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Journal of Controlled Release 65 (2000) 13–17

journal of controlled release

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Cellular delivery of functional peptides to block cytokine gene expression

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Received 17 March 1999; accepted 2 June 1999

Abstract

Advances in molecular and cellular biology have identified the cellular mediators that regulate many disease processes and have facilitated the development of new therapeutic agents that control these events. However, the size, complexity, and cellular inaccessibility of these therapeutic agents make their cellular delivery difficult. Here, we describe an efficient cellular delivery system that exploits the membrane-translocating ability of signal peptides to import functional peptides into cells. Molecular conjugates consisting of the signal import peptide (IP) and nuclear localization sequence (NLS) of the transcription factor NF-κB were synthesized. Electrophoretic mobility shift and enzyme-linked immunosorbent assays were used to assess the inhibitory effects of these synthetic peptides on agonist-induced NF-κB nuclear translocation and transcriptional activation. Our results indicated that the peptides were effective in inhibiting both the nuclear translocation and transcriptional activation of NF-κB. However, their effects required the presence of the IP moiety for efficient cellular entry of the NLS. Structural analysis of IP showed that the hydrophobic domain, and to a lesser extent the N-terminal domain, was responsible for the membrane translocating activity of IP.

Keywords: Peptide; Transport; Transcription factor; NF-κB; TNFα

1. Introduction

Gene expression in eukaryotic cells is governed by nuclear transcription factors. These are proteins that interact with specific DNA regulatory elements and induce or retard the transcriptional rate. NF-κB is a key transcription factor involved in involved in the regulation of many important cytokine genes including TNFα and interleukins [1]. Translocation of this transactivator from the cytoplasm into the nucleus is required for its inducible activity [2]. This translocation is mediated by the nuclear localization signal (NLS) in the amino acid sequence of the nuclear protein [3]. Thus NLS represents a potential target for therapeutic manipulation of cytokine gene expression. Using a series of synthetic peptides comprising the NLS of NF-κB and the import peptide (IP) carrier, we demonstrated that effective inhibition

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0168-3659/00/$ – see front matter
PII: S0168-3659(99)00227-8

of nuclear translocation and transcriptional activation of NF-κB can be achieved. This inhibition required the presence of the cell permeable IP motif for efficient cellular entry. This import peptide has been applied to aid the non-invasive delivery of impermeable molecules such as proteins and nucleic acids into intact cells [4,5].

2. Experimental methods

2.1. Cells and reagents

RAW264.7, A549, SV40, and P39 cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 IU/ml penicillin–streptomycin at 37°C. Peptides were synthesized by a stepwise solid-phase synthesis method. A fluorescent probe was sometimes attached to the C-terminus of the peptides to aid their cellular uptake studies. The peptides were purified by C_{18} reversed-phase HPLC and verified by mass spectrometry. NF-κB (p50) antibody was obtained from Santa Cruz Biotech. (Santa Cruz, CA).

2.2. Cellular uptake studies

Cells (1×10^6/ml) were incubated with fluorescein-labeled peptides (100 μg/ml) at 37°C in DMEM medium. After incubation, they were washed and trypsinized (1 mg/ml, 5 min) to remove surface-bound peptides. The cells were then lysed with 0.1% Triton-X and measured for their fluorescence intensities at the excitation/emission wavelengths of 490/520 nm.

2.3. Confocal laser scanning microscopy

Cells were incubated with fluorescently labeled peptides for 1 h according to the procedure described above. After incubation, the cells were washed, trypsinized, and placed on glass cover slips. They were immediately observed under the Zeiss MC40 confocal fluorescence microscope (Carl Zeiss Inc.) using 515/30 nm band pass filter. The images were obtained with a Zeiss Achroplan 40× water immersion lens with a numerical aperture of 1.40.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method previously described [6]. Nuclear protein–DNA binding reactions were conducted in a reaction mixture containing 3 μg nuclear protein extract, 1 μg Poly dI·dC, 3 μg BSA, and 4×10^4 cpm of ^{32}P-labeled oligonucleotide probe. The NF-κB binding sequence (5′-TGGGATTTTCCCATGAGTCT-3′) was used as a probe. The 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 using T4 kinase. The mixture was resolved on a 5% polyacrylamide gel at 200 V for 90 min, then dried and placed on Kodak X-OMAT film. This film was developed after an overnight exposure at −70°C.

2.5. Enzyme linked immunosorbent assay (ELISA)

Analysis of TNFα levels was performed using the Genzyme TNFα ELISA kit according to the manufacturer's instructions. Absorbance measurements of the enzyme product were performed at the wavelength of 450 nm.

3. Results and discussion

3.1. Design of bifunctional peptides

The IP used in this study was derived from the signal peptide Kaposi fibroblast growth factor [7]. The positively charged N-terminal sequence (n region) and the hydrophobic sequence (h region) of the signal peptide were selected as the membrane transport carrier. The h sequence is known to possess membrane-translocating activity whereas the n sequence promotes peptide–membrane interactions [8]. The absolute requirement of the positively charged n sequence for membrane translocation has not been established and is therefore investigated in this study. The import peptides, with or without the n sequence (designated here as IP^+ and IP), were linked to the NLS of NF-κB p50 and p65 subunits. NF-κB is a heterodimer protein that is frequently composed of these two DNA-binding subunits. The two subunits

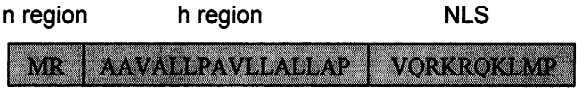

Fig. 1. Sequence of ip$^+$NLS (single-letter amino acid code).

have their individual NLS which are involved in the nuclear translocation of NF-κB [3]. Therefore, if peptides carrying the NLS of p50/p65 are administered into cells, they should be able to compete with NF-κB for its nuclear entry, and thus lead to an inhibition of NF-κB activity. Accordingly, we synthesized bifunctional peptides comprising NLS and IP (ipNLS) or NLS and IP$^+$ (ip$^+$NLS) (see Fig. 1). As controls, peptides containing NLS or IP alone were used.

3.2. Cellular uptake studies

Cellular uptake of various peptides is shown in Fig. 2. The results indicated that the import peptide dramatically increased the uptake of NLS peptide in all cell types tested. This enhancement was dependent on the presence of IP or IP$^+$ since the control NLS peptide was poorly taken up by cells despite its smaller molecular size. The h sequence of the signal peptide was crucial for the translocating activity of the import peptide whereas the n sequence had a modest promoting effect. The n sequence alone did not promote the uptake of NLS peptide (result not shown). MTT assays showed that these peptides were non-toxic at the concentrations used.

3.3. Microscopic studies of peptide uptake

To provide morphologic evidence of peptide uptake, cells were incubated with ip$^+$NLS or NLS at 37°C for 1 h and examined for their cellular distribution using confocal laser scanning microscopy. As seen in Fig. 3, cells treated with ip$^+$NLS exhibited a strong intracellular fluorescence intensity, whereas those treated with NLS showed a very weak fluorescence signal. The results indicated that the IP$^+$ was able to facilitate the cellular entry of NLS and that the NLS by itself was poorly taken up by cells.

3.4. Inhibition of nuclear localization of NF-κB

To test the effect of peptides on nuclear translocation of NF-κB, EMSA studies were conducted. Nuclear proteins were made from RAW264.7 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 was inhibited by ipNLS or ip$^+$NLS, but not by NLS (Fig. 4A). The control peptide ip or ip$^+$ alone was insoluble in the test medium and had no inhibitory effect on the translocation (results not shown). To assess the binding specificity of NF-κB, oligonucleotide competition and antibody supershift assays were conducted (Fig. 4B). The results showed

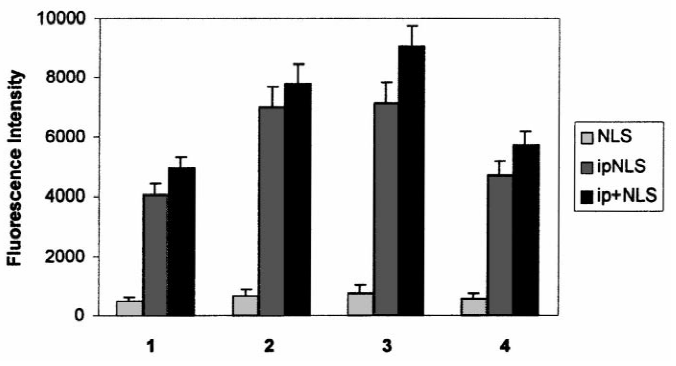

Fig. 2. Cellular uptake of peptides in different cell lines. Cells (1×10^6/ml) were incubated with indicated peptide (μg/ml) in DMEM for 1 h at 37°C. After incubation, the cells were washed and analyzed for cellular fluorescence intensities at 490/520 nm. 1: RAW264.7; 2:A549; 3: P39; 4: Sv40.

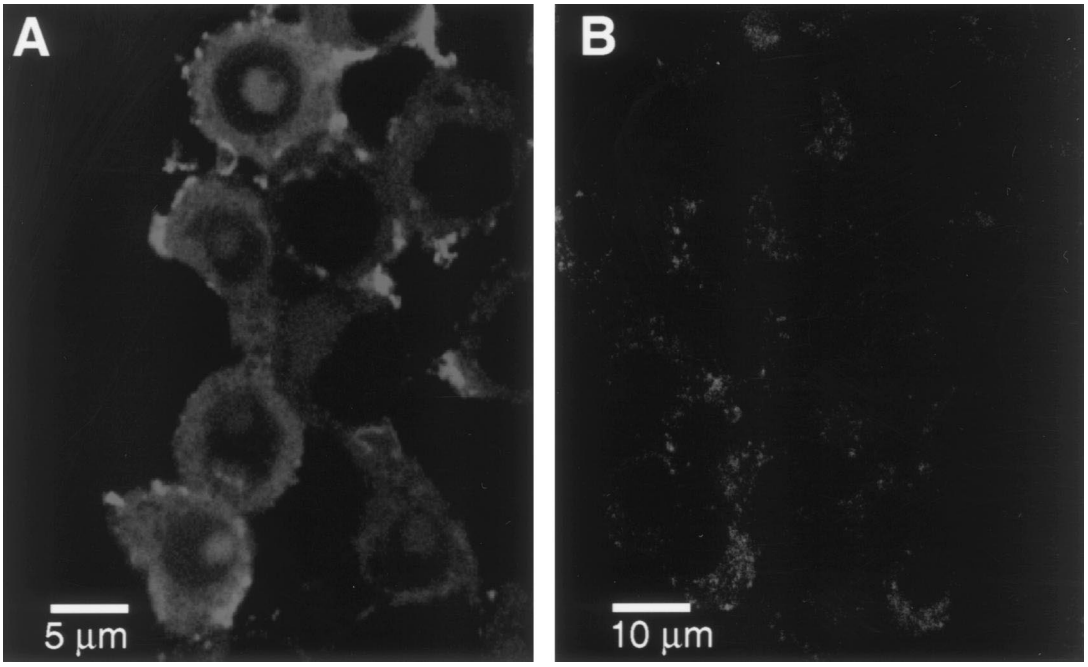

Fig. 3. Confocal fluorescence micrographs of RAW264.7 cells showing cellular peptide distribution. Cells (1×10^6/ml) were incubated with fluorescently labeled ip$^+$NLS (A) or NLS (B), both at 100 μg/ml, in DMEM for 1 h at 37°C. After incubation, the cells were washed, trypsinized (1 mg/ml, 5 min), and analyzed by a 1-μm *Z*-position sectional scanning of the cells.

that the NF-κB binding activity could be competed by a nonlabeled NF-κB oligonucleotide (lane 2) but not by the non-specific AP1 probe (lane 3), and the antibody specific to NF-κB caused a band-shift of the NF-κB complexes (lane 4).

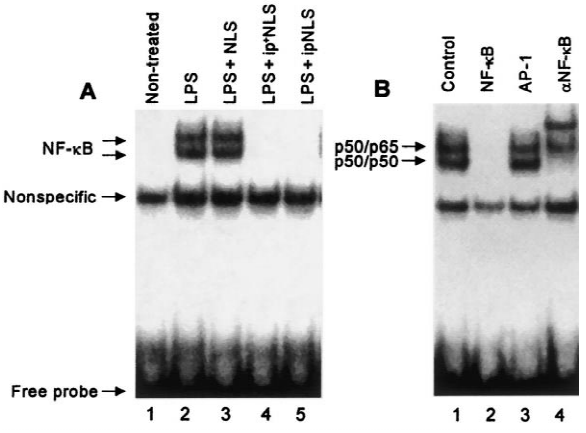

Fig. 4. Effect of peptides on DNA-binding activity of NF-κB. Cells were pretreated with the indicated peptide (100 μg/ml) for 1 h followed by LPS stimulation (10 ng/ml) for 2 h. EMSA studies were conducted as described in Section 2.4.

3.5. Inhibition of TNFα production

The inhibition of nuclear translocation of NF-κB should lead to transcriptional inactivation of NF-κB-dependent genes. The TNFα gene is under the control of NF-κB [1], therefore its expression should be affected by the NF-κB peptides. To test this possibility, ELISA studies were conducted (Fig. 5). The results showed that TNFα was induced by LPS and this induction was inhibited by the peptides ipNLS and ip$^+$NLS, but not by NLS.

4. Conclusions

We have demonstrated in this study that transcriptional inactivation of cytokine gene expression can be achieved using functional peptides that inhibit nuclear translocation of NF-κB. This inhibition

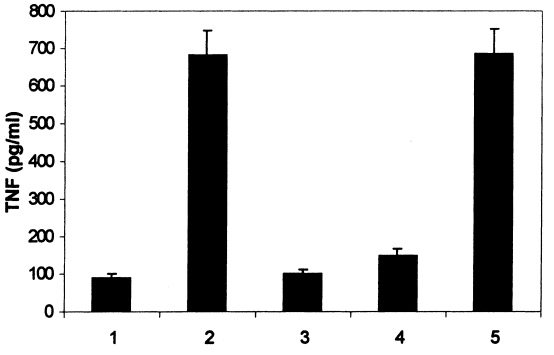

Fig. 5. Inhibition of LPS-induced TNFα production by peptides. Cells were treated with the indicated peptide (100 μg/ml) for 1 h prior to LPS stimulation (10 ng/ml). TNFα levels were detected 6 h after stimulation by ELISA. 1: control; 2: LPS; 3: LPS+ip^{+}NLS; 4: LPS+ipNLS; 5:LPS+NLS.

required the presence of a cell-permeable import peptide for efficient cellular entry. Because this import peptide is derived from the endogenous signal peptide, it is potentially non-toxic and non-immunogenic, thus allowing utilization of this peptide on a repetitive basis. Further studies on the fundamental mechanism of membrane translocation, intracellular trafficking, and potential toxicity of this peptide will shed light on its suitability as a novel drug carrier for bioactive agents.

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