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Glutathione and Thioredoxin: Thiols in Signal Transduction and Gene Regulation

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[17] Redox Regulation of Activation of NF-κB Transcription Factor Complex: Effects of *N*-Acetylcysteine

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Introduction

NF-κB is a heterodimeric transcription factor complex composed, in its classic form, of two DNA-binding subunits: p50 and p65 (reviewed in Ref. 1). NF-κB was first identified as a DNA-binding activity specific for the κB motif in the immunoglobin kappa (κ) enhancer. Cloning of the NF-κB p50 and p65 genes revealed a family of NF-κB/rel proteins that participate in a variety of transcriptionally regulated processes, such as lymphocyte differentiation, responsiveness to cytokines, and embryonic development in *Drosophila*.

NF-κB is present in many cell types in an inactive form in the cytoplasm, bound to a cytoplasmic retention molecule called I-κB (for inhibitor of NF-κB).[2] Nuclear translocation of NF-κB is achieved after stimulation of the cells by many different inducers (cytokines, phorbol esters, viral proteins, oxidants) and presumably involves phosphorylation of I-κB which disrupts its interaction with the p65 NF-κB subunit.[3] The NF-κB complex is then free to migrate to the nucleus and transactivate its various target genes.

Studies from several laboratories have revealed that the signal transduction pathways leading to activation of NF-κB are redox regulated.[4-7] That is, antioxidants such as *N*-acetyl-L-cysteine (NAC) can inhibit activation, whereas oxidants such as hydrogen peroxide (H_2O_2) can directly activate NF-κB. The NAC inhibition of NF-κB activation first implicated a redox-sensitive step in the NF-κB signal transduction pathway.[4] Further studies have found that a wide variety of compounds which share the ability to modulate intracellular oxidant levels also inhibit NF-κB activation. These

[1] G. P. Nolan and D. Baltimore, *Curr. Opin. Gen. Dev.* **2**, 211 (1992).
[2] R. Sen and D. Baltimore, *Cell* (*Cambridge, Mass.*) **47**, 921 (1986).
[3] S. Ghosh and D. Baltimore, *Nature* (*London*) **344**, 678 (1990).
[4] F. J. T. Staal, M. Roederer, L. A. Herzenberg, and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9943 (1990).
[5] T. Kalebic, A. Kinter, G. Poli, M. E. Anderson, A. Meister, and A. S. Famci, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 986 (1991).
[6] S. Mihm, J. Ennen, U. Passara, R. Kurth, and W. Droge, *AIDS* **5**, 497 (1991).
[7] R. Schreck, P. Bieber, and P. Baeuerle, *EMBO J.* **10**, 2247 (1991).

results, coupled with the observations that oxidants such as H_2O_2 are by themselves sufficient to activate NF-κB,[7] suggests oxidants play key roles within NF-κB signaling pathways.

Here we describe detailed methods on the appropriate cell culture conditions to study the influence of oxidants and antioxidants, the preparation of nuclear protein extracts, and the use of gel retardation assays to detect the presence of activated NF-κB in the nucleus. In addition, we briefly describe a reporter gene-based system to assay whether the DNA-bound NF-κB is transcriptionally active.

Methods and Procedures

Principle

The electrophoretic mobility shift assay (EMSA) or gel retardation assay is a powerful method for quantitative and qualitative analysis of protein–DNA interactions. The technique is based on the principle that the electrophoretic mobility of a nucleic acid (e.g., a radiolabeled oligonucleotide) through a polyacrylamide gel is impeded when a protein is bound to it. Thus, free (labeled) oligonucleotide and oligonucleotides that have bound protein can be discerned based on their relative migration in the gel. This principle was originally described by Garner and Rezvin[8] and by Fried and Crothers[9] and extended to the identification of mammalian transcription factors by Singh *et al.*[10] With the EMSA small amounts of a DNA (or RNA)-binding protein can be detected in a heterogeneous mixture of proteins such as a nuclear extract.

Cells and Stimulation Conditions

A variety of cells can be used for measurement of NF-κB. Either constitutive or inducible EMSA-shifted NF-κB complexes are detectable in lymphoid, epithelial, and fibroblast cell lines as well as in primary cells. It is important that the cells are viable, proliferating in logarithmic phase, and grown at the right density. For instance, Jurkat T cells grown at high density (2×10^6 cells/ml) can have about 25% lower glutathione (GSH) levels than cells grown at low density. This density-induced change in GSH can confound redox effects of the compounds actually studied. It is therefore good experimental practice to perform the experiments under standardized culture conditions.

[8] M. M. Garner and A. Revzin, *Nucleic Acids Res.* **9**, 3047 (1981).
[9] M. G. Fried and D. M. Crothers, *Nucleic Acids Res.* **9**, 6505 (1981).
[10] H. Singh, R. Sen, D. Baltimore, and P. A. Sharp, *Nature* (*London*) **319**, 9 (1986).

Cells are incubated with appropriate stimuli (e.g., cytokines, phorbol esters) that activate NF-κB and the redox-regulating compounds (antioxidants, iron chelators, or oxidants) that may inhibit or increase activation. Both the stimuli and inhibitors should be added from stock solutions that are at least 100× concentrated, pH-neutralized, and sterilized before use. If organic solvents [such as dimethyl sulfoxide (DMSO)] are used, it is important to include a control adding the vehicle only. As a first estimate, it is useful to explore the kinetics of activation and inhibition of NF-κB over a time range from 0.5 to 8 hr; subsequently, one can establish a rough titration curve of the redox-active compound.

Preparation of Nuclear Protein Extract

Several methods to prepare nuclear extracts are available, most of which involve salt extraction of whole cell or nuclear homogenates. The original procedure was described by Dignam, Lebovitz, and Roeder and has been modified by others.[11] The nuclear extract method described here works well with 1×10^6 to 2×10^7 cells and is an adaptation from the method described by Schaffner and co-workers.[12] We have compared this method to the original large-scale method and obtained equivalent results for the NF-κB, Oct-1, AP-1, and NF-AT transcription factors (although total protein yield is, of course, lower).

1. After stimulation, the cells are harvested, washed once, and then resuspended in 1.0 ml of cold Tris-buffered saline (TBS). To harvest adherent cells, do not use trypsin but use phosphate-buffered saline (PBS)–EDTA (2.5 m*M*) instead. Subsequent steps are done in the cold room at 4°.
2. Transfer sample to a 1.5-ml Eppendorf tube.
3. Centrifuge 15 sec at 14,000 rpm.
4. Remove supernatant using a drawn out Pasteur pipette and suction.
5. Resuspend (cellular) pellet in 400 μl of buffer A [10 m*M* HEPES, pH 7.8/10 m*M* KCl/2 m*M* $MgCl_2$/0.1 m*M* EDTA/0.5 m*M* dithiothreitol (DTT), supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 0.5 m*M*), antipain (1 μg/ml), leupeptin (0.3 μg/ml), and pepstatin (0.5 μg/ml); DTT and the protease inhibitors are added freshly from stock solutions.].
6. Incubate cells on ice for 15 min.
7. Add 25 μl of 10% Nonidet P-40 (NP-40) solution. This solution should be added to the inner part of the Eppendorf cap. Cap the tubes in

[11] H. Ohlsson and T. Edlund, *Cell* (*Cambridge, Mass.*) **45**, 35 (1986).
[12] E. Schreiber, P. Matthias, M. M. Mueller, and W. Schaffner, *Nucleic Acids Res.* **17**, 6419 (1989).

pairs, invert the tubes, and immediately vortex 10 times for 1 sec. This procedure allows reproducible extraction of nuclear proteins, whereas direct addition of NP-40 can give problems.

8. Centrifuge extract 30 sec at 14,000 rpm. Remove as much as possible of the supernatant (if necessary, centrifuge a second time to remove all liquid). A small white nuclear pellet should be visible.
9. Resuspend pellet in 40 μl of buffer C (50 m*M* HEPES, pH 7.8/50 m*M* KCl/300 m*M* NaCl/0.1 m*M* EDTA/0.5 m*M* DTT/10% (v/v) glycerol; add protease inhibitors as for buffer A). Resuspension is usually somewhat difficult. It is important to assure that clumps are at least somewhat dispersed.
10. Salt-extract nuclear proteins by rotating on a rotator for 30 min.
11. Centrifuge 10 min at full speed (14,000 rpm).
12. Transfer supernatant (nuclear extract) to a new Eppendorf tube. Discard pellet (cell debris).

The extracts can be stored at −70° or used immediately for determination of protein concentration and gel retardation.

Electrophoretic Mobility Shift Assay

The amount of protein obtained can be determined using the Bradford assay (Bio-Rad, Richmond, CA). We routinely use 200 μl of reagent with 2.0 μl of extract and a standard curve of different known concentrations of bovine serum albumin (BSA). We then determine the 560 nm absorbance on a 96-well enzyme-linked immunosorbent assay (ELISA) plate reader, from which the protein concentration in the extract can be calculated. Two micrograms of nuclear extract per condition should be sufficient for detection of NF-κB and other transcription factors by EMSA. Optimal binding of NF-κB is dependent on the final salt concentration in the binding reaction, the concentration of DTT, and the amount of nonspecific competitor DNA.

In our hands, optimal binding occurs under conditions of 70 m*M* salt, 5 m*M* DTT, and 2.0 μg poly(dI-dC) as competitor. Poly(dI-dC) is dissolved in Tris–EDTA buffer (TE)/100 m*M* NaCl, heated to 90°, and slowly cooled (over 30–45 min) to room temperature before use. It may be necessary to vary both the amount of extract and amount of nonspecific competitor to optimize the sensitivity of the assay for detection of NF-κB within different cell types. The radiolabeled probe (see below) is added last; 10,000 counts/min (cpm) should be sufficient for detection after overnight exposure on film. Binding reactions are done at room temperature for 45 min in a final volume of 20–50 μl.

Subsequently, samples are electrophoresed on a 4.5% polyacrylamide gel with 0.25× TBE (Tris–borate–EDTA buffer, pH 8.3) as running buffer

for 1.5–2 hr at 100 V. After electrophoresis, the gel is disassembled, transferred onto two sheets of Whatman (Clifton, NJ) filter paper, and dried. A typical 1.5-mm gel requires about 1 hr of drying under vacuum at 80°. The dried gel is exposed to film overnight at −70° and developed. For most purposes this exposure time should yield a good signal. If necessary, longer exposures should be used.

Labeling of Probe

Many different κB probes can be used. We use a double-stranded probe made by annealing two single-stranded synthetic oligonucleotides with the following sequences.

TCGAGTCAGAGGGGACTTTCCGAG
CAGTCTCCCCTGAAAGGCTCAGC

The unlabeled probe is stored at 100 ng/μl at −20°, and 0.5 μl is used to end-label the probe with Klenow DNA polymerase. Mix 0.5 μl probe, 10 μl GTC 2 m*M* dNTP mixture (2 m*M* dATP, 2 m*M* dGTP, 2 m*M* dTTP),

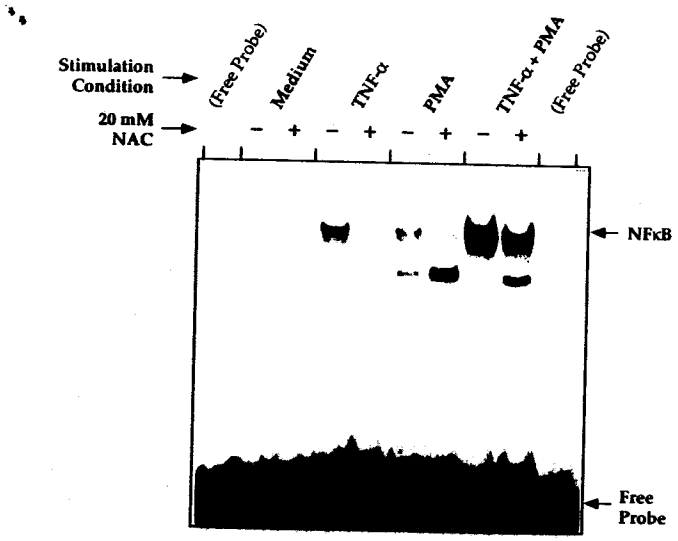

FIG. 1. Inhibition of TNF-α- and PMA-induced NF-κB binding activity by NAC. 293 cells were stimulated with TNF-α, PMA, or both in the presence or absence of 20 m*M* NAC. Nuclear protein extracts were made and EMSAs performed as described in the text. First and last lanes contain free probe without nuclear protein added. The top arrow indicates the inducible NF-κB band; the bottom arrow marks unbound probe. (Reproduced from Staal *et al.*[4] with permission from the publishers.)

30 μl doubly distilled water, 5 μl of 10× Klenow buffer (0.5 m*M* Tris-Cl, pH 7.5, 0.1 *M* $MgCl_2$, 10 m*M* DTT, 0.5 mg/ml BSA), 1 μl Klenow DNA polymerase (100 U/ml), and 4 μl of [α-^{32}P]dCTP (1 mCi/ml). Incubation is for 30 min at room temperature, after which the reaction is stopped by addition of 50 μl TE.

Labeled probe and unincorporated [α-^{32}P]dCTP are separated using a spin column packed with Bio-Rad P6DG or Sephadex G-25. The columns are made using a 1-ml insulin syringe (centrifuge 2 min at 1600 rpm (500 *g*), packed volume should equal about 0.9 ml). Two microliters of the labeled probe is counted using a scintillation counter. A good probe should have at least 25,000–50,000 counts/sec (cps).

Analysis of Results

Typical results of a gel retardation assay are shown in Fig. 1. The presence of NF-κB under stimulated [tumor necrosis factor (TNF), phorbol 12-myristate 13-acetate (PMA)] conditions is visible as a set of two sharp bands, which presumably correspond to the $(p50)_2$ homodimer (lower band) and the p65–p50 heterodimer (upper band). Clearly, if stimulation is done in the presence of 20 m*M* NAC, no activated NF-κB can be found. Similar

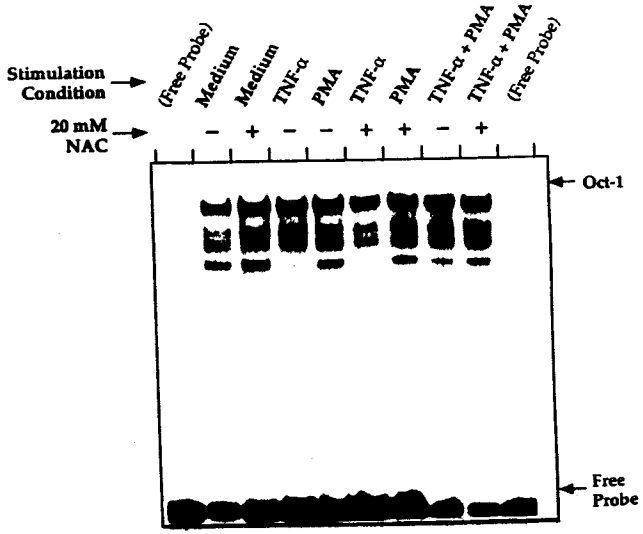

FIG. 2. *N*-Acetylcysteine does not inhibit the Oct-1 transcription factor. The same nuclear protein extracts from Fig. 1 were probed with a Oct-1 probe. The top arrow indicates the constitutive Oct-1 band, the bottom arrow the unbound probe. (Reproduced from Staal *et al.*[4] with permission.)

results can be obtained with many different cell lines and stimulation conditions. Quantitative results can be obtained by scanning the gel with a phosphorimager (radioanalytic imaging system).

It is important to ascertain the specificity of the NF-κB inhibition by antioxidants such as NAC. Therefore, we usually use the same nuclear protein extracts to probe another transcription factor that is not redox-sensitive. The ubiquitous Oct-1 transcription factor can be useful for this purpose. As shown in Fig. 2, conditions that effectively inhibit activation of NF-κB do not affect Oct-1 expression, demonstrating that NAC selectively inhibits NF-κB.

Finally, the presence of activated NF-κB in a nuclear extract does not necessarily mean that NF-κB is transcriptionally active (binding does not imply function). Functional NF-κB can be readily assayed using reporter genes, the transcription of which is directed by an NF-κB-dependent promoter. We have used Jurkat T cells stably transfected with a plasmid containing three κB motifs fused to the *lacZ* (β-galactosidase) gene (Jurkat kB5.2).[4] Cells can be stimulated under the appropriate conditions and assayed for β-galactosidase activity by biochemical means in lysates[13] or by the fluorescence-activated cell sorting (FACS)-Gal assay using a flow cytometer.[14] It is beyond the scope of this chapter to describe these methods in detail, but readers are referred to protocols given elsewhere.[15]

Acknowledgment

Supported in part by NIH CA-42509.

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[14] G. P. Nolan, S. N. Fiering, J. F. Nicolas, and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 2603 (1988).

[15] M. Roederer, S. N. Fiering, and L. A. Herzenberg, *Methods (San Diego)* **2,** 248 (1991).