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IκB Kinase and NF-κB Signaling in Response to Pro-Inflammatory Cytokines

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1. Introduction

Exposure to pro-inflammatory cytokines (TNFα and IL-1) results in activation of signaling cascades that lead to stimulation of activity of the transcription factors AP-1 and NF-κB and induction of genes that are controlled by these transcription factors. Such genes code for cytokines (TNFα, IL-1, IL-2, IL-6, and GM-CSF), chemokines, adhesion molecules, and enzymes (iNOS and X-2) that produce secondary inflammatory mediators ***(1)***.

Transcription factors of the Rel/NF-κB family function as heterodimers and in mammals include RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). Unlike the Rel proteins, which are synthesized in their functional forms, NF-κB1 and NF-κB2 are synthesized as large precursors, p105 and p100, respectively, that require proteolytic processing to produce the mature p50 and p52 subunits ***(2,3)***. In nonstimulated cells, NF-κB dimers are kept inactive in the cytoplasm through interaction with inhibitory proteins, the IκBs. In response to cell stimulation by a variety of stimuli including cytokines, endotoxin (LPS), dsRNA, oxidants and ionizing radiation, a multisubunit complex, the IκB kinase complex (IKK) is rapidly activated and phosphorylates two critical serines (e.g., Ser-32 and Ser-36 in IκBα) in the N-terminal regulatory domain of the IκBs ***(4,5)***. Phosphorylated IκBs are recognized by a specific E3 ubiquitin ligase complex and undergo polyubiquitination, which targets them for rapid degradation by the 26S proteasome ***(6)***. NF-κB dimers, freed from the IκBs, translocate to the nucleus and activate the transcription of specific target genes.

The IKK complex is composed of three subunits: two highly homologous

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catalytic subunits, IKKα and IKKβ and a regulatory subunit, IKKγ (or NEMO) ***(4)***. Biochemical studies and analysis of knockout mice indicate that the catalytic subunit IKKβ and the regulatory subunit IKKγ but not the catalytic subunit IKKα, are absolutely required for IκB phosphorylation and NF-κB activation in response to pro-inflammatory stimuli ***(7–14)***. IKK activation by such stimuli depends on phosphorylation of the IKKβ subunit at two serine residues (Ser-177 and Ser-181) within its activation loop ***(7)***. Despite the conservation of these serines in the IKKα subunit, IKKα does not play an essential role in IKK and NF-κB activation by all major stimuli ***(7)***. Instead, IKKα plays a critical role in developmental processes, in particular terminal differentiation of keratinocytes during formation of the epidermis ***(13,14)***. Recently, IKKα has been found to be involved in activation of a second NF-κB pathway involving processing of NF-κB2 ***(15)***. This pathway is particularly important for B-cell maturation and secondary lymphoid organ formation, key events in the development of adaptive immunity.

This chapter focuses on the basic techniques allowing the study of IKK and NF-κB activation in response to cell stimulation by pro-inflammatory cytokines.

2. Materials

2.1. Stock Solutions

1. Stocks of the reagents are prepared and stored as follows and are stable for at least 6 mo.
   a. Dithiothreitol (DTT) (1 *M*) in 10 m*M* sodium acetate, stored at –80°C.
   b. Phenylmethylsulfonide fluoride (PMSF) (100 m*M*) in ethanol, stored at room temperature.
   c. *p*-nitrophenyl phosphate (*p*NPP) (1 *M*) in H_2O, stored at –20°C.
   d. Na_3VO_4 (100 m*M*) in H_2O, stored at 4°C.
   e. Aprotinin (10 mg/mL) in 10 m*M* HEPES, pH 8.0, stored at –80°C.
   f. Bestatin (10 mg/mL) in H_2O, stored at –80°C.
   g. Leupeptin (10 mg/mL) in H_2O, stored at –80°C.
   h. Pepstatin (10 mg/mL) in ethanol, stored at –80°C.
   i. All protease inhibitors are available from Sigma and Calbiochem (San Diego, CA).
2. Sterile penicillin/streptomycin stock solution and 200 m*M* L-glutamine stock solution are kept at –20°C in 10-mL aliquots. These reagents, as well as Dulbecco's modified Eagle's medium (DMEM), may be obtained from Gibco BRL, Life Technologies, Rockville, MD.
3. Recombinant mouse and human TNFα and IL-1 are available from several commercial sources (BioSource International, Camarillo, CA or R&D Systems, Minneapolis, MN). The lyophilized cytokines are reconstituted at 10 µg/mL in PBS

plus 0.1% bovine serum albumin (BSA, tissue culture grade) and stored at –80°C in 100-µL aliquots.
4. ATP stock solution: 100 m*M* in H_2O, stored at –80°C in 50-µL aliquots.
5. ^{32}P-orthophosphate (cat. no. Nex-53C) is from NEN Life Science Products, Boston, MA).
6. [γ-^{32}P]ATP is from Amersham Pharmacia Biotech, Piscataway, NJ.

2.2. Cell Lysis Buffers

1. Lysis buffer: 50 m*M* Tris-HCl, pH 7.6, 250 m*M* sodium chloride (NaCl), 3 m*M* ethylenediamine tetraacetic acid (EDTA), 3 m*M* ethyleneglycol tetraacetic acid (EGTA), 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet (P-40) (NP40), 10% glycerol, 20 m*M* sodium fluoride (NaF), 40 m*M* β-glycero-3-phosphate. This buffer is prepared as a stock solution, filtered through a 0.45-µm filter unit, and stored at 4°C. Before use, the following should be added: 2 m*M* DTT, 1 m*M* PMSF, 2 m*M* *p*NPP, 1 m*M* sodium orthovanadate (Na_3VO_4), and 10 µg/mL each of aprotinin, bestatin, leupeptin, and pepstatin (*see* **Subheading 2.1.**).
2. RIPA buffer: 20 m*M* Tris-HCl, pH 7.6, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1% NP40, 0.5% (w/v) sodium deoxycholate (DOC), 0.05% sodium dodecyl sulfate (SDS), 10% glycerol, 20 m*M* NaF, 40 m*M* β-glycero-3-phosphate, 2.5 m*M* sodium metabisulfite, 5 m*M* benzamidine, 2 m*M* DTT, 1 m*M* PMSF, 20 m*M* *p*NPP, 1 m*M* Na_3VO_4, and 10 µg/mL each of aprotinin, bestatin, leupeptin, and pepstatin (*see* **Subheading 2.1.**).

2.3. Bacterial Culture Medium and Bacteria Lysis Buffers

1. Lbroth: bacto-tryptone 10 g/L, bacto-yeast extract 5 g/L, NaCl 5 g/L, adjusted to pH 7.5 with NaOH. 100 µg/mL ampicillin and 2% glucose are added.
2. Base buffer: PBS, pH 8.0, 1 m*M* EDTA, 0.5% (v/v) Triton X-100, 5 m*M* DTT, store at 4°C. This buffer is stable for several months if prepared without DTT. DTT can be added just before use (*see* **Subheading 2.1.**).
3. Buffer I : Base buffer containing 0.3 *M* ammonium sulfate [$(NH_4)_2SO_4$].
4. Lysis buffer: Buffer I supplemented with 1 m*M* PMSF and 10 µg/mL of protease inhibitors (aprotinin, bestatin, leupeptin, and pepstatin) (*see* **Subheading 2.1.**).
5. Elution buffer: 100 m*M* Tris-HCl, pH 8.0, 20 m*M* glutathione.
6. Dialysis buffer: 20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 0.2 m*M* EDTA, 10 m*M* β-glycero-3-phosphate and 10% glycerol.

2.4. Kinase Assay

1. Kinase buffer (10X): 200 m*M* HEPES, pH 7.5, 100 m*M* magnesium chloride ($MgCl_2$).
2. Reaction buffer: kinase buffer [1X] supplemented with 20 m*M* β-glycero-3-phosphate, 10 m*M* *p*NPP, 1 m*M* DTT, and 20 µ*M* "unlabeled" ATP. This buffer should be prepared just before use.

2.5. Reagents for Electrophoretic Mobility Shift Assay

1. Oligonucleotide probes:
   NF-κB:5'-GGATCCTCAACAGAGGGGACTTTCCGAGGCCA-3'
   3'- AGTTGTCTCCCCTGAAAGGCTCCGGTCCTAGG-5'
   NF-Y:5'-GTAGGAACCAATGAAATGCGAGG-3'
   3'- TTGGTTACTTTACGCTCCGGATG-5'
   Consensus oligonucleotide sequences for binding of transcription factors are also available from Promega (Madison, WI). The oligonucleotides are diluted at 1 µg/µL in TE buffer (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA) and stored at –20°C.
2. Klenow buffer (1X): 10 m*M* Tris-HCl, pH 7.9, 50 m*M* NaCl, 10 m*M* $MgCl_2$, and 1 m*M* DTT).
3. EMSA buffer (10X): 100 m*M* Tris, pH 7.6, 500 m*M* potassium chloride (KCl), 10 m*M* EDTA, and 50% glycerol, stored at –20°C.
4. Nu-Clean D25 spin columns for probe purification are available from Eastman Kodak Company, Rochester, NY.
5. Poly(dI.dC) is from Amersham Pharmacia Biotech.
6. Gel loading buffer (10X): 250 m*M* Tris-HCl, pH 7.6, 40% glycerol, and 0.2% (w/v) bromophenol blue, stored at 4°C.
7. Acrylamide stock solution (40%): Dissolve 38.71 g of acrylamide and 1.29 g of bis-acrylamide in 100 mL H_2O. Solution is filtered and kept at 4°C.
8. TBE (10X): Dissolve 121.1 g of Tris base, 55 g of boric acid, and 7.4 g of EDTA into 1 L of H_2O. Adjust the pH to 8.3 with solid boric acid.
9. Nondenaturing gel: Mix 36 mL of H_2O, 4.5 mL of acrylamide stock solution, 4.5 mL of TBE 10X, 450 µL of a 10% (w/v) solution of ammonium persulfate (APS) in H_2O, and 45 µL of TEMED.

3. Methods

3.1. Cell Culture and Cytokine Stimulation

1. Adherent cells (HeLa, HEK295, 3T3 fibroblasts) are cultured in DMEM high glucose supplemented with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, and 1% (v/v) penicillin/streptomycin stock solution at 37°C in a 5% CO_2 atmosphere.
2. Tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) stocks solutions are diluted, respectively, at 20 ng/mL and 10 ng/mL final concentration in prewarmed serum-free medium, then added to the cells. Stimulation is performed at 37°C for various periods of time.
3. After stimulation, the cells are placed on ice, medium is removed and the cells are washed twice with ice-cold phosphate-buffered saline (PBS). Cells are harvested in 1 mL PBS/100-mm dish using a cell lifter and pelleted by centrifugation for 5 min at 4°C at 2000 g.
4. Cell pellets are kept frozen at –80°C or directly lysed by resuspension in lysis buffer (400 µL per cell pellet from a 100-mm dish) with incubation on ice for 10–15 min. The lysate is centrifuged at 13,000*g* for 10 min at 4°C.

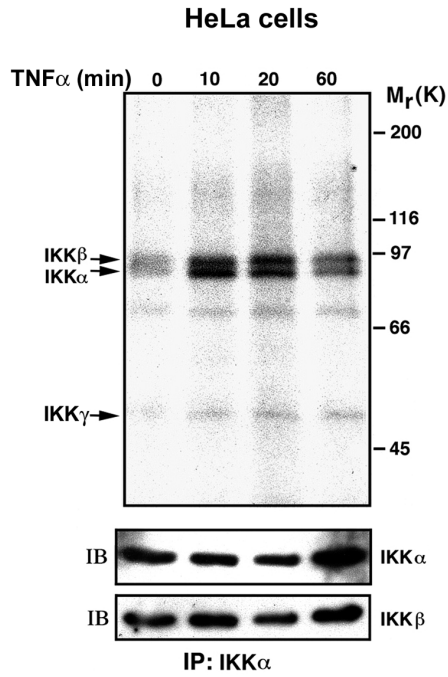

Fig. 1. Cytokine-induced IKK phosphorylation. HeLa cells incubated for 5 h with [^{32}P]orthophosphate were stimulated with TNFα (20 ng/mL) for the indicated times, then lysed. The IKK complex was immunoprecipitated (IP) with an antibody to IKKα (clone # B78-1 from PharMingen), resolved by gel electrophoresis and transferred onto a PVDF membrane. Phosphoproteins were detected by autoradiography. IKKα and IKKβ were identified by immunoblotting (IB) using specific antibodies (anti-IKKα, clone # B78-1 from PharMingen and anti-IKKβ H470 from Santa Cruz Biotechnology).

5. The supernatant is transferred to a clean tube and the protein concentration is determined by Bradford assay *(17)*.
6. The lysates (WCE, whole cell extracts) are stored at –80°C until use. WCEs can be used for analysis of protein composition by western blotting, immune-complex kinase assay and electrophoretic mobility shift assay (EMSA).

3.2. Detection of IKK Phosphorylation by ^{32}P Metabolic Labeling (see *Note 1)*

1. Cells at subconfluence (70–80%) (one 60-mm dish per assay) are washed twice in phosphate-free DMEM and incubated for 1 h in the same medium.
2. The medium is then replaced by labeling medium (4 mL per dish) consisting of phosphate-free DMEM supplemented with 10% dialyzed FBS and 1–2 mCi/mL ^{32}P-orthophosphate. Cells are labeled for 4–5 h at 37°C.
3. TNFα or IL-1 stimulation of the cells is performed as described in **Subheading**

3.1. Cytokines are diluted directly into the labeling medium (20 ng/mL for TNFα and 10 ng/mL for IL-1) and stimulation is performed for 5 to 15 min.

4. Bulk IKK phosphorylation is observed after 5 min following cytokine stimulation (*see* **ref. *7*** and **Fig. 1**). Labeling medium is removed, cells are placed on ice, and washed twice with ice-cold PBS.
5. Cells are lysed by addition of 1 mL of lysis buffer directly to the culture dish. Cells are scraped using a cell lifter, transferred to a microcentrifuge tube and lysed on ice for 10 min. Cellular debris are pelleted by centrifugation at 13,000*g* for 10 min at 4°C.
6. Supernatants are transferred to clean tubes. Cell lysates are clarified by incubation for 1–2 h at 4°C on a rotator in the presence of 10 µL of preimmune serum (if available) or 10 µg of nonspecific control IgG and 50 µL of 50% slurry of protein A/G-Sepharose beads. Beads are pelleted by centrifugation at 13,000*g* for 5 min at 4°C and supernatants are transferred to clean tubes.
7. Immunoprecipitation of the IKK complex is performed by rotating the cell lysate overnight at 4°C after addition of 1–5 µg of anti-IKK antibody (*see* **Note 2**) and 25 µL of 50% of slurry of protein A/G-Sepharose beads. The immune-complexes are collected by centrifugation for 1 min at 13,000*g*, then washed once in lysis buffer containing 300 m*M* NaCl, once in the same buffer supplemented with 2 *M* urea and twice in RIPA buffer. The supernatants are discarded and the immune complexes are resuspended in SDS-loading buffer.
8. The IKK subunits are resolved on a 7.5% SDS-PAGE gel (12X 16 cm) according to a standard procedure ***(17)***. The proteins are transferred onto a nitrocellulose or Immobilon-P polyvinylidene fluoride membrane. The phosphoproteins are detected by exposure of the membrane to Kodak, Biomax-MR film for 2–12 h at –80°C. After exposure, the membrane can be used to detect protein levels by immunoblotting.

3.3. Measurement of IKK Activity by An Immune-Complex Kinase Assay

Rapid and transient activation of the IKK complex in response to cell stimulation by pro-inflammatory cytokines (TNFα and IL-1) can be best measured and quantified by an immune-complex kinase assay using GST-IκBα (1–54) as a substrate (*see* **Fig. 2A**).

Fig. 2. *(opposite page)* IKK and NF-κB activation by TNFα and IL-1. (**A**) HeLa cells (left panel) and LL-2 cells (lung carcinoma cell line, right panel) were stimulated with TNFα and IL-1 for the indicated times. IKK was isolated by immunoprecipitation with anti-IKKα (clone # B78-1, PharMingen) or anti-IKKγ(clone 73-764, PharMingen) and its activity was measured by phosphorylation of GST-IκBα (1-54) as described in **Subheading 3.3.** (**B**) IκBα degradation was analyzed by immunoblotting. IκBα phosphorylation (middle panel) was detected using an antibody directed against P-Ser-32 of IκBα (#9241, New England Biolabs, Beverly, MA). IκBα (bottom panel) was detected by immunoblotting using an anti-IκBα antibody (#IMG-127, Imgenex or # C-21, Santa

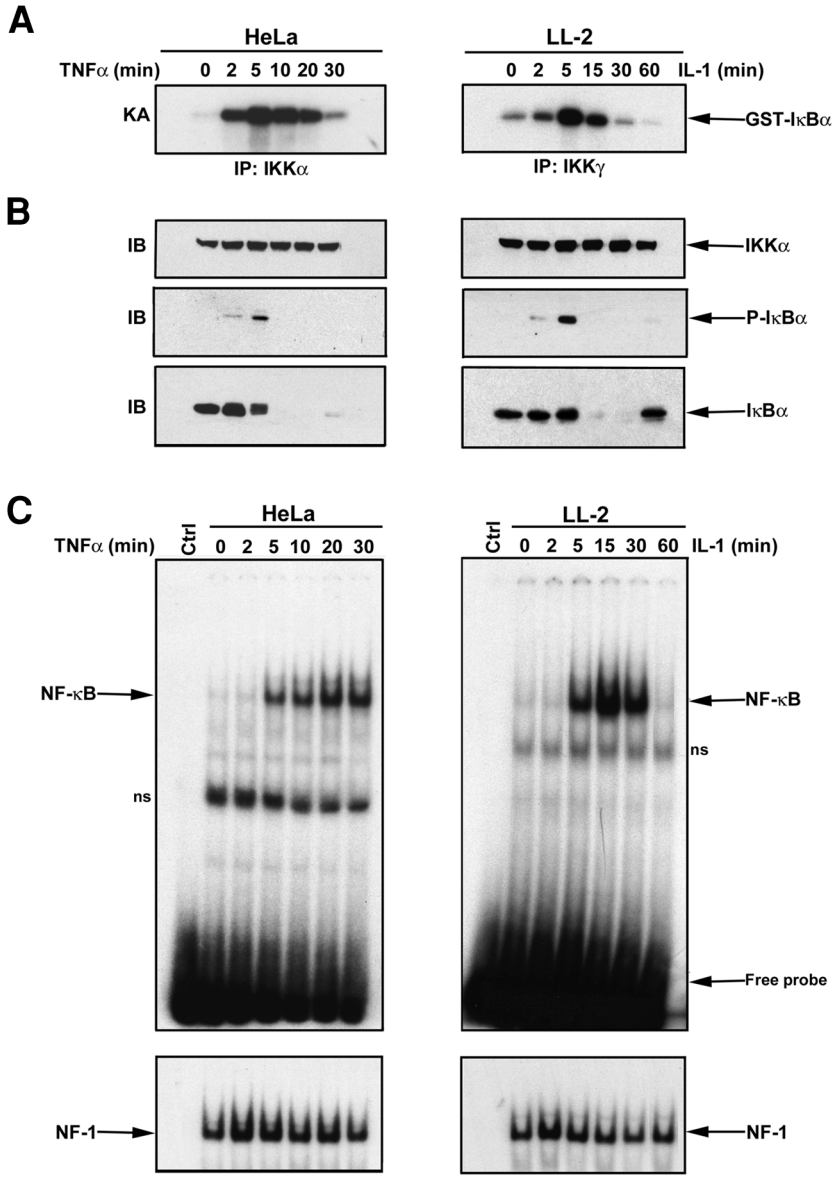

Fig. 2. *(continued)* Cruz Biotechnology). IKKα expression (top panel), used as control for equal protein loading, was determined by immunoblotting using an anti-IKKα antibody (#IMG-136, Imgenex). (**C**) NF-κB DNA-binding activity was measured by electrophoretic mobility shift assay (EMSA). Cells were left untreated or were stimulated with TNFα (left panel) or IL-1 (right panel) for the indicated times, after which WCEs were prepared and NF-κB and NF-Y DNA-binding activities were measured by EMSA as described in **Subheading 3.4.** ns = nonspecific.

3.3.1. Expression and Purification of GST-IkBa (1–54) Fusion Protein in Escherichia coli

1. A cDNA fragment encoding the first 54 N-terminal amino acids (including Ser 32 and Ser 36) of human IκBα was inserted in frame at the 3' end of the GST sequence of a pGEX expression vector (Amersham Pharmacia Biotech) (*see* **Note 3**).
2. BL21 bacteria transformed with this recombinant plasmid are grown at 37°C in 500 mL Lbroth containing 100 µg/mL ampicillin and 2% glucose until the absorbance at 600 nm measured in a 1-cm pathlength cuvet is between 0.4 and 0.5.
3. Protein expression is induced by addition of 2 m*M* isopropyl-β-thiogalactopyranoside (IPTG) for 3 h at 37°C.
4. Bacteria are collected by centrifugation and resuspended on ice in 30 mL of ice-cold bacteria lysis buffer. Lysozyme is added to a final concentration of 1 mg/mL and bacteria are lysed by sonication (Virsonic 600 sonicator). Bacterial debris are removed by centrifugation at 20,000*g* for 20 min at 4°C.
5. The supernatant is transferred to a clean tube and incubated for 1 h at 4°C with 500 µL of 50% slurry of glutathione Sepharose 4B equilibrated with PBS.
6. The beads are collected by centrifugation at 500*g* for 5 min and washed twice with buffer I containing 1 m*M* PMSF, twice with base buffer containing 1 m*M* PMSF and once with PBS (10 bed volumes for each wash). The beads are then transferred to a microcentrifuge tube.
7. The GST fusion protein is eluted by incubation of the beads with two bed volumes of elution buffer for 1 h at 4°C on a rotator. After centrifugation, the supernatant is collected and the beads are subjected to a second 1-h elution step. The supernatants from the two elution steps are combined and dialyzed at 4°C against 2 L of dialysis buffer.
8. The quality and quantity of fusion protein is evaluated by SDS-PAGE followed by Coomassie blue staining ***(17)*** using known amounts of BSA as standard. GST-IκBα (1–54) protein solution is stored at –80°C in 100 µL aliquots.

3.3.2. IKK Immune-Complex Kinase Assay

1. To determine whether IKK activity is induced by a given stimulus, cells are left untreated or are treated for various periods of times with a specific activator (for example TNFα or IL-1) as described in **Subheading 3.1.** WCEs are prepared as described in **Subheading 3.1.**
2. WCEs containing equal amounts of proteins (typically 20–100 µg) from nonstimulated and stimulated cells are aliquoted into prechilled microcentrifuge tubes. The volume is adjusted to 300 µL with cell lysis buffer.
3. Anti-IKK antibody (1 to 2 µg per reaction) (*see* **Note 2**) or control serum (preimmune serum or control IgG) and 20 µL of 50% slurry of protein A/G-Sepharose are added to the lysates. Immunoprecipitation is performed for a minimum of 2 h at 4°C on a rotator.
4. The immune-complexes bound to the beads are collected by centrifugation at 2000*g* for 1 min at 4°C. The supernatants are discarded and the beads are washed once with lysis buffer containing 400 m*M* NaCl, once with the same buffer containing 2 *M* urea and twice with 1X kinase buffer.

5. The immune-complexes are resuspended in a 30-µL reaction buffer as described in **Subheading 2.4.** containing 5µCi of [γ-^{32}P]ATP (6000 Ci/mmole) and 1 µg of GST-IκBα(1–54) substrate (*see* **Subheading 3.3.1**). The kinase reaction is allowed to proceed for 30 min at 30°C.
6. The reactions are stopped by addition of SDS sample loading buffer ***(16)***. The samples are heated at 100ºC for 5 min to denature the proteins and then analyzed by SDS-PAGE (10% gel). Electrophoresis is stopped before the free isotope (which migrates on the gel together with the yellow *p*NPP band) runs off the gel.
7. The bottom of the gel containing the free isotope is cut off and discarded. The gel is stained with Coomassie blue ***(17)***, destained in 10% acetic acid, 10% methanol in H_2O and dried. This step allows visualization of the substrate to verify equal loading.
8. The dried gel is exposed to Kodak X-OMAT-AR films. Alternatively, the kinase assay gel can be directly transferred to a PVDF membrane, which is then exposed to film. This allows further detection of IKK in the immune-complexes by immunoblotting.
9. The IKK activity is determined by the amount of radioactive ^{32}P incorporated into the GST-IκBα(1-54) substrate. Quantification can be performed on a phosphoimager (preferred) or by densitometric scanning of the films.

3.4. Measurement of NF-κB Activation by Electrophoretic Mobility Shift Assay

3.4.1. Oligonucleotide Probe Labeling

1. Complementary oligonucleotides are synthesized according to consensus sequences for binding of transcription factors (*see* **Subheading 2.5.**).
2. 10 µL of both oligonucleotide stock solutions (at 1 µg/µL) are diluted into 100 µL of TE in a microcentrifuge tube. The tube is heated at 80°C for 10 min in a beaker of water, then slowly cooled down to room temperature (about 1 h) to allow annealing of the oligonucleotides. The double-stranded (ds) oligonucleotide stock solution is stored at –20°C.
3. The EMSA probe is prepared by incubation for 20 min at room temperature of 1 µL of ds oligonucleotide stock solution in a 20-µL reaction mixture containing 1X Klenow buffer (*see* **Subheading 2.5.**). 25 µ*M* of each dATP, dTTP, and dGTP, 50 µCi [α-^{32}P]dCTP and 5 units of Klenow enzyme (*see* **Note 4**).
4. The labeling reaction is stopped by addition of 25 µ*M* of unlabelled dCTP and incubation is continued for 5 min at room temperature.
5. The probe is purified on a Nu-Clean D25 spin column (*see* **Note 5**) and stored at –20°C.

3.4.2. Electrophoretic Mobility Shift Assay

1. Aliquots of WCEs containing 10 µg of protein (adjusted to 10 µL with cell lysis buffer) are incubated for 30 min at room temperature in a 20-µL volume reaction containing 2 µL of 10X EMSA buffer (*see* **Subheading 2.5.**), 1 µL of 20 m*M* DTT, 2 µg of poly(dI.dC) and 15,000–30,000 cpm of probe. H_2O is added to bring the volume to 20 µL.

2. The reactions are stopped by addition of 2 µL of 10X gel loading buffer (*see* **Subheading 2.5.**) and the samples are run on a 4% nondenaturing polyacrylamide gel (20 × 20 cm).
3. Electrophoresis is stopped when the free probe (which migrates together with the bromophenol blue band) is 2.5 cm from the bottom of the gel. The gel is fixed (optional) for 15 min in a solution of 10% acetic acid, 10% methanol in H_2O, then dried and exposed to Kodak X-OMAT-AR film for autoradiography (*see* **Fig. 2C**).

4. Notes

1. Precautions must be taken when performing metabolic labeling with ^{32}P-orthophosphate. Work must be carried out behind a 2.5-cm-thick acrylic shield. A thick layer of lead can be attached to the outside of the shield to provide additional protection against radiation. A 1-cm-thick acrylic cell house should be used to provide shielding when the cell dishes are in the incubator or when they are moved in the laboratory. Researchers should wear two layers of gloves during all steps of the labeling procedure.
2. Immunoprecipitation of the entire IKK complex can be performed using an antibody against any of the IKK subunits. Several commercial antibodies have been tested and found to quantitatively immunoprecipitate the IKK complex. We currently use anti-IKKα [clone # B78-1, PharMingen (San Diego, CA), this monoclonal antibody recognizes the human IKKα but not the mouse IKKα], anti-IKKα M280 [Santa Cruz Biotechnology (Santa Cruz, CA)], anti-IKKγ (clones # C73-764 and C73-1794, PharMingen) and anti-IKKβ H470 (Santa Cruz Biotechnology) ***(7,13)***. Intensive washings of the immunoprecipitates in RIPA buffer may disrupt interaction between IKKγ and the IKKα/IKKβ dimers. Therefore, to examine IKKγ phosphorylation, we recommend immunoprecipitating the IKK complex using an antibody against IKKγ.
3. This recombinant plasmid as well as a mutant version in which Ser 32 and Ser 36 have been replaced by Ala (GST-IκBα(1-54)AA) are available from the author's laboratory upon request.
4. If the annealed oligonucleotides do not contain 5' protruding ends, but instead are blunt ended, labeling cannot be performed using Klenow enzyme. Instead T_4 polynucleotide kinase in the presence of [γ-^{32}P] ATP should be used ***(17)***.
5. Unincorporated nucleotides can also be removed by centrifugation through a "self made" 1-mL Sephadex G25 column.

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