely, co-immunoprecipitation of IKKα and IKKγ using identical conditions was observed with either antibody (lanes 5 and 6). We therefore conclude that IKAP is not stably associated with the IKK complex.

Since NF-κB activation by the IKK complex is a cytoplasmic process, components which are involved in this process are expected to reside in the cytoplasm. We analyzed the cytoplasmic and nuclear distribution of IKAP, IKKα, and IKKγ and p65 in HeLa cells (Fig. 3A). Whereas IKKα, IKKγ, IKAP, and p65 were all found predominantly in the cytoplasm (lane 1), a considerable amount of IKAP was also present in the nucleus (lane 2). There, two IKAP bands were detected, both of which were specifically competed by the peptide (data not shown). As IKAP has homology to Eplp1, a yeast protein that tightly binds to the chromatin-associated hyperphosphorylated elongating...
form of RNA polymerase II (pol II) (35), we determined whether IKAP is also found in the chromatin fraction. The pellet obtained after the extraction of nuclei with a buffer containing 400 mM NaCl, which contains the chromosomal DNA and stably associated proteins, was boiled in SDS loading buffer and analyzed by Western blotting. Hyperphosphorylated pol II was specifically eluted from the chromatin fraction using these conditions. In contrast to pol II, IKAP was not found in that fraction (lane 2).

Next, the cellular proteins that are associated with IKKs and IKAP were analyzed by immunoprecipitation from extracts of [35S]methionine pulse-labeled HeLa cells (Fig. 3B). As expected, IKKα as well as IKKγ specifically precipitated a complex consisting of IKKα, β, and -γ (lanes 1–4). No band corresponding to the size of IKAP was detected and no further specific signals were obtained in the range between 25 and 200 kDa. Using an IKAP antibody we specifically co-immunoprecipitated four proteins (lane 6) with sizes of approximately 100, 70, 45, and 39 kDa, none of which co-migrated with the IKKs. These data support that IKKs and IKAP are not stably associated, but that IKAP is part of a different cellular complex with multiple subunits.

Whereas the analysis of the composition of the IKK complex showed that IKAP is not an integral member, it might still function as a potent regulator of IKK activation in response to upstream signaling events. Therefore, we determined the effects of IKAP and IKKγ on NF-kB activation through either overexpression or antisense-mediated down-regulation.

IKAP and IKKγ Affect NF-kB Reporter Activity by Different Mechanisms—Overexpression of IKKγ or IKAP each have been shown to repress cytokine-mediated activation of a NF-kB-dependent reporter, presumably either by disruption of the IKK complex or by competition for upstream activators of the IKK complex (28, 30). To compare the effects of IKAP and IKKγ, either molecule was co-transfected together with an NF-kB-dependent ELAM promoter-luciferase construct (ELAMluc) (Fig. 4A). A thymidine kinase promoter-luciferase reporter (TKluc) was used as internal control. Both, IKAP and IKKγ repressed TNFα-induced activation of NF-kB in the reporter assay (Fig. 4A, upper and middle panel). To test whether the inhibition by IKKγ or IKAP was restricted to cytokine signaling or whether it could also be observed upon overexpression of an IkB kinase, we stimulated NF-kB by overexpression of HA-IKKβ (Fig. 4B). Whereas IKAP inhibited IKKβ-mediated NF-kB activation, IKKγ expression hyper-activated the NF-kB reporter, at least at low concentrations. Strikingly, we observed that the effect of IKAP overexpression was not solely restricted to the NF-kB reporter, but also resulted in a marked reduction of TK promoter activity (Fig. 4, A and B, lower panel). In contrast, IKKγ had no effect on TK promoter activity at concentrations where it efficiently blocked the NF-kB-dependent promoter. Furthermore, IKAP somewhat repressed HA-IKKβ expression, the transcription of which is driven by an β-actin promoter (Fig. 4B, bottom). To determine whether IKAP had a general effect on promoter activity, we tested its effect on cytomegalovirus and Rous sarcoma virus promoter constructs (Fig. 4C). IKAP repressed both reporter constructs to a similar extent while IKKγ had no significant effect. Thus, IKKγ specifically represses NF-kB activation probably by sequestration of upstream activators of the IKK complex. In contrast, repression by IKAP is not restricted to NF-kB-driven promoters but seems to affect promoter activity in general.

Down-regulation of Cellular IKKγ, but Not of IKAP, Inhibits NF-kB Activation—To functionally compare the requirement of cellular IKAP and IKKγ for cytokine-mediated NF-kB activation we reduced the expression levels of both proteins by transient transfection of antisense oligonucleotides (GeneBlocs) in HeLa cells. The effects of the GeneBlocs were first analyzed by RT-PCR. Efficient and specific reduction of the respective mRNAs was observed (Fig. 5A). Next, we tested how down-regulation of IKKγ and IKAP mRNAs affected the respective protein levels and the induction of NF-kB by TNFα (Fig. 5B). Antisense oligonucleotides directed against IKAP (lanes 3–6) significantly decreased IKAP protein levels. Likewise, transfection of an IKKγ GeneBloc (lane 7 and 8) led to a reduction of IKKγ protein levels. The GeneBlocs were specific as they had no effect on expression levels of IKKα or p65. We observed that a decrease of IKKγ protein caused a reduction of NF-kB activation in response to TNFα, as assayed by mobility shift assay (Fig. 5B, upper panel). A similar reduction of IKAP protein did not comparably influence NF-kB activation. As a control, Oct-1 DNA binding activity in the same extracts was not affected by any procedure.

To address the effect of reduced IKKγ or IKAP protein levels on activation of the IKK complex, HeLa cells were transiently transfected with different antisense oligonucleotides and stimulated with either TNFα or IL-1β. We performed an in vitro kinase assay using the immunoprecipitated IKK complex (Fig. 5C, lower panel), which was specifically precipitated by the IKKα antibody (compare Fig. 3C). Transfection of antisense oligonucleotides against IKAP or IKKγ again significantly reduced the steady state amount of each protein but did not change IKKα protein levels (upper panel). The kinase activity of the IKK complex was enhanced in response to TNFα or IL-1β and phosphorylation of IκBα was dependent on the presence of serines 32 and 36, as expected (lanes 1–5). Whereas reduction of IKAP had no effect on IKK activity (lanes 6–8), lowering IKKγ protein levels led to a significant decrease in cytokine inducibility of the IKK complex. We conclude from these experiments that IKAP is not involved to the extent of IKKγ in cytokine-mediated activation of IKKs and subsequent NF-kB activation.

IKKα, IKKβ, and IKKγ Are Sufficient to Constitute a Complex Equivalent to the Endogenous IKK Complex—In gel filtration studies the apparent molecular mass of the IKK complex was determined to be 700–900 kDa (see Fig. 1 and Refs. 26, 27, 30, and 36, and references therein). The cellular IKK complex contains exclusively IKKα, β, and -γ (Fig. 3) and if composed of an IKKα/IKKβ heterodimer and an IKKγ homodimer should have a molecular mass of about 250–300 kDa. We were curious to determine whether the difference between the apparent molecular mass in gel filtration analysis and the theoretical molecular weight was caused by further components or if it resulted from structural properties of the IKK complex. We first tested whether an IKK complex with a large apparent molecular weight could be reconstituted in 293 cells simply by overexpression of IKKα, IKKβ, and IKKγ, 293 cells were transiently transfected and extracts were subjected to gel filtration chromatography (Fig. 6A). Most of the overexpressed HA-IKKαβ eluted in fractions 18–22 and were not integrated into endogenous IKK complexes, which peaked in fraction 14 (see Fig. 1 and data not shown). Co-expression of HA-IKKγ led to a complete shift of HA-IKKαβ to fractions corresponding to an apparent molecular mass of 700 to 900 kDa (Fig. 6A, panels a versus b). Interestingly, the elution peak of HA-IKKγ, when transfected alone, corresponded to more than 600 kDa while co-expression with IKKαβ caused only a slight shift to higher molecular weight fractions (panels b versus c). Probably, IKKγ forms higher order complexes when expressed alone, but integrates into the IKK complex when IKKα and IKKβ are present. As expected from our previous results, overexpression of Flag-IKAP had no effect on the elution profile of IKKα and IKKβ (panels d and e), supporting that IKAP is not a stable member of the IKK complex.
To exclude that a component in 293 cells, which is in vast excess over the endogenous IKKs, is causing the apparent high molecular weight of IKKα/β/γ we analyzed recombinant purified components (Fig. 6B). Complex formation was analyzed by gel filtration and Western blotting. Purified recombinant IKKα peaked in fractions 21 and 22, corresponding to an apparent

Fig. 4. NF-κB-dependent and -independent effects of IKKγ and IKAP overexpression on reporter genes. A and B, effects of Flag-IKAP and HA-IKKγ on NF-κB activation. A, 293 cells were transiently transfected with 0.1 μg of ELAM reporter construct, 0.05 μg of pRLTK reporter as an internal control, and the indicated amounts of Flag-IKAP or HA-IKKγ expression vector. DNA amounts were equalized using empty vector (pcDNA3). 6 h prior to lysis the cells were treated with TNFα or left untreated. Fold activation of the ELAM reporter after normalization with the levels of the thymidine kinase control reporter (upper panel) as well as the activation of each reporter alone (middle and lower panel) are shown. The data represent the mean values and the standard deviation of three independent experiments. B, 293 cells were transfected as in A, except that HA-IKKβ was co-transfected to activate NF-κB. Levels of transfected HA-IKKβ detected by Western blotting are shown at the bottom. The data shown are the mean values of one representative experiment in which each transfection was done in duplicate. C, effects of Flag-IKAP and HA-IKKγ on cytomegalovirus- or Rous sarcoma virus-promoter driven reporter genes. 293 cells were transiently transfected using 0.2 μg of cytomegalovirus or 0.4 μg of Rous sarcoma virus reporter constructs and the indicated amounts of Flag-IKAP or HA-IKKγ; DNA amounts were equalized with empty vector (pcDNA3). The relative luciferase activity is shown. The data represent the mean values and the standard deviation of three independent experiments.
molecular mass of approximately 440–470 kDa. Similar to overexpressed IKKγ, purified IKKγ eluted in fractions 17–18, corresponding to approximately 670 kDa compared to standard proteins. Cross-linking of IKKγ in fraction 18 with sulfo-EGS (ethylene glycol bis(sulfosuccinimidylsuccinate)) indicated a homotrimERIC complex (~150 kDa, data not shown). For the reconstitution of an IKK complex, equal amounts of IKKα and IKKγ, as judged by Coomassie staining (left panel), were mixed and incubated on ice for 45 min. Nearly all IKKα and IKKγ now co-migrated in fractions 14–16, corresponding to an apparent mass of more than 700 kDa. Almost identical results were obtained when IKKβ and IKKγ were used (not shown). These data provide evidence that IKKα and/or IKKβ in conjunction with IKKγ are sufficient to reconstitute a complex with a hydrodynamic property like that of the endogenous IKK complex.

**DISCUSSION**

The IKK complex is required for activation of NF-κB by all physiological inducers tested to date. Recently the two catalytic domains, IKKα and IKKβ, which are responsible for inducible IκB phosphorylation, have been identified but their tight regulation is depending on the association of further regulatory molecules. A number of different proteins have been reported to be contained in or to co-purify with IKK complexes, including NF-κB and IκB proteins, a RelA kinase activity, MEKK1, NIK, mitogen-activated protein kinase phosphatase, and two regulatory components, IKKγ and IKAP (see Refs. 6, 10, 14, and 37–40, for review). However, it has not been determined whether these proteins are substoichiometric or stoichiometric components. Conceptually, the hydrodynamic property of the IKK complex, mimicking a large molecular weight, has been suggested of a number of IKK associated components.

In this study, we have investigated which proteins are part of the IKK complex and have analyzed the relative contribution of two previously proposed regulatory subunits, IKKγ and IKAP. An analysis of the endogenous IKK complex revealed that IKKα, IKKβ, and IKKγ co-elute upon gel filtration, while p65, IκBa, or IKAP peak-eluted in different fractions. Lack of IKKγ in a 70Z/3 mutant cell line decreased the mobility of IKKα, but not that of IKAP. In co-immunoprecipitations from labeled cells IKKα/β associated with IKKγ as the sole further protein, and vice versa, while IKAP was found associated with other proteins of approximately 100, 70, 45, and 39 kDa. The interaction studies clearly suggest that a regular IKK complex contains exclusively stoichiometric amounts of IKKα, -β, and -γ. This was further substantiated by studies employing transfected, in vitro translated or purified, recombinant components: while IKKα or -β and IKKγ were efficiently co-immunoprecipitated, no such strong interaction was detected between IKKα or -β
and IKAP or between IKK\(\alpha\) or IKK\(\beta\) and IKAP. If IKK\(\alpha\) or IKK\(\beta\) and IKAP were the only integral components, the hydrodynamic behavior of such a complex should be the same as that of cellular IKK complexes. We showed with overexpressed and recombinant proteins that an IKK\(\alpha/\beta\) heterodimer or an IKK\(\alpha\) homodimer, respectively, was sequestered by IKK\(\gamma\) into a complex with a gel filtration elution profile similar to that of the endogenous cellular complex. The apparent molecular mass relative to size markers was more than 700–800 kDa. Although the exact stoichiometry of the IKK\(\alpha\), \(\beta\), \(\gamma\) subunits within the complex remains to be determined, this illustrates that no further components are required to explain the gel filtration elution profile of the complex. Of note, the molecular weight determined relative to globular markers is only accurate for proteins with a globular shape. The observed migration of the IKK\(\alpha/\beta/\gamma\) complex suggests that it has an elongated rather than a globular shape.

We found that purified recombinant IKK\(\gamma\) interacts equally well with IKK\(\alpha\) and IKK\(\beta\), while, in striking contrast, Rothwarf et al. (27) and Mercurio et al. (18) detected a direct interaction of IKK\(\gamma\) only with IKK\(\beta\), but not with IKK\(\alpha\). However, in agreement with our results, efficient co-immunoprecipitation of IKK\(\gamma\) and IKK\(\alpha\) was shown with IKK\(\beta\)-deficient embryonic fibroblasts (19).

Whereas the data on the composition of the IKK complex strongly suggest that IKAP is not one of the integral members, the possibility remains that it is an essential weakly associated regulator for IKK activation. Therefore, we determined the contribution of IKK\(\gamma\) and IKAP in functional assays. Overexpression of IKK\(\gamma\) specifically inhibited NF-\(\kappa\)B-dependent reporter activity, while IKAP overexpression inhibited all promoters tested, including NF-\(\kappa\)B-independent reporter genes.

IKK\(\gamma\) overexpression could repress NF-\(\kappa\)B activation by sequestering upstream activators of the IKK complex (27). However, in conjunction with overexpressed IKK\(\beta\), IKK\(\gamma\) could also stimulate NF-\(\kappa\)B reporters, presumably by assembly of even more active complexes containing IKK\(\beta\) homodimers. Reduction of IKK\(\gamma\) levels but not of IKAP, by antisense oligonucleotides significantly reduced cytokine induction of IKK kinase activity and of NF-\(\kappa\)B DNA binding activity. As a possibility, IKAP protein levels could be in vast excess over the components of the IKK complex and only a subpopulation of IKAP, not involved in NF-\(\kappa\)B signaling, could be affected by the Gene-Blocs. However, a comparison of transfected Flag-IKK and Flag-IKK\(\alpha\) with the endogenous proteins, using anti-Flag, anti-IKK\(\alpha\), and anti-IKAP antibodies indicated that the proteins existed in comparable amounts (data not shown).

The repressive effect of IKAP overexpression on cytokine-induced NF-\(\kappa\)B activation in transient transfection assays

![Fig. 6.](image.png)
seems to rely on different or additional mechanisms. The down-regulation of several reporter genes suggests that it may directly act on the level of general transcription, either by direct repression or by titrating out essential components. In line with this, IKAP was also found in the nucleus. Moreover, IKAP is homologous to *Saccharomyces cerevisiae* Elp1/IK3 and to similar proteins in *Schizosaccharomyces pombe* and Arabidopsis *thaliana* (33, 31, and 30% identity, respectively). Yeasts and plants do not have conserved NF-κB proteins or signaling pathways, indicating a more general function of IKAP homologues. In fact, the *S. cerevisiae* protein Elp1 has recently been shown to be part of a pol II elongator complex which contains two further stoichiometric components, Elp2 and Elp3, and associates with hyperphosphorylated pol II (35). The yeast Elp3 subunit is a histone acetylase and inactivation of either Elp1 or Elp3 strongly impairs transcription of glucose-induced genes but not transcription of non-induced genes (35, 41). Could IKAP be part of a human pol II elongator complex? IKAP was not associated with a salt elution-resistant chromat fraction that contained hyperphosphorylated pol II. However, the interaction could have been sensitive to the ionic strength and been lost during the preparation procedure. The migration of IKAP action could have been sensitive to the ionic strength and been lost during the preparation procedure.

The migration of IKAP be part of a human pol II elongator complex? IKAP was not associated with a salt elution-resistant chromat fraction that contained hyperphosphorylated pol II. However, the interaction could have been sensitive to the ionic strength and been lost during the preparation procedure. The migration of IKAP action could have been sensitive to the ionic strength and been lost during the preparation procedure.

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**REFERENCES**

1. Baueule, P. A., and Baltimore, D. (1996) *Cell* 87, 13–20
2. May, M. J., and Ghosh, S. (1997) *Semin. Cancer Biol.* 8, 63–73
3. Luque, I., and Gelinas, C. (1997) *Semin. Cancer Biol.* 8, 103–111
4. Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol.* 14, 469–683
5. Walczyn, F. G., Krapfmann, D., and Scheidereit, C. (1996) *J. Mol. Biol.* 259, 749–769
6. Mercurio, F., and Manning, A. M. (1997) *Curr. Opin. Cell Biol.* 11, 226–232
7. Maniatis, T. (1999) *Genes Dev.* 13, 555–591
8. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B., L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* 278, 860–866
9. Yuan, E., and Karin, M. (1999) *Mol. Cell. Biol.* 19, 4547–4551
10. Dihotano, J. A., Hayakawa, M., Rothwarf, D. M., Zandii, E., and Karin, M. (1997) *Nature* 388, 548–554
11. Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (1997) *Cell* 90, 373–383
12. Woronicz, J. D., Gao, C., Cao, R., Rothe, M., and Goeddel, D. V. (1997) *Science* 278, 866–869
13. Zandii, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) *Cell* 91, 243–252
14. Karin, M., and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* 18, 621–663
15. Li, J., Poet, G. W., Pullen, S. S., Schembeni King, J., Warren, T. C., Marca, K. B., Kohby, M. R., Bartos, R., and Jakes, S. (1998) *J. Biol. Chem.* 273, 30736–30741
16. Zandii, E., Chen, Y., and Karin, M. (1998) *Science* 281, 1360–1363
17. Hennusmeyr, V., Krapfmann, D., Walczyn, F. G., and Scheidereit, C. (1999) *EMBO J.* 18, 4766–4778
18. Mercurio, F., Murray, B. W., Shevchenko, A., Bennett, B. L., Young, D. B., Li, J. W., Paseau, G., Motiwala, A., Zhu, H., Mann, M., and Manning, A. M. (1999) *Mol. Cell. Biol.* 19, 1528–1538
19. Tanaka, M., Fuentes, E. M., Yamaguchi, K., Dunn, M. H., Dalrymple, S. A., Hardy, K. L., and Goeddel, D. V. (1999) *Immunity* 10, 41–42
20. Li, Q., Van Antwerp, D., Mercurio, F., Lee, K. F., and Verma, I. M. (1999) *Science* 284, 321–325
21. Li, Z. W., Chu, W., Hu, Y., Delhase, M., Deerick, T., Ellisman, M., Johnson, R., and Karin, M. (1999) *J. Exp. Med.* 189, 1839–1845
22. Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerick, T., Ellisman, M., Johnson, R., and Karin, M. (1999) *Science* 284, 316–320
23. Li, Q., Lu, Q., Hwang, J. Y., Buscher, D., Lee, K. F., Ispisua Belmonte, J. C., and Verma, I. M. (1999) *Mol. Cell* 3, 6716–6726
24. Krapfmann, D., Emmerich, F., Kordes, U., Scharschmidt, E., Dorken, B., and Scheidereit, C. (1999) *Oncogene* 18, 943–953
25. Rooney, J. W., Emery, D. W., and Ghosh, C. H. (1999) *Immunogenetics* 51, 665–678
26. Coutois, G., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1999) *Cell* 93, 1231–1240
27. Rothwarf, D. M., Zandii, E., Natoli, G., and Karin, M. (1998) *Nature* 395, 297–300
28. Li, Y., Kang, J., Friedman, J., Tarassishin, L., Ye, J., Kovalenko, A., Wallach, D., and Horwitz, M. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1042–1047
29. Li, Y., Kang, J., and Horwitz, M. S. (1998) *Mol. Cell* 18, 1601–1610
30. Cohen, L., Henzel, W. J., and Baueule, C. H. (1998) *Nature* 395, 292–296
31. Krapfmann, D., Walczyn, F. G., and Scheidereit, C. (1996) *EMBO J.* 15, 6716–6726
32. Krapfmann, D., Emmerich, F., Kordes, U., Scharschmidt, E., Dorken, B., and Scheidereit, C. (1999) *Oncogene* 18, 943–953
33. Rooney, J. W., Emery, D. W., and Ghosh, C. H. (1999) *Immunogenetics* 51, 665–678
34. Coutois, G., Whiteside, S. T., Sibley, C. H., and Israel, A. (1997) *Mol. Cell. Biol.* 17, 1441–1449
35. Courtois, G., Whiteside, S. T., Sibley, C. H., and Israel, A. (1997) *Mol. Cell. Biol.* 17, 1441–1449
36. Courtois, G., Whiteside, S. T., Sibley, C. H., and Israel, A. (1997) *Mol. Cell. Biol.* 17, 1441–1449
37. Maniatis, T. (1997) *Science* 278, 818–819
38. Scheidereit, C. (1998) *Nature* 395, 225–226
39. May, M. J., and Ghosh, S. (1999) *Science* 284, 271–273
40. Hatada, E. N., Krapfmann, D., and Scheidereit, C. (2000) *Curr. Opin. Immunol.* 12, 52–58
41. Wittschien, B. O., Otero, G., de Bizemont, T., Fellows, J., Erdjument Bromage, H., Ohba, R., Li, Y., Allis, C. D., Tempst, P., and Svejstrup, J. Q. (1999) *Mol. Cell.* 4, 123–128

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2. J. Svejstrup, personal communication.