Chapter 19

Molecular Targeting of Nuclear Transcriptional Regulators

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Gene regulation by nuclear transcription factors involves intracellular interactions of nuclear transcriptors with specific transport carriers and/or DNA regulatory elements using sequence-specific recognition sites. In this study, we utilized a series of synthetic peptides carrying the nuclear localization sequence of the transcriptor NF-κB p50 and p65 subunits to inhibit NF-κB-mediated gene expression. Gene transfection and gel-shift assays showed that these peptides were effective in inhibiting the nuclear translocation and transcriptional activation of NF-κB. However, their effects required the presence of a cell-permeable peptide which is covalently linked to the inhibitory peptides. These hybrid peptides may be used as novel therapeutic agents in diseases whose etiology is dependent on NF-κB activation.

Gene activation by nuclear transcription factors represents a fundamental process in the cellular control of gene expression. This process involves intracellular interactions of nuclear proteins with specific transport carriers and/or DNA regulatory elements using recognition sequences. By exploiting these recognition sequences, a number of molecular therapeutic approaches has been developed to manipulate pathologic gene expression. In this study, we reported a gene inhibition method which

utilizes nuclear localization sequences (NLS) of the transcription factor NF-κB to regulate the nuclear translocation and transcriptional activity of NF-κB.

NF-κB is a key transcription factor involved in immune and inflammatory disorders. It is frequently composed of two DNA-binding subunits, NF-κB_1 (p50) and RelA (p65) (1, 2). It is normally kept in an inactive form in the cytoplasm by attachment of the inhibitory subunit IκB, which masks the NLS of the NF-κB subunits. The activation of NF-κB is accomplished by phosphorylation of IκB, which triggers the complete degradation of the inhibitor (3). This induced degradation of IκB unmasks the NLS of the NF-κB dimer and allows NF-κB to enter the nucleus, to bind to its DNA target sequence, and to induce transcription. Because nuclear translocation of NF-κB is crucial for its transcriptional activity and because this translocation is dependent on NLS, we rationalize that specific peptide sequences carrying the NLS signal may be used to selectively inhibit the nuclear translocation and thus transcriptional activity of NF-κB. This was tested in the present study using various molecular techniques including electrophoretic mobilty shift assay (EMSA), gene transfection, and enzyme linked immunosorbent assay (ELISA).

Experimental

Cells and Reagents

Macrophage RAW 264.7 cells were used throughout. The cells were maintained in DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine, and 1,000 units/ml penicillin-streptomycin at 37°C in a humidified atmosphere at 5% CO_2. A specific antibody against NF-κB (p50) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Peptides were synthesized by a stepwise solid-phase peptide synthesis method (Quality Controlled Biochemicals, Hopkinton, MA). The peptides were purified by C_{18} reverse-phase HPLC and verified by mass spectrometry.

Nuclear Extracts

Nuclear extracts were prepared as follows; $5x10^7$ cells were treated with 500 µl lysis buffer (50 mM KCl, 0.5% NP-40, 25 mM Hepes, 1 mM PMSF, 10 mg/ml leupeptin, 20 µl/ml aprotinin, 100 mM DTT) on ice for 4 min. Nuclei were pelletted by centrifugation at 14,000 rpm for 1 min and were resuspended in 300 µl extraction buffer (500 mM KCl, 10% glycerol, 25 mM Hepes, 1 mM PMSF, 1 µl/ml leupeptin, 20 µg/ml aprotinin, 100 µM DTT). After centrifugation at 14,000 rpm for 5 min, the

supernatant was harvested and stored at -70°C. The protein concentration of the resulting nuclear protein extract was determined by BCA protein assay reagent (Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assay

The DNA-protein binding reaction was conducted in a 24 μl reaction mixture including 3 μg nuclear protein extract, 1 μg poly dI.dC (sigma), 3 μg BSA, $4x10^4$ cpm of ^{32}P-labeled oligonucleotide probe, and 12 μl of 2xY buffer. The mixture was incubated on ice for 10 min without antibody, or 10 min with antibody, in the absence of radiolabeled probe, then 20 min at room temperature in the presence of radiolabeled probe. The mixture was resolved on a 5% polyacrylamide gel at 200 V for 90 min, then dried and placed on Kodak X-OMAT film (Eastman Kodax, Rochester, NY). This film was developed after an overnight exposure at -70°C. NF-κB binding sequence (5'-TGGGATTTTCCCATGAGTCT-3') was used to synthesize the probe. The synthesized single-stranded oligonucleotide probe was denatured at 80°C for 5 min and annealed with its complementary sequence at room temperature. The double-stranded probe was labeled with ^{32}P-ATP (Amersham, Arlington Heights, IL) using T4 kinase (BRL, Gaithersburgh, MD).

Enzyme Linked Immunosorbent Assay

Analysis of TNFα levels was performed using the Genzyme TNFα ELISA kit (Genzyme Corp., Cambridge, MA) according to the manufacturer's instructions. Absorbance measurements of the enzyme product were performed at the wavelength of 450 nm using the Bio-Rad 500 microplate reader.

Transient Transfection and Luciferase Assay

The reporter plasmid used in this study was generously provided by Dr. J. Ye at the National Institute for Occupational Safety and Health. The luciferase reporter plasmid contains NF-κB binding sites derived from the -615/+15 promoter fragment of TNFα gene. The cells were plated in six-well plates 1 day before transfection. For transient transfection, 1 μg of the reporter DNA was mixed with 6 μg lipofectAMINE (GIBCO BRL) and 2 μg protamine sulfate (Sigma) in 200 μl Optimal medium (GIBCO BRL). The cells were incubated with the mixture of DNA and lipofectAMINE for 6 h at 37°C and then with 4 ml of the complete medium at 37°C for 24 h. After treatment, the cells were harvested and analyzed for luciferase activity using an assay kit (Promega, Madison, WI). To account for potential cytotoxicity caused by the transfecting agents or other test agents, total cell protein was determined and used to normalize the measured luciferase activity.

Results and Discussion

Design of Inhibitory Peptides

NF-κB is a heterodimer nuclear protein that is formed by two common DNA-binding subunits p50 and p65. These two subunits have their individual NLS in the amino acid sequences (4). These NLS are involved in the nuclear translocation of NF-κB: therefore, if peptides carrying the NLS of p50 and/or p65 are administered into cells, they should be able to compete with the NF-κB for the nuclear transport carriers, and thus lead to an inhibition of NF-κB activity. To aid the cellular delivery of NLS peptides, a cell-permeable import peptide (IP) derived from the hydrophobic region of the signal peptide fibroblast growth factor (5) was linked to the NLS peptides. This import peptide has been shown to facilitate the cellular entry of biomolecules into intact cells (6, 7). Accordingly, we synthesized a series of peptides comprising the IP (AAVALLPAVLLALLAP) and various NLS of NF-κB:

IP50	=	IP + NLS of NF-κB p50 (VQRKRQKLMP)
IP65	=	IP + NLS of NF-κB p65 (HRIEEKRKRTYETF)
IP50/65	=	IP + NLS of NF-κB p50 and p65

As comparative controls, the following peptides were used:

IP50M	=	IP + mutated NLS of NF-κB p50 (VQRNGQKLMP)
IP	=	IP
P50	=	NLS of NF-κB p50

Inhibition of Nuclear Translocation of NF-κB

To test the effect of peptides on nuclear translocation of NF-κB, EMSA studies were conducted. Nuclear proteins were made from RAW 264.7 cells after 2-h stimulation with 10 ng/ml of LPS in DMEM. The results showed that nuclear translocation of NF-κB was induced by LPS and this translocation of NF-κB was inhibited by IP50, IP65, and IP50/65 (Fig. 1A). The control peptides P50, IP50M, and IP (insoluble in the test medium) had no effect. To assess the binding specificity of NF-κB in the EMSA studies, oligonucleotide competition and antibody supershift assays were conducted. The results of this study showed that the NF-κB binding activity could be competed by a nonlabeled NF-κB oligonucleotide probe but not by the non-specific AP1 probe, and the antibody specific to the p50 subunit of NF-κB caused a band-shift of the NF-κB complexes (Fig. 1B)). These results indicated the DNA binding specificity of NF-κB under the experimental conditions. The lack of the inhibitory effect of IP50M indicated the sequence specificity for nuclear translocation of IP50. Likewise, the lack of the inhibitory effect of P50 indicated the requirement of the peptide carrier IP for efficient cellular uptake of the NLS peptides. Cellular

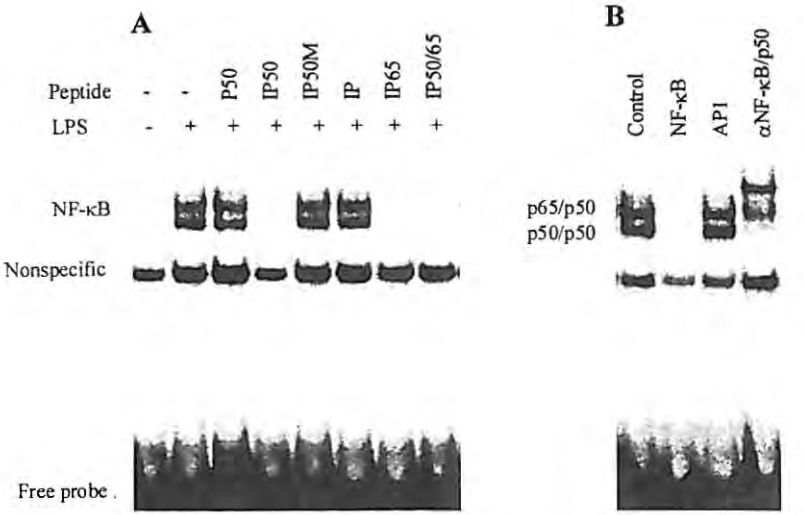

Figure 1. EMSA studies of NF-κB. Cells were pretreated with the indicated peptide (100 μg/ml) for 1 h followed by LPS (10 ng/ml) for 2 h. The assays were performed as described in the Materials and Methods. Competitor and antibody used in each lane as indicated.

uptake studies using fluorescently-labeled peptides in this laboratory supported this conclusion.

Inhibition of LPS-Induced TNFα Gene Expression

The inhibition of nuclear translocation of NF-κB by inhibitory peptides should lead to transcriptional inactivation of NF-κB-dependent genes. The cytokine TNFα gene is one of those genes whose expression is dependent on NF-κB activation (8). To test the potential inhibitory effect of peptides on TNFα gene expression, ELISA studies were conducted. Our results showed that the inhibitory peptides IP50, IP65, and IP50/65 caused a complete inhibition in LPS-induced TNFα expression, whereas the control peptides IP50M had no effect (Fig. 2). These results are in good agreement with the EMSA studies and indicate that the transcriptional inactivation of TNFα gene can lead to an inhibition of its protein expression.

Inhibition of NF-κB-Dependent Reporter Gene Expression

To confirm the inhibitory effect of peptides on NF-κB-dependent gene expression, gene transfection assays using a reporter plasmid were conducted. The reporter plasmid contains luciferase gene under the control of NF-κB gene promoters. Our results indicated that the IP50 was able to inhibit LPS-induced luciferase gene expression in transfected cells, whereas the mutated IP50M had no effect (Fig. 3). The inhibitory effect of IP50 was not due to its cytotoxicity as indicated by trypan blue dye exclusion assay. The percentage of cells viable after treatment with IP50 (100 μg/ml) was 94±4 % which was not significantly different from the nontreated control (97±3 %) ($p < 0.05$).

Conclusions

Using synthetic peptides carrying the NLS of NF-κB, we demonstrated that effective inhibition of the nuclear translocation of NF-κB can be achieved. This inhibition required the presence of a cell-permeable peptide carrier which is linked to the NLS peptides. Control peptides lacking NLS or carrier sequences exhibited no inhibitory effect, as demonstrated by electrophoretic mobility shift assay. ELISA and transient gene transfection assays also showed that these peptides were effective in inhibiting NF-κB-dependent gene expression. These inhibitory peptides may be used as novel therapeutic agents in diseases whose etiology is dependent on NF-κB activation.

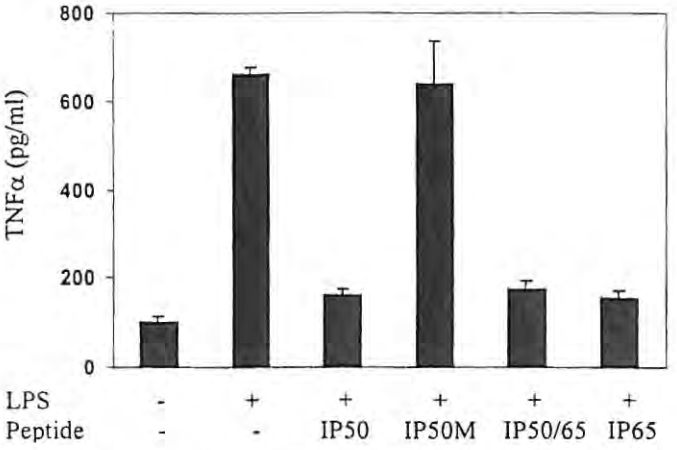

Figure 2. Suppression of LPS-Induced TNF α Production by Inhibitory Peptides. Cells were treated with the indicated peptide (100 μg/ml) for 1 h prior to LPS stimulation (10 ng/ml). TNF α levels were detected 6 h after stimulation by an ELISA assay. The values represent mean ± S.E., n = 4.

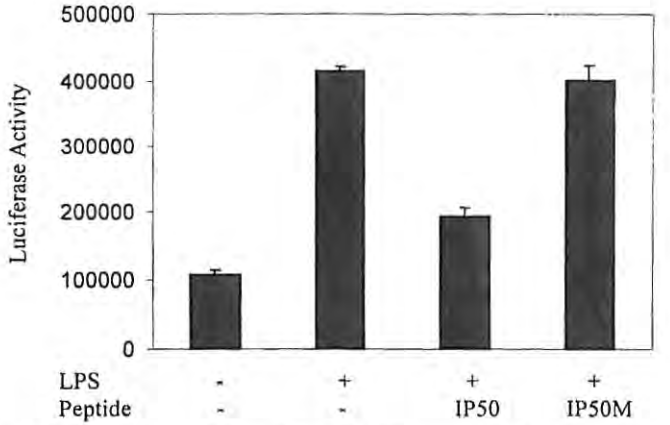

Figure 3. Effect of Inhibitory Peptides on NF-κB-Dependent Luciferase Gene Expression. Transfected cells were treated with peptide (100 μg/ml), followed by LPS stimulation (10 ng/ml). The values represent mean ± S.E., n = 4.

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