Eur. J. Biochem. **259**, 253–261 (1999) © FEBS 1999

All three IκB isoforms and most Rel family members are stably associated with the IκB kinase 1/2 complex

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Nuclear factor kappa B (NF-κB) is an important transcription factor for the genes of many pro-inflammatory proteins and is strongly activated by the cytokines interleukin-1 and tumor necrosis factor (TNF)α under various pathological conditions. In nonstimulated cells, NF-κB is present in the cytosol where it is complexed to its inhibitor IκB. Activation of NF-κB depends on the signal-induced phosphorylation of IκB by specific IκB kinases which initiates the inhibitor's conjugation to ubiquitin and subsequent degradation by the proteasome. We used both TNF-stimulated and okadaic-acid-stimulated HeLa cells to purify three biochemically distinct kinase activities targeting one or both of the two serines (S32 and S36) in IκBα which induce its rapid degradation upon cytokine stimulation. All three activities correspond to known IκB kinases: the mitogen-activated 90 kDa ribosomal S6 kinase ($p90^{rsk1}$), the IκB kinase 1/2 complex (IKK1/2) and casein kinase II (CK II). However, we found that only one of the activities, namely the IKK1/2 complex, exists as a pre-assembled kinase–substrate complex in which the IKKs are directly or indirectly associated with several NF-κB-related and IκB-related proteins: RelA, RelB, cRel, p100, p105, IκBα, IκBβ and IκBε. The existence of stable kinase–substrate complexes, the presence of all three known IκB isoforms in these complexes and our observation that the IKK complex is capable of phosphorylating IκBα-, IκBβ- and IκBε-derived peptides at the respective degradation-relevant serines suggests that the IKK complex exerts a broad regulatory role for the activation of different NF-κB species.

In contrast to previous studies, which locate CK II phosphorylation sites exclusively to the C-terminal PEST sequence of IκBα, we observed efficient phosphorylation of serine 32 in IκBα by the purified endogenous CK II complex. Therefore, both $p90^{rsk1}$ and CK II have the same preference for phosphorylating only one of the two serines which are relevant for inducible degradation.

Keywords: casein kinase II; Iκ B; Iκ B kinase; kinase–substrate complex; NF-κ B.

Tumor necrosis factor (TNF)α and interleukin (IL)-1 are potent pro-inflammatory cytokines that are produced by a variety of cell types. Both cytokines have been implicated in a number of pathological conditions [1,2] and, in many respects, appear to trigger biologically indistinguishable effects by activating the same set of transcription factors. One of the most prominent transcription factors activated by both cytokines is nuclear factor kappa B (NF-κB) [3,4]. This transcription factor occurs as a homodimer or heterodimer composed of subunits belonging to the Rel family of DNA-binding proteins [5,6]. Different NF-κB dimers appear to recognize slightly different promoter sequences, thus enabling a fine-tuned differential regulation of gene expression [7,8]. In most cells, NF-κB proteins are held in the cytoplasm by interaction with an inhibitory protein, IκB: the nuclear localization signal in the Rel homology domain (RHD) of the Rel proteins is masked by the association with IκB, thus preventing the transcription factor's nuclear transfer. When cells are stimulated with TNFα or IL-1, the inhibitor is phosphorylated at two specific serine residues [9–11]. This dual phosphorylation triggers the multi-ubiquitination of IκB and its subsequent degradation by the 26S proteasome [12–14]. The released NF-κB translocates to the nucleus where it activates the transcription of many inflammatory genes. Three IκB isoforms are known, α, β and ε [15–17]; the best characterized is IκBα. However, the exact role of each in the inhibition/activation of NF-κB proteins remains to be determined.

As cytokine-inducible IκB-kinase (IKK) activity tightly controls the activation cascade of κB-dependent genes, much interest has been attributed to the isolation of the corresponding enzymes and the investigation of their regulation. Initially, a large kinase complex with a molecular mass of ~700 kDa was described, which can be independently activated by Ubc4/5-mediated ubiquitination [18] or phosphorylation by mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK-1 [19]). The latter kinase is most often recognized as upstream kinase of the Jun N-terminal kinase stress-activated MAP kinase pathway [20–22]. More recently, two highly homologous IκB kinases, the 85 kDa IKK1 and the 87 kDa IKK2, were purified biochemically and cloned [23–26]. Both kinases appear to have identical substrate specificity and are capable of phosphorylating the degradation-relevant serines at positions 32 and 36 in IκBα, as well as the corresponding serines (at position 19 and

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Abbreviations: CK, casein kinase; DTE, 1,4-dithioerythritol; ERK, extracellular signalregulated kinase; GST, glutathione *S*-transferase; IκB, inhibitor of NF-κB; IKK, IκB kinase; MAP, mitogen-activated protein; MEKK-1, mitogen-activated protein kinase/ERK kinase kinase-1; NF-κB, nuclear factor-κB; NIK, Nf-κB-inducing kinase; PMSF, phenylmethanesulfonyl fluoride; RHD, Rel homology domain; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TNF, tumor necrosis factor.

(Received 14 September 1998, accepted 14 October 1998)

22) in IκBβ. IKK1 and IKK2 might interact with each other through leucine zipper domains located C-terminal to their kinase domains, and they are both contained in a high-molecular-mass protein complex [26]. In addition, IKK1 was also identified in a yeast two-hybrid screen using the recently cloned putative upstream kinase, NF-κB-inducing kinase (NIK), as bait [27]. NIK was originally described as a TNF receptor-associated factor 2-interacting protein that is important for both the TNFα-mediated and IL-1-mediated activation of NF-κB [28], and was later shown to enhance IKK1 and IKK2 activity [25]. It therefore appears that NIK integrates the different signals from the TNF and IL-1 receptor towards activation of NF-κB.

After stimulation of HeLa cells with TNFα, we found three kinase activities that phosphorylated IκBα serine 32: the ≥ 700-kDa IκB kinase 1/2 complex (IKK1/2), the mitogen-activated 90-kDa ribosomal S6 kinase ($p90^{rsk1}$), and casein kinase II (CK II). While $p90^{rsk1}$ has previously been described to phosphorylate IκBα at serine 32 upon mitogen-stimulation, so far CK II has only been reported to constitutively phosphorylate the C-terminal proline/glutamic acid/serine/threonine-rich (PEST) sequence of IκB. However, only the activity of the IKK complex is significantly dependent on TNFα stimulation. In contrast to $p90^{rsk1}$ and CK II, this kinase complex is capable of phosphorylating both degradation-relevant serines in IκBα and exists as a pre-assembled kinase–substrate complex containing all known IκB isoforms and most Rel family members.

EXPERIMENTAL PROCEDURES

Materials

HeLa S3 cells were purchased from the Computer Cell Culture Center (University of Mons, Belgium). All chromatographic media were from Pharmacia. ECL Western blot detection reagents (ECL and ECL Plus) were purchased from Amersham International, and restriction enzymes and other DNA-modifying enzymes from Boehringer Mannheim Corp. Iodoacetyl-LC-biotin and streptavidin-coated 96-well microtiter plates were from Pierce. An expression plasmid for the generation of a glutathione *S*-transferase (GST)–ΔMEKK-1 fusion protein [29] was generously provided by Dr John Kyriakis. All other reagents and chemicals were of the highest quality commercially available.

Expression and purification of recombinant IκBα and IκBβ

The cDNA encoding human IκBα [15] was a kind gift from Dr Roman Urfer (Novartis Pharma AG). IκBβ cDNA [30] was amplified from leukocyte RNA employing reverse transcription (RT) and polymerase chain reaction (PCR) and subsequently cloned into the vector pET17b (Novagen, Inc.). Point mutations were generated with the QuikChange™ site-directed mutagenesis kit (Stratagene). Recombinant proteins were expressed in *Escherichia coli* strain BL21 DE3 pLysS (Novagen, Inc.) according to the supplier's specifications and purified as follows (all steps at 0–4 °C). A 3-g portion of wet cell pellet was resuspended in 10 mL of 50 mM Tris/HCl buffer at pH 8.0, containing 1 mM 1,4-dithioerythritol (DTE), and sonicated on ice (3 × 1 min). The resulting homogenate was centrifuged in a Beckman JA-20 rotor at 18 000 ***g*** for 15 min and the IκB variant was precipitated from the supernatant by the addition of 15% (wt/vol.) ammonium sulfate. After incubation for 15 min and centrifugation in a Beckman JA-20 rotor at 15 000 ***g*** for 12 min, the pellet was dissolved in 20 mL of 50 mM Tris/HCl at pH 8.0, containing 1 mM DTE, and loaded onto a 35-mL Q-Sepharose Fast Flow column equilibrated with the same buffer. Bound proteins were eluted with a linear gradient of 0–1 M NaCl. IκBα-containing fractions, which eluted between 0.55 and 0.6 M salt, were pooled, frozen by dripping the solution into liquid nitrogen and stored at – 80 °C. Typical yields were 2–7 $mg \cdot L^{-1}$ *E. coli* cell culture.

A 6 × His-tagged version of IκBα was constructed by inserting a coding sequence for six successive histidine residues immediately upstream of the stop codon. This IκBα variant was expressed in *E. coli* as described above and purified by affinity chromatography on nickel–nitrilotriacetic acid (NTA; Qiagen) resin following the supplier's specifications.

IKK activity assay

Iodoacetyl-LC-biotin was used to covalently attach a functional biotin moiety to the IκBα substrate via its thiol groups. Labeling was carried out in 0.1 M Tris/HCl at pH 8.0 for 2 h at room temperature and the reaction quenched by the addition of DTE to a final concentration of 5 mM. The kinase reaction was performed in 50 mM Tris/HCl buffer at pH 8.0, containing 10 mM $MgCl_2$, 100 nM okadaic acid and 1 mM DTE. The substrates ATP and biotinylated IκBα were used at concentrations of 10 μM and 0.1 μM, respectively, and the reaction was started by the addition of the IKK-containing fractions. The biotinylated S32 A/S36 A mutant IκBα was used as a negative control in a parallel experiment. After 20–90 min at room temperature the reaction was terminated by the addition of EDTA and the reaction mixtures transferred to streptavidin-coated 96-well microtiter plates. The phosphorylated IκBα was then detected using a phospho-specific IκBα (Ser32) antibody (New England Biolabs) in combination with a horseradish-peroxidase-conjugated anti-rabbit IgG and employment of BM Blue POD substrate (Boehringer Mannheim). Finally, the absorbance of the wells was measured using a Spectra MAX 250 (Molecular Devices) plate reader at 450 nm vs. 650 nm. Alternatively, 10 μCi γ-^{33}P-labeled ATP were added to the kinase reaction. The samples were subjected to SDS/PAGE and the gels were washed twice in 10% (vol./vol.) acetic acid, 40% (vol./vol.) methanol, dried and exposed to a phosphorimager screen.

TNF stimulation of HeLa cells

HeLa cells were maintained in RPMI 1640 medium (GIBCO BRL) supplemented with 5% fetal calf serum (FCS), 446 $mg \cdot L^{-1}$ L-alanyl-L-glutamine, 50 μM mercaptoethanol. For 12 h prior to stimulation, the cells were starved in serum-free RPMI 1640 medium supplemented with L-alanyl-L-glutamine, mercaptoethanol, 100 $IU \cdot mL^{-1}$ penicillin (GIBCO BRL) and 100 $\mu g \cdot mL^{-1}$ streptomycin (GIBCO BRL). The starved cells were stimulated with 50 $ng \cdot mL^{-1}$ TNFα for 5 min at 37 °C, immediately chilled to 4 °C and collected by centrifugation. All subsequent steps were carried out at 4 °C.

Purification of IκB kinases

HeLa cells were suspended in ice-cold extraction buffer (10 mM Tris/HCl at pH 7.5, containing 10 mM KCl, 1 mM EDTA, 1 mM DTE and 100 $\mu g \cdot mL^{-1}$ PMSF) and lysed by 20 strokes in a dounce homogenizer with a pistil B. Centrifugation in a Beckman JA-20 rotor for 30 min at 11 000 ***g*** and then in a Beckman Ti 45 rotor at 180 000 ***g*** for 2 h yielded the postribosomal

supernatant from which the proteins were precipitated with ammonium sulfate in a stepwise manner: addition of ammonium sulfate to a concentration of 8% (wt/vol.), incubation for 15 min and centrifugation in a Beckman JA-20 rotor at 15 000 ***g*** was followed by a second precipitation step at 31% (wt/vol.) ammonium sulfate. After an incubation period of 15 min and centrifugation in a Beckman JA-20 rotor at 15 000 ***g***, the latter pellet was dissolved in 50 mL of 25 mM Tris/HCl buffer at pH 8.0 containing 1 mM DTE, and dialyzed extensively against the same buffer. The protein solution was then loaded onto a 300-mL Q-Sepharose Fast Flow column which was equilibrated with the above dialysis buffer. Bound proteins were eluted with a gradient of 0–1 M NaCl. IKK activity eluted in three distinct peaks and the fractions corresponding to each peak were pooled and the respective kinase further purified.

Peak 1 eluting from the Q-Sepharose column between 100 mM and 200 mM NaCl was subjected to gel-filtration chromatography on a Superdex S200 column in 25 mM Tris/HCl buffer at pH 8.0, containing 150 mM NaCl and 1 mM DTE. Prior to the experiment the column was calibrated with proteins of known molecular mass: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa) and chymotrypsinogen A (25 kDa). Peak 2, which eluted from the Q-Sepharose column between 300 mM and 350 mM NaCl, was concentrated by ultrafiltration with Amicon PM 10 filters and subsequently passed over a Sephacryl S300 column developed in 25 mM Tris/HCl buffer at pH 8.0, containing 150 mM NaCl and 1 mM DTE. The IKK-containing fractions were pooled, loaded onto a MonoQ column and eluted with a linear gradient of 0.15–0.5 M NaCl in 25 mM Tris/HCl, pH 8.0, 1 mM DTE. IKK-containing fractions were then analyzed by analytical gel filtration on a calibrated Superdex S200 column. The fractions corresponding to the third IKK activity peak of the above Q-Sepharose column eluting between 400 and 470 mM NaCl were further purified on a hydroxyapatite column equilibrated with 25 mM Tris/HCl at pH 8.0, 1 mM DTE. Proteins were eluted using a linear gradient of 0–0.5 M potassium phosphate in the same buffer. IκB-containing fractions were pooled and analyzed by gel-filtration chromatography on a calibrated Superdex S200 column.

As an alternative to using freshly stimulated HeLa cells, we applied the above purification scheme to cells which had been stored frozen as a cell pellet at – 80 °C, and which were stimulated after thawing by the addition of 500 nM okadaic acid.

Western blotting

After fractionation by SDS/PAGE, proteins were transferred to Hybond ECL nitrocellulose or Hybond P membranes and probed with the indicated antibodies. Antisera against peptide sequences derived from IKK1 (MERPPGLRPGAGGPY) and IKK2 (MSWSPSLTTQ TRRY), as well as against recombinant IκBβ, were provided by Neosystem (Strasbourg, France). All other primary and secondary antibodies were from Santa Cruz Biotechnologies, Inc. Detection was carried out using the ECL or the ECL Plus chemiluminescence systems.

Peptide phosphorylation assay

Biotinylated 15mer peptides (100 μM) spanning the relevant SxxxS sites of IκBα, IκBβ and IκBε (DDRHDSGLDSMKDEE, DEWCDSGLGSLGPDA, EAQFDSGIESLRALR, respectively) were phosphorylated using biochemically purified IKK activities derived from Q-Sepharose column peaks 1, 2 and 3. The reactions were carried out for 60 min at room temperature in 50 mM Tris/HCl buffer pH 8.0 containing 100 μM vanadate, 20 mM β-glycero-phosphate, 1 mM DTE, 1 μM okadaic acid, 10 mM magnesium chloride, 10 μM ATP and 10 μCi γ-^{33}P-labeled ATP. The reactions were stopped by the addition of 10 mM EDTA. The biotinylated peptides were immobilized on streptavidin-coated polymer beads (Boehringer Mannheim) and washed five times with 50 mM Tris/HCl pH 8.0, 10 mM EDTA and 1 mM DTE. Following the washing procedure, the peptides were eluted from the beads by boiling them for 30 min in 100 mM Tris/HCl pH 6.8, 8% (wt/vol.) SDS, 30% (wt/vol.) glycerol, 200 mM DTE, 0.02% (wt/vol.) Coomassie Blue G 250. The eluates were resolved by electrophoresis using 16.5% Tris/Tricine gels, and the gels were washed twice in 10% (vol./vol.) acetic acid, 40% (vol./vol.) methanol, dried and exposed to a phosphorimager screen. The extent of phosphorylation was quantified by densitometric scanning. Peptides in which both serines are mutated to alanines were used as negative controls, and a nonrelated biotinylated control peptide (GLYQGLST-ATKDTYDALH) with phosphorylated tyrosines was generously provided by the laboratory of Dr Ute Manning-Krieg (Novartis Pharma AG).

In-gel-kinase assay

In-gel-kinase assays were performed as described by Hibi *et al.* [31] with minor modifications. Biochemically purified kinase was applied to a 10% SDS polyacrylamide gel in the presence or absence of 2.5 μM IκBα polymerized into the gel. After electrophoresis, the gel was washed twice for 30 min in 20% (vol./vol.) isopropanol, 50 mM Tris/HCl at pH 7.5, twice for 30 min in buffer A (50 mM Tris/HCl at pH 7.5, 5 mM 2-mercaptoethanol), twice for 30 min in buffer A containing 6 M urea, and then in buffer A containing 0.05% (vol./vol.) Tween-20 and declining urea concentrations (3, 1.5 and 0.75 M, respectively). After a final washing of the gel five times for 1 h in buffer A containing 0.05% (vol./vol.) Tween-20, it was incubated in the same buffer overnight at 4 °C. To carry out the in-gel-kinase reaction, the gel was incubated in 25 mM Tris of pH 7.5, 25 mM β-glycero-phosphate, 25 mM magnesium chloride, 100 μM orthovanadate and 2 mM DTE for 1 h and then in the same buffer containing 10 μM ATP and 10 $\mu Ci \cdot mL^{-1}$ γ-^{33}P-labeled ATP for 1 h at room temperature. Following the kinase reaction, the gel was washed four times in 10% (vol./vol.) acetic acid, 40% (vol./vol.) methanol, dried and exposed to a phosphorimager screen.

RESULTS

Biochemical separation of three distinct IKK activities

To detect cytokine-inducible IKK activities, HeLa cells were stimulated with 50 $ng \cdot mL^{-1}$ TNFα for 5 min at 37 °C or left untreated. Prior to stimulation the cells had been starved for 12 h in serum-free medium to exclude any stimulatory effect of serum factors. The stimulated and nonstimulated cells were disrupted mechanically under hypotonic conditions and the postribosomal supernatants were subjected to an ammonium sulfate precipitation. In a first chromatographic purification step on an anion-exchange matrix the total IKK activity was separated into three distinct pools corresponding to three distinct activity peaks eluting from the column (Fig. 1). The activity in peak 1 was stimulated little by TNFα, and constituted only a minor portion of the total IKK activity in the cytosolic extract. By contrast, the activity in peak 2 displayed the clearest dependence on TNFα induction. The activity eluting with this peak amounted to ~20% of the total IKK activity in the stimulated cytosolic extract. However, the major fraction of the

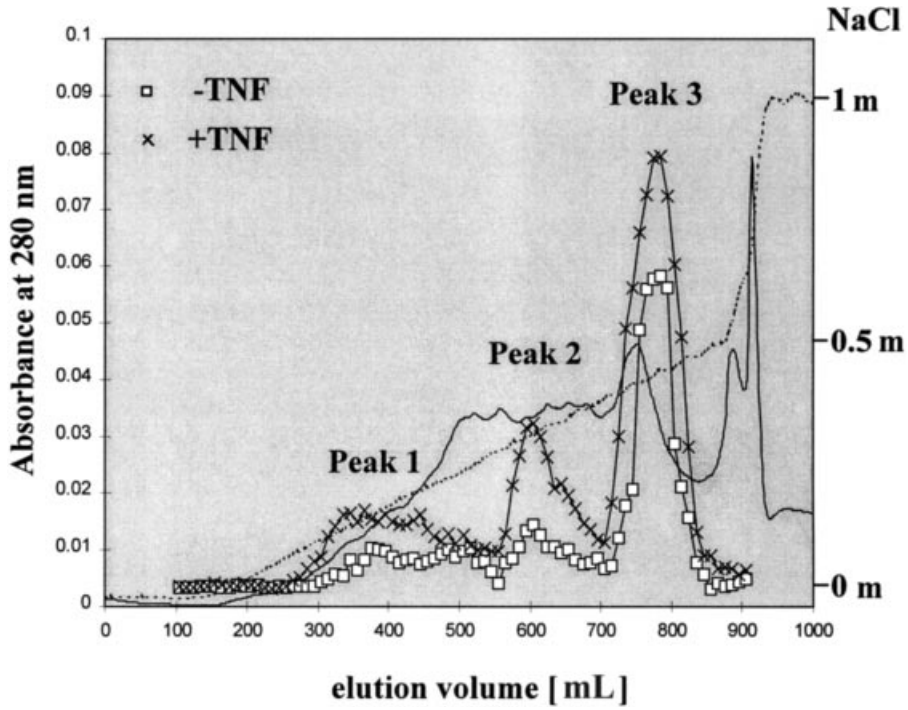

Fig. 1. Induction of the IKK activities by TNFα. Cytosolic extracts of TNFα-stimulated and unstimulated HeLa cells were applied to a Q-Sepharose column and eluted with a gradient of 0–1 M NaCl (dotted line). Elution of the proteins was followed by measuring the absorbance at 280 nm (solid line) and the activity of all individual fractions was determined as described under Experimental Procedures. (×) IKK activity of TNFα-stimulated cell extract; (○) IKK activity of unstimulated cell extract. TNFα-stimulated IKK activity elutes in three distinct peaks, peaks 1–3.

kinase activity eluted in peak 3. The activity in this peak was only stimulated by TNFα to some extent, and its basal, nonstimulated activity already exceeded that of peak 2 after TNFα stimulation.

For convenience, and to further characterize the various IKK activities, all the following experiments were performed using a cytosolic extract that was prepared from a frozen stock of HeLa cells. Prior to extraction, and as an alternative to TNFα stimulation, the cells, when thawed, were exposed to 500 nM okadaic acid for about 5 min at 37 °C. Okadaic acid, an efficient inhibitor of phosphatase 2 A, has been found to induce IκBα phosphorylation and degradation in living cells when added to the extracellular medium [11]. It functions by shifting the balance between an IKK-stimulating phosphorylation and an inactivating dephosphorylation reaction towards the stimulatory side [23]. This procedure generated a semistimulated HeLa cell extract (see below), the IκB activity of which eluted from the anion-exchange column in the same three peaks as for the TNFα-stimulated living cells. However, the peak heights were approximately intermediate with respect to the above profiles obtained for nonstimulated and TNFα-stimulated extracts.

Characterization of the IκB activity from peak 1 by analytical gel filtration

The molecular mass of the IKK activity eluting in peak 1 was determined by analytical gel filtration on a calibrated Superdex S200 column (Fig. 2A). The IκB activity eluted with an apparent molecular mass of about 90 kDa. Interestingly, this molecular mass correlates well with that of the mitogen-activated 90-kDa ribosomal S6 kinase ($p90^{rsk1}$), which was recently shown to function as an IκBα serine 32 phosphorylating enzyme [32,33]. In order to prove the molecular identity of $p90^{rsk1}$ with the 90-kDa IKK eluting in peak 1, we probed all fractions collected from the Q-Sepharose column (data not shown) and from the gel-filtration column, respectively, for the presence of $p90^{rsk1}$ by Western blot analysis using a $p90^{rsk1}$-specific antibody. $p90^{rsk1}$ eluted exclusively in peak 1 of the Q-Sepharose column and, as shown in Fig. 2A, co-eluted exactly with the 90-kDa IκB activity from the analytical gel-filtration column. In an *in vitro* phosphorylation assay (Fig. 2B), various IκBα-derived, IκBβ-derived and IκBε-derived peptides were used to study the substrate specificities of this kinase with amino acid sequences surrounding the dual phosphorylation sites (SxxxS). The purified IKK activity of peak 1 displayed a clear preference for serine 32 (numbering corresponding to the entire amino acid sequence) in IκBα, a similar substrate specificity as described for $p90^{rsk1}$. Serine 36 of IκBα, as well as all the serines in IκBβ and IκBε, were not phosphorylated by the peak 1 kinase. Neither IκBα nor p65 were detectable in association with the purified $p90^{rsk1}$ (data not shown).

Purification and characterization of IκB activity from peak 2

The IκB activity associated with peak 2 from the anion-exchange column appeared to reside on a large protein complex, and was further purified by gel filtration on a Sephacryl S300 column followed by chromatography on a MonoQ column. The resulting activity-containing protein fraction was analyzed on a calibrated analytical gel-filtration column. The IKK activity eluted with an apparent molecular mass of ≥700 kDa, i.e. at the exclusion limit of the Superdex S200 column (Fig. 3A). All individual fractions of the analytical gel-filtration column were probed further for the presence of various known components of the NF-κB activation pathway by Western blotting (Fig. 3B). This analysis revealed that both IKK1 and IKK2, which were recently identified as TNFα-inducible IκB kinases [23–26], co-elute with the IKK activity of peak 2. Further, the α-isoform, β-isoform and ε-isoform of IκB, as well as a series of Rel-family members, namely p65, RelB, cRel, p105 and p100, appear to be associated with this ≥700-kDa kinase complex.

MEKK-1 has been described previously as a potential upstream activator of a 700-kDa IκB kinase complex [34–36]. In order to determine a possible stimulatory effect of MEKK-1 on the isolated IKK complex, we included a constitutively active form of this kinase in our assay. While MEKK-1 alone does not

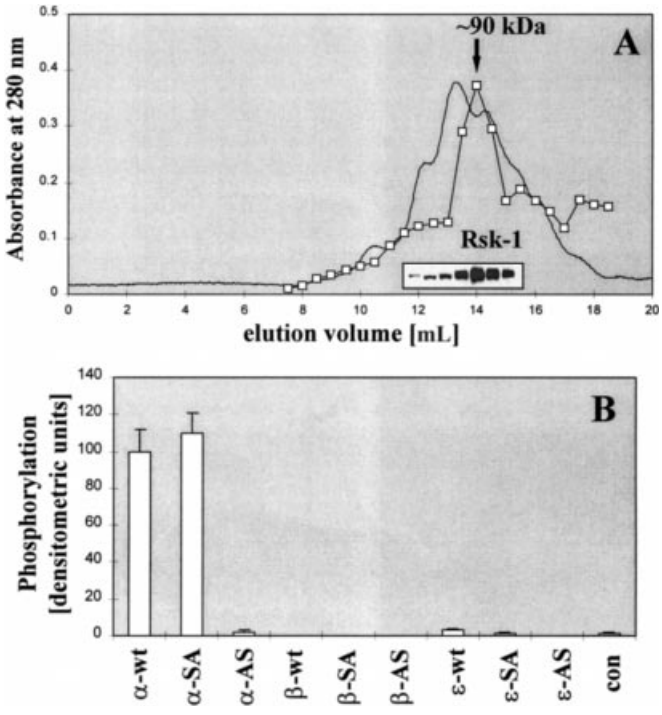

Fig. 2. The IKK activity of peak 1 is mediated by p90^{rsk1}. (A) Fractions from the Q-Sepharose column corresponding to peak 1 were pooled and applied to analytical gel-filtration chromatography on a calibrated Superdex S200 column. Elution of the proteins was followed by measuring the absorbance at 280 nm (solid line). The activity of all protein-containing fractions was determined (○); the apparent molecular mass of the activity peak is indicated above the elution profile. The presence of p90^{rsk1} in the activity-containing fractions was probed by Western blot analysis using a p90^{rsk1}-specific antibody (see inset below elution profile). (B) Biotinylated 15mer peptides spanning the degradation-relevant SxxxS sites of IκBα, IκBβ or IκBε were phosphorylated with γ-^{33}P-labeled ATP using the above IKK activity. The bars indicate the relative incorporation of radioactivity. α-wt, IκBα-derived peptide with wild-type sequence; α-SA, IκBα-derived peptide with serine 36 replaced by alanine; α-AS, IκBα-derived peptide with serine 32 replaced by alanine; β-wt; IκBβ-derived peptide with wild-type sequence; β-SA, IκBβ-derived peptide with serine 23 replaced by alanine; β-AS, IκBβ-derived peptide with serine 19 replaced by alanine; ε-wt, IκBε-derived peptide with wild-type sequence; ε-SA, IκBε-derived peptide with serine 22 replaced by alanine; ε-AS, IκBε-derived peptide with serine 18 replaced by alanine; (all numbers according to full-length protein sequences). Peptides, in which both serines are mutated to alanines (not shown), and a nonrelated peptide (con) were used as negative controls.

phosphorylate IκBα, we found that the activity of the IKK complex towards phosphorylating IκBα was doubled in the presence of MEKK-1 (data not shown). Therefore, the IKK1/2-containing ≥700-kDa complex appears to be semi-activated in the preparation used for these studies, and further activation can be achieved by addition of MEKK-1.

The specificity of the IKK complex towards a panel of IκB-derived peptides was determined in the *in vitro* kinase assay (Fig. 3C). The kinase complex is capable of phosphorylating all three peptides corresponding to the wild-type sequences of IκBα, IκBβ and IκBε (surrounding their respective SxxxS dual phosphorylation motifs) at a comparable rate. However, by using peptide sequences with one or two of the serine residues replaced by alanine(s), we observed a certain preference for the second as compared with the first serine residue of the SxxxS

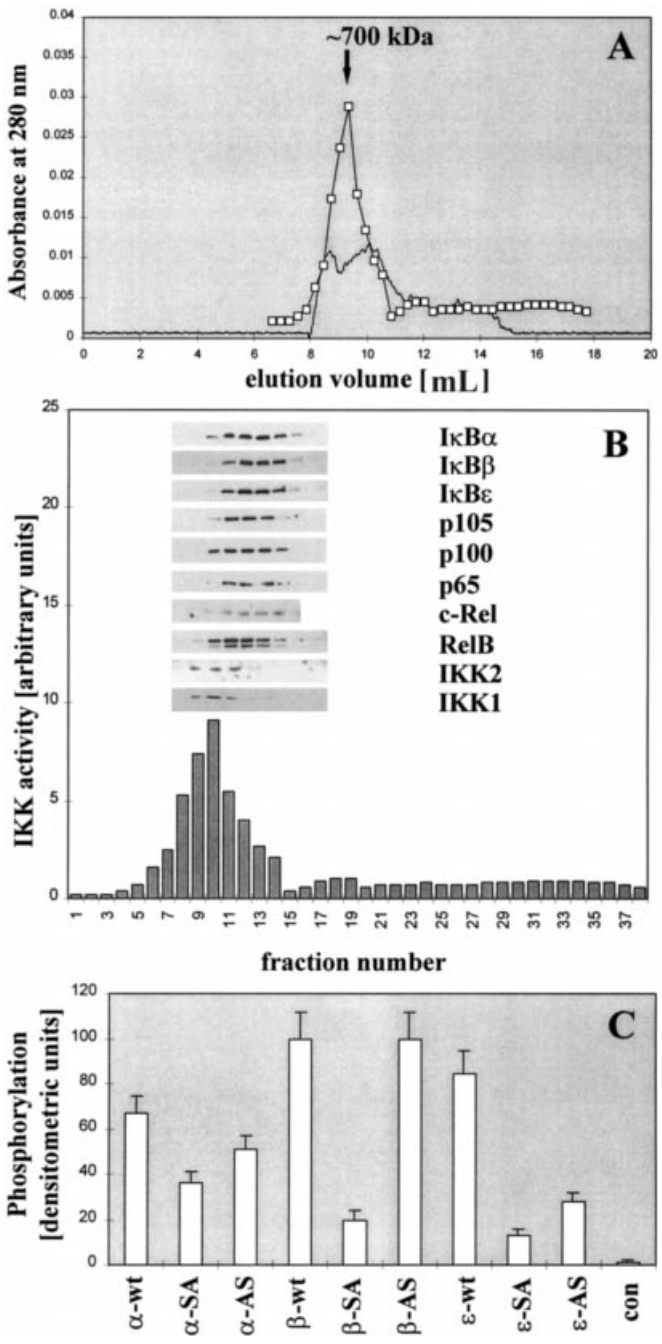

Fig. 3. A ≥ 700 kDa IKK-containing protein complex corresponds to peak 2 IKK activity. IKK activity of peak 2 was further purified and applied to analytical gel-filtration chromatography on a calibrated Superdex S200 column. (A) Elution of the proteins was followed by measuring the absorbance at 280 nm (solid line). The activity of all protein-containing fractions was determined (○); the apparent molecular mass of the activity peak is indicated above the elution profile. (B) Individual fractions of the gel-filtration column were analyzed by Western blot for the presence of NF-κB-related and IκB-related proteins using the indicated antibodies. Relative IKK activity in the fractions is indicated by bars. (C) Biotinylated 15mer peptides spanning the degradation-relevant SxxxS sites of IκBα, IκBβ or IκBε were phosphorylated with γ-^{33}P-labeled ATP using the above purified IKK activity as described in the legend of Fig. 2.

dual-phosphorylation motif in all three peptide series. The most pronounced preference was noticed for the IκBβ peptides in which the serine at position 23 (numbering corresponding to the entire amino acid sequence) was phosphorylated fivefold more efficiently than serine 19.

Purification and characterization of the IKK activity from peak 3

The IKK activity contained in peak 3 of the initial anion-exchange chromatography was further purified by chromatography on hydroxyapatite, followed by a size-exclusion chromatography on Superdex 200. The serine 32 phosphorylating activity eluted from the latter column with an apparent molecular mass of 400 kDa (Fig. 4A). When this activity was analyzed for its specificity towards a panel of IκB-derived peptides in the *in vitro* kinase assay (Fig. 4B), it primarily phosphorylated serine 32 of the IκBα peptide, while IκBβ and IκBε peptides were not phosphorylated to a significant degree.

In order to determine the size of the corresponding polypeptide chain(s) carrying the kinase activity in the 400 kDa complex, eluate fractions from the above-calibrated Superdex 200 column were analyzed in an in-gel-kinase assay (Fig. 4A). For this analysis the proteins were resolved by SDS/PAGE in the presence and absence of IκBα and refolded in the gel. Subsequent to the refolding procedure a kinase reaction with γ-labeled radioactive ATP was performed and radioactively labeled polypeptide chains visualized. In addition to autophosphorylating kinases as identified by this method in the absence of IκBα, a doublet of polypeptide chains with apparent molecular masses of 38 and 42 kDa, respectively, became visible due to their ability to phosphorylate IκBα in the gel. These polypeptide chains co-eluted with the peak 3 IKK activity from the analytical gel filtration column. An apparent high molecular mass in the native state together with molecular masses of 38 and 42 kDa for the corresponding kinase polypeptide chains is reminiscent of CK II [37,38]. So far, however, this kinase is known to phosphorylate IκB only in its C-terminal PEST sequence [39–41]. To test the hypothesis that CK II is indeed contained in the peak 3 IKK preparation and responsible for phosphorylating IκBα at serine 32 all fractions from the analytical gel-filtration column were further analyzed for the presence of the α catalytic subunit of CK II by Western blotting. As shown in Fig. 4A the α subunit co-eluted strictly with the IKK activity which had been purified over three distinct chromatographic columns. Moreover, the enzymatic activity of this pool was efficiently inhibited by the addition of 10 μM heparin sulfate to the *in vitro* kinase assay (data not shown), a hallmark of CK II [42]. Using recombinant full-length wild-type and S32 A/S36 A IκBα variants instead of the peptides in the *in vitro* kinase assay, we found that about twice the amount of

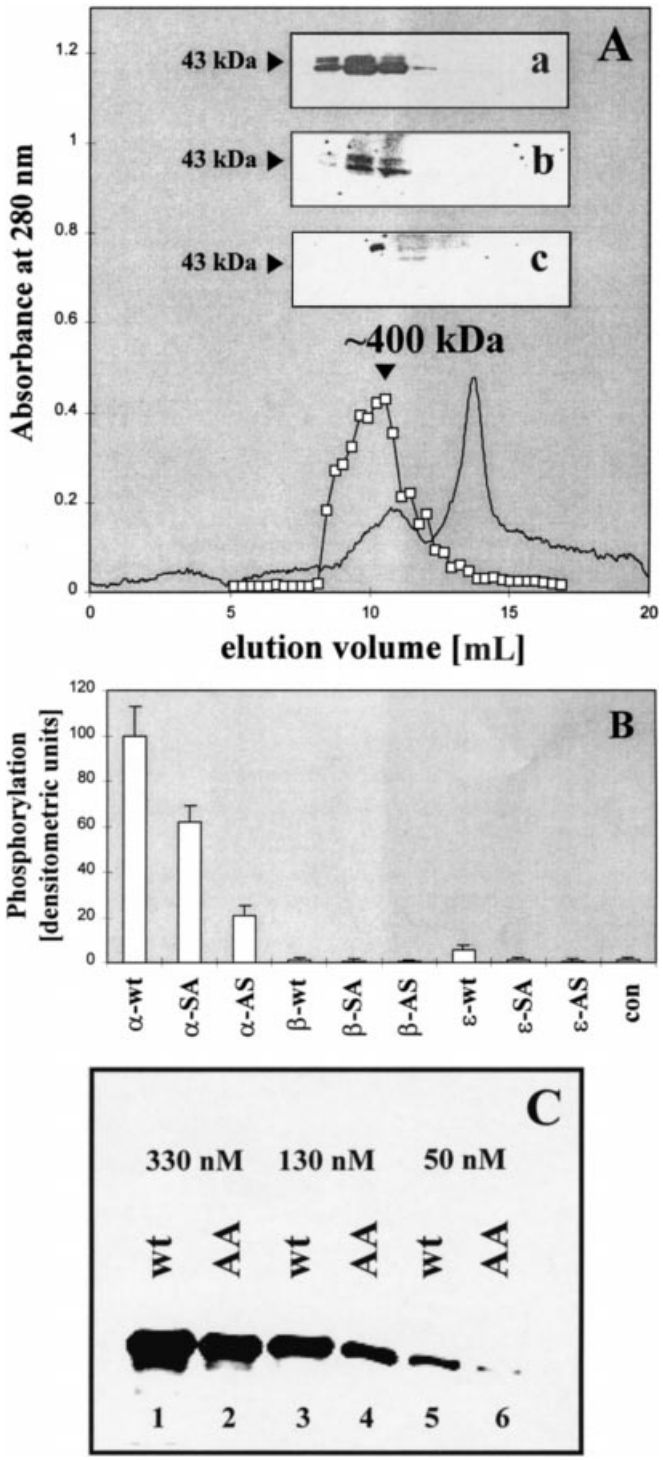

Fig. 4. Peak 3 IKK activity copurifies with CK II. (A) The IKK activity of peak 3 was further purified and applied to analytical gel-filtration chromatography on a calibrated Superdex S200 column. Elution of the proteins was followed by measuring the absorbance at 280 nm (solid line). The activity of all protein-containing fractions was determined (○); the apparent molecular mass of the activity peak is indicated above the elution profile. (Inset a) Individual fractions of the gel-filtration column were analyzed by Western blot for the presence of CK II α/α′ subunits. The same fractions were applied to SDS/PAGE with (inset b) or without (inset c) IκB polymerized into the gel. The resolved proteins were unfolded/refolded from 6 M urea in a stepwise manner by declining the concentration of the denaturant. The kinase assay was carried out in the gel in the presence of γ-^{33}P-labeled ATP. Radioactively labeled polypeptide chains were visualized by exposure to a phosphorimager screen. The apparent molecular mass of the kinase is indicated on the left. (B) Biotinylated 15mer peptides spanning the degradation-relevant SxxxS sites of IκBα, IκBβ or IκBε were phosphorylated with γ-^{33}P-labeled ATP using the above-purified IKK activity as described in the legend of Fig. 2. (C) Full-length recombinant wild-type (lanes 1, 3, 5) or S32A/S36A mutant (lanes 2, 4, 6) IκBα of the indicated concentrations (330 nM, lanes 1, 2; 130 nM: lanes 3, 4; 50 nM: lanes 5, 6) were phosphorylated in the presence of γ-^{33}P-labeled ATP using purified CK II of peak 3. The phosphorylated substrates were subjected to gel electrophoresis, the gel was dried and exposed to a phosphorimager.

radioactivity was incorporated into the wild-type as compared with the mutant protein (Fig. 4C) confirming a significant serine phosphorylation of the IκB N-terminus in addition to phosphorylation of the C-terminal PEST sequence.

Taken together, the above data strongly suggest that the peak 3 IκB kinase is identical to CK II and is able to phosphorylate IκBα on one of its degradation-relevant serines. In contrast to the IKK1/2 complex, however, no association of the purified CK II with IκBα or p65 was detectable (data not shown).

DISCUSSION

The extracted, total IKK activity of TNFα-induced HeLa cells can be separated into three distinct pools using ion-exchange chromatography. The activity that eluted at the lowest ionic strength from the column (cf. Fig. 1), referred to as peak 1 activity, accounts for ~5–10% of the total IKK activity. By combining analytical gel-filtration chromatography with Western blot analysis and *in vitro* enzyme assays, we were able to attribute the IKK activity contained in peak 1 to the presence of $p90^{rsk1}$. This enzyme is commonly placed in the Ras/ MAP kinase cascade [43] as a substrate of the ERKs, $p44^{erk1}$ and $p42^{erk2}$ [44]. Originally, it was described as a kinase that *in vitro* phosphorylates the S6 protein of the ribosomal 40S subunit [45]. However, it is now evident that $p90^{rsk1}$ plays its major role in the induction of several immediate–early genes. $p90^{rsk1}$ has recently been found to specifically phosphorylate IκBα on serine at position 32 in response to 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA [33]). Interestingly, overexpression of a dominant-negative form of $p90^{rsk1}$ does not interfere with IκBα degradation in response to TNFα. This is in good agreement with our finding, that TNFα stimulation of HeLa cells only marginally increases $p90^{rsk1}$ activity and that this activity contributes little to the total IKK activity. The $p90^{rsk1}$ kinase as purified from HeLa cells appears to have a marked preference for phosphorylating IκBα compared with IκBβ and IκBε, and in IκBα particularly targets serine 32 and not serine 36. These observations are explained by the $p90^{rsk1}$ consensus sequence RxxS [46], which is reflected in the region surrounding serine 32 in IκBα, but does not match with the sequence context of serine 36 or the sequence contexts of the relevant serines in IκBβ or IκBε. However, $p90^{rsk1}$-mediated IκBα serine 32 phosphorylation alone is unlikely to cause the inhibitor's degradation, as the proteasome-dependent destruction requires dual phosphorylation of serine 32 and 36 [9–11,13,14,47].

To date, only two kinases have been identified that are capable of inducibly phosphorylating IκBα on both relevant serine residues: IKK1 and IKK2. Both kinases are highly homologous, appear to have the same substrate specificity, and reside on a high-molecular-mass protein complex [23–26]. The IKK activity associated with the second activity peak of the initial anion-exchange column (cf. Fig. 1) appears to correspond to this TNFα-inducible IKK complex. The estimated molecular mass is ≥700 kDa and is in good agreement with previously reported values (ranging between 500 and 900 kDa [18,23,24,26,34]). A model proposing the formation of heterodimers between IKK1 and IKK2, which is based on overexpression of various IKK variants combined with co-immunoprecipitation studies [26], is further substantiated by our finding that endogenous IKK1 and IKK2 copurify strictly and exclusively occur in the same column fractions. The IKK protein complex displays a similar propensity to phosphorylate peptides which contain the dual phosphorylation motifs of IκBα, IκBβ, or IκBε (Fig. 3C). However, a certain preference for the second serine in the SxxxS motif (most notable for the IκBβ peptide, and also observed for the full-length IκBβ protein [27]); was evident. Perhaps this preference might explain the different phosphorylation/degradation kinetics observed for IκBα and IκBβ after TNFα stimulation in certain cell types [11,16,48].

Partial stimulation of the HeLa cell cytosol with okadaic acid allowed us to copurify various endogenous substrates together with the IKK protein complex. In particular, all known IκB family members, i.e. the more recently isolated IκBε in addition to the characterized IKK substrates IκBα and IκBβ, are associated with the IKK complex and thus appear to be regulated by the same upstream kinases. Further, the NF-κB/Rel family members p65/RelA, RelB, cRel, p105 and p100 are contained in this apparently pre-assembled kinase–substrate complex. The latter two Rel proteins are the precursor forms of p50 and p52, respectively. The C-terminal regions of these proteins are highly homologous with the IκB proteins and seem to serve as intramolecular inhibitors [49–51]. Therefore, whereas p65/RelA, RelB and cRel might bind to the IKK complex via their interaction with IκB or IκB-containing proteins, p100 and p105 may well interact directly with the kinase through their IκB regions. The reason for this association is currently unknown. Earlier reports [52–54] suggest that their processing to the mature forms is stimulated by TNF, correlates with their phosphorylation and occurs within a similar time range as does phosphorylation and degradation of IκBα. However, neither p100 nor p105 contain an obvious SxxxS motif, and it now seems that the mature forms are generated by cotranslational processing [55].

Two other proteins have been reported to associate with the IKK complex [24], the mitogen-activated kinase phosphatase-1 (MKP-1) and the mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK-1). Neither of the two proteins copurified with the IKK complex from HeLa cells in our experiments. The kinase activity of the ≥700 kDa IKK complex was, nevertheless, stimulated by a factor of two in the *in vitro* IκB phosphorylation assay by co-incubation with a constitutively active GST–ΔMEKK-1 fusion protein (data not shown). This confirms that MEKK-1 can function *per se* as a direct activator of the IKK complex [34–36].

The kinase responsible for the activity that elutes with peak 3 of the anion-exchange column corresponds to CK II. Casein kinase II has been shown previously to exclusively phosphorylate IκBα at several serine and threonine residues in its C-terminal PEST region [39–41,56], an amino acid sequence rich in prolines, glutamic/aspartic acids, serines and threonines. In contrast to these reports we found that CK II was also able to phosphorylate serine 32 in IκBα, one of the serines involved in its rapid cytokine-induced degradation. This serine residue is contained in a CK II consensus site (SXXE/D, with X being any amino acid [57]). When the full-length wild-type and S32 A/S36A mutant recombinant IκBα's were phosphorylated in the presence of γ-^{33}P-labeled ATP, twice the amount of radioactivity was incorporated into the wild-type as compared with the mutant protein. Therefore, CK II appears to phosphorylate serine 32 almost as efficiently as all other serine and threonine residues of IκBα together. Similar, but without being obviously related to $p90^{rsk1}$, the biochemically purified CK II displayed a remarkable substrate specificity in the *in vitro* peptide phosphorylation assay. It phosphorylates serine 32 of the IκBα-derived peptide far better than serine 36 or any of the other IκBβ-derived and IκBε-derived peptides. However, at present the physiological relevance of this phosphorylation is unclear. In addition to having similar substrate specificities towards serine 32 in IκBα, both $p90^{rsk1}$ and CK II are activated by mitogenic stimuli [44,58,59]. This finding perhaps reflects

the existence of an alternative route to induce NF-κB. If so, and keeping in mind that dual phosphorylation of IκBα at serine 32 and 36 is obligatory for IκBα degradation, it will be interesting to learn how the second serine residue is phosphorylated and which enzyme(s) are involved.

ACKNOWLEDGMENTS

We thank Ute Manning for critical reading of the manuscript and Elizabeth Brooke-Powell, Marie-Josephe Duriatti and Christine Haffner for technical support. We further thank Brian P. Richardson for his unbureaucratic and spontaneous decision to financially support a post-doctorate position for this project.

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