Chapter 2

Detection of IκB Degradation Dynamics and IκB-α Ubiquitination

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Abstract

The NF-κB signaling pathway is a primary regulator of inflammation that has been implicated in the pathogenesis of immune disorders and cancer. This signaling network is strictly regulated; in a nonactivated state, NF-κB transcription factors are sequestered in the cytoplasm by the IκB family of proteins. Various pro-inflammatory stimuli result in the phosphorylation and subsequent ubiquitination of IκBs. These events lead to rapid degradation of IκB and allow translocation of the transcription factors to the nucleus. Therefore, ubiquitination and degradation of IκBs are critical steps in NF-κB pathway activation and can serve as a quantitative parameter to assess pathway activation. In this article, we present a detailed protocol for the quantification of in vivo ubiquitination and turnover of IκB-α in response to a variety of cellular stimuli.

Key words NF-κB, RelA, IκB, Immunoprecipitation, Ubiquitin, Ubiquitination, Ubiquitin-like proteins (UBL)

1 Introduction

The NF-κB transcription factors regulate expression of a large number of genes, which in turn drive a variety of physiological processes including innate and adaptive immunity, apoptosis, and cellular differentiation [1]. Under basal conditions, NF-κB transcription factors are sequestered in the cytoplasm by the inhibitory binding of the so-called classical IκB proteins (IκB-α, IκB-β, and IκB-ε) [2]. In response to a variety of stimuli such as inflammatory cytokines or microbial products, these IκB proteins undergo rapid degradation, enabling NF-κB dimers to translocate to the nucleus and activate gene transcription [3].

The degradation results from two modification events: phosphorylation and subsequent ubiquitination of the IκB proteins [1]. These modifications are executed by corresponding enzyme complexes and lead to proteasomal degradation of IκB. The IκB kinase complex (IKK complex), consisting of the adaptor protein NEMO

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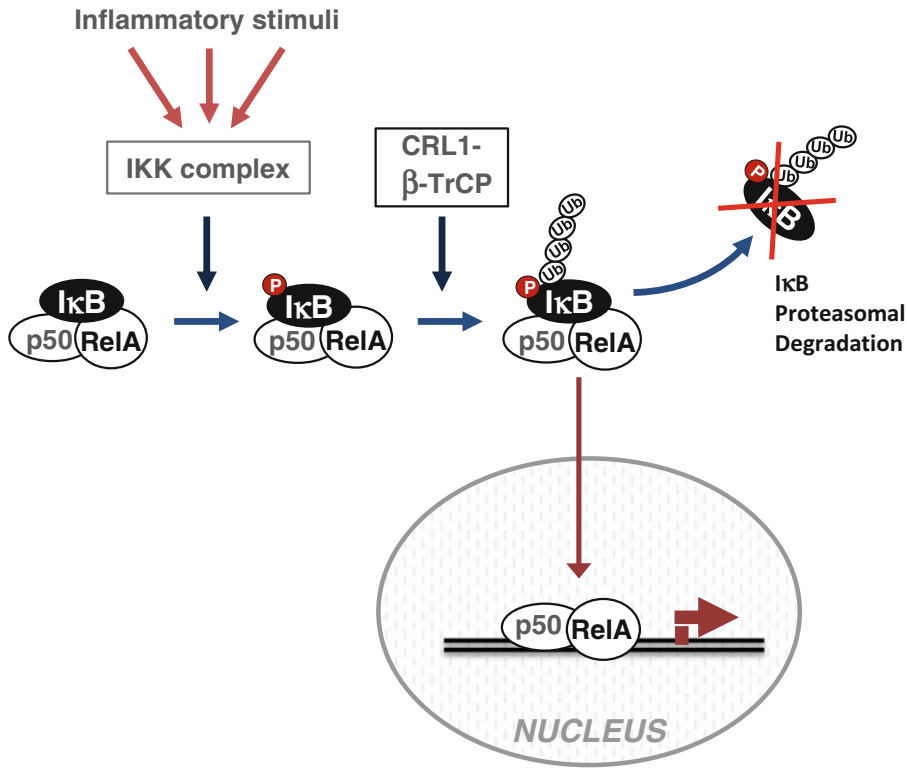

Fig. 1 Schematic representation of IκB-α regulation by phosphorylation and ubiquitination

and the catalytic subunits IKK1 and IKK2, is responsible for the IκB phosphorylation in the classical pathway. Two serine residues in the amino-termini of IκB-α, IκB-β, and IκB-ε are targeted, and the resulting phosphopeptide can be recognized by β-TrCP. This protein serves as the substrate recognition subunit of a multimeric ubiquitin ligase responsible for IκB ubiquitination (Fig. 1), which is known as CRL1-β-TrCP (also containing Skp1, Cul1, and Rbx1) [1].

The interruption of IκB degradation results in significant defects of the NF-κB signaling pathways, and inherited mutations that affect these steps result in developmental and immune defects. Acquired alterations in these pathways are seen in immune disorders and a variety of malignancies. Therefore, enzyme complexes which are involved in phosphorylation and ubiquitination of IκB proteins are key regulators of this pathway and possible targets for drug development. For all these reasons, methods for detecting IκB modifications and degradation are essential tools for understanding the NF-κB pathway. In this regard, IκB phosphorylation and degradation can be detected using a number of commercially available antibodies. However, monitoring IκB ubiquitination in vivo is more challenging due to rapid degradation of the target.

In this article, we review the methods to monitor IκB degradation and a protocol to monitor IκB-α ubiquitination in cells, first developed by Ben-Neriah and colleagues [4] and subsequently used in our laboratory, to study degradation and ubiquitination kinetics within the context of disease-causing mutations affecting the NF-κB pathway.

1.1 Short Method Review

We will describe the method to detect the degradation and endogenous ubiquitination of IκB-α that we typically employ in the laboratory [5], including the antibodies that we find most useful in this regard. Given the rapid degradation of ubiquitinated IκB-α, the detection of this modified form by Western blot of whole cell lysates is not possible in our hands. In addition, direct immunoprecipitation of endogenous IκB-α with a variety of commercially available antibodies proved to be problematic due to low efficiency. Therefore, we utilized a method first described by Ben-Neriah and colleagues [4] for the detection of ubiquitinated IκB-α, which exploits the interaction of phospho-IκB-α (pIκB-α) with the NF-κB subunit p65/RelA. Briefly, cells are treated with the corresponding stimulus (e.g., tumor necrosis factor, TNF, or others) and are then lysed under native conditions. Immunoprecipitation of RelA results in the coprecipitation of NF-κB-associated proteins, including IκB-α. This purified material is then analyzed by Western blotting, which allows for the detection of ubiquitinated IκB-α species using an antibody directed against pIκB-α. Using this approach, monitoring of phosphorylation, ubiquitination, and degradation of IκB-α is feasible and simple (Fig. 2).

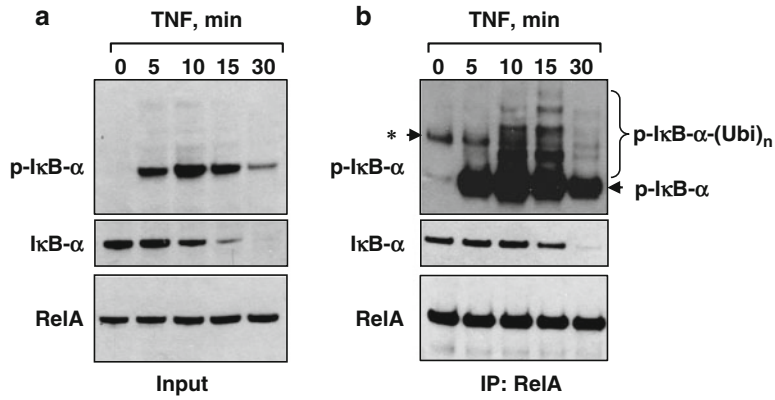

Fig. 2 Purification of ubiquitinated IκB-α from different cell lines as examples of the applicability of the described protocol. Cell lysates from 293 HEK, treated with TNF for indicated times. (**a**) The dynamic of phosphorylation and degradation of IκB-α in total lysates. (**b**) The signal dependent ubiquitination of IκB-α associate with RelA. Maximal ubiquitination rate is observed between 10 and 15 min of stimulation and after dramatically decreased due to proteasomal degradation of IκB-α (30 min sample). RelA serves as loading controls. Asterisk, nonspecific bands

2 Materials

2.1 Reagents

1. Anti-RelA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).
2. Anti-phospho-IκB-α antibody (phospho-Ser32/36; Cell Signaling, Danvers, MA, USA).
3. Anti-IκB-α antibody (Millipore, Billerica, MA, USA).
4. Anti-β-Actin antibody (Sigma, St. Louis, MO, USA).
5. Control rabbit IgG (Santa Cruz Biotechnology).
6. Bradford protein assay.
7. Complete Mini, EDTA free protease/phosphatase inhibitor tablets (Roche, Madison, WI, USA).
8. Dithiotheitol (DTT).
9. Western Lightning Plus-ECL reagent (PerkinElmer, Waltham, MA, USA).
10. Ethylenediaminetetraacetic Acid (EDTA).
11. Glycerol.
12. HEPES.
13. Isopropanol.
14. MegaCD40L soluble recombinant CD40L (Enzo Life Sciences, Farmingdale, NY, USA) (*optional, see* Subheading 3.2).
15. MG-132 (Boston Biochem, Boston, MA, USA).
16. NaCl.
17. Nonfat dry milk.
18. NuPAGE LDS Sample Buffer 4× (Life Technologies, Carlsbad, CA, USA).
19. PBS (phosphate buffered saline), 1× without calcium and magnesium.
20. Phenylmethylsulfonyl fluoride (PMSF).
21. Phorbol 12-myristate 13-acetate (PMA) (*optional, see* Subheading 3.2).
22. Protein A Agarose (Life Technologies).
23. Pre-stained molecular weight marker mix for SDS-PAGE.
24. HRP-linked secondary antibodies (anti-Rabbit IgG and anti-Mouse IgG).
25. Sodium orthovanadate.
26. TNF.
27. Triton X-100.
28. Tween 20.

2.2 Equipment

1. Benchtop microcentrifuge with cooling system for 1.5-mL microcentrifuge tubes.
2. Biophotometer.
3. Cell lifter, 2-cm blade.
4. Filter paper.
5. Inverted microscope.
6. Hypercassette for exposing film.
7. Laminar flow hood and CO_2 incubators for cell culture.
8. Microcentrifuge tubes, 1.5 mL.
9. Rocking platform.
10. Small plastic container for western membrane developing.
11. Serological pipettes and pipette aid.
12. Tissue culture plates, 10-cm (for attached cells) or tissue culture flask, 75 mm (for suspension cells).
13. Tube rotator.
14. X-ray film.

2.3 Cell Lines

The cell line choice will depend on the specific conditions of the experiment. We have utilized this protocol successfully with a variety of cell lines including human embryonic kidney (HEK) 293 cells, HeLa cells, murine, and human immortalized fibroblasts or lymphoblastoid cell lines (LCL). We have not utilized these methods with primary tissues, but they could be adapted to such use.

2.4 Buffers and Stock Solutions

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (RT), unless indicated otherwise. Diligently follow all waste disposal regulations when disposing waste materials. The following solutions can be made ahead of time:

1. 1 M DTT (store at –20 °C).
2. 0.5 M EDTA.
3. 1 M HEPES, pH 7.2 (store at 4 °C).
4. 5 M NaCl.
5. 100 mM PMSF in isopropanol (store at –20 °C).
6. 100 mM sodium orthovanadate (store at –20 °C, *see* **Note 1**).
7. 3× NuPAGE LDS Sample Buffer with 0.3 M DTT: mix 800 µL of 4× NuPAGE LDS loading buffer and 400 µL of 1 M DTT (store at –20 °C).
8. PBS-T buffer: PBS with 0.02 % Tween.
9. 5 % solution of nonfat dry milk in 1× PBS-T (store at 4 °C).

10. Lysis buffer: 25-mM HEPES, 100-mM NaCl, 1-mM EDTA, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100. This buffer can be made ahead of time and stored at RT. Just prior to use, add the following to make "complete" lysis buffer: 1-mM PMSF, 10-mM DTT, 1-mM sodium orthovanadate, protease/phosphatase inhibitor Complete Mini tablet (1 tablet per 10 mL of lysis buffer). Complete lysis buffer can be stored at –20 °C for 4 weeks.

3 Methods

3.1 Cell Culture

Culture conditions and cell numbers will depend on the specific cell line being utilized. For HEK 293, HeLa, and primary human fibroblasts, 75 % confluent 10-cm dishes are sufficient to carry out this protocol. We have also utilized suspension cells (patient-derived lymphoblastoid cell lines, LCL) and found that ~5–7.5 × 10^6 cells per sample are similarly sufficient. In general, we recommend seeding the cells in fresh growth medium 24–48 h prior to cell stimulation.

3.2 Cell Stimulation

Treat cells with appropriate stimuli. For HEK 293, HeLa, U2OS, or NIH 3T3, we usually use recombinant commercial TNF at a concentration of 1,000 U/mL. LCL cells are treated with CD-40L (*see* **Note 2**) at concentration 25–50 ng/mL or with PMA (250 nM). Cells are treated for 5–30 min, depending on specific differences between cell lines (*see* **Note 3**). In order to promote the accumulation of the ubiquitinated form of the IκB-α, cells might be simultaneously treated with a proteasome inhibitor (*see* **Note 4**).

3.3 Preparation of Cell Lysates

1. Keep the plates on ice during this procedure. For cells grown in monolayer, aspirate medium from tissue culture plates, and gently wash at room temperature with 5 mL of 1× PBS. For suspension cells (e.g., LCLs), pellet them for 5 min at 300 g at 4 °C in a conical tube. Remove all supernatant by aspiration, and wash with 5 mL of cold 1× PBS. Repeat the centrifugation, and aspirate the supernatant.
2. Lyse cells with lysis buffer (typically 1.0 mL per 10-cm plate). Scrape the plates with a cell scraper, and collect the lysates and debris in microcentrifuge tubes (*see* **Note 5**).
3. Incubate tubes on ice for 10 min.
4. Remove cell debris by centrifugation of the lysate at 15,000 × *g* for 10 min at 4 °C. Transfer supernatant to a fresh microcentrifuge tube, and determine protein concentration by Bradford assay. Optimal protein concentration is about 1 mg/mL, and if

yield is much higher, lysate should be diluted with cold lysis buffer. Similarly, concentration of protein in different samples should be equalized (*see* **Note 6**). Set aside an aliquot (about 50 μL or 5 % of the supernatant) for IκB-α phosphorylation and degradation analysis.

3.4 Lysates Preclearing

This is an optional step to reduce nonspecific binding of proteins to Protein A agarose (*see* **Note 7**).

1. Equilibrate Protein A agarose: add required amount of the resin (10 μL of 50 % bead slurry per sample) to 200–500 mL of lysis buffer (a minimum of 10× volume of beads). Incubate for 5 min with rotation at 4 °C; afterwards, precipitate beads by centrifugation (300 × *g* for 2 min at 4 °C). Repeat washing two more times.
2. Add equilibrated Protein A agarose to the lysates. Incubate at 4 °C for 30–60 min on a tube rotator. Spin for 10 min (300 × *g*) at 4 °C. Discard pellet (beads), and use cleared supernatant for immunoprecipitation.

3.5 RelA Immunoprecipitation

1. Divide the cell lysate samples into two equal aliquots (approximate 500 μL each), and add 250 ng of primary antibody against RelA or rabbit IgG to the control sample (*see* **Note 8**). Incubate on tube rotator for 3–12 h at 4 °C.
2. Add Protein A agarose beads to each sample (20 μL of 50 % bead slurry, equilibrated with lysis buffer). Incubate samples under rotary agitation for 2–12 h at 4 °C.
3. Microcentrifuge at 300 × *g* for 2 min at 4 °C. Aspirate the supernatant and discard it.
4. Wash pellet three times with 500 μL of 1× lysis buffer on tube rotator for 5 min, precipitate the beads every time by centrifugation (300 × *g* for 2 min at 4 °C). Keep on ice during the washes.
5. Finally, carefully remove as much wash buffer as possible from the beads using a fine tip (e.g., gel loading tips). The pellet contains the RelA-IκB-α complex, which is now ready for analysis by SDS-PAGE followed by Western immunoblotting.

3.6 SDS-PAGE Separation and Western Blotting

1. Resuspend beads in 3× gel loading buffer (1 μL of loading buffer per 1 μL of bead volume). Heat sample for 10 min at 80–95 °C for complex dissociation. Now samples can be stored at –80 °C for several weeks before proceeding with SDS-PAGE separation.
2. Before SDS-PAGE separation, centrifuge tubes for 1 min at maximum speed (>10,000 × *g*) at room temperature. Load maximal amount of immunoprecipitated sample onto a SDS-PAGE gel (*see* **Note 9**). In addition, load input lysates (40–60 μg per sample).

3. The transfer of proteins to nitrocellulose membrane should be performed using a wet or semidry transfer system. After the transfer, proceed to immunodetection steps to detect ubiquitinated forms of IκB-α.

3.7 Immunodetection

After transfer, membrane blocking is performed for 0.5–1 h at room temperature with 5 % solution of fat-free milk in PBS-T buffer with constant gentle agitation.

3.7.1 Immunodetection of pIκB-α

1. Incubate the membrane with anti-phospho (Ser32/36)-IκB-α (dilution 1:1,000 in PBS-T with 2.5 % of nonfat milk) for 1 h at room temperature with gentle agitation (*see* **Note 10**).
2. Wash the membrane with PBS-T buffer three times for 5 min.
3. Apply a secondary HRP-conjugated antibody (dilution 1:4,000 in PBS-T with 2.5 % of nonfat milk) or 1 h at room temperature with gentle agitation.
4. Wash three times for 5 min with PBS-T buffer.
5. Perform ECL-detection using ECL-detection kit according to manufacturer's instruction. A ladder of phosphorylated and ubiquitinated forms of IκB-α will be detectable in late exposures of IP samples (usually, 30–60 min with X-ray film). This step allows detecting phosphorylation and ubiquitination dynamics of IκB-α in samples (Fig. 2a and b, upper blots).

3.7.2 Immunodetection of IκB-α Degradation

1. Wash the membrane for 10 min in PBS-T buffer, and repeat for a total of three times in order to remove chemicals from previous step (*see* **Note 11**).
2. Repeat blocking in 5 % nonfat milk solution in PBS-T for 15 min at room temperature.
3. Re-probe the membrane with anti-IκB-α antibody (dilution 1:1,000 in PBS-T with 2.5 % of nonfat milk).
4. Repeat the procedures as described above. In order to detect the degradation rate of IκB-α, analyze relative amount of the protein in the input samples (Fig. 2a and b, middle blots).

3.7.3 Immunodetection of RelA

1. Wash the membrane for 10 min in PBS-T buffer and repeat for a total of three times in order to remove chemicals from previous steps.
2. Re-probe the same membrane with anti-RelA antibody (dilution 1:1,000 in PBS-T with 2.5 % of nonfat milk), and repeat the procedures as described above. Because RelA is heavier than IκB-α (65 kDa vs. 36 kDa), we do not perform membrane stripping procedure. This step is required to confirm that the amount of immunoprecipitated RelA is equal between loaded samples (Fig. 2a and b, lower blots).

4 Notes

1. Prepare a 100-mM sodium orthovanadate solution in double distilled water. Set pH to 9.0 with HCl, and boil until colorless. Cool down to room temperature, and again set pH to 9.0. Repeat this cycle until the solution remains at pH 9.0 after boiling and cooling. Bring up to the initial volume with water. Store in aliquots at −20 °C.
2. Among the different variants of recombinant CD40L that are commercially available, we recommend to use the recombinant chimeric form known as MegaCD40L (Enzo Life Sciences). This protein consists of two trimeric extracellular domains of mouse CD40L (CD154), which are artificially linked via the collagen domain of ACRP30/adiponectin. In our hands, this form of the CD40L was very effective in stimulating the NF-κB pathway in human lymphocytes.
3. The dynamic of IκB-α degradation varies in different cell lines. We found that the optimal time of stimulation for most cell lines, including HEK 293 and HeLa cells, is 15–20 min. In general, we recommend using more than two time points (e.g., 0, 5, 10, 15, and 30 min of stimulation, Fig. 2). On the other hand, LCLs show very quick IκB-α degradation, and the optimal time to detect ubiquitinated forms of IκB-α is around 5–10 min of stimulation [5].
4. In the case of proteasomal blockade, we routinely use MG-132, at a concentration of 40 μM for 3–5 h. In the case of ligand stimulation, we use standard time points for the ligand. Alternatively, co-expression of the known E3 ligase for the target protein might be useful.
5. The volumes in this protocol are given for a single 10-cm plate ($2–4 \times 10^6$ adherent cells) or $5–7 \times 10^6$ LCLs (due to the smaller volume of the latter). For larger-scale experiments, all the volumes should be increased proportionally.
6. The supernatant is the cell lysate. If necessary, lysate can be stored at −80 °C for several months.
7. This step removes proteins, which may potentially bind nonspecifically to the agarose beads during immunoprecipitation. It is worthwhile to evaluate the amount of nonspecific proteins under your exact experimental conditions. This step may result in a lower level of background and improve signal-to-noise ratio.
8. The amount of applied antibody may vary, so we advise to begin with an average amount as a guideline. Usually, we use 1 μg of antibody per total lysate from a 10-cm plate (HeLa, HEK 293) or 5×10^6 LCL cells and 2 μg per total lysate from 15-cm plate (HeLa, HEK 293) or 10×10^6 LCL cells. There is no need to further increase the amount of cells for the detection of IκBα ubiquitination.

9. We routinely use premade NuPAGE Bis–Tris Mini Gels from Life Technologies. Briefly, 12-wells 4–12 % gradient gel (1.0 mm) are loaded with 20–25 μL of samples (~50 μg of total protein per lane); run conditions are set up according to the manufacturer's recommendations.
10. The membrane can be incubated with the antibodies overnight (either with a primary or a secondary). In many cases, this step will be reached at the end of the day, and thus, we routinely performed the binding overnight at 4 °C without any apparent detriment to the procedure.
11. In this case, there is no need for a stripping procedure, since the used antibodies are generated from different animal species (anti-phospho (Ser32/36)-IκB-α antibody is raised from a mouse, while anti-IκB-α is raised from a rabbit). Hence, the corresponding bands are detected by different secondary antibodies and are not overlapped.

References

1. Hayden MS, Ghosh S (2012) Genes Dev 26: 203–234
2. Basak S, Kim H, Kearns JD, Tergaonkar V, O'Dea E, Werner SL, Benedict CA, Ware CF, Ghosh G, Verma IM, Hoffmann A (2007) Cell 128:369–381
3. Hoffmann A, Natoli G, Ghosh G (2006) Oncogene 25:6706–6716
4. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E (2006) Nat Cell Biol 8:1327–1336
5. Starokadomskyy P, Gluck N, Li H, Chen B, Wallis M, Maine GN, Mao X, Zaidi IW, Hein MY, McDonald FJ, Lenzner S, Zecha A, Ropers HH, Kuss AW, McGaughran J, Gecz J, Burstein E (2013) J Clin Invest 123: 2244–2256

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